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Acclimation of cotton (Gossypium) to abiotic stress

Armeanu, Katrin

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Acclimation of Cotton (*Gossypium*) to Abiotic Stress

PhD Thesis

by

Katrin Armeanu

School of Biological Sciences

University of Wales

Bangor

Supervisor: Dr. John Gorham

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Abstract

Cotton (*Gossypium hirsutum*) was assessed for its acclimation potential to heat, chilling, and salinity stress. Pretreatment at 40°C for 6 days reduced the extent of decrease in CO_2 assimilation on exposure to 49°C. This was associated with an increase in transpiration relative to the experimental control level, which did not occur in control-pretreated plants. The acclimating heat-pretreatment induced an increase in transpiration, as determined on the final day of treatment, and an increase in stomatal conductance on subsequent return to control temperature. This indicated an increase in plant hydraulic conductance, suggesting the involvement of transpirational cooling in the observed phenomenon of heat acclimation. Pretreatment at low temperatures decreasing gradually from 18 to 13°C by 1°C per day followed by recovery in control temperature for 1 day reduced the extent of chronic photoinhibition during subsequent treatment at 11°C under moderate incident light. This was associated with a reduced extent of leaf death in response to treatment at 11°C. The low temperature pretreatment induced photosynthetic responses of predominantly acclimative nature, as indicated by photo-protective photoinhibitory quenching and a slight increase in net rate of CO_2 uptake following the initial decline. Subsequent recovery in control temperature for 1 day induced an increase in F_m, suggesting increased yield of electron transport through photosystem II. The same chilling pre-treatment partially protected photosynthetic processes on exposure to 49°C, as indicated by a reduced extent of decrease in maximum quantum yield of PSII and net rate of CO_2 uptake. Pretreatment at 120 mol m^{-3} NaCl reduced the extent of increase in leaf sap Na^+ concentration on exposure to 300 mol m^{-3} NaCl. Increased leaf succulence explained part of this phenomenon, whereas the reduced extent of increase in leaf Na^+ concentration on a dry weight basis suggested the involvement also of other mechanisms of control of leaf Na^+ concentrations.

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1 Literature Review

1.1 Cotton (*Gossypium*)

1.1.1 Morphology and development

The cotton plant forms a small bush reaching about 1 metre in height (Langer & Hill, 1991). It has a strong tap-root which can reach a depth of 3 metres (Rehm & Espig, 1991). The shoot system shows two types of branching: The main axis is monopodial. At the base of each main axis leaf two buds may develop, a true axillary and an accessory bud. In the lower part of the stem the true axillary buds develop into monopodial vegetative branches. Higher up on the stem the accessory buds form sympodial fruiting branches (Rehm & Espig, 1991, Langer & Hill, 1991). All axes carry alternate leaves with long petioles and laminae separating, to various extents in different varieties, into five lobes (Franke, 1992).

The flowers occur singly. The calyx consists of five sepals and is reduced and cup-shaped. The corolla is composed of five large petals which are folded in the bud and last only for about a day once the bud has opened. As the flowers occur on several fruiting branches of different ages, flowering can take place over a range of time. The calyx is subtended by an epicalyx composed of three leafy, deeply toothed bracts, which persist beyond maturity. The filaments of the 100 to 150 stamina are grown together forming a tube, which surrounds the style. The ovary is superior and consists of three to five united carpels, each containing 8 to 12 ovules. Pollination mainly occurs in the form of self-pollination, but the flower also contains nectaries and can be pollinated by insects and humming birds, where present (Langer & Hill, 1991).

After pollination the fruits grow quickly. They reach their final size after about 20 days and maturity after a further 25 to 45 days. The fruit is an ovoid capsule referred to as boll. It contains 20 to 40 seeds. The outer epidermis cells of the seed integument develop two types of single-celled hairs: long hairs which are loosely attached and called lint, and much shorter and strongly attached hairs referred to as fuzz or linters. Lint hairs are the fibres for which cotton is cultivated. The hairs initially contain protoplasm. When the seeds ripen, their hairs die and hair walls collapse, leaving a narrow lumen inside, which contains

the remainder of the protoplasm. The hair walls consist of spiral layers of cellulose fibres. On maturity the ball walls open in the middle of each carpel and the lint emerges (Rehm & Espig, 1991, Langer & Hill, 1991).

Upland cotton (*Gossypium hirsutum*) is distinguished by the length of its lint hairs which is up to 2.5 cm in short-staple and 3.4 cm in long-staple cultivars (Langer & Hill, 1991).

1.1.2 Ecophysiology

The environmental requirements of the cotton plant have to be seen in the background of its tropical origin. Rehm & Espig (1991) describe cotton as a warmth-loving plant, with optimum temperatures of 35°C for sowing and 27°C for further development, and extremely sensitive to frost. High amounts of sunshine promote flowering and fruit setting. Cotton is considered to be relatively drought resistant, owing to its deep-reaching root system. Cotton cultivation requires 600 to 1500 mm rainfall, and deep soil with sufficient drainage. The plant's nutrient requirements are moderate due to its high nutrient uptake ability. In general, cotton is characterised as relatively salt tolerant (salt contents of 0.5 – 0.6 % cause no damage), although tolerance varies among cultivars.

1.1.3 Geographical radiation and diversification of the genus

The evolutionary diversification of the genus *Gossypium* will be outlined briefly in the following, according to Wendel (1995).

The genus *Gossypium* consists of species of shrubs and small trees distributed in arid or seasonally arid tropical and subtropical regions. Taxonomically, based on morphological differences, it is commonly divided into 39 diploid (2n = 26) and six tetraploid (2n = 4x = 52) species, grouped into 4 subgenera and 8 sections. Using cytogenetic criteria like chromosome size differences and interspecific meiotic pairing behaviour, seven diploid genomic groups (A-G) are distinguished. Molecular analysis of the chloroplast genome allows determination of phylogenetic relationships and estimation of the timing of divergence events.

Gossypium is believed to have originated in the Old World. About 25 to 30 million years ago the genus split into an Australian and an African branch. The

Australian branch comprises the 16 diploid C-genome species, the African branch the three diploid B-genome species, four E genome, one F genome, and two A-genome species. The latter are two of the cultivated species, *G. arboreum and G. herbaceum.*

About 6 – 11 million years BP long-distance dispersal of African species to the Americas led to evolution of the 13 diploid New World D-genome species, which probably originated in north-western Mexico. The New World tetraploid species are allopolyploids, as they contain one genome similar to that of Old World A-genome diploids and one similar to that of New World D-genome diploids. Based on molecular analysis it is thought that polyploid formation occurred only once and probably in the Pleistocene, about 1 – 2 million years ago. The identity of the A- and D-genome donors is uncertain. So far *G. raimondii* from Peru *and G. herbaceum* are considered to be the closest living models of D- and A-genome donors, respectively. The A-genome donor is believed to have been introduced to the Americas via transatlantic dispersal or alternatively via pre-agricultural dispersal to Asia followed by transpacific transport. Geographic radiation of the polyploids resulted in diversification into an early branch now represented by *G. mustelinum* (restricted to a small region of north-east Brazil) and two later branches, one branch comprising *G. hirsutum* (distributed in meso-America) and *G. tomentosum* (Hawaiian Islands), the other *G. barbadense* (north-west South America) and *G. darwinii* (Galapagos Islands). The sixth tetraploid species, *G. lanceolatum*, is now considered as a variant of *G. hirsutum.*

1.1.4 Domestication

Four species of *Gossypium* were domesticated independently in different parts of the world for their seed fibre and oil: the Old World diploids *G. arboreum and G. herbaceum*, and the New World tetraploids *G. hirsutum* and *G. barbadense.* A brief summary of what is known about the domestication of these four species is given in the following according to Wendel (1995).

The oldest remains of cloth and yarn made from old world cottons were found in India and Pakistan and date to 4300 years BP. *Gossypium herbaceum* was cultivated from Ethiopia to western India, *Gossypium arboreum* from China and Korea westward, including India, to northern Africa. It is believed these two species were independently domesticated from different wild ancestors.

G. barbadense is thought to have been originally domesticated in north-western South America. Remains of seed, fibre, fruit, yarn, fishing nets, and fabrics were found in central coastal Peru and date to 5500 years BP. It was later distributed across the Andes and northward to the Caribbean, Amazonia, and northern South America.

The oldest archeobotanical remains of *G. hirsutum* were found in the Tehuacan valley of Mexico and date to 4000 to 5000 years BP. However, it is thought that these cotton forms were introduced, and not originally domesticated there. The indigenous geographical range of the species, covering most of meso-America and the Caribbean, shows two broad genetic centres of diversity, one in southern Mexico-Guatemala, one in the Caribbean. It is therefore thought that *G. hirsutum* could have been domesticated more than once in different parts of its range. The modern Upland cultivars are believed to have been developed from semi-domesticated forms near the Mexican-Guatemalan border.

1.1.5 Development of modern cultivars by human selection and breeding

In the period of the European exploration of the New World the Old World diploid cottons were gradually replaced by agronomically superior New World tetraploid cottons.

The development of modern cotton cultivars will only be described in detail for one of the domesticated species, *G. hirsutum*, which was used in the author's research. Wendel (1995) summarises the fragmentary knowledge about the development of the four categories of modern *G. hirsutum* cultivars (Acala, Delta, Plains, and Eastern) in the south-eastern USA, whose derivates make a large contribution to world-wide cotton cultivation. In the late 1700s "green seed" and "black seed" cultivars were grown on the first plantings of commercial scale. The green-seeded stocks were superior regarding lint qualities, yield and disease resistance, but their lint was difficult to gin. When this problem was overcome by the invention of Whitney's saw gin, the green-seeded stocks were mainly grown. From the early 1800s onwards daylength neutral Mexican highland stocks were introgressed with the green and black seed stocks leading to further improvement of lint qualities, yield, disease resistance and ease and earliness of harvest. Additional introductions from the early 1900s onwards aimed at producing

varieties with early maturity (to decrease susceptibility to the boll weevil) and good adaptation to environmental conditions in the cultivation area. Most of the original green and black seed germplasm is thought to have been replaced by later Mexican introductions in the nineteenth and twentieth century. Breeding of modern Upland cultivars for the above desired agronomical traits led to severe reduction in genetic variation, although in comparison to other crops modest levels are still present.

Development of modern cultivars of all four domesticated species can be described as the result of parallel and/or convergent human selection for similar agronomical characteristics to produce varieties which are high yielding, day-length neutral, annual and having improved fibre qualities, from originally tropical perennial ancestors with shorter, sparser fibre (Wendel, 1995).

1.1.6 Modern cotton cultivation

The four domesticated cotton species supply the world's most important textile fibre and second most important oil and meal seed. Over 90 per cent of the world's cotton production today is supplied by *G. hirsutum*, referred to as Upland cotton. It is grown in tropical and temperate latitudes from 47° N in the Ukraine and 37° N in the USA to 32° S in South America and Australia. *G. barbadense* (Extra-long staple, Pima or Egyptian cotton) produces longer strong and fine fibres, but, due to its lower yield, contributes less than 10 per cent to total world production. It is cultivated in regions of the Commonwealth of Independent States, Egypt, Sudan, India, USA and China. The Old World domesticated species make only a small contribution to modern cotton production, but are still occasionally cultivated: *G. arboreum* in India and Pakistan, *G. herbaceum* in certain regions of Africa and Asia (Wendel, 1995).

Cultivar improvement today focuses, in addition to the traditional agronomical characteristics of high yield, improved fibre quality, early maturity, and disease resistance, on characteristics alleviating environmental damage caused by chemical agricultural inputs as well as locally on resistance to pests and disease, cold, drought and high soil salinity (Rehm & Espig, 1991, Wendel, 1995).

1.2 The concept of plant stress response

1.2.1 Biological stress and strain

Levitt (1980) was among the first to define a concept of biological stress in analogy to concepts developed earlier for mechanical stress. In mechanical terms, stress produces a state of strain in a non-living body, which is manifested in a change in dimension of the body. Elastic strain is completely reversible. Beyond a certain point, which is specific for the body, the strain becomes partially irreversible, and the irreversible component is called plastic strain. By analogy, biological stress is defined as any environmental factor capable of inducing a potentially injurious strain in a living organism. This strain can occur as physical or chemical change and can be elastic or plastic.

Environmental stresses are currently grouped into abiotic stresses, caused by excess or deficit in the physical or chemical environment, and biotic stresses, imposed by other organisms (Buchanan *et al.*, 2000).

1.2.2 Stress-induced injury and stress resistance

Stress-induced injury in plants can arise in three different ways (Levitt, 1980): (a) Brief exposures (seconds or minutes) to severe stress can induce a direct plastic strain, which produces so-called direct stress injury. (b) Moderate stress levels can induce an elastic strain, which does not induce injury initially, but with continued exposure (hours or days) can give rise to a plastic strain, which then results in a type of injury referred to as indirect stress injury. (c) Longer exposure to a (primary) stress can induce a second stress, which in turn can result in either direct or indirect so-called secondary stress injury.

According to Levitt (1980) the stress resistance of a plant can be measured as the stress necessary to produce a specific strain, which can be elastic or plastic. This specific strain is often set as 50% lethality. On a functional basis, two mechanisms of resistance are distinguished: (a) Resistance achieved by avoiding thermodynamic equilibrium with the stress is termed stress avoidance. (b) The ability to enter thermodynamic equilibrium with the stress without suffering injury is referred to as stress tolerance. These two functional mechanisms can be applied at the level of stress, elastic strain, or plastic strain. Thus stress can be avoided or tolerated, and stress tolerance in turn can occur as avoidance or tolerance of

elastic or plastic strains. The mechanism of tolerance to plastic strains is unique to living organisms as compared to non-living matter, as the former can possess mechanisms to repair thermodynamically irreversible strains by active expenditure of metabolic energy.

Lichtenthaler (1996) presented a model (summarised and modified from earlier stress concepts) showing the type of plant stress response as dependent on the severity and duration of the external stress factor, as well as on internal plant resistance. Plant stress response is thought to follow the following four-phase kinetics:

a) Alarm phase: Plant metabolic activities and physiological functions decrease. If the stress intensity exceeds the threshold of plant constitutive resistance (resistance minimum), acute damage and eventually death occur. If the threshold of resistance is not exceeded, the plant will activate its stress coping mechanisms.

b) Restitution and resistance phase: Stress coping mechanisms are put into action. They are classified into a) repair processes leading to homeostatic restitution of the initial physiological state and b) hardening processes establishing a new physiological standard optimal under the new environmental conditions (resistance maximum).

c) End phase (stage of exhaustion): When the stress dose, the product of stress intensity and duration, exceeds the threshold of maximum resistance set by the capacity of stress coping mechanisms, metabolic activities and physiological functions decline. Ultimately chronic damage and finally cell death can occur. It is noted that the assumed stress dose – stress effect relationship has not been proven for all types of stress.

d) Regeneration phase: If the stress is removed, before senescence processes become dominant, the plant will regenerate and move to a new physiological standard between the stress minimum and maximum, the relative position of which depends on the stage of exhaustion at which the stress is removed.

Thus in Lichtenthaler's model (1996) the term acute damage is equivalent to that of direct stress injury introduced by Levitt (1980), and accordingly chronic damage to that of indirect stress injury.

1.2.3 Adaptation and acclimation

In contrast to non-living matter, living organisms can increase their stress resistance, a process originally termed adaptation (Levitt, 1980). Levitt distinguishes stable and unstable adaptation. While stable adaptation is defined as having arisen in evolution, unstable adaptation is dependent on the developmental stage and growth environment of an organism, and the hereditary potential of the organism for such adaptation. Based on the reversibility of the strain to which resistance is developed, two subtypes of adaptation are distinguished, which can occur in the case of stable and unstable adaptation.

(a) Elastic adaptation allows an organism to live, grow, complete its life cycle, and regenerate in the presence of stress.

(b) Plastic adaptation may not permit growth and may just prevent a plastic strain, which normally causes injury to a not adapted organism.

Unstable adaptation is thought to develop in organisms, which have the according hereditary potential, in response to continued exposure to a small stress, which produces only elastic strain. The elastic strain can induce secondary changes, which lead to:

(a) elastic adaptation resulting in decrease of the elastic strain to a constant low value and/or

(b) plastic adaptation resulting in resistance to a plastic strain, while the elastic strain remains constant.

In most cases unstable adaptation is thought to involve tolerance, only in some cases avoidance mechanisms.

In the model presented by Lichtenthaler (1996) outlined above the plant minimum resistance level would be to some extent equivalent to the term resistance due to stable adaptation, as defined by Levitt (1980). Maximum resistance achieved by repair and hardening processes would accordingly be to some extent equivalent to resistance as a result of elastic and plastic unstable adaptation.

The terminology describing these changes in organisms to decrease or prevent stress-induced strain has since changed. Although there seems to be considerable variation in the literature, the current use of terminology can be summarised roughly as follows. According to Buchanan *et al.* (2000) adaptations

are defined as genotypically determined traits for stress resistance, which result from evolutionary selection for enhanced environmental fitness of a population of organisms. These traits are constitutive, *i.e.* they are expressed whether the plants are stressed or not. In contrast, acclimation is the adjustment of individual organisms in response to changing environmental factors resulting in altered steady-state physiology (homeostasis). In principle, Pearce (1999) uses similar definitions, but adaptation is seen in a broader sense as possession of genes conferring greater stress resistance. Some of these genes are expressed constitutively, and confer a permanent state of greater resistance. Acclimation is defined as expression of the genetic potential under inductive conditions, and in this sense is viewed as also genotypically determined.

Many investigations have been undertaken to elucidate the mechanism of induction of acclimation as defined above. From the cellular to the whole plant level the sequence of events leading to acclimation is now understood as follows. Initially the plant recognises a stress at the cellular level followed by activation of signal transduction pathways, which transmit information within cells and throughout the plant. These pathways lead to changes in gene expression that result in biochemical and physiological changes and are integrated into a response by the whole plant, involving modification of growth and development. (Buchanan *et al.*, 2000, Grover *et al.*, 2001). Many stress-induced changes in metabolism and development are the result of such changes in gene expression. In general accumulation of gene products can be the result of transcriptional activation of gene expression as well as posttranscriptional mechanisms, the latter leading to increases in the amounts of specific mRNAs, increased translation, stabilization of proteins or changes in protein activities (Buchanan *et al.*, 2000).

1.3 Common elements in plant stress responses

1.3.1 Photoinhibition & photo-oxidation – a commonality of high light stress and the combination of moderate light with stresses impairing energy utilisation

1.3.1.1 Photoinhibition

In the context of fluorescence analysis, Osmond (1994) defined photoinhibition as inhibition of the light reactions of photosynthesis as a result of

the unavoidable harvesting of excess photons. It describes all processes that lower the efficiency of photosynthetic energy utilisation (photon yield). Photoinhibition is closely linked to the dark reactions of photosynthesis. The efficiency of photosynthesis is highest in limiting light conditions, when the rates of the dark reactions are least limiting and lowest in conditions of photon excess, when the rates of the dark reactions are maximum (P_m) and most limiting. Osmond (1994) distinguishes two classes of photoinhibition based on their relaxation times.

1. Dynamic photoinhibition is rapidly relaxing and readily reversible.
2. Chronic photoinhibition is more slowly reversible and occurs in response to sustained exposure to excess photons. It consists of two stages, and any photoinhibited state may involve both of them, depending on the cumulative exposure to excess photons.

a) Initially photoinhibition is associated with a decrease in photon yield, but little change in photosynthetic capacity. It results in a proportional reduction of photosynthetic efficiency of all cells, chloroplasts or PSII centres in the tissue (analogous to dynamic photoinhibition). This response is viewed as a mechanism of photon protection.

b) After prolonged exposure to high PPFD photoinhibition is associated with a proportional decrease in both photon yield and photosynthetic capacity. It is due to a complete inactivation of photosynthesis in a proportion of cells or chloroplasts in the tissue. This form of chronic photoinhibition is attributed to photon damage.

The condition of photon excess can occur in response to many environmental factors. Huner *et al.* (1998) describe the processes contributing to over-excitation of PSII as follows. In general, PSII excitation pressure is dependent on the balance between absorption and utilisation of excitation energy. These two processes can be estimated as follows. The rate of excitation of PSII under light-limiting conditions is given as $\sigma_{PSII} * I$, where σ_{PSII} is the functional absorption cross-sectional area of PSII, and I is the incident photon flux (μmol photons m^{-2} s^{-1}). The rate of utilisation of excitation energy through electron transport and metabolism under light-saturating conditions is given as $n * 1/\tau$, where n is the number of components acting as sinks for electrons, τ is the life-time and $1/\tau$ the turnover rate of theses electron sinks. Inequality of these two processes of the form $\sigma_{PSII} * I > n * 1/\tau$ causes over-excitation of PSII. Such an

imbalance can result from excessive incident photon flux I (high light conditions), from reduction in the turnover rate of electron sinks $1/\tau$ (as caused by low temperature), and from reduced sink capacity n. The latter can be caused by impairment of CO_2 assimilation via limitation of CO_2 supply (drought) or impairment of N and S reduction (macronutrient deficiency). According to Osmond (1994) environmental conditions, which themselves impair photosynthetic function, as restricted CO_2 supply, low or high temperature, inadequate nutrition and pathogens, have the potential to cause chronic photoinhibition, all of which (except low temperature) would also fall into the category of factors reducing sink capacity n as classified by Huner *et al.* (1998).

In molecular studies, the term photoinhibition is confined to damage to PSII reaction centres (Maxwell & Johnson, 2000) and is defined as the decrease in efficiency and/or maximum rate of photosynthesis caused by photooxidative damage to the photosynthetic apparatus (Niyogi, 1999).

1.3.1.2 Oxidative damage

Photoinhibition (as defined in molecular studies) is the result of oxidative damage of certain target molecules, mainly the D1 protein of the PSII reaction centre and the Calvin cycle enzymes fructose-1,6-bisphosphatase and glyceraldehyde-3-phosphate dehydrogenase, by photo-produced reactive molecules. The inactivation of these target molecules in turn reduces the capacity to utilise excitation energy and further increases photo-production of reactive molecules. Photo-produced reactive molecules are triplet chlorophyll (^{3}Chl) and active oxygen species (AOS). AOS include a) 3 types of reduced species of dioxygen, which are superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^\cdot$), and b) excited singlet oxygen ($^1O_2$) (Asada, 1994).

In general, the potentially damaging reactive molecules can be generated at three sites in the photosynthetic apparatus (Niyogi, 1999).

a) In the light-harvesting complex associated with PSII ^{3}Chl can be formed through intersystem crossing from chlorophyll in the singlet excited state (^{1}Chl), which usually transfers the excitation energy to neighbouring chlorophylls and ultimately to the reaction centre. The yield of ^{3}Chl formation depends on the average lifetime of ^{1}Chl in the antenna. ^{3}Chl is relatively long-lived and can interact with O_2 resulting in the formation of $^1O_2$. $^1O_2$ in the

reaction centre can cause oxidation of lipids, proteins, and pigments in the immediate vicinity (Niyogi, 1999). Polyunsaturated lipids (like those in the thylakoid membrane) are susceptible to so called lipid peroxidation, which can be initiated by reaction of $^1O_2$ with the methylene groups resulting in the formation of conjugated dienes, lipid peroxy radicals, and hydroperoxides. The peroxy radicals can induce a chain reaction of peroxidation by abstracting H from other unsaturated fatty acids in the thylakoid membrane (Smirnoff, 1995).

b) In the PSII reaction centre the reversible primary charge separation between P680 and pheophytin can result in the formation of triplet P680 (^{3}P680), which in turn can interact with O_2, generating $^1O_2$. P680 and the secondary donor Y_Z^+ (in the oxidised state) can also oxidise pigments and proteins in close vicinity. $^1O_2$ and $P680^+$ can cause oxidation of lipids, critical pigment factors, and PSII subunits, particularly the D1 protein (Niyogi, 1999).

c) In PSI the acceptor side of P700 can reduce O_2 to O_2^- (Niyogi, 1999). Dismutation of O_2^- results in formation of hydrogen peroxide (see below). H_2O_2 can be further reduced to form $OH^\cdot$ via the metal-catalysed (iron, copper, and other transition metals) Haber-Weiss or Fenton reaction: $O_2^- + Fe^{3+} \rightarrow O_2 + Fe^{2+}$ and $Fe^{2+} + H_2O_2 \rightarrow OH^\cdot + OH^- + Fe^{3+}$. While H_2O_2 is relatively stable, $OH^\cdot$ is extremely reactive and short-lived (Smirnoff, 1995). On the acceptor (stromal) side of PSI O_2^-, H_2O_2 and $OH^\cdot$ can lead to oxidation of key enzymes of the photosynthetic carbon metabolism like phosphoribulokinase, fructose-1,6-bisphosphatase, and NADP-glyceraldehyde-3-phosphate dehydrogenase (Niyogi, 1999).

However, O_2^- and H_2O_2 are not only produced in the photosynthetic apparatus, but also during respiratory electron transport, oxidation of glycolate during photorespiration, and oxidation of xanthine and glucose (Noctor & Foyer, 1998).

1.3.2 Stress resistance to photo-oxidative damage

1.3.2.1 Photoprotective acclimation

Photoprotection defines the array of mechanisms, which have evolved in oxygenic photosynthetic organisms to prevent net damage to the photosynthetic

apparatus by excessive light (Niyogi, 1999). These can occur at different levels as described in the following.

1.3.2.1.1 Thermal dissipation of excess absorbed light energy

This type of acclimation results in an adjustment of the functional absorption cross-sectional area of PSII σ_{PSII} (Huner *et al.*, 1998). According to their time scale of induction and relaxation two processes involving thermal dissipation of excess excitation energy are distinguished, which are measured as nonphotochemical quenching of chlorophyll fluorescence (NPQ).

a) ΔpH-dependent high energy state quenching (qE) can be rapidly induced on a time sale of minutes (Huner *et al.*, 1998), relaxes within minutes under dark conditions, and is the major component of NPQ under most conditions (Maxwell & Johnson, 2000). In principle qE is equivalent to the term dynamic photoinhibition, as defined by Osmond (1994). Niyogi (1999, 2000) describes qE as follows. It is caused by a decrease in thylakoid lumen pH, which a) activates violaxanthin de-epoxidase (VDE), the lumenal enzyme, which converts violaxanthin to zeaxanthin (and antheraxanthin) as part of the xanthophyll cycle, and b) may also result in protonation of certain proteins in the PSII light harvesting complex, LHCB4 and LHCB5. Both binding of protons and zeaxanthin are thought to cause a conformational change, which switches the light harvesting complex into a state in which it efficiently de-excites singlet chlorophyll and dissipates excitation energy as heat. The decrease in lumen pH is caused by the build-up of a high thylakoid ΔpH in response to PSII over-excitation. Strong correlations between zeaxanthin accumulation and qE have been observed in a variety of plant species. Mutant analysis confirms that the majority of qE absolutely requires de-epoxidation of violaxanthin to zeaxanthin, and also implies a possible (direct or indirect) role for lutein. The biophysical mechanism of de-excitation of singlet chlorophyll is still unclear and could be due to transfer of excitation energy to the singlet state of zeaxanthin or to allosteric effects of xanthophylls on LHC structure.
b) Photoinhibitory quenching (qI) is a long-term mechanism of sustained thermal dissipation (Niyogi, 2000), which relaxes over a time-scale of hours (Maxwell & Johnson, 2000). This type of quenching can be related to a)

photoprotective processes, which cause quenching in the light-harvesting antenna of PSII and b) damage to PSII reactions centres, which results in quenching within PSII reaction centres (Maxwell & Johnson, 2000). Part of qI is thought to be mechanistically similar to qE. Overwintering evergreens have been shown to develop sustained, only slowly reversible thermal dissipation. Development of qI in these plants is associated with zeaxanthin accumulation and recovery with conversion of zeaxanthin back to violaxanthin (Niyogi, 2000). According to Huner *et al.* (1998) evergreen plants show an increase in the total xanthophyll pool size and zeaxanthin content in response to days or months of low temperatures, which results in sustained capacity of thermal dissipation.

1.3.2.1.2 Adjustment in light-harvesting antenna size

Reduction in the PSII antenna size is an acclimative mechanism resulting in adjustment of the functional absorption cross-sectional area of PSII σ_{PSII}, which occurs over a longer time scale (hours to days) (Huner *et al.,* 1998). This adjustment results from changes in gene expression and/or degradation of light harvesting complex proteins (Niyogi, 1999).

1.3.2.1.3 Adjustment in photosynthetic capacity

Increases in the capacity of photosynthetic electron transport and CO_2 fixation are long-term acclimative processes usually occurring over a period of several days, which result from altered gene expression and enzyme activities. Adjustment in the maximum rate of photosynthesis has been observed in response to growth in different light environments (Niyogi, 1999, 2000). It results in adjustment of electron sink capacity as defined by Huner *et al.* (1998).

1.3.2.1.4 Alternative electron sinks

As photoinhibition results from an imbalance between energy absorption and utilisation, alternative electron acceptors, *i.e.* in addition to the major chloroplastic electron sinks of C, N, and S reduction, can help to prevent over-excitation of PSII. The following processes have been suggested to function as such alternative electron sinks.

a) **Photorespiration:** Particularly under CO_2 limitation photorespiration can maintain linear electron transport by utilising reducing power and chemical

potential energy. It is thought be important in preventing light-induced damage to the photosynthetic apparatus. Mutation and inhibitor analysis have shown that blocking photorespiration results in inhibition of photosynthesis and photo-oxidative damage (Niyogi, 1999).

b) **Photoreduction of oxygen by photosystem I:** The combined system of the Mehler reaction, the reactions catalysed by the thylakoid-bound isozymes of SOD and APX, and the regeneration of ascorbate from the MDA radical *via* direct photo-reduction by reduced ferredoxin in PSI (see below) is called the Mehler-ascorbate peroxidase reaction, pseudocyclic electron transport or water-water cycle. The term water-water cycle originates in the fact that in this cycle electrons generated in PSII from the oxidation of H_2O are used to reduce O_2 to H_2O at PSI. This cycle maintains electron transport and contributes to the generation of ΔpH for ATP synthesis without producing NADPH or net O_2 or utilising ATP. Generation of ΔpH may also play a role in the maintenance of nonphotochemical quenching. However, the importance of this cycle as a photoprotective mechanism *in vivo* is still unclear (Niyogi, 1999, 2000).

c) **Chlororespiration:** Chloroplasts appear to have a respiratory chain which transfers electrons from NAD(P)H to O_2 *via* the plastoquinone pool. An *Arabidopsis* chloroplast protein similar to the alternative oxidase of mitochondria appears to be involved in oxidation of reduced plastoquinol, which is necessary for carotenoid biosynthesis. The chlororespiratory oxidase could function in maintaining electron transport and allowing carotenoid biosynthesis to proceed, which is required for the repair of photodamaged PSII (Niyogi, 2000).

1.3.2.2 Scavenging of reactive oxygen species

1.3.2.2.1 Antioxidant molecules in the thylakoid membrane

β-carotene in the PSII reaction centre can quench $^1O_2$ (but not ^{3}P680), while xanthophylls bound to the light-harvesting complex quench ^{3}Chl and $^1O_2$. Carotenoids have been shown to inhibit lipid peroxidation and stabilise membranes (Niyogi, 1999).

α-tocopherol (vitamin E) is thought to have the following functions in preventing/alleviating oxidative stress: It can (a) physically quench $^1O_2$, (b)

chemically scavenge $^1O_2$, O_2^-, and $OH^\cdot$, producing tocopheryl quinones and quinone epoxides, (c) react with lipid radicals resulting in its oxidation to the α–chromanoxyl radical, thereby terminating lipid peroxidation chain reactions, and (d) influence the state of the thylakoid membrane by firstly decreasing its ion permeability (facilitating maintenance of ΔpH), and secondly by increasing its stability through binding free fatty acids produced by de-esterification of phospholipids by O_2^- (Smirnoff, 1995, Niyogi, 1999).

1.3.2.2.2 Soluble antioxidant molecules in the chloroplast

(1) Ascorbate (vitamin C) is believed to have the following roles in preventing/alleviating oxidative stress in the chloroplast (Smirnoff, 2000): Ascorbate in the stroma a) prevents oxidative damage by scavenging of O_2^-, $OH^\cdot$, and $^1O_2$, b) scavenges H_2O_2 in a reaction catalysed by ascorbate peroxidase (APX), and c) regenerates α-tocopherol from its α–chromanoxyl radical. Lumenal ascorbate a) donates electrons to PSII and b) acts as cofactor of violaxanthin de-epoxidase (VDE). The primary oxidation product of ascorbate produced in these reactions is the monodehydroascorbate radical (MDA), which is relatively stable (which is probably why ascorbate is such an important free radical scavenger). MDA then disproportionates to ascorbate and dehydroascorbate (DHA). DHA is unstable and is irreversibly converted to other compounds, if its formation exceeds the capacity of the ascorbate regeneration systems (see below). Ascorbate is one of the most abundant chloroplast metabolites. Its concentration in chloroplasts is estimated to be in the range of 10 - 230 mol m^{-3}. The concentration of ascorbate in the thylakoid lumen is not known, but ascorbate is thought to move across the thylakoid membrane by diffusion and not by a carrier system, which would result in a low lumenal concentration. The exceptionally high concentration of ascorbate in the stroma could therefore reflect the need to maintain a high lumenal ascorbate concentration necessary for the operation of VDE as part of the xanthophyll cycle. The ascorbate pool is mostly reduced in normal metabolism (90%), but becomes oxidised in response to oxidative stress (Smirnoff, 2000).

(2) The tripeptide glutathione (GSH, γ-glutamylcysteinylglycine) is the second of the major soluble antioxidants, its concentration in the chloroplast is 3-4 mol m^{-3} (Smirnoff, 2000). It is very stable due to the γ-glutamyl linkage and a

powerful reductant owing to the nucleophilic nature of the central cysteine, rendering it efficient in scavenging peroxides (May *et al.*, 1998). GSH can scavenge $^1O_2$ and $OH^{\cdot}$, protect thiol groups in stromal enzymes, and plays a role in the regeneration of α-tocopherol and ascorbate *via* the glutathione-ascorbate cycle (see below) (Niyogi, 1999). Its oxidation results in formation of oxidised glutathione (GSSG), in which two GSH molecules are joined by a disulphide bond. Under normal conditions the GSH pool is highly reduced (90%) (Smirnoff, 1995).

1.3.2.2.3 Antioxidant enzymes

(1) In PSI reduced ferredoxin can reduce O_2 generating superoxide (Mehler reaction) (Smirnoff, 2000). Superoxide dismutase (SOD) catalyses the (also spontaneously occurring) disproportionation of superoxide: $O_2^{\cdot-} + O_2^{\cdot-} + 2\ H^+ \rightarrow H_2O_2 + O_2$. SOD thereby lowers the steady-state concentration and in turn diffusion distance of $O_2^{\cdot-}$. Three types of SOD isozymes are distinguished according to their prosthetic metals: CuZn, Mn, and Fe SOD. In the chloroplast the SOD in the stroma is the CuZn form in most species, or the Fe form in some higher plants. The thylakoid-bound SOD is of the Mn type. Thus thylakoid-bound SOD scavenges $O_2^{\cdot-}$ released from the PSI reaction centre at the site of its production, producing H_2O_2 and O_2, and thereby prevents generation of the most reactive AOS species $OH^{\cdot}$ (Asada, 1994).

(2) H_2O_2 can inactivate Calvin cycle enzymes and participate in the Haber-Weiss or Fenton reaction, and also has to be efficiently scavenged. Two types of enzymes can detoxify H_2O_2.

(a) Catalase is localised in the peroxisomes and catalyses the disproportionation of H_2O_2, produced by glycolate oxidase as part of the photorespiratory metabolism, to oxygen and water. Catalase does not depend on electron donors for scavenging, but cannot lower the concentration of H_2O_2 as effectively as peroxidase (see below) due to its higher K_m.

(b) Ascorbate peroxidase (APX) is a heme enzyme (like other plant peroxidases) and catalyses the reduction of H_2O_2 to water, using ascorbate as electron donor, which is oxidised to the monodehydroascorbate radical (MDA): 2 ascorbate + $H_2O_2 \rightarrow$ 2 MDA + 2 H_2O. APX exists in multiple

isoforms which are bound to the thylakoid membrane or soluble in the chloroplast stroma and the cytosol. The thylakoid-bound APX form can scavenge almost all of the H_2O_2 generated by PSI at the site of its production (Asada, 1994).

(3) As the rate of ascorbate biosynthesis is relatively sluggish, the rate of its oxidation in the chloroplast exceeds that of its transport into the chloroplast (the last step of its biosynthesis takes place in the mitochondria), and DHA is unstable, ascorbate regeneration systems are necessary to maintain sufficient ascorbate concentration in the chloroplast (Smirnoff, 1995, 2000). Ascorbate, the electron donor of APX, can be regenerated in three different ways: (a) The MDA radical can be directly photo-reduced in the thylakoids by reduced ferredoxin in PSI in the so-called water-water cycle: MDA + reduced ferredoxin → ascorbate + ferredoxin. (b) In the stroma the MDA radical can be enzymatically reduced to ascorbate by the FAD enzyme monodehydroascorbate reductase (MDHAR), which uses NAD(P)H as electron donor: 2 MDA + NAD(P)H → 2 ascorbate + NAD(P). (c) MDA radicals, which are not reduced in either of the two pathways, disproportionate to ascorbate and dehydroascorbate (DHA): MDA + MDA → ascorbate + dehydroascorbate. A set of enzymes in the stroma comprising the glutathione-ascorbate cycle can regenerate ascorbate from DHA as follows. DHA is reduced to ascorbate by the thiol enzyme dehydroascorbate reductase (DHAR), using reduced glutathione (GSH) as electron donor. Finally oxidised glutathione (GSSG) can be reduced back to GSH by glutathione reductase (GR), using NADPH produced in photosynthesis as electron donor (Asada, 1994, Niyogi, 1999).

Two different scavenging systems seem to scavenge O_2^- and H_2O_2 in different compartments of the chloroplast: The thylakoidal scavenging system is composed of PSI-associated peripheral or membrane-bound SOD, thylakoid-bound APX, and photoreduction of MDA via ferredoxin and is the primary scavenging system for reactive molecules at the site of their production. The stromal scavenging system consists of stromal SOD, stromal APX, MDAR and DHAR, the latter two occurring only in the stroma, and scavenge reactive molecules, which have diffused to the stroma. The primary function of these

systems is the rapid removal of reactive molecules to prevent inactivation of stromal enzymes (Asada, 1994).

1.3.2.3 Repair or de novo synthesis of damaged molecules

The repair system for inactivated PSII involves selective degradation of damaged proteins (mainly D1) and incorporation of newly synthesised proteins to reconstitute functional PSII (Niyogi, 1999). In general, inactivation of PSII reaction centres during photoinhibition is not caused by a loss, *i.e.* net degradation, of D1 protein (Giardi *et al.*, 1997), but D1 degradation is synchronised with resynthesis and replacement of D1 (Krause, 1994). The observation that addition of inhibitors of chloroplast protein synthesis strongly increased photoinhibition led to the view that inactivation of PSII centres occurs simultaneously with PSII repair *via* D1 turnover. The extent of photoinhibition after a prolonged time in strong light at room temperature is thus thought to reflect the balance between these two processes (Krause, 1994).

Turnover of the D1 protein is, however, not the only process thought to be involved in reactivation of inactivated PSII centres during chronic photoinhibition. A model suggested by Krause & Weis (1991) and Krause (1994) describes the inactivation of PSII reaction centres as a sequence of two steps. (a) The first step is reversible, and fast recovery (within an hour) can take place, involving direct reactivation of PSII without turnover of D1. The mechanism of this inactivation is unclear and possibly involves a conformational change of the reaction centre complex. (b) In the second phase the D1 protein is marked for degradation. The active oxygen species $^1O_2$ and possibly O_2^- are thought to be involved in the marking process. Slow recovery (lasting several hours) results in complete reactivation of PSII and proceeds via the D1 repair cycle.

Krause (1994) and Osmond (1994) note in this context that during chronic photoinhibition PSII centres with sustained inactivation in both stages are thought to be able to trap excitation energy and act as conduits for thermal dissipation of excess photons. Thus even this accumulation of inactivated centres can be regarded as some type of photoprotection. They are thought to retain good excitation connectivity with remaining functional centres and thereby provide shading to the functional centres.

1.3.3 Plant water deficit

Plant water deficit occurs, when more water is lost by a plant through transpiration than is taken up by the roots. It is a component not only of drought, but also of salinity, freezing and chilling stress (Bray, 1997).

1.3.3.1 Cellular water deficit, plasmolysis, and cytorrhysis

Plant water relations in the absence of stress can be described as outlined briefly in the following. Water movement from the soil into plant roots and up to leaves is driven by gradients in water potential (Thomas, 1997). Water moves spontaneously from regions of higher chemical potential to those of lower chemical potential. Water potential is defined as $\Psi = (\mu_W - \mu_W^o) / V_W$. μ_W is the chemical potential of water, and is defined as $\mu_W = \delta G / \delta n_W$, where δG is the change in Gibbs free energy in the system, and δn_W the change (by addition or removal) in the number of moles of water, at constant temperature and pressure. Ψ is expressed as difference between the chemical potential of water in the system and the chemical potential of pure free water at the same temperature, at atmospheric pressure, and at reference elevation (μ_W^o). Dividing by the partial molal volume of water (V_W) allows expression in pressure units (Pascal). At the reference point, Ψ is zero and water is freely available. Negative values of Ψ reflect decreased availability of water (Jones, 1992).

In plant cells the total water potential can be described as $\Psi = \Psi_p + \Psi_\pi$. Here Ψ_π stands for osmotic or solute potential and is related to the concentration of dissolved solutes as follows: $\Psi_\pi = - R T c_s$, where R is the universal gas constant, T the temperature of the solution, and c_s the solute concentration of the system expressed as osmolality, i.e. the number of osmol, which each contain Avogadro's number of osmotically active particles, per mass of solvent. It is always negative and results in absorption of water into the cell and cell expansion. Ψ_p is the pressure or turgor potential and is defined as difference in hydrostatic pressure from the reference point. The characteristic rigid cell walls of plant cells resist cell expansion, and extend elastically, thus generating a positive internal hydrostatic pressure. The difference in pressure inside and outside the cell wall is termed turgor pressure (P) (Jones, 1992).

The Höfler-Thoday diagram describes the development of cellular water deficit in mature tissues by interrelating changes in cell volume and cell water potential and its components as dependent on relative water content (the water content as fraction of the water content at full turgidity). In a fully turgid cell the relative water content is 1, $\Psi = 0$, and $\Psi_\pi = -\Psi_p$. As the cell loses water and the relative water content decreases, turgor pressure decreases approximately linearly, and osmotic potential decreases curvilinearly with decreasing cell volume. When Ψ_p reaches 0, it usually remains close to zero with further decreases in relative water content (Jones, 1992).

The slope of the response curve of cell water potential and its components to decreasing relative water content depends on the bulk modulus of elasticity of the tissue (ε_B). ε_B describes the cell wall rigidity in a tissue, and is defined as $\varepsilon_B = dP / (dV / V)$, where P stands for pressure and V for cell volume. It depends primarily on the elasticity/rigidity of the cell walls, but also on tissue structure and interactions between cells. In general, ε_B increases with rigidity of the cell walls and decreases with size of cells in a tissue (Jones, 1992). A more flexible leaf develops a weaker gradient between leaf and environment at a given relative water content, but reaches the point of zero turgor at a lower relative water content than a stiffer leaf (Thomas, 1997).

The relationship between the components of cellular water potential depends on cellular solute concentration, cell wall rigidity, and cell size, and varies with species, growth conditions and developmental stage (Lawlor, 1995). It thus reflects adaptation and acclimation responses. In contrast, the relative water content represents a normalised form of water content (relative to the fully hydrated state), which reflects relative changes in cell volume, and is therefore suited for comparison of different species and tissues, and for relating cellular water status to metabolism (Lawlor & Cornic, 2002).

The point of zero turgor is associated with leaf wilting and can lead to two possible events. (a) Plasmolysis, the separation of the cell membrane from the cell wall, is observed, when tissues are placed in hypertonic solutions. Due to this observation the point of zero turgor is also referred to as point of limiting plasmolysis. (b) Cytorrhysis, the collapse of cells, occurs in aerial tissues (in which capillary forces at the interface of air and water prevent plasmolysis), when

the tension in the cell wall generated by decreasing osmotic potential cannot be supported anymore (Jones, 1992).

1.3.3.2 ABA-induced responses

The plant hormone abscisic acid (ABA) is one of the major signals involved in plant responses to drought and acts (a) as a long-distance signal transported in the xylem at the whole plant level, and (b) as a signalling molecule inducing gene expression at the cellular level. Signalling by ABA is, however, not only involved in responses to drought, but also to other environmental stresses inducing plant water deficit. These include salinity, freezing, and chilling, as well as responses to stresses that lack a water deficit component, such as hypoxia, wounding, and light, but not the response to heat stress (Bray, 1997).

1.3.3.2.1 ABA signalling at the whole plant level

ABA has been implicated in two plant physiological responses to soil drying, which result in conservation of water: inhibition of shoot expansion growth and stomatal closure (Wilkinson & Davies, 2002).

(1) Cell expansion growth generally depends on water as outlined in the following. Volumetric cell growth depends on two processes (Jones, 1992). (a) Water uptake into the cell is driven by the difference in total water potential across the membrane, and the contribution of osmotic potential is determined by the degree of semi-permeability of the membrane (*i.e.* permeability to water and impermeability to solutes). The latter is described by the reflection coefficient (σ), which is zero for a completely permeable and one for a perfectly semi-permeable membrane (Jones, 1992). This definition of the reflection coefficient (σ) does not, however, consider the control of membrane permeability by aquaporins (water channel proteins) (Thomas, 1997). Volume flux density of water across the membrane can accordingly be described as $J_v = L_p (\sigma \Delta \Psi_\pi - P)$, where the term $\sigma \Delta \Psi_\pi - P$ describes the effective driving force for water flow, and L_p is the proportionality constant referred to as hydraulic conductance. Multiplying J_v with the cell surface area (A) and dividing by the cell volume (V), yields the relative rate of cell volume increase: $(1/V) (dV / dt) = L_v (\sigma \Delta \Psi_\pi - P)$, where t is time and L_v the volumetric hydraulic conductance, which is defined as L_p multiplied by (A/V).

(b)The relative rate of cell volume increase is also dependent on the cell wall extensibility (Φ), which is the rate of irreversible cell expansion, when turgor has exceeded the yield threshold (Y), beyond which expansion occurs: $(1/V)(dV / dt) = \Phi (P - Y)$.

Thus the effective relative rate of cell volume increase, as dependent on cell water uptake and rheological properties of the cell wall, can be described as: $(1/V)(dV / dt) = (\Phi L_v / (\Phi + L_v))(\sigma \Delta \Psi_\pi - Y)$. Under conditions of limiting water supply volumetric cell growth is determined by cell water uptake in order to exceed the yield threshold of turgor, whereas under abundant water supply it is dependent on the rheological properties of the cell wall (Jones, 1992). The biophysical processes contributing to volumetric cell growth under unstressed conditions undergo dynamic changes and interactions as follows (Thomas, 1997). The cell wall extensibility can be modified by changes in the synthesis of cell wall material or in the activity of enzymes like xyloglucan endotransglycosylase and peroxidases, or by loosening of fibres due to hormonal changes leading to proton extrusion. Irreversible cell expansion causes firstly a decrease in turgor pressure, and secondly a decrease in osmotic gradient as a result of further water uptake. The loss in osmotic gradient is then counteracted by accumulation of solutes resulting from increased solute import, stronger inhibition in cell growth than import, or decrease in the degree of polymerisation of solutes.

As one of the earliest responses following imposition of soil drought, before any changes in shoot water status occur, shoot cell wall plasticity decreases, due to changes in either cell wall extensibility (Φ) or yield threshold of turgor pressure (Y), and cell expansion in shoots is reduced. In contrast, cell wall properties of root cells are less affected by soil drought (Thomas, 1997). Exposing growing roots and shoots to comparable reductions in water potential has also been shown to result in greater reduction of shoot as compared to root growth. The involvement of ABA in these growth processes was demonstrated by application of an inhibitor of carotenoid biosynthesis, resulting in reduction of endogenous ABA concentrations. The treatment increased shoot and decreased root elongation during growth under low water potential. The differential growth responses of plant shoot and roots are considered to be of adaptive value, as reduction in shoot growth results in a decrease in the transpiring leaf area, while maintenance of root

growth allows continued water uptake (Davies *et al.*, 1993), but they result in decrease overall plant biomass production.

(2) Exposure of plants to soil drying results in reduction of stomatal conductance. This can occur in response to (a) a root dehydration-induced ABA signal transported to the leaves via the xylem stream, (b) an additional leaf-sourced ABA signal generated in response to leaf dehydration under severe drought, (c) a leaf-sourced ABA signal produced in response to high vapour pressure deficit (VPD) or high photosynthetic photon flux density (PPFD) without detectable changes in leaf water status, or (d) direct dehydration effects of high VPD on epidermal and/or guard cells (Wilkinson & Davies, 2002). Stomatal closure is regarded as acclimation response to drought, as it reduces water loss through transpiration (Thomas, 1997), but it concurrently limits photosynthetic CO_2 assimilation.

The definite involvement of an ABA signal from the roots in these two physiological responses was demonstrated in early studies. A positive correlation was found between ABA concentration in the leaf xylem sap and reductions in leaf growth rate and leaf conductance under soil drought. Exogenous application of ABA, resulting in similar xylem ABA concentrations, produced similar responses. Some experiments showed that application of pressure to plant roots in drying soil, to counteract drought-related changes in soil water potential, could maintain shoot water relations comparable to control plants, but did not prevent reductions in leaf conductance. This was interpreted as evidence against a role of shoot dehydration in the induction of this response. In a different study roots of individual apple trees were split, and one part exposed to drying, the other to well-watered substrate. This treatment caused a decrease in leaf growth, although the well-watered section of roots allowed abundant water supply to the plant. Removal of the root section exposed to soil drying resulted in recovery of leaf growth rate to control levels, thus implicating the concurrent removal of a root-sourced inhibitor of leaf growth. (Davies *et al.*, 1993).

The processes involved in root-sourced ABA signalling are now understood in more detail. Under soil drought roots can increase their ABA content by uptake of ABA from the surrounding soil solution, ABA synthesis in the roots, release of ABA from conjugated forms already present in the roots, or arrival of shoot-sourced ABA in the phloem. ABA is transported across the root

towards the xylem with the flux of water in the symplast or apoplast. Its movement across the root endodermis is termed apoplastic by-pass, as ABA filtration at the endodermis increases its local concentration to values leading to "solvent drag". From the apoplast ABA can be loaded directly into the xylem, from the symplast it is loaded *via* specialised xylem parenchyma cells. In the case of the induction of stomatal closure, ABA is eventually transferred from the xylem vessels to the leaf apoplast. It is then carried apoplastically or symplastically by the transpiration stream within the leaf mesophyll, and finally reaches ABA receptors in the plasma membrane of stomatal guard cells in the epidermis. Binding of ABA to the receptors induces a signal transduction cascade, which involves increases in cytoplasmic calcium caused by release from external and internal stores, and results in loss of K^+ and Cl^- ions from stomatal guard cells, leading to stomatal closure (Wilkinson & Davies, 2002).

Although increased ABA concentrations in the xylem sap in response to soil drought have been shown to result in increased ABA concentrations in different compartments of the leaf, the xylem ABA concentrations are not always related to the degree of stomatal closure in response to soil drought. Two types of processes are thought contribute to this variation in sensitivity of leaf conductance to given concentrations of ABA in the xylem sap (Wilkinson & Davies, 2002).

(a) The ABA signal can be modified along its path from source to target, mainly as a result of pH changes induced by soil drying. The underlying principle is that ABA, due to its anionic character, tends to accumulate in alkaline as opposed to acidic cell compartments. Mild soil drought-induced dehydration of root tissue results in generation of an ABA signal in roots. ABA itself can increase root hydraulic conductivity in species with dominantly symplastic radial transport across the root (with strongly suberized roots) by inducing opening of inwardly directed aquaporins. Soil drying reduces the rate of ABA degradation in roots, and leads to alkalisation of the apoplast and acidification of the cytoplasm. The pH changes result in reduced loss of ABA from the roots, compartmentalisation of shoot-sourced ABA in the apoplast and release of ABA, which may have been synthesised or stored in the cytoplasm, from this compartment to the apoplast. Soil drying has also been shown to result in reduced activity of H^+-ATPases in the plasma membranes of xylem parenchyma cells, thereby increasing xylem sap pH. This promotes loading of

ABA from the root and from stores in the stem parenchyma (the latter only in the early phase of soil drying) into the xylem. Soil drought-induced increase in the pH of xylem and apoplastic sap can also lead to reduced sequestration of ABA into the symplast of leaf mesophyll and epidermis, thus allowing more of ABA arriving from the xylem to reach the stomatal guard cells. Under severe soil drought, which results in dehydration of shoot tissues, synthesis of ABA also occurs in leaf cells. Leaf ABA synthesis increases the ABA signal penetrating to the guard cells, and at the same time enhances the effective concentration of xylem-sourced ABA reaching the guard cells by filling up the sympastic reservoir of potential ABA sequestration. In addition, leaf dehydration causes acidification of the cytoplasm, which transfers ABA to its protonated lipophilic form, in which it can cross the plasma membrane and thus leave the symplast (Wilkinson & Davies, 2002).

(b) The sensitivity of target cells to the final ABA signal can be modified, and this is mainly related to other components involved either in the signalling cascade (calcium) or in the response (potassium) or the presence of organic acids (e.g. malate) or other phytohormones (cytokinins).

1.3.3.2.2 ABA signalling at the cellular level

Many genes, which show increased expression in response to drought, can also be induced by exogenous ABA treatment, and ABA has therefore been implicated in regulation of these genes *in vivo* (Ingram & Bartels, 1996). Recognition of ABA by cellular receptors induces a signal transduction pathway, which is thought to involve a protein kinase/phosphatase cascade and calcium. ABA-induced gene expression involves specific *cis*-elements (regulatory elements on the same strand as the coding region) in the promoter region. One such element is the ABA-response element (ABRE) with the consensus sequence RYACGTGGYR (where R is a nucleotide with purine base and Y a nucleotide with pyrimidine base), which contains the G-box core sequence ACGT. Promoter *cis*-elements generally interact with *trans*-acting DNA-binding proteins termed transcription factors, which facilitate transcription of the protein-coding region of the gene. ABRE is bound by basic leucine zipper-type transcription factors. In some genes ABA-induced gene expression requires the presence of specific coupling elements in addition to ABRE, which also interact with sequence-

specific DNA-binding proteins (transcriptional activators). Specificity of gene regulation is related to (a) the sequences flanking the G-box core element and (b) specific coupling elements and transcriptional activators (Bray, 1997).

1.3.4 Stress resistance to damage induced by plant water deficit

1.3.4.1 Compatible solutes

Compatible solutes are defined as low-molecular-weight organic compounds, which are highly soluble in water and non-toxic, *i.e.* neutral with respect to perturbation of cellular functions even at high concentrations, and are accumulated in plants in response to salinity, drought or low temperature stress (Chen & Murata, 2002; Sakamoto & Murata, 2002). They include polyols and sugars such as mannitol, sorbitol, and trehalose; betaines; and amino acids such as proline (Chen & Murata, 2002). Hare *et al.* (1998) grouped compatible solutes occurring in plants in two main categories: (a) polyhydroxylic compounds (saccharides and polyhydric alcohols) and (b) zwitterionic alkylamines (amino acids and quarternary ammonium compounds). Accumulation of compatible solutes is considered as an evolutionary ancient trait, as stress-induced accumulation of compatible solutes occurs in all biological kingdoms. Different compatible solutes are thought to have evolved in different taxa within the plant kingdom, however, there is no clear correlation between distribution of specific compatible solutes and broad taxonomic divisions (Hare *et al.*, 1998).

1.3.4.1.1 Glycinebetaine

1.3.4.1.1.1 Molecular features

Betaines are quarternary ammonium compounds with a fully methylated nitrogen atom and include glycinebetaine, prolinebetaine, β-alaninebetaine, choline-O-sulfate, and 3-dimethylsulfoniopropionate (Chen & Murata, 2002). Glycinebetaine (N,N,N-trimethylglycine) is a dipolar, but (at physiological pH) electrically neutral molecule (Sakamoto & Murata, 2000). It can interact with hydrophilic domains in macromolecules and is extremely soluble in water due to its dipole character, while its hydrocarbon moiety consisting of three methyl

groups allows interaction with hydrophobic macromolecule domains (Sakamoto & Murata, 2002).

1.3.4.1.1.2 Biosynthesis

Pathways of glycinebetaine biosynthesis differ between biological organisms. (a) Many plants, animals, and micoorganisms synthesise glycinebetaine in a two-step oxidation from choline *via* the toxic intermediate betainealdehyde. In *E. coli* and other bacteria, as well as in animals two dehydrogenases, choline dehydrogenase (CDH) and betaine aldehyde dehydrogenase (BADH), catalyse the oxidation reactions. In higher plants ferredoxin-dependent choline monooxygenase (CMO) and NAD^+-dependent BADH, which are both soluble and localised in the chloroplast stroma, catalyse glycinebetaine synthesis. (b) In the soil bacterium *Arthrobacter* glycinebetaine synthesis proceeds as a one-step oxidation of choline, catalysed by a single enzyme, choline oxidase (COD), and this reaction is accompanied by generation of H_2O_2. (c) The halophilic microorganisms *Ectothiorhodospira halochloris* and *Actinopolyspora halophilia* synthesise glycinebetaine from glycine by three-step methylation of the amino residue. These reactions are catalysed by glycine sarcosine methyltransferase (GSMT) and sarcosine dimethylglycine methyltransferase (SDMT), using *S*-adenosylmethionine as methyl group donor (Sakamoto & Murata, 2000, 2002; Chen & Murata, 2002). Choline is synthesised from ethanolamine via successive N-methylation reactions, which are catalysed by *N*-methyltransferases. Routes differ between plant species, and *N*-methylation can occur on the free base, phospho-base or phosphatidyl-base. Ethanolamine is ultimately derived from the amino acid serine (Rhodes & Hanson, 1993).

1.3.4.1.1.3 Stress-induced accumulation

The ability to accumulate glycinebetaine is widespread among higher plants and found in distantly related plant families, but may occur only sporadically among the genera of one family. Species that can accumulate glycinebetaine to (potentially) osmotically significant levels, are referred to as (natural) accumulators. They generally have glycinebetaine concentrations of 5 to 100 μmol g^{-1} dry weight in the absence of stress and 40 to 400 μmol g^{-1} dry weight in response to drought or salinity. So-called non-accumulating species

have glycinebetaine concentrations of < 1 μmol g^{-1} dry weight (Rhodes & Hanson, 1993).

Natural accumulators show an increase in glycinebetaine concentrations in response to long-term exposure to salinity or drought, and accumulation of glycinebetaine has also been observed in response to low temperature stress. The accumulation results from (a) decreased dilution of the glycinebetaine pool due to a stress-induced reduction in leaf growth rate, and (b) increased biosynthesis in leaves. The latter is associated with increased activities of CMO, BADH and key enzymes of choline synthesis. Glycinebetaine degradation is not thought to be involved (Rhodes & Hanson, 1993). Stress-induced accumulation occurs mainly in mature leaves, which is attributed to the localisation of its synthesis in chloroplasts. On stress relief leaf glycinebetaine concentrations decrease, and this is thought to be due to translocation to different plant organs, not to degradation (Hare *et al.*, 1998). Glycinebetaine was also detected in several genera of the Malvaceae. *Gossypium* species were found to have leaf glycinebetaine levels of > 20 μmol g^{-1} fresh weight. Concentrations of glycinebetaine in expressed leaf sap increased with increasing degree of salinity treatment (125 and 200 mol m^{-3} NaCl) as well as in response to cyclical drought. Increases in concentrations were still apparent, when expressed on a dry weight basis, indicating that they were not solely due to leaf dehydration (Gorham, 1996).

1.3.4.1.1.4 Suggested roles

Several hypotheses have been proposed, attributing adaptive roles to stress-induced glycinebetaine accumulation. They are outlined in the following.

(1) Osmotic adjustment is defined as reduction of the cellular osmotic potential due to net accumulation of solutes, which may allow maintenance of a sufficient water potential gradient favouring water uptake as well as maintenance of turgor (Bray, 1997). Concentrations of glycinebetaine accumulated by natural accumulators in response to long-term salinity or drought stress are generally linearly related to leaf osmotic potentials. However, the observed concentrations of compatible solutes are only sufficient to be implicated in osmotic adjustment if it is assumed that they are localised exclusively in the cytoplasm. Glycinebetaine was preferentially localised in the

cytoplasm of leaf cells in halophytic chenopods, and in chloroplasts of salinity-treated spinach plants and *Suaeda*. The term "compatible" in compatible solutes refers to the absence of perturbing effects on cellular functions, and *in vitro* studies have shown that glycinebetaine affects enzyme activity and translation less than equivalent concentrations of inorganic ions. There is evidence that inorganic ions, with potentially perturbing effects on protein native structure, are preferentially localised in the vacuole (Rhodes & Hanson, 1993).

(2) Glycinebetaine has also been implicated in osmoprotection, *i.e.* the stabilisation of structures and functions of macromolecules. Two types of mechanisms of protection have been suggested. (a) Glycinebetaine is preferentially excluded from the surface of proteins, thus maintaining the protein hydration shell and stabilising protein native structure. (b) The hydrophobic moiety of glycinebetaine binds to hydrophobic protein domains, thus making it more accessible to water and preventing denaturation (Sakamoto & Murata, 2002).

I*n vitro* experiments, mostly using very high concentrations of glycinebetaine (1 mol dm^{-3}), have demonstrated protective effects of glycinebetaine under various environmental conditions as follows (reviewed by Sakamoto & Murata, 2002). (a) Glycinebetaine can protect the photosystem II (PSII) complex. It prevents the dissociation of extrinsic polypeptides from the oxygen-evolving PSII complex on exposure to high salt concentrations, thus protecting it from inactivation. In addition, it can also protect the PSII complex from inactivation in response to heat stress. (b) Glycinebetaine can protect enzyme function. It has been shown to protect the enzymes Rubisco and malate dehydrogenase from salt-induced inhibition, as well as several enzymes and protein complexes from heat-induced destabilisation. (c) Glycinebetaine protects membranes against destabilisation in response to heat, freezing and certain chemicals, and from chilling-induced lipid peroxidation.

Studies on transgenic plants allow the direct assessment of protective effects *in vivo*. Genes of enzymes involved in the pathway of glycinebetaine biosynthesis have been cloned and introduced in originally non-accumulating species (Sakamoto & Murata, 2000). In general most

transgenic plants expressing genes for the synthesis of compatible solutes accumulate only low levels of the respective compounds, which are considered insufficient for osmotic adjustment, but still result in increased stress tolerance (Chen & Murata, 2002). Transgenic plants engineered to produce glycinebetaine accumulated it to levels of 50 to 100 mol m^{-3} at most. Protection of the following cellular functions has been demonstrated (as reviewed by Sakamoto & Murata, 2002). (a) Transformation of *Arabidopsis* with COD targeted to the chloroplasts resulted in increased tolerance of the oxygen-evolving PSII complex to salinity as well as light, chilling and freezing stress. Transgenic *Arabidopsis* plants showed less reduction in PSII activity on exposure to strong light as compared to wild-type plants, and inhibition of chloroplastic protein synthesis decreased this difference. In addition, the transgenic plants showed more rapid recovery of PSII activity under weak light. Thus glycinebetaine appeared to promote PSII repair processes. (b) Transgenic plants expressing enzymes of glycinebetaine synthesis also showed protection of enzyme functions compared to wild-type plants. Engineering of the cyanobacterium *Synechococcus* with the *bet* operon from *E.coli* (encoding CDH and BADH) protected Rubisco from salinity-induced inactivation, and this effect was attributed to a role of glycinebetaine in stabilising the native enzyme conformation. (c) Protection of membranes was shown in *Synechococcus* over-expressing COD, which led to accumulation of glycinebetaine in the cytosol. This resulted in a decrease of the phase transition temperature of plasma membrane lipids. Genetic engineering of glycinebetaine synthesis by introducing genes for COD has been criticised, however, for ignoring the generation of H_2O_2, which is associated with the reaction catalysed by this enzyme, as H_2O_2 has been implicated in stress-related signal transduction (Hare *et al.*, 1998).

(3) Several compatible solutes including mannitol, sorbitol, and proline can act as scavengers of active oxygen species (AOS) *in vitro*. In addition engineering of plants to express genes involved in biosynthesis of a number of compatible solutes also resulted in increased tolerance to oxidative stress. However, this is not the case for glycinebetaine (Chen & Murata, 2002).

(4) The environmental stress factors drought, salinity, and low temperature all involve plant water deficit, which is caused by reduced water uptake or decreased hydraulic conductance. Plant water deficit generally induces stomatal closure, which results in a decrease in intercellular CO_2 concentrations, which in turn can lead to increased PSII excitation pressure and increased relative rates of photorespiration. Finally, adjustments in carbon and nitrogen allocation and storage follow (Bohnert & Sheveleva, 1998). It has been suggested that the adaptive value of the accumulation of compatible solutes lies in the utilisation of precursor compounds, which accumulate under stress conditions as a result of such metabolic disruptions/shifts. The up-regulation of particular metabolic pathways involved in the synthesis of compatible solutes may have been selected for in evolution due to the biocompatible features of their products. Glycinebetaine has been suggested to play the following roles in metabolic adjustment (Hare *et al.*, 1998). (a) Increased rates of photorespiration (at least as proportion of net photosynthetic rate) may provide a substantial alternative sink for electrons. However, the photorespiratory reaction catalysed by glycolate oxidase in the peroxisomes generates H_2O_2. If the production of H_2O_2 exceeds the capacity of catalase to destroy it, and if peroxisomal membrane integrity is disrupted by lipid peroxidation, H_2O_2 may leak into other cellular compartments. It has been hypothesised that up-regulation of glycinebetaine synthesis may contribute to suppression of excessive generation of H_2O_2 in photorespiration by removal of serine, the initial precursor of glycinebetaine, from the cycle. In photosynthetic tissues in the light, photorespiration is the major source of serine. (b) Drought and salinity stress also induce enhanced *S*-adenosylmethionine (SAM)-dependent methyl group transfer. The synthesis of the glycinebetaine precursor compound choline from ethanolamine involves such SAM-dependent *N*-methylation reactions. Thus glycinebetaine accumulation may serve in utilisation of excess methyl groups.

1.4 Temperature stress

The temperature dependence of growth follows an optimum curve. It was recognised early that the growth inhibition below the optimum temperature is due to the positive correlation between the rate of chemical reactions and temperature, while growth inhibition above the optimum temperature is caused by injury (Levitt, 1980).

This optimum curve describing the temperature dependence of growth is due to the general nature of enzyme-catalysed reactions. They show an increase in rate with increasing temperature up to a certain temperature optimum, above which further increases in temperature cause a rapid decrease in rate. High temperature results in increased vibration of enzyme molecules, until they finally vibrate so violently that their hydrogen bonds, which maintain tertiary and quarternary structure, break, resulting in enzyme denaturation (Barbor *et al.*, 1997).

Thermal stress is generally defined to occur in a given plant species, when plant temperature exceeds the upper and lower thresholds of the optimal thermal range of that species. The optimal thermal range can be determined on different levels. (a) As the optimal temperature for whole-plant growth and development (Mahan *et al.*, 1997). (b) As the optimal temperature for enzyme function. The conservation of apparent Michaelis-Menten constants (K_m) of substrates and co-factors for homologous enzymes at the physiological temperatures of the organism (as discussed below) seems to justify a definition of the optimal thermal range in terms of enzyme function. Thermal kinetic windows (TKWs) of optimal enzyme function are defined as the temperature range over which the apparent K_m is within 200 % of the minimum apparent K_m of that enzyme, and are used as indicators of the optimal thermal range for metabolism (Burke, 1995). (c) As the optimal temperature for the reappearance of PSII variable or maximum fluorescence following illumination. TKWs and the optimal temperature range for variable fluorescence reappearance were found to be strongly correlated (Burke, 1995). Optimal thermal ranges determined in relation to enzyme function and variable fluorescence recovery were also in general agreement with those related to whole-plant growth (Mahan *et al.*, 1997).

1.5 Long-term temperature adaptation and acclimation in nature

1.5.1 Adjustments in the thermal dependence of photosynthesis

In field studies it was observed that the temperature dependence of photosynthesis in different plant species generally reflects an adaptation to their native habitat. Plants native to predominantly cool environments have superior photosynthetic rates and a photosynthetic temperature optimum at lower temperatures than plants native to habitats with prevailing warm temperatures during the period of active growth, and *vice versa*. Plants from habitats with temperature variations during the growing season generally show shifts in their temperature optimum for photosynthesis, which parallel the seasonal changes in prevailing temperature (Berry & Björkman, 1980).

To investigate variation in acclimation potential, *i.e.* the genetically determined ability to acclimate, plants native to different habitats were exposed to different growth temperature regimes in long-term studies in controlled environments, and leaves developed in the new temperature regime were assessed for their thermal dependence of photosynthesis (Berry & Björkman, 1980). When plants restricted in their natural distribution to cool environments were grown in a hot temperature regime, their optimum temperature for photosynthesis shifted to higher temperatures, but their photosynthetic rate was reduced at all measurement temperatures. In plants from environments with prevailing warm temperatures during the period of active growth, long-term exposure to a cool temperature regime also resulted in a reduction in photosynthetic rate at all measurement temperatures. In contrast, plants from habitats with large temperature variations during the growing season, *e.g.* evergreen desert shrubs, exhibited a shift in temperature optimum of photosynthesis corresponding to the growth temperature regime, and the photosynthetic rates at the respective photosynthetic optimum temperatures were similar, irrespective of the growth temperature. This last group of plants also showed a shift in thermal characteristics of photosynthesis in mature fully-developed leaves corresponding to changes in growth temperature. This type

of adjustment was found to require at least several days to several weeks of growth in the new temperature regime.

Above authors drew the general conclusion that plants from habitats with large temperature variations during the growing season tend to have a greater potential for acclimation to a wide temperature range compared to plants from habitats with stable temperatures during the period of active growth.

The authors attributed the shift in the optimum temperature of photosynthesis to two different processes with separate mechanistic bases. (a) Adjustment to low temperature was thought to be generally associated with increases in the capacity of temperature-limited photosynthetic enzymes. (b) Adjustment to high temperature was thought to involve an increase in heat stability of the photosynthetic apparatus. Adaptation and acclimation to low or high temperature were found to be generally related to reduced photosynthetic performance at the respective other end of the temperature scale.

1.5.2 Adjustments in the thermal dependence of enzyme function

Studies in animals and plants have demonstrated the conservation of the apparent Michaelis-Menten constants (K_m) of substrates and co-factors for homologous enzymes from species native to differing thermal habitats. Minimum apparent K_m values are conserved at the average physiological temperature of the organism, and this is thought to reflect a biochemical adaptation to habitat temperature. Critical thermal maxima, but not minima, appear to be co-adapted with optimal temperatures. The significance of conservation of the apparent K_m is thought to lie in the fact that enzymes operating at a substrate concentration close to the apparent K_m instead of near the potential V_{max} maintain a reserve capacity for increase in rate in response to regulatory signals (Burke, 1995).

Acclimation of enzyme function in response to seasonal variation in temperature has been observed to occur in two ways: (a) Increases in enzyme concentration can compensate for temperature effects on enzyme kinetics and are thought to play a major role in seasonal acclimation. Studies in *Spirodela* for example showed a general increase in K_m values for malate dehydrogenase (MDH) with temperature. Acclimation to higher temperatures resulted in increased MDH levels, which appeared to be a rate-compensating mechanism. (b)

Synthesis of isozymes with altered kinetic characteristics has been shown to occur during cold hardening. For example, glutathione reductase (GR) from cold-acclimated spinach had a lower K_m for oxidised glutathione than GR from non-hardened spinach, when assayed at low temperature, and the reverse was true when assayed at high temperature (Burke, 1995).

In general, enzymes of plant populations native to environments with large temperature changes appear to exhibit less thermal dependence of K_m values (ensuring adequate enzyme function at a large range of temperatures) than enzymes from populations growing in climates with relatively constant temperatures (Burke, 1995).

1.6 Plant responses to chilling temperature

Chilling generally refers to non-freezing temperatures of 0 to 12°C (Allen & Ort, 2001). Based on their response to low temperature plants are classified as follows. Chill-susceptible plants are damaged by temperatures below 12°C. Chill-tolerant but freezing-susceptible plants are able to acclimate to temperatures below 12°C, but unable to survive freezing, and freeze-tolerant plants are able to acquire tolerance to temperatures significantly below freezing by acclimation (Pearce, 1999). Many plant species of tropical or sub-tropical origin are chilling-sensitive, while plants of temperate climatic regions are generally chilling-tolerant (Krause, 1994).

1.6.1 Mechanisms underlying chilling-induced damage

1.6.1.1 Alteration of the relative rates of metabolic reactions

Low temperature generally slows the rate of chemical reactions. Plant metabolic reactions differ in their activation energy, and the higher the activation energy, the higher the extent of inhibition by low temperature. Therefore, reactions with lower levels of activation energy will be enhanced relative to those with higher levels, resulting in changes in relative rates of metabolic reactions. While such alterations tend to be reversible in chilling-resistant plants, they may result in injury in chilling-sensitive plants (Levitt, 1980).

1.6.1.2 Lipid phase transition

Early reviews on chilling injury describe the hypothesis relating chilling injury to solidification of membrane lipids as follows. Low temperatures can induce phase transition of membrane lipids from the liquid crystalline to the solid crystalline or gel state. The phase change to the solid state results in decrease of the lipid surface area by 33%, and thus contraction of the membrane layer. This transition occurs rapidly and is completely reversible. The transition temperature is dependent on degree of saturation, length of the fatty acid chains, and the head group of lipids (Levitt, 1980). As biological membranes consist of a complex mixture of lipids, the phase change does not occur at an exact point, but over a temperature range of several degrees. Within this temperature range liquid and solid phase separate, a process termed lateral phase separation (Berry & Björkman, 1980).

However, it was established later that most cellular lipids in higher plants, due to their high degree of unsaturation, undergo phase transition only below 0°C. Only phosphatidyl glycerol, which occurs mainly in thylakoid membranes, comprises high-melting point molecular species with low degrees of unsaturation of fatty acids, resulting in transition temperatures between 0 and 30°C (Wilson, 1997).

1.6.1.3 Protein denaturation

Low temperature induces weakening of hydrophobic protein bonds (Levitt, 1980). Hydrophobic bonds determine protein tertiary structure and stabilise multimeric protein complexes, and their weakening can affect soluble enzymes by causing (a) reversible changes in conformation (denaturation), and (b) reversible or irreversible dissociation into subunits (Wilson, 1997).

1.6.2 Impairment of photosynthesis by chilling in the dark

1.6.2.1 Disruption of carbohydrate metabolism

Carbohydrate metabolism is thought to be particularly chill-sensitive. Accumulation of triose phosphates as a result of impaired carbohydrate metabolism restricts the return of inorganic phosphate to the chloroplast, and thus

can inhibit photosynthesis by limiting photophosphorylation (Leegood, 1995, Allen & Ort, 2001).

Impairment of carbohydrate metabolism has been shown to occur in two ways in chilling-sensitive plants (Allen & Ort, 2001). (a) Chilling can inhibit night-time mobilisation of starch in leaves. (b) In tomato, chilling under low light (50 μmol photons m^{-2} s^{-1}) disrupts the stable circadian rhythm in sucrose phosphate synthase activity. On return to control temperature the rhythm resumes, but with a phase shift corresponding to the duration of the chilling. The shift could result in incongruity between sucrose synthesis and Calvin cycle activity. However, the extent to which end-product inhibition actually contributes to chilling-induced inhibition of photosynthesis has not been resolved.

1.6.2.2 Inactivation of Rubisco

In chilling-sensitive species chilling in the dark as well as in the light induces a decrease in the activity of Rubisco, which is thought to play a role in the chilling-induced decline in CO_2 assimilation. The loss of activity was originally attributed to damage to the Rubisco protein, but more recently to disruption of Rubisco activation (Allen & Ort, 2001).

1.6.2.3 Stomatal responses

Low temperature in the root zone can induce water deficit in the shoot by increasing resistance to water uptake into the roots. Soil temperature does not fluctuate as rapidly as air temperature, but soil still undergoes diurnal, weather-dependent and seasonal changes in temperature (Fennell & Markhart, 1998). Two types of stomatal response to chilling have been observed in chilling-sensitive species. (a) Chilling of whole potted plants can cause the stomata to remain locked open and unable to respond normally to leaf water deficit. (b) In contrast, chilling of only the shoots, which represents chilling in the field more closely (due to the high specific heat capacity of the soil), induces stomatal closure. The latter response is observed in response to chilling in the dark as well as in the light. Stomatal closure could induce a reduction in the internal leaf CO_2 concentration (c_i) and in turn a decrease in CO_2 assimilation rate. Alternatively, it could be an indirect response to a rise in c_i due to chill-induced inhibition of mesophyll photosynthesis. Based on investigations involving A/c_i-curves, inhibition of CO_2

assimilation in response to chilling is generally thought to be the result of a combination of stomatal and non-stomatal effects (Allen & Ort, 2001).

1.6.3 Impairment of photosynthesis by chilling in the light

Chilling concurrently with incident light affects the same processes as chilling in the dark, but induces additional responses and enhances effects, which occur during chilling in the dark (Allen & Ort, 2001).

1.6.3.1 Chilling-induced photoinhibition

Although chill-tolerant and susceptible species differ in their ability to acclimate to low temperature and photoinhibition, chilling is thought to induce photoinhibition in both types of plants by a similar mechanism, which is outlined in the following according to Krause (1994).

Photoinhibition, manifested as a decrease in optimal quantum yield of photosynthesis, at low temperatures results from complete inactivation of a fraction of PSII reaction centres. These "silent" PSII centres do not contribute to linear electron transport or variable chlorophyll fluorescence. However, they are still thought to trap excitation energy and convert it to heat, thus protecting the photosynthetic apparatus from gross photooxidation in unhardened plants. The following factors contribute to the enhancement of photoinhibtion at chilling temperatures:

(1) Chilling decreases the rate of carbon assimilation. This decrease in utilisation of excitation energy results in over-excitation of PSII.

(2) Chilling reduces the rates of D1 repair processes. As D1 degradation is generally synchronised with the resynthesis and replacement of D1, the rate of degradation is reduced, and damaged D1 is only thought to be marked for degradation during chilling. The actual proteolysis of D1 occurs on return to control temperature. While addition of an inhibitor of chloroplast protein synthesis strongly enhances photoinhibition at higher temperatures, this effect is absent at low temperatures. It is thought that chilling also reduces the rate of inactivation of PSII centres *per se*, and that this compensates for the reduction in rate of repair.

(3) Low temperatures reduce the rate of development, but not the final extent, of energy-dependent quenching (qE) and of zeaxanthin synthesis. Thus chilling

slows down the photo-protective process of the otherwise rapidly inducible dissipation of excess excitation energy as heat.

(4) Chilling may reduce the rates of enzyme reactions involved in scavenging of active oxygen species.

Regarding the D1 repair cycle it has to be noted that in winter rye leaves the addition of linomycin (an inhibitor of chloroplastic protein synthesis) caused severe photoinhibition at 22°C and also enhanced photoinhibition at 4°C, although to a much lesser extent than at 22°C. Thus D1 synthesis and replacement took place also at low temperatures in this species (Streb *et al.*, 1999). The mechanisms contributing to disruption of the D1 repair cycle at chilling temperatures are now better understood and include (a) changes in D1 gene expression and (b) decreased membrane fluidity, which slows down the diffusion of marked D1 to non-appressed thylakoid membrane regions (Allen & Ort, 2001).

1.6.3.2 Disruption of the light activation of stromal bisphosphatases

In the chilling-sensitive species tomato and bean, restriction of the light activation of enzymes of the regenerative phase of the Calvin cycle by the ferredoxin-thioredoxin system occurs very early in the sequence of events in response to chilling (Leegood, 1995). In fact in tomato inhibition of the activation of the stromal bisphosphatases sedoheptulose 1,7-bisphosphatase (SBPase) and chloroplast fructose 1,6-bisphosphatase (FBPase) has been shown to be the primary restriction on photosynthesis during chilling in the light (Allen & Ort, 2001).

1.6.3.3 Disruption of antioxidant regeneration

In the chilling-susceptible maize plant photosynthesis is inhibited irreversibly by temperatures below 10°C. Chilling decreases the rate of CO_2 assimilation, but does not affect either the relationship between the quantum efficiency of PSII or linear electron transport and the quantum efficiency for CO_2 assimilation or the relationship between PSI and PSII efficiencies. In addition chilling does not influence the co-ordination between development of NPQ and changes in photochemical efficiency. Thus chilling does not cause photoinhibition or increased potential for photosynthetic generation of active oxygen species

(AOS). Nevertheless, AOS are thought to be generated periodically as a consequence of endogenous circadian rhythms of metabolism. In C_4 plants regeneration of ascorbate and reduced glutathione occurs only in the mesophyll cells, where reducing power is abundant (whereas bundle sheath thylakoids are deficient in PSII and NADP-ferredoxin reductase). Chilling generally slows down transport processes, and inhibition of transport processes between mesophyll and bundle sheath cells results in restriction of antioxidant regeneration, leading to oxidative damage in bundle sheath cells. In addition, maize has a low capacity to increase synthesis of reduced glutathione (GSH) in response to chilling. Thus chilling sensitivity of maize is attributed to restriction in antioxidative defence (Foyer *et al.*, 2002).

Increased oxidative potential due to restriction in antioxidant regeneration during chilling can have (a) direct effects in causing oxidative damage, and also (b) indirect effects by changing the redox state of the chloroplast stroma, which in turn can affect activation of enzymes of the Calvin cycle by the ferredoxin-thioredoxin system (Allen & Ort, 2001).

1.7 Plant resistance to chilling

1.7.1 Unsaturation of membrane lipids

Early studies demonstrated a general correlation between lipid properties and thermal habitat preference of species, indicating the involvement of lipid properties in adaptation to temperature. Species native to cool environments were found to have membrane lipids with phase separation temperatures near or below 0°C, species native to warm environments had separation temperatures from 5 to 15°C, and evergreen species from temperate regions showed seasonal alterations in lipid properties (Berry & Björkman, 1980). This shift in phase transition temperature to lower values was thought to be achieved by increased contents of unsaturated fatty acids in membrane lipids (Levitt, 1980).

In an early study, Wilson & Crawford (1974a) found no correlation between constitutive chilling resistance in seven chilling-susceptible and six chilling-resistant plant species and the degree of unsaturation of total fatty acids extracted from their leaves. In the same study they showed that chilling-susceptible species like *Gossypium hirsutum*, *Phaseolus vulgaris*, *Saintpaulia grandiflora*, and *Cucumis sativus* could acquire resistance to chilling at 5°C, 95%

RH for 48 hours by pretreatment with 12°C, 95% RH for 4 days. The chilling treatment resulted in necrosis and curling after return to control temperature for 1 to 10 days in 79 to 100% of leaves per plant in unhardened, and 16 to 33% of leaves per plant in hardened plants. However, the hardening treatment did not alter the degree of unsaturation of total leaf fatty acids. In a later study Wilson & Crawford (1974b) distinguished between the individual classes of lipids, which make up the total leaf lipids including (a) polar lipids, which are grouped into phospholipids and glycolipids and (b) neutral lipids, and investigated changes in the fraction of the polar lipids. The chilling-susceptible species *Phaseolus vulgaris* and *Gossypium hirsutum* and the chilling-resistant species *Hordeum vulgare* were exposed to a hardening treatment of 12°C at 95% RH for 4 days and to subsequent severe chilling at 5°C. It was found that actually control plants of chilling-resistant as well as chilling-sensitive plants maintained at 25°C exhibited a decrease in the percentage of linoleic acid and of total unsaturated acids (*i.e.* linoleic and linolenic acid) with time. In the two chilling-sensitive species a difference in plant age of 4 days also resulted in a higher percentage of necrotic leaf area in the older plants in response to chilling at 5°C, 85% RH for 24 hours and return to control temperature for 48 hours. Hardening induced an increase in the percentage of linoleic acid and total unsaturated fatty acids in all phospholipids in *Gossypium hirsutum* and *Phaseolus vulgaris*, whereas *Hordeum vulgare* exhibited only small changes in the percentage of unsaturated fatty acids in the phospholipids. Thus in the chilling-susceptible species hardening resulted in an increase in the degree of unsaturation in phospholipids above the level before hardening, whereas in the chilling-resistant species it only prevented the decrease in degree of unsaturation with plant age. Neither chilling-susceptible nor chilling-resistant plants showed changes in the fatty acid composition of the glycolipids at 25 or 12°C. In the chill-susceptible species *Gossypium hirsutum* and *Phaseolus vulgaris* chilling at 5°C for 24 hours caused a decrease in percentage of linoleic acid in all phospholipids in both hardened and unhardened plants. However, hardened pants maintained higher absolute values of linoleic acid percentage due to the large increase in percentage that had occurred during hardening. In unhardened chill-susceptible plants chilling at 5°C did not cause decreases in the percentage of linolenic acid in any of the phospholipids. In the chilling-resistant *Hordeum vulgare* chilling at 5°C induced an increase in the percentage of linoleic

acid and of total unsaturated acids in some of the phospholipids in hardened as well as unhardened plants. These changes were interpreted as part of the acclimation process to below freezing temperatures. The fatty acid composition of glycolipids was not altered in response to chilling at 5°C in either chilling-susceptible or chilling-resistant species. As phospholipids constitute only 22 % of the total leaf fatty acids, changes in the degree of unsaturation of phospholids had not been detected in the previous study. Thus it was shown that in *Gossypium hirsutum* and *Phaseololus vulgaris* hardening at temperatures slightly above the chilling range, which resulted in increased resistance to subsequent exposure to severe chilling temperatures, was associated with increases in the degree of unsaturation of phospholipids. However, later studies revealed that protection from this type of leaf injury in response to chilling at 5°C, 85% RH by chill-hardening at 12°C, 85% RH was primarily related to prevention of leaf dehydration, and that increased fatty acid unsaturation of membrane lipids was apparently not involved (Wilson, 1978).

It was established later that lipid phase transitions do not occur in bulk lipids, but only in a small fraction of total lipids, namely the phospholipid phosphatidyl glycerol (PG), which is the only cellular lipid with a low enough degree of unsaturation to yield phase transition temperatures above 0°C (Wilson, 1997). A positive correlation was found between constitutive chilling sensitivity and the level of saturated and *trans*-monounsaturated molecular species of PG in many herbaceous and woody plants. In addition it was shown that a downward shift in growth temperature induced an increase in the level of unsaturated fatty acids in membrane glycerolipids in microorganisms as well as plants, thus indicating the involvement of fatty acid unsaturation in acclimation to low temperature. This increase in degree of lipid unsaturation can compensate for the chilling-induced decrease in lipid fluidity (Nishida & Murata, 1996).

Studies involving genetic manipulation of enzymes of fatty acid metabolism have confirmed the role of unsaturation of membrane lipids in low temperature tolerance. In the cyanobacterium *Synechocystis* sp. PCC6803 disruption of the desA gene (encoding Δ12 acyl-lipid desaturase) in the mutant Fad6, which is already defective in expression of the desD gene (encoding a Δ6 desaturase) produced cells referred to as Fad6/desA::Km^r, which only contained saturated and monounsaturated lipids. They had a lower growth rate at 22°C, but

not at 34°C, than the wild-type. Exposure of wild-type cells grown at 34°C to low temperatures of 30, 20, and 10°C resulted in increasing inhibition of oxygen evolution with decreasing temperature, and this inhibition was enhanced in Fad6/desA::Km^r (Nishida & Murata, 1996). When *Anacystis nidulans* R2-SPc, a group 1 strain of cyanobacteria unable to produce polyunsaturated fatty acids, was transformed with the desA gene of *Synechocystis* PCC6803, it was consequently able to desaturate 16:1(9) to 16:2(9,12) at 22°C, and 16:1(9) as well as 18:1(9) to 16:2(9,12) and 18:2(9,12), respectively, at 34°C in all lipid classes. Exposure of cells grown at 34°C to low temperatures from 16 to 0°C for 1 hour at each temperature caused considerably less irreversible inhibition of oxygen evolving activity in transformed than in wild-type cells. Cells grown at 22°C showed generally less inhibition, and differences between wild-type and mutant cells were thus smaller (Wada *et al.*, 1994). In a study on higher plants tobacco was transformed by overexpressing cDNAs for the plastid-localised glycerol-3-phosphate acyltransferase (GPAT) from squash and *Arabidopsis*. GPAT generally shows higher specificity to unsaturated fatty acid-ACP (*i.e.* acyl-carrier protein, a protein cofactor) in chilling-resistant than chilling-sensitive plants. Tobacco has a chilling sensitivity and degree of fatty acid saturation of phosphatidylglycerol (PG) intermediate between those of squash and *Arabidopsis*, and transformation with the cDNA for squash and *Arabidopsis* GPAT resulted in decreased and increased levels of *cis*-unsaturated molecular species of PG in tobacco leaves, respectively. When leaf discs were exposed to chilling at 1°C under strong illumination for 4 hours, tobacco plants transformed with the squash cDNA showed enhanced, and those transformed with the *Arabidopsis* cDNA decreased, inhibition of photosynthesis compared to the wild-type (Nishida & Murata, 1996).

1.7.2 Photoprotection during chilling in the light

1.7.2.1 Adjustment in photosynthetic capacity

Winter cereals like winter rye and wheat acquire freezing tolerance during prolonged exposure to low, non-freezing temperatures and exhibit active growth and development in this acclimation period. During cold acclimation these plants also acquire increased tolerance to photoinhibition. It was shown that this was not due to changes in leaf optical properties, non-photochemical quenching or

capacity of PSII repair. Instead these plants exhibited increases in photosynthetic capacity as a result of increased transcription and translation of the major regulatory enzymes of photosynthetic carbon metabolism; Rubisco, stromal and cytosolic fructose bisphosphatase and sucrose phosphate synthase. These enzymes showed increased activation as well as activity (Huner *et al.*, 1998). Leegood (1995) described two stages of acclimation of enzymes of carbon assimilation and carbohydrate metabolism to low temperature in chilling-tolerant species. Immediately after transfer to low temperature Rubisco and stromal fructose bisphosphatase show increases in capacities, which maintain (but do not increase) photosynthetic capacity in a compensatory fashion. The increase in capacity can result from increased light-activation, increase in substrate concentration or temperature–dependent changes in kinetic parameters. In contrast, sucrose phosphate synthase is not activated in this initial phase, and the rate of sucrose synthesis can thus inhibit photosynthesis by restricted return of inorganic phosphate to the chloroplast. In spite of the inhibition of sucrose synthesis leaf sucrose content increases due to decreased export. In the longer term (over several days), photosynthetic capacity increases. Enzymes of the Calvin cycle show increases in capacity as a result of upregulated gene expression. Over several days also sucrose phosphate synthase shows increases in enzyme levels, but not activation state, and partitioning of carbon into sucrose increases. This alteration in the relationship between photosynthesis and carbohydrate synthesis is viewed as part of the acclimation process to freezing temperatures.

In spinach, hardening was shown to result in changes in the kinetic properties of Rubisco. The enzyme from hardened plants had a decreased activation energy of ribulose-1,5-bisphosphate (RuBP) carboxylation, and the Michaelis-Menten constant (K_m) for RuBP was decreased at 5°C and increased at 20°C as compared to the enzyme from unhardened plants. A similar response to hardening was found for Rubisco from winter rye, regarding the K_m for CO_2 (Krause, 1994).

Chilling-sensitive species, however, tend to show a disruption of the light activation of Rubisco and the stromal bisphosphatases by the ferredoxin-thioredoxin system very early in the sequence of events following chilling. This has been shown for tomato and bean. The resulting inability to adjust

photosynthetic capacity in response to low temperature is thought to be a major factor responsible for chilling-sensitivity (Leegood, 1995).

1.7.2.2 Increase in alternative electron sinks

Comparison of the relative rates of PSII electron transport and of CO_2 assimilation indicates an increase in alternative electron sinks in some studies on chilling-susceptible species. Photorespiration is generally not thought to play a role in photoprotection in chilling-sensitive species, as these species often show inhibition of Rubisco carboxylase and thus by necessity also oxygenase activity. Only if stomatal closure, resulting in decreased CO_2 concentration in the leaf intercellular air space, is the primary cause of chilling-induced inhibition of photosynthesis, could photorespiration contribute to photoprotection. In addition chilling does not appear to induce increased cyclic electron transport around PSI. The Mehler-ascorbate peroxidase reaction, also termed the water-water cycle, in which electrons generated in PSII from the oxidation of H_2O are finally used to reduce O_2 to H_2O at PSI, could possibly contribute to photoprotection at low temperatures. However, the extent of this contribution is currently unclear (Allen & Ort, 2001).

1.7.2.3 Increase in energy dissipation as heat

In the chilling-tolerant spinach plant cold acclimation resulted in an increase in the rate, but not the capacity, of development of ΔpH-dependent high energy state quenching (qE) in isolated chloroplasts. Cold acclimation was also associated with increases in the total xanthophyll content in spinach, and with increases in violaxanthin and zeaxanthin contents in winter and spring wheat (Krause, 1994).

In evergreen plants, which cease to grow in winter, cold acclimation to freezing temperatures is not associated with increased tolerance to photoinhibition (Krause, 1994). In the short-term they develop ΔpH-dependent high energy state quenching (qE) (within minutes of exposure to excess light), accompanied by reversible conversion of violaxanthin to zeaxanthin. However, in the long-term (over days or months of extensive periods of low temperature) they show a sustained reduction in photosynthetic efficiency and capacity, and sustained, only slowly reversible thermal dissipation of energy, termed photoinhibitory quenching

(qI). This is accompanied by an increase in pool size of total xanthophylls and content of zeaxanthin (Huner *et al.*, 1998).

Reversible down-regulation of PSII efficiency associated with ΔpH-dependent high energy state quenching has also been observed in chilling-susceptible plants like maize and tomato (Allen & Ort, 2001).

1.7.2.4 Increase in the capacity to scavenge active oxygen species

In spinach leaves cold acclimation has been shown to result in increased activities of superoxide dismutase, ascorbate peroxidase, monodehydroascorbate reductase, and glutathione reductase and increased levels of the soluble antioxidants ascorbate and glutathione (Krause, 1994). In winter rye cold hardening, which protected PSII from photoinhibition and catalase from photo-inactivation at low temperature, was also associated with increases in the levels of α-tocopherol, reduced and oxidised ascorbate and glutathione and in the activities of superoxide dismutase, glutathione reductase, and guaiacol peroxidase. However, the loss of the acquired tolerance of PSII and catalase to chilling-induced photoinactivation during dehardening was not paralleled by concomitant decreases in ascorbate, glutathione, α-tocopherol, or superoxide dismutase. It was thus concluded that this acquired tolerance was not related to antioxidant compounds (Streb *et al.*, 1999). When unhardened leaves of winter rye were incubated with precursor substrates of ascorbate and glutathione, they exhibited increased levels of these antioxidants as well as increased activities of superoxide dismutase, catalase and glutathione reductase. Although this treatment resulted in decreased photo-oxidation of catalase and chlorophyll in response to treatment with paraquat (which mediates superoxide formation), it did not alleviate either paraquat-induced inactivation of PSII, or low temperature-induced photo-inactivation of PSII and catalase. Tolerance to low temperature-induced photo-damage appeared to be rather determined by adjustment of carbon assimilation, as treatment with glycoaldehyde (which interferes with carbon assimilation) enhanced low temperature-induced photoinactivation of PSII and catalase in cold-acclimated plants (Streb & Feierabend, 1999).

1.7.2.5 Increase in capacity for repair of damaged PSII reaction centres

In the study involving transformation of tobacco by overexpressing cDNAs for the plastid-localised glycerol-3-phosphate acyltransferase (GPAT) from squash and *Arabidopsis* (see 1.7.1) plant responses to chilling at 1°C under strong illumination for 4 hours and subsequent recovery at 17°C under weak illumination for 2 hours were investigated in detail. The process of photoinactivation at low temperatures occurred at the same rate in plants transformed with the cDNA for squash GPAT and the control plasmid, when an inhibitor of protein synthesis was added (blocking D1 synthesis). Recovery took place, if photoinhibition did not exceed a certain level, and plants transformed with the cDNA for squash GPAT (resulting in reduced levels of *cis*-unsaturated molecular species of phosphatidyl glycerol) showed much slower recovery than plants transformed with the control plasmid. Similar responses were found in strains of the cyanobacterium *Synechocystis* sp. PCC6803 differing in degree of unsaturation of their membrane lipids. It was therefore suggested that unsaturation of membrane lipids plays a role in the recovery process from photoinhibition, probably by accelerating either processing or assembly of the precursor D1 protein with the PSII complex (Nishida & Murata, 1996).

Cyanobacteria are thought to acquire elevated tolerance to photoinhibition by increasing their capacity to repair damaged PSII centres during acclimation. Exposure to low temperature, which induces increased tolerance to chilling and photoinhibition at low temperature, is also associated with increased fatty acid unsaturation of thylakoid lipids, which is thought to accelerate D1 replacement (Huner *et al.*, 1998).

1.7.3 Prevention of chilling-induced dehydration

Wilson & Crawford (1974a,b) found in two studies summarised above (see 1.7.1) that *Phaseolus vulgaris* and *Gossypium hirsutum* could acquire tolerance to chilling at 5°C, 95% RH for 48 hours by pretreament with 12°C, 95% RH for 4 days. The chill-hardening treatment was associated with an increased percentage of linoleic acid and total unsaturated fatty acids in phospholipids. In following studies it was shown that the same type of chilling tolerance could be induced in *Phaseolus vulgaris* by withholding water at control temperatures for 4

days. This drought-hardening did not result in an increased degree of fatty acid unsaturation of phopholipids. In contrast, chill-hardening at 12°C at 100% RH did not induce chilling tolerance. In addition, *Phaseolus vulgaris* leaves could also be protected from chilling injury for 9 days by maintenance of plants at 100% RH (as a result of enclosing them in polythene bags) during exposure to 5°C. It was shown that chilling at 5°C, 85% RH results in decreased rates of root water absorption and rapid opening of the stomata in this species. Chill-hardening at 12°C, 85% RH also induced decreased root water absorption and opening of stomata, resulting in temporary leaf wilting, but to a lesser extent than at 5°C, and conditioned stomata to close on exposure to 5°C. In comparison, drought-hardening caused stomatal closure, and stomata remained closed on exposure to 5°C. It was concluded that protection of leaves from chilling injury was primarily related to prevention of leaf dehydration. However, the factors causing the more slowly-induced leaf injury occurring after 9 days in plants maintained in 100% RH during chilling at 5°C were not resolved (Wilson, 1978).

A mechanism for this type of sensitisation of stomata to close in response to low temperature by chill-hardening has been proposed. At chilling temperatures stomata show reduced sensitivity to ABA, but at the same time guard cells appear to be more sensitive to apoplastic calcium. Pretreatment with chilling temperatures is thought to replenish internal cellular stores of calcium in guard cells, thus conditioning stomata for closure in response to a subsequent ABA signal (Wilkinson & Davies, 2002).

It has also been shown that root hydraulic conductivity can acclimate rapidly to low temperature in *Spinacia oleracea* (Fennell & Markhart, 1998). When detached root systems of plants that had been grown at 20°C were exposed to chilling at 5°C, hydraulic flux through the root system decreased initially, but started to recover within 1 to 3 hours after reaching 5°C. It continued to rise for another 8 hours, and then remained constant at a level of 50 to 60 % of the values at 20°C. Root hydraulic conductivity (which is determined under applied pressure, thus eliminating effects of temperature on osmotic potential gradients) increased during exposure to 5°C for 7 days, when it reached levels of about 50% of the values at 20°C. In addition, when just the roots of intact plants were chilled at 5°C, stomatal aperture decreased initially, but then increased gradually over the next 6 hours, and stomatal response continued to recover over the next 2 days. These

changes thus paralleled responses of hydraulic flux and hydraulic conductivity in detached root systems. The possible mechanisms implicated in this rapid acclimation were: (a) changes in the composition of the rate-limiting membranes (degree of fatty acid unsaturation, membrane lipid to protein ratio, sterol content, or fatty acid chain length) and (b) changes related to aquaporins (water channel proteins).

1.7.4 Cold-regulated genes

Cold-regulated (COR) genes, also referred to as low-temperature-induced (LTI), cold acclimation-specific (CAS), cold-induced (Kin) or responsive to desiccation (RD), are a set of genes, which are strongly (50 to 100 fold) induced by low temperature (Xin & Browse, 2000). Most of these genes are induced during cold acclimation, a process in which temperate plants increase their tolerance to freezing temperatures in response to a period of low non-freezing temperatures. They are, therefore, thought to play a role in freezing tolerance. These genes, however, have not been implicated in chilling tolerance to date (Foyer *et al.*, 2002).

1.8 High temperature induced injury

1.8.1 Mechanisms underlying heat-induced damage

1.8.1.1 Alteration of the relative rates of metabolic reactions

High temperature generally increases rates of chemical reactions. As plant metabolic reactions differ in their activation energies, high temperature can result in metabolic imbalance (Levitt, 1980).

1.8.1.2 Protein denaturation

Protein denaturation is generally defined as unfolding of the molecule resulting in loss of activity. Studies with pure proteins have allowed the distinction of two phases. (a) Denaturation is reversible and due to disruption of secondary and tertiary protein structure by breaking of hydrogen bonds (their strength remains constant, while that of hydrophobic bonds increases with temperature). (b) Aggregation follows denaturation, is thermodynamically irreversible, and is due to the oxidation of SH-groups resulting in the formation of intermolecular disulfid-bonds. In general, an enzyme can show decreased activity

at a lower temperature than loss of conformation, the latter being determined by its stability. Proteins differ in their heat-sensitivity, and specific, particularly sensitive proteins may be responsible for heat-induced injury (Levitt, 1980).

1.8.1.3 Increased fluidity of membrane lipids

Although high temperatures do not induce phase transition of membrane lipids, which occurs at temperatures below the temperature optimum for growth, they generally increase lipid mobility. This can result in conformational changes of membrane-bound proteins and, beyond a certain threshold temperature, in disruption of the lipid bilayer and lipid vesicle formation, leading to membrane destruction and cell death (Levitt, 1980).

1.8.2 Inhibition of photosynthesis

1.8.2.1 Increase in dark respiration and photorespiration

The temperature optimum for respiration is considerably higher than that for photosynthesis. The temperature compensation point is defined as the temperature at which photosynthesis and respiration are equally rapid. Beyond this point respiration rate continues to rise, while photosynthetic rate continually decreases, resulting in an exponential rise in the rate of starvation (Levitt, 1980).

In C_3 plants rate and optimum temperature of light-saturated photosynthesis are increased at rate-saturating CO_2 and at decreased O_2 concentrations compared to normal atmospheric concentrations of these gases (Berry & Björkman, 1980). This is attributed to the rise in rate of photorespiratory CO_2 release with increasing temperature. At ambient CO_2 and O_2 concentrations the enzyme Rubisco catalyses both, carboxylation and oxygenation, of RuBP, and these two reactions are competitive. High temperature promotes the oxygenation reaction as follows. (a) About one-third of the change is attributed to the more rapid decrease in solubility of CO_2 than that of O_2 in water with increasing temperature. (b) About two thirds are attributed to changes in the kinetic parameters and specificity factor of Rubisco with increasing temperature in the range between 5 and 40°C, due to the higher activation energy of the oxygenation as compared to the carboxylation reaction. In normal air the ratio of the rates of photorespiration to photosynthesis rises from about 0.1 at 10°C to about 0.3 at 40°C. Additional water stress, which causes a decrease in intercellular CO_2

concentration, can result in ratios of 0.6. Increased photorespiration also results in increased release of NH_4^+, which can cause loss of nitrogen, if it is not efficiently re-fixed (Leegood, 1995).

1.8.2.2 Irreversible damage to the photosynthetic apparatus in the thylakoid membrane

Temperatures just beyond the threshold for irreversible damage cause specific lesions in the photosynthetic apparatus. The heat stability of cell membranes and of dark respiration exceeds that of photosynthesis and the heat resistance of the chloroplast envelope is greater than that of the thylakoid membrane. It was therefore concluded that the heat-induced inhibition of whole leaf photosynthesis is caused by a direct effect of heat on chloroplast function. *In vivo* and *in vitro* studies identified PSII activity and noncyclic photophosphorylation as the components most closely related to inactivation of chloroplast function, while PSI activity was more heat-resistant. The heat inactivation of noncyclic photophosphorylation was interpreted as a consequence of inactivation of PSII electron transport and uncoupling of photophosphorylation from electron transport (Berry & Björkman, 1980).

The high sensitivity of PSII to heat has been attributed to the following components. (a) The water-splitting apparatus of PSII is considered particularly heat sensitive, heat injury being possibly caused by the loss of manganese. (b) Changes in the association between the light-harvesting pigments and the reaction centre complexes are thought to occur at a lower temperature than loss in electron transport capacity, and are thus considered especially heat-sensitive. The light-harvesting complex is thought to become physically dissociated from the PSII core due to decreasing strength of hydrophilic and increasing strength of hydrophobic interactions with increasing temperature. The threshold temperature for this dissociation could be related to the fluidity of thylakoid membrane lipids (Berry & Björkman, 1980).

It is now generally accepted that components of the photosynthetic system in the thylakoid membranes, probably PSII, are the primary site of thermal damage resulting in inactivation of photosynthesis (Havaux & Tardy, 1996).

1.8.2.3 Inactivation of soluble enzymes of photosynthetic carbon assimilation

Temperatures resulting in irreversible inhibition of photosynthesis are associated with inactivation of soluble enzymes of photosynthetic carbon metabolism. In *Atriplex sabulosa*, *Tidestromia oblongifolia* and *Nerium oleander* (the latter two growing in Death Valley, California) only three of the fourteen enzymes isolated from plants treated at a series of high temperatures had heat stabilities consistently correlated to those of photosynthesis; NADP glyceradehyde-3P dehydrogenase, ribulose-5P kinase, and NADP malate dehydrogenase. As these enzymes are light-activated, their inactivation was considered as a likely consequence of inhibition of PSII activity. The heat stability of Rubisco isolated from above three species, as well as spinach, *Hedera helix*, and various grasses by far exceeded that for whole leaf photosynthesis (Berry & Björkman, 1980).

However, other studies have shown that CO_2 assimilation activity is reversibly inhibited by moderately high temperatures. Rubisco activation was identified as a primary site of inhibition. Heat-induced inhibition of Rubisco activation was attributed to the heat-lability of activase, which denatured at temperatures above 40°C. Changes in the light-saturated initial activity of Rubisco in response to heat stress have been shown to be due to changes in the activity of Rubisco activase. While the activity of fully activated Rubisco was demonstrated to be very heat-stable, the activity of isolated activase was shown to be extremely heat-sensitive. In studies on cotton and wheat the decrease in CO_2 uptake rate in response to increasing temperature was strongly correlated to a concomitant decline in Rubisco activation and concomitant increase in qN, while F_v/F_m started to decrease at higher temperatures. It was concluded that Calvin cycle activity is more heat-sensitive than PSII activity in these two species (Law & Crafts-Brandner, 1999).

1.8.3 Damage to cellular membrane systems

Early visual observations by light and electron microscopy identified nuclear, chloroplast, mitochondrial, ER, golgi and plasma membranes as the primary site of injury in response to heat shock. Injury to the plasma membrane is manifested as ion leakage from heat-shocked cells. The threshold temperature for

the occurrence of ion leakage considerably exceeds that of inactivation of photosynthesis (by up to 11°C), and the chloroplast envelope is less heat-sensitive than the photosynthetic systems in the thylakoid membranes. Cellular membrane systems were generally ranked according to increasing heat stability as follows: chloroplast < mitochondria < plasma membrane (Levitt, 1980).

1.9 Resistance to high temperature induced injury

1.9.1 Heat avoidance

1.9.1.1 Transpirational cooling

Leaf temperature may exceed air temperature due to the strong absorption of radiant energy by leaves. Most plants maintain their leaf temperature below air temperature by maintaining high stomatal conductance in response to high temperature. The process of evaporation of water from leaves driven by transpiration absorbs heat and can lower the leaf temperature. Conservative estimates indicate that leaf temperature can be lowered by 2 to 5°C, but under extreme conditions of temperature and vapour pressure deficit even greater cooling effects are possible. The importance of transpirational cooling has been demonstrated by restricting plant water supply and by the use of antitranspirants (Levitt, 1980).

Factors determining plant capacity for transpirational cooling relate to the efficiency of the root system to extract water from the substrate and include: (a) factors which determine the substrate volume explored by the root system, such as root length, degree of branching, rooting depth, and root elongation rate, and (b) radial and axial hydraulic conductivity of the root system (Mahan *et al.*, 1997).

1.9.1.2 Decrease in absorption of radiant energy

A decrease in leaf absorption of radiant energy can be achieved by (a) increased reflectance due to increase in leaf pubescence, refractive index discontinuities (dependent on cell size, shape, and number) (Levitt, 1980), and wax depositions (Viswanathan & Khanna-Chopra, 1996) (b) increased transmissivity through changes in leaf orientation, and (c) protective external layers of high heat tolerance, which may be cells with high water content (filtering out infrared radiation) or a thick cuticle (Levitt, 1980).

1.9.1.3 Increase in conductive heat loss

In general small leaves, leaflets of compound leaves or dissected leaves have thinner boundary layers, which are more conducive to heat transfer to the environment than bigger undivided leaves (Mahan *et al.*, 1997).

1.9.2 Heat tolerance

1.9.2.1 Prevention of the heat-induced increase in respiration

Plants native to habitats with large fluctuations in temperature, *e.g.* deserts, show seasonal adjustments of dark respiration. Acclimation to high temperature involves reduction in respiration rates and decreased thermal dependence of respiration (Levitt, 1980).

Prevention of the increase in photorespiration in response to high temperature has been achieved on an evolutionary scale by the development of C_4 metabolism. However, acclimation of C_3 plants to high temperature does not appear to involve modifications of photorespiratory mechanisms (Berry & Björkman, 1980).

1.9.2.2 Stabilisation of cellular proteins by heat shock proteins

Several classes of heat shock proteins (HSPs) are distinguished based on their approximate molecular weights in kDa: HSP110, HSP90, HSP70, HSP60, and low molecular weight (LMW) HSPs. Ubiquitin is also referred to as a HSP. Exposure of seedlings to temperatures 5 or more degrees above their optimal growing temperatures results in transcription and translation of a small set of specific heat shock proteins, while normal protein synthesis is repressed. This molecular response to high temperature is highly conserved among biological organisms. Among eukaryotes the major HSPs are highly homologous, and in some cases they also show sequence similarity to prokaryotic HSPs. The molecular mechanism of heat-induced gene induction is also similar among eukaryotes. Features unique to plants are the occurrence of HSP70, HSP60, and LMW HSPs in chloroplasts and the abundance and diversity of LMW HSPs (Vierling, 1991).

The production of HSPs is thought to be involved in the phenomenon of acquired thermotolerance, *i.e.* the short-term development of tolerance to

otherwise lethal high temperature treatments by pretreatment with non-lethal high temperatures. It was shown that in etiolated soybean seedlings the development of thermotolerance is correlated with the rate of synthesis of LMW HSPs and that the acquired thermotolerance parallels LMW HSP accumulation (Vierling, 1991). Other studies with wheat, sorghum, pearl millet, and maize confirmed a correlation between development of thermotolerance of seedling growth and the kinetics of HSP synthesis. In addition, correlations were found between intraspecific differences in thermotolerance of cultivars of wheat and maize and corresponding differences in quantity or quality of accumulated HSPs (Viswanathan & Khanna-Chopra, 1996). While moderate pretreatment temperatures result in transcription and translation of HSPs and parallel development of thermotolerance, more severe high temperatures cause HSP gene induction, but the development of thermotolerance requires a recovery period, during which actual HSP synthesis takes place. The heat-shock response is a transient phenomenon. During prolonged high temperature treatment of soybean at 40°C active HSP gene transcription occurs only in the first 2 hours, and HSP mRNAs persist for about 9 hours, but decline rapidly on return to control temperature. HSPs may, however, persist for hours to days after return to control temperature, possibly aiding repair processes or ensuring survival of future heat shocks. It is thought that HSPs allow biochemical and structural adjustments in the plant to proceed, but are not involved in the complex metabolic readjustments during long-term heat hardening (Pollock *et al.*, 1993).

HSP expression also occurs in natural environments. Soybeans showed LMW HSP expression on hot days. In field-grown cotton increased leaf temperatures in response to recurring water stress were correlated with accumulation of HSPs. Gradual changes in temperature typical for natural diurnal temperature courses have also been mimicked in the laboratory in a study with pea seedlings. Plants showed accumulation of LMW HSPs that was directly proportional to the midday leaf temperature. Chloroplast and cytoplasmic LMW HSPs had half-lives of 52 and 37 hours following heat stress relief. In general, HSP production is thought to be important in plant structures that are not efficiently cooled by transpiration, like reproductive structures with low stomatal densities and emerging seedlings, which have not developed a full transpiration stream (Vierling, 1991).

HSPs are encoded by multi-gene superfamilies, with different members occurring in specific subcellular compartments, including cytoplasm, endoplasmatic reticulum, chloroplast, and mitochondria. HSP70 and HSP60 homologs are present constitutively and at elevated temperatures. HSP family members that are expressed in the absence of heat stress, are referred to as HSP cognates (HSCs) (Vierling, 1991).

In eukaryotes HSP100/Clp proteins, HSP90, HSP70, HSP60/chaperonins, calnexin, nucleoplasmin, LMW HSPs and co-chaperones HSP40/DnaJ, GrpE, and Cpn60 are classified as molecular chaperones. Molecular chaperones are defined as proteins that bind to and stabilise substrate proteins with otherwise unstable conformation, and, by controlled binding and release, facilitate their correct fate, regarding either folding, oligomeric assembly, transport to specific subcellular compartments or disposal by degradation, *in vivo* (Sun *et al.*, 2002). Ubiquitin is involved in protein turnover by conjugating to other proteins and marking them for degradation (Vierling, 1991).

LMW HSPs, also referred to as small heat shock proteins (sHSPs), have molecular weights of 15 to 42 kDa, and are the most dominant proteins synthesised in plants in response to high temperature. They are divided into six classes, three of which occur in cytoplasm or nucleus, and three in plastids, endoplasmatic reticulum, and mitochondria. Distinguishing structural features of sHSPs include the conserved 90-amino acid carboxyl-terminal called α-crystallin domain (ACD) with the consensus I and II domains and the divergent amino-terminal domain, which in plastid-localised sHSPs contains a methione-rich consensus III domain, which is thought to form an amphipatic α-helix tertiary structure. In the absence of stress, sHSPs are not present in vegetative tissues, and are only produced during embryogenesis, germination, pollen development, and fruit maturation. sHSPs are generally produced in response to sub-lethal high temperatures, some are induced by osmotic stress, and some by oxidative stress. Under non-stress conditions sHSPs form homo-oligomeric structures, with dimers as building blocks. High temperature causes conformational changes in sHSPs resulting in dissociation of the oligomeric complex, and sHSP dimers are then thought to reassemble with denatured substrate proteins into larger complexes. By binding selectively non-native substrate proteins, sHSPs are thought to prevent their aggregation and create a reservoir of refoldable proteins in an ATP-

independent mechanism, which they then pass to HSP70 and co-chaperones for ATP-dependent refolding (Sun *et al.*, 2002).

1.9.2.3 Protection of photosynthetic electron transport

1.9.2.3.1 Chloroplastic heat shock proteins

In a biochemical assay Heckathorn *et al.* (1998) provided the first direct evidence that chloroplast-localised HSPs are involved in photosynthetic thermotolerance. They showed that chloroplasts isolated from tomato plants pretreated at 43°C for 6 hours exhibited less reduction in PSII electron transport compared to chloroplasts from non-pretreated control plants, when assayed at 47°C. This acclimation of heat-pretreated plants could be prevented by addition of antibodies specific to sHSPs. In addition, a sHSP from *Chenopodium album* localised in the thylakoid lumen was observed to specifically interact with and protect the PSII oxygen evolving complex from heat-induced damage (Sun *et al.*, 2002). The fact that some sHSPs can be induced by oxidative stress, and that overexpression of a sHSP in *Arabidopsis* resulted in increased tolerance to a combination of heat and high light stress (potential conditions for photoinhibition) suggest that sHSPs might play a role in oxidative stress tolerance (Sun *et al.*, 2002).

1.9.2.3.2 Xanthophylls

Havaux (1993) showed that pretreatment of *Solanum tuberosum* leaf discs with 35°C for 2 hours resulted in increased PSII thermotolerance. The temperature at which chlorophyll fluorescence starts to rise, the temperature of peak fluorescence, and the temperature at which the maximal quantum yield for PSII photochemistry (F_v/F_m) is reduced, were shifted from control values of 38, 45, and 38°C to values of 43, 52, and 43°C, respectively. This type of PSII thermotolerance could be fully acquired within one hour, and, on return to control temperature, was lost at a comparably slower rate than its acquisition.

The acclimation phenomenon was neither attributed to de novo protein synthesis (due to lack of effect of protein synthesis inhibitors), nor changes in the lipid composition of thylakoid membranes (which would require more time). The pretreatment at 35°C for 2 hours in darkness was shown to cause specific changes in the effectiveness of blue versus red/orange light on the relative quantum yield

of oxygen evolution and PSII photochemistry, in leaf absorption spectra, xanthophyll conversion, and non-photochemical quenching of chlorophyll fluorescence. These changes were interpreted as a result of reduced excitation energy transfer from accessory carotenoid pigments to antenna chlorophylls, related to a desorption of violaxanthin from LHCII and migration towards the membrane-anchored violaxanthin de-epoxidase (which precedes photoconversion of violaxanthin to zeaxanthin). Violaxanthin desorption from LHCII could allow a conformational change resulting in increased heat-stability or alternatively cause a change in lipid properties of the thylakoid membrane, resulting in membrane reinforcement, decreased lipid fluidity, and reduced membrane permeability (Havaux & Tardy, 1996). The authors also observed that heat-pretreated leaves showed a stronger reduction in the quantum yield of photosynthetic electron transport at chilling temperature compared with control leaves, and that brief exposure to strong white light could induce PSII thermostability qualitatively similar to that induced by heat pretreatment.

1.9.2.4 Protection of Rubisco activation

Law & Crafts-Brandner (1999) found that in cotton and wheat CO_2 assimilation, Rubisco activation, and photochemical efficiency of PSII are reduced less and nonphotochemical quenching of chlorophyll fluorescence is increased less, when the leaf temperature of an individual leaf is increased gradually by 2.5°C every hour than when different leaves are each exposed to different points in the temperature range tested, but with temperatures being imposed rapidly (at 1°C per minute). CO_2 assimilation and Rubisco activation were strongly correlated during rapid and gradual heat stress. Gradually imposed heat stress also induced acclimation of photochemical efficiency of PSII, but for both rapid and gradual heat treatment Calvin cycle activity was more sensitive to high temperature than photochemical efficiency of PSII.

Interestingly, treatment of cotton plants at 41/37°C day/night temperature also resulted in *de novo* synthesis of HSPs after one hour, and after three hours a new form of Rubisco activase with an apparent molecular weight of 46-kDa was synthesised (Law *et al.*, 2001). Synthesis of this heat-induced new activase form still occurred after 24 and 48 hours of heat treatment, but declined rapidly on return to control conditions. It accumulated to about 5% of the total activase pool

after 24 hours at the high temperature treatment. The heat-induced form of activase is thought to have a role in the acclimation of photosynthesis to heat stress, possibly by preventing dissociation of the two subunits that make up the mature constitutive activase protein.

1.9.2.5 Saturation of membrane lipids

A number of studies have demonstrated a correlation between high temperature tolerance and degree of saturation of membrane fatty acids. In the thermophilic eukaryotic alga *Cyanidium caldarium* growth at 55°C induced increased heat tolerance and a higher degree of saturation of membrane fatty acids compared to growth at 20°C (Levitt, 1980). In *Atriplex lentiformis* and *Nerium oleander* high temperature acclimation induces a shift of the temperature at which a steep rise in the level of F_o fluorescence occurs (which indicates irreversible heat inactivation of PSII activity) to higher values. In both species, photosynthetic acclimation also results in reduced fluidity of the polar lipids of the thylakoid membrane, associated with an increase in saturation of fatty acids of this lipid fraction. In addition, the rise in F_o fluorescence was shown to always occur at the same degree of lipid fluidity, regardless of species and growth temperature (Berry & Björkman, 1980).

However, the increase in PSII thermotolerance observed in the above study could also be due to other changes induced by the acclimation temperature. More recent studies involving genetic manipulation of enzymes of fatty acid metabolism have questioned the role of saturation of membrane lipids in high temperature tolerance. In the cyanobacterium *Synechocystis* sp. PCC6803 disruption of the desA gene (encoding Δ12 acyl-lipid desaturase) in the mutant Fad6, which is already defective in expression of the desD gene (encoding a Δ6 desaturase), resulted in reduced growth at 22°C, but not at 34°C. However, decreased unsaturation of membrane lipids was not associated with changes in high temperature tolerance of photosynthesis (Nishida & Murata, 1996). When *Anacystis nidulans* R2-SPc, which is a group 1 strain of cyanobacteria and unable to produce polyunsaturated fatty acids, was transformed with the desA gene of *Synechocystis* PCC6803, it was consequently able to desaturate 16:1(9) to 16:2(9,12) at 22°C, and 16:1(9) as well as 18:1(9) to 16:2(9,12) and 18:2(9,12), respectively, at 34°C in all lipid classes. Wild-type and transformant (with higher

lipid unsaturation) showed identical profiles of high temperature inactivation of oxygen evolution, and for both growth at 34°C resulted in inactivation of oxygen evolution at higher temperatures than growth at 22°C (Wada *et al.*, 1994). In a study on higher plants tobacco was transformed by overexpressing cDNAs for the plastid-localised glycerol-3-phosphate acyltransferase (GPAT) from squash and *Arabidopsis*. GPAT generally shows higher specificity to unsaturated fatty acid-ACP (*i.e.* acyl-carrier protein, a protein cofactor) in chilling-resistant than chilling-sensitive plants. Tobacco has a chilling sensitivity and degree of fatty acid saturation of phosphatidylglycerol (PG) intermediate between those of squash and *Arabidopsis*. Transformation with the cDNA for squash and *Arabidopsis* GPAT resulted in decreased and increased levels of cis-unsaturated molecular species of PG in tobacco leaves, respectively. Transformation with the squash cDNA did not, however, influence high temperature inactivation of oxygen evolution (Nishida & Murata, 1996).

1.10 Impairment of physiological and metabolic functions in response to soil drought

1.10.1 Cellular water deficit, plasmolysis, and cytorrhysis

This topic has been discussed in section 1.3.3 dealing with plant water deficit as common element in plant stress responses.

1.10.2 ABA-induced responses

The plant hormone abscisic acid (ABA) is one of the major signals involved in plant responses to drought and has been discussed in section 1.3.3 about plant water deficit as common element in plant stress responses.

1.10.3 Impairment of photosynthesis

1.10.3.1 Reduction in the net rate of CO_2 uptake – stomatal and non-stomatal limitations

Changes in net photosynthetic rate in response to progressively decreasing RWC and restrictions effective at different stages of cellular water loss are outlined in the following according to an excellent recent review by Lawlor & Cornic (2002).

The net rate of CO_2 uptake per unit leaf area (A) is determined by 2 major factors: (a) CO_2 supply to Rubisco, which depends on ambient CO_2 concentration (c_a) and stomatal or gas phase conductance for CO_2 (g_s). (b) Metabolic limitations described by the mesophyll or liquid phase conductance (g_m). Determination of maximum A under saturating CO_2 and light in fully turgid leaves (A_{pot}) by means of A/c_i response curves allows distinction between stomatal and metabolic limitations of A.

This method has identified two types of responses, which appear to be related to variation in dehydration-sensitivity of particular metabolic processes or cellular water relations dependent on species and growth conditions. The type 1 response is characterised by a distinct transition between stomatal and metabolic limitations: Decreases in RWC in the range of 100 to 75 % result in reductions in A, that can be fully reversed by large c_a, *i.e.* A_{pot} of control leaves can be fully restored. Thus in this RWC range reductions in A are due to stomatal limitations. Further decreases in RWC < 75 % result in a steeper decrease in A, associated with a progressive decline in A_{pot}. A is therefore limited by metabolic restrictions, the extent of which increases progressively with decreasing RWC. However, even in this range stomatal limitations still exert some control over A. In contrast, a progressive transition between stomatal and metabolic limitations of A is typical for the type 2 response. With decreasing RWC, A_{pot} decreases linearly, and thus stomatal control declines and metabolic control increases progressively.

The following mechanism has been implicated in the metabolic limitation of A. The basic restriction appears to be the reduction of leaf ATP concentration at low RWC, probably resulting from decreased ATP synthetic capacity due to loss of Coupling Factor CF1-CF0, also termed ATP synthase. RuBP synthesis generally depends on supply of ATP and NADPH as well as Calvin cycle function. Low RWC induces an increase in the ratio 3-phosphoglycerate (3-PGA) / ribulose 1,5-bisphosphate (RuBP), indicating that regeneration of RuBP is inhibited. This is thought to be due to ATP deficiency, whereas enzyme activity does not appear to be limiting. Although enzymes of the Calvin cycle are thus not thought to be directly responsible for the metabolic limitation of A, Rubisco activity has been shown to decline with decreasing RWC, and this is thought to result from restriction of Rubisco activation, which is dependent on Rubisco activase and ATP.

Determinations of (a) the CO_2 concentration in the leaf intercellular air space (c_i) by gas exchange measurements and (b) the CO_2 concentration at the active sites of Rubisco in the chloropasts (c_c) by measurement of fluorescence parameters and oxygen isotope exchange have shown the following general response pattern. In the type 1 response decreasing RWC in the range of 100 to 75 % results in a decline in c_i and c_c, which may drop to the compensation point, indicating that A_{pot} is not inhibited. In the type 2 response decreasing RWC in this range also causes a decline in c_i, but not to the compensation point, showing that A_{pot} is progressively inhibited, but that g_s is decreased more than A_{pot}. In both response types further decreases in RWC < 75 % result in a rise of c_i. In addition the CO_2 compensation point, determined by gas exchange measurements, has been shown to rise slightly with small decreases in RWC, and substantially with further cellular water loss, and this increase is O_2-insensitive. The rise in CO_2 compensation point is thought to reflect an increase in CO_2 evolution by dark respiration as a proportion of A (while dark respiration remains constant or decreases slightly in absolute terms) with decreasing RWC. The rise in c_i at low RWC is accordingly attributed to a stronger proportional decrease of A as compared to respiration or g_s. CO_2 evolution by dark respiration at low RWC results from consumption of stored carbohydrates as opposed to immediate photosynthate.

1.10.3.2 Effects on photosynthetic light reactions

Decreasing RWC in the physiologically relevant range (100 to 50 %) does not impair photon capture, excitation energy transfer, charge separation, reduction of acceptors or electron transport. The maximum photochemical efficiency of PSII (measured as dark-adapted F_v/F_m) is also insensitive to decreasing RWC in this range, indicating that photoinhibition does not occur. The relative concentration of open PSII centres (qP) declines only at RWC < 75 %. These results indicate that electrons are transferred to alternative sinks at low RWC (Lawlor & Cornic, 2002).

1.10.4 Impairment of carbohydrate synthesis

Responses of carbohydrate metabolism to decreasing RWC can be described as two consecutive phases. (a) At RWC in the range of 100 to 80 % the

activity of sucrose phosphate synthase (SPS) may decrease. There appears to be no effect on the capacity of carbohydrate translocation out of the leaf. However, reductions in expansion growth (see above) decrease sink capacity, and if the limitation in total demand exceeds that in total A, contents of starch and sucrose may increase. The ratio starch/sucrose increases, and concentrations of hexoses tend to be low. (b) At RWC at or below 75 % synthesis of starch and sucrose are severely reduced. The reduction in sucrose synthesis results from decreased flux of triosephosphate to the cytoplasm due to a decline in A as well as decreased activity of SPS. The activity of SPS decreases rapidly even in response to small changes in RWC, and inhibition is thought to depend on RWC, reduced c_i or alterations in substrate/effector concentrations related to the reduced flux of triosephosphates. Increased invertase activity leads to consumption of storage carbohydrates, and concentrations of hexoses increase. The latter are ultimately consumed in respiration and synthesis of amino acids (Lawlor & Cornic, 2002).

1.11 Drought resistance

1.11.1 Avoidance of plant water deficit

Plant water deficit occurs when more water is lost by a plant through transpiration than is taken up by the roots (Bray, 1997), and can thus be avoided by increased water uptake and decreased evaporative water loss, as outlined in the following.

1.11.1.1 Maintenance of water uptake

1.11.1.1.1 Root growth

The ABA-induced differential growth responses of plant shoot and roots are regarded as adaptive responses. They result in an increased root / shoot ratio (Thomas, 1997), and maintenance of root growth allows continued water uptake (Davies *et al.*, 1993).

In deep soil, deep root systems can improve access to water, but not to plant nutrients, which are concentrated in the top soil. Deep rooting may be constitutive or induced by soil drought. In contrast, in shallow soil a smaller root system with lower growth rate and narrow xylem vessels may help to ration water

supply. Other root characters such as vertical distribution, branching, diameter, and presence of root hairs may also influence water uptake (Thomas, 1997).

1.11.1.1.2 Aquaporins

The term aquaporins refers to a family of membrane proteins involved in water transport (Bray, 1997). In *Arabidopsis* 23 putative water channel proteins, which are located in the plamalemma or tonoplast, have been identified (Bohnert & Sheveleva, 1998). Genes encoding putative water channel proteins are induced in response to drought stress in *Arabidopsis thaliana* and the resurrection plant *Craterostigma plantagineum*, thus pointing to a role in regulating water flux under drought stress conditions (Ingram & Bartels, 1996).

Aquaporin activity is thought to be controlled by: (a) channel protein phosphorylation, (b) alteration in degree of oligomerisation, or (c) channel protein removal and either subsequent re-insertion or replacement by newly synthesised protein (Bohnert & Sheveleva, 1998). Phosphorylation of aquaporins increases their water transport capacity, and changes in apoplastic water potential can alter aquaporin phosphorylation status (Bray, 1997). In species with dominantly symplastic radial transport across the root (with strongly suberized roots), ABA signals generated in roots in response to soil drying may induce opening of inwardly directed aquaporins, resulting in increased root hydraulic conductivity (Wilkinson & Davies, 2002).

1.11.1.1.3 Cell wall characteristics

Acclimation to drought often involves an increase in the bulk modulus of elasticity (ε_B), reflecting increased tissue stiffness. Increased stiffness results in a faster decrease in cellular water potential with decreasing RWC, facilitating maintenance of water potential gradients, which drive water uptake (Thomas, 1997).

1.11.1.2 Reduction in water loss

Stomatal closure reduces water loss through transpiration and is therefore regarded as a mechanism of rapid acclimation to drought resulting in tissue water conservation. However, long-term acclimation is thought to be related to reduced drought sensitivity of stomata and maintenance of transpiration (see below) (Thomas, 1997, Yordanov *et al.*, 2000).

Cuticular conductance to water diffusion can be reduced by epicuticular wax layers, which confer a glaucous appearance to leaves. A correlation between genotypic variation in leaf waxiness, either constitutive or drought-induced, and drought resistance has been shown for some species. Leaf rolling decreases transpirational water loss by reducing the leaf surface area exposed to the environment (Thomas, 1997).

Absorption of radiation and thus transpiration load as dependent on leaf temperature can be reduced by (a) above mentioned epicuticular wax layers or leaf pubescence, which may increase reflectance (Thomas, 1997), and (b) leaf wilting, which results in reduced leaf surface area as a consequence of turgor loss-induced leaf shrinking. In addition leaf wilting decreases the leaf area exposed to radiation, as it is generally associated with leaf orientation in parallel to solar rays, with dicotyledonous leaves hanging down and monocotyledonous leaves rolling or folding vertically (Lawlor & Cornic, 2002).

1.11.2 Avoidance of turgor loss

1.11.2.1 Cell wall characteristics

A decrease in the bulk modulus of elasticity (ε_B), due to either reduced cell wall rigidity or changes in cell size, reflects increased leaf flexibility, and results in a slower decline in turgor potential with decreasing RWC (Thomas, 1997).

1.11.2.2 Osmotic adjustment

Thomas (1997) described osmotic adjustment as reduction in tissue osmotic potential in response to plant water deficit. Two types are distinguished based on their mechanisms. (a) Passive osmotic adjustment is solely the result of tissue water loss. (b) Active osmotic adjustment results from active accumulation of osmotically active compounds. In contrast, Bray (1997) defines osmotic adjustment as reduction of the cellular osmotic potential due to net accumulation of solutes (thus not including the passive component due to tissue water loss). It may allow maintenance of a sufficient water potential gradient favouring water uptake as well as maintenance of turgor. In addition, osmotic adjustment may lead to an absolute increase in turgor on leaf re-hydration overnight or after rain (Thomas, 1997). The biochemical mechanisms of active or net accumulation of

organic osmotically active compounds have been discussed in detail for sugars (see 1.10.4) and the compatible solute glycinebetaine (1.3.4.1.1).

1.11.3 Prevention of photosynthetic inhibition

1.11.3.1 Stomatal conductance

Although stomatal closure reduces water loss through transpiration and is, therefore, regarded as a rapid mechanism of acclimation to drought resulting in tissue water conservation, long-term acclimation is thought to be related to reduced sensitivity of stomata to drought (Thomas, 1997, Yordanov *et al.*, 2000).

Constitutive drought tolerance can be related to two types of stomatal response patterns. (a) Adjustment of stomatal conductance to diurnal fluctuations in evaporative load to maximise water use efficiency (WUE), which can be defined either as ratio of dry weight gain based on whole plant, shoot, or harvestable dry weight to plant evaporative water loss or alternatively as ratio of CO_2 fixed to H_2O transpired by a leaf. Under soil drought this is achieved by high stomatal conductance in the morning and evening and stomatal closure around midday (Thomas, 1997). (b) Rapid increase in stomatal conductance on relief of drought stress (Yordanov *et al.*, 2000).

1.11.3.2 Photoprotection

1.11.3.2.1 Reduced absorption of radiation energy

Leaf wilting reduces the functional absorption area for incident radiation of leaves as a result of (a) decreased leaf surface area as a consequence of turgor loss-induced leaf shrinking and (b) decrease in leaf area exposed to radiation. Wilted leaves are generally orientated in parallel to solar rays, with dicotyledonous leaves hanging down and monocotyledonous leaves rolling or folding vertically (Lawlor & Cornic, 2002).

1.11.3.2.2 Thermal dissipation of excitation energy

The inhibition of the net rate of CO_2 uptake (A) with decreasing RWC due to either stomatal or metabolic limitation, although accompanied by an increase in electron consuming photorespiration as a proportion of A (see below), results in a reduction in total electron sink capacity. This leads to PSII over-excitation, causing build-up of a high thylakoid ΔpH. The latter activates ΔpH-dependent

high energy state quenching (qE), which is a major component of nonphotochemical quenching of chlorophyll fluorescence (NPQ). Thus the reduction in A with decreasing RWC is associated with a steep rise in NPQ (Lawlor & Cornic, 2002).

1.11.3.2.3 Alternative electron sinks

1.11.3.2.3.1 Photorespiration

The ratio of rates of photorespiration / net photosynthesis increases by up to 50 % in response to soil drought, as determined by fluorescence and oxygen isotope studies, while the absolute rate of photorespiration may decrease. Oxygen exchange analysis reveals three phases in the response of photorespiration to decreasing RWC. (a) Initially the ratio of O_2 uptake / gross O_2 evolution increases with the decrease in the CO_2 concentration in the leaf intercellular air space (c_i) in response to reduced stomatal conductance. (b) The above ratio then remains constant. (c) Finally the ratio of O_2 uptake / gross O_2 evolution and gross O_2 evolution decrease concomitantly. This is thought to be due to the rise in c_i resulting from the increase in CO_2 evolution by dark respiration as a proportion of the net rate of CO_2 uptake (A) and the metabolic limitation of A at low RWC, which is associated with decreased CO_2 re-assimilation. Thus photorespiration is a substantial alternative sink for electrons under decreasing RWC, but is inhibited, when A is severely restricted due to metabolic limitations (Lawlor & Cornic, 2002).

1.11.3.2.3.2 Mehler-ascorbate peroxidase reaction

The Mehler-ascorbate peroxidase reaction, also referred to as the water-water cycle, is not thought to play a major role in consumption of electrons at decreasing RWC (Lawlor & Cornic, 2002).

1.11.3.2.3.3 Mitochondrial respiration

The mitochondrial electron chain generally transports electrons from the citric acid cycle or exogenous NAD(P)H, and transport is coupled to ATP synthesis. It accepts electrons released by photorespiration as well as the chloroplastic electron chain. In the latter case, NADPH is fed into the citric acid cycle *via* formation of malate. The contribution of mitochondrial citric acid cycle respiration to consumption of electrons from the photosynthetic electron chain

varies with progressively decreasing RWC. (a) In the initial phase of cellular water loss photorespiration plays a major role in utilisation of electrons, and the contribution of mitochondrial citric acid cycle respiration is thought to be minor. (b) When the RWC decreases to levels, which result in complete inhibition of net rate of CO_2 uptake (A) due to metabolic limitation and concomitantly in inhibition of photorespiration, mitochondrial respiration may become an important alternative electron sink (Lawlor & Cornic, 2002).

1.12 Impairment of physiological and metabolic functions in response to salinity

1.12.1 Reduction in plant growth and stomatal conductance

The adverse effects of NaCl salinity on plant function are commonly divided into three components: (a) Osmotic stress resulting from decreased soil osmotic potential due to the presence of dissolved NaCl. (b) Plant nutrient imbalance due to interference of Na^+ with the uptake of specific plant nutrients. (c) Salt toxicity resulting from inhibition of enzyme activity by Na^+ and Cl^- (Glenn *et al.*, 1997). The contribution of these different component stresses to plant responses to salinity are outlined in the following, in most parts according to a model proposed by Munns (1993, 2002), which resolves the action of specific component stresses with time of salinity exposure.

1.12.1.1 Osmotic stress

1.12.1.1.1 Gradual imposition of salinity – ABA-induced responses

The initial responses of plants to salinity exposure on a time-scale of days are generally attributed to effects of salt outside the plant, *i.e.* the decrease in soil osmotic potential. These responses show strong similarities to the ABA-induced rapid plant responses under soil drying, as described in the following according to Munns (1993, 2002).

(1) After several days of exposure to salinity the rate of leaf appearance and final leaf size decline as a result of changes in cell expansion and cell division, and shoot growth is more reduced than root growth. It has been shown that Na^+ and Cl^- concentrations in growing leaf and root cells are generally below the threshold levels for enzyme toxicity (see below), thus indicating that salt-

specific effects are unlikely to occur at this stage. In tissues with rapid cell division such as shoot and root apices salt concentrations are generally relatively low. In expanding tissues expansion of vacuoles is thought to allow continued uptake and dilution of salts, thus also resulting in relatively low cellular salt concentrations. It was shown that application of pressure to plant roots in saline soil to counteract salinity-induced changes in soil water potential over periods of up to 8 days did not prevent reductions in growth in a variety of species. In addition, plant exposure to salinity did not affect cellular turgor in expanding leaf cells. These results indicate that the reduction in leaf growth under salinity was probably induced by hormonal signals from the root, and not by hydraulic signals originating in the shoot.

(2) Stomatal conductance of plants under salinity is reduced. The following evidence against the involvement of leaf dehydration in the induction of this response has been found (Munns, 1993). Applying pressure to plants to counteract salinity-induced changes in soil water potential resulted in increased leaf water potential and leaf (but not root) turgor, but did not prevent the salinity-induced decrease in transpiration rate in wheat and barley. In fully-expanded leaves of salinity-treated plants turgor values lower, equal or higher than control values have been observed. Comparisons of varieties differing in salt tolerance have shown that salt sensitivity is often related to higher leaf turgor values and also greater reduction in stomatal conductance. Thus it was suggested that a hormonal signal from the root acts as trigger for stomatal closure.

1.12.1.1.2 Sudden imposition of salinity – osmotic shock

Sudden (one step) transfer of plants to a NaCl solution, which has an osmotic pressure higher than cell turgor, induces rapid cellular dehydration (osmotic shock) and plasmolysis. This occurs only in cells that are in direct contact with a plasmolysing solution, such as in roots of hydroponically grown plants. During plasmolysis the protoplast shrinks, and the plasma membrane detaches from large sections of the cell wall. The plasma membrane is stretched into strands, which remain tethered to the cell wall at particular sites. Plasmolysis may induce fluxes of abnormal amounts of salt and consequently salt-specific effects in two ways. (a) The space between the plasma membrane and the cell wall

may fill with solution, thus opening up an apoplastic pathway for radial movement of salt across the root. (b) The plasma membrane may become leaky, allowing unregulated flux of salt in or out of the protoplast (Munns, 2002).

Studies on a variety of species have shown that sudden imposition of salinity results in greater inhibition of growth and higher salt concentrations in shoots as compared to gradual imposition. Munns (2002) suggests that the more adverse effects of sudden imposition (salt shock) in these studies may be due to plasmolysis of root cells and possibly movement of salt through artefactual apoplastic pathways.

1.12.1.2 Salt toxicity

Plant responses at later stages of salinity exposure are attributed to salt-specific effects, *i.e.* effects of salt within leaves. Salt-specific injury may occur after several days to weeks, dependent on salinity level, genotypically determined ability of salt exclusion or compartmentation, and environmental factors such as temperature, relative humidity and nutrient availability (Munns, 2002).

Plants show visible injury (yellowing) and eventually death of older leaves, which have been transpiring the longest, and older leaves are progressively lost. If the rate of leaf death exceeds the rate of production of new leaves, the proportion of injured leaves increases. This results in inhibition of growth of younger leaves due to decreased supply of assimilates. Then initiation of the reproductive phase may be accelerated in order to be completed, as long as sufficient photosynthate is available (Munns, 2002).

The following mechanisms of salt-specific injury have been described. In general, plant water loss through transpiration exceeds water utilisation for cell expansion by a factor of 30 to 70. Thus the salt concentration arriving in leaves through the transpiration stream will increase by this factor, and salt levels will build up progressively with time (Munns, 2002). When the cellular ability to sequester arriving Na^+ and Cl^- in the vacuole is exceeded, newly arriving ions may build up in two different compartments.

(a) Salt may accumulate in the cell wall, and this has been demonstrated for rice and pea. Salt transport through the leaf mesophyll was originally thought to be apoplastic, later symplastic. In the latter case it is thus assumed that efflux of excess ions to the apoplast occurs. Increasing salt concentrations in the cell

wall result in a concomitant decrease in cell turgor and volume. The latter in turn causes an increase in the salt concentration in the cell, leading to further salt efflux to the apoplast. These processes lead to rapid cell dehydration (Munns, 1993).

(b) Salts may build up in the cytoplasm, and salt concentration will rise at a steeper rate than in the vacuole due to the comparatively small volume of the cytoplasm (Munns, 1993). When cytoplasmic salt concentrations exceed a certain threshold level, activities of many enzymes are inhibited. This threshold concentration is about 100 mol m^{-3} for Na^+, and probably a similar value for Cl^- (Munns, 2002). Although individual plant enzymes differ in their salt sensitivity, the increase in the cytoplasmic salt concentration occurs so rapidly (at a rate of up to 10 mol m^{-3} h^{-1}), that all enzymes would be poisoned within a very short interval of time (a maximum of one day) (Munns, 1993).

Whether salt-specific injury occurs *via* salt accumulation in the cell wall and cell dehydration or build up of salt in the cytoplasm and salt poisoning, cell death occurs within a few days from the point where the cellular ability to sequester salt in the vacuole is exceeded (Munns, 1993).

1.12.1.3 Nutritional imbalance

NaCl salinity can induce Ca^{2+} deficiency by inhibiting uptake and transport of Ca^{2+}. High Na^+ concentrations may result in removal of Ca^{2+} from its binding sites at the external plasma membrane surface (Marschner, 1995). NaCl-induced Ca^{2+} deficiency can induce rapid and severe inhibition of root growth, and low levels of supplemental Ca^{2+} partially prevent this growth reduction (Munns, 2002). Addition of Ca^{2+} has also been shown to result in increased shoot growth and decreased shoot Na^+ content in a variety of species under NaCl salinity. A possible mechanism of the ameliorating effect of supplemental Ca^{2+} has been demonstrated (Marschner, 1995): Addition of Ca^{2+} to external salt solution (NaCl and KCl) causes a shift in the K^+/Na^+ selectivity of roots in favour of K^+ uptake. This effect occurs in Na^+ excluding as well as including species. In general, Ca^{2+} plays a role in membrane stabilisation and maintenance of a high electropotential across the membrane by reacting with negatively charged groups on the membrane surface. Supplemental Ca^{2+} may thus increase K^+/Na^+

selectivity by enhancement of Na^+ efflux processes or general effects on plasma membrane integrity.

Transport of Na^+ across root cell plasma membranes is thought to be mediated by the same carrier systems that transport K^+. Physiological studies implicate the operation of two different carrier systems. (a) A system with high selectivity for K^+ over Na^+ mediates uptake of univalent cations at external cation concentrations of up to 1 mol m^{-3}. (b) At higher concentrations a carrier system with higher V_{max} and lower affinity for K^+ is activated. Na^+ may thus compete with K^+ for intracellular influx, particularly at high salinity levels (Glenn *et al.*, 1997, Hasegawa *et al.*, 2000). K^+ is an essential plant nutrient, and a cytoplasmic concentration of about 130 mol m^{-3} is considered to be the threshold level for maintenance of enzyme function and membrane integrity. However, the implication of K^+ deficiency as a limiting factor for plant growth under salinity has been questioned, based on the following observations. Halophytes may maintain absolute levels of K^+ uptake at salinity levels of up to 1000 mol m^{-3}, and many glycophytes maintain high selectivity for K^+ uptake and preferentially accumulate K^+ in vacuoles at salinity levels of up to 200 mol m^{-3}. Supplemental K^+ did not result in increased salt tolerance (Glenn *et al.*, 1997).

1.13 Resistance to salinity stress

Crop species may be classified according to their growth response to increasing substrate salinity (Marschner, 1995). (a) Halophytes show stimulation of growth at moderate to high levels of NaCl salinity. (b) Growth of glycophytes may be unaffected at low levels of NaCl salinity, but is inhibited with increasing salinity. Halophytes show higher levels of NaCl resistance, determined as dry matter production in response to increasing salinity, and exhibit different mechanisms of salinity adaptation than glycophytes (Glenn *et al.*, 1997). The following generalisations can be made (Munns, 2002): Halophytes are characterised by efficient exclusion of salts at the root level, and effective compartmentalisation of the residual salt, which has entered the transpiration stream, in the vacuoles of leaf cells. Glycophytes may also have the ability to exclude salt efficiently from the transpiration stream, but are not as effective as halophytes in compartmentalisation of salt in leaves. Most glycophytes, however, have a poor ability to exclude salt.

In the following mechanisms of salt resistance in glycophytes and halophytes are reviewed. However specialised structures confined to halophytes, such as salt glands or bladders, are not discussed.

1.13.1 Avoidance of excessive salt accumulation in leaves

1.13.1.1 Avoidance of excessive salt transport to leaves

1.13.1.1.1 Reduction in water flux through the plant

Similar to plant responses under soil drying, plant exposure to salinity induces rapid reduction in stomatal conductance. Stomatal closure is regarded as a short-term mechanism of acclimation to salinity stress, as it reduces transpiration, and thereby ion flux to the leaves. However, stomatal closure concurrently reduces CO_2 uptake and photosynthetic CO_2 assimilation. It is thus not viewed as a suitable long-term mechanism of salinity tolerance (Hasegawa *et al.*, 2000).

Exposure to high levels of salinity may cause reductions in root hydraulic conductivity as has been shown for maize, and involvement of changes in number or specific water transport activity of aquaporins have been suggested. However, studies investigating the possible role of aquaporins in plants roots under salinity are scarce, and thus their role in control of water uptake under salinity is unclear (Hasegawa *et al.*, 2000).

1.13.1.1.2 Salt exclusion in roots

The radial movement of water and ions across the root cortex to the stele may occur in two parallel pathways: the symplast and the apoplast. However, the radial and transverse walls of the endodermis are surrounded by a band of hydrophobic suberin incrustations, the Casparian band, which constrains or blocks the apoplastic flux into the stele. In some species the exodermis, a cell layer below the rhizodermis, may also contain suberin incrustations and act as barrier to apoplastic flux in older roots. Symplastic transport of ions can thus begin at the rhizodermis or exodermis, but at the latest at the endodermis (Marschner, 1995).

The roots of most species exclude a high proportion of NaCl in the external solution from entering the transpiration stream, while admitting water. This mechanism of avoiding accumulation of salt to excessive levels in leaves is common to halophytes and glycophytes (Glenn *et al.*, 1997, Munns, 2002). However, halophytes and glycophytes differ in their predominant mechanisms of

salt exclusion in roots (Marschner, 1995): In halophytes salt exclusion occurs as a result of anatomical adjustments: their endodermal Casparian bands are several times thicker than those of glycophytes, and some have a second endodermis in the inner cortex. In glycophytes restriction of salt transport to the stele is predominantly related to membrane properties of root cells. Plants may have different capacities for exclusion of Na^+ and Cl^-, and the following mechanisms have been observed. (a) Na^+ exclusion may be due to increased efflux and sequestration into the vacuole. Enhanced activity of Na^+/H^+ antiport and H^+-ATPase in plasma membrane and tonoplast of root cells in response to salinity have been demonstrated. (b) Genotypical differences in the degree of chloride exclusion are often related to specific differences in the lipid composition of root membranes.

1.13.1.1.3 Resorption of salt from the xylem

Along the pathway of xylem flux from the roots to the leaves solutes can be resorbed or unloaded from the xylem. Reabsorbed solutes can be (a) stored transiently or permanently in xylem parenchyma and other stem tissue or (b) re-translocated to the roots by xylem-phloem transfer. Phloem and xylem are only separated by a few cells in the vascular bundles, and transfer can occur via xylem parenchyma transfer cells. Some species, like bean and castor bean, retain Na^+ in the roots and the lower part of the stem, thus preventing translocation to the leaves. It has been demonstrated that application of radio-labelled Na^+ to mature primary leaves of bean results in translocation to the basal zones of the roots, where Na^+ efflux may occur (Marschner, 1995). However, this mechanism of resorption in the xylem and retranslocation to the roots appears to be restricted to the more salt-sensitive species among glycophytes. The more resistant glycophyte species exhibit more efficient exclusion at the root level or take up and compartmentalise ions in leaves for osmotic adjustment (Glenn *et al.*, 1997).

1.13.1.2 Dilution of accumulated salts

Higher intrinsic growth rates may reduce salt accumulation as a result of greater shoot biomass production per unit salt uptake (Glenn *et al.*, 1997, Munns, 2002), and a correlation between differences in salinity resistance among rice varieties and differences in intrinsic growth rates has been shown (Glenn *et al.*,

1997). High shoot/root ratios may also reduce the relative rate of salt accumulation in the shoot (Munns, 2002).

The storage capacity of mesophyll cells for Na^+ and Cl^- may be increased by increasing cellular water content as a result of vacuolar expansion (succulence). This has been demonstrated for halophyte and glycophyte species (Marschner, 1995, Glenn *et al*, 1997).

1.13.2 Restriction of salt movement into growing tissues

Halophytes show a characteristic gradient of ion accumulation between old and young leaves under saline conditions, with Na^+ concentration decreasing and K^+ concentration increasing with decreasing leaf age. Sugar beet, a halophilic crop plant, exhibits a similar gradient of Na^+ and K^+ between old and young leaves, resulting from low Na^+ and K^+ import *via* the xylem, but high import of K^+ originating from older leaves *via* the phloem. Also glycophyte species like wheat have been shown to restrict import of Na^+ and Cl^- into young leaves, inflorescences, and seeds (Marschner, 1995).

The mechanism underlying this preferential salt accumulation in older tissues is still debated (Hasegawa *et al.*, 2000). It may be an acclimation response, in which older leaves act as ion sinks, thus restricting salt translocation to meristematic and actively growing cells. Alternatively, exclusion from these tissues may be a consequence of transpirational and expansive fluxes, degree of connection to the vascular system, and cell morphology. Thus the oldest leaves have transpired the longest, and meristematic cells are not directly connected to the vasculature, and have little vacuolation, making them inconducive to ion storage.

1.13.3 Salt uptake into leaves for osmotic adjustment and compartmentalisation

Salinity involves an osmotic stress component, as the presence of dissolved NaCl decreases the soil osmotic potential, and can thus induce plant water deficit (Marschner, 1995). Osmotic adjustment, defined as reduction of the cellular osmotic potential due to net accumulation of solutes, is therefore required to maintain a sufficient water potential gradient favouring water uptake as well as maintenance of turgor (Bray, 1997). Under salinity stress this may occur in two ways:

(1) Accumulation of salt in cells. Halophilic bacteria adjust by taking up salt from the external solution and accumulating it in their protoplasm to higher levels than in the external solution. Their enzymes are highly salt tolerant (Glenn *et al.*, 1997). However, neither halophytic nor glycophytic species among higher plants have enzymes that are tolerant of high levels of NaCl salinity (Munns, 2002).

(2) Accumulation of organic solutes. The synthesis of organic solutes in leaves is metabolically expensive, and has been estimated to require about 34 ATP for mannitol, 41 for proline, 50 for glycinebetaine, and 52 for sucrose. In comparison accumulation of Na^+ consumes only 3.5 ATP (Munns, 2002).

It is now generally accepted that in most higher plants osmotic adjustment of the large central vacuole typical for plant cells (which has a volume at least 20 times higher than that of the cytoplasm) occurs via accumulation of Na^+ and K^+ salts. Glycophytic plants at the lower end of salinity resistance tend to have a high K^+/Na^+ selectivity of root uptake systems and preferentially accumulate K^+ in vacuoles. Halophytes, in contrast, preferentially accumulate Na^+ in their vacuoles, and may even develop levels of leaf osmotic pressure several times higher than that of the external NaCl solution (Glenn *et al.*, 1997). Toxic ions are thus partitioned away from the cytoplasm via energy-dependent transport into the vacuole (Hasegawa *et al.*, 2000).

In both halophytes and glycophytes, organic solutes (compatible solutes) accumulate in the cytosol, and in the lumen, matrix or stroma of organelles, contributing to osmotic adjustment in these compartments (Hasegawa *et al.*, 2000). Categories and general features of compatible solutes and in particular the molecular features, biosynthesis, mechanism of stress-induced accumulation, and suggested roles of glycinebetaine have been discussed earlier (section 1.3.4.1.1).

Determination of inorganic ions and quarternary ammonium compounds (QACs) in leaf extracts versus isolated chloroplasts of salinity-treated (300 mol m^{-3} NaCl) spinach plants indicated such preferential accumulation of Na^+ and Cl^- in the vacuole and of QACs in the chloroplast (Marschner, 1995). X-ray microanalysis of cultured tobacco cells has also provided direct evidence for sequestration of Na^+ and Cl^- in the vacuole. Indirect evidence arises from the observed high concentrations of Na^+ and Cl^- in normally functioning leaves,

which often exceed the threshold level for enzyme toxicity *in vitro* (Munns, 2002).

The high salinity resistance of halophytes has been attributed to the following mechanisms: (a) They take up a higher proportion of NaCl from the external salt solution, and the roots often have lower NaCl concentrations than the shoot (Glenn *et al.*, 1997, Hasegawa *et al.*, 2000). (b) Their compartmentalisation system for Na^+ and Cl^- is more responsive to salinity. A comparison of ion transport activities in the plasmalemma and tonoplast of root cells of the halophyte *Atriplex nummularia* and the glycophyte cotton showed that the halophyte had higher Na^+/H^+ antiporter activity, and salinity exposure induced an increase in activity (Glenn *et al.*, 1997). (c) They have a more effective co-ordination of salt compartmentalisation with processes controlling growth and ion flux across the plasma membrane, as continued vacuolar ion sequestration requires continued production of new vacuoles, *i.e.* growth (Hasegawa *et al.*, 2000).

Although salinity resistance in glycophytes is generally mainly associated with salt exclusion, this is a relative classification in comparison to halophytes (Marschner, 1995). Ion uptake patterns of glycophytes at the higher end of salinity resistance, such as barley, may resemble those of halophytes, and these species also use NaCl for osmotic adjustment (Glenn *et al.*, 1997). Even within species salinity tolerance of varieties may be related to both, predominant salt exclusion and salt compartmentalisation in leaves, as has been shown for wheat and tomato (Glenn *et al.*, 1997, Marschner, 1995).

1.13.4 Ion transport systems involved in salinity resistance

Solute transport across cellular membranes can generally occur in two ways. (a) A solute can be transported along a chemical potential gradient (*i.e.* a gradient in its concentration), which can be maintained by adsorption of the solute at charged groups or incorporation into organic structures on one side of the membrane. This is termed passive transport. (b) A solute can be transported against such a chemical potential gradient, and this process has to be coupled, directly or indirectly, to an energy-consuming pump in the membrane. It is thus termed active transport. ATP-driven H^+ pumps (proton motive force) transport

protons across membranes and thereby generate a gradient in pH and electropotential (*i.e.* differences in millivolts measured by means of a microelectrode). Proton pumps in the plasma membrane transport H^+ from the cytoplasm into the apoplasm, proton pumps in the tonoplast translocate H^+ from the cytoplasm into the vacuole. The resulting characteristic differences in pH are the driving force for secondary energised transport of ions along the electrochemical gradient (Marschner, 1995).

The following transport systems are thought to mediate ion homeostasis during salinity (Hasegawa *et al.*, 2000).

(1) Salt influx across the plasma membrane: Na^+ transport could occur along the Na^+ chemical potential gradient and the large inside negative gradient in electropotential. In general Na^+ is transported by K^+ transport systems, which also have more or less affinity for Na^+. Transport could thus be mediated by inward rectifying K^+ channels, Na^+-K^+ symporters, K^+ transporters, voltage-dependent outward-rectifying cation channels, and voltage-independent cation channels, but the exact nature of transport has not been resolved. Uptake of Cl^- is thought to be mediated by different mechanisms with time of salinity exposure. Initially, following dissipation of the H^+ electrochemical gradient by rapid Na^+ influx, Cl^- may be transported passively through anion channels along its chemical potential gradient. After re-establishment of the membrane electropotential gradient, transport is thought to be mediated by energy-dependent Cl^--H^+ symport.

(2) Salt sequestration in the vacuole: Na^+ transport into the vacuole is mediated by energy-dependent Na^+/H^+ antiporters. Cl^- influx into the vacuole is thought to be coupled to the H^+ gradient, and anion permeable channels and H^+/anion antiporters have been suggested.

(3) Na^+ efflux: Na^+ transport across the plasma membrane to the apoplast is mediated by energy-dependent Na^+/H^+ antiporters.

Primary energised process: The electrochemical gradient, which drives secondary energised transport involved in efflux and vacuolar sequestration of salt ions, is generated by the plasma membrane H^+-ATPase and tonoplast H^+-ATPase and H^+-pyrophosphatase. Activities of plasma membrane H^+-ATPase and tonoplast H^+-ATPase have been shown to increase on salinity exposure.

1.14 Objectives of the present study

The purpose of the present study was to address the following two questions:

1. Can cotton (*Gossypium hirsutum*) acquire increased resistance to high levels of the abiotic stress factors heat, chilling, and salinity following pretreatment with low to moderate levels of the same stress factors?

The rationale behind this approach was based on the general model of plant response to environmental stress as summarised by Lichtenthaler (1996). Exposure of plants to stress at an intensity not exceeding the threshold of constitutive resistance of that species (resistance minimum) activates mechanisms for coping with stress. These consist of (a) repair processes leading to homeostatic restitution of the initial physiological state and (b) hardening processes establishing a new physiological state that is optimal under the new environmental conditions. This is associated with an increase in stress resistance (resistance maximum). Both mechanisms are described by the term acclimation, which has been defined as adjustment of individual organisms in response to changing environmental factors resulting in altered steady-state physiology (Buchanan *et al.*, 2000), or as expression of genes conferring greater stress resistance under inductive conditions (Pearce, 1999). In the present study I tested whether pretreatment at low to moderate stress levels induced such acclimation by comparing responses of stress- and control-pretreated plants to subsequent treatment at high levels of the same stress factor, approaching or exceeding the threshold of constitutive resistance. Studies aimed at unveiling the mechanisms underlying acclimation generally assess plant responses to sub-lethal stress levels, during which these mechanisms are induced (Grover *et al.*, 2001). To further investigate potential mechanisms of acclimation in the present study, plant responses to stress-pretreatment at low to moderate stress intensity were thus also monitored.

In the present study the potential of cotton to acclimate to heat was assessed with respect to photosynthetic parameters: photosynthetic gas exchange and maximum quantum yield of photosystem II (PSII). It has been shown previously that exposure of species originating from habitats with large temperature variations during the growing season can shift their temperature

optimum of photosynthetic CO_2 uptake, while maintaining similar photosynthetic rates, in parallel to long-term changes in growth temperature in leaves developed in the new temperature regime. In some of these species exposure to changes in growth temperature on a time-scale of days to weeks also results in corresponding shifts of the thermal characteristics of photosynthesis in mature fully-developed leaves. Acclimation of photosynthesis to high temperature was attributed to increased heat stability of the photosynthetic apparatus (Berry & Björkman, 1980). Havaux (1993) demonstrated that short-term heat-pretreatment on a time-scale of hours increased thermotolerance of PSII in potato leaves, and this was associated with de-sorption of violaxanthin from the light harvesting complex II during pretreatment (Havaux & Tardy, 1996). Heckathorn *et al.* (1998) observed a similar acclimative effect of short-term heat-pretreatment on a time-scale of hours on heat resistance of PSII electron transport in tomato leaves, and they provided direct evidence that small heat shock proteins (sHSPs) were involved in this phenomenon. Studies aiming to evaluate the potential of cotton to acclimate to heat have shown that long-term growth at moderately elevated temperatures increased heat stability of PSII and photosynthetic gas exchange (Seemann *et al.*, 1984) and that gradual (2.5°C per hour) versus rapid (1°C per minute) imposition of heat stress resulted in increased heat resistance of net rate of CO_2 uptake, Rubisco activase activity, and PSII stability (Law & Crafts-Brandner, 1999). In the present study heat pretreatment at moderately elevated temperature and of a duration of 6 to 7 days, thus intermediate between pretreatment periods used in previous studies on cotton, was employed, and potential mechanisms underlying acclimation effects were identified.

Evaluation of acclimation potential of cotton to low temperatures in the present study focused on resistance to photoinhibition in response to chilling concurrently with low incident light. It has long been recognised that temperate plants can acquire increased resistance to temperatures below freezing during a period of low non-freezing temperatures, a process termed cold-acclimation. The period of low non-freezing temperatures is associated with a strong induction of cold-regulated (COR) genes, and much progress has been made in recent years in assigning functions to these genes and in elucidating the signal transduction pathways leading to changes in gene expression (Xin & Browse, 2000). Many herbaceous plants with the ability to cold-acclimate acquire increased resistance to

photoinhibition during cold acclimation (Krause, 1994). Increased resistance to photoinhibition can be brought about by increased thermal dissipation of excitation energy, increased energy utilisation by photosynthesis or alternative electron sinks, increased antioxidant capacity in the chloroplast, and increased capacity to repair inactivated PSII (Niyogi, 1999, 2000). Cotton, however, originates from tropical ancestors (Wendel, 1995) and is classified as chilling-sensitive (Kornyeyev *et al.*, 2002). Chilling-sensitive species generally have a low ability to acclimate to low temperatures (Krause, 1994, Allen & Ort, 2001). In the present study I tested whether a chilling pretreatment regime involving a gradual decrease in temperature, resembling the step-wise reduction in temperature applied by Somersalo & Krause (1989, 1990) to evaluate the potential of the temperate spinach plant to acquire increased resistance to photoinhibition, could also increase resistance to photoinhibition in the chilling-sensitive cotton plant. It was demonstrated that cotton can acclimate to chilling-induced photoinhibition, and photosynthetic responses of acclimative natures were identified in the initial phase of chilling-pretreatment.

The acclimation potential of cotton to salinity was assessed on two levels:

(a) Pretreatment of young (23-day old) plants at moderate salinity for 26 days was assessed for its potential to protect shoot dry matter production and restrict leaf Na^+ accumulation in response to high salinity. There are only few reports on successful acclimation of plant shoot growth and Na^+ accumulation to salinity, which generally involve stress-pretreatment of very young plants. Amzallag *et al.* (1990, 1993) demonstrated that pretreatment of 5- and 8-day old plants *of Sorghum bicolor* at moderate salinity for 20 days allowed resumption of shoot growth on exposure to high salinity, whereas pretreatment in control conditions or at moderate salinity, but in older plants, led to plant death. Acclimation of shoot growth was correlated with reduced accumulation of Na^+ in shoots. Similarly, Umezawa *et al.* (2000) found that pretreatment of 8-day old plants of *Glycine max* at moderate salinity for 23 days resulted in increased rates of plant survival and reduced extents of Na^+ accumulation in leaves and roots during subsequent treatment at high salinity. The present study demonstrated some potential also in cotton to acquire the ability to restrict salinity-induced Na^+ accumulation in leaves during salinity-pretreatment, and confirmed the crucial role of plant age at initiation of salinity-pretreatment in this response.

(b) I tested whether pretreatment of mature, 47-day old, plants at moderate salinity for 18 days could increase salinity-resistance of photosynthetic gas exchange. Plaut & Federman (1991) had shown previously that exposure of 25-day old cotton plants to gradually (over 5-8 days) versus rapidly (over 1-2 days) imposed salinity treatment decreased the extent of reduction in photosynthetic CO_2 uptake, and this was associated with higher levels of osmotic adjustment. The present study confirmed that cotton could similarly acquire increased salinity resistance of photosynthetic gas exchange during more long-term pretreatment at moderate salinity.

2. Can cotton (*Gossypium hirsutum*) acquire increased resistance to high levels of heat or drought stress by pretreatment at gradually decreasing low temperatures?

The purpose of these experiments was to evaluate the "general adaptation syndrome hypothesis", which was originally formulated for animals, but has more recently also been adopted for plant systems, and states that pre-exposure to sub-lethal levels of one stress may induce increased resistance to a different stress. It is based on the observation that plant acclimation responses to different environmental stress factors share common elements on a molecular and biochemical level, and on the feasibility of an evolutionary advantage of multiple stress resistance (Leshem & Kuiper, 1996). Based on the fact that drought, salinity, freezing, and chilling stress all involve a plant water deficit component, they induce overlapping acclimative responses, which often involve signalling by the plant hormone abscisic acid (Bray, 1997). The accumulation of compatible solutes, including polyols and sugars such as mannitol, sorbitol, and trehalose, betaines, and amino acids such as proline, is such a common response to these stresses, which has received particular research attention, but the exact roles of these compounds are still debated (Chen & Murata, 2002, Sakamoto & Murata, 2002). In principle, all environmental stress factors, applied concurrently with low to moderate incident light, with the potential to impair excitation energy utilisation, may involve photoinhibition as a common element (Osmond, 1994, Huner *et al.*, 1998) and may thus induce overlapping acclimative responses of photoprotection and antioxidant defence.

In the present study assessment of the potential of chilling pretreatment to induce increased resistance to soil drying was complicated by adverse effects of

stress-pretreatment on shoot growth resulting in differences in plant size of control- and stress-pretreated plants. To address the problem associated with comparing responses of plants differing in size to a stress factor involving resource supply, effects of plant size on plant response to soil drying were statistically removed as described by Osório *et al.* (1998).

In the present study evidence is presented that pretreatment of cotton at chilling temperatures can partially protect PSII activity and photosynthetic gas exchange during treatment at high levels of heat stress. This appears to be the first report on acquirement of cross-resistance to chilling-induced photoinhibition as well as heat-induced impairment of photosynthesis as a result of pretreatment at chilling temperatures. Photosynthetic acclimation to long-term changes in growth temperature generally involves a shift in the temperature optimum of photosynthesis and is thus by necessity associated with decreased performance at the respective other end of the temperature scale (Berry & Björkman, 1980). Similarly, increased heat resistance of PSII activity in response to short-term heat pretreatment on a time-scale of hours was shown to be associated with decreased resistance of this parameter to low temperatures in potato leaves (Havaux & Tardy, 1996). However, a number of links at a cellular level have been suggested between high and low temperature stress, which include the induction of heat shock proteins (Viswanathan & Khanna-Chopra, 1996), processes related to photoinhibition (Feierabend *et al.*, 1992), and mechanisms of antioxidative defence (Badiani *et al.*, 1997). In the present study the possible role of glycinebetaine accumulation observed during chilling pretreatment in the acquirement of increased photosynthetic heat resistance is discussed.

2 Materials and Methods

2.1 Plant material

Seeds of the variety CIM 443 of *Gossypium hirsutum* developed in the Central Cotton Institute of Multan in Pakistan were used in all experiments.

2.2 Growth conditions

Plant seeds were sown in John Innes No. 1 compost in plastic sowing trays consisting of 12 compartments (P12, Plantpak, Maldon, U.K.), each of which had a volume of 100 cm^3. After several weeks of growth (as specified below) they were transplanted (one plant per pot) to 1.6-dm^3 plastic pots filled with the same substrate. They were germinated and grown in Pen-y-Ffridd field station, greenhouse No. 2, which is naturally lit and supplemented with artificial light from 400 W sodium lamps, at 25°C and at a 16-hour photoperiod, on greenhouse benches supplied with capillary matting, and watered daily (control environment).

At intervals specified below, plants were treated with plant nutrient solution prepared (unless otherwise stated) from the macronutrient fertilizer Phostrogen (Phostrogen Ltd., Corwen, Clwyd, U.K.) and micronutrients as used for Hoagland's medium (Hoagland & Arnon, 1950). In most experiments phostrogen was used at a concentration of 2 g dm^{-3} and Hoagland's micronutrients at half strength, resulting in plant nutrient concentrations as shown in table 2.1.

Table 2.1 Concentrations of plant nutrients in the nutrient solution used in most experiments.

a) Macronutrients (mol m^{-3}) at 2 g phostrogen dm^{-3}					
K	**Na**	**NO_3**	**PO_4**	**Ca**	**Mg**
9.52	0.40	11.76	2.36	1.04	1.28
b) Micronutrients (g m^{-3}) as in half strength Hoagland's medium					
B	**Mn**	**Zn**	**Cu**	**Mo**	
0.250	0.250	0.025	0.010	0.005	

For treatment with high and low temperature plants were transferred to a temperature-controlled Vindon growth cabinet, with temperatures set as specified and a 12-hour photoperiod. The photosynthetic photon flux density (PPFD) of 260 – 320 μmol m^{-2} s^{-1} (measured at plant height) was provided by high pressure sodium and metal halide lamps. For high temperature treatment plants were

placed in trays which were filled with water throughout treatment to avoid soil moisture deficit. To prevent occurrence of increased water vapor pressure deficit during high temperature treatment cabinet floor and moisture-holding mats underneath the cabinet shelves were kept wet throughout treatment to maintain relative humidity in the cabinet > 80%.

Drought treatment consisted of slowly-induced soil moisture deficit and was imposed by keeping plants in the greenhouse control environment, but placing them on a metal grid to drain off remaining water, and withholding water for periods of time as specified. Before the start of treatment the following measures were taken to slow down the drying process: plants were transplanted to bigger pots (pot size as specified), and the substrate surface was covered with gravel (to reduce evaporation from the soil).

For salt treatment plants were kept in the greenhouse control environment, but treated with salt solutions containing NaCl at concentrations as specified, $CaCl_2$, added as to achieve a ratio of Ca^{2+} to Na^{+} of 1:10, as well as plant nutrients in the form of phostrogen at 1 g dm^{-3} and micronutrients as in half strength Hoagland's medium. Final salinity levels were imposed gradually, with salinity increasing stepwise by a maximum of 40 – 75 mol m^{-3} (as specified) per day. Salinity was imposed **a)** by applying salt solutions manually to the substrate surface or **b)** by flooding plants with salt solutions in an automatic flood bench system. This consisted of solution reservoirs of 300-dm^3 capacity, each supplying solution to 2 plant tubs, each of which accommodated 3 – 4 plants. Before start of salinity treatment, plants were transplanted to bigger pots (pot size as specified). To prevent leaching of substrate by application of salt solutions, substrate surfaces were covered with gravel, and in the case of salt application by flooding, bottoms of pots were lined with mesh tissue. In the case of manual application of solutions pots were placed on a metal grid to drain off excess solution.

2.3 Chlorophyll fluorescence

2.3.1 Theory of chlorophyll fluorescence measurements

Fluorescence phenomena in general were first described as light emission, with emission bands shifted to longer wavelengths than absorption bands (so called Stokes' shift), by Stokes in 1852 who called the phenomenon "dispersive

reflexion" or "fluorescence". The landmark in research on fluorescence of chlorophyll was set by Kautsky & Hirsch who in 1931 published the following observations on chlorophyll fluorescence following illumination of dark-adapted leaves: Chlorophyll fluorescence first rises rapidly to a maximum, then decreases, and finally reaches a steady level. The rising section was unaffected by low temperature or poisoning with HCN and therefore attributed to primary photochemical reactions. The decreasing portion was observed to be inversely related to an increase in the rate of CO_2 assimilation. In general these changes in chlorophyll a fluorescence intensity upon illumination of a dark-adapted leaf or a suspension of higher plant, algal or cyanobacterial cells with continuous light are currently described as fluorescence induction, fluorescence transient, or just Kautsky effect (Govindjee, 1995).

At room temperature about 90% of chlorophyll a fluorescence originates in PSII complexes. Fluorescence emission spectra show a major fluorescence band at 683 – 685 nm and its vibrational satellite at 720 – 735 nm, as well as a minor shoulder at 693 – 695 nm, when reaction centres are closed (see below), all originating in PSII core chlorophyll a protein complexes. Only a minor emission at 705 – 715 nm emanates from PSI (core or antenna). At low temperature (77 K) an increase in fluorescence emission from PSI is observed. Fluorescence emission spectra show two major fluorescence bands at 685 and at 695 nm originating in PSII core complexes. Two other major bands at 720 and 740 nm are assigned to originate in PSI core and PSI light harvesting complex, respectively. However, changes in photochemistry are only reflected in PSII fluorescence, *i.e.* variable fluorescence originates strictly in PSII. Explanations as to why PSI complexes are only weakly fluorescent and do not contribute to variable fluorescence include the following: energy transfer to P700 (the reaction centre chlorophyll a of PSI) is more irreversible; the immediate electron acceptor or donor to P700 does not act as chemical quencher of chlorophyll a; the physico-chemical nature of PSI antenna chlorophyll a favours de-excitation by heat dissipation over fluorescence (Govindjee, 1995).

Chlorophyll a fluorescence is a minor of several pathways competing in the deactivation of excited chlorophyll. Govindjee (1995) describes these interrelations as follows: The quantum yield of chlorophyll a fluorescence Φ_f is measured as a) the total number of photons emitted (F) divided by the total

number of photons absorbed (I_a) or b) as life time of fluorescence (τ) divided by the theoretical intrinsic lifetime of fluorescence, when the only pathway of de-excitation is fluorescence (τ_o). In theoretical terms Φ_f is defined as the rate constant of fluorescence (k_f) divided by the sum of the rate constants of all pathways competing in de-excitation of chlorophyll, *i.e.* fluorescence, heat dissipation (k_h), excitation energy transfer (k_t), quenching by quenchers (k_q), and photochemistry (k_p). Krause & Weis (1991) roughly quantify Φ_f as about 30% for chlorophyll a in solution, and for chlorophyll a in the photosynthetic apparatus as around 3% at maximum and around 0.6% at minimum fluorescence.

The fluorescence transient is generally divided into 2 sections according to their time scale (Govindjee, 1995): 1) the fast transient (up to 1 s) consisting of a fluorescence rise from O (origin) to I (inflection), then a decrease to D (dip), followed by a rise to P (peak); 2) the slow transient (up to several minutes) in which fluorescence decreases from P to S (quasi-steady state), then rises to M (a maximum), and finally declines to T (terminal steady state).

The fast transient on illumination is easier to interpret than the slow one (Govindjee, 1995) and reflects the successive, but overlapping reduction of the electron acceptor pool of photosystem II, including mainly Q_A (one-electron acceptor-bound plastoquinone), Q_B (two-electron acceptor-bound plastoquinone), and PQs (mobile plastochinone molecules). It is generally accepted that of these the redox state of Q_A is the major determinant of fluorescence changes. When all Q_A is oxidised, the reaction centres are said to be in an open state, and Φ_f is minimal, when Q_A is fully reduced, reaction centres are said to be closed, and maximum Φ_f is achieved (Krause & Weis, 1991).

The decline of chlorophyll fluorescence in the slow transient is called fluorescence quenching and generally denotes all processes lowering Φ_f below its maximum. It is subdivided into a) photochemical quenching which is caused by the re-oxidation of reduced Q_A and b) non-photochemical quenching comprising all quenching mechanisms not directly related to the redox state of Q_A (Krause & Weis, 1991). While photochemical quenching is mainly due to the light-induced activation of enzymes in carbon metabolism and the opening of stomata, non-photochemical quenching is mainly caused by an increased efficiency of energy dissipation as heat (Maxwell & Johnson, 2000).

The practical procedure of a typical chlorophyll fluorescence measurement is described according to Maxwell & Johnson (2000). The measurement is started by switching on the measuring light and measuring minimal fluorescence (F_o). Then a saturating flash of light is applied, giving a measure of the maximum fluorescence in the dark-adapted state (F^o_m). After that actinic light (light to drive photosynthesis) is switched on. At certain intervals further saturating flashes of light are applied giving measures of fluorescence maxima in the light (F'_m). The steady-state value of fluorescence immediately prior to a flash is called terminal fluorescence (F_t). After a flash actinic light is temporarily removed (preferably whilst applying far-red light) giving a measure of minimal fluorescence in the light (F'_o). The basic principle underlying this measurement is the "light doubling" technique: a high intensity, short duration flash of light is applied to transiently close all PSII reaction centres and thereby temporarily reduce the contribution of photochemical quenching to zero. The duration of the flash has to be short enough not to induce changes in non-photochemical quenching or long-term changes in efficiency of photochemistry. F_m is therefore the fluorescence yield in the absence of any photochemical quenching.

A relationship between measured fluorescence parameters and quantum yield of photochemistry of PSII (Φ_p) was initially derived as follows (Govindjee, 1995). At low light intensities most Q_A is in the oxidised state, and chlorophyll a fluorescence yield (Φ_f) is minimal: (1) $\Phi_f{}^{min}$ (at F_o) = $k_f / (k_f + k_h + k'_p)$. At high light intensities all Q_A is reduced, therefore k'_p is minimal and chlorophyll a Φ_f is maximal: (2) $\Phi_f{}^{max}$ (at F_m) = $k_f / (k_f + k_h)$. Dividing (1) by (2) yields: $\Phi_f{}^{min} / \Phi_f{}^{max} = [k_f / (k_f + k_h + k'_p)] \times [(k_f + k_h) / k_f] = (k_f + k_h) / (k_f + k_h + k'_p)$. Adding and subtracting k'_p in the numerator: $(k_f + k_h + k'_p - k'_p) / (k_f + k_h + k'_p) = 1 - [k'_p / (k_f + k_h + k'_p) = 1 - \Phi_p{}^{max}$. Thus $\Phi_p{}^{max} = 1 - (\Phi_f{}^{min} / \Phi_f{}^{max}) = (\Phi_f{}^{max} - \Phi_f{}^{min}) / \Phi_f{}^{max}$ which is equivalent to F_v (variable fluorescence) / F_m. This calculation assumes that k_t and k_q (see above) can be ignored and that none of the rate constants k_f, k_h, and k'_p change between F_o and F_m.

The F_v/F_m ratio of chlorophyll fluorescence $F_v/F_m = (F_m - F_o) / F_m$ therefore gives (as derived above) the intrinsic or maximum efficiency of PSII, *i.e.* it indicates the quantum efficiency of PSII, if all PSII centres were open. It can be measured a) after dark-adaptation (calculated from F^o_o and F^o_m) or b) in the light (calculated from F'_o and F'_m). Changes in F_v/F_m are due to changes in the

efficiency of non-photochemical quenching (Maxwell & Johnson, 2000). Dark-adapted values of F_v/F_m fall into a narrow range of 0.832 ± 0.004 for different species and ecotypes (Krause & Weis, 1991). A decrease in dark-adapted F_v/F_m below this range indicates stress, in particular photoinhibition (Maxwell & Johnson, 2000). The F_v/F_m ratio interrelates two other fluorescence parameters: $F_v/F_m = \Phi_{PSII} / qP$. The efficiency of PSII photochemistry is calculated as $\Phi_{PSII} = (F'_m - F_t) / (F'_m)$. It measures the proportion of light absorbed by chlorophyll a associated with PSII and used in photochemistry, and therefore relates to achieved (as opposed to maximum) efficiency. Under laboratory conditions it is linearly related to the efficiency of carbon fixation. However, under stress conditions efficiency of PSII photochemistry and efficiency of CO_2 fixation may deviate due to electron flux to alternative sinks, e.g. photorespiration, pseudocyclic electron transport (Mehler-ascorbate peroxidase pathway) or nitrogen metabolism. Photochemical quenching qP is calculated as $(F'_m - F_t) / (F'_m - F'_o)$ and indicates the proportion of open PSII reaction centres (Maxwell & Johnson, 2000).

Non-photochemical quenching (NPQ) is calculated as $(F^o_m - F'_m) / F'_m$. It indicates the change in efficiency of heat dissipation relative to the dark-adapted state. In the laboratory the dark-adapted value of F_m (F^o_m) is measured after overnight to 24-hour dark-adaptation, in the field a predawn measurement of F_m can be used. As F^o_m is sensitive to the stress history of a plant, NPQ of leaves with different histories resulting in differing dark-adapted values of F_v/F_m should not be directly compared (Maxwell & Johnson, 2000).

The different component processes of NPQ can be distinguished by the rates at which they relax after a period of illumination. Fast relaxing NPQ is reversed within a few minutes and comprises a) high energy state quenching (qE) and b) state transition quenching (qT). Slowly relaxing quenching is reversed only in a matter of hours and is due to photoinhibition (qI) (Maxwell & Johnson, 2000). High energy state quenching (qE) capacity was found to be related to intrathylakoid acidification (due to light-driven proton translocation across the thylakoid membrane) and to zeaxanthin content and is based on increased heat dissipation in PSII (Krause & Weis, 1991). State transition quenching (qT) is caused by a reduced absorption cross section of PSII relative to PSI. It only makes a minor contribution to NPQ and reaches a maximum under low light, but is suppressed under high light conditions. qT involves phosphorylation of part of

light harvesting complex (LHC) II leading to transition of PSII from state 1 to state 2. LHCII is consequently detached from the core antenna of PSII and moves from appressed to non-appressed membrane regions. If in β-form, light energy absorbed by phosphorylated LHCII can be transferred to PSI, a process termed spillover (Krause & Weis, 1991). Photoinhibitory quenching (qI) is related to photoinhibition of photosynthesis in response to either high light exceeding light saturation of photosynthetic CO_2 assimilation and the capacity of qE or in response to moderate light in combination with conditions limiting energy utilisation in carbon metabolism or affecting repair processes (Krause & Weis, 1991). qI can be due to a) photoprotective processes and b) damage to the reaction centres of PSII (Maxwell & Johnson, 2000). Photoprotective processes are related to the presence of zeaxanthin, occur in the light-harvesting antenna of PSII, and can be identified by a change in the F_o level of quenching. Damage to the reaction centres of PSII results in quenching within the PSII reaction centre and no change in the F_o level of quenching.

2.3.2 Measurement of F_v/F_m with the Hansatech Fluorescence Monitoring System

The Hansatech Fluorescence Monitoring System (Hansatech Instruments Ltd., King's Lynn, UK) consists of a control unit housing electronics, optics, and light sources and a fibre optic interface module which links the control unit to the plant sample. A fibre-optic adapter, designed to shield the sample from ambient light during measurement, was attached to the end of the fibre optic.

Before measurement leaf samples were dark-adapted for 20 minutes using plastic dark-adaptation leaf-clips provided. They contain a foam pad to support the sample and a metal sliding shutter which is closed for dark-adaptation. After the dark-adaptation period the fibre-optic / adapter assembly was sealed over the dark-adaptation clip and the shutter plate opened for fluorescence measurement.

The measurement started with application of the measuring light, a modulating beam of 1.8 μs duration pulses, provided by a pulsed amber LED light source. The integrated amount of radiation incident upon the sample produced by the modulating beam is < 0.05 μmol photons m^{-2} s^{-1} and therefore considered too small to cause significant physiological changes. Pulse peak tracking electronics

in the instrument amplify and measure only pulsed fluorescence signals, while they discard ambient light and non-pulsed fluorescence, a technique termed modulated fluorometry. Minimal fluorescence (F_o) was determined by averaging the fluorescence signal produced in response to only the modulating beam over a period of 1.6 s.

Maximal fluorescence (F_m) was then measured by applying a saturating light pulse of 10800 µmol photons m^{-2} s^{-1} of a duration of 0.3 to 5 s, provided by an internal halogen saturation lamp. Variable fluorescence (F_v) and F_v/F_m were automatically calculated.

2.4 Leaf gas exchange

2.4.1 Enclosure method

The basic principle underlying the measurement of gas exchange by a leaf is the so-called enclosure technique: a leaf is enclosed in a chamber through which air is pumped, and the changes in concentrations of CO_2 and water vapour, due to CO_2 assimilation and transpiration, respectively, in the air flowing across that chamber are measured.

In an open or differential system leaf assimilation rate (A) is calculated as $A = [u (c_e - c_o)] / s$, where c_e and c_o are the measured concentrations of CO_2 at the entrance and at the outlet of the chamber, respectively, u denotes the known mole flow of air through the chamber, and s the projected surface area of the leaf. Dilution of CO_2 concentration due to increased water vapour concentration produced by transpiration is accounted for by multiplying with $(1 - w_o) / (1 - w_e)$, where w_o and w_e are the measured water vapour concentrations at the outlet and at the entrance of the chamber, respectively (Long & Haellgren, 1993). Leaf transpiration rate (E) can be calculated similarly.

2.4.2 Infrared gas analysis

The measurement principle of infrared gas analysis is based on the fact that hetero-atomic gas molecules like CO_2 and H_2O absorb infrared (IR) light at specific IR wavebands. CO_2 shows a major absorption band at 4.25 µm and secondary peaks at 2.66, 2.77, and 14.99 µm.

The basic parts of an infrared gas analyser (IRGA) are an IR source, two parallel cells termed the analysis and the reference cell, each containing a gas inlet and outlet, and a detector. An air supply unit supplies air of controlled CO_2 concentration and humidity at a controlled flow rate to the leaf chamber. Air which has passed through the chamber is then pumped through the analysis cell, air coming directly from the air supply unit through the reference cell. Measurement of CO_2 concentrations in reference and analysis air will give the CO_2 concentrations at the entrance (c_e) and outlet of the chamber (c_o), respectively, used in the calculation of assimilation rate as outlined above. The IR source is a spiral of nichrome alloy or tungsten, heated to 600 – 800°C through a low voltage circuit. Two parallel beams of IR, produced by two IR sources in series or by splitting the beam of a single source, pass through reference and analysis cell to the detector. Non-dispersive (NDIR) instruments use broad-band IR as produced by the source, while dispersive (DIR) instruments pass the source radiation through a monochromator before entering the cells (Long & Haellgren, 1993).

When IR light is passed through the cells, any CO_2 present will absorb IR at the characteristic wavebands and reduce the intensity of the IR beam reaching the detector at these wavelengths. The reduction in intensity of a light beam of a particular wavelength λ is expressed as absorbance $A_\lambda = \log [I_{o(\lambda)} / I_{(\lambda)}]$, where $I_{o(\lambda)}$ denotes the initial intensity of the light beam and $I_{(\lambda)}$ the intensity after passing the cell. The absorbance of a substance at a particular wavelength λ is proportional to the concentration of that substance c, a relationship called Beer-Lambert law: $A_\lambda = \varepsilon_\lambda \times c \times b$, where b is the length of the optical path and ε_λ the molar absorption coefficient at the wavelength λ, a constant for the absorbing substance (Lewis & Evans, 1997).

2.4.3 Analysis of gas exchange measurements: calculation of g_s and c_i

The fluxes of water vapour out of the leaf and CO_2 into the leaf, as related to transpiration and photosynthetic assimilation, respectively, can be described by analogy to Ohm's Law. The driving force for the flux of water vapour out of the leaf is the steep gradient of water vapour concentration between the air in the leaf

intercellular air spaces (which is almost saturated with water vapour due to the huge surface area of mesophyll cells exposed to the intercellular space) and the atmosphere (Lambers *et al.*, 1998). A net influx of CO_2 into the leaf occurs due to the steep gradient of CO_2 concentration between the sites of CO_2 assimilation within the mesophyll and the atmosphere. Applying Fick's Law of diffusion, which describes the molecular flux along a one-dimensional diffusion pathway as ratio of concentration difference to the diffusion resistance across the pathway, transpiration and assimilation rate can be expressed as follows: $E = (w_i - w_a) / \Sigma r'$, where w_i and w_a are the water vapour concentrations at the mesophyll cell surface and in the bulk air, respectively, and $A = (c_a - c_c) / \Sigma r$ with c_a and c_c being the CO_2 concentrations in the bulk air and at the site of carboxylation, respectively. $\Sigma r'$ and Σr are the total resistances to transfer of water vapour and CO_2, respectively. The total resistance can be divided into several component resistances in analogy to resistors in an electrical circuit. Flux can also be related to conductance to gas diffusion (g) which is the reciprocal of resistance (Long & Haellgren, 1993).

From these considerations of diffusion, the total gas phase conductance of the leaf to H_2O diffusion (g'_l) can therefore be calculated using the measured flux of water vapour (E) and the measured water vapour concentration in the bulk air (w_a), and assuming that the air at the mesophyll cell surface is saturated with water vapour at the leaf temperature T ($w_{s[T]}$) as: $g'l = E / (w_{s[T]} - w_a)$. The corresponding total gas phase conductance of the leaf to CO_2 diffusion (g_l) can be derived from this by dividing by the ratio of the binary diffusivities of water vapour / air and CO_2 / air which is assumed to be 1.6 for stomatal and 1.37 for boundary layer conductance (due to additional turbulent transfer): $1/g_l = 1.61 / g'_s = 1.37 / g'_b$, where g'_s and g'_b are stomatal and boundary layer conductance to H_2O diffusion, respectively. g'_b in the leaf chamber is measured during the production process in the factory using an area of wet filter paper as leaf simulant. This value of g'_b can be used for leaves of similar shape to the laminar simulant, but has to be re-evaluated for leaves of different form. g'_s is calculated as $g'_s = g'_l \times g'_b / (g'_b - g'_l)$ (Long & Haellgren, 1993).

Finally the CO_2 concentration in the leaf intercellular air space (c_i) can be calculated from the measured flux of CO_2 (A), the CO_2 concentration in the bulk air (c_a), and the calculated value of total gas phase conductance of the leaf to CO_2

diffusion (g_l) as: $c_i = c_a - A / g_l$ (Long & Haellgren, 1993). In addition the mass flow of gases out of the leaf, due to a pressure gradient resulting from domination of gas efflux (mainly water vapour) over influx, has to be accounted for by including the transpiration rate (E) in the calculation: $c_i = [(g_l - E/2) \times c_a - A] / (g_l + E/2)$ (Long & Haellgren, 1993).

Distinguishing stomatal and mesophyll limitations to CO_2 assimilation is not straightforward. Mesophyll resistance is calculated as gradient in CO_2 concentration between the intercellular air space (c_i) and the site of carboxylation (c_c) divided by the assimilation rate (A). By assuming c_c to be zero, Σr can be regarded as total diffusion resistance to transfer of CO_2. Theoretically the relative limitation by stomata could then be calculated as ratio of stomatal resistance to total leaf resistance. However, this definition can only be used under CO_2-limiting conditions, but is incorrect, as CO_2 saturation is approached (the response of A to increasing c_i is of hyperbolic or asymptotic nature). In a different approach the relative limitation by stomata is calculated as the relative reduction of the actual assimilation rate (A) measured at atmospheric CO_2 concentration c_a below the potential rate which would occur at infinite stomatal conductance (A_o), *i.e.* the value of A extrapolated from the A/c_i response curve at c_i corresponding to c_a above (Long & Haellgren, 1993, Jones, 1992). However, although this method has the advantage of not making the assumption of linearity of the A/c_i response, Jones (1992) criticises the assumption of infinite stomatal conductance as unrealistic, and suggests control analysis to resolve stomatal and mesophyll limitations. Control coefficients (C) reflect the response of steady-state fluxes (J) to small changes in certain parameters (b_i), both of which are defined as fractional changes to allow independence from measurement units: $C_{bi} = (\delta J / J) / (\delta b_i / b_i)$. Stomatal limitation can accordingly be defined as relative sensitivity of A to a small change in stomatal resistance.

2.4.4 Measurement of leaf gas exchange with the CIRAS-1 Combined Infrared Gas Analysis System and the CIRAS-1 Parkinson Leaf Cuvette with Automatic Control System for the Ciras Cuvette Environment

The CIRAS-1 Combined infrared gas analysis system (PP Systems, Hitchin, Hertfordshire, UK) was used to measure transpiration, stomatal conductance, net rate of CO_2 uptake, and CO_2 mole fraction in the leaf intercellular air space. Its basic parts are: a) The CIRAS-1 Differential CO_2/H_2O Infrared Gas Analyser, which combines 4 independent infrared gas analysers (IRGAs), *i.e.* separate ones for the measurement of CO_2 and water vapour concentration and for analysis and reference cell. The IRGAs use broad-band IR (non-dispersive) and work as absolute absorptiometers, with microprocessor control and linearisation. Gas is pumped through analysis and reference cells by integral DC pumps fitted with mass flow controllers at 100 cm^3 min^{-1}. b) The Cuvette Air Supply Unit, which supplies air at a flow rate controlled by a mass flowmeter to the leaf cuvette. An air conditioning system allows control of the concentrations of CO_2 (0 to 2000 ppm) and water vapour (0 to saturating vapour pressure at the air temperature). CO_2 concentration is regulated by adding defined amounts of CO_2 from a compressed gas cylinder (a replaceable CO_2 cartridge with about 8-hour running time) to ambient air, which has been passed through a column of CO_2-absorbing sodalime. Humidity is adjusted (to below ambient) by passing a defined proportion of the air stream through a column containing the drying agent drierite (anhydrous calcium sulphate).

The CIRAS-1 Combined infrared gas analysis system was operated in combination with the CIRAS-1 Parkinson Leaf Cuvette with Automatic Control System for the Ciras Cuvette Environment (PP Systems, Hitchin, Hertfordshire, UK). The cuvette is made from aluminium alloy, the cuvette window from interference filter (Calflex 3000), which removes most IR radiation above 0.75 microns, the leaf seal from foam gaskets. It is supplied with an air circulation fan (to maximise leaf boundary layer conductance). The cuvette is also fitted with sensors for measuring photosynthetic active radiation (a fully cosine corrected, filtered silicon cell) and air and leaf temperature (Betatherm 100K6 precision

thermistors). The Automatic Control System for the Ciras Cuvette Environment allows regulation of leaf temperature and light intensity. The light unit consists of halogen bulbs (specifications for main bulbs M35 12V 20W G4, for smaller axial bulbs 6V 115mA), and is supplied with a cooling fan. Light intensity can be adjusted to photon flux densities (PFD) of 15 to 2000 μmol m^{-2} s^{-1}. Leaf temperature is controlled by peltier elements built into the chamber walls.

Gas exchange rates were measured in an open system under controlled cuvette environment conditions. CO_2 concentration and relative humidity of air entering the cuvette were set to 350 ppm and 5%, respectively. Photon flux density was set to 1500 μmol m^{-2} s^{-1}, leaf temperature to mimic ambient conditions. Measurements of gas exchange were taken after establishment of steady-state rates of gas exchange (up to 5 minutes).

2.4.5 Significance of the measured leaf assimilation rate

Leaf assimilation rate (A) is measured as net rate of CO_2 uptake per unit leaf area (Long & Haellgren, 1993). As such it is equivalent to net photosynthesis (P_n) which is defined as the difference between the gross rate of CO_2 fixation (P_g) and the rate of respiratory CO_2 loss (R), the latter being the sum of dark respiration and photorespiration (Jones, 1992).

The general nature of the processes of gross photosynthesis, dark respiration, and photorespiration and their contributions to the measured net photosynthetic rate shall be outlined briefly as follows, according to Buchanan *et al.* (2000).

Gross photosynthesis results from the Calvin cycle reactions which are also termed dark reactions of photosynthesis, although they also occur in the light and depend on the light reactions for high-energy products and for co-ordinating regulatory signals. The Calvin cycle proceeds through 13 steps in three phases. In the carboxylation phase the enzyme ribulose bisphosphate carboxylase/oxygenase (Rubisco) catalyses the carboxylation of the five-carbon sugar ribulose 1,5-bisphosphate (RuBP) and the immediate cleaving of the C_6 intermediate into two molecules of 3-PGA. In the two-step reductive phase 3-PGA is converted to the triose phosphate glyceraldehyde 3-phosphate (GAP), using ATP and NADPH. In the last and largest phase, involving 10 of the 13 Calvin cycle enzymes, RuBP is regenerated, using ATP. Looking at the balance, carboxylation of three molecules

of RuBP yields six molecules of 3-PGA, which are phosphorylated and reduced to GAP. Five of the six GAP and three ATP are used to regenerate the three RuBP molecules. The net product, the remaining GAP, can be used for the synthesis of carbohydrates or other cellular constituents.

Dark respiration involves the controlled oxidation of reduced organic substrates, including carbohydrates, lipids, proteins, amino acids, and organic acids, to CO_2 and H_2O. The first phase is glycolysis, in which carbohydrates are oxidized to organic acids in the cytosol. In the citric acid cycle occurring in the mitochondrial matrix these organic acids are completely oxidized to CO_2. The electrons released during this cylce are transported via a chain of multiprotein complexes in the inner mitochondrial membrane, and ultimately transferred to O_2, reducing it to H_2O. During this electron transport a proton electrochemical gradient across the inner membrane is created which is used by the ATP synthase complex to produce ATP. This chemical bond energy can be used to drive metabolic reactions of plant growth, development and maintenance. Metabolic intermediates of the primary pathways of respiration also form the substrates for the synthesis of nucleic, amino, and fatty acids and many secondary metabolites. Respiration is regulated by the availability of ADP and P_i.

In general the entire plant shows high values of dark respiration during the night. In the light all non-photosynthetic tissues respire, while respiration of photosynthetic tissues is difficult to measure, as respiratory CO_2 release is masked by photosynthetic CO_2 uptake. Darkened leaves show respiration rates in a range of only 5 to 10 % of maximum photosynthetic rates. Indirect measurements of CO_2 output from leaves and determination of CO_2 compensation points led to the conclusion that light slows the rate of carbon flux through the citric acid cycle by about 50 %. Theoretical considerations include that on the one hand photosynthesis is thought to increase the cytosolic ATP/ADP ratio leading to inhibition of respiration. On the other hand the requirement of metabolic intermediates of respiration in many biosynthetic pathways, as well as the requirement of high cytosolic ATP concentrations for sucrose synthesis are arguments for the necessity of some respiration in photosynthetic tissues in the light.

Photorespiration involves the light-dependent uptake of O_2 and evolution of CO_2 during photosynthesis (three molecules of O_2 are taken up for every CO_2

released). The first step is the oxygenase reaction catalysed by the enzyme Rubisco, in which molecular oxygen reacts with RuBP producing an unstable C_5 intermediate, which breaks down to 3-PGA and 2-phosphoglycolate. 2-phosphoglycolate cannot be used in the Calvin cycle. In the second phase, the C_2 oxidative photosynthetic carbon cycle, two molecules of 2-phosphoglycolate are therefore converted to one molecule of CO_2 and one of 3-PGA, which can return to the Calvin cycle. Thus 75 % of the carbon in 2-phosphoglycolate is salvaged in the form of 3-PGA, while 25 % is lost as CO_2. The reactions of the C_2 cycle occur in three different organelles: the chloroplasts, peroxisomes, and mitochondria.

The inhibition of gross photosynthesis by photorespiration arises due to a) the release of CO_2 in the C_2 cycle and b) the prevention of the carboxylation reaction of Rubisco. Theoretical considerations show that, as the rate of carboxylation to oxygenation is about 3 to 1 under current atmospheric conditions, and 0.5 molecules of CO_2 are released for each oxygenation, the rate of photorespiratory CO_2 production should form about 16 % of gross photosynthetic CO_2 uptake. Measurements using oxygen isotopes to discriminate between photosynthetic and photorespiratory CO_2 exchange showed in fact rates of photorespiratory CO_2 production in the range of 18 to 27 % of gross photosynthetic CO_2 uptake. Photorespiration prevents the carboxylation reaction of Rubisco, as for every CO_2 released two Rubisco oxygenation reactions take place, thus preventing CO_2 from being fixed instead. Replacing these two O_2 molecules by CO_2 would cause an additional increase of photosynthetic rate by 40% (assuming photorespiratory CO_2 release to be roughly 20 % of gross photosynthetic CO_2 uptake). Experiments investigating net photosynthetic rates in C_3 plants at conditions of 1 to 2 % O_2 showed increases by 50 to 70 %, corresponding well to the above calculations.

2.5 Soil moisture content

Volumetric soil water content was measured with the Theta Probe Type ML1-UM-2 (Delta-T Devices Ltd., Cambridge, UK). Its main parts are a power supply and an analogue output signal display, connected via a cable to the electronics, which are protected by waterproof housing, the latter having 4 stainless steel rods attached to it. For measurement the steel rods are inserted into the soil until they are fully covered.

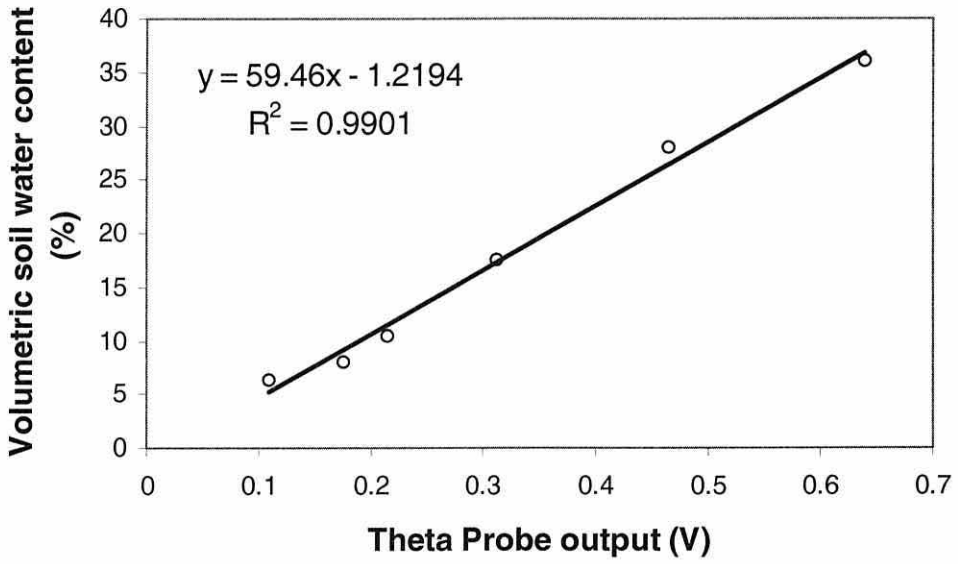
The Theta Probe measures the volumetric soil moisture content by applying a 100 MHz signal to the soil via the steel rods, using a special transmission line, the impedance of which changes with the impedance of the soil. Soil impedance is generated by a) the apparent dielectric constant and b) ionic conductivity, however, at the signal frequency used the influence of ionic conductivity can be ignored. The change in impedance creates a voltage standing wave, which again changes the voltage produced by a crystal oscillator. This signal is used to derive the apparent dielectric constant of the soil.

The volumetric soil water content (θ_V) is defined as $\theta_V = V_W / V_S * 100$, where V_W is the volume of water in a soil sample and V_S the total volume of the sample. Volumetric as opposed to gravimetric soil water content is used, as the volume of the soil sample can be assumed to remain constant with addition of water, so that the bulk density of the soil can be ignored. The relationship between the output in Volt produced by the Theta Probe and the volumetric soil moisture content is approximately linear in an output range of 0 to 0.9 V, beyond which it becomes excessively non-linear. As this relationship depends on the particular composition of the soil, the Theta Probe has to be calibrated for the specific soil used in an experiment.

The Theta Probe was used in the above mentioned probe output range of 0 to 0.9 V, in which the output is approximately linearly related to the volumetric soil water content. Therefore a slope and offset conversion, using a linear-fit equation from experimental data involving the same substrate as used in all following experiments, was used to derive values of volumetric soil water content. In a preliminary experiment seeds of *Gossypium hirsutum* var. CIM 443 were sown in 2.5-l pots filled with John Innes No. 1 compost on April 1st, 1999. When 58 days old, six plants were exposed to slowly induced drought by withholding water for 9 days. Before start of drought treatment plant pots were covered with plastic foam lids to reduce evaporation from the soil surface and slow down the drying process. On days 2, 3, 4, 6, and 9 after initiation of drought treatment, the substrate of one plant each was assessed as follows (except on day 3, when the substrate of two plants was assessed). a) Six Theta Probe measurements per plant, spread evenly across the substrate, were taken, the average of which formed the value for each plant. b) Then plant shoots were cut off at the stem base, and the volumetric water content of the plant substrate was determined as $\theta_V = (FW_s -$

DW_s) / V_s * 100, where FW_s denotes the fresh weight (kg), V_s the volume (l) at the time of taking the fresh weight, and DW_s the dry weight (kg) after oven-drying at 60°C for a minimum of 48 hours of the sample. Finally a linear regression was fitted to data pairs of Theta Probe output and volumetric soil water content for each plant (Figure 2.1). The linear-fit equation was used for slope and offset conversion of Theta Probe outputs in all experiments involving this technique.

Figure 2.1 Plot of Theta Probe output versus volumetric soil water content in the substrate of six plants of *Gossypium hirsutum* after exposure to varying periods of soil moisture deficit. The line is fitted by linear regression, and equation and regression coefficient (R^2) are shown.



2.6 Leaf water relations

Leaf water content (WC) and relative water content (RWC), two parameters expressing the amount of water in a plant sample, were determined as described by Beadle *et al.* (1993): WC = (FW – DW) / DW * 100 and RWC = (FW – DW) / (TW – DW) * 100, where FW denotes fresh weight at the time of sampling, DW dry weight after oven-drying at 70°C, and TW turgid weight. The method of obtaining turgid weight as described by the above authors (floating leaf samples on water at the light compensation point, until they reach constant weight) was modified for this study as follows. Directly after measurement of fresh weight leaf samples were placed between two sheets of tissue paper, which had been soaked in water, and were kept in darkness at 5°C for a period of 18 hours to 8 days (13 days in one instant). The duration of this period was constant for groups of pretreated plants, which were subjected to the same subsequent treatment including, in most cases, the experimental controls they were compared to.

Leaf samples used for the measurement of leaf water content and relative water content were entire leaf laminae or, in most experiments, single leaf lobes of medium-aged leaves.

2.7 Plant growth

Shoot height was measured as distance between stem base and shoot apex. Shoot dry matter production was determined by harvesting plant shoots, separating them into stem, leaves, and reproductive organs (flowers and fruits), and then oven-drying plant components at 70°C for a minimum of 48 hours.

2.8 Leaf sap extraction

Leaf samples consisted of leaf lobes of medium-aged leaves. They were placed in 1.5 cm^3 polyethylene microcentrifuge tubes, and frozen and stored at approximately -18°C. The freezing process causes damage to plant cell membranes, making the cell sap available for extraction.

After thawing sap was extracted by centrifuging plant samples in their microcentrifuge tubes, which had been pierced at the bottom (and at the top to allow pressure equilibration) at 10 000 g for 10 minutes, collecting the sap in another 1.5 cm^3 polyethylene microcentrifuge tube placed underneath. Extracted sap was frozen and stored at approximately -18°C until analysed. For analysis samples were left to thaw and then briefly vortexed.

2.9 Concentration of cations and glycinebetaine in leaves

2.9.1 High performance ion exchange chromatography

A Dionex 2000i chromatograph (Dionex (UK) Ltd, Camberley, Surrey) was used to measure concentrations of cations and glycinebetaine in leaf sap samples. Samples were injected by means of the Spark-Holland "Marathon" autosampler fitted with a 5 mm^3 PEEK sample loop (with a capacity of 96 vials). In principle a loop injector consists of a metal loop of a small fixed volume, which is filled with the sample. By means of a valve switching system the eluant flow from the pump can be channelled either directly onto the column or via the loop to the column. In this way the sample can be transferred to the column without interrupting the column flow (Wilson & Walker, 1994). A Shodex differential

refractometer was used as detector. A differential refractometer monitors the presence of analytes in the column effluent by continuously measuring the difference in refractive index between pure solvent and column effluent, and has a sensitivity of around 0.1 μg (Boyer, 1993). The detector was linked to a Shimadzu CR5A plotting integrator.

Cations as well as quarternary ammonium compounds (QACs) including glycinebetaine were separated by ion exchange chromatography. Ion exchange chromatography is designed to separate charged compounds by creating an ion-exchange equilibrium between a stationary ion exchanger and a mobile electrolyte phase (Wilson & Walker, 1994). Ion-exchange column packings with charged functional groups are prepared by chemically bonding the ionic groups to the support matrix via silicon atoms or by using polystyrene-divinylbenzene resins (Boyer, 1993).

2.9.2 Determination of cations in expressed leaf sap

For HPLC analysis 12 mm^3 leaf sap was diluted in an autoinjector vial with 1.5 cm^3 eluant (see below). Concentrations of Na^+, K^+, Ca^{2+} and Mg^{2+} in leaf sap samples were determined using a CS12 cation exchange column and a self-regenerating cation suppresser operated in auto-regeneration model. The column was operated at 50°C. 20 mol m^{-3} methane sulphonic acid in deionized water (> 18 MOhms) was used as eluant at a flow rate of 1 cm^3 min^{-1}.

Ion concentrations were quantified by measuring peak heights on the chart output produced by the integrator, and converting to values of concentration by using external standards. External standards contained Na^+ and K^+ at 250 mol m^{-3} and Ca^{2+} and Mg^{2+} at 100 mol m^{-3} and were diluted and analysed in the same way as leaf samples. Two standards each were run in intervals of ten leaf samples.

2.9.3 Determination of glycinebetaine in expressed leaf sap

Leaf sap samples were prepared for analysis by HPLC by dilution of 20 mm^3 of leaf sap with 1.5 cm^3 eluant (see below) in an autoinjector vial. Quarternary ammonium compounds and carbohydrates were separated using a

250 x 4 mm Sarasep Na^+-form carbohydrate column. The column was operated at 80°C at a flow rate of 0.6 cm^3 min^{-1}, using 25 mol m^{-3} Na_2SO_4 as eluant.

Separated compounds were quantified by measuring peak heights on the chart output produced by the integrator. Concentrations of glycinebetaine were inferred from external standards, which contained glycinebetaine, dimethylglycine, sucrose, fructose, glucose, and proline, each at concentrations of 100 mol m^{-3}, and were diluted and analysed as leaf sap samples.

2.10 Statistical data analysis

Effects of environmental stress treatments on the physiological parameters studied were investigated by 2-tailed independent samples t-test for comparison of two groups, and by single-factor analysis of variance (ANOVA) for comparison of more groups. When the ANOVA showed a significant difference between groups, multiple pair wise comparisons between individual groups were performed by Tukey's test. To remove the effect of plant size on certain physiological parameters measured in response to drought treatment, the effect of differences in soil moisture deficit as a result of differences in plant size was removed by single-factor analysis of covariance (ANCOVA). The assumptions associated with ANCOVA, including a linear relationship between the dependent variable and the covariate and the homogeneity of these regression slopes for all groups, were tested as described by Pallant (2001). SPSS for Windows (Version 10.0) was used for all calculations.

3 Effects of pretreatment with moderate heat stress on resistance to severe heat stress

3.1 Experiment 1

3.1.1 Experimental protocol

The objective of this experiment was to investigate whether pretreatment with moderately elevated temperature would affect response to subsequent exposure to severe heat stress. As the aim was to test two pretreatment temperatures, but only one temperature-controlled growth cabinet was available, the experiment was split into two consecutive phases.

Two sets of seeds of *Gossypium hirsutum* var. CIM 443 were sown as described in 2.1 on June 21st and July 1st 1999, respectively. Seedlings were transplanted to 1.6-dm^3 plastic pots 18 and 25 days after sowing for the first and second set, respectively.

Both sets of plants were exposed to pretreatment, when they were 36 days old. Plants of the first set were randomly assigned to either treatment with high temperature of 40°C for 7 days or treatment with control temperature of 25°C for the same period of time. Plants of the second set were treated with 45°C for 7 days. Response to pretreatment was assessed by measuring gas exchange parameters and F_v/F_m of chlorophyll fluorescence on the final day (day 7) of pretreatment.

Directly after pretreatment (without recovery) differently pretreated plants of both sets of plants were exposed to treatment with severe heat stress of 50°C for 3 days, except some plants of the first set assigned to control pretreatment, which were maintained in control temperature, and formed the experimental control. Plant response to severe heat stress was investigated on day 3 of treatment by assessment of visible leaf injury and measurement of gas exchange and chlorophyll fluorescence parameters. As exposure of plants that had been pretreated with control temperature to 3 days of severe heat stress resulted in leaf death, their response to severe heat treatment could only be characterised by assessment of visible leaf injury.

Experimental control plants, which were part of the first set of plants, were measured only once in the course of the experiment, on the day which

corresponded to day 3 of severe heat stress treatment of plants assigned to stress treatments.

3.1.2 Results

3.1.2.1 Plant response to pretreatment at moderately elevated temperature

When 36-day old plants of *Gossypium hirsutum* were exposed to moderately elevated temperature of either 40°C or 45°C for 7 days, they showed significant changes in gas exchange parameters on the last day of this period in comparison to control plants (maintained at 25°C), and these changes differed for the two temperature regimes tested (Table 3.1). Exposure to 40°C induced a significant increase in transpiration rate, whereas net assimilation rate, stomatal conductance, and intercellular CO_2 concentration were comparable to control plants. In contrast, treatment with 45°C resulted in a significant decrease in net assimilation rate, leaf stomatal conductance, and intercellular CO_2 concentration, but transpiration rate was unaltered. Maximum quantum yield of PSII, determined as ratio of variable to maximum chlorophyll fluorescence (F_v/F_m) at the end of treatment, was not significantly altered by treatment with either 40 or 45°C (Table 3.4).

Table 3.1 Response of transpiration (E), stomatal conductance (g_s), net rate of CO_2 uptake (A) and CO_2 mole fraction in the leaf intercellular air space (c_i) in *Gossypium hirsutum* to treatment with moderately elevated sublethal temperature (40 or 45°C for 7 days). Heat-treated and control plants (maintained at 25°C) were 43 and 46 days old, respectively, at the time of measurement. Means ± standard error for 5 (Control) or 10 (40 and 45°C treatment) plants. Where there are no letters in common between treatment means, they differ significantly ($P < 0.05$).

	E ($mmol\ m^{-2}\ s^{-1}$)	**g_s** ($mmol\ m^{-2}\ s^{-1}$)	**A** ($\mu mol\ m^{-2}\ s^{-1}$)	**c_i** ($\mu mol\ mol^{-1}$)
25°C	8.2 ± 0.37 a	468 ± 45.9 a	13.5 ± 0.22 a	286 ± 4.7 a
40°C	12.8 ± 0.58 b	554 ± 53.2 a	12.1 ± 0.46 a	294 ± 4.9 a
45°C	9.0 ± 0.92 a	186 ± 22.9 b	8.1 ± 0.94 b	256 ± 3.0 b

3.1.2.2 Plant response to treatment at 50°C

To assess the potential of above treatments with moderately elevated temperature to induce thermotolerance, plants pretreated in this way as well as

plants maintained at control temperature for the same period of time were subsequently exposed to high temperature of 50°C. Assessment of visible injury after 3 days of exposure to 50°C revealed marked differences between plants from different pretreatments (Table 3.2). Treatment with 50°C for 3 days resulted in death of plants previously maintained at control temperature. Pretreatment at both 40 and 45°C alleviated plant injury on exposure to treatment with 50°C: injury of main stem leaves was reduced, and only young branch leaves died. However, the degree of protection conferred differed between the two pretreatment temperatures. The 40°C pretreatment resulted in turgor maintenance of all main stem leaves in the majority of plants (9 out of 10), whereas in plants pretreated with 45°C on average 38% of main stem leaves died, only the remaining ones maintained turgor.

Table 3.2 Variation in visible leaf injury in *Gossypium hirsutum* plants after exposure to 50°C for 3 days. Plants had previously been pretreated with moderately elevated sublethal temperature (40 or 45°C for 7 days) or maintained in control conditions (25°C) for the same period of time. Combined sequential temperature treatments are indicated as pretreatment temperature / subsequent (lethal high) temperature.

	Visible leaf injury
25/50°C	All leaves dead
40/50°C	9 plants: main stem leaves turgid, all young branch leaves dead; 1 plant: all leaves dead
45/50°C	17 – 60 % (38 % on average) of main stem leaves dead, all young branch leaves dead

Those plants, which survived treatment at 50°C for 3 days, *i.e.* plants previously exposed to 40°C and 45°C, were also assessed regarding gas exchange parameters and maximum quantum yield of PSII (determined as F_v/F_m ratio of chlorophyll fluorescence). Plants previously exposed to moderately elevated temperature exhibited significant decreases in all of the gas exchange parameters assessed in comparison to experimental control plants (Table3.3). Transpiration was decreased by about 40%, stomatal conductance by about 80%, net assimilation rate decreased to values around zero, and the intercellular

concentration of CO_2 increased by about 20 %. However, there was no significant difference between responses of plants pretreated with 40°C and 45°C.

Table 3.3 Variation in transpiration (E), stomatal conductance (g_s), net rate of CO_2 uptake (A) and CO_2 mole fraction in the leaf intercellular air space (c_i) in *Gossypium hirsutum* after exposure of plants, which had been pretreated at 40 or 45°C for 7 days, to 50°C for 3 days. Control plants were maintained at 25°C throughout the experiment. Combined sequential temperature treatments are indicated as pretreatment temperature / subsequent temperature. Mean for number of replicates as indicated ± standard error. Means followed by the same letter are not significantly different at $P < 0.05$.

	E ($mmol\ m^{-2}\ s^{-1}$)	**g_s** ($mmol\ m^{-2}\ s^{-1}$)	**A** ($\mu mol\ m^{-2}\ s^{-1}$)	**c_i** ($\mu mol\ mol^{-1}$)
25/25°C [1]	8.2 ± 0.37 a	468 ± 45.9 a	13.48 ± 0.22 a	286 ± 4.7 a
40/50°C [2]	4.6 ± 0.36 b	81 ± 6.5 b	-0.52 ± 0.29 b	345 ± 6.5 b
45/50°C [3]	5.2 ± 0.87 b	101 ± 17.3 b	-0.17 ± 0.59 b	349 ± 24.7 b

[1] n = 5
[2] n = 10
[3] n = 7 (for the 3 remaining plants of this treatment leaves in the measurement position had died)

Pretreatment with both levels of moderately elevated temperature resulted in a significant decrease in F_v/F_m on exposure to 50°C for 3 days as compared to plants maintained at control temperature for the duration of the experiment (Table 3.4). However, the extent of this decrease was significantly greater in plants of the 45°C pretreatment than the 40°C pretreatment.

Table 3.4 Variation of F_v/F_m of chlorophyll fluorescence in *Gossypium hirsutum* after **(a)** pretreatment with 40 or 45°C for 7 days and **(b)** subsequent exposure of pretreated plants to 50°C for 3 days. Plants were 43 (a) and 46 (b) days old, respectively, at the time of measurement. Control plants were maintained at 25°C and measured when 46 days old. Mean for number of replicates as indicated ± standard error. Within columns a and b, different letters indicate a significant difference ($P < 0.05$) between treatments.

Pretreatment Temperature (°C)	**(a)** F_v/F_m in response to moderately elevated sublethal temperature (pretreatment)	**(b)** F_v/F_m of pretreated plants following subsequent exposure to high lethal temperature
	See left	**50°C**
40	0.731 [2] ± 0.004 a	0.644 [2] ± 0.009 b
45	0.739 [2] ± 0.008 a	0.593 [3] ± 0.023 c
	In comparison to control conditions **25°C**	
25	0.756 [1] ± 0.006 a	0.756 [1] ± 0.006 a

[1] Mean for 5 plants
[2] Means for 10 plants
[3] Mean for 7 plants (for the 3 remaining plants of this treatment leaves in the measurement position had died)

3.2 Experiment 2

3.2.1 Experimental protocol

Seeds of *Gossypium hirsutum* var. CIM 443 were sown as described in section 2.1 on August 11th 2000. 33 days after sowing seedlings were transplanted to 1.6-dm^3 plastic pots. Plants were first treated with plant nutrient solution containing phostrogen at 2 g dm^{-3} and micronutrients as in half strength Hoagland's solution 25 and 34 days after sowing, from then on about every second day.

For assignment to stress treatments plants were arranged in a randomized block design stratified for height (to minimise the influence of plant size on stress responses) as follows. They were sorted according to height, divided into 4 strata of increasing height, and from each stratum plants were randomly assigned to all of the combinations of pretreatment / subsequent treatment which are outlined in Table 3.5.

Table 3.5 Experimental design consisting of **(1)** 2 levels of pretreatment: exposure to high temperature of 40°C for 6 days or to control temperature for the same period of time and **(2)** the following modes of subsequent treatment: **a)** no further treatment, but assessment of plant response to pretreatment; **b)** exposure to severe levels of heat stress; **c)** maintenance in control conditions throughout the experiment (applies only to control-pretreated plants, forming the experimental control). The number of replicates for each combination of pretreatment / subsequent treatment is shown.

(2) Subsequent treatment	**(1) Pretreatment**	
	Control	40°C
a) Assessment of plant response to pretreatment		
-	5	5
b) Subsequent treatment with severe stress		
Heat	5	5
c) Maintenance in control conditions		
Ct	4	

Plants were subjected to pretreatment when they were 53 days old. It consisted of exposure to either 40°C for 6 days or control temperature of 25°C for the same period of time. Plants assigned to pretreatment with 40°C were transferred to a temperature-controlled Vindon growth cabinet, and spatial sections of plants were rotated in daily intervals during treatment (to minimize effects of environmental heterogeneity). To assess plant response to pretreatment gas exchange, chlorophyll fluorescence, and leaf water relation parameters (water content and relative water content) were measured at 2 points of time: a) on the final day (day 6) of pretreatment and b) after 2 to 6 days (as specified) of recovery in control conditions. In addition shoot height and above ground dry matter production were measured after 2 and 6 days of recovery, respectively, following pretreatment.

Plants pretreated with either 25°C or 40°C were subsequently subjected to treatment with severe heat stress of 49°C for 2 days. They were transferred to a temperature-controlled Vindon growth cabinet, and, as for heat pretreatment, spatial sections of plants were rotated in daily intervals during treatment. For assessment of plant response to severe heat stress, gas exchange and chlorophyll fluorescence parameters of heat-treated plants were measured at 2 points of time within the period of heat stress, after 22 and 41 hours and after 19 and 24 hours of

treatment, respectively. Experimental control plants were assessed for these parameters at only one of the 2 points of time. Leaf water relation parameters (water content and relative water content) were measured after 46 hours of treatment.

3.2.2 Results

3.2.2.1 Plant responses to pretreatment at 40°C

Exposure of plants to elevated temperature of 40°C for 6 days resulted in a significant increase in transpiration rate to more than twice the value of control plants, while net assimilation rate, stomatal conductance, and intercellular CO_2 concentration were not significantly altered compared to plants maintained at control temperature (Table 3.6). Water use efficiency of photosynthesis (determined as ratio net assimilation rate / transpiration rate) was significantly reduced as a result of the increase in transpiration rate in plants treated at 40°C.

Transfer of plants heat-treated for 6 days to control temperature of 25°C for 4 days resulted in a decrease in transpiration rate to values comparable to control plants. Stomatal conductance was, however, significantly increased by 7 % in comparison to control plants (Table 3.6).

Table 3.6 Transpiration (E), stomatal conductance (g_s), net rate of CO_2 uptake (A), CO_2 mole fraction in the leaf intercellular air space (c_i), and water use efficiency of net photosynthesis (A/E) in *Gossypium hirsutum* after exposure to 40°C (Heat) for 6 days (day 6 after initiation of treatment) and subsequent recovery in control conditions of 25°C for 4 days (day 10 after initiation of treatment). Control plants were maintained at 25°C. Each value is the mean ± standard error for 5 plants; the value for each plant is the average of measurements for 2 adjacent medium-aged leaves. For each day and variable control and heat treatment were compared by a t-test. Asterisks *, * *, * * * represent significance at P = 0.05, 0.01, and 0.001, respectively; ns = nonsignificant at P = 0.05.

Day [1]		**E** (mmol m^{-2} s^{-1})	**g_s** (mmol m^{-2} s^{-1})	**A** (μmol m^{-2} s^{-1})	**c_i** (μmol mol^{-1})	**A/E**
6	Control	5.1 ± 0.14	312 ± 16.1	17.3 ± 1.61	241 ± 10.6	3.4 ± 0.28
	Heat	10.8 ± 0.20	348 ± 12.8	16.7 ± 1.07	241 ± 6.2	1.5 ± 0.07
	Significance of t-test between treatments					
		* * *	ns	ns	ns	* * *
10	Control	5.5 ± 0.09	367 ± 8.0	21.6 ± 0.8	213 ± 5.1	3.9 ± 0.10
	Heat	5.8 ± 0.04	392 ± 6.3	20.2 ± 0.8	224 ± 5.4	3.5 ± 0.16
	Significance of t-test between treatments					
		ns	*	ns	ns	ns

[1] day after initiation of treatment

Treatment with 40°C for 6 days induced a slight decrease in F_v/F_m of chlorophyll fluorescence in comparison to control plants, although this was not significant (Table 3.7). The decrease was mainly due to a significant decrease in F_m (by 14 %), while F_o was slightly, but not significantly reduced compared to the control. Transfer of heat-treated plants to control conditions for 2 days resulted in complete recovery of F_m to control values.

Table 3.7 F_v/F_m, F_o, and F_m of chlorophyll fluorescence in *Gossypium hirsutum* after exposure to high temperature of 40°C (Heat) for 6 days (day 6 after initiation of treatment) and subsequent recovery in control conditions of 25°C for 2 days (day 8 after initiation of treatment). Control plants were maintained at 25°C. Each value is the mean ± standard error for 5 plants; the value for each plant is the average of measurements for 2 adjacent medium-aged leaves. Statistical testing as in Table 3.6.

Day [1]		**F_v/F_m**	**F_o**	**F_m**
6	Control	0.819 ± 0.005	383 ± 7.7	2128 ± 41.6
	Heat	0.803 ± 0.007	359 ± 16.2	1832 ± 80.3
	Significance of t-test between treatments			
		ns	ns	*
8	Control	0.821 ± 0.005	388 ± 26.7	2160 ± 95.0
	Heat	0.808 ± 0.005	408 ± 14.5	2130 ± 81.4
	Significance of t-test between treatments			
		ns	ns	ns

[1] day after initiation of treatment

After exposure to 40°C for 6 days plants had slightly decreased values of relative water content (RWC) compared to control plants, but this difference disappeared after subsequent return to control temperature for 6 days (Table 3.8). In contrast, heat treatment did not significantly alter values of water content (WC) compared to those of control plants (Table 3.8).

Table 3.8 Leaf water content (WC) and relative water content (RWC) in *Gossypium hirsutum* **a)** after treatment with high temperature of 40°C (Heat) for 6 days (day 6 after initiation of treatment) and **b)** after subsequent recovery in control conditions for 6 days (day 12 after initiation of treatment). Control plants were maintained at 25°C. Mean ± standard error for 5 plants. For each day and variable control and heat treatment were compared by a t-test. Statistical testing as in Table 3.6.

Day [1]	**Treatment**	**WC (%)**	**RWC (%)**
a) *6*	Control	386 ± 32.6	90 ± 0.9
	Heat	426 ± 14.9	86 ± 0.7
	Significance of t-test between treatments		
		ns	*
b) *12*	Control	337 ± 25.1	84 ± 1.1
	Heat	365 ± 26.8	85 ± 0.9
	Significance of t-test between treatments		
		ns	ns

[1] day after initiation of treatment

Shoot height, determined after 6 days of heat treatment at 40°C and 2 days of recovery in control temperature, was significantly decreased to 81 % of the value of control plants (Table 3.9). Shoot dry matter production was determined after 6 days of recovery in control temperature following heat treatment at 40°C. Overall shoot dry matter production of heat-treated plants was reduced to 74 % of that of control plants, but this difference was not significant. The decrease was mainly due to the significant reduction in stem dry matter production to 62 % of control values, whereas dry weights of leaves and flowers were not significantly different from the control (Table 3.9).

Table 3.9 Variation in shoot height and above ground dry matter production (whole shoot and individual contributions of stem, leaves and flowers) in *Gossypium hirsutum* in response to exposure to high temperature of 40°C (Heat) for 6 days and subsequent recovery in control temperature of 25°C for 2 days (measurement of shoot height) and 6 days (measurement of above ground dry matter production). Mean ± standard error for 5 plants. Statistical testing as in Table 3.6.

	Shoot height (cm plant^{-1})	**Dry matter production (g plant^{-1})**			
		Stem	**Leaves**	**Flowers**	**Shoot**
Control	83.6 ± 2.5	10.6 ± 0.9	14.8 ± 1.0	0.18 ± 0.07	25.6 ± 1.9
Heat	67.8 ± 2.3	6.6 ± 0.8	12.3 ± 1.4	0.07 ± 0.05	19.0 ± 2.3
	Significance of t-test between treatments				
	* *	*	ns	ns	ns

3.2.2.2 Plant response to treatment at 49°C

When plants treated at 40°C for 6 days as well as control plants of the same age were subjected to treatment at 49°C, differently pretreated plants showed marked differences in response (Figure 3.1a). Plants previously treated at 40°C had a significantly (about 3-fold) higher transpiration rate compared to experimental control plants, the latter being maintained in control temperature for the entire duration of the experiment, and this increase was apparent after both, 22 and 41 hours of treatment at 49°C. In contrast, transpiration rates of plants pretreated at control temperature were not significantly different from those of the experimental controls, when measured after 22 or 41 hours of treatment at 49°C. Differently pretreated plants were significantly different from each other with respect to their transpiration rates, and this was the case for both times of measurement.

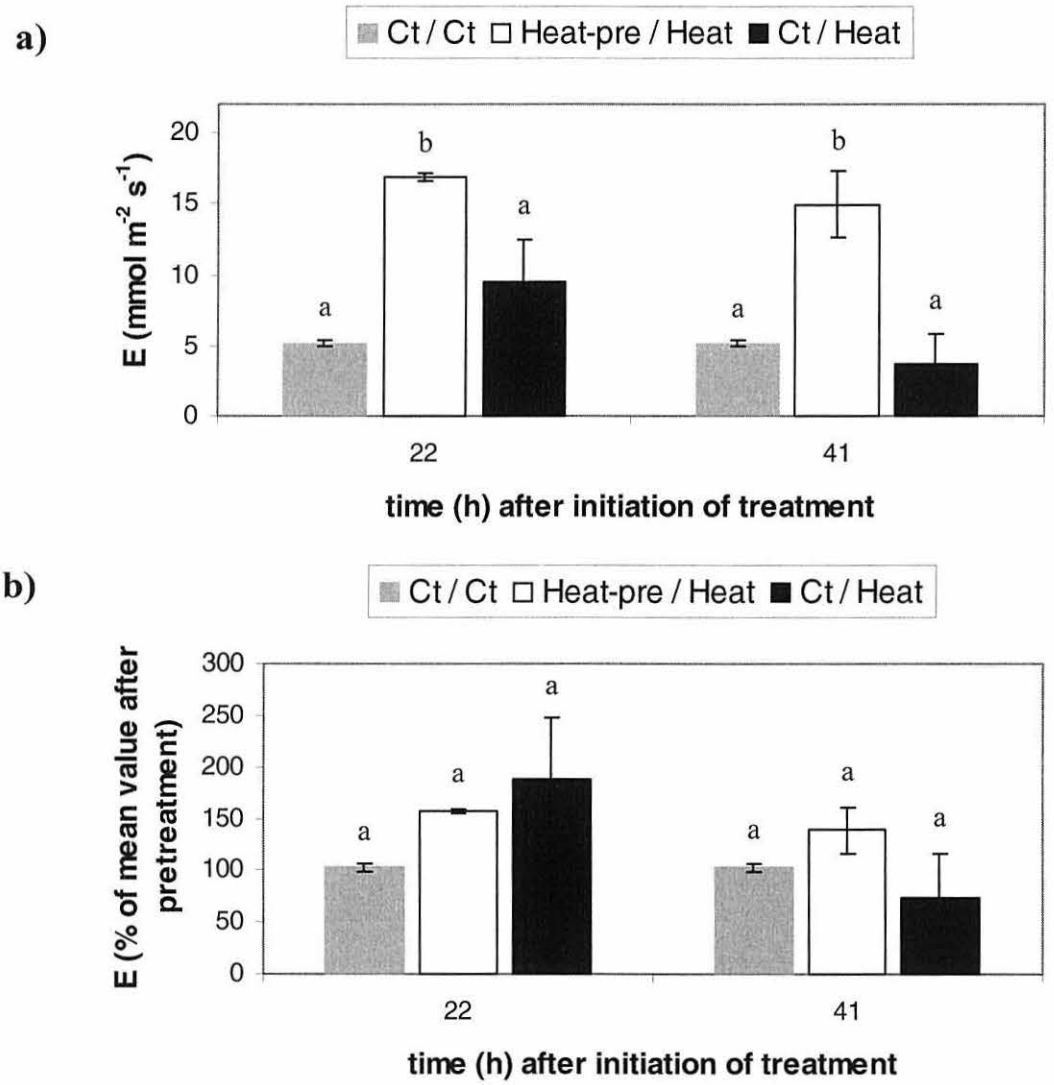
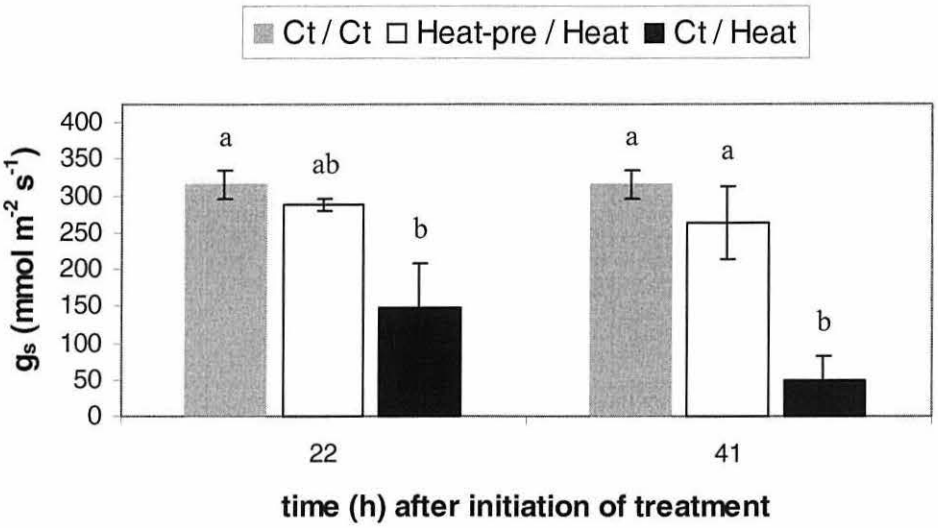


Figure 3.1 Transpiration (E) in *Gossypium hirsutum* after treatment with high temperature of 49°C for 22 and 41 hours (Heat). Plants had been pretreated with high temperature of 40°C for 6 days (Heat-pre) or with control temperature for the same period of time (Ct). Experimental control plants (Ct / Ct) were maintained in control temperature of 25°C throughout the experiment. **a)** Absolute values of transpiration measured after heat treatment; **b)** values as in a as % of the mean value after pretreatment. The measurement of response to pretreatment was carried out on a different set of plants (see Table 3.6) which had been exposed to the same pretreatment regime. Mean ± standard error for 5 (heat treatments) and 4 (experimental control) plants. The value for each plant is the average of measurements for 2 medium-aged leaves which were adjacent (except 2 replicates of Ct / Heat measured after 41 hours of treatment for which leaves in the second measurement position were too dry for measurement). A one-way analysis of variance was performed between combined treatments. After 22 hours of treatment only heat-treated plants were measured and compared with experimental control plants measured after 41 hours of treatment. Means followed by the same letters are not significantly different ($P < 0.05$).

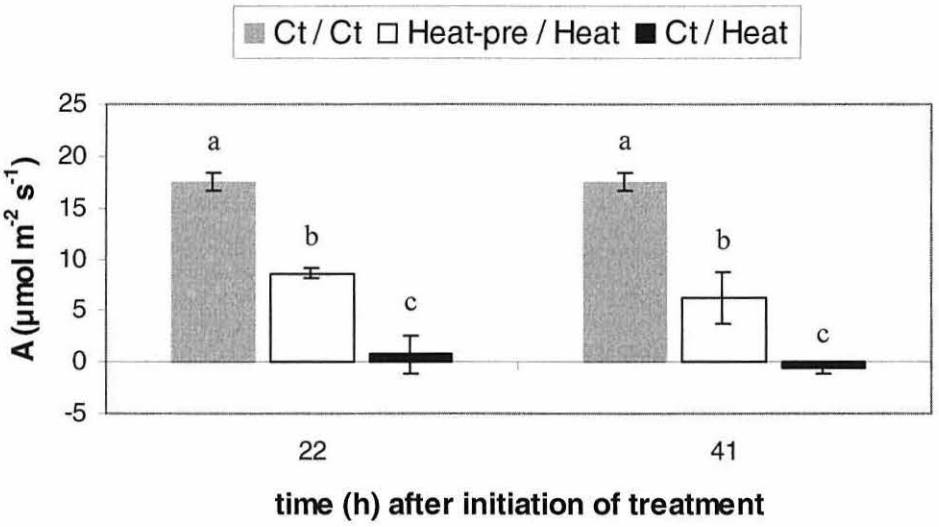
As pretreatment at 40°C induced a significant increase in transpiration rate, transpiration rates of differently pretreated plants in response to treatment at 50°C were also expressed as percentage of the mean value for this parameter after pretreatment (Figure 3.1b). As can be seen the increase in transpiration rate of plants pretreated at 40°C relative to both, plants pretreated at control temperature and experimental control plants, disappears. This indicates that the relative increase in transpiration rate exhibited by plants pretreated at 40°C on exposure to 49°C can be explained by the effect of the pretreatment at 40°C on transpiration rate.

Pretreatment also influenced the response of other gas exchange parameters to treatment at 49°C. In plants pretreated at control temperature, stomatal conductance was significantly reduced to 47 % and 15 % of the experimental control level after 22 and 41 hours of treatment at 49°C, respectively (Figure 3.2a). In contrast, stomatal conductance values of plants pretreated at 40°C did not differ significantly from those of experimental control plants, whether measured after 22 or 41 hours of treatment at 49°C. This resulted in a significant difference between plants pretreated at control and moderately elevated temperature after 41 hours of exposure to 49°C. Exposure to high temperature of 49°C induced a significant decrease in net CO_2 assimilation rate in plants from both pretreatment temperature regimes, as compared to experimental control plants (Figure 3.2b). However, pretreatment had an effect on the extent of this reduction. Net CO_2 assimilation rates of plants pretreated at control temperature were close to zero, when measured after exposure to 49°C for 22 and 41 hours. In contrast, net CO_2 assimilation rates of plants pretreated at 40°C were reduced to 49 and 36 % of experimental control values after 22 and 41 hours of treatment at 49°C, respectively. There was thus a significant difference between differently pretreated plants at both times of measurement carried out in the course of the high temperature treatment. In addition control-pretreated plants exhibited a significant increase in the intercellular CO_2 concentration by 1.7 and 2 fold, determined after 22 and 41 hours of exposure to 49°C, respectively, in comparison to experimental control plants, whereas plants pretreated at 40°C showed only a slight increase that was not significant (Figure 3.2c).

a)


Ct / Ct □ Heat-pre / Heat ■ Ct / Heat
g_s (mmol m^{-2} s^{-1})
400
350
300
250
200
150
100
50
0
a
ab
b
a
a
b
22
41
time (h) after initiation of treatment

b)


Ct / Ct □ Heat-pre / Heat ■ Ct / Heat
A (µmol m^{-2} s^{-1})
25
20
15
10
5
0
-5
a
b
c
a
b
c
22
41
time (h) after initiation of treatment

c)

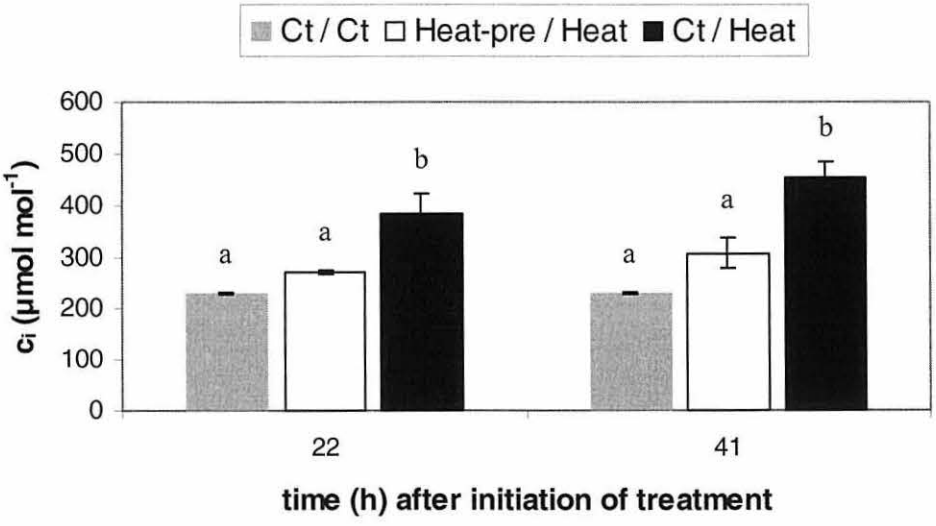
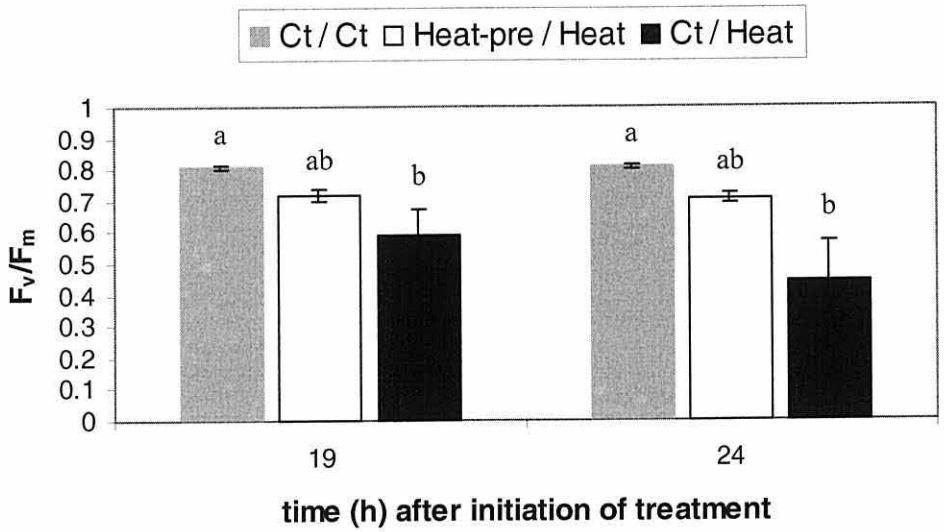

Ct / Ct □ Heat-pre / Heat ■ Ct / Heat
c_i (µmol mol^{-1})
600
500
400
300
200
100
0
a
a
b
a
a
b
22
41
time (h) after initiation of treatment

Figure 3.2 **a)** Stomatal conductance (g_s), **b)** net rate of CO_2 uptake (A), and **c)** CO_2 mole fraction in the leaf intercellular air space (c_i) in *Gossypium hirsutum*. Experimental treatments and statistical testing as in Figure 3.1.

Exposure of plants to 49°C decreased F_v/F_m of chlorophyll fluorescence by 27 and 11 %, in control- and heat-pretreated plants, respectively, after 19 hours of treatment, in comparison to experimental control plants, and the respective decreases were 45 and 12 % after 24 hours of treatment (Figure 3.3a). The decrease in control-pretreated plants was significant compared to the experimental control, whereas the intermediate values for heat-pretreated plants were not significantly different from either experimental control or control-pretreated plants. The decrease in F_v/F_m was due to an increase in the F_o level of fluorescence by 60 and 39 % in control- and heat-pretreated plants, respectively, after 19 hours of treatment, and the respective increases were 77 and 63 % after 24 hours of treatment (Figure 3.4a). As observed for F_v/F_m, the increase was significant for control-pretreated plants compared to experimental control plants, whereas the intermediate values of heat-pretreated plants were not significantly different from either experimental controls or control-pretreated plants.

As heat pretreatment itself induced a slight decrease in F_v/F_m, F_o and F_m, which was significant in the case of F_m, chlorophyll fluorescence parameters were also expressed as percentage of the mean value for these parameters after pretreatment. This enhanced the increase in F_o in heat-pretreated plants, resulting in a significant difference from values of experimental control plants after treatment at 49°C for 24 hours (Figure 3.4b). This indicates that the difference in response of differently pretreated plants with respect to F_o on exposure to 49°C may be partly a consequence of the decrease in F_o induced by pretreatment at 40°C. However, expression of F_v/F_m values as percentage of the mean values after the respective pretreatment did not alter the relative differences between treatments regarding this parameter (Figure 3.3b). Exposure to 49°C did not induce significant changes in the F_m level of chlorophyll fluorescence in plants from either pretreatment, and expression of F_m as percentage of the mean value after pretreatment did not significantly alter relative treatment differences (data not shown).

a)



b)

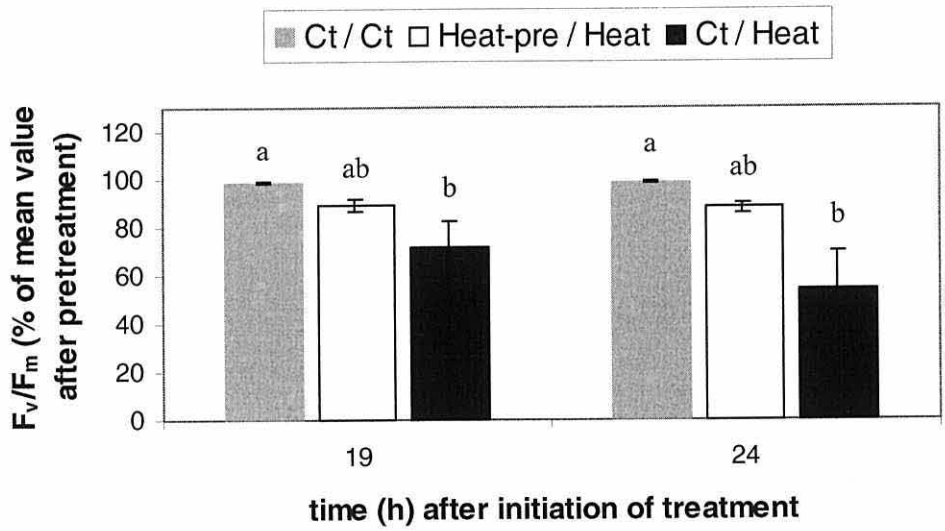
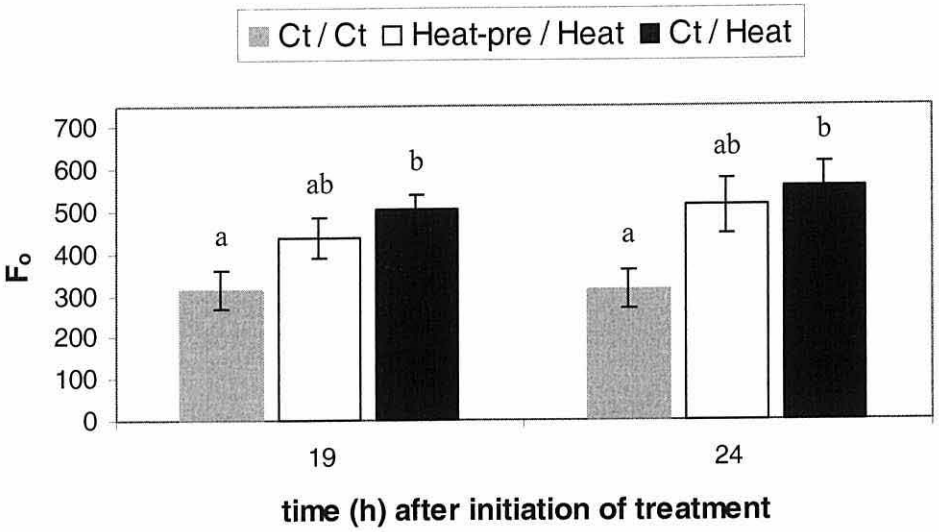


Figure 3.3 F_v/F_m of chlorophyll fluorescence in *Gossypium hirsutum* after treatment with high temperature of 49°C for 19 and 24 hours (Heat). Pretreatments and experimental control treatment as in Figure 3.1. **a)** Absolute values of F_v/F_m measured after heat treatment; **b)** values as in a as % of the mean value after the respective pretreatment. The measurement of response to pretreatment was carried out on a different set of plants (see Table 3.7) which had been exposed to the same pretreatment regime. Mean ± standard error for 5 (heat treatments) and 4 (experimental control) plants. The value for each plant is the average of measurements for 3 (measurement after 19 hours of treatment) and 2 (measurement after 24 hours of treatment, except 1 replicate of Ct / Heat for which the leaf in the second measurement position was too dry for measurement) adjacent medium-aged leaves. A single factor analysis of variance was performed between combined treatments. After 24 hours of treatment only heat-treated plants were measured and compared with experimental control plants measured after 19 hours of treatment. Means followed by different letters are significantly different ($P < 0.05$).

a)



b)

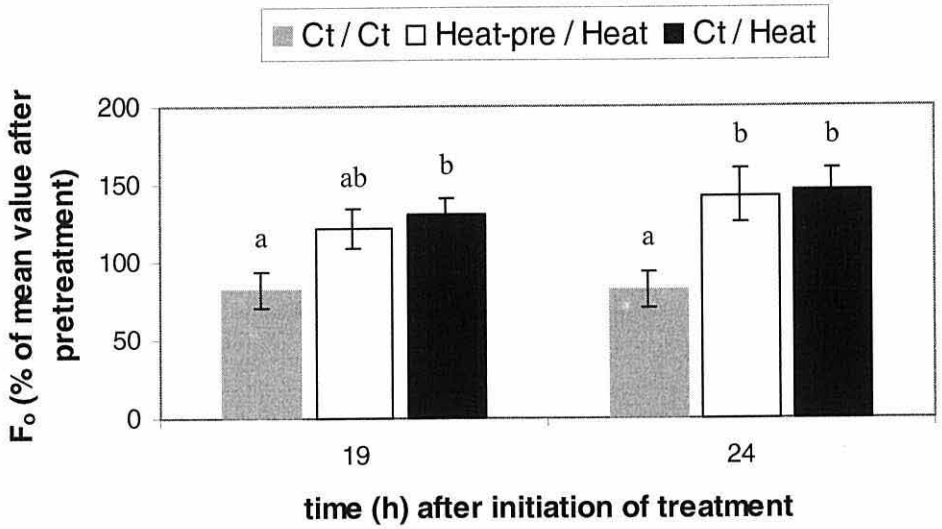


Figure 3.4 F_o of chlorophyll fluorescence in *Gossypium hirsutum.* Experimental treatments, expression as absolute values and relative to values after pretreatment, and statistical testing as in Figure 3.3.

Determination of water content and relative water content after exposure to 49°C for 46 hours revealed large differences between differently pretreated plants (Table 3.10a). In plants pretreated at control temperature water content and relative water content were reduced significantly by 87 and 75 % compared to experimental control plants, respectively, whereas in plants pretreated at 40°C the respective reductions amounted to only 16 and 24 % and were not significant. This resulted in a significant difference between differently pretreated plants with respect to these two water relation parameters. As pretreatment itself induced changes in water content and relative water content, which were significant in the case of relative water content, values for these parameters in response to treatment at 49°C were also expressed as percentage of the mean values after pretreatment (Table 3.10b). This did not significantly alter the relative differences between treatments, indicating that the large differences between differently pretreated plants regarding these parameters on exposure to 49°C did not result from a direct continuation of pretreatment-induced changes in these parameters.

Table 3.10 Leaf water content (WC) and relative water content (RWC) in *Gossypium hirsutum* in response to treatment at 49°C for 46 hours (Heat). Pretreatments and experimental control treatment as in Figure 3.1. Leaf water relation parameters are presented as **a)** absolute values measured after heat treatment and **b)** values measured after heat treatment as % of the mean value after the respective pretreatment. The measurement of response to pretreatment was carried out on a different set of plants (see Table 3.8) which had been exposed to the same pretreatment regime. Mean ± standard error for 5 (heat treatments) and 4 (experimental control) plants. For each variable a single factor analysis of variance was performed between combined treatments. Means followed by different letters are significantly different ($P < 0.05$).

	WC (%)	**RWC (%)**
a) *Absolute values measured after heat treatment*		
Ct / Ct	376 ± 20.0 a	89 ± 1.2 a
Heat-pre / Heat	316 ± 58.0 a	68 ± 8.7 a
Ct / Heat	50 ± 5.1 b	22 ± 0.7 b
b) *Values as in a as % of the mean value after pretreatment*		
Ct / Ct	97 ± 5.2 a	99 ± 1.3 a
Heat-pre / Heat	74 ± 13.6 a	79 ± 10.1 a
Ct / Heat	13 ± 1.3 b	24 ± 0.8 b

3.3 Discussion

3.3.1 Physiological responses of non-acclimated plants to treatment at 49°C

Exposure of *Gossypium hirsutum* to temperatures of 49 to 50°C resulted in severe inhibition of plant physiological processes in plants not previously acclimated to high temperature (non-acclimated). Experiment 2 shows plant responses in the period between 22 and 41 hours of exposure to 49°C. In non-acclimated plants net CO_2 assimilation rate (A) reached values slightly above zero after 22 hours and values slightly below zero after 41 hours of treatment. The intercellular CO_2 concentration (c_i) showed a 1.7- and 2-fold increase after 22 and 41 hours of treatment, respectively. The decrease in A to values around zero associated with an increase in c_i is likely to result from a stronger proportional decrease in photosynthesis compared to respiratory processes, as observed for responses of A to relatively severe drought stress (Lawlor & Cornic, 2002). Values of A close to zero indicate that the rate of photosynthetic dark reactions was similar to that of respiratory processes in these plants. This is in general agreement with studies on the variation in heat stability of cellular processes. Photosynthesis has been identified as one of the most heat-sensitive cellular processes, and the heat stability of cell membranes and dark respiration far exceeds that of photosynthesis (Berry & Björkman, 1980). In addition it is well known that photorespiration increases as a proportion of A with increasing temperature due to decreased solubility of CO_2 relative to O_2 and increased specificity of Rubisco for O_2 with increasing temperature (Leegood, 1995). Regarding the inhibition of photosynthesis, most research effort appears to have focused on the nature of the irreversible inhibition of photosynthetic processes and has identified PSII as the primary target of this injury (see below). However, the temperature dependence of CO_2 saturated net CO_2 uptake rates consists of two phases: (a) a gradual and reversible decline and (b) a rapid and irreversible decline, which occurs above a certain threshold temperature (Seemann *et al.*, 1984). The reversible inhibition of Rubisco activation has been identified as primary lesion in the heat induced inhibition of Calvin cycle activity. While fully activated Rubisco activity is very heat-stable, the activity of Rubisco activase is extremely heat-sensitive (Law & Crafts-Brandner, 1999).

Exposure of non-acclimated plants to 49°C also resulted in a significant reduction in the maximum quantum yield of PSII, determined as dark-adapted F_v/F_m ratio of chlorophyll fluorescence, to 73 % of the values of experimental control plants after 19 hours and 55 % of the experimental control values after 24 hours of treatment. The decrease in F_v/F_m was due to a significant increase in the F_o level of chlorophyll fluorescence.

A rise in the F_o level of fluorescence is generally regarded as result of irreversible damage to the photosynthetic apparatus. Schreiber & Berry (1977) determined the temperature dependence of the F_o level of chlorophyll fluorescence, yielding a so-called fluorescence- temperature or F-T curve, and found that the threshold temperature for a sharp increase in F_o corresponded to the threshold temperature for a sudden change in the quantum yield for CO_2 fixation. In a study on *Larrea divaricata* Armond *et al.* (1978) found similar threshold temperatures for sudden transitions in the following processes: CO_2-saturated photosynthesis, light-saturated electron transport rates, and F_o level of chlorophyll a fluorescence. A more recent study illustrates the relationship between the temperature dependence of net CO_2 exchange and F_o level fluorescence (Seemann *et al.*, 1984). CO_2-saturated net CO_2 uptake initially decreased gradually with increasing temperature, reaching stable values at each new temperature. When temperature exceeded a certain threshold level, net CO_2 uptake rate became unstable with time, decreasing continuously without further change in temperature, and could not be fully recovered to initial values. The sudden increase in F_o level fluorescence occurred at a similar temperature as the time-instability of net CO_2 uptake rate, and this agreement was shown in a number of species. Havaux (1993) demonstrated how F-T curves relate to the maximum quantum yield of PSII, determined as dark-adapted F_v/F_m ratio of chlorophyll fluorescence. Exposure of leaves of *Solanum tuberosum* to temperatures slightly above the threshold temperature for the sudden rise in F_o level fluorescence, extracted from an F-T curve, induced a rapid decline of F_v/F_m. In contrast, temperatures slightly below the threshold temperature resulted in only minor changes in F_v/F_m over a period of more than 8 hours.

From studies showing that heat specifically affects the quantum yield of CO_2 fixation measured under light-limiting conditions, it was concluded that the thylakoid membrane constitutes the primary target of heat inactivation (Schreiber

& Berry, 1977). In a study on *Larrea divaricata* Armond *et al.* (1978) investigated the threshold temperatures for various photosynthetic processes. They found that the threshold temperature for inhibition of light-limited electron transport was much lower than that for changes in CO_2-saturated photosynthesis, light-saturated electron transport rates, and F_o level of chlorophyll a fluorescence. The wave length dependence of F_o level fluorescence and light-limited whole chain electron transport changed with increasing temperature. Together these results indicated a block in excitation energy transfer from chlorophyll b to chlorophyll a as primary lesion in the photosynthetic apparatus. *In vivo* and *in vitro* studies have shown that heat-inhibition of PSII activity occurs at lower temperatures than that of PSI activity (Berry & Björkman, 1980). There appears to be general agreement in the literature now that PSII is the primary target of heat damage. Particularly sensitive components are the association between light-harvesting complex II and PSII complex and the water-splitting/oxygen-evolving system (Havaux & Tardy, 1996).

The significant decrease in F_v/F_m associated with a significant increase in F_o in the present study can therefore be regarded as indication of irreversible damage to PSII. There is, however, a major difference between the studies cited above and the present one. F-T curves assess the temperature dependence of the F_o level of fluorescence by increasing leaf temperature at a rate of 1°C/minute (Schreiber & Berry, 1977). In contrast, in the present study the dark-adapted F_v/F_m and F_o were measured after comparatively long-term exposure to a constant high temperature for 19 and 24 hours.

46 hours of treatment at 49°C also resulted in a large significant decrease in water content and relative water content to 13 and 25 % of levels of the experimental control (Experiment 2). Rapid leaf dehydration may be a consequence of damage to the plasma membrane. The latter has a higher heat stability than the chloroplast membranes (Levitt, 1980), and injury to the plasma membrane thus occurs towards the end of the heat-induced sequence of events ultimately leading to cell death. In experiment 1 exposure to the slightly higher temperature of 50°C for 3 days resulted in death of all leaves in non-acclimated plants, demonstrating that this combination of temperature and exposure time was lethal to non-acclimated plants.

3.3.2 Effects of heat pretreatment on plant response to treatment at 49°C

Pretreatment of *Gossypium hirsutum* plants at moderately elevated temperatures for 6 to 7 days alleviated heat injury on exposure to 49 and 50°C on several levels. (a) Exposure to 40°C for 6 days partially protected dark reactions of photosynthesis, as shown by the maintenance of net rates of CO_2 uptake at 49 and 36 % of the experimental control level after 22 and 41 hours of treatment at 49°C, respectively, in heat-pretreated plants, whereas non-acclimated plants had values around zero at these times (experiment 2). In addition it prevented the significant increase in intercellular CO_2 concentration occurring in non-acclimated plants on exposure to 49°C, confirming that photosynthesis decreased less as a proportion of respiratory processes in heat-pretreated plants. (b) Heat-pretreatment at 40°C prevented the decrease in stomatal conductance on exposure to 49°C, shown by the maintenance of stomatal conductance at experimental control levels even after 41 hours of treatment, while stomatal conductance of non-acclimated plants was significantly reduced to 15 % of the experimental control level at this time (experiment 2). (c) Heat-pretreatment at 40°C partially protected PSII activity. The maximum quantum yield of PSII was reduced by 11 % compared to the experimental control after 19 hours at 49°C in heat-pretreated plants and did not decrease much further within the next few hours, exhibiting 12 % reduction after 24 hours of treatment. In contrast, maximum quantum yield of PSII was reduced to a greater extent in non-acclimated plants, and also continued to decrease between 19 and 24 hours of treatment, with the relative reductions amounting to 27 and 45 % after 19 and 24 hours of treatment, respectively. However, the difference between heat-pretreated and non-acclimated plants was not statistically significant ($P < 0.05$). (d) Heat pretreatment at 40°C and 45°C for 7 days partially protected main stem leaves from rapid dehydration and death in response to treatment at 50°C for 3 days (experiment 1).

Acclimation of plant photosynthesis to high temperature by pretreatment with moderately elevated temperature has been demonstrated in a variety of species, and studies investigating this phenomenon generally fall into three categories, based on the duration of the acclimation treatment. (a) Long-term acclimation on a time-scale of months. Species native to environments with

temperature variation during the growing season have been shown to shift their thermal optimum for photosynthetic CO_2 uptake in parallel to seasonal changes in prevailing temperature. This has been confirmed in studies in controlled environments for evergreen desert shrubs. Long-term growth in a high temperature regime induces a shift in the thermal optimum of photosynthetic CO_2 uptake to a higher temperature, with similar photosynthetic rates at the new optimum temperature in leaves developed in the acclimating growth temperature (Berry & Björkman, 1980). For example comparison of plants of *Larrea divaricata* grown at 20, 32 and 45°C showed that growth at higher temperatures shifted the temperature dependence curve of light-saturated electron transport and CO_2-saturated net photosynthetic rate as well as the threshold temperature for the rise in F_o level fluorescence to higher temperatures. In addition growth at the highest temperature prevented the block in excitation energy transfer between chlorophyll b and a (Armond *et al.*, 1978). (b) Short-term acclimation on a time-scale of minutes to hours. Havaux (1993) demonstrated that pretreatment of leaf discs of *Solanum tuberosum* at 35°C in the dark for 2 hours shifted the threshold temperature for a sudden rise in F_o level fluorescence from 38 to 43°C. This was accompanied by a shift in the temperature dependence curve of maximum photon yield of PSII towards higher temperatures. This type of PSII thermotolerance could be acquired within 1 hour. Valladares & Pearcy (1997) observed rapid adjustments in the thermal stability of PSII on a time-scale of hours in field-grown plants of the chaparral sclerophyll *Heteromeles arbutifolia*. The threshold temperature for the sudden rise in F_o level fluorescence exhibited diurnal changes, which paralleled the diurnal fluctuations in air temperature. (c) Intermediate acclimation periods on a time-scale of days to weeks. It was shown that when plants, which are native to environments with large temperature fluctuations during the growing season, are grown in a high temperature regime for several days to several weeks, the thermal characteristics of photosynthesis shift to higher temperatures in mature fully-developed leaves (Berry & Björkman, 1980).

In cotton acclimation of photosynthesis to high temperature by pretreatment with moderately elevated temperature has been demonstrated for two of the categories defined above. (a) Seemann *et al.* (1984) showed that long-term growth of *Gossypium hirsutum* in a 40/32°C as compared to 20/15°C day/night temperature regime shifted the threshold temperature for irreversible inhibition of

net rates of CO_2 uptake from 45.0 to 46.7°C, and that for the sudden rise in F_o level fluorescence from 45.2 to 48.6°C. (b) Law & Crafts-Brandner (1999) compared the effects of a gradual increase in temperature of individual leaves of *Gossypium hirsutum* by 2.5°C every hour from 27.5 to 45°C with the effects of exposure of different leaves, each to different points in the same temperature range tested, but with temperatures being imposed rapidly (at 1°C per minute). Gradual heat treatment induced a shift in T_{50} values (temperatures causing 50 % reduction) of the rates of several photosynthetic processes, expressed as proportion of the control, to higher temperatures, as compared to rapidly imposed heat treatment. T_{50} for net CO_2 uptake was increased from 41.1 to 42.4°C, T_{50} for initial Rubisco activity (related to Rubisco activase activity) from 40.8 to 43.3°C, and T_{50} for F_v/F_m from 42.5 to 45.0°C. The acclimation phenomenon observed in the present study differs from the previous work on cotton with respect to the acclimation period, which extended over 6 and 7 days in experiment 2 and 1, respectively.

The heat acclimation of whole leaf photosynthesis observed in the present study is in general agreement with the acclimation phenomena, involving short- and long-term acclimation treatments, described in the studies above. However, it differs with respect to the photosynthetic processes, which were protected by acclimation. In most studies protection of photosynthetic processes was shown to occur at the level of PSII. In the present study pretreatment at 40°C for 6 days strongly alleviated the proportional reduction in photosynthetic CO_2 uptake relative to respiratory processes on exposure to 49°C for 41 hours, whereas protection of PSII activity occurred to a lesser extent. The difference between non-acclimated and acclimated plants with respect to PSII activity on exposure to 49°C was not statistically significant. However, the protection of photosynthetic processes observed in the present study shows strong similarity to the acclimation of photosynthesis described in the study by Law & Crafts-Brandner (1999) on cotton. Close inspection of their data reveals that acclimation resulted in protection of the dark reactions, which was particularly pronounced at the higher temperatures in the range tested, while the extent of protection of PSII activity, determined as dark-adapted F_v/F_m, was highest at 42.5°C, but only small at 45°C.

3.3.3 Dependence of acclimation on pretreatment temperature

Two pretreatment temperature regimes, 40 and 45°C, each applied for 7 days, differed with respect to their ability to alleviate heat injury on exposure to 50°C (experiment 1). Both pretreatment regimes reduced the degree of heat-induced leaf injury. However, pretreatment at 40°C prevented rapid dehydration of all main stem leaves, whereas pretreatment at 45°C protected only an average of 62 % of main stem leaves per plant. Pretreatment at 45°C also resulted in significantly greater inhibition of PSII activity, determined as dark-adapted F_v/F_m, on exposure to 50°C for 3 days, as compared to pretreatment at 40°C. The differences in acclimating effect of the two pretreatment temperature regimes point to the existence of a temperature optimum for the induction of thermotolerance. Chen *et al.* (1982) assessed the dependence of acquired heat resistance on the temperature of pretreatment (applied for 24 hours) in tomato, bean, potato, and soybean, and came to a similar conclusion. Heat resistance was expressed as heat killing time, defined as the time required to cause 50 % injury to leaves on transfer to a water bath at 50°C, and viability was determined based on tissue ion leakage. Pretreatment temperatures above a certain threshold were found to increase heat resistance dramatically, but the acclimating effect started to decrease again above a certain temperature. The reduced acclimating effect of pretreatment at 45°C in comparison to 40°C in the present study was probably due to increased heat injury at 45°C. Exposure to treatment at 45°C for 7 days resulted in a significant decrease in rate of net CO_2 uptake, stomatal conductance, and intercellular CO_2 concentration, compared to experimental control plants, whereas these parameters were not affected by pretreatment at 40°C. Law & Crafts-Brandner (1999) investigated the response of net CO_2 uptake rate to a range of rapidly induced high temperatures (increasing at 1°C per minute) in *Gossypium hirsutum* and found that temperatures above 42.5°C induced complete inhibition of the CO_2 exchange rate. In the present study treatment at 45°C for 7 days did not result in complete inactivation, but only a large reduction in net CO_2 uptake by 40% compared to the experimental control level, probably due to acclimation processes taking place in the longer treatment period. In spite of this the results of Law & Crafts-Brandner (1999) clearly demonstrate the potential of temperatures

above this threshold of 42.5°C, which separates the two pretreatment temperatures, to induce serious inactivation of net CO_2 uptake in cotton.

3.3.4 Mechanism of acclimation

Pretreatment at 40°C for 6 days caused a more than 2-fold increase in transpiration rate compared to control plants (experiment 2). A significant increase in transpiration rate was also observed in response to treatment at 40°C for 7 days (experiment 1). In addition treatment at 40°C for 6 days induced a large (about 3-fold) increase in transpiration on exposure to 49°C in comparison to experimental control plants, which appeared to be a continuation of the effect of pretreatment on this parameter. In contrast non-acclimated plants exhibited transpiration rates comparable to those of the experimental control. Pretreatment at 40°C for 6 days prevented the reduction in stomatal conductance on exposure to 49°C. The increase in transpiration and maintenance of high stomatal conductance suggest increased transpirational cooling in heat-acclimated plants during the acclimation treatment as well as on exposure to 49°C. Transpirational cooling is regarded as heat avoidance mechanism, based on the fact that the process of evaporation of water from leaves absorbs heat and can lower the leaf temperature (Levitt, 1980). The increase in transpirational cooling observed in plants acclimated at 40°C may have been due to specific alterations in root morphology and/or hydraulic conductivity of the root system, as specific characteristics of these parameters are regarded as crucial determinants of plant capacity for transpirational cooling (Mahan *et al.*, 1997). The slight, but significant increase in stomatal conductance detected in plants treated at 40°C for 6 days after return to control temperature for 4 days lends further support to the proposed contribution of transpirational cooling to heat avoidance in heat-acclimated plants in the present study. In the tree species *Xylia xylocarpa* pretreatment at 40/20°C day/night temperatures, which reduced the inhibition of net photosynthesis and the increase in electrolyte leakage on exposure to 50°C, also resulted in increased stomatal conductance, when measured at 25°C in comparison to plants pretreated at lower temperatures. When measured at the pretreatment temperature, stomatal conductance was not altered (Saelim & Zwiazek, 2000). The authors suggested that the high temperature pretreatment may have induced modifications of cell membranes, which in turn affected plant hydraulic conductance, leading to

increased stomatal conductance. It has to be noted, however, that this high temperature pretreatment resulted in lower stomatal conductance on exposure to 50°C as compared to plants pretreated at lower temperatures, which stands in marked contrast to the results of the present study. Interestingly, transpirational cooling has been implicated in intra- and interspecific differences in heat resistance in cotton. In a historical series of *Gossypium barbadense*, which encompasses 40 years of selection for increased yields in hot environments, a strong positive correlation was found between lint yield and stomatal conductance of lines (Lu *et al.*, 1998). Selection for higher yield and heat resistance was apparently associated with selection pressures for higher stomatal conductance (independent from higher photosynthetic rates), and resulted in altered stomatal responses to temperature in advanced lines. Higher stomatal conductance appeared thus to be of adaptive value and was implicated in leaf evaporative cooling. Similarly, the higher lint yield and heat resistance in an advanced cultivar of *Gossypium hirsutum* compared to two cultivars of *Gossypium barbadense* was attributed to its higher stomatal conductance, photosynthetic rate, and smaller leaf areas (Lu *et al.*, 1997).

However, treatment at 45°C for 7 days induced a significant decrease in stomatal conductance and did not result in increased transpiration in comparison to experimental control plants, but still conferred some degree of thermotolerance (experiment 1). Thus it seems likely that mechanisms other than transpirational cooling also contributed to the acclimating effect of treatment at moderately elevated temperature observed in the present study.

An attractive mechanism for the protection of Calvin cycle activity in cotton has been proposed (Law *et al.*, 2001). Treatment at 41/37°C day/night temperature induced the synthesis of a new form of Rubisco activase. Synthesis first occurred after 3 hours and continued over 48 hours of treatment. It was suggested that this new activase form may protect the constitutive activase protein by preventing dissociation of its subunits.

Heat shock proteins (HSPs) are likely to have contributed to the acclimation response in the present study. They have been strongly implicated in the induction of resistance of seedling growth to otherwise lethal high temperature by short-term pretreatment with nonlethal high temperature, a phenomenon termed acquired thermotolerance (Vierling, 1991). Heckathorn *et al.* (1998)

provided direct evidence that low molecular weight HSPs (sHSPs) were involved in the acclimation of PSII activity to high temperature of 47°C by pretreatment at 43°C for 6 hours in tomato. In seedlings HSP synthesis is induced by exposure to temperatures 5 or more degrees above their optimal growing temperature (Vierling, 1991). The thermal kinetic window for optimal enzyme function in cotton lies in the temperature range of 23.5 to 32.0°C (Lu *et al.*, 1997). Fender & O'Connell (1989) investigated the potential of a range of pretreatment temperatures from 25 to 48°C, each applied for 2 hours, on *in vivo* HSP synthesis (assessed by labelling studies) in seedlings of *Gossypium hirsutum* and *Gossypium barbadense*. They found that HSP synthesis first occurred at 37°C and was maximal at 45°C, whereas temperatures above 45°C completely inhibited protein synthesis. Burke *et al.* (1985) showed that soil drought in field-grown cotton, resulting in midday canopy temperatures above 38°C for 36 days, induced accumulation of 8 polypeptides with molecular weights corresponding to those of HSPs. A more recent study confirmed that increasing leaf temperature of *Gossypium hirsutum* to 41°C resulted in *de novo* synthesis of HSPs after 1 hour, and synthesis was still apparent after 24 hours of treatment (Law *et al.*, 2001). Thus theoretical considerations as well as experimental evidence regarding the temperature for HSP induction in cotton imply that the pretreatment temperatures of 40 and 45°C used in the present study resulted in synthesis of HSPs.

4 Effects of pretreatment at low temperature on resistance to severe heat, chilling or drought stress

4.1 Experiment 1

4.1.1 Experimental protocol

Seeds of *Gossypium hirsutum* var. CIM 443 were sown as described in section 2.1 on May 25th 2000. Seedlings were transplanted to 1.6-dm^3 plastic pots 26 days after sowing. Plants were treated with nutrient solutions containing micronutrients as in half strength Hoagland's solution and phostrogen at varying concentrations about every second day from 25 days after sowing onwards. Phostrogen concentrations used were 1, 3, and 5 g dm^{-3} on days 25, 26, and 28 after sowing, respectively, then 1 g dm^{-3} from day 31 to 36 after sowing; after that phostrogen was used at 2 g dm^{-3}.

Plants were randomly assigned to combinations of pretreatment and subsequent treatment which are summarised in table 4.1.

Table 4.1 Experimental design consisting of **(1)** 2 levels of pretreatment: treatment with low temperature for 7 days decreasing from 18 to 12°C by 1°C per day (Cold-pre) or with only control temperature of 25°C for the same period of time (Control) and **(2)** the following modes of subsequent treatment: **a)** no further treatment, but assessment of plant response to pretreatment; **b)** exposure to a severe stress level of cold; **c)** maintenance in control conditions throughout the experiment (applies only to control-pretreated plants, forming the experimental control). The number of replicates for each combination of pretreatment / subsequent treatment is shown.

(2) Subsequent treatment	**(1) Pretreatment**	
	Control	Cold-pre
***a)** Assessment of plant response to pre-treatment*		
-	5	5
***b)** Subsequent treatment with severe stress*		
Cold	5	5
***c)** Maintenance in control conditions*		
Control	4	

When plants were 45 days old, they were exposed to pretreatment with low temperature for 7 days decreasing from 18 to 12°C by 1°C per day and

subsequent recovery in control conditions for 8 to 9 days (as specified below) or alternatively with only control temperature of 25°C for the same period of time. During pretreatment application of plant nutrient solutions was continued normally as outlined above. The time course of plant response to pretreatment was monitored by measuring chlorophyll fluorescence parameters daily during low temperature treatment and on days 3 and 4 of recovery in control conditions. Variation in gas exchange parameters over time in response to pretreatment was investigated by taking measurements every to every second day during low temperature treatment and on days 3 and 4 of recovery in control conditions. Sampling of leaves for measurement of water content, relative water content, and concentrations of cations was carried out at 2 points of time during treatment: a) on the final day (day 7) of cold treatment and b) on day 6 of recovery in control conditions. Shoot height and above ground dry matter production were measured on day 6 and 9, respectively, of recovery following low temperature treatment.

Plants assigned to the treatment with severe cold stress were subjected to above described pretreatment with low temperature and subsequent recovery in control conditions for 8 days or treatment with control temperature for the same period of time. 6 days before the start of severe cold treatment plants were transplanted to 4-dm^3 pots. The severe cold treatment consisted of exposure to 8°C for 11 days. During the cold treatment, plant nutrient solutions (composed as outlined above) continued to be applied, but in intervals of 3 days. The time course of plant response to treatment was assessed by taking measurements of chlorophyll fluorescence parameters daily from day 1 to 3, then daily from day 7 to 9, and a final measurement on day 11 after initiation of the treatment. Experimental control plants were assessed for these parameters at the same points of time except on day 7, for which values of cold-treated plants were compared with measurements of experimental control plants taken on day 8 of cold treatment. Variation in gas exchange parameters with time in response to treatment was investigated by taking measurements on days 2, 4, and 11 after initiation of treatment. After 10 days of recovery in control conditions following cold treatment the degree of cold-induced plant injury was assessed.

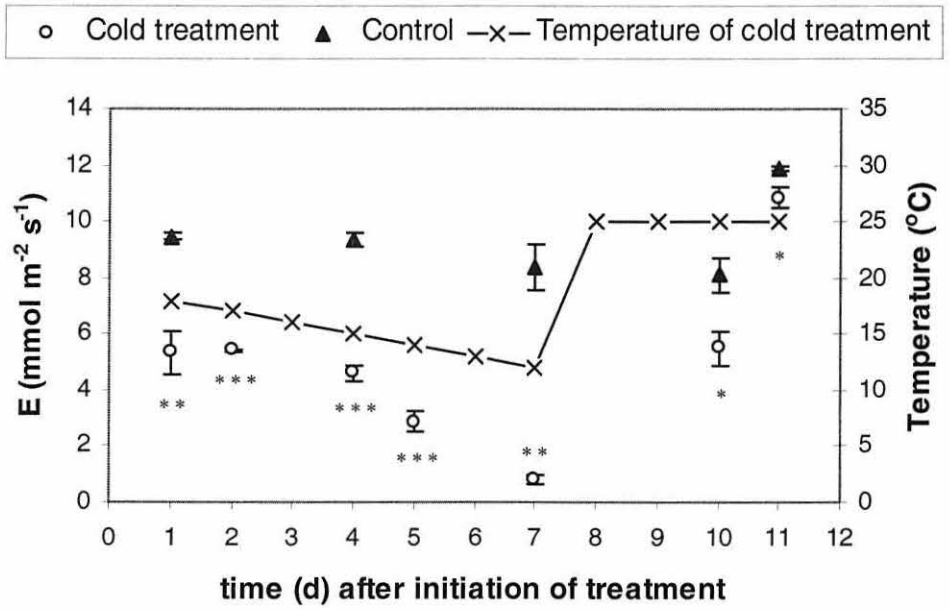
4.1.2 Results

4.1.2.1 Effect of pretreatment at gradually decreasing low temperature

Exposure of 45-day old *Gossypium hirsutum* plants to low temperature for 7 days decreasing gradually from 18 to 12°C by 1°C per day induced significant changes in a variety of physiological parameters.

Responses of transpiration and net rate of CO_2 uptake to low temperature were significantly different from those of the control throughout the applied temperature range (Figure 4.1). The response of net rate of CO_2 uptake occurred in several phases (Figure 4.1b). Exposure to 18°C on day 1 resulted in a large reduction in net rate of CO_2 uptake to 49% of the control level. On day 2 (at 17°C) net rate of CO_2 uptake recovered to 58% of the control level, and on day 4 (at 15°C) it showed a similar level of reduction as on day 1. On day 5 (at 14°C) net rate of CO_2 uptake was reduced further to 41 % of the control level. It decreased steeply towards day 7 (at 12°C), on which it amounted to only 15 % of the control. The kinetics of the response of transpiration rate resembled those of net rate of CO_2 uptake (Figure 4.1a). Exposure to 18°C and 17°C on day 1 and 2, respectively, induced reductions in transpiration rate to 56 and 57%, respectively, of the control level. On day 4 (at 15°C) transpiration rate showed a further reduction to 49% of the control. Towards day 5 (at 14°C) transpiration rate decreased more steeply to a value of 31% of the control level. A further massive decrease occurred towards day 7 (at 12°C), when rates amounted to only 10% of the control level. The two parameters differed in response to return to control temperature for 4 days following low temperature treatment (Figure 4.1). Net rate of CO_2 uptake recovered to control levels in the 4 day period, and rates were not significantly from those of control plants, when measured after 3 and 4 days of recovery (Figure 4.1b). Transpiration recovered gradually, but to a lesser extent than the net rate of CO_2 uptake in the 4 day period (Figure 4.1a). Rates amounted to 68 and 92% of the control level on days 3 and 4 after return to control temperature, respectively, and were significantly different from those of the control in both occasions (Figure 4.1a).

a)



b)

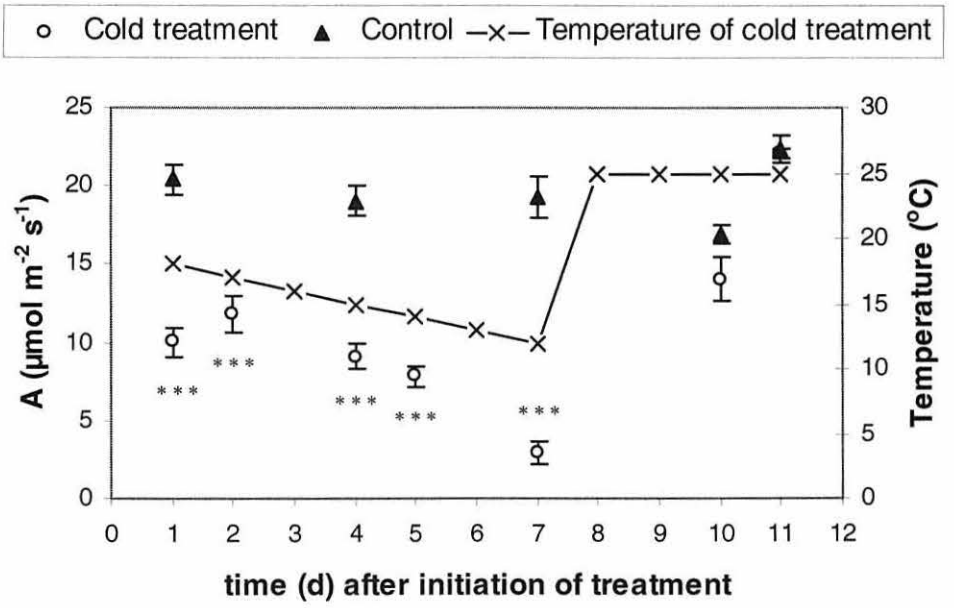
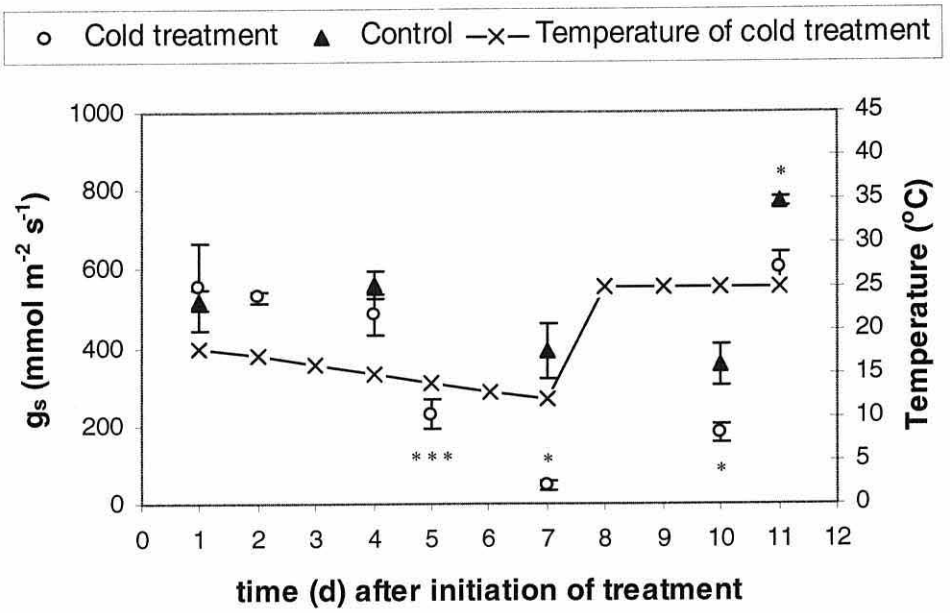


Figure 4.1 Variation in **a)** transpiration (E) and **b)** net rate of CO_2 uptake (A), in *Gossypium hirsutum* after treatment with low temperature for 7 days decreasing from 18 to 12°C by 1°C per day and subsequent recovery in control conditions for 4 days. Control plants were maintained at 25°C. Mean ± standard error for 5 plants (except control treatment on day 7, for which n = 4). For each day control and cold treatment were compared by a t-test. On days 2 and 5 only cold-treated plants were measured and compared with control measurements from days 1 and 4, respectively. Asterisks *, * *, * * * represent significance at P = 0.05, 0.01, and 0.001, respectively.

Responses of stomatal conductance and intercellular CO_2 concentration to low temperature differed significantly from those of control plants only during part of the treatment period (Figure 4.2). Stomatal conductance was not affected in the first half of low temperature treatment, but exhibited a significant decrease to 41% of the control level on day 5 (at 14°C), and a further steep drop towards day 7 (at 12°C), when it amounted to only 12 % of the control (Figure 4.2a). The intercellular CO_2 concentration was significantly increased by 17 to 20% relative to control plants in the first 4 days treatment, with low temperature decreasing from 18 to 15°C, but then fell to the control level on days 5 (14°C) to 7 (12°C) of low temperature treatment (Figure 4.2b). On subsequent return to control temperature stomatal conductance and intercellular CO_2 concentration differed in their kinetics of recovery (Figure 4.2). Stomatal conductance recovered gradually, increasing to 51 and 78% of the control level on days 3 and 4, respectively, after transfer to control temperature, but values remained significantly different from the control (Figure 4.2a). The intercellular CO_2 concentration initially decreased significantly to a value 20% below the control level on day 3, but then recovered to the control level on day 4 after return to control temperature (Figure 4.2b).

a)



b)

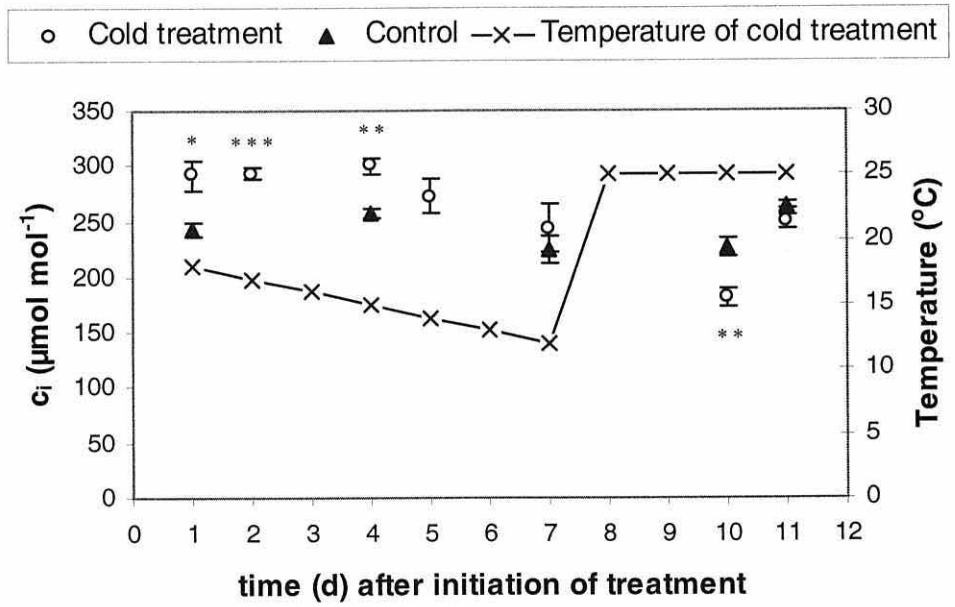


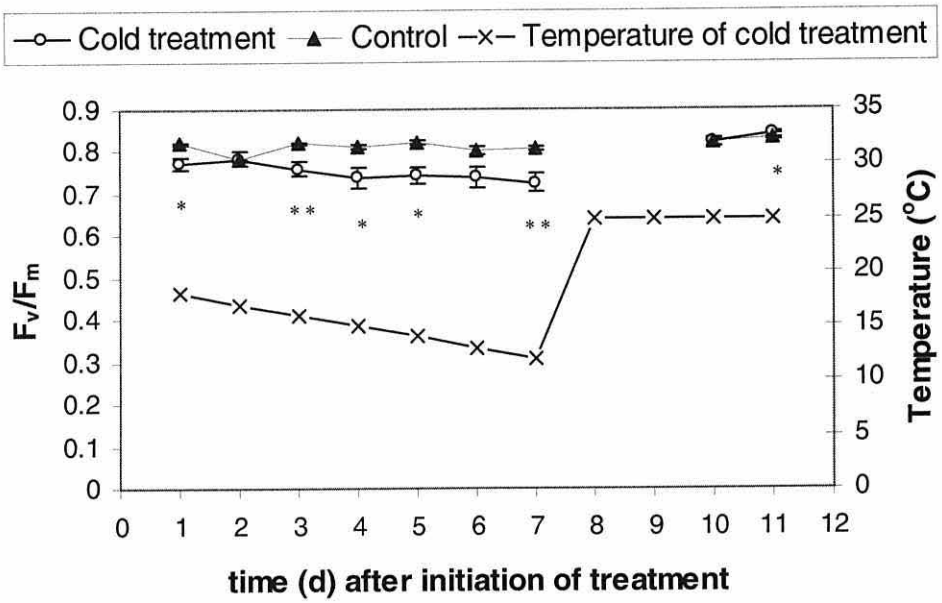
Figure 4.2 Variation in **a)** leaf stomatal conductance (g_s) and **b)** CO_2 mole fraction in the leaf intercellular air space (c_i) in *Gossypium hirsutum.* Experimental treatments as in Figure 4.1. Mean ± standard error for 5 plants (except control treatment on day 7, for which n = 4). Statistical testing as in Figure 4.1.

Exposure to low temperature decreasing from 18 to 12°C by 1°C per day induced a decrease in the F_v/F_m ratio of chlorophyll fluorescence relative to the control, and this decrease was significant on days 1, 3, 4, 5, and 7 of treatment. The difference between low temperature-treated and control plants exhibited a tendency to increase slightly in the course of the treatment period, with reductions amounting to 5% on day 1 (18°C) to 10% on day 7 (12°C) of treatment (Figure 4.3a). The response kinetics of the F_v/F_o ratio of chlorophyll fluorescence to the low temperature treatment were similar to those of F_v/F_m, but revealed greater differences between low temperature-treated and control plants (Figure 4.3b). Low temperature treatment induced a significant reduction in F_v/F_o relative to the control throughout the treatment period except on day 2, and reductions ranged from 19% on day 1 (18°C) to 30% on day 7 (12°C) of treatment relative to the control (Figure 4.3b).

The F_m and F_o level of chlorophyll fluorescence, used for the calculation of F_v/F_m and F_v/F_o, showed a differential response to treatment at low temperature (Figure 4.4). F_o tended to be reduced below the control level in the first 4 days, and then increased above the level of control plants in the following 3 days of low temperature treatment (Figure 4.4a). F_o was significantly decreased relative to the control on days 1 and 4 of treatment, with reductions amounting to 8 and 11%, respectively, and significantly increased relative to the control on days 5 and 7 of treatment, the increase amounting to 13 and 17%, respectively. Low temperature treatment significantly reduced F_m relative to the control level throughout the treatment period except on day 2 (Figure 4.4b). The extent of reduction varied, ranging from 14% to 31%.

On return to control temperature F_v/F_m and F_v/F_o not only recovered to control levels, with no significant difference between previously low temperature–treated and control plants on day 3, but further increased significantly above the control level on day 4 after transfer to control temperature (Figure 4.3). The increase relative to the control was 2% for F_v/F_m, and was more pronounced for F_v/F_o, amounting to 10%. This trend was paralleled by F_m, which had recovered to the control level after 3 days and increased to a value 8% above the control level on day 4 after transfer to control temperature. In contrast, F_o was not significantly different from the control, when determined either on day 3 or 4 after return to control temperature (Figure 4.4).

a)



b)

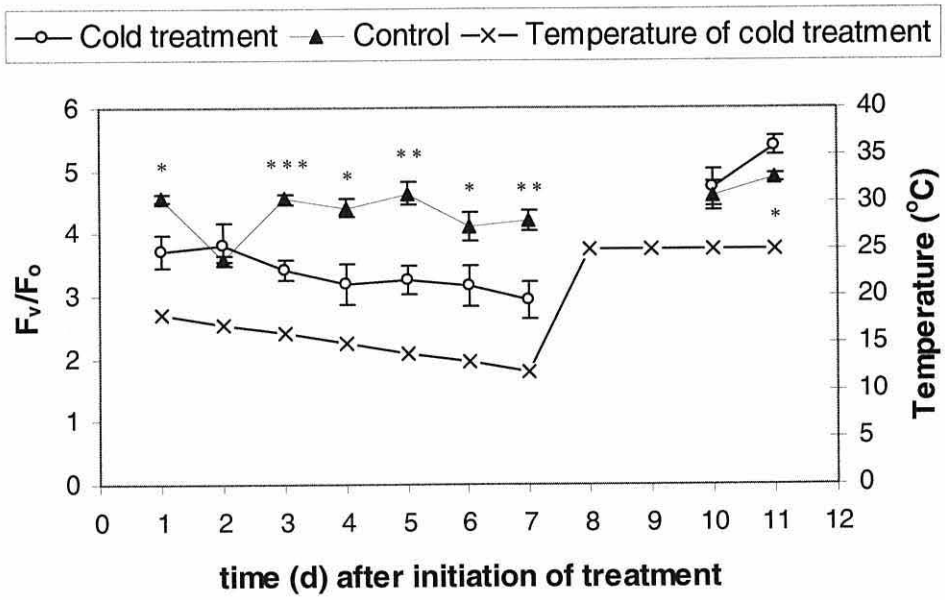
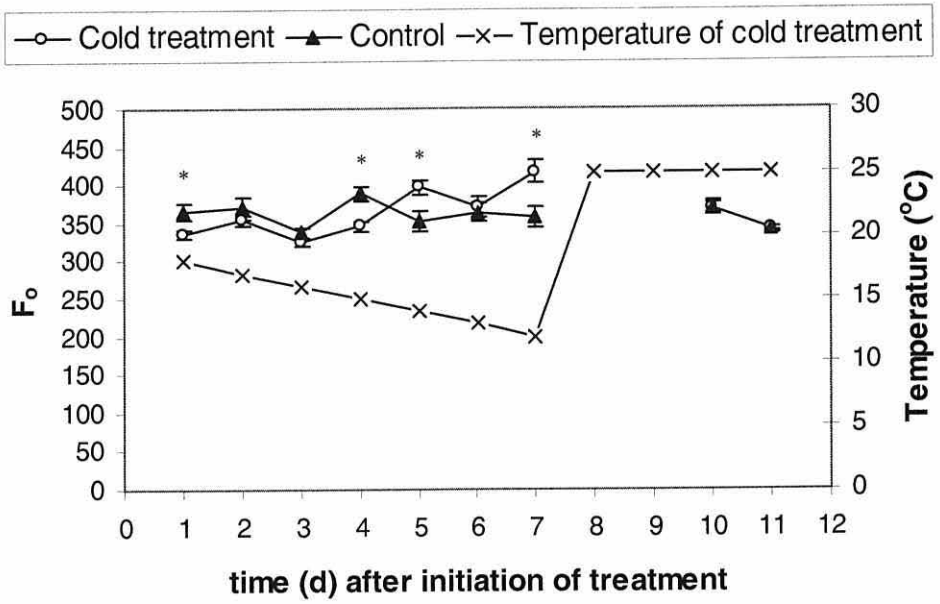


Figure 4.3 Responses of **a)** F_v/F_m and **b)** F_v/F_o of chlorophyll fluorescence in *Gossypium hirsutum*. Experimental treatments as in Figure 4.1. Mean ± standard error for 5 plants; the value for each plant is the average of measurements for 2 young adjacent leaves. For each day control and cold treatment were compared by a t-test. Asterisks *, * *, * * * represent significance at P = 0.05, 0.01, and 0.001, respectively.

a)



b)

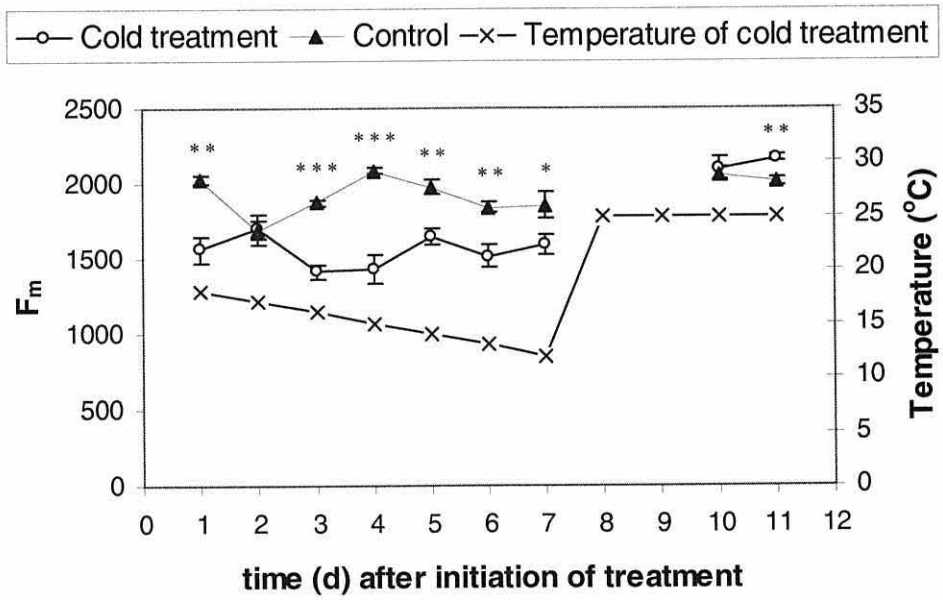


Figure 4.4 Responses of **a)** F_o and **b)** F_m of chlorophyll fluorescence in *Gossypium hirsutum*. Experimental treatments as in Figure 4.1. Mean ± standard error for 5 plants; the value for each plant is the average of measurements for 2 young adjacent leaves. Statistical testing as in Figure 4.3.

Shoot dry matter production was significantly reduced to 61% of the control level, when determined after 9 days of recovery in control temperature following low temperature treatment (Table. 4.2). This was due to significant reductions in stem and leaf dry weight to 52 and 69%, respectively, of the control level. Flower dry weight was also reduced by 48% relative to the control, but this was not statistically significant ($P < 0.05$). The decrease in shoot dry matter production was associated with a significant reduction in shoot height, determined after 6 days of recovery in control temperature, to 82% of the control level (Table 4.2).

Table 4.2 Variation in shoot height and above ground dry matter production (whole shoot and individual contributions of stem, leaves and flowers) in *Gossypium hirsutum* after treatment with low temperature for 7 days decreasing from 18 to 12°C by 1°C per day and subsequent recovery in control conditions for 6 (measurement of height) and 9 days (measurement of dry matter production). Control plants were maintained at 25°C. Mean ± standard error for 5 plants. For each variable control and cold treatment were compared by a t-test. Asterisks *, * *, * * * represent significance at P = 0.05, 0.01, and 0.001, respectively; ns = nonsignificant at P = 0.05.

	Shoot height (cm plant^{-1})	**Dry matter production (g plant^{-1})**			
		Stem	**Leaves**	**Flowers**	**Shoot**
Control	86.4 ± 1.61	15.5 ± 0.98	17.8 ± 1.38	0.82 ± 0.26	34.1 ± 2.40
Cold	70.5 ± 2.42	8.1 ± 0.66	12.2 ± 0.73	0.43 ± 0.06	20.7 ± 1.43
Significance of t-test between treatments					
	* *	* * *	* *	ns	* *

Low temperature treatment induced changes in leaf water relation parameters, and this was apparent during treatment as well as on return to control temperature (Table 4.3). Leaf water content, determined on the last day of treatment, was increased by 25% relative to the control, whereas relative water content was slightly, but not significantly reduced. Re-assessment of leaf water relation parameters after 6 days of recovery in control temperature showed that leaf water content as well as relative water content were significantly increased by 35 and 3%, respectively, relative to the control.

Table 4.3 Leaf water content (WC) and relative water content (RWC) in *Gossypium hirsutum* **a)** after treatment with low temperature for 7 days decreasing from 18 to 12°C by 1°C per day and **b)** after subsequent recovery in control conditions for 6 days (day 13 after initiation of treatment). Control plants were maintained at 25°C. Each value is the mean ± standard error for 5 plants. Statistical testing as in Table 4.2.

Day[1]	Treatment	WC (%)	RWC (%)
a) *7*	Control	280 ± 25.8	90.0 ± 1.42
	Cold	350 ± 15.7	87.5 ± 2.98
Significance of t-test between treatments			
		*	ns
b) *13*	Control	314 ± 14.2	90.1 ± 0.58
	Cold	424 ± 29.3	93.1 ± 1.02
Significance of t-test between treatments			
		*	*

[1] day after initiation of treatment

Low temperature treatment also induced some changes in leaf cation concentrations (Table 4.4). Concentrations of K^+ and Mg^{2+} in the leaf sap decreased significantly by 32 and 38%, respectively, relative to the control, whereas those of Na^+ and Ca^{2+} exhibited an increase by 20 and 19%, respectively, but the increases were not statistically significant ($P < 0.05$) (Table 4.4a). The changes in Na^+ and K^+ concentrations caused a significant decrease in the K^+/Na^+ ratio. When cation concentrations, determined in the leaf sap, where expressed on a dry weight basis, the significant difference between low temperature-treated and control plants regarding K^+ and Mg^{2+} concentrations disappeared, indicating that the decrease in concentration was a result of dilution due to increased leaf water content (Table 4.4b). However, expression on a dry weight basis enhanced the difference between low temperature-treated and control plants with respect to Na^+ and Ca^{2+} concentrations, resulting in a statistically significant increase (by 50%) for Na^+ relative to the control. After return to control temperature for 6 days previously low temperature-treated plants showed leaf sap cation concentrations comparable to those of control plants (Table 4.4a), but expression on a dry weight basis revealed significant increases in Ca^{2+} and Mg^{2+} concentrations by 58 and 53%, respectively (Table 4.4b).

Table 4.4 Variation in concentrations of Na^+, K^+, Mg^{2+}, and Ca^{2+}, and ratio of K^+ / Na^+ in leaves of *Gossypium hirsutum* after treatment with low temperature for 7 days decreasing from 18 to 12°C by 1°C per day (day 7 after initiation of treatment) and after subsequent recovery in control conditions for 6 days (day 13 after initiation of treatment). Control plants were maintained at 25°C. **a)** Ion concentration measured in leaf sap. **b)** Ion concentration measured as in a converted to a dry weight basis via the measured leaf water content. Determinations of water content (see Table 4.3) were carried out within 2 hours of sampling for leaf ion concentrations using a leaf adjacent to the one sampled for the measurement of ion concentrations. Mean ± standard error for 5 plants. Statistical testing as in Table 4.2.

		Ions in leaves				
Day[1]		**Na^+**	**K^+**	**K^+ / Na^+**	**Ca^{2+}**	**Mg^{2+}**
		a) Concentration in leaf sap (mol m^{-3})				
7	Control	4.4 ± 0.0	102 ± 9.0	22.9 ± 2.0	237 ± 9.0	29 ± 2.3
	Cold	5.3 ± 0.5	69 ± 6.9	13.1 ± 0.5	283 ± 30.6	18 ± 1.0
	Significance of t-test between treatments					
		ns	*	* *	ns	* *
13	Control	7.3 ± 0.0	89 ± 10.1	12.2 ± 1.4	356 ± 14.9	30 ± 0.6
	Cold	6.8 ± 0.5	79 ± 10.2	12.0 ± 1.8	417 ± 25.0	35 ± 2.8
	Significance of t-test between treatments					
		ns	ns	ns	ns	ns
		b) Concentration as in a converted to dry weight basis (µmol g^{-1}DW) via leaf water content				
7	Control	12 ± 1.1	276 ± 10		674 ± 93	81 ± 8
	Cold	18 ± 1.7	240 ± 23		991 ± 115	64 ± 3
	Significance of t-test between treatments					
		*	ns		ns	ns
13	Control	23 ± 1.0	276 ± 29		1119 ± 63	94 ± 5
	Cold	29 ± 3.3	334 ± 45		1765 ± 134	144 ± 7
	Significance of t-test between treatments					
		ns	ns		* *	* * *

[1] day after initiation of treatment

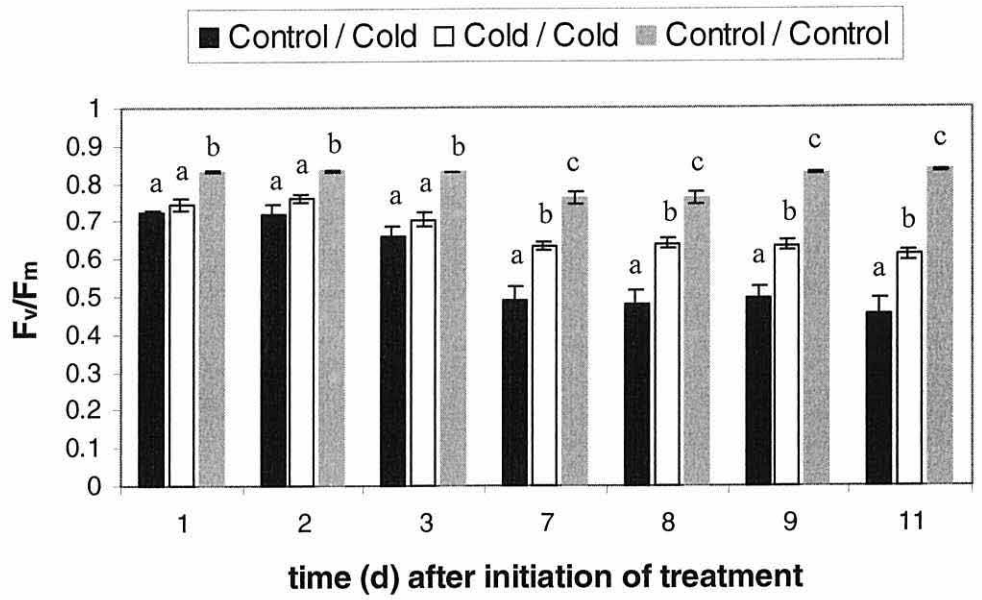
4.1.2.2 Effect of pretreatment at gradually decreasing low temperature on plant response to treatment at 8°C

Plants pretreated at low temperature decreasing from 18 to 12°C by 1°C per day followed by 8 days of recovery in control temperature (cold-pretreated plants) and plants of the same age, which had been maintained at control

temperature (control-pretreated plants), were subsequently treated at 8°C for 11 days.

Assessment of chlorophyll fluorescence parameters in the initial phase of treatment (days 1 to 3) and at a later stage, when plant injury had further progressed (days 7 to 11), revealed differences in the responses of cold- and control-pretreated plants (Figure 4.5 and 4.6). Treatment at 8°C caused a significant decline in F_v/F_m and F_v/F_o for plants from both pretreatments throughout the treatment period, but pretreatment influenced the extent of decrease (Figure 4.5). In the initial phase of treatment control- and cold pretreated plants did not differ significantly with respect to these parameters, with relative reductions ranging from 9 to 20% for F_v/F_m and 36 to 57% for F_v/F_o, but cold-pretreated plants tended to maintain slightly higher values. However, determination of F_v/F_m and F_v/F_o in the period from day 7 to 11 revealed significantly greater reductions in control- as compared to cold-pretreated plants. On day 7 reductions in F_v/F_m amounted to 35 and 16% for control- and cold-pretreated plants, respectively, and the respective values for F_v/F_o were 65 and 43%. Within the 5-day period from day 7 to 11 F_v/F_m and F_v/F_o exhibited only minor changes with further progression of treatment.

a)



b)

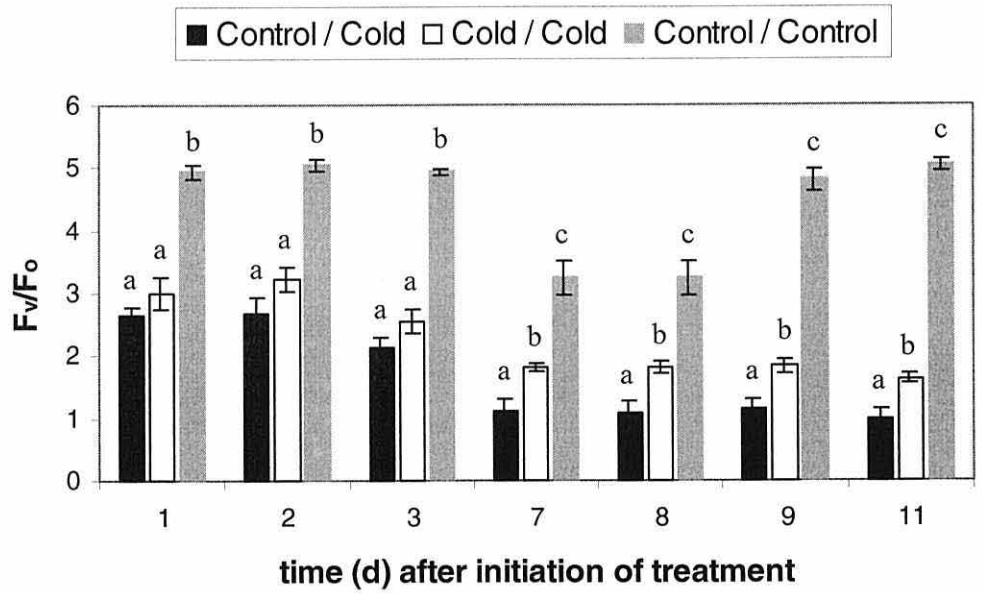


Figure 4.5 **a)** F_v/F_m and **b)** F_v/F_o of chlorophyll fluorescence in *Gossypium hirsutum* on exposure to low temperature of 8°C for 11 days. Plants had been pretreated with low temperature for 7 days decreasing from 18 to 12°C by 1°C per day and then recovered in control temperature of 25°C for 8 days or had been pretreated with only control temperature of 25°C for the same period of time. Experimental control plants were maintained in control conditions of 25°C throughout the experiment. Each value is the mean ± standard error for 5 (cold-treated) and 4 (experimental control) plants. The value for each plant is the average of measurements on 2 adjacent young leaves on days 1 and 2, on subsequent days it is the average of measurements on all medium-aged leaves for cold-treated plants (i.e. 3 - 5 for cold / cold and 5 - 6 for control / cold) and of 2 medium-aged leaves for experimental control (control / control) plants. For each day a one-way analysis of variance was performed between combined treatments. On day 7 only cold-treated plants were measured and compared with measurements on experimental control plants from day 8. Means followed by the same letters are not significantly different at $P < 0.05$.

Treatment at 8°C had a differential effect on F_o and F_m, which are the basic parameters used for the calculation of F_v/F_m and F_v/F_o (Figure 4.6). The kinetics of the response of F_m resembled those of F_v/F_m and F_v/F_o (Figure 4.6b). Exposure to 8°C induced a significant decrease in F_m throughout the treatment period, except on day 2, compared to experimental control plants, and this occurred in plants from both pretreatments. In the initial phase of treatment responses of control- and cold-treated plants did not differ significantly, and relative reductions in comparison to the experimental control ranged from 24 to 36% on days 1 and 3. In the period from day 7 to 11 of treatment cold-pretreated plants maintained significantly higher values of F_m as compared to control-pretreated plants. On day 7 the relative reductions in F_m compared to the experimental control amounted to 29 and 52% for cold- and control-pretreated plants, respectively. F_o showed a significant increase in response to exposure to 8°C compared to the experimental control, when determined on days 1 to 3 as well as 9 and 11 of treatment in plants from both pretreatments (Figure 4.6a). Cold-pretreatment significantly reduced the extent of this increase on the first day of treatment, when relative increases compared to experimental control plants amounted to 12 and 25% in cold- and control-pretreated plants, respectively. However, pretreatment had no significant effect on response of F_o to 8°C on the following days of treatment.

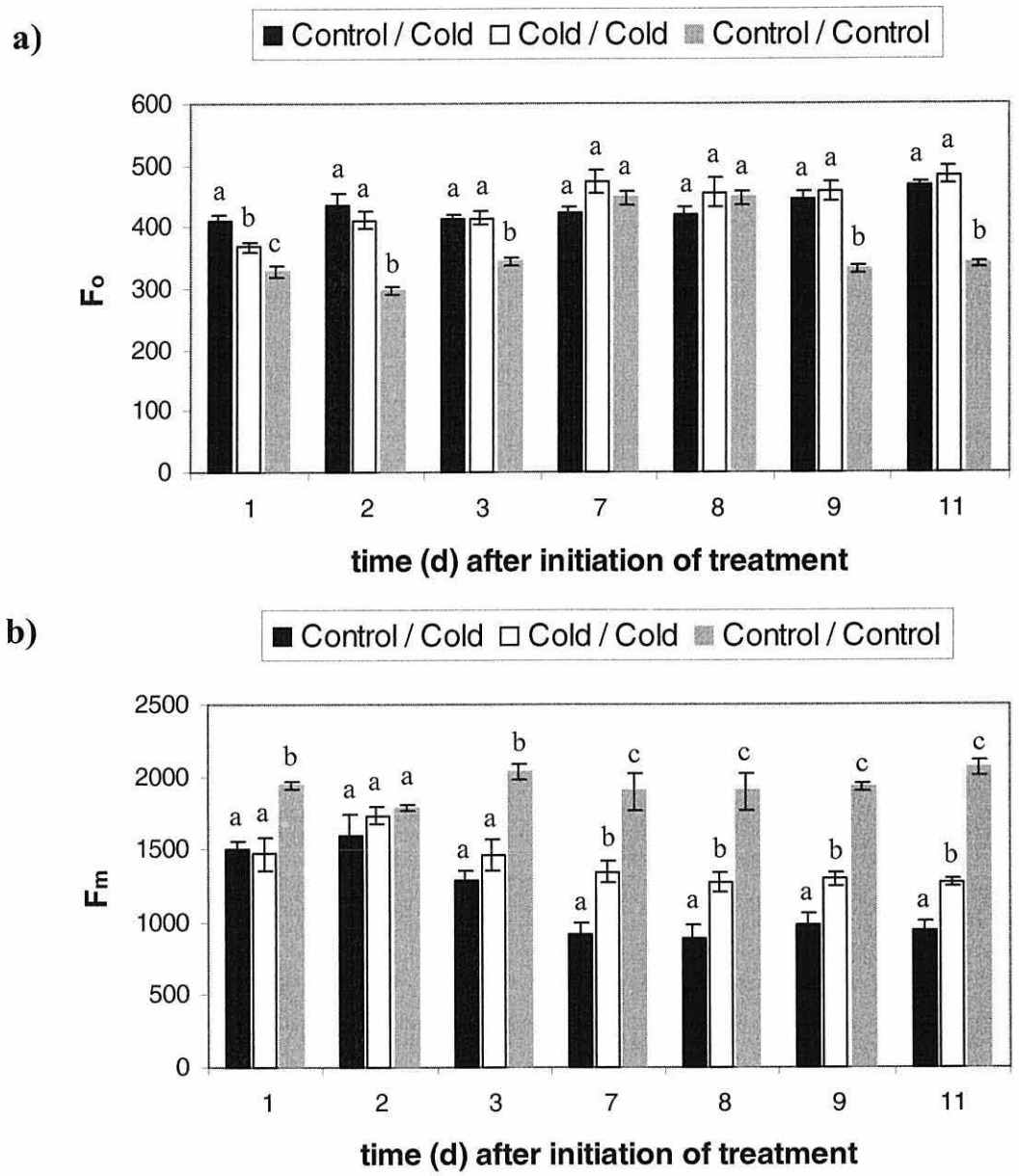


Fig.4.6 **a)** F_o and **b)** F_m of chlorophyll fluorescence in *Gossypium hirsutum.* Experimental treatments, measures of descriptive statistics, and statistical testing as in Figure 4.5.

The effect of treatment at 8°C for 11 days on gas exchange parameters is illustrated in Table 4.5. Determination of gas exchange parameters on day 2 of treatment showed significant reductions in transpiration, stomatal conductance and net rate of CO_2 uptake in response to treatment at 8°C for plants from both pretreatments, whereas the intercellular CO_2 concentration was unaltered relative to experimental control plants. Single factor analysis of variance comparing responses of experimental control, control-pretreated and cold-pretreated plants, did not detect a significant difference in responses of control- and cold-pretreated plants with respect to transpiration, stomatal conductance, and net rate of CO_2 uptake. A t-test comparing only responses of control- and cold-pretreated plants with respect to these parameters did find a significant difference between the two groups. However, reductions in transpiration, stomatal conductance, and net rate of CO_2 uptake in response to treatment at 8°C were very large for plants from both pretreatments, and thus differences between differently pretreated plants were very small in absolute terms. Transpiration rate and stomatal conductance were reduced to 5 and 3% of the experimental control level for cold- and control-pretreated plants, respectively, and the respective values regarding net rate of CO_2 uptake were 10 and 3%. With progression of treatment at 8°C, transpiration, stomatal conductance, and net rate of CO_2 uptake, determined on days 4 and 11, remained greatly reduced, and neither analysis of variance, comparing all treatments, nor a t-test, comparing only plants treated at 8°C, detected a significant difference between control- and cold-pretreated plants. Values of leaf intercellular CO_2 concentration, determined on days 4 and 11 of treatment at 8°C, remained unaltered in comparison to those of experimental control plants in plants from both pretreatments.

Table 4.5 Responses of transpiration (E), stomatal conductance (g_s), net rate of CO_2 uptake (A) and CO_2 mole fraction in the leaf intercellular air space (c_i) in *Gossypium hirsutum* to exposure to low temperature of 8°C for 2, 4, and 11 days. Pretreatments and experimental control treatment as in Figure 4.5. Mean ± standard error for 5 (cold-treated) and 4 (control / control) plants. Measurements were taken for 1 leaf per plant. For each day a single factor analysis of variance was performed between combined treatments. Means followed by different letters are significantly different ($P < 0.05$).

Day[1]	**Temperature treatment**	**E** (mmol m^{-2} s^{-1})	**g_s** (mmol m^{-2} s^{-1})	**A** (µmol m^{-2} s^{-1})	**c_i** (µmol mol^{-1})
2	*Control / Control*	9.3 ± 1.59 a	459 ± 119.9 a	21.2 ± 2.59 a	214 ± 22.8 a
	Cold / Cold	0.5 ± 0.07 b[2]	24 ± 3.6 b[2]	2.1 ± 0.42 b[2]	216 ± 12.6 a
	Control / Cold	0.3 ± 0.01 b[2]	13 ± 0.5 b[2]	0.6 ± 0.28 b[2]	282 ± 36.2 a
4	*Control / Control*	11.5 ± 0.35 a	686 ± 65.3 a	24.9 ± 0.49 a	246 ± 5.0 a
	Cold / Cold	0.5 ± 0.05 b	23 ± 2.8 b	1.6 ± 0.54 b	247 ± 30.6 a
	Control / Cold	0.4 ± 0.06 b	22 ± 3.3 b	1.3 ± 0.33 b	263 ± 14.9 a
11	*Control / Control*	8.7 ± 0.58 a	451 ± 64.8 a	19.1 ± 1.24 a	242 ± 8.2 a
	Cold / Cold	0.2 ± 0.05 b	12 ± 2.4 b	-0.3 ± 0.30 b	334 ± 75.3 a
	Control / Cold	0.3 ± 0.04 b	13 ± 2.2 b	0.0 ± 0.16 b	354 ± 25.6 a

[1] Day after initiation of treatment

[2] A t-test comparing only cold treatments (cold / cold and control / cold) showed a significant difference ($P < 0.05$) for measurements of E, g_s and A on day 2.

Plant injury was assessed after 10 days of recovery in control temperature following treatment at 8°C for 11 days. Cold-treatment induced severe injury resulting in death of old leaves in plants from both pretreatments. The degree of plant injury was classified by assignment of an injury score consisting of 3 levels, which were based on survival of apex, medium-aged leaves, and branches (Table 4.6). Although the assessment of plant injury was only of semi-quantitative nature, thus making the calculation of average values debatable, the average over the replicates of each treatment clearly showed that control- and cold-treated plants did not differ with respect to cold-induced injury.

Table 4.6 Variation in cold-induced plant injury (expressed as injury score) in *Gossypium hirsutum* after treatment with low temperature of 8°C for 11 days and recovery in control conditions (25°C) for 10 days. Pretreatments and experimental control treatment as in Figure 4.5. The score of cold-induced plant injury is derived as follows: * old leaves dead, but apex, medium-aged leaves and young branches green; * * apex, medium-aged and old leaves dead, but young branches green; * * * plants dead.

	Degree of cold-induced plant injury (expressed as injury score):	
	For 5 replicates of each treatment	Average for treatment
Cold / Cold	2 plants: * 3 plants: * * *	* *
Control / Cold	1 plant: * 3 plants: * * 1 plant: * * *	* *

4.2 Experiment 2

4.2.1 Experimental protocol

Seeds of *Gossypium hirsutum* var. CIM 443 were sown as described in section 2.1 on September 13th 2000. Seedlings were transplanted to 1.6-dm^3 pots 37 to 38 days after sowing. Plants were first treated with plant nutrient solution containing phostrogen at 2 g dm^{-3} and micronutrients as in half strength Hoagland's solution 17, 24, 30, 35, and 39 days after sowing, from then on about every second day.

For assignment to stress treatments plants were arranged in a randomized block design stratified for height (to minimise the influence of plant size on stress responses) as follows. They were sorted according to height, divided into 4 strata of increasing height, and from each stratum plants were randomly assigned to all of the combinations of pretreatment / subsequent treatment which are outlined in Table 4.7.

Table 4.7 Experimental design consisting of **(1)** 2 levels of pretreatment: exposure to low temperature for 6 days decreasing from18°C to 13°C by 1°C per day (Cold-pre) or to control temperature of 25°C for the same period of time (Control) and **(2)** the following modes of subsequent treatment: **a)** no further treatment, but assessment of plant response to pretreatment; **b)** exposure to severe stress levels of heat, cold, or drought; **c)** maintenance in control conditions throughout the experiment (applies only to control-pretreated plants, forming the experimental control). The number of replicates for each combination of pretreatment / subsequent treatment is shown.

(2) Subsequent treatment	**(1) Pretreatment**	
	Control	Cold-pre
***a)** Assessment of plant response to pre-treatment*		
-	5	5
***b)** Subsequent treatment with severe stress*		
Heat	5	5
Cold	5	5
Drought	5	5
***c)** Maintenance in control conditions*		
Control	3 x 4 [a]	

[a] 3 experimental control treatments consisting of 4 replicates each for comparison with each of the 3 severe stress treatments.

When plants were 57 days old, they were subjected to pretreatment with either low temperature for 6 days decreasing from 18°C to 13°C by 1°C per day and subsequent recovery in control conditions for 0 to 5 days (as specified below for different combinations of pretreatment / subsequent treatment) or alternatively with only control temperature of 25°C for the same period of time. For low temperature treatment plants were transferred to a temperature-controlled Vindon growth cabinet. During treatment spatial sections of plants were rotated in daily intervals (to minimize effects of environmental heterogeneity), and application of plant nutrient solutions was continued normally as outlined above. To assess plant

response to pretreatment, gas exchange, chlorophyll fluorescence, and leaf water relation (water content and relative water content) parameters were measured at 2 points of time in the course of pretreatment: a) on the final day (day 6) of cold treatment and b) after subsequent recovery in control temperature for 1 to 3 days (as specified). Shoot height and above ground dry matter production were measured after 5 days of recovery in control conditions following cold treatment. Medium-aged leaves were sampled for determination of leaf sap concentrations of glycine betaine on the final day of low temperature pretreatment (day 6) and after 3 days of subsequent recovery in control temperature.

Plants assigned to treatment with severe heat stress were pretreated with low temperature for 6 days decreasing from 18°C to 13°C by 1°C per day and subsequent recovery in control conditions for 1 day or alternatively with only control temperature of 25°C for the same period of time. They were then subjected to treatment with high temperature of 49°C for 46 hours. Assessment of plant response to heat treatment included measurement of gas exchange and chlorophyll fluorescence parameters 27 and 29 hours after initiation of treatment, respectively, and values of heat-treated plants were compared with measurements for experimental control plants taken 2 and 1 days later, respectively. Leaf water relation parameters (water content and relative water content) were measured after 45 hours of treatment.

Plants assigned to treatment with severe cold stress were subjected to pretreatment with low temperature for 6 days decreasing from 18°C to 13°C by 1°C per day and subsequent recovery in control conditions for 1 day or alternatively with only control temperature of 25°C for the same period of time. Subsequent severe cold treatment consisted of exposure to low temperature of 11°C for 5 days and subsequent recovery in control conditions of 25°C for 15 days. For low temperature treatment plants were transferred to a temperature-controlled Vindon growth cabinet. During treatment spatial sections of plants were rotated in daily intervals (to minimise effects of environmental heterogeneity), and application of plant nutrient solutions was continued normally as outlined above. The time course of plant response to severe cold treatment was investigated by measuring chlorophyll fluorescence parameters daily during the 5-day period of cold treatment and on day 1 of subsequent recovery in control conditions. Experimental control plants were only measured every second day

(days 2 and 4) of cold treatment and on day 1 of recovery in control conditions, and measurements of cold-treated plants taken on days 1, 3, and 5 of cold treatment were compared with measurements for experimental control plants from days 2, 2, and 4, respectively. Gas exchange parameters were measured at 2 points of time during treatment: a) on the final day (day 5) of cold treatment and b) on day 2 of subsequent recovery in control conditions. Experimental control plants were only measured at the first point of time. Leaves were sampled for determination of water content, relative water content, and glycine betaine concentration on the final day (day 5) of cold treatment. After 15 days of recovery in control conditions following cold treatment plants were assessed for extent of severe leaf injury, and shoot height and above ground dry matter production were measured.

Plants assigned to treatment with severe drought stress were pretreated with low temperature for 6 days decreasing from 18°C to 13°C by 1°C per day (no recovery period) or alternatively with only control temperature of 25°C for the same period of time. Directly before the start of the drought treatment, plants were transplanted to 7.5-dm^3 pots. Severe drought was imposed by withholding water for 14 days. From day 4 of drought treatment onwards plants were rotated in daily intervals (to minimise effects of environmental heterogeneity). Development of soil moisture deficit was investigated by measuring volumetric soil water content in the plant substrate in intervals of 1 to 2 days from day 4 to 14 (days 4, 5, 6, 7, 9, 10, 11, 13, and 14) after initiation of treatment. Measurements for experimental control plants were taken less frequently (on days corresponding to days 4, 5, 6, 7, 10, 13, and 14 of treatment for drought-treated plants). Similarly the time course of plant response to drought treatment was assessed by measuring gas exchange parameters in intervals of 1 to 2 days from day 4 to 14 (days 4, 5, 6, 7, 9, 10, 11, 12, and 14) after initiation of treatment. Experimental control plants were assessed for these parameters less frequently (on the days corresponding to days 5, 7, 10, and 14 of treatment for drought-treated plants). Leaf water relation parameters (water content and relative water content) were measured on the final day (day 14) of drought treatment. Finally shoot height and above ground dry matter production were measured after 10 and 11 days, respectively, of recovery in well-watered control conditions following drought treatment.

4.2.2 Results

4.2.2.1 Effect of pretreatment at gradually decreasing low temperature

Treatment of 57-day old plants of *Gossypium hirsutum* at low temperatures decreasing from 18 to 13°C by 1°C per day over a 6-day period induced significant changes in a variety of physiological parameters.

Gas exchange parameters were assessed on the final day of low temperature treatment (day 6) and after subsequent recovery in control temperature for 1 day (Table 4.7). Transpiration rate, stomatal conductance, and net rate of CO_2 uptake exhibited significant reductions to 8, 11, and 13%, respectively, of the control level when determined on day 6 of low temperature treatment. Determination of intercellular CO_2 concentration and water use efficiency of net photosynthesis on day 6 of treatment showed no significant difference in the response of these parameters relative to the control. Return of low temperature-treated plants to control temperature for 1 day resulted in a partial recovery of transpiration, stomatal conductance and net rate of CO_2 uptake to 53, 40, and 53%, respectively, of the control level, with values remaining significantly reduced below those of control plants.

Table 4.7 Variation in transpiration (E), stomatal conductance (g_s), net rate of CO_2 uptake (A), CO_2 mole fraction in the leaf intercellular air space (c_i), and water use efficiency of net photosynthesis (A/E) in *Gossypium hirsutum* after exposure to low temperature for 6 days decreasing from 18°C to 13°C by 1°C per day (day 6 after initiation of treatment) and subsequent recovery in control conditions for 1 day (day 7 after initiation of treatment). Control plants were maintained at 25°C. Each value is the mean ± standard error for 5 plants; the value for each plant is the average of measurements for 2 adjacent medium-aged leaves. For each day and variable control and cold treatment were compared by a t-test. Asterisks *, * *, * * * represent significance at P = 0.05, 0.01, and 0.001, respectively; ns = nonsignificant at P = 0.05.

Day [1]		**E** (mmol m^{-2} s^{-1})	**g_s** (mmol m^{-2} s^{-1})	**A** (μmol m^{-2} s^{-1})	**c_i** (μmol mol^{-1})	**A/E**
6	Control	7.6 ± 0.14	325 ± 6.8	16 ± 0.6	230 ± 2.7	2.2 ± 0.07
	Cold	0.6 ± 0.07	36 ± 4.7	2 ± 0.3	280 ± 20.4	2.7 ± 0.58
	Significance of t-test between treatments					
		* * *	* * *	* * *	ns	ns
7	Control	7.8 ± 0.10	324 ± 10.2	17 ± 0.5	220 ± 3.5	2.2 ± 0.06
	Cold	4.1 ± 0.43	128 ± 16.1	9 ± 0.9	202 ± 7.6	2.3 ± 0.12
	Significance of t-test between treatments					
		* * *	* * *	* * *	ns	ns

[1] day after initiation of treatment

The effects of low temperature treatment on chlorophyll fluorescence parameters are illustrated in Table 4.8. On the final day of treatment F_v/F_m was significantly reduced by 13% compared to the control, and this was due to a significant decrease in F_m by 32% relative to the control, while F_o was not significantly affected. On return to control temperature for 1 day F_v/F_m completely recovered to the level of control plants. However, this was not simply due to recovery of F_m, but resulted from significant increases in both, F_o and F_m, by 10 and 15%, respectively, relative to the level of control plants.

Table 4.8 F_v/F_m, F_o, and F_m of chlorophyll fluorescence in *Gossypium hirsutum* after exposure to low temperature for 6 days decreasing from18°C to 13°C by 1°C per day (day 6 after initiation of treatment) and subsequent recovery in control conditions for 1 day (day 7 after initiation of treatment). Control plants were maintained at 25°C. Each value is the mean ± standard error for 5 plants; the value for each plant is the average of measurements for 3 adjacent medium-aged leaves. Statistical testing as in Table 4.7.

Day [1]		**F_v/F_m**	**F_o**	**F_m**
6	Control	0.793 ± 0.011	426 ± 24.4	2075 ± 67.9
	Cold	0.693 ± 0.023	401 ± 9.1	1402 ± 110.0
	Significance of t-test between treatments			
		* *	ns	* *
7	Control	0.797 ± 0.014	395 ± 13.4	1981 ± 87.1
	Cold	0.809 ± 0.002	435 ± 4.8	2285 ± 10.2
	Significance of t-test between treatments			
		ns	*	* *

[1] day after initiation of treatment

The response of shoot dry matter production and shoot height increase to low temperature treatment was assessed after 5 days of recovery in control temperature following low temperature treatment (Table 4.9). Shoot dry matter production exhibited a significant reduction to 75% of the control level. This was due to significant reductions in stem and leaf dry matter per plant to 71 and 76%, respectively, of the control level, whereas flower dry matter (exhibiting very low values for both treatments) did not differ significantly from the control. Shoot height showed a significant, but less pronounced reduction by 10% relative to the control.

Table 4.9 Variation in shoot height and above ground dry matter production (whole shoot and individual contributions of stem, leaves and flowers) in *Gossypium hirsutum* after exposure to low temperature for 6 days decreasing from 18°C to 13°C by 1°C per day and subsequent recovery in control conditions of 25°C for 5 days (day 11 after initiation of treatment). Control plants were maintained at 25°C. Each value is the mean ± standard error for 5 plants. Statistical testing as in Table 4.7.

	Shoot height (cm plant^{-1})	**Dry matter production (g plant^{-1})**			
		Stem	**Leaves**	**Flowers**	**Shoot**
Control	94.7 ± 2.0	9.7 ± 0.7	14.4 ± 0.9	0.13 ± 0.04	24.2 ± 1.5
Cold	85.2 ± 2.6	6.9 ± 0.4	11.0 ± 0.5	0.14 ± 0.07	18.1 ± 0.9
Significance of t-test between treatments					
	*	*	* *	ns	* *

Low temperature treatment for 6 days induced a significant reduction in relative water content by 21% relative to the control, when determined on the final day of treatment, but did not cause significant changes in water content (Table 4.10). Subsequent return to control temperature for 3 days resulted in partial recovery of relative water content, but it remained significantly reduced below the control level by 4% (Table 4.10).

Table 4.10 Leaf water content (WC) and relative water content (RWC) in *Gossypium hirsutum* **a)** after treatment with low temperature for 6 days decreasing from 18 to 13°C by 1°C per day (day 6 after initiation of treatment) and **b)** after subsequent recovery in control conditions for 3 days (day 9 after initiation of treatment). Control plants were maintained at 25°C. Mean ± standard error, with number of replicates as indicated. Statistical testing as in Table 4.7.

Day [1]	**Treatment**	**WC (%)**	**RWC (%)**
a) 6	Control	412 ± 25.2 [2]	96 ± 1.4 [3]
	Cold	368 ± 11.6 [2]	76 ± 0.5 [2]
	Significance of t-test between treatments		
		ns	* * *
b) 9	Control	436 ± 15.3 [2]	93 ± 0.9 [2]
	Cold	416 ± 11.5 [2]	89 ± 0.9 [2]
	Significance of t-test between treatments		
		ns	*

[1] day after initiation of treatment
[2] n = 5
[3] n = 4

Low temperature-treated plants accumulated glycinebetaine to significantly (3-fold) higher concentrations in the leaf sap, when determined on the final day of treatment, as compared to control plants (Table 4.11a). To assess net accumulation of glycinebetaine independent of effects of passive dehydration/dilution, concentrations determined in expressed leaf sap were also expressed on a dry weight basis. This did not greatly modify relative treatment differences, with low temperature-treated plants exhibiting significantly (2.8-fold) higher concentrations as compared to the control. Return of low-temperature treated plants to control temperature for 3 days resulted in decrease of leaf glycinebetaine concentrations to values not significantly different from the control, and this was apparent when expressed as concentration in leaf sap or related to dry weight (Table 4.11b).

Table 4.11 Variation in glycinebetaine concentration in leaves of *Gossypium hirsutum* **a)** after treatment with low temperature for 6 days decreasing from 18 to 13°C by 1°C per day (day 6 after initiation of treatment), **b)** after subsequent recovery in control conditions for 3 days (day 9 after initiation of treatment). Control plants were maintained at 25°C. Concentrations are indicated as determined in expressed sap as well as converted to a dry weight basis via the measured leaf water content. Water content was measured within 1 hour of sampling for leaf glycinebetaine concentrations using an adjacent leaf. Mean ± standard error for 5 plants. Statistical testing as in Table 4.7.

Day [1]	**Treatment**	**Glycinebetaine in leaves**	
		In expressed sap mol m^{-3}	On dry weight basis µmol g^{-1}
a) 6	Control	50 ± 3.6	204 ± 12.9
	Cold	152 ± 17.5	562 ± 74.7
	Significance of t-test between treatments		
		* *	* *
b) 9	Control	40 ± 5.6	175 ± 24.0
	Cold	47 ± 5.3	195 ± 24.5
	Significance of t-test between treatments		
		ns	ns

[1] day after initiation of treatment

4.2.2.2 Effect of pretreatment at low temperature on plant response to treatment at 49°C

Plants pretreated at low temperature decreasing from 18 to 13°C by 1°C per day followed by recovery in control temperature for 1 day (cold-pretreated) and plants of the same age, which had been maintained in control temperature (control-pretreated) were subjected to treatment at 49°C for 1 day.

Heat-treatment induced significant changes in chlorophyll fluorescence parameters (Table 4.12). F_v/F_m was significantly decreased in plants from both pretreatments in comparison to the experimental control, but control- and cold-pretreated plants differed significantly with respect to the extent of this decrease, with relative reductions amounting to 61 and 21 % in control- and cold-pretreated plants, respectively (Table 4.12a). The response of F_v/F_m was paralleled by equivalent changes in F_m. In contrast, F_o exhibited a different response pattern: Values of F_o were increased by 41 and 51% relative to the experimental control level in control- and cold-pretreated plants, respectively. While F_o values of cold-

pretreated plants differed significantly from those of the experimental control, the intermediate values of control-pretreated plants were not significantly different from either cold-pretreated or experimental control plants.

Tab 4.12 F_v/F_m, F_o, and F_m of chlorophyll fluorescence in *Gossypium hirsutum* after exposure to high temperature of 49°C for 29 hours. Plants had previously been pretreated with either low temperature for 6 days decreasing from18°C to 13°C by 1°C per day and then control temperature for 1 day (Cold) or alternatively with control temperature for the same period of time (Ct). Experimental control plants (Ct / Ct) were maintained in control temperature of 25°C throughout the experiment. Chlorophyll fluorescence parameters are presented as **a)** absolute values measured after heat treatment and **b)** values measured after heat treatment as % of the mean value after pretreatment. The measurement of response to pretreatment was carried out on a different set of plants (see Table 4.8). At the time of measurement heat-treated plants were 65 and experimental control plants 66 days old. Mean ± standard error for 5 (heat-treatments) and 4 (experimental control) plants, the value for each plant being the average of measurements for 2 adjacent medium-aged leaves. For each variable a one-way analysis of variance was performed between combined treatments. Means followed by different letters are significantly different ($P < 0.05$).

	F_v/F_m	F_o	F_m
	***a)** Absolute values measured after heat treatment*		
Ct / Ct	0.811 ± 0.006 a	408 ± 11 a	2168 ± 75 a
Cold / 49°C	0.639 ± 0.014 b	617 ± 53 b	1693 ± 86 b
Ct / 49°C	0.320 ± 0.062 c	577 ± 48 ab	870 ± 84 c
	***b)** Values as in a as % of the mean value after the respective pretreatment*		
Ct / Ct	102 ± 0.7 a	103 ± 2.9 a	109 ± 3.8 a
Cold / 49°C	79 ± 1.7 b	142 ± 12.1 ab	74 ± 3.8 b
Ct / 49°C	40 ± 7.8 c	146 ± 12.1 b	44 ± 4.2 c

As low temperature pretreatment itself induced significant changes in chlorophyll fluorescence parameters, responses to treatment at 49°C were also expressed as percentage of the mean value after the respective pretreatment (Table 4.12b). This did not greatly modify relative treatment differences with respect to F_v/F_m and F_m. However, when F_o was expressed as percentage of the respective pretreatment level, relative increases in comparison to the experimental control amounted to 42 and 38% for control- and cold-pretreated plants, respectively. In this case only values of control-pretreated plants differed significantly from those of the experimental control, whereas the intermediate values of cold-pretreated

plants were not significantly different from those of either control-pretreated or experimental control plants.

Gas exchange parameters exhibited significant changes in response to heat treatment (Table 4.13). Transpiration rate and stomatal conductance were significantly reduced as compared to the experimental control in plants from both pretreatments. Although differently pretreated plants showed slight differences in the extent of these reductions, with relative reductions in transpiration rate amounting to 53 and 45% for control- and cold treated plants, respectively, and corresponding reductions in stomatal conductance being 63 and 53%, these differences were not statistically significant ($P < 0.05$) (Table 4.13a). However, pretreatment had a significant effect on the response of net rate of CO_2 uptake to heat treatment. Exposure to 49°C for 1 day caused a significant decrease in the net rate of CO_2 uptake in plants from both pretreatments, but control-pretreated plants exhibited values slightly below zero, whereas cold-pretreated plants maintained positive net rates of CO_2 uptake at 17% of the control level. Heat treatment induced a significant increase in intercellular CO_2 concentration in plants from both pretreatments. The relative increase compared to the experimental control was greater in control-pretreated plants (71%) than cold-pretreated plants (44%), but this difference was not statistically significant ($P < 0.05$).

As cold-pretreatment itself significantly affected gas exchange parameters, responses of these parameters to treatment at 49°C were also expressed as percentage of the mean value after the respective pretreatment (Table 4.13b). This changed relative treatment differences regarding the response of transpiration rate and stomatal conductance to heat treatment: values of control-pretreated plants were significantly reduced, whereas those of cold-pretreated plants did not differ significantly from those of the experimental control. Expression relative to values after pretreatment did not greatly modify relative treatment differences with respect to responses of net rate of CO_2 uptake or intercellular CO_2 concentration to heat treatment.

Table 4.13 Variation in transpiration (E), stomatal conductance (g_s), net rate of CO_2 uptake (A), and CO_2 mole fraction in the leaf intercellular air space (c_i) in *Gossypium hirsutum* after treatment with high temperature of 49°C for 27 hours. Pretreatments and experimental control treatment as in Table 4.12. Gas exchange parameters are indicated as **a)** absolute values measured after heat treatment and **b)** values measured after heat treatment as % of the mean value after the respective pretreatment. The measurement of response to pretreatment was carried out on a different set of plants (see Table 4.7). At the time of measurement heat-treated plants were 65 and experimental control plants 67 days old. Mean ± standard error for 5 (heat-treatments) and 4 (experimental control) plants, the value for each plant being the average of measurements for 2 adjacent medium-aged leaves. Statistical testing as in Tab. 4.12.

	E ($mmol\ m^{-2}\ s^{-1}$)	$\mathbf{g_s}$ ($mmol\ m^{-2}\ s^{-1}$)	**A** ($\mu mol\ m^{-2}\ s^{-1}$)	$\mathbf{c_i}$ ($\mu mol\ mol^{-1}$)
	a) *absolute values measured after heat treatment*			
Ct / Ct	7.3 ± 0.23 a	315 ± 13.9 a	16.9 ± 0.93 a	228 ± 4.4 a
Cold / 49°C	4.0 ± 0.41 b	148 ± 18.4 b	2.8 ± 0.47 b	329 ± 9.6 b
Ct / 49°C	3.4 ± 0.70 b	116 ± 31.6 b	-0.6 ± 0.43 c	391 ± 28.2 b
	b) *Values as in a as % of the mean value after the respective pre-treatment*			
Ct / Ct	93 ± 3.0 a	97 ± 4.3 a	97 ± 5.4 a	103 ± 2.0 a
Cold / 49°C	98 ± 10.1 a	116 ± 14.4 a	30 ± 5.1 b	163 ± 4.8 b
Ct / 49°C	44 ± 9.0 b	36 ± 9.7 b	-3 ± 2.5 c	178 ± 12.8 b

Determination of leaf water content and relative water content after 2 days of exposure to 49°C revealed significantly different responses of control- and cold-pretreated plants (Table 4.14). Heat treatment induced significant reductions in these parameters in plants from both pretreatments relative to the experimental control, but the extent of this reduction was significantly greater in control-pretreated plants (amounting to 86 and 65% for water content and relative water content, respectively) as compared to cold-pretreated plants (with respective reductions of 70 and 46%).

Table 4.14 Water content (WC) and relative water content (RWC) in *Gossypium hirsutum* after treatment with high temperature of 49°C for 45 hours. Pretreatments and experimental control treatment as in Table 4.12. Mean ± standard error for 5 (heat-treatments) and 4 (experimental control) plants. Statistical testing as in Tab. 4.12.

	WC (%)	**RWC (%)**
Ct / Ct	410 ± 16.7 a	91 ± 1.1 a
Cold / 49°C	125 ± 15.4 b	49 ± 5.1 b
Ct / 49°C	56 ± 2.7 c	32 ± 0.2 c

4.2.2.3 Effect of pretreatment at low temperature gradually decreasing from 18 to 13°C by 1°C per day on plant response to treatment at 11°C

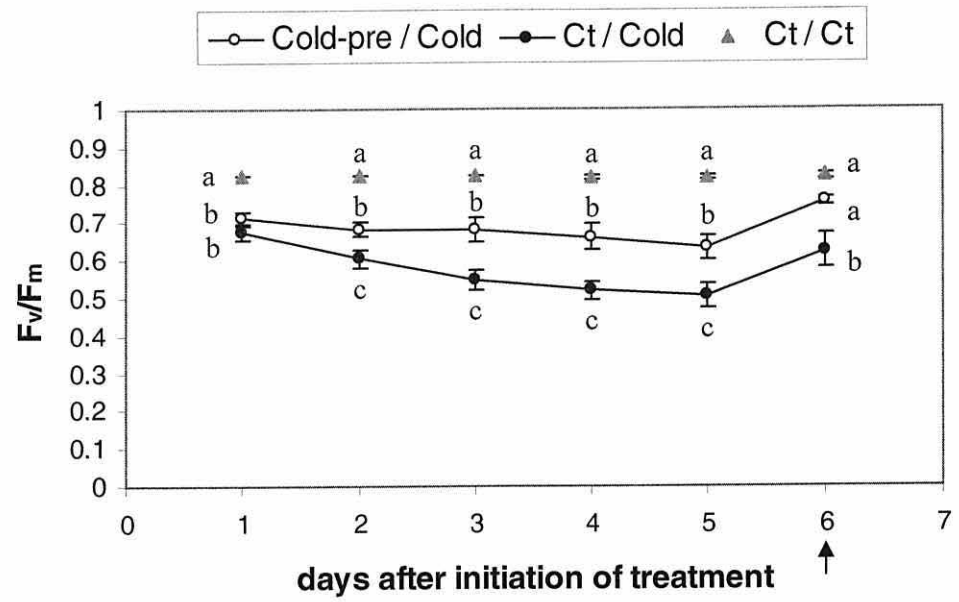
Plants pretreated at low temperature decreasing from 18 to 13°C by 1°C per day followed by recovery in control temperature for 1 day (cold-pretreated) and plants of the same age, which had been maintained in control temperature (control-pretreated) were subsequently exposed to low temperature of 11°C for 5 days.

On exposure to low temperature of 11°C for 5 days significant differences emerged between control- and cold-pretreated plants with respect to responses of chlorophyll fluorescence parameters (Figure 4.7). A significant decrease in F_v/F_m relative to the experimental control occurred in plants from both pretreatments throughout the treatment period, but the extent of reduction was significantly greater in control- as compared to cold-pretreated plants (Figure 4.7a). Plants from both pretreatments exhibited a gradual decrease in F_v/F_m with increasing exposure time. Values of control-pretreated plants declined from 82% of the experimental control level on day 1 to 62% on day 5 of treatment, whereas those of cold-pretreated plants decreased from 86% to 77% of the control level in the same period of time. The response pattern of F_m closely paralleled that of F_v/F_m (Figure 4.7b). In contrast, signifcant changes in F_o relative to the experimental control occurred only on day 2 and 3 of low temperature treatment (Figure 4.7c). Cold-pretreated plants exhibited a significant increase by 22 and 17% relative to the experimental control on days 2 and 3 of treatment, respectively, whereas

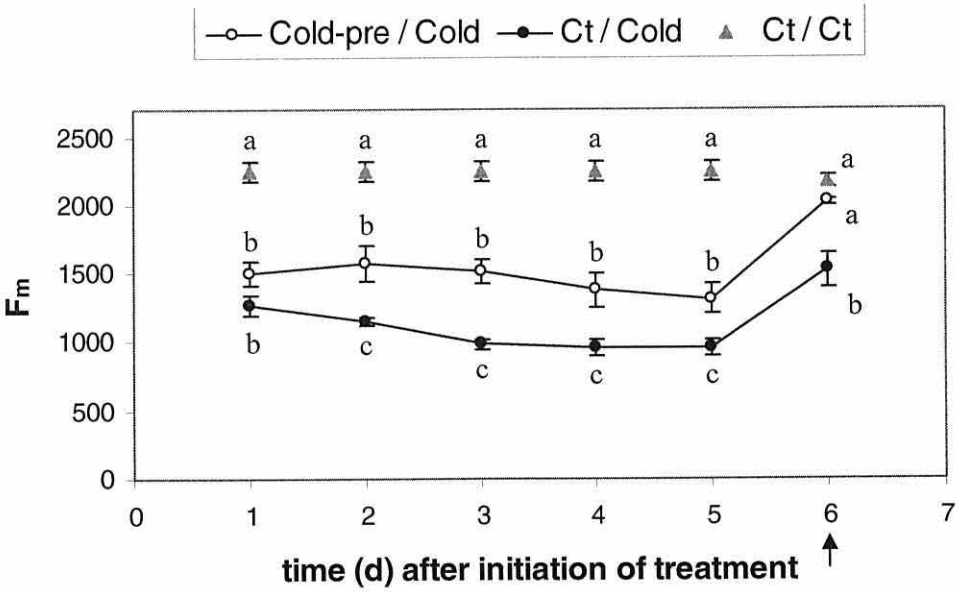
values of control-pretreated plants increased only slightly (by 8%) and were not significantly different from either experimental control or cold-pretreated plants.

Subsequent return to control temperature for 1 day resulted in some recovery of F_v/F_m in plants from both pretreatments, but control- and cold-pretreated plants differed significantly in the extent of this recovery (Figure 4.7a). Values of control-pretreated plants rose to 76% of the experimental control level, but remained significantly reduced below that level, whereas values of cold-pretreated plants recovered to 92% of the control level and were not significantly different from those of experimental control plants. The response of F_m closely paralleled that of F_v/F_m (Figure 4.7b). F_o exhibited a different response pattern: While plants from both pretreatments showed F_o levels not significantly different from those of the experimental control on the final day of low temperature treatment, F_o increased significantly by 22 and 27% in control- and cold-pretreated plants, respectively, on return to control temperature for 1 day (Figure 4.7c).

a)


Cold-pre / Cold
Ct / Cold
Ct / Ct
Fv/Fm
days after initiation of treatment

b)


Cold-pre / Cold
Ct / Cold
Ct / Ct
Fm
time (d) after initiation of treatment

c)

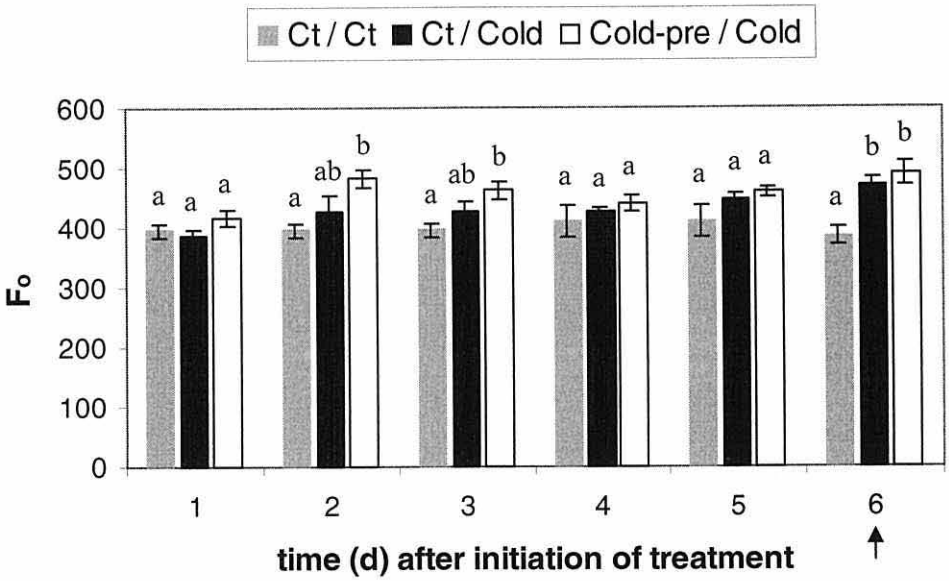
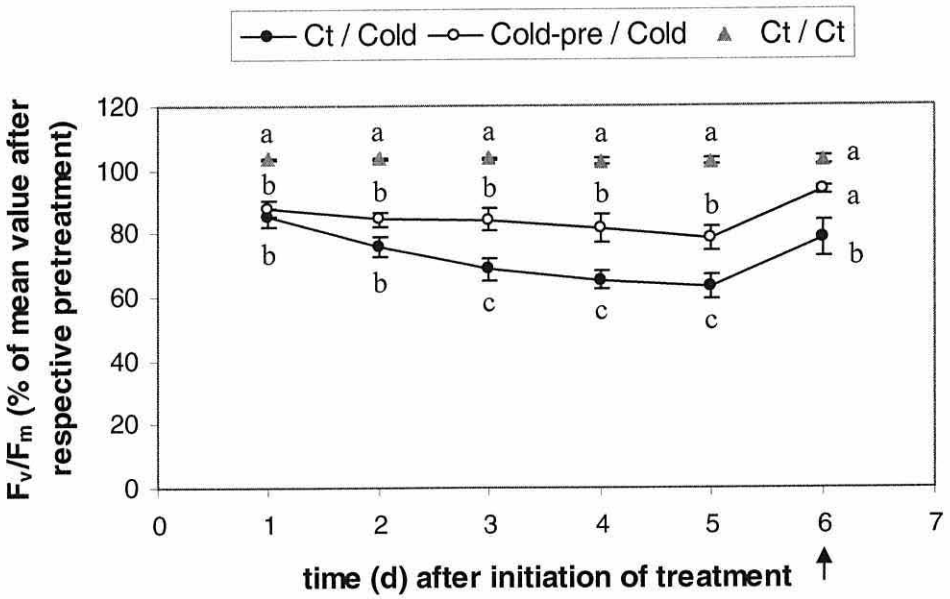

Ct / Ct
Ct / Cold
Cold-pre / Cold
Fo
time (d) after initiation of treatment

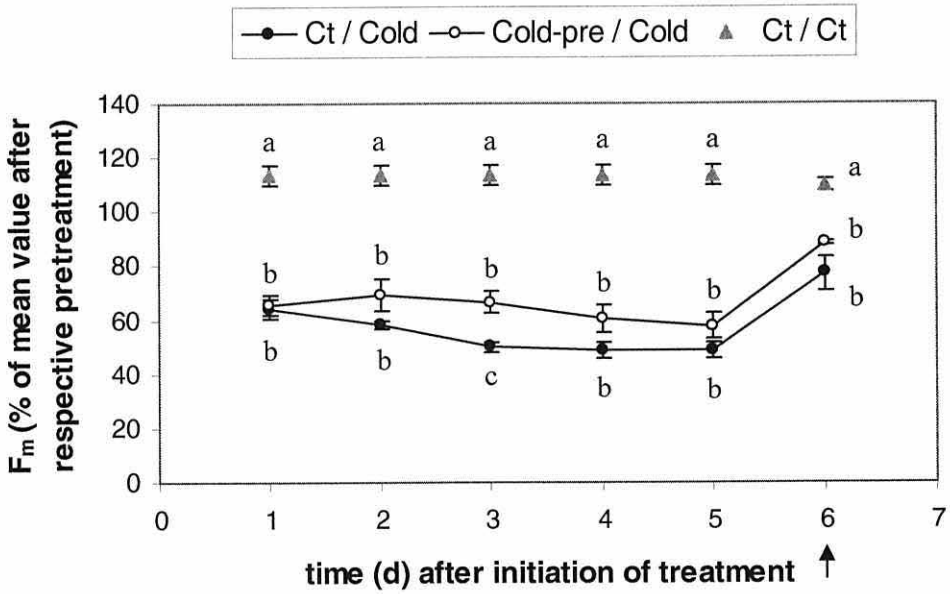
Figure 4.7 **a)** F_v/F_m, **b)** F_m, and **c)** F_0 of chlorophyll fluorescence in *Gossypium hirsutum* in response to exposure to low temperature of 11°C for 5 days (Cold). Black arrows mark subsequent recovery in control conditions of 25°C for 1 day. Plants had been pretreated with either low temperature for 6 days decreasing from18°C to 13°C by 1°C per day and then control temperature for 1 day (Cold-pre) or alternatively only with control temperature for the same period of time (Ct). Experimental control plants were maintained in control temperature of 25°C throughout the experiment. Each value is the mean ± standard error for 5 (cold-treatments) and 4 (experimental control) plants, the value for each plant being the average of measurements for 3 adjacent medium-aged leaves. For each day and variable a one-way analysis of variance was performed between combined treatments. On days 1, 3, and 5 only cold-treated plants were measured and compared with measurements for experimental control plants from days 2, 2 and 4, respectively. Means followed by different letters are significantly different ($P < 0.05$).

To correct for the contribution of effects remaining from low temperature pretreatment on the levels of chlorophyll fluorescence parameters observed in response to treatment at 11°C, the latter values were expressed as percentage of the values after pretreatment (Figure 4.8). This diminished most of the differences between control- and cold-pretreated plants with respect to F_m and F_o. Treatment at 11°C induced significant reductions in F_m relative to the experimental control throughout the treatment period, but although control-pretreated plants still showed a tendency to slightly greater reductions as compared to cold-pretreated plants, a significant difference between differently pretreated plants occurred only on day 3 of treatment (Figure 4.8b). The significant difference between differently pretreated plants on return to control temperature also disappeared, with values of plants from both pretreatments remaining significantly reduced below the experimental control level. When F_o was expressed relative to the level after pretreatment, significant differences between treatments in the period of low temperature treatment disappeared (Figure 4.8c). On return to control temperature following treatment at 11°C plants from both pretreatments still showed an increase in F_o, but this was only statistically significant in control-pretreated plants, whereas the intermediate levels of cold-pretreated plants did not differ significantly from either experimental control or control-pretreated plants. Expressing F_v/F_m relative to the values after pretreatment did not greatly affect relative treatment differences on exposure to 11°C or on return to control temperature for 1 day (Figure 4.8a). Only on day 2 of low temperature treatment the significant difference between control- and cold-pretreated plants disappeared. Comparison of relative treatment differences with respect to chlorophyll fluorescence parameters, when expressed as absolute values or relative to the values after pretreatment, allows the following conclusions. The higher values of F_m and F_o in cold-pretreated as compared to control-pretreated plants on exposure to low temperature at 11°C as well as on return to control temperature may be largely attributed to the significant increases in both parameters, which occurred after 1 day of recovery following cold-pretreatment. This increase in both parameters used for the calculation of F_v resulted in overall unaltered F_v/F_m values after 1 day of recovery following cold-pretreatment, and this explains why expression of F_v/F_m relative to values after pretreatment did not greatly modify relative treatment differences.

a)


Ct / Cold
Cold-pre / Cold
Ct / Ct
F_v/F_m (% of mean value after respective pretreatment)
time (d) after initiation of treatment

b)


Ct / Cold
Cold-pre / Cold
Ct / Ct
F_m (% of mean value after respective pretreatment)
time (d) after initiation of treatment

c)

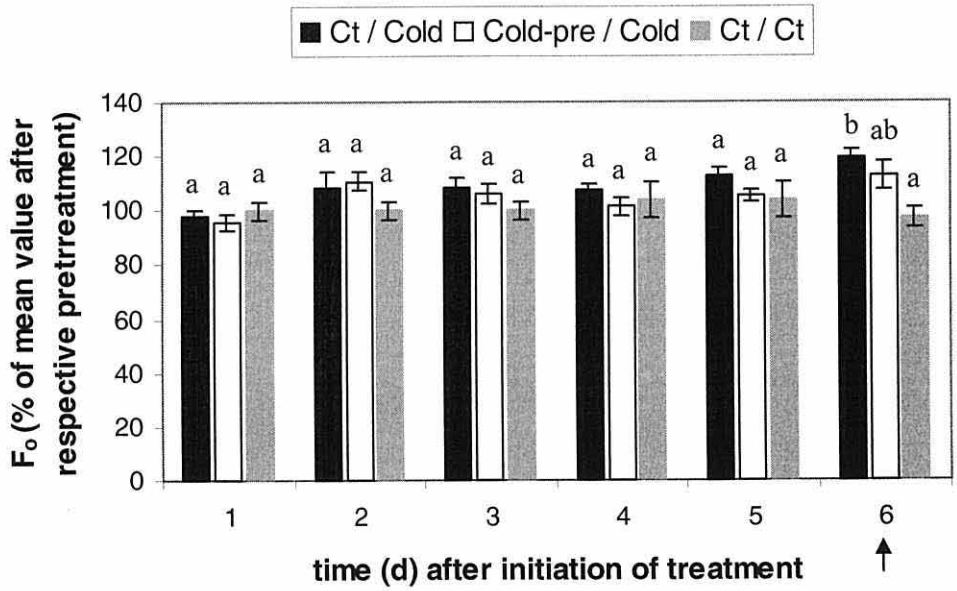

Ct / Cold
Cold-pre / Cold
Ct / Ct
F_o (% of mean value after respective pretreatment)
time (d) after initiation of treatment

Figure 4.8 **a)** F_v/F_m, **b)** F_m, and **c)** F_o of chlorophyll fluorescence in *Gossypium hirsutum* expressed as % of the mean value after the respective pretreatment. Experimental treatments, measures of descriptive statistics and statistical testing as in Figure 4.7. Response to pretreatment was measured in a different set of plants (see table 4.8).

The responses of gas exchange parameters, assessed on the final day of low temperature treatment and after subsequent return to control temperature for 2 days, are illustrated in Table 4.15. Control- and cold-pretreated plants did not differ significantly with respect to responses of gas exchange parameters, when determined on the final day of low temperature treatment (Table 4.15a). Transpiration rates and stomatal conductance were greatly reduced to only 4 to 6% of the control level, the net rate of CO_2 uptake exhibited values around zero, and the intercellular CO_2 concentration was increased by 1.5- to 2-fold as compared to experimental control plants. However, pretreatment had a significant effect on the response of all gas exchange parameters determined on return to control temperature for 2 days (Table 4.15b). Transpiration rate of cold-pretreated plants slightly recovered to 11% of the experimental control level, whereas that of control-pretreated plants amounted to only 7% of the experimental control level. Stomatal conductance remained at 6% of the experimental control level in cold-pretreated plants, but was further reduced to 3% of the experimental control level in control-pretreated plants. While cold-pretreated plants exhibited small (6% of the experimental control level), but positive net rates of CO_2 uptake, control-pretreated plants had negative rates. This was associated with a significantly higher intercellular CO_2 concentration in control- as compared to cold-pretreated plants.

Table 4.15 Variation in transpiration (E), stomatal conductance (g_s), net rate of CO_2 uptake (A), and CO_2 mole fraction in the leaf intercellular air space (c_i) in *Gossypium hirsutum* in response to treatment with low temperature (Cold): **a)** after exposure to low temperature of 11°C for 5 days; **b)** after subsequent recovery in control conditions of 25°C for 2 days. Pretreatments and experimental control treatment as in Figure 4.7. Each value is the mean ± standard error for 5 (cold-treatments) and 4 (experimental control) plants. The value for each plant is the average of measurements for 2 medium-aged leaves adjacent to each other (except in one replicate of treatment Ct / Cold on day 7 in which the measured leaves were 1 node apart, as the leaf in between them had been shed). For each day and variable cold-treatments were compared by a t-test. Asterisks *, * *, * * * represent significance at P = 0.05, 0.01, and 0.001, respectively; ns = nonsignificant at P = 0.05.

Day [1]		**E** (mmol m^{-2} s^{-1})	**g_s** (mmol m^{-2} s^{-1})	**A** (µmol m^{-2} s^{-1})	**c_i** (µmol mol^{-1})
a) 5	Ct / Ct	8.34 ± 0.21	347 ± 15.9	18.55 ± 0.59	226 ± 1
	Ct / Cold	0.37 ± 0.07	20 ± 4.4	-0.52 ± 0.59	463 ± 77
	Cold-pre / Cold	0.38 ± 0.05	20 ± 2.6	0.36 ± 0.26	330 ± 20
	Significance of t-test between cold-treatments				
		ns	ns	ns	ns
b) 7	Ct / Cold	0.58 ± 0.03	11 ± 0.5	-1.24 ± 0.35	516 ± 47
	Cold-pre / Cold	0.90 ± 0.05	20 ± 1.7	1.04 ± 0.16	281 ± 11
	Significance of t-test between cold-treatments				
		* *	* *	* * *	* *

[1] day after initiation of treatment

Exposure to low temperature at 11°C for 5 days did not significantly affect leaf water content, but caused a significant reduction in relative water content (by 27%) in cold-pretreated plants, whereas values of control-pretreated plants were not significantly different from those of the experimental control (Table 4.16).

Table 4.16 Variation in leaf water content (WC) and relative water content (RWC) in *Gossypium hirsutum* after exposure to low temperature of 11°C for 5 days. Pretreatments and experimental control treatment as in Figure 4.7. Mean ± standard error for 5 (cold-treatments), 4 (experimental control, measurement of WC), and 2 (experimental control, measurement of RWC) plants. For each variable a single factor analysis of variance was performed between combined treatments. Means followed by different letters are significantly different ($P < 0.05$).

	WC (%)	**RWC (%)**
Ct / Ct	419 ± 48.3 a	97 ± 1.5 a
Ct / Cold	365 ± 21.2 a	82 ± 4.0 a
Cold-Pre / Cold	317 ± 6.7 a	71 ± 1.8 b

Treatment at 11°C induced a significant rise in glycinebetaine concentration in expressed leaf sap relative to the experimental control level in plants from both pretreatments, but pretreatment had a significant effect on the extent of this increase (Table 4.17). Cold-pretreated plants exhibited a 6.4-fold increase, control-pretreated plants only a 3.9-fold rise relative to the experimental control level. However, expression of concentrations determined in expressed leaf sap on dry weight basis, using values of water content measured in the same leaves, diminished the difference between control- and cold-pretreated plants. Plants from both pretreatments exhibited a significant increase in concentration relative to the experimental control level, and the extent of this increase was higher in cold-pretreated (4.7-fold) as compared to control-pretreated plants (3.4-fold), but this difference was not statistically significant ($P < 0.05$).

Table 4.17 Variation in glycine betaine concentration in leaves of *Gossypium hirsutum* after exposure to low temperature of 11°C for 5 days. Pretreatments and experimental control treatment as in Figure 4.7. Concentrations are indicated as determined in expressed sap as well as converted to dry weight basis via the measured leaf water content. Water content was measured within 1 hour of sampling for leaf glycinebetaine concentrations using an adjacent leaf. At the time of measurement cold-treated plants were 69 and experimental control plants 66 days old. Mean ± standard error, number of replicates as indicated. Statistical testing as in Table 4.16.

Treatment	**Glycinebetaine in leaves**	
	In expressed sap mol m^{-3}	On dry weight basis µmol g^{-1}
Ct / Ct [1]	40 ± 5.6 a	175 ± 24.0 a
Ct / Cold [2]	156 ± 17.6 b	599 ± 70.9 b
Cold-Pre / Cold [1]	257 ± 17.9 c	816 ± 65.0 b

[1] n = 5
[2] n = 3

Pretreatment had a significant effect on the degree of leaf injury induced by low temperature treatment at 11°C (Table 4.18). Leaf injury was classified based on the proportion of main stem leaves, which had died after 15 days of recovery in control temperature following low temperature treatment at 11°C for 5 days. Control-pretreated plants showed death of 56% of the total number of main stem leaves per plant, whereas the respective value for cold-pretreated plants was only 7%.

Table 4.18 Severe leaf injury (% of dead main stem leaves, shed or still attached to the stem, $plant^{-1}$) in *Gossypium hirsutum* in response to exposure to low temperature of 11°C for 5 days and recovery in control conditions of 25°C for 15 days (Cold). Pretreatments and experimental control treatment as in Figure 4.7. Mean ± standard error for 5 (cold-treatments) and 4 (experimental control) plants. Statistical testing as in Table 4.16.

	Dead main stem leaves (% $plant^{-1}$)
Ct / Ct	1.5 ± 1.5 a
Cold-Pre / Cold	6.5 ± 3.0 a
Ct / Cold	56.3 ± 12.3 b

The responses of shoot height and shoot dry matter production to low temperature treatment at 11°C for 5 days were assessed after 15 days of recovery in control temperature following treatment and are shown in Table 4.19. Low temperature treatment at 11°C greatly reduced shoot height and shoot dry matter production. Differently pretreated plants did not differ significantly with respect to the extent of the reduction in shoot height, total shoot dry matter production, stem or leaf dry matter production (Table 4.19a). In control-pretreated plants low temperature treatment also caused a significant reduction in dry matter production of reproductive organs to only 9% of the control level. In contrast, values of cold-pretreated plants were reduced to a lesser extent (by 54% relative to the experimental control level) and did not differ significantly from either experimental control or control-pretreated plants.

To assess changes in shoot height and shoot dry matter production in response to low temperature treatment at 11°C independently from reductions caused by pretreatment itself, values observed in response to treatment at 11°C were expressed as percentage of the values after pretreatment (Table 4.19b). This did not greatly modify relative treatment differences with respect to shoot height or dry matter production of stem and reproductive organs. However, expression of leaf dry matter production as percentage of values after the respective pretreatment showed that leaf dry matter of experimental control and cold-pretreated plants had increased by 69 and 29%, respectively, whereas that of control-pretreated plants had actually decreased by 25% since pretreatment, indicating the loss of leaves. When expressed as percentage of the value after the respective pretreatment, leaf dry matter production of cold-pretreated plants did not differ significantly from that of the experimental control, whereas the values for control-pretreated plants were significantly different from experimental control as well as cold-pretreated plants. The relative changes in leaf dry matter production were reflected in total shoot dry matter production, which increased by 102% and 35% in experimental control and cold-pretreated plants, respectively, but decreased by 13% in control-pretreated plants relative to the value after pretreatment. Low temperature treatment at 11°C significantly reduced shoot dry matter production, expressed as percentage of the value after pretreatment, in plants from both pretreatments relative to the experimental control level, and values of control- and cold-pretreated plants were significantly different.

Table 4.19 Shoot height and above ground dry matter production (whole shoot and individual contributions of stem, leaves and reproductive organs) in *Gossypium hirsutum* after exposure to low temperature of 11°C for 5 days and recovery in control conditions of 25°C for 15 days (Cold). Pretreatments and experimental control treatment as in Figure 4.7. **a)** Absolute values of shoot height and above ground dry matter production measured after cold treatment. **b)** Values as in a as % of the mean value in response to only pretreatment. Measurement of response to pretreatment was carried out on a different set of plants (see Table 4.9), which had been exposed either to low temperature for 6 days decreasing from 18°C to 13°C by 1°C per day and subsequent recovery in control temperature of 25°C for 5 days or alternatively to only control temperature for the same period of time (11 days). Mean ± standard error for 5 (cold-treatments) and 4 (experimental control) plants. Statistical testing as in Table 4.16.

	Shoot height (cm plant^{-1})	**Dry matter production (g plant^{-1})**			
		Stem	**Leaves**	**Flowers / fruits**	**Shoot**
	***a)** as absolute values found after cold treatment*				
Ct / Ct	119 ± 3.0 a	23.1 ± 0.9 a	24.4 ± 0.9 a	1.37 ± 0.37 a	48.9 ± 2.0 a
Cold-Pre / Cold	94 ± 2.2 b	9.5 ± 0.5 b	14.3 ± 0.6 b	0.63 ± 0.20 ab	24.4 ± 1.2 b
Ct / Cold	97 ± 4.1 b	10.0 ± 1.3 b	10.9 ± 2.5 b	0.13 ± 0.09 b	21.0 ± 3.8 b
	***b)** as % of the mean value after the respective pre-treatment*				
Ct / Ct	126 ± 3.1 a	238 ± 9.0 a	169 ± 6.3 a	1021 ± 272 a	202 ± 8.1 a
Cold-Pre / Cold	111 ± 2.6 b	137 ± 7.4 b	129 ± 5.5 a	451 ± 144 ab	135 ± 6.5 b
Ct / Cold	103 ± 4.3 b	104 ± 13.4 b	75 ± 17.5 b	94 ± 66 b	87 ± 15.8 c

4.2.2.4 Effect of pretreatment at low temperature on plant response to soil drying

Plants pretreated at low temperature decreasing from 18 to 13°C by 1°C per day (cold-pretreated) and plants of the same age, which had been maintained in control temperature (control-pretreated) were subsequently exposed to gradual soil drying.

Determination of the volumetric water content in the plant substrate revealed differences between differently pretreated plants with respect to the rate at which they depleted water in the substrate after cessation of watering (Figure 4.9). On day 4 of the soil drying period the volumetric water content of control-pretreated plants was reduced by 61% compared to the experimental control value, whereas the respective reduction for cold-pretreated plants amounted to only 42%. In the following days values of volumetric soil water content decreased only slightly in the substrate of control-pretreated plants, but declined steadily and at a steeper rate in the substrate of cold-pretreated plants. Thus the difference between differently pretreated plants diminished with increasing time of soil drying, and was statistically significant from day 4 to 9 of the soil drying period, but became negligible from day 10 onwards.

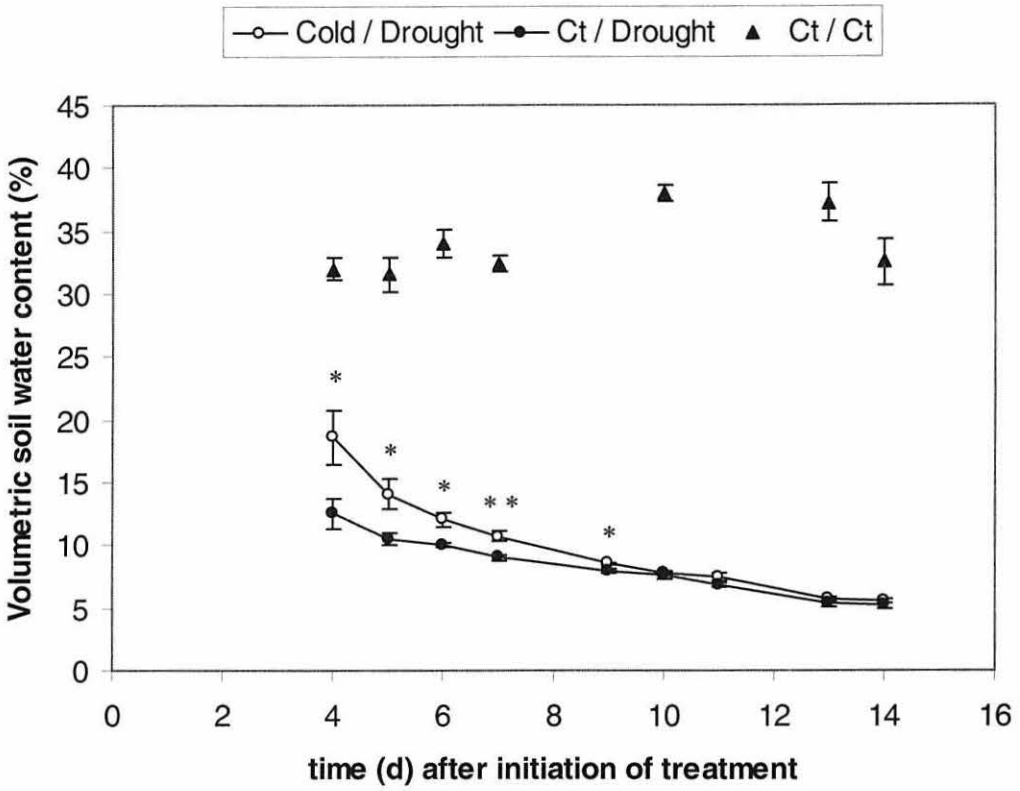
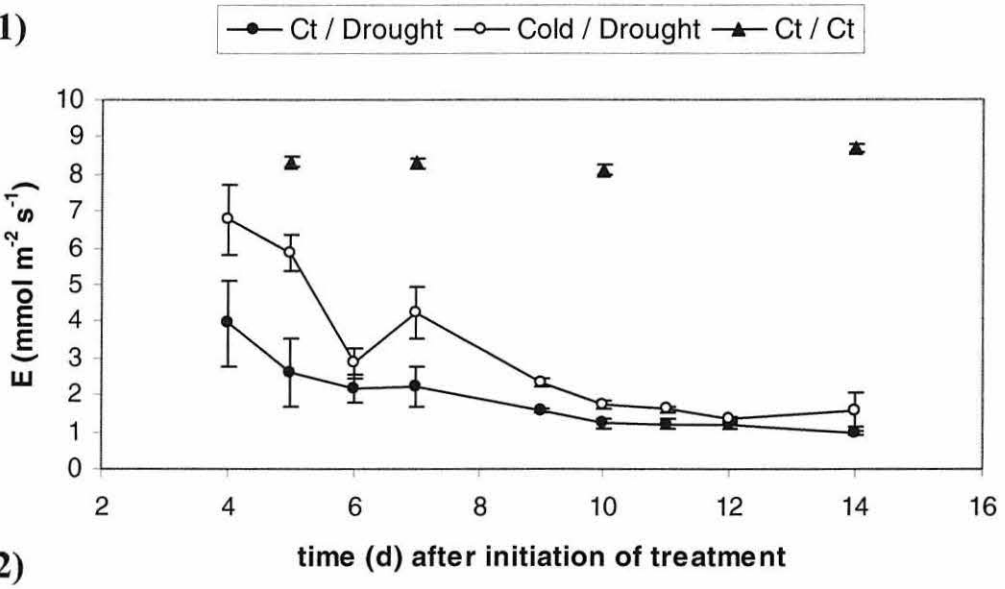


Figure 4.9 Variation in volumetric soil water content in the substrate of *Gossypium hirsutum* in response to withholding water (Drought) for 14 days. At the beginning of treatment plants were 63 days old. They had previously been pretreated with either low temperature for 6 days decreasing from 18°C to 13°C by 1°C per day (Cold) or alternatively with control temperature for the same period of time (Ct). Experimental control plants were maintained in control conditions (25°C and well-watered) throughout the experiment. Mean ± standard error for 5 (drought treatments) and 4 (experimental control) plants, the value for each plant being the average of 3 measurements spread evenly across the substrate surface. For each day drought treatments were compared by a t-test. Asterisks *, * *, * * * represent significance at P = 0.05, 0.01, and 0.001.

Pretreatment had a significant effect on the responses of transpiration, stomatal conductance, and net rate of CO_2 uptake to progressive soil drying, and the following response pattern was common to these three parameters (Figure 4.10 - 4.12). Throughout most of the period from day 4 to 11 cold-pretreated plants exhibited considerably higher levels of these gas exchange parameters as compared to control-pretreated plants. The difference between differently pretreated plants was particularly large on days 4 and 5 of the drying period. In the case of transpiration and net rate of CO_2 uptake this difference was largest on day 5. Values of transpiration were reduced to 31 and 71% of the experimental control level in control- and cold-pretreated plants, respectively (Figure 4.10). The corresponding values for net rate of CO_2 uptake were 27 and 71% (Figure 4.12). For stomatal conductance the difference was largest on day 4, with control- and cold-pretreated plants exhibiting values of 39 and 79%, respectively, of the experimental control value (Figure 4.11). In the following days values of these parameters decreased only slowly in control-pretreated plants, but continually and at a steeper rate in cold-pretreated plants. Thus the difference between differently pretreated plants diminished with progressive drought development and was negligible and not statistically significant from day 12 of the drying period onwards.

(1)



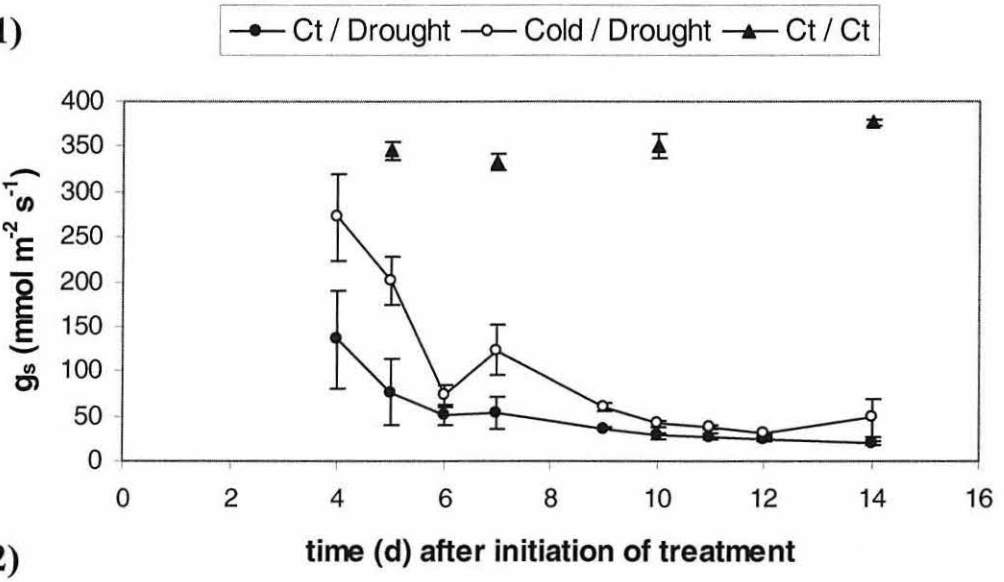
(2)

	Dependent variable: E								
Days [a]	4	5	6	7	9	10	11	12	14
a) *Significance of one-way ANOVA*									
Pretreatment	ns	*	ns	ns	* * *	*	*	ns	ns
b) *Significance of one-way ANCOVA*									
Soil wc d4	* *	*	na [b]	na [d]	na [c]	na [d]	na [d]	na [d]	na [d]
Pretreatment	ns	ns	na [b]	na [d]	na [c]	na [d]	na [d]	na [d]	na [d]

[a] Days after initiation of drought treatment on which E was measured
[b] – [d]: Low correlation between dependent variable and covariate ($R^2 < 0.4$) for
[b] Cold / Drought
[c] Ct / Drought
[d] both drought treatments

(1) Figure 4.10 Variation in transpiration (E) in *Gossypium hirsutum.* Experimental treatments as in Figure 4.9. Mean ± standard error for 5 (drought treatments) and 4 (experimental control) plants, the value for each plant being the average of measurements for 2 adjacent medium-aged leaves.
(2) Table 4.20 **a)** Significance levels of analysis of variance (ANOVA) with transpiration (E) from Figure 4.10 as dependent variable, and pretreatment (comparison of only drought treatments) as independent variable. **b)** Significance levels of analysis of covariance (ANCOVA) with dependent and independent variables as in a, and with volumetric soil water content measured on day 4 (soil wc d4) as covariate (to control for the effect of plant size). Asterisks *, * *, * * * represent significance at P = 0.05, 0.01, and 0.001, respectively; ns = nonsignificant at P = 0.05; na = result of ANCOVA not available due to preconditions (as indicated) not being met;

(1)



(2)

	Dependent variable: g_s								
Days [a]	4	5	6	7	9	10	11	12	14
a) *Significance of one-way ANOVA*									
Pretreatment	ns	*	ns	ns	* * * [e]	*	*	ns	ns
b) *Significance of one-way ANCOVA*									
Soil wc d4	* *	*	na [b]	na [d]	ns [e]	na [d]	na [d]	na [c]	na [d]
Pretreatment	ns	ns	na [b]	na [d]	ns [e]	na [d]	na [d]	na [c]	na [d]

[a] Days after initiation of drought treatment on which g_s was measured

[b] – [e]: Low correlation between dependent variable and covariate ($R^2 < 0.4$) for

[b] Cold / Drought

[c] Ct / Drought

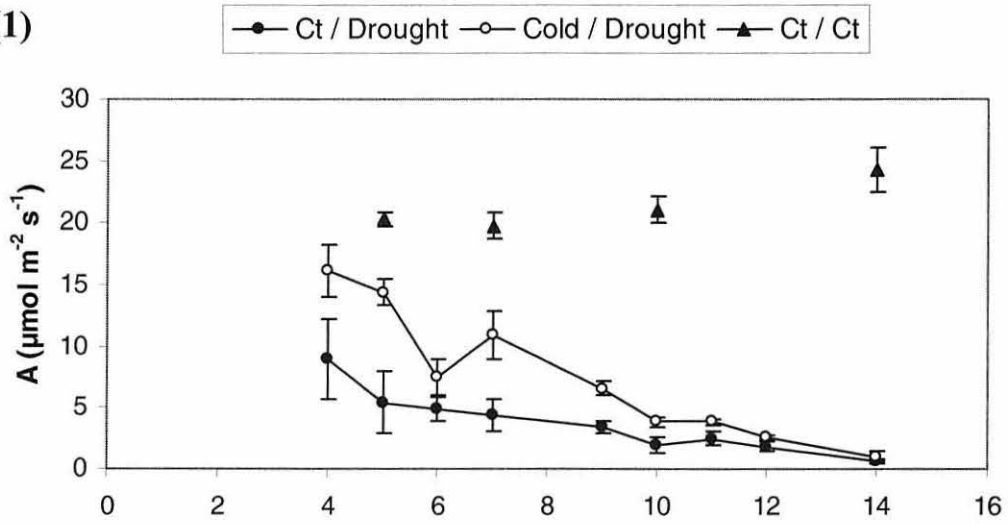
[d] both drought treatments

[e] Cold / Drought, the value for 1 replicate considered as outlier was removed (resulting in $R^2 > 0.4$);

(1) Figure 4.11 Variation in stomatal conductance (g_s) in *Gossypium hirsutum.* Experimental treatments as in Figure 4.9. Mean ± standard error for 5 plants (drought treatments, except Cold / Drought on day 9 for which n = 4 due to removal of an outlier) and 4 plants (experimental control). The value for each plant is the average of measurements for 2 adjacent medium-aged leaves.

(2) Table 4.21 Significance levels of analysis of variance **(a)** and covariance **(b)** as in Table 4.20, but with stomatal conductance (g_s) from Figure 4.11 as dependent variable.

(1)



(2)

	Dependent variable: A								
Days [a]	4	5	6	7	9	10	11	12	14
a) *Significance of one-way ANOVA*									
Pretreatment	ns	*	ns	*	* * [e]	*	*	ns	ns
b) *Significance of one-way ANCOVA*									
Soil wc d4	* *	ns	na [b]	na [d]	* [e]	na [d]	na [d]	na [d]	na [d]
Pretreatment	ns	ns	na [b]	na [d]	ns [e]	na [d]	na [d]	na [d]	na [d]

[a] Days after initiation of drought treatment on which A was measured

[b] – [e]: Low correlation between dependent variable and covariate ($R^2 < 0.4$) for

[b] Cold / Drought

[c] Ct / Drought

[d] both drought treatments

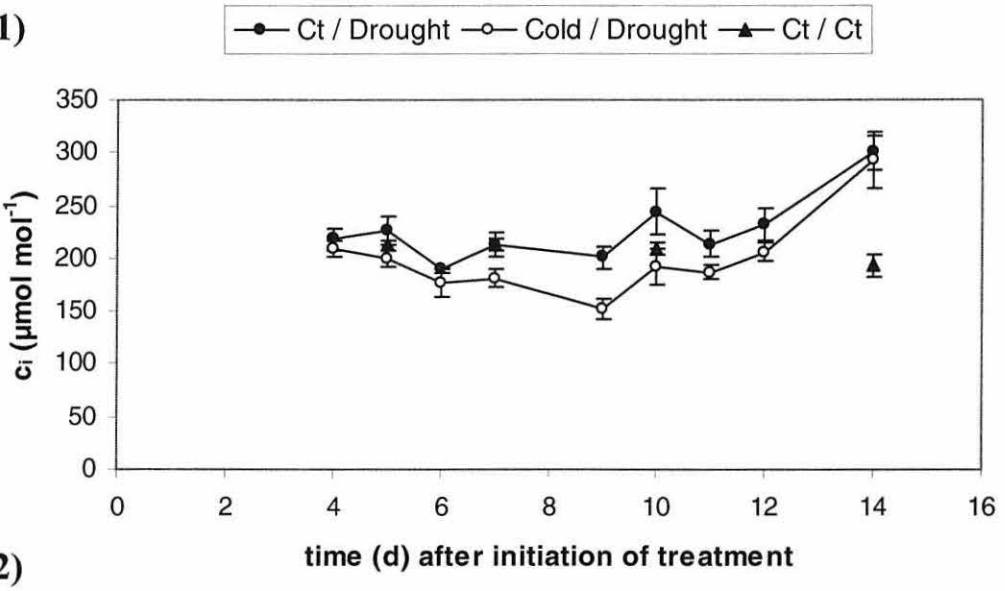
[e] Cold / Drought, the value for 1 replicate considered as outlier was removed (resulting in $R^2 > 0.4$);

(1) Figure 4.12 Variation in net rate of CO_2 uptake (A) in *Gossypium hirsutum.* Experimental treatments as in Figure 4.9. Mean ± standard error for 5 plants (drought treatments, except Cold / Drought on day 9 for which n = 4 due to removal of an outlier) and 4 plants (experimental control). The value for each plant is the average of measurements for 2 adjacent medium-aged leaves.

(2) Table 4.22 Significance levels of analysis of variance **(a)** and covariance **(b)** as in Table 4.20, but with net rate of CO_2 uptake (A) from Figure 4.12 as dependent variable.

Intercellular CO_2 concentration (c_i) exhibited the following response pattern to soil drying (Figure 4.13): From day 4 to 12 of the soil drying period values of control-pretreated plants did not differ significantly from those of the experimental control. In contrast, values of cold-pretreated plants showed a tendency to decrease in this period, and were significantly reduced below values of the experimental control as well as those of control-pretreated plants on day 9 of the soil drying period. On day 14 plants from both pretreatments exhibited a significant increase in c_i by 52 to 55% compared to the experimental control level. Pretreatment influenced responses of water use efficiency of photosynthesis (WUE) to soil drying (Figure 4.14). From day 4 to 9 of the soil drying period cold-pretreated plants tended to maintain higher values of WUE, and the difference between differently pretreated plants was significant on days 7 and 9, but neither cold- nor control-pretreated plants differed significantly from the experimental control. From day 10 to 11 of the soil drying period values of control-pretreated plants were significantly reduced to 56 and 72%, respectively, of the experimental control level. The intermediate values of cold-pretreated plants did not differ significantly from either experimental control or control-pretreated plants in this period. From day 12 to 14 of soil drying values of WUE of plants from both pretreatments were significantly reduced relative to the experimental control level, and pretreatment did not affect the extent of this decrease.

(1)



(2)

	Dependent variable: c_i								
Days [a]	4	5	6	7	9	10	11	12	14
a) *Significance of one-way ANOVA*									
Pretreatment	ns	ns	ns	ns	* *	ns	ns	ns	ns
b) *Significance of one-way ANCOVA*									
Soil wc d4	na [b]	na [e]	na [d]	na [c]	na [e]	na [e]	na [e]	na [e]	na [e]
Pretreatment	na [b]	na [e]	na [d]	na [c]	na [e]	na [e]	na [e]	na [e]	na [e]

[a] Days after initiation of drought treatment on which c_i was measured

[b] – [e]: Correlation between dependent variable and covariate

[b] low ($R^2 < 0.4$) for Ct / Drought; sufficient ($R^2 > 0.4$), but positive (unrelated to plant size) for Cold / Drought

[c] positive (unrelated to plant size) for Cold / Drought

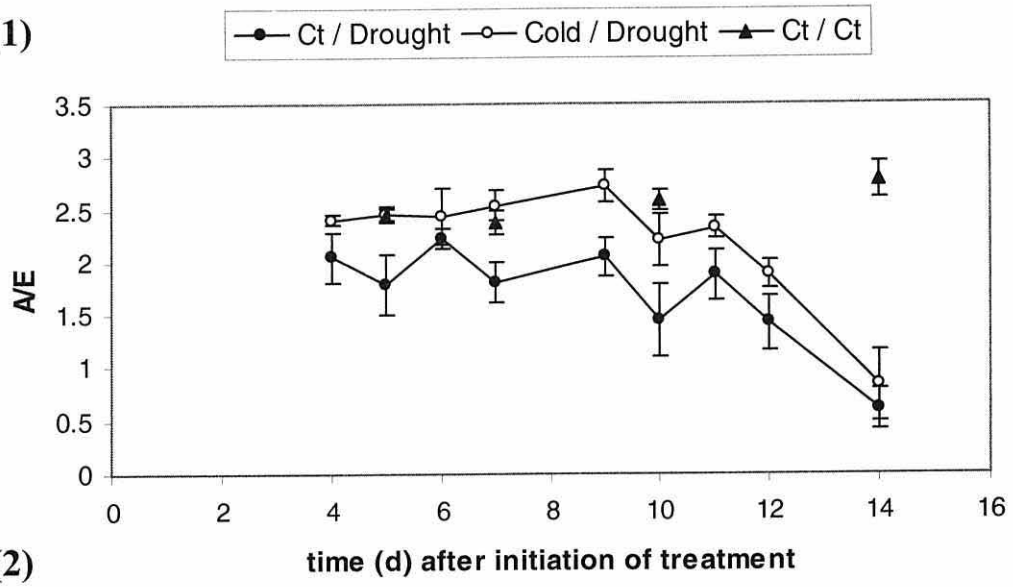
[d] low ($R^2 < 0.4$) for Cold / Drought

[e] low ($R^2 < 0.4$) for both drought treatments

(1) Figure 4.13 Variation in CO_2 mole fraction in the leaf intercellular air space (c_i) in *Gossypium hirsutum*. Experimental treatments as in Figure 4.9. Mean ± standard error for 5 (drought treatments) and 4 plants (Ct / Ct). The value for each plant is the average of measurements for 2 adjacent medium-aged leaves.

(2) Table 4.23 Significance levels of analysis of variance **(a)** and covariance **(b)** as in Table 4.20, but with CO_2 mole fraction in the leaf intercellular air space (c_i) from Figure 4.13 as dependent variable.

(1)



(2)

	Dependent variable: A/E								
Days [a]	4	5	6	7	9	10	11	12	14
a) *Significance of one-way ANOVA*									
Pretreatment	ns	ns	ns	*	* [e]	ns	ns	ns	ns
b) *Significance of one-way ANCOVA*									
Soil wc d4	na [b]	na [d]	na [c]	na [b]	* [e]	na [d]	na [d]	na [d]	na [d]
Pretreatment	na [b]	na [d]	na [c]	na [b]	ns [e]	na [d]	na [d]	na [d]	na [d]

[a] Days after initiation of drought treatment on which A/E was measured

[b] – [e]: Correlation between dependent variable and covariate:

[b] negative (unrelated to plant size) for Cold / Drought

[c] low ($R^2 < 0.4$) for Cold / Drought

[d] low ($R^2 < 0.4$) for both drought treatments

[e] negative (unrelated to plant size) for Cold / Drought; the value for 1 replicate considered as outlier was removed (resulting in positive correlation);

(1) Figure 4.14 Variation in water use efficiency of net photosynthesis (A/E) in *Gossypium hirsutum*. Experimental treatments as in Figure 4.9. Mean ± standard error for 5 plants (drought treatments, except Cold / Drought on day 9 for which n = 4 due to removal of an outlier) and 4 plants (experimental control). The value for each plant is the average of measurements for 2 adjacent medium-aged leaves.

(2) Table 4.24 Significance levels of analysis of variance **(a)** and covariance **(b)** as in Table 4.20, but with water use efficiency of net photosynthesis (A/E) from Figure 4.14 as dependent variable.

In a subsidiary experiment responses of gas exchange parameters to soil drying were shown to be related to plant size (see appendix 1). This relationship was quantified for the net rate of CO_2 uptake, and a strong correlation between the response of net rate of CO_2 uptake to soil drying and plant size was demonstrated. Increments in plant size had been created by suitable increments in plant age. In the present experiment cold-pretreatment at temperatures decreasing from 18 to 13°C by 1°C per day followed by 5 days of recovery in control temperature caused significant reductions in shoot height by 10% and in shoot dry matter production by 25% (Table 4.9). It was therefore investigated whether the difference between control- and cold-pretreated plants with respect to the response of gas exchange parameters to soil drying could be due to differences in plant size. For this purpose analysis of covariance (ANCOVA) with gas exchange parameters as dependent variable, pretreatment as independent variable, and a covariate related to plant size was performed. Volumetric soil water content measured on the day, on which the highest relative difference of this parameter between the plant groups differing in plant size occurred, was used as covariate. A strong correlation between this parameter, which was shown to be related to plant size, and the net rate of CO_2 uptake measured on particular days in the soil drying period had been demonstrated in the subsidiary study mentioned above (see appendix 1). In the present study volumetric soil water content was measured in the period from day 4 to 14 of the drying period, and the largest relative difference between differently pretreated plants regarding this parameter occurred on day 4.

When compared by one-way analysis of variance (ANOVA), control- and cold-pretreated plants differed significantly with respect to transpiration rate on days 5, 9, 10, and 11 of the drying period (Table 4.20). The assumptions of ANCOVA were satisfied only on days 4 and 5. ANCOVA using transpiration rate determined on days 4 and 5 as dependent variables, pretreatment as independent variable, and volumetric soil water content measured on day 4 as covariate showed a significant effect for the covariate, whereas the effect of pretreatment disappeared. Differently pretreated plants showed significant differences with respect to stomatal conductance on days 5, 9, 10, and 11 of the soil drying period, as determined by ANOVA (Table 4.21). Regarding the net rate of CO_2 uptake, cold-pretreated plants exhibited significantly higher values as compared to control-pretreated plants on days 5, 7, 9, 10, and 11, when compared by one-way

ANOVA (Table 4.22). As described for transpiration, ANCOVA was performed for measurements of stomatal conductance (Table 4.21) and net rate of CO_2 uptake (Table 4.22) on particular days in the drying period, when the specific assumptions for this analysis were satisfied. For both parameters ANCOVA revealed a significant effect of the covariate in some cases, whereas the significant effect of pretreatment generally disappeared.

Although cold-pretreated plants tended to have lower values of intercellular CO_2 concentration as compared to control-pretreated plants through most of the treatment period, a significant effect of pretreatment with respect to this parameter occurred only on day 9 of the drying period (Table 4.23). As the assumptions of ANCOVA were not satisfied for this particular measurement, the effect of plant size could not be determined. Control- and cold-pretreated plants showed significant differences with respect to water use efficiency of net photosynthesis on days 7 and 9 (Table 4.24). ANCOVA was performed for the measurement on day 9, when the assumptions were satisfied, and showed a significant effect of the covariate, while the significant effect of pretreatment disappeared.

Determination of shoot height and shoot dry matter production 14 days after the cessation of watering revealed significant differences between control- and cold-pretreated plants (Table 4.25). Cold-pretreated plants showed significantly lower values of shoot dry matter production, with relative reductions for cold- and control-pretreated plants as compared to the experimental control amounting to 53 and 46%, respectively (Table 4.25a). This was associated with significantly lower values of stem and leaf dry matter production in cold-pretreated as compared to control-pretreated plants, whereas dry matter production of reproductive organs was not significantly different in plants from different pretreatments. Control- and cold-pretreated plants did not differ significantly with respect to shoot height. However, pretreatment itself significantly affected shoot height and shoot dry matter production. To assess changes in shoot height and shoot dry matter production in response to progressive soil drying independently from reductions caused by pretreatment itself, values observed in response to soil drying were expressed as percentage of the values after pretreatment (Table 4.25b). This revealed a significant difference between control- and cold-pretreated plants with respect to shoot height. Values of

cold-pretreated plants had increased by 13% relative to the pretreatment level, while the respective increase was only 4% in control-pretreated plants. Similarly, shoot dry matter had increased by 67% in cold-pretreated, but only 45% in control-pretreated plants relative to the values after pretreatment, and the difference between differently pretreated plants was statistically significant. The difference in shoot dry matter production was due to greater increases in dry matter of stem, leaves, and reproductive organs in cold- as compared to control-pretreated plants relative to the values exhibited after pretreatment, but a statistically significant difference occurred only with respect to stem dry matter production.

Table 4.25 Variation in shoot height and above ground dry matter production (whole shoot and individual contributions of stem, leaves and reproductive organs) in *Gossypium hirsutum* in response to withholding water for 14 days (Drought). Shoot height and above ground dry matter production were measured after subsequent recovery in control conditions for 10 and 11 days, respectively. Pretreatments and experimental control treatment as in Figure 4.9. **a)** Absolute values of shoot height and above ground dry matter production; **b)** Values as in a as % of the mean value in response to only pretreatment. Mean ± standard error for 5 (drought-treatments) and 4 (experimental control) plants. For each variable drought-treatments were compared by a t-test. Asterisks *, * *, * * * represent significance at P = 0.05, 0.01, and 0.001, respectively; ns = nonsignificant at P = 0.05.

	Shoot height (cm plant^{-1})	**Dry matter production (g plant^{-1})**			
		Stem	**Leaves**	**Flowers / fruits**	**Shoot**
	a) as absolute values found after cold treatment				
Ct / Ct	129 ± 2.2	29.7 ± 2.0	32.4 ± 2.0	2.43 ± 0.57	64.6 ± 4.4
Ct / Drought	99 ± 2.3	13.6 ± 0.7	20.9 ± 0.8	0.59 ± 0.19	35.1 ± 1.6
Cold / Drought	96 ± 1.3	11.4 ± 0.3	17.7 ± 0.7	1.20 ± 0.22	30.3 ± 0.9
Significance of t-test between drought treatments					
	ns	*	*	ns	*
	b) as % of the mean value after the respective pretreatment [1]				
Ct / Ct	137 ± 2.4	307 ± 21.1	225 ± 14.1	1814 ± 426.4	267 ± 18.2
Ct / Drought	104 ± 2.4	141 ± 6.9	145 ± 5.4	439 ± 142.2	145 ± 6.4
Cold / Drought	113 ± 1.6	164 ± 4.9	161 ± 6.4	858 ± 157.8	167 ± 5.0
Significance of t-test between drought treatments					
	*	*	ns	ns	*

[1] Measurement of response to pretreatment was carried out on a different set of plants which had been exposed to either low temperature for 6 days decreasing from 18°C to 13°C by 1°C per day and subsequent recovery in control conditions of 25°C for 5 days or to control temperature for the same period of time (see Table 4.9).

Drought treatment caused a significant decrease in water content and relative water content, determined after 14 days of exposure to progressive soil drying, in plants from both pretreatments as compared to the experimental control (Table 4.26). The extent of the relative reduction was comparable in control- and cold-pretreated plants, amounting to around 30% for both parameters.

Table 4.26 Water content (WC) and relative water content (RWC) in *Gossypium hirsutum* after withholding water for 14 days (Drought). Mean ± standard error for 5 (drought treatments) and 4 (experimental control) plants. Pretreatments and experimental control treatment as in Figure 4.9. For each variable a one-way analysis of variance was performed between combined treatments. Means followed by the same letters are not significantly different ($P < 0.05$).

	WC (%)	RWC (%)
Ct / Ct	400 ± 18.0 a	95 ± 1.7 a
Cold / Drought	283 ± 4.9 b	67 ± 1.2 b
Ct / Drought	270 ± 10.8 b	63 ± 1.4 b

4.3 Discussion

4.3.1 Plant responses to low temperature pretreatment

4.3.1.1 Acclimation and injury in response to a gradual decrease in temperature

The pretreatment regimes used in experiment 1 and 2 both involved a gradual decrease in temperature by 1°C per day, starting at 18°C, but they differed in the minimum temperature reached, which was 13°C in experiment 2 and 12°C in experiment 1. This was associated with a difference in length of the treatment period, being 6 and 7 days in experiments 2 and 1, respectively. The responses of gas exchange and chlorophyll fluorescence parameters to the individual steps of temperature involved in the sequence of gradually decreasing temperatures were monitored only in experiment 1. It is assumed that responses observed in the first 6 days of this sequence in experiment 1 are equivalent to responses occurring in the course of pretreatment in experiment 2. It has to be noted, however, that experiment 1 and 2 differed with respect to the age of plants at the onset of pretreatment, being 45 and 57 days in experiment 1 and 2, respectively, and this

difference might have slightly modified plant response to low temperature treatment.

Investigation of the responses of gas exchange and chlorophyll fluorescence parameters to gradually decreasing temperatures from 18°C to 12°C by 1°C per day (experiment 1) revealed two distinct phases. (a) Response kinetics associated with the initial phase from day 1 to 4 may be summarised as follows. Exposure to low temperature on day 1 induced significant reductions in net rate of CO_2 uptake and transpiration to around 50 to 60% of the control level, and a significant increase in intercellular CO_2 concentration. With further progression of treatment until day 4 values of the above parameters remained relatively stable at these reduced/increased levels or showed only slight changes. Net rate of CO_2 uptake even exhibited a slight increase on day 2 compared to the level on day 1. Stomatal conductance was not affected in this period. Low temperature-induced significant reductions in F_v/F_m and F_v/F_o were associated with reductions in F_m and a decline in F_o. (b) Response patterns typical for the second phase from day 5 to 7 may be described as follows. Stomatal conductance exhibited a sudden significant decrease to 41% of the control level on day 5 and a further massive reduction to only 12% of the control level on day 7. Net rate of CO_2 uptake and transpiration started to decrease more steeply, reaching levels of 41 and 31%, respectively, of the control level, on day 5, and showed a further massive decrease towards day 7, when values amounted to only 15 and 10%, respectively, of the control level. The previously increased intercellular CO_2 concentration fell to the control level on day 5, and values remained comparable to those of the control until day 7. Reductions in F_v/F_m and F_v/F_o in the period from day 5 to 7 were due to decreased F_m and an increase in F_o.

The following picture emerges with respect to the differential limitations underlying reductions in transpiration and net rate of CO_2 uptake in the two response phases. In the initial phase reductions appear to be caused by metabolic restrictions. The reduction in net rate of CO_2 uptake accompanied by an increase in intercellular CO_2 concentration in this phase suggests a proportional dominance of respiratory processes over carbon assimilation. The steeper reductions in net rate of CO_2 uptake and transpiration in the second phase were associated with large reductions in stomatal conductance, thus indicating the contribution of stomatal limitations. The reduction in stomatal conductance would have led to a

decrease in intercellular CO_2 concentration, counteracting the effect of a proportional dominance of respiratory processes over carbon assimilation rate observed in the previous phase on this parameter, resulting in overall levels of intercellular CO_2 concentration comparable to the control.

In both phases reductions in maximum quantum yield of PSII, as indicated by a decline in F_v/F_m, were associated with reductions in F_m. However, the two phases differed with respect to changes in F_o, which exhibited a decline in the initial phase and an increase relative to the control level in the second phase of treatment. The mechanism underlying low-temperature-induced photoinhibition, the general interpretation of the dark-adapted chlorophyll fluorescence parameters F_v/F_m and F_v/F_o, as well as the distinction between processes involving photon damage and photoprotection based on changes in F_o level fluorescence are discussed in detail in section 4.3.2. Yang *et al.* (2001) describe the level of F_o at any point of time as the net result of two types of processes, photoprotection and photon damage, which may occur simultaneously, and both of which influence F_o, although in opposite directions. The direction of change in F_o thus indicates the dominant process. These authors investigated the response of *Gossypium hirsutum* plants, which had been grown in the shade at 40% of full sunlight for several weeks, to sudden exposure to full sunlight. After transfer to full sunlight plants showed a decrease in F_v/F_m over 4 days, but then a gradual increase in the following days, resulting in partial recovery of the initial level in the shade. This was associated with a rise in F_o over the first 4 days, and a gradual decrease in the following days. The authors concluded that the increase in F_o in the initial phase after exposure to full sunlight indicated dominance of photodamage, whereas photoprotective processes governed the decline in the later phase. The changes in F_o observed in the present study as part of low temperature-induced photoinhibition may thus be interpreted accordingly as follows. In the initial phase (days 1 to 4) the gradual decrease in temperature from 18 to 15°C induced photoprotective processes of thermal dissipation, as indicated by the decline in F_o. With further exposure to low temperature decreasing from 14 to 12°C in the second phase (days 5 to 7) processes of photon damage became dominant, leading to the observed increase in F_o. In the study by Yang *et al.* (2001) acclimation of *Gossypium hirsutum* to photoinhibition was associated with an increase in the net rate of CO_2 uptake above the initial level in the shade, increased violaxanthin de-

epoxidation, increased total xanthophyll cycle pool size, and increased capacity of non-photochemical quenching. Similar mechanisms may have contributed to the protective components of photoinhibitory quenching observed in the initial phase of low temperature treatment in the present study on the same species. However, the present study differs from that by Yang *et al.* (2001) with respect to the nature of the photoinhibitory treatment, which may influence the acclimation mechanisms employed. While Yang *et al.* (2001) exposed low light-acclimated plants to excessive incident photon flux, photoinhibition in the present study resulted from treatment at low temperature concurrently with low incident light (260 to 320 μmol photons m^{-2} s^{-1}).

In conclusion, the two phases identified in the response of gas exchange and chlorophyll fluorescence parameters to gradually decreasing temperatures from 18°C to 12°C by 1°C per day (experiment 1) may be characterised as follows. (a) Responses in the initial phase from day 1 to 4 reflect primarily acclimation to treatment at low temperature in combination with low incident light. This is apparent from the decline in F_v/F_m associated with a decrease in F_o, indicating photoinhibitory quenching of mainly photoprotective nature. The increase in net rate of CO_2 uptake between days 1 and 2 of treatment indicates some acclimation of photosynthetic dark reactions. (b) Responses in the second phase from day 5 to 7 reflect the onset of more severe injury. This is suggested by the decline in F_v/F_m associated with an increase in F_o, indicating photoinhibitory quenching primarily as a result of damage to PSII reaction centres, and the steep decline in net rate of CO_2 uptake and transpiration, associated with a sudden steep reduction in stomatal conductance.

4.3.1.2 Differential effects of pretreatment regimes differing in minimum temperature on shoot growth and recovery of gas exchange and PSII activity

The differential effects of the two pretreatment regimes investigated, both of which involved a gradual decrease in temperature by 1°C per day starting at 18°C, but differed in the minimum temperature reached, being 12°C and 13°C in experiment 1 and 2, respectively, may be identified by differences in recovery. Plants from both pretreatment regimes showed a gradual recovery of gas exchange parameters. In both cases F_m recovered to values significantly above the control level. However, this occurred after different times of recovery. In experiment 1

F_v/F_m exhibited values comparable to the control on day 3, but significantly increased to values 2% above the control level on day 4 of recovery. The parameter F_v/F_o, which similarly to F_v/F_m determines changes in the efficiency of non-photochemical quenching, but is more sensitive than the latter at higher values (Maxwell & Johnson, 2000), showed values increased by 10% compared to the control on day 4. Increases in F_v/F_m and F_v/F_o were due to a corresponding significant increase in F_m, whereas F_o was not significantly different from the control. In experiment 2 F_m was significantly increased by 15% relative to the control level after 1 day of recovery. This was associated with a significant increase also in F_o by 10% relative to the control level, resulting in an overall value of F_v/F_m comparable to that of control plants.

Differential effects of the two pretreatment regimes were also apparent with respect to their effects on shoot height and dry matter production. However, experiment 1 and 2 differed slightly with respect to the recovery periods following cold pre-treatment, after which these parameters were assessed, as well as with respect to plant age at the initiation of low-temperature treatment (see 4.3.1.1). In experiment 1 shoot height was determined after 6 days and shoot dry matter production after 9 days of recovery, whereas in experiment 2 both, shoot height and dry matter production, where assessed after 5 days of recovery. The pretreatment regime used in experiment 1 induced greater reductions in shoot dry matter production as compared to the temperature regime applied in experiment 2. Reductions relative to the control level amounted to 39 and 25% in experiment 1 and 2, respectively. This trend was also apparent with respect to shoot height, which was determined after comparable periods of recovery in the two experiments (6 and 5 days in experiment 1 and 2, respectively). Reductions relative to the control level amounted to 18% and 10% in experiment 1 and 2, respectively.

4.3.1.3 Accumulation of glycinebetaine

In the present study treatment at temperatures decreasing gradually from 18 to 13°C by 1°C per day induced a significant 3-fold increase in glycinebetaine concentration in the leaf sap. When expressed on a dry weight basis, low-temperature-treated plants exhibited a still significant 2.8-fold increase in concentration relative to the control, indicating that only a minor part of the

accumulation was due to the slightly lower water content in cold- as compared to control-pretreated plants.

Plant species with the ability to accumulate glycinebetaine to potentially osmotically significant levels are termed (natural) accumulators, and are found in distantly related plant families across the plant kingdom (Rhodes & Hanson, 1993). An extensive study on glycinebetaine accumulation in the Malvaceae revealed a widespread ability to accumulate this compound in this plant family. Accumulation to levels ranging from 0.5 to 4.6% of dry weight in shoot organs was demonstrated in almost all species investigated, originating from 18 genera, including *Gossypium*, from all 5 tribes of this family (Blunden *et al.*, 2001). In the present study the unstressed control plants of *Gossypium hirsutum* exhibited concentrations of glycinebetaine in extracted leaf sap of 40 to 50 mol m^{-3}. These concentrations are slightly higher than those reported by Gorham (1996) who investigated concentrations of glycinebetaine in expressed sap of a number of species of the Malvaceae and found an average concentration of 28.7 mol m^{-3} in young fully expanded leaves of unstressed *Gossypium hirsutum* cv. Acala SJ2 plants.

Accumulation of glycinebetaine in response to long-term drought and salinity has been demonstrated for a variety of species, and levels of glycinebetaine in leaves were generally linearly related to leaf osmotic potential (Rhodes & Hanson, 1993). Gorham (1996) exposed a variety of *Gossypium* spp. to salinity and cyclical drought stress and observed an increase in concentrations of glycinebetaine in expressed leaf sap. In contrast, there are only a few reports on accumulation of glycinebetaine in response to low temperature. Kishitani *et al.* (1994) investigated differences in low temperature-induced glycinebetaine accumulation between near-isogenic lines of two winter cultivars of barley, with each line differing in one of three major genes determining the spring-related growth habit. Treatment at 5°C for 8 days resulted in accumulation of glycinebetaine particularly in young leaves. Lines with the winter-related growth habit exhibited higher concentrations of glycinebetaine in young leaves as compared to those of the spring type growth habit, and leaf betaine levels were associated with a lower degree of leaf freezing injury on exposure to –5°C for 2 days. It was concluded that accumulation of glycinebetaine in response to low non-freezing temperatures might contribute to the acquisition of freezing tolerance

in the process of seasonal cold acclimation. However, the difference in extent of freezing-induced leaf injury between the two winter cultivars was not related to levels of glycinebetaine. While low temperature-induced accumulation of glycinebetaine has thus been shown in freeze-tolerant plants during the process of cold acclimation to freezing temperatures, there appear to be no corresponding reports on glycinebetaine accumulation in response to low temperature in chilling-sensitive plants. In the present study a 6-day exposure to a gradual decrease in temperature from 18 to 13°C by 1°C per day induced a 3-fold increase in glycinebetaine concentration in the leaf sap in chilling-sensitive *Gossypium hirsutum* plants. Gorham (1996) observed an increase of similar extent (3.7-fold) in glycinebetaine concentration in the leaf sap of the same species in response to cyclical drought.

4.3.2 Plant responses to treatment at constant low temperature of 8°C and 11°C

4.3.2.1 Photoinhibition in non-acclimated plants

Physiological responses of mature non-acclimated plants of *Gossypium hirsutum* to treatment at two levels of low temperature, 8 and 11°C, concurrently with low incident light (260 to 320 μmol photons m^{-2} s^{-1}) were investigated. Both levels of low temperature significantly affected dark and light reactions of photosynthesis. (a) Exposure of 60-day old plants to 8°C for 11 days (experiment 1) resulted in large reductions in the net rate of CO_2 uptake to only 3% of the experimental control level, when determined on day 2, and values remained very low throughout treatment. Values of intercellular CO_2 concentration were not significantly altered relative to the experimental control at any time during treatment. F_v/F_m was reduced by 13% compared to the experimental control on day 1, and the extent of reduction increased with increasing exposure time, reaching 46% on day 11 of treatment. F_v/F_o exhibited even greater changes relative to the experimental control as compared to F_v/F_m, with reductions amounting to 46% on day 1 and decreasing further with exposure time, reaching 80% on day 11 of treatment. The response kinetics of F_m paralleled those of F_v/F_m and F_v/F_o, exhibiting significant reductions compared to the experimental control level throughout most of the treatment period, with relative reductions amounting to 24% on day 1 and reaching 54% on day 11 of treatment. F_o exhibited a

significant increase relative to the experimental control level in the initial phase (days 1 to 3) and at a later stage of treatment (days 9 and 11). Relative increases amounted to 25% on day 1 and 37% on day 11 of treatment, with the maximum increase (47%) occurring on day 2 of treatment. (b) Treatment of 64-day old plants at low temperature of 11°C for 5 days (experiment 2) induced a large reduction in net rate of CO_2 uptake to levels around zero and a 2-fold increase in intercellular CO_2 concentration, when determined on the final day of treatment. F_v/F_m was significantly reduced relative to the experimental control level throughout treatment, with the extent of reduction increasing from 18% on day 1 to 38% on day 5 of treatment. The response kinetics of F_m paralleled those of F_v/F_m, whereas F_o was not significantly altered relative to the experimental control level at any time during low temperature treatment.

The ratios F_v/F_m and F_v/F_o generally reflect changes in the efficiency of non-photochemical quenching of chlorophyll fluorescence (Maxwell & Johnson, 2000). Non-photochemical quenching (NPQ) involves the dissipation of excess excitation energy as heat, and generally comprises two types of processes, which can be distinguished based on their induction and relaxation time (Maxwell & Johnson, 2000). (a) ΔpH-dependent high energy state quenching (qE) can be rapidly induced on a time-scale of minutes and relaxes within minutes under dark conditions. (b) Photoinhibitory quenching (qI) is a sustained type of NPQ, which relaxes over a time-scale of hours. In the present study chlorophyll fluorescence parameters were determined after twenty minutes of dark adaptation (experiments 1 and 2). Dark-adapted fluorescence parameters reflect only photoinhibitory quenching, also termed chronic photoinhibition, based on the fact that qI is slowly reversible, whereas ΔpH-dependent high energy state quenching (qE) relaxes during dark adaptation (Osmond, 1994). In the present study exposure to low temperatures of 8°C for 11 days (experiment 1) and 11°C for 5 days (experiment 2) concurrently with low incident light resulted in significant reductions in dark-adapted values of F_v/F_m throughout the respective treatment periods, thus indicating that these treatments induced chronic photoinhibition. Responses of dark-adapted chlorophyll fluorescence parameters to treatment at 8°C were also expressed as F_v/F_o (experiment 1). F_v/F_o exhibited similar response kinetics, but greater changes as compared to F_v/F_m. This is in general agreement with the notion that F_v/F_o, similar to F_v/F_m, determines changes in the efficiency of non-

photochemical quenching, but is more sensitive than the latter at higher values (Maxwell & Johnson, 2000). This fact was used in the detection of very small changes in dark-adapted chlorophyll fluorescence parameters in response to low temperature pretreatment (see above).

In the present study photoinhibition occurred in *Gossypium hirsutum* in response to treatment at low temperatures of 8°C (experiment 1) and 11°C (experiment 2) concurrently with low incident light (260 to 320 μmol photons m^{-2} s^{-1}). It is well known that photoinhibition may not only be induced by excessive incident photon flux, but in general by environmental conditions, which result in an imbalance between absorption and utilisation of excitation energy. Low temperature can cause such an imbalance by reducing the turnover rate of electron sinks (Huner *et al.*, 1998). In the present study treatment of *Gossypium hirsutum* at 8°C (experiment 1) and 11°C (experiment 2) resulted in a reduction in net rate of CO_2 uptake to values close to zero, when determined on day 2 and day 5 of treatment, respectively. In the case of treatment at 11°C for 5 days (experiment 2) this was also associated with a large (2-fold) rise in intercellular CO_2 concentration (c_i). Thus both treatment temperatures resulted in a massive reduction in the turnover rate of carbon assimilation, a major plant electron sink. The increase in c_i in response to treatment at 11°C (experiment 2) reflects the occurrence of respiratory processes, whereas the lack of such an increase in response to treatment at 8°C (experiment 1) probably indicates that not only photosynthesis, but also respiratory processes were inhibited at this temperature. In addition to reducing the turnover rate of carbon assimilation, low temperature may also promote photoinhibition by slowing down the rates of protective and repair processes as follows (Krause, 1994). (a) Chilling can reduce the rates of photoprotective processes, such as the rate of development of ΔpH-dependent high energy state quenching, zeaxanthin synthesis, and enzyme reactions involved in scavenging of active oxygen species. (b) Chilling decreases the rates of D1 repair processes, and it is thought that some of the damaged D1 is only marked for degradation, but not actually degraded during low temperature treatment. Chilling exerts its effect on the D1 repair cycle at the level of D1 gene expression and reduction in membrane fluidity, and the latter results in a reduced rate of diffusion of marked D1 to non-appressed thylakoid membrane regions (Allen & Ort, 2001).

Photoinhibitory quenching, also referred to as chronic photoinhibition, can involve photoprotective processes and/or photon damage. Studies investigating the effect of inhibitors of chloroplast-encoded protein synthesis and xanthophyll conversion on responses of dark-adapted fluorescence parameters to excessive incident photon flux imply the following interpretation of changes in dark-adapted F_v/F_m and F_o (Osmond, 1994). (a) A decline in F_v/F_m associated with a decrease in F_o indicates dominance of photoprotective processes. This involves a decrease in photon yield without concomitant decline in photosynthetic capacity, and results in proportional reduction of photosynthetic efficiency in all cells, chloroplasts or PSII centres in the tissue. (b) A decline in F_v/F_m associated with an increase in F_o indicates dominance of photon damage. This is due to complete inactivation of photosynthesis in a proportion of cells or chloroplasts in the tissue, and involves a proportional decline in photon yield as well as photosynthetic capacity. Chilling-induced photoinhibition results from a complete inactivation of a fraction of PSII reaction centres, which are "silent", *i.e.* they do not contribute to linear electron transport or variable chlorophyll fluorescence, and this leads to a quenching of maximum variable fluorescence (Krause, 1994). This author suggests diagnosis of chilling-induced photoinhibition based on a decline in F_v or F_v/F_m, but criticises the interpretation of changes in F_o as proposed by Osmond (1994) (see above). He points out that not only irreversible, but also rapidly reversible increases in F_o have been observed in response to low temperature treatment, and that the reversible ones may result from stabilised Q_A^-, not sustained damage to PSII. However, the inhibitor studies reviewed by Osmond (1994) lend strong support to the suggested interpretation of changes in dark-adapted F_o, thus justifying application of these guidelines to the results of the present study. Treatment at 8°C (experiment 1) induced a significant rise in F_o relative to the experimental control level in the first three days and on days 9 and 11 of treatment, whereas treatment at 11°C (experiment 2) did not significantly affect F_o at any time during low temperature treatment. This indicates that treatment at 8°C resulted in photon-damage to PSII reaction centres from an early stage of treatment onwards, and that treatment at 8°C is likely to have caused relatively more damage than treatment at 11°C. The contribution of protective processes and damage to the low temperature-induced decrease in F_v/F_m may also be estimated from the kinetics of recovery on return to control temperature, which was assessed for plants treated at

11°C for 5 days (experiment 2). After 1 day of recovery dark-adapted values of F_v/F_m had only recovered to 76% of the experimental control level and were still significantly lower than values of the experimental control, indicating that treatment at 11°C for 5 days induced severe irreversible damage to PSII.

Kornyeyev *et al.* (2002) conducted a study with some resemblance to the present one, investigating the responses of *Gossypium hirsutum* to treatment at low temperature of 10°C concurrently with moderate incident light with respect to chlorophyll fluorescence parameters. However, this study shows the following differences to the present one. Kornyeyev *et al.* (2002) used detached leaves and leaf discs, a higher incident photosynthetic photon flux density (500 µmol photons m^{-2} s^{-1}), a longer dark-adaptation period preceding the determination of F_v/F_m (3 hours), and they pre-incubated leaves with linomycin (an inhibitor of chloroplastic protein synthesis) for 3 hours in darkness. In addition they only assessed plant response over a period of 6 hours. These authors resolved the allocation of excitation energy into photochemistry, thermal dissipation, and energy in excess of the first two processes, and further resolved thermal dissipation into constitutive (occurring in control leaves), regulated (mainly qE) and photoinhibitory (corresponding to qI) components. After 3 hours of treatment only 4% of absorbed photon energy were used in photochemistry. The proportion of excitation energy dissipated as heat increased rapidly during the first 60 minutes of treatment, and then gradually, dissipating 80% of absorbed photon energy as heat after 6 hours of treatment. The regulated component of thermal dissipation increased rapidly, reaching its maximum after 80 minutes of treatment, whereas the photoinhibitory component increased gradually throughout the 6-hour treatment period. The study by Kornyeyev *et al.* (2002) confirms the trend of a gradual increase in photoinhibitory quenching with increasing time of exposure to low temperature observed in the present study in *Gossypium hirsutum*, and in addition suggests that the low temperature treatments of the present study may have induced ΔpH-dependent high energy state quenching (qE).

4.3.2.2 Responses of non-acclimated plants with respect to water relation parameters and leaf injury

Treatment at 8°C (experiment 1) resulted in large reductions in transpiration and stomatal conductance to only 3% of the experimental control

level on the second day of treatment, and values remained low throughout the treatment period. It caused severe plant injury, determined after recovery in control temperature for 10 days following low temperature treatment, with some variation among replicates, ranging from injury of only old leaves to death of the entire shoot. Treatment at 11°C (experiment 2) also induced massive reductions in transpiration and stomatal conductance, assessed on day 5 of treatment, to 4 and 6%, respectively, of the experimental control level, but did not significantly affect either leaf water content or relative water content. Determination of leaf injury after 15 days of recovery in control temperature following treatment revealed death of around 56% of main stem leaves.

Wilson & Crawford (1974a, 1974b) also found leaf injury in a number of chilling-sensitive species, including *Gossypium hirsutum*, *Phaseolus vulgaris*, *Saintpauli grandiflora*, and *Cucumis sativus*, in response to treatment at low temperature of 5°C, 95%RH for 48 hours. This treatment resulted in necrosis and curling of leaves, determined after recovery in control conditions for 1 to 10 days, in 79 to 100% of leaves per plant. Plant responses possibly contributing to this type of chilling injury were further investigated in follow-up studies on *Phaseolus vulgaris*. These studies showed that the above chilling treatment caused a decline in rates of root water absorption and rapid opening of stomata, leading to leaf dehydration (Wilson, 1978). The "locking-open" of stomata described in these studies stands in marked contrast to responses observed in the present study, which showed that treatment of *Gossypium hirsutum* at low temperatures of 8°C (experiment 1) and 11°C (experiment 2) induced large reductions in stomatal conductance and transpiration. The observed stomatal closure as well as the fact that treatment at 11°C for 5 days did not significantly affect leaf water content or relative water content indicate that leaf injury observed in the present study was not caused by leaf dehydration. The different results obtained in the present study as compared to those presented by Wilson (1978) and Wilson & Crawford (1974a, 1974b) may be due to the difference in treatment temperature (11°C and 8°C in the present study versus 5°C in the studies by Wilson and Wilson & Crawford).

Leaf injury following chronic photoinhibition, as observed in the present study, is more likely to be due to oxidation of chlorophyll and destruction of chloroplasts as a consequence of excessive irreversible photon damage (Osmond, 1994). Such chilling-induced photo-oxidation of pigments and lipids on a large

scale typically occurs in chilling-sensitive, but not in chilling-tolerant species (Krause, 1994). Low temperature treatment at 8°C for 11 days induced a higher degree of plant injury as compared to treatment at 11°C for 5 days, indicating that the lower temperature of 8°C, possibly in combination with the longer exposure time, resulted in a greater extent of photo-oxidative damage.

4.3.2.3 Responses of cold-pretreated plants

Two low temperature regimes involving a gradual decrease in low temperature by 1°C per day (a) from 18°C to 12°C followed by 8 days of recovery in control temperature (experiment 1) and (b) from 18°C to 13°C followed by 1 day of recovery in control temperature (experiment 2) were tested for their potential to induce acclimation to subsequent treatment at constant low temperature of 8°C (experiment 1) or 11°C (experiment 2), respectively. Both experiments demonstrated partial protection from photoinhibition by pretreatment at low temperature on exposure to subsequent low temperature treatment. However, experiment 1 and 2 exhibited marked differences with respect to the kinetics of protective effects.

In experiment 1 cold-pretreatment exerted only limited protective effects on photosynthesis in the initial phase of treatment at 8°C, manifested as reduced degree of increase in F_o on day 1 and a slight reduction in extent of decrease in net rate of CO_2 uptake determined on day 2 of treatment. The extent of difference between cold- and control-pretreated plants with respect to net rate of CO_2 uptake was small compared to the large extent of reductions relative to the experimental control plants apparent in plants from both pretreatments. At later stages of treatment (days 7 to 11) cold-pretreatment alleviated the decline in F_v/F_m and F_v/F_o. The protective effect of pretreatment on F_v/F_m and F_v/F_o was related to F_m, which exhibited a lesser degree of reduction in cold- as compared to control-pretreated plants relative to the experimental control in this period of time. However, the protective effects on photosynthesis were not associated with protection from low-temperature-induced plant injury assessed after recovery in control temperature for 10 days.

In experiment 2 cold-pretreatment reduced the degree of decrease in F_v/F_m throughout most of the treatment period (days 2 to 5) at 11°C, and this was due to maintenance of higher values of F_m in cold- as compared to control-pretreated

plants. Cold-pretreated plants showed recovery of dark-adapted F_v/F_m values to 92% of the experimental control level within 1 day of return to control temperature following treatment at 11°C for 5 days, whereas values of control-pretreated plants only recovered to 76% of the experimental control level in the same time. Differently pretreated plants did not differ in the slope of increase in F_v/F_m on return to control temperature. The response pattern of F_v/F_m during recovery was essentially paralleled by that of F_m, but in the case of F_m cold-pretreated plants exhibited a slightly higher slope of increase on return to control temperature as compared to control-pretreated plants. The recovery responses collectively suggest that cold-pretreatment partially protected PSII from irreversible damage induced by treatment at 11°C. Alleviation of irreversible leaf injury by cold-pretreatment was also apparent from a small degree of recovery of values of net rate of CO_2 uptake in cold-pretreated plants within 2 days after return to control temperature, whereas values of control-pretreated plants remained negative. Control-pretreated plants also exhibited significantly higher values of intercellular CO_2 concentration as compared to cold-pretreated plants, indicating dominance of respiratory processes in these plants. In addition, cold-pretreatment reduced low temperature-induced death and loss of leaves. This was apparent from (a) a lower percentage of leaves, which had died in cold- as compared to control-pretreated plants, and (b) from the increase in leaf dry matter production by 29% in cold-pretreated plants, whereas control-pretreated plants exhibited a reduction by 25% relative to the pretreatment value, both after return to control temperature for 15 days.

While in experiment 2 cold-pretreatment significantly reduced the decrease in F_v/F_m which occurred in non-acclimated plants on exposure to low temperature of 11°C throughout most of the treatment period, in experiment 1 cold-pretreatment only alleviated this decline after several days of treatment at 8°C. In addition, in experiment 2 cold-pretreatment reduced the extent of low temperature-induced leaf injury, whereas this was not the case in experiment 1. It was concluded that only experiment 2 demonstrated a significant protective effect of cold-pretreatment, manifested as reduced extent of photoinhibition as well as irreversible leaf damage as compared to control-pretreated plants on exposure to subsequent cold treatment. Therefore in the following section only the acclimation phenomenon observed in experiment 2 will be discussed in more detail.

A number of studies have demonstrated the acquirement of increased resistance to low temperature-induced photoinhibition during cold-acclimation, as indicated by a reduction in the extent of decrease in F_v/F_m in cold-acclimated as compared to non-acclimated plants, as was observed in the present study.

The cold-pretreatment procedure used in experiment 2 in the present study particularly resembles that applied by Somersalo & Krause (1989, 1990). As in the present study mature plants were subjected to acclimation treatment, which consisted of gradually decreasing temperatures over a period of several days. Somersalo & Krause (1989) investigated the effect of pretreatment of *Spinacia oleracea* at low temperatures decreasing in steps of two days each from 18/15, 15/10, 10/3, 3/1 to 1/1°C (day/night) at a photon flux density (PFD) of 260-300 μmol m^{-2} s^{-1}on resistance to low temperature-induced photoinhibition. Cold-pretreatment significantly increased frost tolerance and prevented the decrease in F_v/F_m and quantum yield of O_2 evolution, which occurred in non-acclimated plants (grown at 18°C) on exposure to 4°C under moderate incident light (PFD of 550 μmol m^{-2} s^{-1}). In a follow-up study Somersalo & Krause (1990) further characterised the observed acclimation phenomenon by determining electron transport activities in thylakoids isolated from cold-acclimated and non-acclimated plants by means of biochemical assays. Cold-pretreatment was shown to prevent the decrease in quantum yield and maximal rate of PSII-driven electron transport occurring in non-acclimated plants on exposure to the above described photoinhibitory treatment for 3 hours. However, in the study by Somersalo & Krause (1989) on spinach the decline in F_v/F_m in non-acclimated plants was due to a decrease in F_v as well as an increase in F_o. Cold-pretreatment almost completely prevented the decrease in F_v and the increase in F_o on exposure to the photoinhibitory treatment. In contrast, in experiment 2 of the present study on cotton, the decline in F_v/F_m in non-acclimated plants on exposure to treatment at 11°C was due to a decrease in F_m, whereas F_o was not altered. Cold-pretreatment alleviated, but did not completely prevent, the decrease in F_v/F_m by reducing the extent of reduction in F_m.

However, studies by Boese & Huner (1992) and Hurry & Huner (1992) demonstrated a similar response pattern as found in the present study. The present study differs from that by Boese & Huner (1992) and Hurry & Huner (1992) with respect to the duration of the acclimation period. In the present study mature

plants were subjected to cold-pretreatment for several days, whereas Boese & Huner (1992) and Hurry & Huner (1992) assessed the effects of long-term growth of plants over several weeks on resistance to photoinhibition in leaves newly developed in the respective growth temperature. Boese & Huner (1992) investigated the effects of long-term growth of spinach under 5, 16, and 25°C at PFD of 250 μmol m^{-2} s^{-1} on susceptibility to photoinhibition and compared plants of equivalent developmental stages by adjusting plant age correspondingly. Growth at the lower temperatures reduced the extent of decrease in F_v/F_m on exposure to 5°C under high incident PFD of 1200 μmol photons m^{-2} s^{-1}, but not 500 or 800 μmol m^{-2} s^{-1}, and the difference increased with exposure time (assessed over 9 hours). The lesser degree of decrease in F_v/F_m in plants grown at lower temperatures was due to maintenance of higher values of F_v in low temperature- as compared to high temperature-grown plants, whereas F_o was not significantly altered by the photoinhibitory treatment. On transfer of plants treated at 5°C and a PFD of 1200 μmol photons m^{-2} s^{-1} to a higher temperature of 20°C and a PFD of 20 μmol m^{-2} s^{-1}, plants from different growth temperatures exhibited similar rates of recovery of F_v/F_m, and recovery was complete within 24 hours. Hurry & Huner (1992) compared the effects of long-term growth of winter and spring cultivars of wheat at 20/16°C and 5/5°C (day/night), both at PFD of 250 μmol m^{-2} s^{-1}, on susceptibility to photoinhibition, and plants of equivalent developmental stages were compared. Cold-acclimation reduced the extent of decrease in F_v/F_m on exposure to 5°C at PFD of 1200 μmol photons m^{-2} s^{-1}, and the winter wheat cultivars acquired higher resistance to photoinhibition than the spring wheat cultivar during cold-acclimation. The decrease in F_v/F_m was due to a decrease in F_v, whereas F_o was not altered relative to the values after pretreatment. Application of an inhibitor of protein synthesis demonstrated that the difference between cold-acclimated and non-acclimated plants and between winter and spring cultivars with respect to resistance to photoinhibition was not due to photosystem II (PSII) repair processes involving *de novo* protein synthesis. On return to 20°C at PFD of 50 μmol photons m^{-2} s^{-1} cold-acclimated and non-acclimated plants showed biphasic recovery kinetics, an initial fast and a second slow phase of recovery, and cold-acclimation resulted in an increased rate of the initial fast phase.

The main factors distinguishing the present study from the ones mentioned above are: (a) Temperate plants like spinach and wheat can acquire increased resistance to temperatures below freezing in a period of low non-freezing temperatures, a process termed cold acclimation (Xin & Browse, 2000). Resistance to photoinhibition during such periods of low temperatures may thus be a crucial factor determining progression of the acclimation process and winter survival in the field (Hurry & Huner, 1992), and many herbaceous plants with the ability to cold-acclimate acquire increased resistance to photoinhibition during cold acclimation (Krause, 1994). In contrast, cotton, the subject of the present study, is a chilling sensitive species (Kornyeyev *et al.*, 2002), and Pearce (1999) defines chilling-sensitive plants as those which are damaged by temperatures below 12°C. Although the mechanisms underlying photoinhibition in chilling-tolerant and -sensitive species appear to be similar (Krause, 1994), chilling-sensitive plants are generally thought to have a low ability to acclimate to chilling or freezing temperatures (Krause, 1994, Allen & Ort, 2001). (b) In experiment 2 in the present study plants were left to recover for 1 day in control temperature after cold-acclimation treatment at gradually decreasing temperatures from 18°C to 13°C by 1°C per day, before being subjected to subsequent low temperature treatment at 11°C. In contrast, Somersalo & Krause (1989, 1990), Boese & Huner (1992), and Hurry & Huner (1992) directly transferred plants from short-term cold-acclimation treatment or long-term low growth temperature to the photoinhibitory treatment.

However, it has been shown that some potential to acquire increased resistance to photoinhibition during growth at chilling temperatures also exists in chilling-sensitive plants. Greer & Laing (1989) investigated the effects of long-term growth of kiwifruit (*Actinidia deliciosa*) at 30/25, 25/20, 20/15, 15/10°C (day/night) at a PFD of 700 μmol m^{-2} s^{-1} on the resistance of leaves developed in the respective growth temperature to photoinhibition. The extent of photoinhibition on exposure to PFD of 1500 μmol m^{-2} s^{-1}, determined as the change in F_v relative to the pretreatment level, tended to decrease with decreasing growth temperature, when determined at exposure temperatures of 25 and 20 °C, but not at 10°C. The differential effect of growth temperature on the extent of photoinhibition was due to differences in F_o. Plants grown at 20/15 and 15/10 °C exhibited a lower degree of increase in F_o as compared to plants grown at the

higher growth temperatures, when determined at 20 or 10°C, and they showed a decrease in F_o, when determined at 25°C. In contrast, plants grown at 30/25 and 25/20 °C exhibited an increase in F_o at all exposure temperatures. In addition, plants grown at 15/10°C showed a higher rate and extent of recovery at a PFD of 20 μmol photons m^{-2} s^{-1} relative to the pretreatment level compared to plants grown at the higher temperatures. The present study agrees with that by Greer & Laing (1989) with respect to the fact that both demonstrate a reduced extent of photoinhibition in chilling-sensitive plants acclimated to lower as compared to higher temperatures. However, the studies differ with respect to the duration of the acclimation period as well as the changes in F_o and F_m underlying changes in F_v. While the present study involved cold-pretreatment of mature plants over several days, Greer & Laing (1989) determined the effects of long-term growth at different temperatures over several weeks on resistance to photoinhibition in leaves newly developed in the respective growth temperature. In the study by Greer & Laing (1989) the reduced extent of photoinhibition in plants grown at lower as compared to higher temperatures was due to a smaller degree of increase or even a decrease in F_o. In contrast, the cold-pretreatment used in experiment 2 in the present study alleviated the decrease in F_v by reducing the extent of decrease in F_m.

4.3.2.4 Mechanism of acclimation to low temperature

In experiment 2 in the present study significant reductions in F_v/F_m relative to the experimental control level in control-pretreated plants on exposure to 11°C were due to parallel changes in F_m, whereas F_o was not significantly altered. Pretreatment at temperatures decreasing gradually by 1°C per day from 18°C to 13°C followed by 1 day of recovery in control temperature reduced the degree of decrease in F_v/F_m, and this was due to maintenance of higher values of F_m in cold- as compared to control-pretreated plants.

Boese & Huner (1992) found that resistance to low temperature-induced photoinhibition in spinach leaves changed with leaf developmental age. Susceptibility to photoinhibition remained low before and at full expansion, but increased after that. In the present study the possible contribution of effects of leaf age on differences between cold- and control-pretreated plants with respect to chlorophyll fluorescence parameters was minimised as follows. The average of

measurements on at least 2 leaves in experiment 1 and 3 leaves in experiment 2 (the youngest fully expanded and adjacent next older leaves) was taken as the value for each plant.

Hurry & Huner (1992) found a similar pattern of partial protection from photoinhibition by cold-pretreatment in wheat as observed in the present study on cotton. Wheat plants grown at 5/5°C (day/night) exhibited a reduced extent of decrease in F_v/F_m on exposure to 5°C at PFD of 1200 µmol photons m^{-2} s^{-1} as compared to plants grown at 20/16°C (day/night). The reduction in the degree of decrease in F_v/F_m in cold-acclimated plants was due to reduced decrease in F_m, whereas F_o remained unaltered relative to the pretreatment level. Winter wheat cultivars were shown to acquire higher resistance to photoinhibition during cold-acclimation than the tested spring wheat cultivar. The mechanism underlying the acquirement of increased resistance to photoinhibition during cold acclimation in winter cereals has been resolved, and lies in an increased capacity of oxidation of Q_A^- by the Cytochrome f/b_6 complex via the PQH_2 pool, the rate-limiting step of photosynthetic electron transport. This is due to an increase in photosynthetic capacity, but not efficiency during growth at low temperatures, which is associated with increased expression of genes encoding central enzymes of photosynthetic carbon metabolism (Huner *et al.*, 1998). Similarly, winter and spring wheat cultivars were shown to differ in their ability to increase photosynthetic capacity during cold acclimation. Plants of the winter as well as the spring cultivar grown at 5/5°C (day/night) for several weeks exhibited increased light-saturated rates of CO_2 assimilation as compared to plants grown at 20/16°C (day/night) on exposure to 5°C. Only in winter cultivars this was associated with increased CO_2-saturated rates of CO_2 assimilation. The apparent higher photosynthetic capacity of cold-acclimated winter as compared to spring wheat was attributed to increased synthesis of sucrose and fructans in leaves, and increased sink capacity for sucrose in crowns, which showed increased rates of fructan synthesis (Savitch *et al.*, 2000). In the study by Hurry & Huner (1992) on wheat the pretreatment regime, which increased resistance to low-temperature-induced photoinhibition, caused itself differential changes in dark-adapted chlorophyll fluorescence parameters. Winter wheat cultivars showed an increase in F_o by 50 to 60% associated with an increase in F_m by 20 to 25%, whereas the spring cultivar exhibited an increase only in F_o but not F_m. In experiment 2 in the

present study similar responses of F_o and F_m were observed after 1 day of recovery in control temperature following cold-pretreatment: F_m and F_o were significantly increased by 15 and 10%, respectively, relative to the control level. This resulted in no net change in F_v/F_m as compared to the control level. Expression of values of cold- and control-pretreated plants with respect to F_m and F_o in response to treatment at 11°C relative to the values after pretreatment showed that the changes in response to recovery from pretreatment for 1 day could explain most of the differences between cold- and control-pretreated plants on exposure to 11°C. The apparent increase in the yield of electron transport through PSII, as indicated by the increase in F_m, might thus have contributed to the increased resistance of cold-pretreated cotton plants to photoinhibition. This does not appear to be due to increased photosynthetic capacity, as cold- and control-pretreated plants did not differ with respect to net rates of CO_2 uptake after treatment at 11°C for 5 days, which were close to zero in both cases. Rather cold-pretreatment may have induced an increased capacity of alternative electron sinks, and this could have caused the increase in F_m above the control level, when the major plant electron sink, carbon assimilation, recovered on return of plants to control temperature following cold-pretreatment. Photorespiration, the water-water cycle and chlororespiration have been suggested to act as alternative electron sinks under photoinhibitory conditions (Niyogi, 1999, 2000).

However, acquirement of increased resistance to photoinhibition during low temperature acclimation treatment may also result from other mechanisms of photoprotection, including increased levels of soluble antioxidants and activities of antioxidant enzymes or an increase in the capacity to repair damaged PSII reaction centres (Krause, 1994). These mechanisms may have equally contributed to the reduced degree of photoinhibition in cold- as compared to control-pretreated plants of *Gossypium hirsutum* on exposure to 11°C concurrently with low incident light observed in experiment 2 in the present study.

The ability of cotton to accumulate glycinebetaine to osmotically significant levels (Blunden *et al.*, 2001, Gorham, 1996) provides yet another potential mechanisms which might have contributed to the observed acquirement of increased resistance to low temperature-induced photoinhibition during cold-pretreatment in the present study. Evidence for a role of glycinebetaine in alleviating low temperature-induced photoinhibition *in vivo* has been provided by

studies involving genetic engineering of glycinebetaine synthesis. Hayashi *et al.* (1997) transformed *Arabidopsis thaliana* with the gene for choline oxidase from *Arthrobacter globiformis* together with the coding sequence for the transit peptide of the small subunit of Rubisco, resulting in accumulation of glycinebetaine in leaves to levels of 1.0 μmol g^{-1} fresh weight. This was associated with increased resistance to low temperature-induced photoinhibition, as indicated by a reduced extent of decrease in dark-adapted values of F_v/F_m as compared to the wild-type on exposure to 5°C at a PFD of 250 μmol m^{-2} s^{-1} over 4 days. Transformed plants also exhibited a reduced degree of leaf injury on return to control temperature following exposure to 5°C at a PFFD of 250 μmol m^{-2} s^{-1} for 7 days. In a follow-up study the factors contributing to the reduced susceptibility to photoinhibition in transformed plants were further investigated (Alia *et al.*, 1999). Transformed plants exhibited a reduced extent of decrease in dark-adapted F_v/F_m on exposure to 10°C at a PFD of 1200 μmol m^{-2} s^{-1} as compared to the wild-type. Addition of an inhibitor of chloroplastic protein synthesis showed that this was due to an increased capacity of PSII repair processes involving protein synthesis in transformed plants. This was also apparent from an increased rate of recovery of dark-adapted F_v/F_m in control temperature and at PPFD of 70 μmol m^{-2} s^{-1} in transformed as compared to wild-type plants, when both had previously been photoinhibited to the same extent. Increased resistance to photoinhibition in transformed plants was not associated with changes in the composition of membrane lipids, but with increased levels of H_2O_2 as by-product of the choline oxidase reaction, and increased activity of catalase and ascorbate peroxidase in response to low temperature stress. In spite of this the above observations suggested that glycinebetaine may have increased the rate of PSII recovery processes, which involve protein synthesis, following photoinhibition. Chen *et al.* (2000) provided the first report implicating glycinebetaine in alleviation of chilling injury in a chilling-sensitive plant. They showed that exogenous supply of glycinebetaine at 2.5 mol m^{-3} to suspension-cultured cells of maize resulted in increased cell survival during subsequent treatment at 4°C for 7 days. Supply of 2.5 mol m^{-3} glycinebetaine to roots of maize seedlings also prevented leaf injury occurring in control plants in response to chilling at 4°C for 3 days.

In the present study the significant increase in glycinebetaine concentration in response to cold-pretreatment at gradually decreasing

temperatures may have contributed to increased resistance to photoinhibition only in the initial phase of subsequent treatment at 11°C concurrently with low incident light. On exposure to 11°C both control- and cold-pretreated plants showed a significant increase in leaf sap glycinebetaine concentration, when determined on day 5 of treatment. Cold-pretreated plants exhibited significantly higher concentrations of glycinebetaine as compared to control-pretreated plants in expressed leaf sap, and two processes may have contributed to this. (a) Cold-pretreated plants exhibited lower levels of leaf water content as compared to control-pretreated plants. When concentrations were expressed on a dry weight basis, the difference between differently pretreated plants was reduced and not significant. (b) Although glycinebetaine concentrations in the leaf sap were comparable to those of control plants after recovery in control temperature for 3 days following pretreatment at gradually decreasing temperatures, considerable levels might still have been present after recovery for only one day, before plants were exposed to treatment at 11°C. Stress-induced accumulation of glycinebetaine in cotton generally leads to comparatively large concentrations in the leaf sap, indicating that glycinebetaine is probably not only localised in cytoplasm and chloroplasts, but predominantly in the vacuole in this genus (Gorham, 1996). Thus it is unlikely that the additional glycinebetaine present in cold-pretreated plants would have further enhanced possible osmoprotective effects of the already large concentration observed in control-pretreated plants in response to treatment at 11°C.

The difference in potential of the pretreatment regimes used in experiment 1 and 2 to induce acclimation to low temperature-induced photoinhibition may be explained as follows. The pretreatment regimes used in experiment 1 and 2 both involved a gradual decrease in temperature by 1°C per day, starting at 18°C, but they differed in the minimum temperature reached, which was 13°C in experiment 2 and 12°C in experiment 1. The responses with respect to chlorophyll fluorescence and gas exchange parameters in the second phase of pretreatment in experiment 1 (from day 5 to 7) were shown to reflect the onset of more severe injury as compared to the initial phase of treatment (see above). The pretreatment regime in experiment 2 differed from that in experiment 1 in that it did not involve the final day of this treatment, thus inducing potentially less injury. This was confirmed by growth data, which showed that the pretreatment regime used in

experiment 1 induced much greater reductions in shoot dry matter production (see above) compared to that used in experiment 2. In addition experiment 1 and 2 also differed with respect to the temperature regime of the subsequent low temperature treatment. Treatment at 8°C applied in experiment 1 appeared to cause a greater extent of photon-damage to PSII reaction centres, as indicated by a significant rise in F_o relative to the experimental control from an early stage of treatment onwards. In contrast, treatment at 11°C in experiment 2 did not significantly affect F_o at any time during treatment. Thus treatment at 8°C may have exceeded the threshold of the increased resistance possibly acquired in the course of cold-pretreatment.

4.3.3 Plant responses to treatment at 49°C

4.3.3.1 Effects of cold-pretreatment

Pretreatment of *Gossypium hirsutum* at gradually decreasing temperatures from 18°C to 13°C by 1°C per day followed by 1 day of recovery in control temperature increased plant resistance to treatment at high temperature of 49°C with respect to the following physiological processes (experiment 2). (a) Cold-pretreatment partially protected PSII activity. This was apparent from a reduced extent of decrease in dark-adapted F_v/F_m in cold- as compared to control-pretreated plants, with reductions relative to the experimental control amounting to 21 and 61% in cold- and control-pretreated plants, respectively, after 29 hours of treatment at 49°C. The difference between differently pretreated plants with respect to F_v/F_m was due to equivalent relative treatment differences in F_m. The higher F_o values observed in cold- as compared to control-pretreated plants on exposure to 49°C may be a direct consequence of the increase in F_o, which occurred after 1 day of recovery following cold-pretreatment, as indicated by expression of responses to treatment at 49°C relative to values after pretreatment. Expression of values of F_m relative to the values after pretreatment did not greatly modify relative treatment differences, indicating that the strongly reduced extent of decrease in cold- as compared to control-pretreated plants could not be explained by the significant increase in F_m after 1 day of recovery following cold-pretreatment. (b) Cold-pretreatment partially protected photosynthetic dark reactions. After treatment at 49°C for 27 hours the net rate of CO_2 uptake was reduced to values below zero in control-pretreated plants, whereas cold-pretreated

plants exhibited positive rates at 17% of the experimental control level. Differences between cold- and control-pretreated plants with respect to net rate of CO_2 uptake were not associated with corresponding differences in transpiration or stomatal conductance. Plants from both pretreatments exhibited significant decreases in these parameters and did not differ with respect to the extent of reductions. The relative reductions in transpiration rate and stomatal conductance observed in cold-pretreated plants in response to treatment at 49°C could be explained by the only partial recovery of these parameters after 1 day of recovery in control temperature following pretreatment at gradually decreasing temperatures. However, net rates of CO_2 uptake exhibited a similar degree of only partial recovery on return to control temperature for 1 day following cold-pretreatment, but cold-pretreated plants still exhibited higher values as compared to control-pretreated plants in response to subsequent treatment at 49°C. (c) Cold-pretreatment partially protected leaves from rapid dehydration, as shown by the significantly reduced extent of decrease in values of water content and relative water content, determined after treatment at 49°C for 45 hours, in cold-as compared to control-pretreated plants.

Acquirement of increased resistance of photosynthesis to high temperature in response to pretreatment at gradually decreasing low temperatures, as observed in the present study, stands in marked contrast to other phenomena of photosynthetic acclimation to temperature reported in the literature. These generally involve a shift in the optimum temperature of photosynthetic processes corresponding to the temperature of the acclimation treatment. Acclimation of photosynthesis to either high or low temperatures is thus by necessity associated with decreased photosynthetic performance at the respective other end of the temperature scale. This has been demonstrated for acclimation treatments of varying duration. (a) Long-term growth of plants, which originate from habitats with large temperature variations during the growing season, under different temperature regimes induced shifts in the photosynthetic temperature optimum in leaves developed in the new temperature regime, while similar photosynthetic rates were maintained at the different temperature optima (Berry & Björkman, 1980). Also mature fully developed leaves were shown to exhibit adjustments in thermal characteristics of photosynthesis during growth at different temperatures over several days to weeks. (b) Havaux & Tardy (1996) demonstrated acclimation

to high temperature during short-term heat-pretreatment in *Solanum tuberosum*. Pretreatment of mature detached leaves at 35°C in darkness for 2 hours shifted the threshold temperature for a sudden rise in F_o level fluorescence to higher temperatures, and this was associated with changes in the temperature dependence of the actual quantum yield of PSII photochemistry. Heat-pretreated plants exhibited greater reductions in this parameter in response to temperatures below 18°C, but reductions of a lesser extent in response to temperatures above 37°C as compared to control-pretreated plants, when heated from 2 to 30 and 30 to 50°C at 1°C per minute.

However, concomitant increases in freezing and heat resistance with respect to irreversible leaf damage have been shown to occur in the process of cold acclimation, a period of low non-freezing temperatures, in which temperate plants acquire increased freezing resistance. Palta *et al.* (1981) investigated the effect of cold acclimation at 5/2°C (day/night) for 15 days on relative levels of freezing and heat resistance in *Solanum* species differing in constitutive resistance to heat and freezing stress. Relative values of acquired freezing resistance were defined as freezing temperature, those of acquired heat resistance as time at 50°C, in each case leading to 50% loss in leaf viability (based on ion leakage and tissue capacity to reduce TTC). This was shown to correspond to the onset of irreversible visible tissue injury. In the cultivated species *Solanum tuberosum* with high constitutive heat-, but low freezing-resistance cold acclimation did not increase freezing resistance and resulted in unaltered or even decreased levels of heat resistance (depending on the viability test used). In contrast, the non-cultivated species *Solanum commersonii* "Oka 5040" with high constitutive levels of freezing as well as heat resistance exhibited a 3-fold increase in freezing resistance associated with a 2-fold increase in heat resistance.

Protective effects of pretreatment at moderately elevated temperatures against injury induced by subsequent chilling treatment have been demonstrated in a number of chilling-sensitive species and have been reviewed by Sabehat *et al.* (1998). Pre-exposure of green tomato fruits to 38°C was shown to prevent tissue injury on subsequent exposure to 2°C for several days or 55°C for several minutes. Treatment at moderately elevated temperatures also reduced chilling-induced injury in hypocotyls of mung bean and seeds and cotyledons of cucumber. In all cases acquirement of increased resistance to chilling injury was correlated with *de*

novo synthesis of heat shock proteins (HSPs). Although providing a link between heat and chilling resistance in chilling-sensitive plants, these studies demonstrate only acquirement of chilling resistance by previous exposure to heat treatment, but not the reverse, *i.e.* acquirement of heat resistance by pretreatment at chilling temperatures, as observed in the present study.

4.3.3.2 Mechanism of increased resistance to high temperature as a result of pretreatment at low temperature

In the present study pretreatment of *Gossypium hirsutum* at gradually decreasing temperatures from 18°C to 13°C by 1°C per day followed by 1 day of recovery in control temperature increased plant resistance to subsequent treatment at 49°C (experiment 2), and protection occurred specifically at the level of photosynthesis. Cold-pretreatment strongly reduced the extent of decrease in F_v/F_m on exposure to 49°C, by reducing the decrease in F_m, indicating specific protection of the yield of electron transport through photosystem II (PSII). Cold-pretreatment also reduced the extent of decrease in net rate of CO_2 uptake on exposure to 49°C, but the extent of protection was small as compared to that of PSII activity.

The protective effect of low temperature treatment, specifically at the level of photosynthetic activity, during subsequent treatment at 49°C observed in the present study may be related to the ability of cotton to accumulate glycinebetaine to osmotically significant levels (Blunden *et al.*, 2001, Gorham, 1996). In experiment 2 of the present study pretreatment at gradually decreasing temperatures from 18 to 13°C by 1°C per day induced a significant 3-fold increase in glycinebetaine concentration in the leaf sap. Plants were left to recover in control temperature for 1 day before being subjected to subsequent treatment at 49°C. Although glycinebetaine concentrations in the leaf sap were comparable to those of control plants after recovery in control temperature for 3 days following pretreatment at gradually decreasing temperatures, considerable levels were probably still present after recovery for only 1 day (see 4.3.2, mechanism of acclimation to low temperature).

Interestingly, a number of studies have demonstrated a protective effect of glycinebetaine on photosynthetic reactions in the thylakoid membrane against heat inactivation *in vitro*. Allakhverdieva *et al.* (1999) found that glycinebetaine

protected oxygen evolving activity of PSII in isolated spinach thylakoid membranes and shifted the half-maximal inactivation temperature ($t_{1/2}$) of this activity from 39 to 50°C. Electron transport by PSII particles, from which extrinsic proteins maintaining the oxygen-evolving complex had been removed, exhibited a higher $t_{1/2}$ of 49°C, indicating higher heat stability of the reaction centre as compared to the oxygen evolving complex of PSII. Glycinebetaine also protected this activity, shifting $t_{1/2}$ to 55°C. PSI-mediated electron transport activity of isolated thylakoid membranes exhibited a higher $t_{1/2}$ (55°C) as compared to PSII electron transport, which was shifted to 63°C in the presence of glycinebetaine. Glycinebetaine conferred only slight protection to electron transport activity of isolated PSI particles. It was thus suggested that the protective effect of glycinebetaine on PSI electron transport occurred on the donor side of PSI. Allakhverdiev *et al.* (2003) purified PSII reaction centre complexes consisting of the D1/D2 heterodimer and Cyotchrome b559 (D1/D2/Cytb559 complex), and compared effects of addition of various compatible solutes, including glycinebetaine, proline, sucrose, and trehalose, on $t_{1/2}$ of light-induced electron transport activity mediated by these complexes. While proline and sucrose exhibited some protective effect against heat-induced inactivation, when supplied at concentrations above 500 mol m^{-3}, glycinebetaine was generally the most effective, even when supplied at concentrations < 500 mol m^{-3}. Protective effects of glycinebetaine against heat damage have also been demonstrated *in vivo*. Transformation of *Arabidopsis thaliana* with the gene for choline oxidase from *Arthrobacter globiformis* together with the coding sequence for the transit peptide of the small subunit of Rubisco resulted in increased plant resistance to high temperatures during imbibition of seeds, germination, and seedling growth (Alia *et al.*, 1998).

In the light of these studies it seems possible that the accumulation of glycinebetaine during cold-pretreatment observed in the present study played a major role in the protective effect of this pretreatment against heat inactivation of PSII activity.

4.3.4 Plant responses to soil drying

4.3.4.1 Responses of plants previously grown in control conditions

When 63-day old control-pretreated plants of *Gossypium hirsutum* were exposed to soil drought by withholding water (experiment 2), plants depleted soil water rapidly, as indicated by the steep decrease in volumetric soil water content. On day 4 after initiation of treatment it was already reduced to 39% of the experimental control level, and it exhibited a further slower decrease in the following days, reaching 16% of the experimental control level on day 14.

When first determined on day 4 of the drying period, net rate of CO_2 uptake was reduced to 44% of the experimental control level. It exhibited a further steep decline to 27% on day 5, and from then on a much slower gradual decrease, reaching 4% of the experimental control level on day 14. The response kinetics of transpiration and stomatal conductance generally resembled those of net rate of CO_2 uptake to soil drying. However, from day 10 to 14 of the drying period control-pretreated plants exhibited a significant decrease in water use efficiency of photosynthesis (WUE) relative to the experimental control level, thus indicating that net rate of CO_2 uptake declined to a greater extent than transpiration. WUE ranged from 51 to 72% of the experimental control level between days 10 and 12, and decreased again steeply towards day 14, when it amounted to 22% of the experimental control level. Intercellular CO_2 concentration exhibited a sudden significant increase by 55% relative to the experimental control level on day 14. After exposure to soil drying for 14 days relative water content was significantly reduced compared to the experimental control and amounted to 63%.

Lawlor & Cornic (2002) distinguished two phases in the response of gas exchange parameters to a progressive decrease in relative water content (RWC) as a result of soil drying. (a) Decreases in RWC in the range of 100 to 75% induce reductions in net rate of CO_2 uptake initially via stomatal limitations. Stomatal restrictions may play the major role in this phase or may be gradually superseded by metabolic limitations. As long as stomatal closure poses the main restriction to photosynthetic CO_2 assimilation, the reduction in net rate of CO_2 uptake is accompanied by a decline in intercellular CO_2 concentration. (b) Further decreases in RWC result in a steeper decrease in net rate of CO_2 uptake, which is mainly due to metabolic limitations, and their extent increases progressively with decreasing

RWC. In this phase intercellular CO_2 concentration starts to rise as a result of a stronger proportional decrease in photosynthetic CO_2 assimilation as compared to dark respiration or stomatal conductance. Stomatal and metabolic limitations of net rate of CO_2 uptake may be distinguished by analysis of the response of net rate of CO_2 uptake to increasing intercellular CO_2 concentration (A/c_i curve) (Long & Hällgren, 1993; Lawlor & Cornic, 2002). Although this time-consuming analysis was not conducted in the present study, some conclusions about the nature of photosynthetic limitations are possible. In the present study responses of gas exchange parameters were monitored from day 4 to 14 of the drying period onwards. On day 4 control-pretreated plants already exhibited a large decrease in net rate of CO_2 uptake, and although this was associated with a decline in stomatal conductance, values of intercellular CO_2 concentration remained comparable to those of experimental control plants. This indicates that metabolic limitations probably already played a major part in the reduction of net rate of CO_2 uptake at this stage. The reductions in WUE from day 10 onwards probably indicate the onset of metabolic restrictions to photosynthetic CO_2 assimilation of a more severe nature. The sharp rise in intercellular CO_2 concentration on day 14 suggests that photosynthetic CO_2 assimilation was relatively more reduced than dark respiration and stomatal conductance at this point. The relative water content of 63%, determined on the final day (day 14) of the soil drying period in control-pretreated plants, clearly falls into the range of RWC values defining phase 2 of the model by Lawlor & Cornic (2002). This confirms the emerging conclusion that the drought treatment applied in the present study resulted in severe inhibition of photosynthesis, in which metabolic restrictions played a major role.

4.3.4.2 Responses of cold-pretreated plants

Pretreatment at gradually decreasing temperatures from 18°C to 13°C appeared to increase plant resistance to subsequent soil drying with respect to the following parameters. (a) Cold-pretreatment reduced the degree of soil drought-induced reductions in net rate of CO_2 uptake, transpiration, and stomatal conductance, and the difference between control- and cold-pretreated plants was significant on most days in the period of day 5 to 11 of soil drying. The extent of this difference decreased progressively with continued soil drying. This was due to the fact that in cold-pretreated plants these parameters decreased gradually

throughout most of the drying period, whereas in control-pretreated plants they had already reached very low levels on day 6 of the soil drying period and decreased only slowly after that. (b) In cold-pretreated the intercellular CO_2 concentration tended to decrease between day 4 and 9, and was significantly reduced by 27% relative to the experimental control level, and also significantly lower as compared to values of control-pretreated plants, on day 9 of the soil drying period. In the following days it showed a tendency to rise. A decline in intercellular CO_2 concentration accompanying a reduction in net rate of CO_2 uptake indicates limitation of photosynthetic CO_2 assimilation predominantly by reductions in stomatal conductance, and is characteristic of phase 1 of the model by Lawlor & Cornic (2002) (see above). Thus cold-pretreatment appeared to delay the onset of metabolic restrictions to photosynthetic CO_2 assimilation. (c) Cold-pretreated plants maintained values of water use efficiency of net photosynthesis (WUE) comparable to those of experimental control plants from day 4 to 11, while values of control-pretreated plants were significantly reduced relative to the experimental control on day 10 and 11 of the soil drying period. This resulted in significantly higher values of WUE in cold- as compared to control-pretreated plants on day 7 and 9 of the soil drying period. (d) Cold-pretreated plants exhibited significantly greater increases in shoot height and stem dry matter production, determined after recovery in well-watered conditions for 10 to 11 days following soil drought treatment for 14 days, relative to the pretreatment level as compared to control-pretreated plants.

However, cold-pretreatment did not prevent the sudden rise in intercellular CO_2 concentration on day 14, which indicates the relative dominance of dark respiration over CO_2 assimilation (Lawlor & Cornic, 2002) or the rapid reduction in WUE in the period from day 12 to 14 of soil drying. In addition cold-pretreatment did not alleviate the reduction in leaf water content or relative water content, determined on the final day (day 14) of soil drying.

Roberts & Zwiazek (1999) investigated the effect of pre-exposure of 5-week old plants of *Picea glauca* to periodic chilling treatments at 5°C over 16 weeks to subsequent exposure to soil drying, imposed by withholding water, and obtained essentially similar results as described in the present study. Cold-pretreated plants maintained higher values of midday shoot water potential, determined after 9 days of soil drying, and showed faster rates of recovery of this

parameter after re-watering. Although chilling reduced the rate of shoot height increase in proportion to the frequency of periodic chilling treatments, cold-pretreated plants tended to have higher rates of shoot height increase during subsequent growth in the field for 16.5 weeks. This diminished the initial difference between control- and cold-pretreated with respect to shoot height.

The effect of cold-pretreatment on drought resistance does not appear to have been investigated previously in cotton. However, several studies have demonstrated that *Gossypium hirsutum* can be acclimated to soil drought by pre-exposure to cyclic soil drying or by slowly- as compared to rapidly-induced soil drought. Ackerson & Herbert (1981) found that pre-exposure of plants to 5 stress cycles, each consisting of withholding water for 24 to 48 hours, increased plant resistance to subsequent soil drying. Drought-pretreated plants maintained higher rates of photosynthesis at low values of leaf water potential, although rates at high values of leaf water potential were reduced as compared to control-pretreated plants. In addition, drought-pretreatment shifted the dependence curve of turgor potential and leaf gas phase conductance on leaf water potential to lower (more negative) values of leaf water potential in drought- as compared to control-pretreated plants. The maintenance of higher values of turgor potential was attributed mainly to net accumulation of solutes. Wise *et al.* (1992) exposed plants to 3 cycles of drought, each consisting of withholding water for 5 days, interspersed by 3-day re-watering periods, and compared responses of plants during the first and third drying cycle, which were regarded as non-acclimated and acclimated responses, respectively. By beginning of the third drying cycle leaf water potential had decreased, but the relationship between net rate of CO_2 uptake and leaf water potential had shifted to lower values of leaf water potential. Thus drought-acclimated plants maintained photosynthetic rates at control levels at leaf water potentials, which caused large reductions in net rate of CO_2 uptake in non-acclimated plants. Plaut & Federman (1991) imposed levels of soil water potential of -0.3, -0.6, and -0.9 MPa either within 5, 7, and 8 days, respectively, (acclimating treatment) or within 1, 2, and 2.5 days, respectively, (non-acclimating treatment). Acclimation induced only a slight reduction in the extent of decrease in net rate of CO_2 uptake in response to soil water deficit, when determined at a CO_2 partial pressure (pCO_2) of 34 Pa, and this was not associated with differences in transpiration. However, the extent of the difference between

acclimated and non-acclimated plants with respect to this parameter increased, when determined at a nearly saturating pCO_2 of 70 Pa. In addition the reduction in leaf water potential with decreasing soil water potential occurred at a steeper rate in acclimated as compared to non-acclimated plants, but this was associated with a much steeper decrease in leaf osmotic potential, thus indicating osmotic adjustment.

4.3.4.3 Mechanism of cold-induced drought resistance and future perspectives

Cold-pretreatment reduced the extent of water depletion in the substrate, as indicated by significantly higher values of volumetric soil water content in cold- as compared to control-pretreated plants in the period from day 4 to 9 of soil drying. In control-pretreated plants values had already reached very low levels on day 5 and decreased only slowly after that, whereas in cold-pretreated plants they continued to decrease gradually and at a steeper rate from day 4 to 10 of the drying period. Thus the extent of the difference between differently pretreated plants decreased progressively with time of soil drying.

The reduced extent of soil water depletion in cold- as compared to control-pretreated plants indicates reduced plant water loss by transpiration. This may result from two factors. (1) Differences in plant transpiration per unit leaf area. Bryla & Duniway (1998) investigated the effects of pretreatment of safflower and spring wheat plants with soil drought, imposed by withholding water for several days, on resistance to subsequent exposure to soil drying. In response to re-watering in between the two drought cycles, drought-acclimated plants initially exhibited reduced rates, but with progression of soil drying after re-watering increased rates of transpiration as compared to non-acclimated plants. This was associated with corresponding differences in water uptake, calculated from changes in volumetric soil water content, between drought-acclimated and non-acclimated plants. However, in the present study the decreased rate of soil water depletion in cold-pretreated plants was clearly not due to a reduction in plant water loss by transpiration. Cold-pretreated plants exhibited higher levels of volumetric soil water content concomitantly with higher rates of transpiration and stomatal conductance as compared to control-pretreated plants during soil drying. (2) Differences in plant transpirational area. Cold-pretreatment caused significant

reductions in shoot height and shoot dry matter production by 10 and 25%, respectively, when determined after 5 days of recovery in control conditions following low temperature treatment. Osório *et al.* (1998) addressed the problem associated with comparisons of responses of plants of the same age, but different size, to stresses involving resource supply, like soil drying. They found that removing the effect of plant size by stepwise analysis of covariance (ANCOVA) also eliminated the significant effect of soil water deficit on dry matter allocation patterns in 3 different genotypes of *Eucalyptus globulus*, which had previously been detected by conventional biomass allocation analysis. Instead, large differences between the tested genotypes emerged.

In the present study the response kinetics of net rate of CO_2 uptake, transpiration, and stomatal conductance to soil drying over a 14-day period closely resembled those of volumetric soil water content in the substrate. This suggested that differences in extent of soil water depletion between differently pretreated plants might have caused the corresponding differences in gas exchange parameters. To further investigate this possibility, the effects of plant size on the response of gas exchange parameters to soil drying were removed statistically using the method applied by Osório *et al.* (1998). In a subsidiary experiment, in which increments in plant size were created by increments in plant age, a close relationship between responses of gas exchange parameters to soil drying and parameters describing plant size (shoot height and dry matter production) had been demonstrated (see appendix 1). In addition, the rate of water depletion, determined as reduction in volumetric soil water content, was shown to depend on plant size. In the present study volumetric soil water content measured on the day, on which the highest relative difference with respect to this parameter between the plant groups differing in plant size occurred, was introduced as covariate into the analysis. A strong correlation between this parameter and the net rate of CO_2 uptake measured on particular days in the soil drying period had been demonstrated in the subsidiary study mentioned above (see appendix 1). When responses of gas exchange parameters on individual days in the drying period were analysed by ANCOVA, the significant effect of pretreatment generally disappeared for those measurement days, on which the specific assumptions for ANCOVA were satisfied. This indicates that cold-pretreatment appeared to reduce the extent of soil drought-induced reductions in net rate of CO_2

uptake, transpiration, and stomatal conductance as well as to delay the onset of metabolic limitations to CO_2 assimilation rate via reductions in shoot height and dry matter production. This in turn resulted in a lower rate of water depletion in the substrate, thus slowing the rate of drought stress development.

Roberts & Zwiazek (1999) did not resolve the mechanisms underlying the increase in drought resistance of *Picea glauca* plants as a result of pre-exposure to periodic chilling treatments at 5°C (see above), but suggested the involvement of either cold-induced reductions in shoot height or differences in parameters determining cellular water balance. As to the studies on acquirement of increased drought resistance in response to drought acclimation treatments in cotton (see above), Ackerson & Herbert (1981) observed reductions in plant height in response to drought-pretreatment, and this difference in plant size between drought- and control-pretretaed plants was not adjusted. However, Wise *et al.* (1992) compared responses of plants during the first and third of three drying cycles, which were regarded as non-acclimated and acclimated responses, respectively. In this experimental set-up differences in plant size are unlikely to have contributed to the observed phenomenon of acclimation of photosynthesis to soil drying. Plaut & Federman (1991) investigated effects of variation in the rate of soil drought imposition on drought resistance, and differences in plant size were thus not involved. In these studies on cotton acclimation of photosynthesis at the level of stomatal sensitivity to low leaf water potentials (Ackerson & Herbert, 1981) as well as at the chloroplast level (Plaut & Federman, 1991) have been demonstrated.

In the present study the phenomenon of acquirement of increased resistance of photosynthesis to soil drying by pretreatment at low temperature could be fully accounted for by effects of cold-pretreatment on plant growth. However, this might not necessarily reflect a lack of potential of cold-pretreatment to induce drought resistance in cotton, but rather the lack of technical sophistication in experimental set up and physiological methods used to detect differences in plant response to soil drying in the present study. The following technical adjustments are suggested: (a) Reductions in water potential in the plant rooting medium may not only be achieved by withholding water from soil-grown plants, but also by addition of polyethylene glycol (PEG), a non-ionic, long-chain, non-penetrating, inert polymer, to the nutrient solution of hydroponically-grown

plants. The use of PEG allows plant exposure to precisely defined soil water potentials (Nepomuceno *et al.*, 1998). These authors confirmed differences between 4 genotypes of *Gossypium hirsutum* with respect to drought resistance, which had been identified in the field mainly by differences in yield under soil drought, on a physiological level by subjecting plants to PEG-induced water deficit. (b) Differences in plant growth parameters between acclimated and non-acclimated plants can be balanced by adjusting plant age of non-acclimated plants accordingly. Matthews & Boyer (1984) investigated the effect of pre-exposure of 1 week-old plants of *Helianthus annuus* to 25% soil water content over several weeks on responses of photosynthesis to subsequent soil drying. Responses of acclimated plants were compared to those of well-watered plants of the same age (age control) and to those of well-watered plants of the same developmental stage, defined by plant height and leaf number, and thus of younger age (developmental control). (c) The pressure-volume technique could be used to assess acclimation mechanisms at the level of leaf water relations. Maury *et al.* (2000) investigated differences in constitutive and induced drought resistance between three genotypes of *Helianthus annuus*. They used pressure-volume curves to estimate leaf osmotic potential at full turgor (π^{100}) and at the turgor-loss point (Ψ_{tlp}), relative water content at the turgor-loss point (RWC_{tlp}), apoplastic water fraction (AWF), bulk elastic modulus (BEM), and relative symplastic solute content (RNs^{PV}). The RWC_{tlp} gives information about plant tolerance to internal water deficit, and the genotype, which exhibited the highest constitutive drought resistance with respect to photosynthesis, had the lowest RWC_{tlp}, and this was associated with a lower BEM. However, a different genotype acquired the highest drought resistance of photosynthesis during drought-pretreatment, and exhibited a decrease in Ψ_{tlp} and π^{100} during drought pretreatment as well as subsequent drought treatment. The decrease in π^{100} was associated with an increase in relative symplastic solute content during drought-pretreatment and subsequent treatment. This was the only genotype, which showed a significant decrease in RWC_{tlp} in response to the second drought stress. The genotype with the highest constitutive drought resistance with respect to photosynthesis exhibited higher cumulative osmotic adjustment during drought-pretreatment and subsequent drought treatment than the genotype with the highest acquired drought resistance of photosynthesis. However, as this was associated with an increase in BEM during

the second drought treatment, an actual increase in RWC_{tlp} occurred in response to the second drought treatment. The study by Maury *et al.* (2000) demonstrates that the pressure-volume technique would allow a detailed resolution of the contribution of mechanisms, which are related to maintenance of turgor pressure at the level of tissue water relations, to plant drought resistance.

5 Effects of pretreatment with moderate salinity on resistance to severe salinity stress

5.1 Experiment 1

5.1.1 Experimental protocol

Seeds of *Gossypium hirsutum* var. CIM 443 were sown as described in section 2.1 on Feb. 28th 2000. Seedlings were transplanted to 1.6-dm^3 pots 18 days after sowing.

Plants were randomly assigned to combinations of pretreatment and subsequent treatment which are summarised in table 5.1.

Table 5.1 Experimental design consisting of **(1)** 3 levels of pretreatment: treatment with NaCl solutions of final concentrations of 80 and 120 mol m^{-3} or with control solution and **(2)** the following modes of subsequent treatment: **a)** no further treatment, but assessment of plant response to pretreatment; **b)** exposure to high levels of salinity; **c)** maintenance in control conditions throughout the experiment (applies only to control-pretreated plants, forming the experimental control). The number of replicates for each combination of pretreatment / subsequent treatment is shown.

(2) Subsequent treatment	**(1) Pretreatment**		
	Control	80 mol m^{-3} NaCl	120 mol m^{-3} NaCl
***a)** Assessment of plant response to pretreatment*			
-	5	5	5
***b)** Subsequent treatment with severe stress*			
Salinity	5	5	5
***c)** Maintenance in control conditions*			
Control	5		

When plants were 23 days old, they were exposed to pretreatment with either NaCl solutions of final concentrations of 80 and 120 mol m^{-3} for 21 to 26 days (as specified below for different combinations of pretreatment / subsequent treatment) or alternatively with control solution for the same period of time. Final salinity levels were imposed gradually increasing stepwise by 40 mol m^{-3} per day. Salt solutions were composed as described earlier in materials & methods, control solution contained only plant nutrients in the same amount and composition as the salt solutions. Solutions were applied manually to the substrate surface, using a

volume of about 300 cm^3, every second day. Control plants were occasionally treated with water instead of control solution and occasionally with additional water. Response to pretreatment was assessed by measuring chlorophyll fluorescence parameters on day 22, and gas exchange parameters on days 22 and 23 after initiation of pretreatment. Leaves were sampled for measurement of water content and concentrations of cations after 24 days of treatment. Shoot height and aboveground dry matter production were measured on day 25 of pretreatment.

Plants assigned to treatment with severe salinity stress were pretreated with either NaCl solutions of final concentrations of 80 or 120 mol m^{-3} for 26 days or with control solution. They were subsequently subjected to salinity imposed by flooding of plant substrate with NaCl solutions of a final concentration of 300 mol m^{-3} twice a day for 31 days, using an automatic floodbench system. The final salinity level of 300 mol m^{-3} was imposed gradually increasing stepwise by 75 mol m^{-3} per day. For assessment of plant response to salinity, leaf samples for measurement of concentrations of cations were taken 30 days after initiation of treatment. Leaf water content as well as shoot height and above ground dry matter production were measured after 31 days of treatment.

Experimental control plants were transplanted to 2.5-dm^3 pots 46 days after sowing. They were treated with control solution about every second day from 23 to 50 days after sowing. After that they were treated with plant nutrient solution containing phostrogen at 2 g dm^{-3} and micronutrients as in half strength Hoagland's solution 52, 53, 54, and 77 and 78 days after sowing, and in between with solution containing phostrogen at 5 g dm^{-3} and micronutrients as in half strength Hoagland's solution 72, 73, 74, and 76 days after sowing.

5.1.2 Results

5.1.2.1 Effects of pretreatment at two levels of moderate salinity

23-day old plants of *Gossypium hirsutum* were treated at two different levels of moderate salinity, 80 and 120 mol m^{-3} NaCl, for up to 25 days, and responses to salinity were assessed with respect to a variety of physiological parameters.

Exposure to moderate salinity for 22 days induced a significant increase in F_v/F_m by 2% relative to the control level in plants treated at both salinity levels

(Table 5.2). The rise in F_v/F_m was associated with a decrease in F_o by around 10% compared to the control in plants treated at both salinity levels, although this was not statistically significant ($P < 0.05$).

Table 5.2 Responses of F_v/F_m, F_o and F_m of chlorophyll fluorescence in *Gossypium hirsutum* to treatment at 80 and 120 mol m^{-3} NaCl for 22 days (NaCl 80 and NaCl 120, respectively). Salt solutions contained added calcium (ratio 1:10 to Na) and plant nutrients. Control plants were treated with solution containing no added NaCl, but plant nutrients in the same amount and composition as the salt solutions. Mean ± standard error for 5 plants. For each variable a one-way analysis of variance was performed between treatments. Means followed by the same letters are not significantly different at $P < 0.05$.

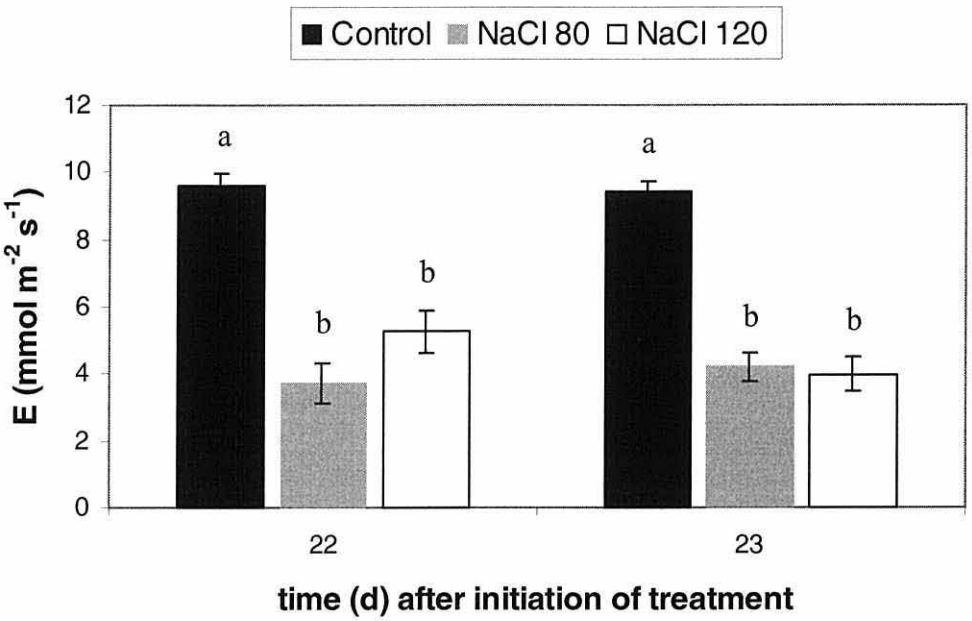
	F_v/F_m	F_o	F_m
Control	0.837 ± 0.003 a	256 ± 11.0 a	1569 ± 42.1 a
NaCl 80	0.852 ± 0.001 b	230 ± 8.8 a	1557 ± 54.6 a
NaCl 120	0.856 ± 0.002 b	232 ± 8.2 a	1610 ± 45.8 a

Moderate salinity decreased levels of transpiration (Figure 5.1a), net rate of CO_2 uptake (Figure 5.1b), and stomatal conductance (Figure 5.2a) relative to the control, when determined on days 22 and 23 of treatment, and reductions were mostly statistically significant (except for plants treated at 120 mol m^{-3} NaCl with respect to net rate of CO_2 uptake on day 23). The level of moderate salinity did not significantly affect the extent of decrease in these parameters, and relative reductions ranged from 45 to 61% for transpiration, 25 to 46% for net rate of CO_2 uptake, and 67 to 79% for stomatal conductance. Moderate salinity treatment induced a significant decrease in intercellular CO_2 concentration (c_i) in plants treated at both levels of moderate salinity relative to the control level (Figure 5.2b). The level of moderate salinity only affected the extent of decline on day 23, when relative reductions amounted to 30 and 43% in plants treated at 80 and 120 mol m^{-3} NaCl, respectively.

The proportionally greater reductions in transpiration as compared to net rate of CO_2 uptake in response to moderate salinity treatment resulted in a significant rise in water use efficiency of photosynthesis in plants treated at both levels of moderate salinity (Figure 5.3). Plants treated at 80 and 120 mol m^{-3} did not differ significantly with respect to the extent of increase, which ranged from

31 to 38%, when determined on day 22, and 54 to 76% relative to the control level, when determined on day 23 of treatment.

a)



b)

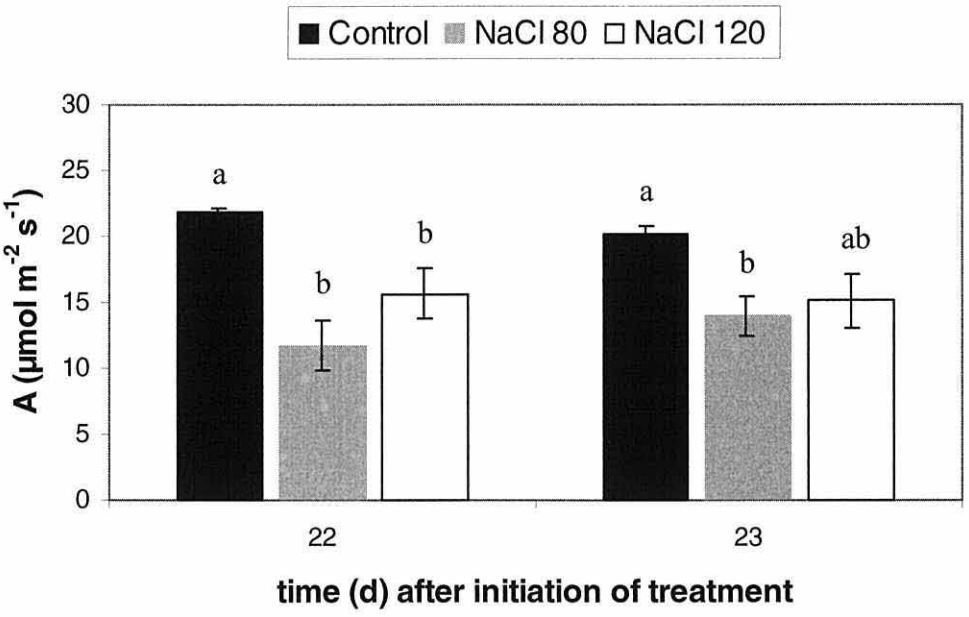
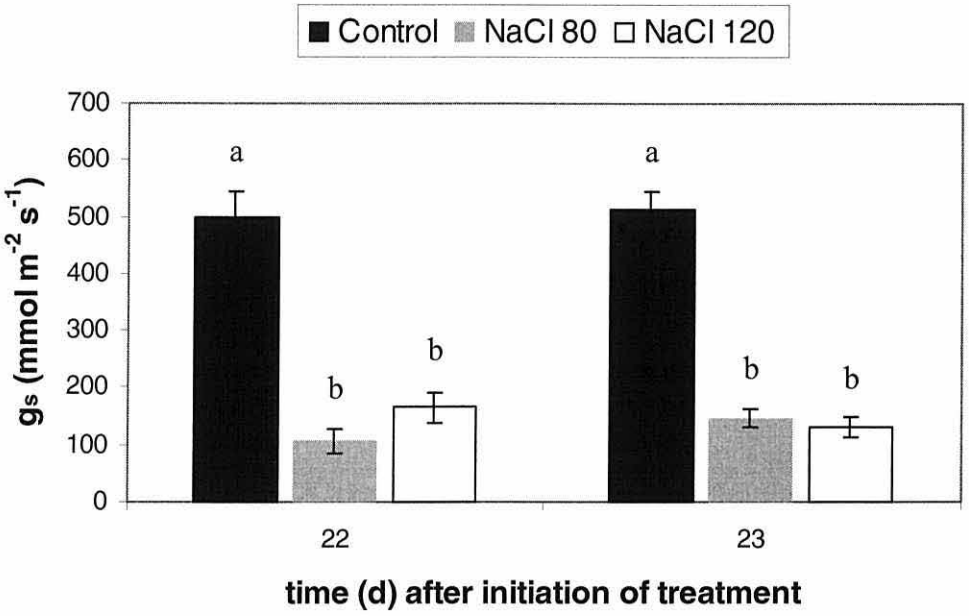


Figure 5.1 Variation in **a)** transpiration (E) and **b)** net rate of CO_2 uptake (A) in *Gossypium hirsutum* in response to treatment at 80 or 120 mol m^{-3} NaCl for 22 and 23 days (NaCl 80 and NaCl 120, respectively). Control treatment and composition of salt and control solutions as in table 5.2. Mean ± standard error for 5 plants. Measurements were taken for 1 leaf per plant. For each variable and day a single factor analysis of variance was performed between treatments. Means followed by the same letters are not significantly different at $P < 0.05$.

a)



b)

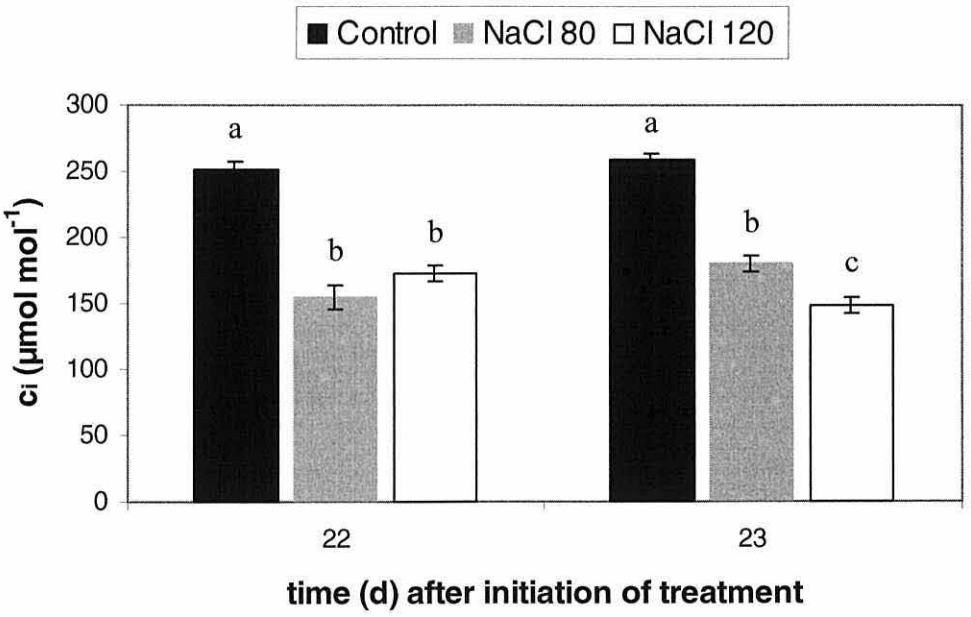


Figure 5.2 Variation in **a)** stomatal conductance (g_s) and **b)** CO_2 mole fraction in the leaf intercellular air space (c_i) in *Gossypium hirsutum*. Treatments and statistical testing as in figure 5.1.

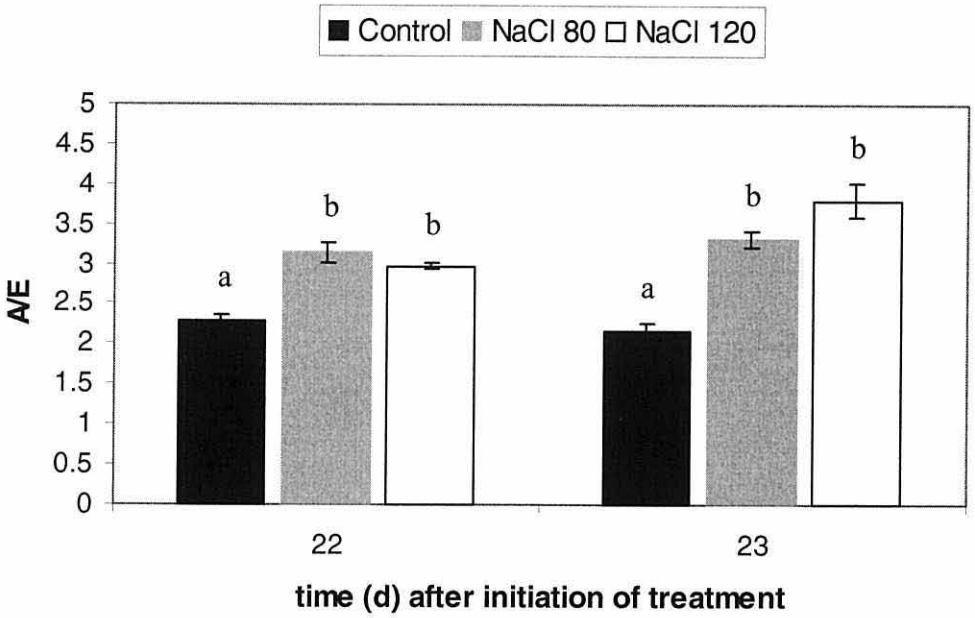


Figure 5.3 Variation in instantaneous water use efficiency of net photosynthesis (A/E) in *Gossypium hirsutum.* Treatments and statistical testing as in figure 5.1.

Moderate salinity caused significant changes in shoot growth parameters, when determined after 25 days of treatment (Table 5.3). Plants treated at both levels of moderate salinity exhibited a significant decrease in shoot height and shoot dry matter production compared to the control, and the extent of reduction was significantly higher in plants treated at 120 as compared to 80 mol m^{-3} NaCl. Relative reductions in shoot height amounted to 22 and 28% relative to the control level in plants treated at 80 and 120 mol m^{-3}, respectively, and the respective values for shoot dry matter production were 28 and 41%. The salinity-induced decreases in shoot dry matter production were associated with significant reductions in stem and leaf dry matter production in plants treated at both levels of moderate salinity, whereas dry matter production of reproductive organs was not significantly affected by salinity treatment. The level of moderate salinity had a significant effect on the extent of decrease in leaf dry matter production, with relative reductions amounting to 30 and 44% relative to the control level in plants subjected to salinity treatment at 80 and 120 mol m^{-3} NaCl, respectively. In contrast, plants treated at 80 and 120 mol m^{-3} NaCl did not differ significantly with respect to the degree of decrease in stem dry matter production, and relative reductions ranged from 24 to 34%.

Table 5.3 Variation in shoot height and above ground dry matter production (whole shoot and individual contributions of stem, leaves and flowers) in *Gossypium hirsutum* in response to treatment at 80 or 120 mol m^{-3} NaCl for 25 days (NaCl 80 and NaCl 120, respectively). Composition of salt and control solutions, descriptive statistics, and statistical testing as in table 5.2.

	Shoot height (cm plant^{-1})	**Dry matter production (g plant^{-1})**			
		Stem	**Leaves**	**Flowers**	**Shoot**
Control	43.9 ± 0.43 a	2.9 ± 0.09 a	6.6 ± 0.12 a	0.06 ± 0.028 a	9.6 ± 0.18 a
NaCl 80	34.4 ± 0.68 b	2.2 ± 0.07 b	4.6 ± 0.13 b	0.04 ± 0.033 a	6.9 ± 0.13 b
NaCl 120	31.6 ± 0.75 c	1.9 ± 0.13 b	3.7 ± 0.12 c	0.13 ± 0.056 a	5.7 ± 0.28 c

Determination of leaf water content after 24 days of treatment at moderate salinity revealed a significant rise in this parameter in plants treated at both levels of moderate salinity compared to the control (Table 5.4). Plants treated at 80 and

120 mol m^{-3} NaCl did not differ significantly with respect to the extent of this increase, which ranged from 24 to 31% relative to the control level.

Table 5.4 Water content of leaves (WC) of *Gossypium hirsutum* after treatment at 80 or 120 mol m^{-3} NaCl for 24 days (NaCl 80 and NaCl 120, respectively). Composition of salt solutions and control solution as in Table 5.2. Each value is the mean ± standard error for 5 plants. Statistical testing as in table 5.2.

	WC (%)
Control	289 ± 9.0 a
NaCl 80	378 ± 12.1 b
NaCl 120	357 ± 4.1 b

Treatment at moderate salinity induced significant changes in concentrations of cations in expressed leaf sap, when assessed after 24 days of treatment (Table 5.5a). Plants treated at both levels of moderate salinity exhibited a significant rise in Na^+ concentration and a significant drop in K^+ concentration as compared to the control, and the level of moderate salinity did not significantly affect the extent of these changes. Relative increases in Na^+ concentration ranged from 81 to 91% and reductions in K^+ concentration from 46 to 51% relative to the control level. The salinity-induced changes in Na^+ and K^+ concentration resulted in a significant drop in K^+/Na^+ ratio in plants treated at both levels of moderate salinity. Plants treated at 80 and 120 mol m^{-3} did not differ significantly with respect to the extent of this reduction, and exhibited values of around 30% of the control level. Moderate salinity treatment induced a significant increase in the concentration of Mg^{2+} by 41% relative to the control level in plants treated at 80 mol m^{-3} NaCl. Values of plants treated at 120 mol m^{-3} NaCl were only slightly increased by 15% relative to the control, and did not differ significantly from those of either control plants or plants exposed to 120 mol m^{-3} NaCl. Treatment at moderate salinity did not significantly affect concentrations of Ca^{2+} in expressed leaf sap in plants treated at either level of moderate salinity.

Plants treated at both levels of moderate salinity exhibited an increase in leaf water content compared to control plants (Table 5.4). To remove the effects of passive dehydration/dilution of leaf cation concentrations as a result of changes in leaf water content, cation concentrations determined in expressed leaf sap were

converted to a dry weight basis (Table 5.5b). Expression on a dry weight basis enhanced the increase in Na^+ concentration and diminished the reduction in K^+ concentration in response to treatment at both levels of moderate salinity compared to the control. The level of moderate salinity did not significantly affect the extent of relative increases/reductions in these ions, with relative increases in Na^+ concentration amounting to 134% in plants from both levels of salinity treatment, and relative reductions in K^+ concentration ranging from 29 to 39%. Expression on a dry weight basis revealed a significant increase in Ca^{2+} concentration in response to treatment at both levels of moderate salinity, which was not observed, when concentrations were expressed as determined in the leaf sap. Plants treated at 80 and 120 mol m^{-3} NaCl did not differ significantly with respect to the extent of increase in Ca^{2+} concentration, which ranged from 42 to 55%. When expressed on a dry weight basis, significant increases in Mg^{2+} concentration occurred in plants treated at both levels of moderate salinity, but the extent of this increase was significantly greater in plants treated at 80 mol m^{-3} (86%) as compared to plants treated at 120 mol m^{-3} NaCl (46%).

Table 5.5 Variation in concentration of Na^+, K^+, Mg^{2+}, and Ca^{2+}, and ratio of K^+ / Na^+ in leaves of *Gossypium hirsutum* in response to treatment at 80 or 120 mol m^{-3} NaCl for 24 days (NaCl 80 and NaCl 120, respectively). Composition of salt and control solutions as in table 5.2. **a)** Ion concentration measured in leaf sap. **b)** Ion concentration as in a converted to a dry weight basis via the measured leaf water content. Determination of leaf water content (see Table 5.4) was carried out within 1 hour of sampling for leaf ion concentrations, using a leaf adjacent to the one sampled for the measurement of ion concentrations. Mean ± standard error for 5 plants. Statistical testing as in table 5.2.

	Ions in leaves				
	Na^+	**K^+**	**K^+ / Na^+**	**Ca^{2+}**	**Mg^{2+}**
	***a)** Concentration measured in leaf sap (mol m^{-3})*				
Control	4.3 ± 0.00 a	145 ± 4.4 a	34 ± 1.0 a	207 ± 11.5 a	39 ± 3.1 a
NaCl 80	7.8 ± 0.86 b	78 ± 4.4 b	11 ± 1.3 b	245 ± 15.0 a	55 ± 3.2 b
NaCl 120	8.2 ± 0.28 b	71 ± 5.4 b	9 ± 0.4 b	238 ± 9.0 a	45 ± 2.3 ab
	***b)** Concentration measured as in a converted to dry weight basis (μmol g^{-1} DW)*				
Control	12.5 ± 0.4 a	417 ± 7.7 a		597 ± 31.1 a	111 ± 6.9 a
NaCl 80	29.3 ± 3.2 b	295 ± 13.8 b		928 ± 68.7 b	207 ± 16.9 c
NaCl 120	29.3 ± 1.2 b	255 ± 20.3 b		850 ± 30.0 b	162 ± 9.3 b

5.1.2.2 Effects of pretreatment at two levels of moderate salinity on plant response to treatment at severe salinity

Plants pretreated at moderate salinity of either 80 or 120 mol m^{-3} for 26 days (salt-pretreated) and plants of the same age, which had been maintained in control conditions (control-pretreated), were subjected to salinity treatment at 300 mol m^{-3} NaCl.

Treatment at 300 mol m^{-3} NaCl caused a decrease in shoot height and dry matter production, when determined after 31 days of treatment, in plants from all pretreatments relative to the experimental control level, but pretreatment had a significant effect on the extent of reduction (Table 5.6a). The extent of decrease in shoot height and shoot dry matter production relative to the experimental control level increased with increasing NaCl concentration experienced during pretreatment. Relative reductions in shoot height amounted to 35, 51, and 56% in plants pretreated at control conditions, 80, and 120 mol m^{-3} NaCl, respectively, and the respective values for shoot dry matter production were 72, 79, and 83%. The differences between differently-pretreated plants with respect to these parameters were significant. The effect of salinity level experienced during pretreatment on shoot dry matter production in response to treatment at 300 mol m^{-3} NaCl was reflected in corresponding trends with respect to leaf and stem dry matter production, whereas pretreatment did not significantly affect dry matter production of reproductive organs. However, differences between differently pretreated plants with respect to stem and leaf dry matter production were only statistically significant between control- and salt-pretreated plants, whereas the only small differences between plants treated at 80 and 120 mol m^{-3} were not significant.

To assess changes in shoot height and dry matter production in response to treatment at 300 mol m^{-3} NaCl independently from reductions observed in response to pretreatment itself (Table 5.3), values in response to treatment at 300 mol m^{-3} NaCl were expressed as percentage of those after pretreatment (Table 5.6b). When expressed relative to the pretreatment level, control- and salt-pretreated plants did not differ significantly with respect to shoot height and total shoot and leaf dry matter production in response to exposure to 300 mol m^{-3} NaCl. However, salt-pretreated plants tended to have lower increases in stem dry matter production relative to the pretreatment level as compared to control-pretreated

plants, and this effect was more pronounced and statistically significant only for plants pretreated at 80 mol m^{-3} NaCl. The intermediate values of plants pretreated at 120 mol m^{-3} NaCl did not differ significantly from those of control-pretreated plants or plants pretreated at 80 mol m^{-3} NaCl. Expression relative to the pretreatment level revealed a significant effect of pretreatment on production of reproductive organs in response to treatment at 300 mol m^{-3} NaCl. Control-pretreated plants and plants pretreated at 120 mol m^{-3} NaCl exhibited significantly lower values as compared to plants pretreated at 80 mol m^{-3} NaCl.

Determination of leaf water content revealed significant differences in responses of differently pretreated plants to treatment at 300 mol m^{-3} NaCl for 31 days (Table 5.7a). Plants from all pretreatments exhibited increased values of water content relative to the control level after treatment at 300 mol m^{-3}, but pretreatment had significant effects on the extent of this increase. Values of water content were significantly higher in plants pretreated at 80 mol m^{-3} NaCl as compared to control-pretreated plants, whereas the intermediate values of plants pretreated at 120 mol m^{-3} NaCl did not differ significantly from plants pretreated at 80 mol m^{-3} NaCl or control conditions.

Table 5.6 Variation in shoot height and above ground dry matter production (whole shoot and individual contributions of stem, leaves and reproductive organs) in *Gossypium hirsutum* after treatment at 300 mol m^{-3} NaCl for 31 days (NaCl 300). Plants had previously been pretreated at 80 or 120 mol m^{-3} NaCl for 26 days (NaCl 80 and NaCl 120, respectively) or with control solution for the same period of time (Ct). Salt solutions contained added calcium (Ca / Na ratio 1:10) and plant nutrients, control solution only plant nutrients in the same amount and composition as the salt solutions. Experimental control plants (Ct/Ct) were treated with water and plant nutrients throughout the experiment. Shoot height and above ground dry matter production are expressed **a)** as absolute values measured after salt treatment and **b)** as fold increase / decrease relative to the mean value in response to only pretreatment. The measurement of response to pretreatment was carried out on a different set of plants (see Table 5.3) which had been exposed to the respective pretreatments for 25 days. Mean ± standard error for 5 plants. All salt-treatments (NaCl 300) were compared by a single factor analysis of variance. Means followed by the same letters are not significantly different ($P < 0.05$).

	Shoot height (cm plant^{-1})	**Dry matter production (g plant^{-1})**			
		Stem	**Leaves**	**Flowers / fruits**	**Shoot**
	a) *as absolute values found after salt treatment*				
Ct/Ct	79.4 ± 2.0	24.4 ± 1.7	29.6 ± 3.0	7.4 ± 1.4	61.4 ± 3.9
Ct/NaCl 300	51.4 ± 0.7 a	7.4 ± 0.2 a	6.5 ± 0.3 a	3.1 ± 0.5 a	16.9 ± 0.4 a
NaCl 80/NaCl 300	39.2 ± 0.5 b	4.7 ± 0.2 b	4.6 ± 0.1 b	3.5 ± 0.5 a	12.9 ± 0.3 b
NaCl 120/NaCl 300	35.2 ± 0.6 c	4.3 ± 0.2 b	4.0 ± 0.2 b	2.3 ± 0.3 a	10.7 ± 0.4 c
	b) *fold increase / decrease relative to mean value after pretreatment*				
Ct/Ct	1.8 ± 0.05	8.4 ± 0.60	4.5 ± 0.45	115 ± 21.4	6.4 ± 0.41
Ct/NaCl 300	1.2 ± 0.02 a	2.6 ± 0.06 a	1.0 ± 0.04 a	48 ± 8.1 b	1.8 ± 0.05 a
NaCl 80/NaCl 300	1.1 ± 0.01 a	2.1 ± 0.09 b	1.0 ± 0.03 a	97 ± 13.5 a	1.9 ± 0.04 a
NaCl 120/NaCl 300	1.1 ± 0.02 a	2.3 ± 0.10 ab	1.1 ± 0.06 a	18 ± 2.5 b	1.9 ± 0.06 a

As salt-pretreatment itself induced significant increases in leaf water content, responses of plants to treatment at 300 mol m^{-3} NaCl were also expressed relative to the pretreatment level to extract changes resulting solely from treatment at 300 mol m^{-3} NaCl (Table 5.7b). When expressed as percentage of the values after pretreatment, values of salt-pretreated plants were comparable to those of the experimental control, and only control-pretreated plants exhibited a significant increase relative to the experimental control level as well as in comparison to salt-pretreated plants. This indicates that the increases in water content relative to the experimental control observed in salt-pretreated plants after treatment at 300 mol m^{-3} NaCl reflected the increases which occurred during salt-pretreatment, whereas control-pretreated plants showed a net increase in water content in response to treatment at 300 mol m^{-3} NaCl.

Table 5.7 Leaf water content in *Gossypium hirsutum* after treatment at 300 mol m^{-3} NaCl for 31 days (NaCl 300). Pretreatments, experimental control treatment, and composition of salt and control solutions as in table 5.6. Water content is expressed as **a)** absolute values measured after salt treatment and **b)** percentage of the mean value in response to only pretreatment. The measurement of response to pretreatment was carried out on a different set of plants (see Table 5.4), which had been exposed to the respective pretreatments for 24 days. Mean ± standard error for 5 plants. A single factor analysis of variance was performed between combined treatments. Means followed by different letters are significantly different ($P < 0.05$).

	Water content (%)	
	***a)** as absolute values found after salt treatment*	***b)** as % of the mean value after pretreatment*
Control / Control	259 ± 10.9 a	89.6 ± 3.8 a
Control / NaCl 300	310 ± 8.7 b	107.4 ± 3.0 b
NaCl 80 / NaCl 300	353 ± 4.0 c	93.4 ± 1.1 a
NaCl 120 / NaCl 300	338 ± 8.2 bc	94.8 ± 2.3 a

Concentrations of cations in the leaf sap, determined after treatment at 300 mol m^{-3} NaCl for 30 days, are presented in Table 5.8a. Plants from all pretreatments showed a significant increase in leaf sap Na^+ concentration compared to the experimental control, but pretreatment affected the extent of this increase. Plants pretreated at control conditions, 80, and 120 mol m^{-3} NaCl exhibited increases in leaf sap Na^+ concentrations by 70-, 66-, and 52-fold, respectively. Control-pretreated plants had significantly higher concentrations of

Na^+ than plants pretreated at 120 mol m^{-3} NaCl, and the intermediate values of plants pretreated at 80 mol m^{-3} NaCl did not differ significantly from plants pretreated at either control conditions or 120 mol m^{-3} NaCl. Treatment at 300 mol m^{-3} NaCl induced a significant increase in leaf sap K^+ concentration in control-pretreated plants by 81% compared to the experimental control, and values were also significantly higher compared to those of salt-pretreated plants. Plants pretreated at 120 mol m^{-3} NaCl showed a significant decrease in leaf sap K^+ concentration by 29% relative to the experimental control level. In contrast, values of plants pretreated at 80 mol m^{-3} were only slightly decreased (by 13%) compared to the experimental control, and did not differ significantly from either experimental control plants or plants pretreated at 120 mol m^{-3} NaCl. The large increases in leaf sap Na^+ concentrations in response to treatment at 300 mol m^{-3} NaCl resulted in significant reductions in K^+/Na^+ ratio in plants from all pretreatments. Values were reduced to only 2.3 and 1.4% of the experimental control level in control- and salt-pretreated plants respectively, and differences between differently pretreated plants were not statistically significant ($P < 0.5$). Treatment at 300 mol m^{-3} NaCl induced a significant rise in leaf sap concentrations of Ca^{2+} and Mg^{2+} relative to the experimental control in plants from all pretreatments. Pretreatment did not significantly affect the extent of rise, and relative increases ranged from 59 to 77% for concentrations of Ca^{2+}, and 85 to 103% for concentrations of Mg^{2+} in expressed leaf sap.

To eliminate effects of passive dehydration or dilution due to changes in leaf water content, cation concentrations determined in expressed leaf sap were converted to a dry weight basis (Table 5.8b). This diminished the differences between differently pretreated plants in response to treatment at 300 mol m^{-3} NaCl. Plants from all pretreatments exhibited a significant rise in Na^+ concentration, and values were increased 84-, 89-, and 68-fold in plants pretreated at control conditions, 80, and 120 mol m^{-3} NaCl, respectively, but differences between differently pretreated plants were not statistically significant ($P < 0.05$). When expressed on a dry weight basis, salt-pretreated plants showed K^+ concentrations comparable to those of the experimental control, whereas values of control-pretreated plants were significantly increased by 117% relative to the experimental control level, and were also significantly higher compared to values of salt-pretreated plants. Expression on a dry weight basis did not greatly modify

relative treatment differences with respect to leaf concentrations of Ca^{2+} and Mg^{2+}, but enhanced the extent of rise in concentrations of these cations in plants from all pretreatments, with relative increases ranging from 109 to 117% for Ca^{2+} and 146 to 169% for Mg^{2+}.

Concentrations on a dry weight basis were also expressed relative to the levels after pretreatment to assess changes in response to treatment at 300 mol m^{-3} NaCl independently from changes, which occurred during pretreatment (Table 5.8d). Experimental control plants showed a decrease in K^+ concentration relative to the level determined at the time of pretreatment, whereas K^+ concentrations of plants treated at 300 mol m^{-3} NaCl remained similar to the pretreatment level (salt-pretreated) or increased relative to the level after pretreatment (control-pretreated plants). Thus when K^+ concentrations were expressed relative to the pretreatment level, plants from all pretreatments exposed to 300 mol m^{-3} NaCl exhibited significantly higher values as compared to the experimental control, and values of control-pretreated plants were significantly higher than those of salt-pretreated plants. Concentrations of Ca^{2+} and Mg^{2+} exhibited only minor changes relative to the level at the time of pretreatment in experimental control plants, whereas concentrations of these cations tended to increase relative to the pretreatment level in response to treatment at 300 mol m^{-3} NaCl in plants from all pretreatments. When expressed relative to the pretreatment level, plants pretreated at control conditions and 120 mol m^{-3} NaCl exhibited significantly higher values with respect to Ca^{2+} and Mg^{2+} concentrations as compared to experimental control plants, and the extent of this increase relative to the experimental control level was greater in plants pretreated at control conditions as compared to 120 mol m^{-3} NaCl. In contrast, values of plants pretreated at 80 mol m^{-3} were only slightly, but not significantly higher than those of the experimental control. While leaf Na^+ concentration remained unaltered relative to the time of pretreatment in experimental control plants, it increased greatly in response to salinity treatment at 300 mol m^{-3} NaCl in plants from all pretreatments, and the extent of increase was significantly greater in control- as compared to salt-pretreated plants. The lower extent of relative increase in salt-pretreated plants was due to significant increases in Na^+ concentration in response to salinity pretreatment. However, in absolute terms the increase in Na^+ concentration in response to moderate salinity treatment was very small compared to the extent of increase in response to treatment at 300

mol m^{-3} NaCl. During plant exposure to NaCl-salinity, levels of Na^+ and Cl^- in leaves generally build up progressively with time due to the fact that plant water loss through transpiration exceeds water utilisation for cell expansion by a factor of 30 to 70 (Munns, 2002). To assess progressive accumulation of Na^+ in response to treatment at 300 mol m^{-3} NaCl independently from Na^+ accumulation during pretreatment, leaf Na^+ concentrations on a dry weight basis were also expressed as absolute increase in concentration since pretreatment (Table 5.8c). When expressed in this way, differently pretreated plants did not differ significantly with respect to the extent of increase in Na^+ concentration in response to treatment at 300 mol m^{-3} NaCl.

Table 5.8 Concentration of Na^+, K^+, Ca^{2+}, Mg^{2+}, and ratio of K^+ / Na^+ in leaves of *Gossypium hirsutum* after treatment at 300 mol m^{-3} NaCl for 30 days (NaCl 300). Pretreatments, experimental control treatment, and composition of salt and control solution as in table 5.6. **a)** Ion concentration measured in leaf sap. **b)** Ion concentration as in a converted to a dry weight basis via the measured leaf water content. **c)** Absolute difference between ion concentrations on a dry weight basis determined after treatment at 300 mol m^{-3} NaCl and the mean value after the respective pretreatment. **d)** Fold increase / decrease in concentration on a dry weight basis relative to the mean value after pretreatment. The measurement of response to pretreatment was carried out on a different set of plants (see Table 5.5), which had been exposed to the respective pretreatments for 24 days. Leaf water content (see Table 5.7) was measured 1 day after sampling for leaf sap ion concentrations using an adjacent leaf. Mean ± standard error for 5 plants. Statistical testing as in table 5.7.

	Ions in leaves				
	Na^+	K^+	K^+ / Na^+	Ca^{2+}	Mg^{2+}
	a) Concentration in leaf sap (mol m^{-3})				
Ct/Ct	5 ± 0.9 a	102 ± 7.7 b	21.5 ± 3.25 a	202 ± 24.2 a	34 ± 3.0 a
Ct/NaCl 300	348 ± 22.0 c	185 ± 3.4 c	0.5 ± 0.03 b	357 ± 10.6 b	69 ± 4.1 b
NaCl 80/NaCl 300	329 ± 31.6 bc	89 ± 9.0 ab	0.3 ± 0.01 b	322 ± 26.5 b	63 ± 5.0 b
NaCl 120/NaCl 300	262 ± 15.6 b	72 ± 6.8 a	0.3 ± 0.02 b	324 ± 10.8 b	69 ± 1.7 b
	b) Concentration as in a converted to dry weight basis (µmol g^{-1} DW)				
Ct/Ct	13 ± 1.7 a	264 ± 23.4 a		525 ± 71.9 a	87 ± 8.8 a
Ct/NaCl 300	1087 ± 100.5 b	574 ± 21.0 b		1106 ± 36.7 b	214 ± 11.0 b
NaCl 80/NaCl 300	1161 ± 113.3 b	314 ± 31.4 a		1137 ± 89.8 b	224 ± 16.7 b
NaCl 120/NaCl 300	885 ± 53.2 b	241 ± 19.6 a		1096 ± 47.0 b	234 ± 1.0 b
	c) Absolute increase in concentration (µmol g^{-1} DW as in b) compared to the mean value after pretreatment				
Ct/Ct	1 ± 1.7 a				
Ct/NaCl 300	1074 ± 100.5 b				
NaCl 80/NaCl 300	1131 ± 113.3 b				
NaCl 120/NaCl 300	856 ± 53.2 b				
	d) Fold increase / decrease in concentration (µmol g^{-1} DW as in b) relative to the mean value after pretreatment				
Ct/Ct	1 ± 0.1 a	0.6 ± 0.06 a		0.9 ± 0.12 a	0.8 ± 0.08 a
Ct/NaCl 300	87 ± 8.1 c	1.4 ± 0.05 c		1.9 ± 0.06 c	1.9 ± 0.10 c
NaCl 80/NaCl 300	40 ± 3.9 b	1.1 ± 0.11 b		1.2 ± 0.10 ab	1.1 ± 0.08 a
NaCl 120/NaCl 300	30 ± 1.8 b	0.9 ± 0.08 b		1.3 ± 0.06 b	1.4 ± 0.01 b

5.2 Experiment 2

5.2.1 Experimental protocol

Seeds of *Gossypium hirsutum* var. CIM 443 were sown as described in section 2.1 on Sep 29th 2000. Seedlings were transplanted to 1.6-dm^3 pots 37 days after sowing. Reserve plants from the same sowing date were transplanted to 1.6-dm^3 pots 44 days after sowing to replace some of the plants transplanted at the earlier date, which had been severely damaged by thrips. Plants were first treated with plant nutrient solution containing phostrogen at 2 g dm^{-3} and micronutrients as in half strength Hoagland's solution 16, 21, 33, and 37 days after sowing, from then on about every second day.

Table 5.9 Experimental design consisting of **(1)** 2 levels of pretreatment: treatment with NaCl solutions of a final concentration of 100 mol m^{-3} (NaCl 100) or with plant nutrients and water (Control) and **(2)** the following modes of subsequent treatment: **a)** no further treatment, but assessment of plant response to pretreatment; **b)** exposure to high stress levels of salinity; **c)** maintenance in control conditions throughout the experiment (applies only to control-pretreated plants, forming the experimental control). The number of replicates for each combination of pretreatment / subsequent treatment is shown.

(2) Subsequent treatment	**(1) Pretreatment**	
	Control	NaCl 100
***a)** Assessment of plant response to pretreatment*		
-	5	5
***b)** Subsequent treatment with severe stress*		
Salinity	5	5
***c)** Maintenance in control conditions*		
Control	4	

For assignment to control and stress treatments plants were arranged in a randomized block design stratified for height (to minimise the influence of plant size on stress responses) as follows. They were sorted according to height, divided into 4 strata of increasing height, and from each stratum plants were randomly assigned to all of the combinations of pretreatment / subsequent treatment which are outlined in Table 5.9.

When plants were 47 days old, they were subjected to pretreatment with NaCl solutions of a final concentration of 100 mol m^{-3} for 17 to 28 days (as specified below for different combinations of pretreatment / subsequent treatment) or alternatively with control solution for the same period of time. Final salinity levels were imposed gradually increasing stepwise by a maximum of 40 mol m^{-3} NaCl per day. Salt solutions were composed as described in section 2.1, control solution contained only plant nutrients in the same amount and composition as the salt solutions. Solutions were applied manually to the substrate surface, using a volume of about 300 cm^3 every day. Plant response to pretreatment was assessed by measurement of gas exchange parameters on days 18 and 21 and chlorophyll fluorescence parameters on day 20 of pretreatment. Leaves were sampled for measurement of water content, relative water content, and concentrations of cations 20 days after initiation of pretreatment. Shoot height and above ground dry matter production were measured 24 and 28 days after initiation of pretreatment, respectively.

Plants assigned to treatment with severe salinity stress were subjected to pretreatment with either NaCl solutions of a final concentration of 100 mol m^{-3} for 18 days or alternatively with control solution for the same period of time. Before start of subsequent treatment plants were transplanted to 5-dm^3 pots. Severe salinity treatment consisted of manual application of NaCl solutions of a final concentration of 300 mol m^{-3} to the plant substrate surface, using a volume of about 500 cm^3 once daily for 14 days. The final salinity level of 300 mol m^{-3} was imposed gradually increasing stepwise by 75 mol m^{-3} per day. During treatment plants were rotated in about daily intervals (to minimize effects of environmental heterogeneity). The time course of plant response to severe salinity treatment was monitored by measuring gas exchange parameters on days 7, 11, 13, and 14 of treatment; experimental control plants were only assessed for these parameters on day 11 of treatment. Leaf samples for measurement of water content, relative water content, as well as concentrations of cations were taken 13 days after initiation of treatment. Finally shoot height and above ground dry matter production were measured after 14 days of treatment.

5.2.2 Results

5.2.2.1 Effects of pretreatment at moderate salinity

47-day old plants of *Gossypium hirsutum* were exposed to treatment at moderate salinity of 100 mol m^{-3} NaCl for up to 28 days, and plant response to salinity was assessed with respect to a variety of physiological parameters.

Exposure to moderate salinity at 100 mol m^{-3} NaCl for 20 days caused a significant increase in F_v/F_m of chlorophyll fluorescence by 3% compared to the control. This was associated with a significant reduction in F_o by 13% relative to the control level, whereas values of F_m were not significantly affected (Table 5.10).

Table 5.10 F_v/F_m, F_o, and F_m of chlorophyll fluorescence in *Gossypium hirsutum* in response to treatment at 100 mol m^{-3} NaCl for 20 days (NaCl 100). Salt solutions contained added calcium (ratio 1:10 to Na) and plant nutrients. Control plants were treated with solution containing no added NaCl, but plant nutrients in the same amount and composition as the salt solutions. Mean ± standard error for 5 plants, the value for each plant being the average of measurements for 2 adjacent medium-aged leaves. For each variable treatments were compared by a t-test. Asterisks *, * *, * * * represent significance at P = 0.05, 0.01, and 0.001, respectively; ns = nonsignificant at P = 0.05.

	F_v/F_m	F_o	F_m
Control	0.798 ± 0.006	405 ± 11.9	2010 ± 39.2
NaCl 100	0.825 ± 0.003	353 ± 13.8	2018 ± 68.1
Significance of t-test between treatments			
	* *	*	ns

Treatment at 100 mol m^{-3} NaCl induced significant reductions in transpiration, stomatal conductance, net rate of CO_2 uptake, and intercellular CO_2 concentration, when determined after 18 and 21 days of treatment (Table 5.11). A similar extent of reductions was observed on both days of measurement, and relative reductions amounted to around 66, 80, 56, and 22% for transpiration, stomatal conductance, net rate of CO_2 uptake, and intercellular CO_2 concentration, respectively. The proportionally greater reductions in transpiration as compared to net rate of CO_2 uptake resulted in an increase in water use efficiency of photosynthesis by 19% relative to the control level on both days of measurement, and this increase was statistically significant on day 18 of treatment (Table 5.11).

Table 5.11 Variation in transpiration (E), stomatal conductance (g_s), net rate of CO_2 uptake (A), CO_2 mole fraction in the leaf intercellular air space (c_i), and instantaneous water use efficiency of net photosynthesis (A/E) in *Gossypium hirsutum* in response to treatment at 100 mol m^{-3} NaCl (NaCl 100) for 18 and 21 days. Control treatment and compositions of salt and control solutions as in table 5.10. Each value is the mean ± standard error for 5 plants; the value for each plant is the average of measurements for 2 adjacent medium-aged leaves. Statistical testing as in table 5.10.

Day [1]		**E** (mmol m^{-2} s^{-1})	**g_s** (mmol m^{-2} s^{-1})	**A** (μmol m^{-2} s^{-1})	**c_i** (μmol mol^{-1})	**A/E**
18	Control	7.5 ± 0.17	326 ± 13.0	15.6 ± 0.6	241 ± 2.2	2.1 ± 0.05
	NaCl 100	2.6 ± 0.18	71 ± 5.7	6.8 ± 0.7	188 ± 6.7	2.5 ± 0.12
	Significance of t-test between treatments					
		* * *	* * *	* * *	* * *	*
21	Control	8.3 ± 0.07	374 ± 6.5	17.2 ± 0.6	247 ± 2.0	2.1 ± 0.06
	NaCl 100	2.7 ± 0.45	72 ± 14.0	7.5 ± 1.6	191 ± 12.1	2.5 ± 0.23
	Significance of t-test between treatments					
		* * *	* * *	* * *	* *	ns

[1] day after initiation of treatment

Plants treated at 100 mol m^{-3} NaCl exhibited significant reductions in shoot height by 23% and shoot dry matter production by 22% relative to the control level, when determined after 24 and 28 days of treatment, respectively (Table 5.12). The salinity-induced decrease in shoot dry matter production was due to significant reductions in stem and leaf dry matter production by 26 and 25%, respectively, whereas dry matter production of flowers increased by 103% relative to the control, however, this increase was not statistically significant ($P < 0.05$).

Table 5.12 Variation in shoot height and above ground dry matter production (whole shoot and individual contributions of stem, leaves and flowers) in *Gossypium hirsutum* in response treatment at 100 mol m^{-3} NaCl (NaCl 100). Shoot height was measured after 24 days, above ground dry matter production after 28 days of salinity treatment. Control treatment and compositions of salt and control solutions as in table 5.10. Mean ± standard error for 5 plants. Statistical testing as in table 5.10.

	Shoot height (cm plant^{-1})	**Dry matter production (g plant^{-1})**			
		Stem	**Leaves**	**Flowers**	**Shoot**
Control	80.0 ± 2.8	11.3 ± 1.0	17.2 ± 1.6	0.70 ± 0.31	29.2 ± 2.5
NaCl 100	61.7 ± 2.4	8.4 ± 0.3	12.9 ± 0.5	1.42 ± 0.31	22.8 ± 0.8
Significance of t-test between treatments					
	* *	*	*	ns	*

Treatment at moderate salinity of 100 mol m^{-3} NaCl induced a significant drop in relative water content by 9% relative to the control level, whereas values of leaf water content increased by 11% compared to the control, however, this increase was not statistically significant ($P < 0.05$) (Table 5.13).

Table 5.13 Response of leaf water content (WC) and relative water content (RWC) in *Gossypium hirsutum* to treatment at 100 mol m^{-3} NaCl (NaCl 100) for 20 days. Control treatment and compositions of salt and control solutions as in table 5.10. Mean ± standard error for 5 plants. Statistical testing as in table 5.10.

Treatment	**WC (%)**	**RWC (%)**
Control	334 ± 17.3	87 ± 0.8
NaCl 100	371 ± 10.5	79 ± 0.6
Significance of t-test between treatments		
	ns	* * *

The effects of treatment at moderate salinity of 100 mol m^{-3} NaCl for 20 days on concentrations of cations in expressed leaf sap are presented in Table 5.14a. Salinity-treated plants exhibited a significant 8-fold increase in leaf sap Na^+ concentration, whereas leaf sap K^+ concentration was not significantly altered as compared to control plants, and this resulted in a significant reduction in K^+/Na^+ ratio by 87% relative to the control level. Treatment at 100 mol m^{-3} NaCl induced significant increases in leaf sap concentrations of Ca^{2+} and Mg^{2+}, in each case by 36% relative to the control level.

To eliminate effects of passive dehydration/dilution on leaf cation concentrations, concentrations determined in expressed leaf sap were converted to a dry weight basis (Table 5.14b). This enhanced the extent of increase in leaf concentrations of Na^+, Ca^{2+}, and Mg^{2+} in response to salinity. When expressed on a dry weight basis, plants treated at 100 mol m^{-3} NaCl exhibited a significant 9-fold increase in Na^+ concentration, and significant increases in concentrations of Ca^{2+} and Mg^{2+} amounted to 51% each relative to the control level. Expression on a dry weight basis did not affect relative treatment differences with respect to K^+ concentration, and values of salinity-treated plants did not differ significantly from those of the control.

Table 5.14 Variation in concentration of Na^+, K^+, Ca^{2+}, and Mg^{2+}, and ratio of K^+ / Na^+ in leaves of *Gossypium hirsutum* in response to treatment at 100 mol m^{-3} NaCl (NaCl 100) for 20 days. Control treatment and composition of salt and control solutions as in table 5.10. **a)** Ion concentration measured in leaf sap. **b)** Ion concentration as in a converted to a dry weight basis via the measured leaf water content. Leaf water content (see Table 5.13) was determined within 1 hour of sampling for leaf ion concentrations using an adjacent leaf. Statistical testing as in table 5.10.

	Ions in leaves				
	Na^+	**K^+**	**K^+ / Na^+**	**Ca^{2+}**	**Mg^{2+}**
	a) Concentration in leaf sap (mol m^{-3})				
Control	6 ± 0.0	143 ± 7.1	24.0 ± 1.2	143 ± 7.9	74 ± 4.0
NaCl 100	49 ± 9.1	136 ± 13.3	3.1 ± 0.4	194 ± 9.5	101 ± 3.5
Significance of t-test between treatments					
	* *	ns	* * *	* *	* *
	b) Concentration as in a converted to dry weight basis (µmol g^{-1}DW) via leaf water content				
Control	20 ± 1.0	475 ± 23.7		473 ± 10.2	246 ± 7.7
NaCl 100	181 ± 32.4	505 ± 49.6		716 ± 26.3	372 ± 7.0
Significance of t-test between treatments					
	* *	ns		* * *	* * *

5.2.2.2 Effects of pretreatment at moderate salinity on plant response to treatment at severe salinity

Plants previously exposed to pretreatment at 100 mol m^{-3} NaCl (salt-pretreated) and plants of the same age, which had been maintained in control conditions (control-pretreated), were exposed to treatment at 300 mol m^{-3} NaCl for up to 14 days.

Treatment at 300 mol m^{-3} NaCl induced significant changes in gas exchange parameters, when determined after 7 and 11 days of treatment (Table 5.15). On both days of measurement treatment at 300 mol m^{-3} NaCl significantly reduced transpiration, stomatal conductance, and net rate of CO_2 uptake in plants from both pretreatments. On day 7 differently pretreated plants differed significantly with respect to the extent of reductions in these parameters. Relative reductions in transpiration amounted to 73 and 81% in salt- and control-pretreated plants, respectively, and the respective values for stomatal conductance were 82 and 88%, and for net rate of CO_2 uptake 65 and 83%. On day 7 of treatment at 300 mol m^{-3} NaCl salt-pretreated plants exhibited a decrease in intercellular CO_2 concentration by 18%, and control-pretreated plants an increase by 18% relative to the experimental control level. Although the differences between differently pretreated plants and the experimental control were not statistically significant ($P < 0.05$), control- and salt-pretreated plants differed significantly from each other with respect to intercellular CO_2 concentration. When determined on day 11, plants from both pretreatments exhibited a significant decline in transpiration, stomatal conductance, and net rate of CO_2 uptake, but pretreatment did not significantly affect the extent of this decrease. Relative reductions in transpiration, stomatal conductance, and net rate of CO_2 uptake ranged from 76 to 82%, 86 to 90, and 74 to 83%, respectively. On day 11 values of intercellular CO_2 concentration were not significantly affected by treatment at 300 mol m^{-3} NaCl in plants from either pretreatment.

Table 5.15 Variation in transpiration (E), stomatal conductance (g_s), net rate of CO_2 uptake (A), and CO_2 mole fraction in the leaf intercellular air space (c_i) in *Gossypium hirsutum* after treatment at 300 mol m^{-3} NaCl for number of days as indicated (NaCl300). Plants had been pretreated at 100 mol m^{-3} NaCl for 18 days (NaCl100) or with control solution for the same period of time (Ct). Salt solutions contained added calcium (Ca / Na ratio 1:10) and plant nutrients, control solution only plant nutrients in the same amount and composition as the salt solutions. Experimental control plants were treated with water and plant nutrients throughout the experiment. Mean ± standard error for 5 (salt treatments) and 4 (experimental control) plants; the value for each plant is the average of measurements for 2 adjacent medium-aged leaves. For each day a one-way analysis of variance was performed between combined treatments. Means followed by different letters are significantly different ($P < 0.05$).

Day [1]	**Treatment**	**E** (mmol m^{-2} s^{-1})	**g_s** (mmol m^{-2} s^{-1})	**A** (µmol m^{-2} s^{-1})	**c_i** (µmol mol^{-1})
7	Ct/Ct	8.3 ± 0.09 a	326 ± 5.0 a	17.1 ± 0.89 a	239 ± 5.6 ab
	NaCl100/NaCl300	2.2 ± 0.16 b	58 ± 5.5 b	6.0 ± 0.66 b	197 ± 7.6 a
	Ct/NaCl300	1.6 ± 0.05 c	40 ± 2.3 c	2.9 ± 0.15 c	282 ± 16.2 b
11	Ct/Ct	8.3 ± 0.09 a	326 ± 5.0 a	17.1 ± 0.89 a	239 ± 5.6 a
	NaCl100/NaCl300	2.0 ± 0.14 b	47 ± 4.4 b	4.4 ± 0.51 b	203 ± 8.7 a
	Ct/NaCl300	1.5 ± 0.26 b	34 ± 6.8 b	2.9 ± 0.98 b	234 ± 26.2 a

[1] day after initiation of treatment

Plants from both pretreatments exhibited significant reductions in shoot height and dry matter production of leaves compared to the experimental control, when determined after 14 days of treatment at 300 mol m^{-3} NaCl (Table 5.16a). Control- and salt-pretreated plants did not differ significantly with respect to the extent of reduction in shoot height, with relative reductions ranging from 16 to 20%. However, salt-pretreated plants showed significantly greater reductions in leaf dry matter production as compared to control-pretreated plants, with reductions relative to the experimental control level amounting to 18 and 37% in control- and salt-pretreated plants, respectively. After 14 days of treatment at 300 mol m^{-3} NaCl control-pretreated plants exhibited values of dry matter production of stems and reproductive organs comparable to those of the experimental control (Table 5.16a). In contrast, salt-pretreated plants showed a significant reduction in stem dry matter production by 31%, and an increase in production of reproductive organs by 126% relative to the experimental control level, however, this increase was not statistically significant ($P < 0.05$). Control-pretreated plants did not differ significantly from the experimental control with respect to total shoot dry matter production, determined after 14 days of treatment at 300 mol m^{-3} NaCl. In contrast, salt-pretreated plants showed a significant reduction by 32% relative to the experimental control level, and values were also significantly different from those of control-pretreated plants.

To assess changes in shoot height and shoot dry matter production in response to treatment at 300 mol m^{-3} NaCl independently from changes which occurred during pretreatment, values observed in response to treatment at 300 mol m^{-3} NaCl were expressed as percentage of the values after pretreatment (Table 5.16b). This diminished relative treatment differences with respect to shoot growth parameters. Experimental control plants and control- and salt-pretreated plants exposed to 300 mol m^{-3} NaCl showed comparable values of dry matter production of stems and reproductive organs. However, plants from both pretreatments exposed to 300 mol m^{-3} NaCl exhibited significantly lower values of leaf dry matter production relative to the pretreatment level as compared to experimental control plants. While leaf dry matter increased by 19% relative to the pretreatment level in experimental control plants, it remained at the level observed after pretreatment in plants treated at 300 mol m^{-3} NaCl. Total shoot dry matter production increased by 15% in experimental control plants, but remained

around the level after pretreatment in plants treated at 300 mol m^{-3} NaCl, however, differences between treatments were not statistically significant with respect to this parameter ($P < 0.05$). Shoot height exhibited a slight increase relative to the pretreatment level in experimental control plants and salt-pretreated plants exposed to 300 mol m^{-3} NaCl, but an apparent decrease in control-pretreated plants on exposure to 300 mol m^{-3} NaCl. Thus when expressed relative to the level after pretreatment, control-pretreated showed significantly lower values as compared to experimental control- and salt-pretreated plants. However, plant response to pretreatment was assessed in a different set of plants, which had been exposed to pretreatment for 24 days, whereas plants assessed for their response to treatment at 300 mol m^{-3} NaCl had been pretreated for only 18 days. The relatively large difference of 6 days is likely to have caused the apparent decrease in shoot height, when responses to treatment at 300 mol m^{-3} NaCl were expressed as percentage of those to pretreatment.

Table 5.16 Variation in shoot height and above ground dry matter production (whole shoot and individual contributions of stem, leaves and reproductive organs) in *Gossypium hirsutum* after treatment at 300 mol m^{-3} NaCl for 14 days (NaCl 300). Pretreatments, experimental control treatment, and composition of salt and control solutions as in table 5.15. **a)** Absolute values of shoot height and above ground dry matter production; **b)** Values as in a as % of the mean value in response to only pretreatment. Mean ± standard error for 5 (salt treatments) and 4 (experimental control) plants. Statistical testing as in table 5.15.

	Shoot height (cm plant^{-1})	**Dry matter production (g plant^{-1})**			
		Stem	**Leaves**	**Flowers / fruits**	**Shoot**
	a) as absolute values found after salt treatment				
Ct / Ct	85.1 ± 2.4 a	12.7 ± 0.8 a	20.5 ± 1.1 a	0.58 ± 0.23 a	33.8 ± 2.1 a
Ct / NaCl 300	71.6 ± 1.4 b	12.1 ± 0.5 a	16.8 ± 0.8 b	0.59 ± 0.18 a	29.5 ± 1.5 a
NaCl 100 / NaCl 300	68.2 ± 2.4 b	8.8 ± 0.6 b	12.9 ± 0.3 c	1.31 ± 0.24 a	23.0 ± 1.0 b
	b) as % of the mean value after the respective pretreatment [1]				
Ct / Ct	106 ± 3.0 a	112 ± 7.0 a	119 ± 6.5 a	82 ± 32.4 a	115 ± 7.0 a
Ct / NaCl 300	90 ± 1.8 b	107 ± 4.8 a	98 ± 4.8 b	84 ± 25.0 a	101 ± 5.0 a
NaCl 100 / NaCl 300	111 ± 3.9 a	105 ± 7.2 a	100 ± 2.4 b	92 ± 17.0 a	101 ± 4.2 a

[1] The assessment of response to pretreatment was carried out on a different set of plants (see Table 5.12) which had been treated at 100 mol m^{-3} NaCl or with control solution for 24 days (measurement of shoot height) and 28 days (measurement of above ground dry matter production).

Treatment at 300 mol m^{-3} NaCl for 13 days did not significantly affect leaf water content in either control- or salt-pretreated plants relative to the experimental control level (Table 5.17a). In contrast, plants from both pretreatments showed a significant decline in relative water content, when determined after 13 days of exposure to 300 mol m^{-3} NaCl, as compared to the experimental control (Table 5.17a). Pretreatment did not significantly affect the extent of this decrease, with relative reductions ranging from 5 to 7%.

To separate changes in leaf water relation parameters in response to treatment at 300 mol m^{-3} NaCl from changes, which occurred during pretreatment, responses of water content and relative water content to exposure to 300 mol m^{-3} NaCl were also expressed as percentage of the values after pretreatment (Table 5.17b). Experimental control plants and salt-pretreated plants exposed to 300 mol m^{-3} NaCl showed a slight increase in relative water content compared to the level after pretreatment, whereas values of control-pretreated plants treated at 300 mol m^{-3} NaCl remained unaltered relative to the pretreatment level. Thus when expressed relative to the level after pretreatment, control-pretreated plants exposed to 300 mol m^{-3} NaCl exhibited significantly lower values as compared to experimental control plants as well as salt-pretreated plants subsequently treated at 300 mol m^{-3} NaCl. Expression relative to the level after pretreatment did not greatly modify relative treatment differences with respect to water content.

Table 5.17 Leaf water content (WC) and relative water content (RWC) in *Gossypium hirsutum* after treatment at 300 mol m^{-3} NaCl for 13 days (NaCl 300). Pretreatments, experimental control treatment, and composition of salt and control solutions as in table 5.15. **a)** Absolute values measured after salt treatment; **b)** Values as in a as % of the mean value in response to the respective pretreatment. Mean ± standard error for 5 (salt treatments) and 4 (experimental control) plants. Statistical testing as in table 5.15.

Treatment	**WC (%)**	**RWC (%)**
***a)** as absolute values found after salt treatment*		
Ct / Ct	286 ± 12.4 a	91 ± 0.7 a
Ct / NaCl 300	309 ± 8.8 a	86 ± 1.5 b
NaCl 100 / NaCl 300	324 ± 9.3 a	85 ± 1.3 b
***b)** as % of the mean value after pretreatment* [1]		
Ct / Ct	86 ± 3.7 a	105 ± 0.8 a
Ct / NaCl 300	93 ± 2.6 a	99 ± 1.7 b
NaCl 100 / NaCl 300	87 ± 2.5 a	109 ± 1.6 a

[1] The assessment of response to pretreatment was carried out on a different set of plants (see Table 5.13) which had been treated with 100 mol m^{-3} NaCl or control solution for 20 days.

Determination of cation concentrations in expressed leaf sap after treatment at 300 mol m^{-3} NaCl for 13 days revealed significant differences in responses of control- and salt-pretreated plants (Table 5.18a). Treatment at 300 mol m^{-3} NaCl induced a 6- and 14-fold rise in leaf sap Na^+ concentration relative to the experimental control level in control- and salt-pretreated plants, respectively, however, this increase was statistically significant ($P < 0.05$) only for salt-pretreated plants. While salt-pretreated plants maintained values of leaf sap K^+ concentration comparable to those of the experimental control, control-pretreated plants exhibited a significant increase in K^+ concentration by 67% relative to the experimental control level, and values were also significantly higher than those of salt-pretreated plants. Plants from both pretreatments exhibited significant reductions in K^+/Na^+ ratio relative to the experimental control level, but pretreatment significantly affected the extent of these reductions. The lower extent of Na^+ accumulation in control- as compared to salt-pretreated plants associated with an increase in K^+ concentration only in control-pretreated plants resulted in a greater reduction in K^+/Na^+ ratio in salt- as compared to

control-pretreated plants. Reductions relative to the experimental control level amounted to 70 and 92% in control- and salt-pretreated plants, respectively. Treatment at 300 mol m^{-3} NaCl induced increases in leaf sap Ca^{2+} concentration by 53 and 125% relative to the experimental control level in salt- and control-pretreated plants, respectively, however, only the increase in control-pretreated plants was statistically significant ($P < 0.05$). Both control- and salt-pretreated plants exhibited a significant rise in leaf sap Mg^{2+} concentration in response to treatment at 300 mol m^{-3} NaCl, but pretreatment significantly affected the extent of this rise, with relative increases amounting to 146 and 213% in salt- and control-pretreated plants, respectively.

To eliminate effects of passive dehydration/dilution on leaf cation concentrations, concentrations determined in expressed leaf sap were converted to a dry weight basis (Table 5.18b). When expressed on a dry weight basis, plants from both pretreatments exhibited a slightly greater extent of increase in Na^+ concentration, when determined after treatment at 300 mol m^{-3} NaCl. Control- and salt-pretreated plants showed 7- and 16-fold higher values as compared to the experimental control, but again only the increase in salt-pretreated plants was statistically significant ($P < 0.05$). Expression on a dry weight basis also enhanced relative treatment differences with respect to leaf Ca^{2+} and Mg^{2+} concentrations. Treatment at 300 mol m^{-3} NaCl caused a significant increase in concentrations of Ca^{2+} and Mg^{2+} relative to the experimental control level in plants from both pretreatments, but the extent of increase was significantly greater in control- as compared to salt-pretreated plants. Relative increases in Ca^{2+} concentration amounted to 71 and 141% in salt- and control-pretreated plants, respectively, and the respective values for Mg^{2+} concentration were 166 and 225%. Expression on a dry weight basis did not greatly modify relative treatment differences with respect to K^+ concentration.

Leaf cation concentrations on a dry weight basis were also expressed as percentage of the value after the respective pretreatment to assess changes in leaf cation concentrations in response to treatment at 300 mol m^{-3} NaCl independently from changes, which occurred during pretreatment (Table 5.18d). Experimental control and salt-pretreated plants exposed to 300 mol m^{-3} NaCl exhibited a slight increase in Ca^{2+} concentration by 14 and 28%, respectively, relative to the pretreatment level, whereas control-pretreated plants showed a large rise in Ca^{2+}

concentration by 173% relative to value after pretreatment on exposure to 300 mol m^{-3} NaCl. Thus when expressed relative to the pretreatment level, control-pretreated plants subsequently treated at 300 mol m^{-3} NaCl exhibited significantly higher values of Ca^{2+} concentration as compared to experimental control and salt-pretreated plants exposed to 300 mol m^{-3} NaCl, and values of the latter two did not differ significantly. Experimental control and salt-pretreated plants exposed to 300 mol m^{-3} NaCl showed a decrease in Mg^{2+} concentration relative to the pretreatment level, and the extent of decrease was significantly lower in salt-pretreated as compared to experimental control plants. In contrast, control-pretreated plants subsequently treated at 300 mol m^{-3} NaCl maintained values close to the level after pretreatment. Thus values of leaf Mg^{2+} concentration expressed relative to the pretreatment level were significantly greater in control-pretreated plants subsequently treated at 300 mol m^{-3} NaCl than in experimental control as well as salt-pretreated plants exposed to 300 mol m^{-3} NaCl. Plants from all experimental treatments exhibited a decrease in K^+ concentration relative to the pretreatment level, however, this decrease was of a significantly lesser extent in control-pretreated plants exposed to 300 mol m^{-3} NaCl as compared to experimental control and salt-pretreated plants exposed to 300 mol m^{-3} NaCl. Experimental control plants exhibited a decrease in Na^+ concentration by 28%, salt- and control-pretreated plants an increase by 25 and 388% relative to the pretreatment level, respectively, on exposure to 300 mol m^{-3} NaCl. Thus when expressed relative to the level after pretreatment, only control-pretreated plants showed a significant increase relative to the experimental control level, whereas values of salt-pretreated plants did not differ significantly from those of the experimental control. The leaf K^+/Na^+ ratio decreased relative to the pretreatment level in plants from all treatments, but the extent of decrease was greater in plants exposed to 300 mol m^{-3} NaCl as compared to experimental control plants, and again greater in control-pretreated as compared to salt-pretreated plants. Only values of control-pretreated plants differed significantly from those of the experimental control, whereas the intermediate values of salt-pretreated plants were not significantly different from either experimental control or control-pretreated plants.

During plant exposure to NaCl-salinity, levels of Na^+ and Cl^- in leaves generally build up progressively with time due to the fact that plant water loss

through transpiration exceeds water utilisation for cell expansion by a factor of 30 to 70 (Munns, 2002). To assess progressive accumulation of Na^+ in response to treatment at 300 mol m^{-3} NaCl independently from Na^+ accumulation during pretreatment, leaf Na^+ concentrations on a dry weight basis were also expressed as absolute increase in concentration since pretreatment (Table 5.18c). When expressed in this way, differences between experimental treatments were not statistically significant ($P < 0.05$).

Table 5.18 Concentration of Na^+, K^+, Ca^{2+}, Mg^{2+}, and ratio of K^+ / Na^+ in leaves of *Gossypium hirsutum* after treatment at 300 mol m^{-3} NaCl for 13 days (NaCl 300). Pretreatments, experimental control treatment, and composition of salt and control solutions as in table 5.15. **a)** Ion concentration measured in leaf sap. **b)** Ion concentration as in a converted to a dry weight basis via the measured leaf water content. **c)** Absolute difference between ion concentration on dry weight basis in response to treatment at 300 mol m^{-3} NaCl and mean concentration determined directly after the respective pretreatment. **d)** Ion concentration on dry weight basis as % of the mean value after pretreatment. Leaf water content (see table 5.17) was measured within 2 hours of sampling for leaf sap ion concentration using an adjacent leaf. The assessment of response to pretreatment was carried out on a different set of plants (see table 5.14) which had been treated with 100 mol m^{-3} NaCl or control solution for 20 days. Mean ± standard error for 5 (salt treatments) and 4 (experimental control) plants. Statistical testing as in table 5.15.

	Ions in leaves				
	Na^+	K^+	K^+ / Na^+	Ca^{2+}	Mg^{2+}
	a) Concentration in leaf sap (mol m^{-3})				
Ct / Ct	5 ± 0.0 a	76 ± 6.7 a	15.3 ± 1.35 a	186 ± 15.7 a	24 ± 2.0 a
Ct / NaCl 300	31 ± 8.9 a	119 ± 12.1 b	4.6 ± 0.69 b	419 ± 31.4 b	75 ± 3.2 c
NaCl 100 / NaCl 300	69 ± 10.3 b	68 ± 6.9 a	1.2 ± 0.32 c	285 ± 27.5 a	59 ± 3.1 b
	b) Concentration as in a converted to dry weight basis (µmol g^{-1}DW) via leaf water content				
Ct / Ct	14± 0.6 a	221 ± 26.3 a		537 ± 63.2 a	71 ± 8.3 a
Ct / NaCl 300	97 ± 29.2 a	369 ± 40.8 b		1292 ± 92.3 c	231 ± 9.8 c
NaCl 100 / NaCl 300	226 ± 36.7 b	218 ± 18.4 a		918 ± 80.2 b	189 ± 8.3 b
	c) Absolute increase in concentration (µmol g^{-1} DW as in b) compared to the mean value after pre-treatment				
Ct / Ct	-6 ± 0.6 a				
Ct / NaCl 300	77 ± 29.2 a				
NaCl 100 / NaCl 300	46 ± 36.7 a				
	d) Values as in b as % of the mean value after the respective pretreatment				
Ct / Ct	72 ± 3.1 a	46 ± 5.5 a	64 ± 5.6 a	114 ± 13.4 a	29 ± 3.4 a
Ct / NaCl 300	488 ± 146.8 b	78 ± 8.6 b	19 ± 2.9 b	273 ± 19.5 b	94 ± 4.0 c
NaCl 100 / NaCl 300	125 ± 20.3 a	43 ± 3.6 a	38 ± 10.4 ab	128 ± 11.2 a	51 ± 2.2 b

5.3 Discussion

5.3.1 Imposition of salinity

Salinity may affect plant growth and physiology via different mechanisms, which may be roughly grouped into osmotic effects due to a salinity-induced reduction in soil osmotic potential, salt-specific effects due to accumulation of salt in the plant, and plant nutritional imbalance in the form of Ca^{2+} and K^+ deficiency (Glenn *et al.*, 1997). In the present study two types of salinity-induced damage related to osmotic and nutritional effects, which are unlikely to occur in natural environments, and the underlying mechanisms of which have been characterised in detail, were prevented as follows.

(1) Osmotic shock was prevented by gradual imposition of NaCl-salinity in increments of 40 mol m^{-3} NaCl per day during pretreatment at moderate salinity and 75 mol m^{-3} NaCl per day during subsequent treatment at severe salinity. Early studies established the crucial effect of the rate of stress imposition on plant response to salinity. Storey & Wyn Jones (1978) exposed 3-week old barley plants growing in nutrient solution to salinity of (a) 200 mol m^{-3} NaCl, imposed gradually in increments of 25 mol m^{-3} NaCl every third day over 24 days, (b) 50, 100, 150, 200, and 250 mol m^{-3} NaCl, imposed gradually in 2 increments of 25 mol m^{-3} NaCl every second day followed by daily increments of 50 mol m^{-3} NaCl, and maintenance of final salinity levels for 12 days, and (c) 250 mol m^{-3} NaCl imposed in one step for a period of 4 days. Treatment (a) resulted in progressive increases in concentrations of Na^+ and Cl^- associated with a progressive decrease in K^+ concentration in shoots and roots. In shoots the increase in Na^+ concentration was equivalent to, in roots it exceeded the decrease in K^+ concentration. Treatment (b) resulted in a progressive increase in Na^+ concentration in shoots which compensated for reductions in K^+ concentration at salinity levels of 50 to 150 mol m^{-3} NaCl and exceeded these reductions at levels of 200 to 250 mol m^{-3} NaCl. In roots the increase in Na^+ concentration exceeded the reduction in K^+ concentration at all salinity levels. Treatment (c) induced rapid efflux of K^+ from roots, rapid increases in shoot Na^+ concentration to values exceeding those in response to treatments (a) and (b), and a much greater extent of increase in Na^+

concentration in shoots as compared to roots. Sudden imposition of NaCl solutions of an osmotic potential exceeding plant turgor potential can induce plasmolysis in root cells, which are in direct contact with the NaCl solution, and cytorrhesis in leaf cells surrounded by air. Plasmolysis in turn may allow fluxes of abnormal amounts of salt through plant roots (Munns, 2002). The increase in NaCl concentration in daily increments of 40 to 75 mol m^{-3} NaCl in the present study resembles the salinity regime in treatment (b) in the study by Storey & Wyn Jones (1978). It results in a gradual imposition of the osmotic component of salinity stress, and is thus assumed to allow plant osmotic adjustment, preventing osmotic shock.

(2) In the present study NaCl-salinity was imposed concomitantly with supplemental Ca^{2+} in a ratio of 1:10 to Na^+. Marschner (1995) described the interference of high concentrations of NaCl with the function of Ca^{2+} in plant cells as follows. Ca^{2+} is involved in stabilisation of membranes and maintenance of a high electropotential across the membrane by reacting with negatively charged groups on the membrane surface. NaCl can induce Ca^{2+} deficiency by inhibiting uptake and transport of Ca^{2+} and in addition may remove Ca^{2+} from its binding sites at the external surface of the plasma membrane. Supplemental Ca^{2+} has been shown to diminish NaCl salinity-induced reductions in root growth (Munns, 2002) and to enhance K^+/Na^+ selectivity of roots (Marschner, 1995). This ameliorative effect of supplemental Ca^{2+} on root K^+/Na^+ selectivity has also been demonstrated in cotton (Zhong & Läuchli, 1994). These authors subjected 2-day old seedlings of *Gossypium hirsutum* to 150 mol m^{-3} NaCl, imposed in increments of 50 mol m^{-3} NaCl within 24 hours at two levels of Ca^{2+}, supplied in a ratio of 1:15 or 1:150 to Na^+. They found that supplemental Ca^{2+} in a ratio of 1:15 to Na^+ prevented the reduction in K^+ deposition rates, reduced the extent of increase in Na^+ deposition rates, and increased the ratio of K^+ to Na^+ deposition rate in the apical region of the root, all of which occurred in plants exposed to 150 mol m^{-3} NaCl with supplemental Ca^{2+} in a ratio of only 1:150 to Na^+. It was suggested that the protective effect of Ca^{2+} specifically in the meristematic cells of low degree of vacuolation in the root apical region indicated a role of Ca^{2+} in increasing K^+/Na^+ selectivity of the plasma membrane.

5.3.2 Plant responses to treatment at moderate salinity

Plants of *Gossypium hirsutum* were exposed to three pretreatment regimes at moderate salinity, which differed with respect to the following factors. (a) Plant age at the initiation of salinity exposure. Plants were subjected to salinity treatment, when they were 23 (experiment 1) and 47 (experiment 2) days old. (b) Level of moderate salinity. NaCl concentrations applied were 80 or 120 mol m^{-3} NaCl (experiment 1) and 120 mol m^{-3} NaCl (experiment 2).

5.3.2.1 Leaf succulence

Exposure of 23-day old plants to 80 or 120 mol m^{-3} NaCl for 24 days (experiment 1) and of 47-day old plants to 100 mol m^{-3} NaCl for 20 days (experiment 2) induced an increase in leaf water content relative to the control level. However, this increase was statistically significant only in plants first subjected to moderate salinity, when 23 days old (experiment 1). In experiment 1 the two levels of moderate salinity tested, 80 and 120 mol m^{-3} NaCl, did not differ significantly with respect to the extent of increase in leaf water content they induced. Relative increases ranged from 24 to 31% in plants first subjected to moderate salinity, when 23 days old (experiment 1), whereas it amounted to only 11% in plants, which were 47 days old, when moderate salinity treatment was initiated (experiment 2).

Brugnoli & Björkman (1992) reported a similar increase in leaf water content, expressed as percentage of fresh weight, by 8% relative to the control, for *Gossypium hirsutum* plants germinated and grown in 26% seawater, which contained Na^+ at a concentration of 131 mol m^{-3}. An increase in water content of leaf mesophyll cells, associated with an increase in vacuolar and cell size, has been observed in a variety of halophytic and glycophytic species in response to salinity, and this phenomenon is termed leaf succulence (Marschner, 1995, Glenn *et al.*, 1997). The higher degree of salinity-induced succulence in plants subjected to moderate salinity, when 23 (experiment 1) as compared to 47 (experiment 2) days old, is probably due to the higher capacity of expanding as compared to mature tissues to increase cell and vacuolar size.

5.3.2.2 Leaf ion relations

In experiment 1 the two levels of moderate salinity tested (80 and 120 mol m^{-3} NaCl) did not differ significantly in their effect on leaf concentrations of Na^+,

K^+, and Ca^{2+}. Comparison of responses of leaf cation relations to moderate salinity in experiment 1 and 2 revealed a profound effect of plant age at initiation of moderate salinity treatment. Exposure of 23-day old plants to 80 or 120 mol m^{-3} NaCl for 24 days (experiment 1) and exposure of 47-day old plants to 100 mol m^{-3} NaCl for 20 days (experiment 2) induced significant increases in Na^+ concentration. However, in experiment 2 increases relative to the control level amounted to 717% in extracted leaf sap and 805% on a dry weight basis, whereas the respective increases were only 81 to 91% and 134% in experiment 1.

The accumulation of Na^+ in leaves observed on exposure of 47-day old plants to 100 mol m^{-3} NaCl (experiment 2) reflects the progressive build-up of Na^+ ions in leaves, which generally occurs on plant exposure to NaCl-salinity due to the fact that water loss through transpiration exceeds water utilisation in cell expansion (Munns, 2002). The observed significant decrease in relative water content (this parameter was only determined in experiment 2), associated with a slight (although not statistically significant) increase in leaf water content, indicates osmotic adjustment, probably as a result of accumulation of Na^+ and Cl^- in leaves. Osmotic adjustment in response to soil drought may induce an absolute increase in turgor pressure on re-hydration (Thomas, 1997), and thus it may have increased the turgid weight, *i.e.* the weight at full leaf hydration, and thereby decreased the relative water content in the present study.

Plants subjected to moderate salinity, when 23 days old (experiment 1), exhibited a much lower degree of increase in leaf Na^+ concentrations on a dry weight basis as compared to plants exposed to salinity, when aged 47 days (experiment 2), although treatment duration in experiment 1 exceeded that in experiment 2 by 4 days. The difference in degree of Na^+ accumulation observed in response to moderate salinity between experiment 1 and 2 may be explained by differences in plant developmental stage and the predominant developmental stage of leaves at initiation of salinity treatment. The majority of leaves were still expanding in 23-day old plants, but were mature and fully-expanded in 47-day old plants. The increase in vacuole volume during expansion growth allows continued uptake and dilution of salts, thus expanding tissues tend to have relatively low cellular salt concentrations (Munns, 1993).

In both, experiment 1 and 2, Na^+ concentrations in expressed leaf sap rose significantly relative to the control, but remained far below Na^+ concentrations in

the external salt solutions applied to the plant substrate. This stands in marked contrast to findings by Brugnoli & Björkman (1992), who germinated and grew *Gossypium hirsutum* in 26% seawater containing Na^+ at a concentration of 131 mol m^{-3} and observed an increase in Na^+ concentration in bulk leaf water, which was proportional to the Na^+ concentration in the substrate solution.

Moderate salinity treatment in experiment 1 induced significant reductions in K^+ concentration by 46 to 51% in expressed leaf sap, and 29 to 39% on a dry weight basis, whereas the pretreatment regime in experiment 2 did not significantly affect leaf K^+ concentration. In the study by Brugnoli & Björkman (1992) germination and growth of plants of *Gossypium hirsutum* in 26% seawater resulted in a reduction in K^+ concentration only, when expressed as concentration in bulk leaf water, but not on a dry weight basis. However, in spite of the reduction in leaf K^+ concentration in experiment 1, the greatly reduced extent of leaf Na^+ accumulation in experiment 1 as compared to experiment 2 resulted in higher K^+/Na^+ ratios. In experiment 1 and 2 K^+/Na^+ ratios were reduced to 26 to 32% and 13% of the control level, respectively.

The differences between the study by Brugnoli & Björkman (1992) and the present one with respect to changes in leaf Na^+ and K^+ concentrations in response to moderate salinity may be due to differences in developmental stage of plants at initiation of treatment and to differences in treatment duration. Brugnoli & Björkman (1992) investigated the effects of long-term growth in moderate salinity from the stage of germination onwards, whereas in the present study 23- and 47-day old plants were subjected to salinity over a period of only 3 to 4 weeks.

In experiments 1 and 2 moderate salinity induced significant increases in Ca^{2+} concentration on a dry weight basis of a similar extent (around 50%), indicating that plant shoots did not suffer from Ca^{2+} deficiency.

5.3.2.3 Photosynthesis

Net rate of CO_2 uptake, transpiration, stomatal conductance, and intercellular CO_2 concentration were determined on day 22 and 23 of exposure of 23-day old plants to 80 or 120 mol m^{-3} NaCl (experiment 1) and on day 18 and 21 of treatment of 47-day old plants at 100 mol m^{-3} NaCl (experiment 2). All three pretreatment regimes of moderate salinity caused reductions in these parameters

relative to the control level, which were statistically significant in most instances ($P < 0.05$). The two levels of salinity tested in experiment 1 (80 and 120 mol m^{-3} NaCl) did not differ greatly with respect to the extent of induced reductions in these parameters. Only, when determined on day 23, values of intercellular CO_2 concentration were significantly lower in plants treated at 120 as compared to 80 mol m^{-3} NaCl. Initiation of moderate salinity treatment at a plant age of 47 as compared to 23 days tended to enhance the extent of reductions in net rate of CO_2 uptake, transpiration, and stomatal conductance and diminish the decrease in intercellular CO_2 concentration. In all three pretreatment regimes proportionally greater reductions in transpiration as compared to net rate of CO_2 uptake caused an increase in water use efficiency of photosynthesis, and this was statistically significant ($P < 0.05$) on most days of measurement. Initiation of moderate salinity treatment at a plant age of 23 as compared to 47 days tended to enhance the extent of this increase.

The response pattern of gas exchange parameters to exposure to moderate salinity observed in the present study closely resembles that reported by Brugnoli & Björkman (1992). These authors found a decline in net rate of CO_2 uptake, transpiration, stomatal conductance, and intercellular CO_2 concentration relative to the control in plants of *Gossypium hirsutum* germinated and grown in 26% seawater. Reductions in stomatal conductance and transpiration were proportionally greater than those in net rate of CO_2 uptake, resulting in an increase in water use efficiency of photosynthesis. The authors also estimated the relative contribution of stomatal and non-stomatal limitations of photosynthesis by calculating net rates of CO_2 uptake at constant intercellular CO_2 concentrations and rates of energy flow through photosynthetic carbon reduction/oxygenation cycles. They demonstrated that plant exposure to 26% seawater resulted in inhibition of net rates of photosynthesis mainly due to stomatal limitations. In the present study all pre-treatment regimes induced proportionally greater reductions in stomatal conductance as compared to net rate of CO_2 uptake, and this resulted in a decrease in intercellular CO_2 concentration. In analogy to the study by Brugnoli & Björkman (1992) this may be interpreted as indication of an inhibition of net rate of CO_2 uptake via stomatal closure. The tendency of the pretreatment regime in experiment 2 to induce lower extents of reduction in intercellular CO_2 concentration as well as of increase in water use efficiency of photosynthesis as

compared to the pretreatment regimes in experiment 1 suggests a relatively greater contribution of non-stomatal limitations in experiment 2. The increase in water use efficiency of photosynthesis observed in response to all pretreatment regimes at moderate salinity in the present study may be of adaptive value. Brugnoli & Björkman (1992) interpreted the increase in this parameter in plants of *Gossypium hirsutum* germinated and grown in 26% seawater as a mechanism to reduce the amount of salt uptake per amount of carbon assimilated.

Pretreatment of 23-day old plants at 80 or 120 mol m^{-3} NaCl for 22 days (experiment 1) and 47-day old plants at 100 mol m^{-3} NaCl for 20 days (experiment 2) induced a significant increase in F_v/F_m. In experiment 1 the level of moderate salinity (80 or 120 mol m^{-3} NaCl) did not significantly affect the extent of this increase. Relative increases amounted to 2% and 3% in experiment 1 and 2, respectively. In all three pretreatment regimes this was associated with a decrease in F_o relative to the control level, which amounted to around 10% at both levels of moderate salinity tested in experiment 1 and to 13% in experiment 2, but was statistically significant ($P < 0.05$) only in experiment 2. F_m was not significantly affected by moderate salinity in either pretreatment regime.

The increase in F_v/F_m observed in the present study in response to all pretreatment regimes at moderate salinity stands in marked contrast to results obtained by Brugnoli & Björkman (1992). These authors found no effect of germination and growth of *Gossypium hirsutum* in 26% seawater on F_v/F_m, photon yield of oxygen evolution at rate-limiting photon flux densities, or non-photochemical quenching. However, determination of the light response of the epoxidation state of xanthophyll cycle pigments revealed that at photon flux densities higher than 1200 μmol m^{-2} s^{-1} (which exceeded the growth photon flux density) salinity-treated plants exhibited a higher extent of conversion of violaxanthin to zeaxanthin as compared to the control. The authors concluded that at the growth photon flux density photoinhibition was prevented not by thermal energy dissipation, but by alternative electron sinks, possibly including the water-water cycle or ATP consumption in salt ion transport processes.

It is well known that reduction of the capacity of CO_2 assimilation as electron sink due to limitation of CO_2 supply (as a result of stomatal closure) may cause an imbalance between absorption and utilisation of excitation energy and thus lead to photoinhibition (Huner *et al.*, 1998). Although treatment at moderate

salinity induced an inhibition of net rate of CO_2 uptake by stomatal closure in the present study as well as in the study by Brugnoli & Björkman (1992), photoinhibition, which is generally diagnosed from a decrease in dark-adapted F_v/F_m (Osmond, 1994), did not occur in either case. Instead a significant increase in dark-adapted F_v/F_m was observed in response to all three pretreatment regimes at moderate salinity in the present study. This was associated with a decrease in F_o, which was particularly pronounced in experiment 2. In the context of photoinhibition a decrease in dark-adapted F_o indicates photo-protective processes of thermal dissipation (Osmond, 1994). Photoinhibitory quenching of chlorophyll fluorescence of a photo-protective nature causes quenching within the light-harvesting antenna of PSII (Maxwell & Johnson, 2000). It is thus possible that the decrease in F_o in response to moderate salinity observed in the present study indicates the activation of such photo-protective processes involving energy dissipation as heat in the light-harvesting antenna of PSII, although it was not associated with a decrease in F_m and F_v/F_m. The fact that the pretreatment regime in experiment 2 induced a higher extent of reduction in F_o and also resulted in a higher extent of reduction in net rate of CO_2 uptake, thus posing a greater potential for photoinhibition, as compared to the pretreatment regimes in experiment 1 lends further support to this idea.

5.3.2.4 Shoot growth

Exposure of 23-day old plants to 80 or 120 mol m^{-3} NaCl for 25 days (experiment 1) and exposure of 47-day old plants to 100 mol m^{-3} NaCl for 24 to 28 days (experiment 2) caused significant reductions in shoot height and shoot dry matter production. In both experiments the decrease in shoot dry matter production was due to significant reductions in stem and leaf dry matter production. Initiation of moderate salinity treatment at a plant age of 23 days as compared to 47 days tended to enhance the extent of reductions in leaf and total shoot dry matter production. In experiment 1, in which two levels of moderate salinity (80 and 120 mol m^{-3} NaCl) were tested for their effect on growth parameters, the extent of reduction in shoot height and shoot dry matter production increased significantly with increasing salinity. This was associated with a significant effect of the level of moderate salinity on leaf, but not stem dry matter production.

The reduction in shoot growth in response to moderate salinity observed in the present study is in general agreement with results obtained by Brugnoli & Björkman (1992). These authors found that germination and growth of *Gossypium hirsutum* in 26% seawater containing 131 mol m^{-3} Na^+ resulted in a reduction of plant relative growth rate to 54% of the control level. This was due to reductions in leaf area development and to a lesser extent to reductions in net assimilation rate. Reductions in shoot growth in the initial phase of salinity exposure are generally attributed to reductions in soil osmotic potential, and are thought to be induced by hormonal signals from the root, while visible leaf injury and leaf death occurring at later stages are signs of salt toxicity (Munns, 2002).

In the present study the two levels of moderate salinity tested in experiment 1 (80 and 120 mol m^{-3} NaCl) did not differ significantly with respect to their effect on gas exchange, dark-adapted chlorophyll fluorescence, leaf water relation or leaf cation relation parameters (except regarding leaf concentrations of Mg^{2+}). However, plants treated at 80 and 120 mol m^{-3} NaCl exhibited significant differences in shoot height and total shoot and leaf dry matter production. The specific sensitivity of responses of shoot growth to the small difference in the two NaCl concentrations applied is in agreement with the notion that salinity-induced reductions in growth generally precede reductions in photosynthesis (Munns, 1993).

5.3.3 Plant responses to treatment at high salinity

5.3.3.1 Non-acclimated plants

Experiment 1 and 2 both involved plant exposure to a high salinity level of 300 mol m^{-3} NaCl, but they differed with respect to three factors. (a) Experiment 1 and 2 assessed plant responses over a different time-scale of exposure to salinity. In experiment 1 effects of long-term growth over a period of 30 to 31 days on plant growth, leaf water-, and cation relations were investigated. In contrast, experiment 2 focused on more short-term effects on gas exchange parameters as well as growth and leaf water- and cation relations over a period of only 14 days. (b) The two experiments differed with respect to plant age at the initiation of high salinity treatment, being 49 days in experiment 1 and 65 days in experiment 2. (c) In experiment 1 high salinity was imposed by means of a flood bench system, in experiment 2 by manual application of salt solutions to the substrate.

Exposure of 49-day old plants to 300 mol m^{-3} NaCl for 31 days (experiment 1) resulted in large reductions in shoot height and shoot dry matter production by 35% and 72%, respectively. The latter was associated with reductions in dry matter production of stem, leaf, and reproductive organs by 70, 78, and 58%, respectively. Leaf water content increased significantly by 20% relative to the control level, when determined after 31 days of treatment. Na^+ concentrations in expressed leaf sap increased significantly by 70-fold (to 348 mol m^{-3}) in expressed leaf sap and by 84-fold on a dry weight basis in response to treatment at 300 mol m^{-3} NaCl for 30 days. K^+ concentrations increased significantly by 81% and 117% in expressed leaf sap and on a dry weight basis, respectively. Leaf K^+/Na^+ ratio was reduced to 2.3% of the experimental control level.

The results of experiment 1 in the present study closely resemble those obtained by Brugnoli & Björkman (1992) who investigated effects of germination and long-term growth of *Gossypium hirsutum* in 55% seawater, which contained 277 mol m^{-3} Na^+. Plant relative growth rate decreased by 83% relative to the control level. This was due to reductions in leaf area development by 50% and net assimilation rate by up to 66%. Salinity treatment induced an increase in leaf water content by 12% and a 26-fold increase in Na^+ concentration in bulk leaf water (to 362 mol m^{-3}), which corresponded to a 51-fold increase on a dry weight basis. Leaf K^+ concentration was reduced by 33%, when expressed as concentration in bulk leaf water, but increased by 24%, when expressed on a dry weight basis. Leaf K^+/Na^+ ratio declined to 2.6% of the control level.

In summary the present study and that by Brugnoli & Björkman (1992) show the following common response pattern of *Gossypium hirsutum* plants to long-term growth under NaCl-salinity at around 300 mol m^{-3}. (a) Salinity treatment induced large reductions in plant dry matter production. (b) Na^+ concentrations in the bulk leaf water increased greatly in response to salinity treatment and exceeded Na^+ concentrations in the external salt solution to some extent. (b) Increased leaf succulence, indicated by an increase in leaf water content, resulted in dilution of accumulated leaf Na^+ levels, as apparent from the greater extent of salinity-induced increase in leaf Na^+ concentration, when expressed on a dry weight as compared to a leaf bulk water basis. (c) Salinity treatment induced an increase in leaf K^+ concentration on a dry weight basis, thus

indicating selective accumulation of K^+. (d) The large extent of Na^+ accumulation in leaves resulted in a reduction in leaf K^+/Na^+ ratio to 2 to 3% of the control level.

Exposure of 65-day old plants to 300 mol m^{-3} NaCl for 14 days (experiment 2) induced significant reductions in shoot height by 16% and in leaf dry matter production by 18%. As dry matter production of stems and reproductive organs was not significantly altered relative to the experimental control level, total shoot dry matter production was reduced by only 13% relative to the experimental control level, and this was not statistically significant ($P < 0.05$). Determination of leaf water relation parameters after 13 days of treatment revealed a significant reduction in relative water content by 5% associated with no significant change in water content relative to the experimental control level. Leaf Na^+ concentrations were increased by 6- (to 31 mol m^{-3}) and 7-fold in expressed leaf sap and on a dry weight basis, respectively, when determined after 13 days of treatment. K^+ concentrations exhibited a significant increase by 57% relative to the experimental control level in expressed leaf sap, which corresponded to a 67% increase on a dry weight basis. Leaf K^+/Na^+ ratio was reduced to 30% of the experimental control level.

Responses of plants to treatment at 300 mol m^{-3} NaCl in experiment 2 thus showed marked differences from those observed in experiment 1 and in the study by Brugnoli & Björkman (1992). The salinity treatment in experiment 2 induced a much lower extent of decrease in shoot height and shoot dry matter production, did not result in increased leaf succulence, and induced a lower extent of increase in leaf Na^+ concentration and decrease in leaf K^+/Na^+ ratio. However, in agreement with the studies above, it induced a significant increase in K^+ concentration on a dry weight basis. The differential responses observed in experiment 2 and the above studies may be due to differences with respect to technique of salinity imposition and duration of salinity treatment. Brugnoli & Björkman (1992) imposed salinity by means of a hydroponic flowing nutrient culture system, which continually pumped seawater solutions through the rooting medium. In experiment 1 of the present study the substrate of soil-grown plants was flooded twice a day with salt solutions by means of a flood bench system. Salinity imposition in experiment 2 of the present study occurred via manual application of salt solutions at a volume of about 500 cm^3 to the plant substrate

surface once a day. Thus the greater volumes and higher frequency of application of salt solutions and the longer duration of salinity treatments used in the study by Brugnoli & Björkman (1992) and in experiment 1 as compared to experiment 2 of the present study would have enhanced the impact of salinity treatment.

In experiment 2 also responses of gas exchange parameters in response to short-term exposure to salinity were investigated. Transpiration, stomatal conductance, and net rate of CO_2 uptake were significantly reduced by 81, 88, and 83%, respectively, when determined after 7 days of treatment at 300 mol m^{-3} NaCl, and remained at these greatly reduced levels, when determined on day 11 of treatment. Values of intercellular CO_2 concentration tended to be slightly increased above those of the experimental control (by 18%), but this difference was not statistically significant ($P < 0.05$).

The only slightly higher extent of reductions in stomatal conductance as compared to net rate of CO_2 uptake and the apparent slight increase in intercellular CO_2 concentration indicate that non-stomatal limitations played a major role in the inhibition of net rate of CO_2 uptake observed in the present study. This is in general agreement with findings by Brugnoli & Björkman (1992) who reported reductions in stomatal conductance and net rate of CO_2 uptake in response to germination and growth of *Gossypium hirsutum* in 55% seawater (containing 277 mol m^{-3} Na^+) by 81 and 60%, respectively. Values of net rate of CO_2 uptake estimated for a constant intercellular CO_2 concentration were reduced by 42% relative to the control, thus implicating contribution of non-stomatal factors in the inhibition of net rates of CO_2 uptake at this salinity level.

5.3.3.2 Responses of salt-pretreated plants

In the present study three pretreatment regimes at moderate salinity were assessed for their potential to induce increased tolerance to treatment at high salinity of 300 mol m^{-3} NaCl in *Gossypium hirsutum*. Plants were exposed to pretreatment at 80 or 120 mol m^{-3} NaCl for 26 days, when they were 23 days old (experiment 1) or to 100 mol m^{-3} NaCl for 18 days, when they were 47 days old (experiment 2). Pretreatment salinity was generally imposed by daily increments of a maximum of 40 mol m^{-3} NaCl, high salinity in daily increments of 75 mol m^{-3} NaCl. In the following differential effects of the 3 pretreatment regimes on plant responses to subsequent treatment at 300 mol m^{-3} NaCl are discussed.

(1) Pretreatment at 120 mol m^{-3} NaCl for 26 days, initiated, when plants were 23 days old, influenced plant response to treatment at 300 mol m^{-3} NaCl for 31 days with respect to the following physiological parameters (experiment 1). (a) It slightly enhanced the increase in water content observed in response to exposure to 300 mol m^{-3} NaCl relative to the experimental control level. Relative increases in water content amounted to 31 and 20% in plants pretreated at 120 mol m^{-3} NaCl and control conditions, respectively, however, this difference between differently pretreated plants was not statistically significant ($P < 0.05$). Expression relative to values after pretreatment revealed that in salt-pretreated plants the relatively higher values of water content as compared to experimental control plants in response to treatment at 300 mol m^{-3} NaCl were mainly a consequence of an increase in this parameter during pretreatment and not of a net increase on exposure to 300 mol m^{-3} NaCl. (b) It significantly reduced the extent of increase in leaf sap Na^+ concentration in response to treatment at 300 mol m^{-3} NaCl. Plants pretreated at 120 mol m^{-3} NaCl and at control conditions exhibited a 52- and 70-fold increase, respectively, in leaf sap Na^+ concentration relative to the experimental control level. Leaf Na^+ concentrations on a dry weight basis were 68- and 84-fold higher compared to the experimental control level in plants pretreated at 120 mol m^{-3} NaCl and at control conditions, respectively, and the difference between plants from these 2 pretreatments was not statistically significant anymore ($P < 0.05$). (c) It prevented the increase in K^+ concentration, expressed on a dry weight basis, which occurred in control-pretreated plants on exposure to 300 mol m^{-3} NaCl. (d) It slightly reduced the extent of increase in stem dry matter production relative to the pretreatment level in comparison to control-pretreated plants, but this effect was not statistically significant.

Pretreatment at 120 mol m^{-3} NaCl thus appeared to influence the control of Na^+ concentration in leaves, as indicated by the reduced extent of increase in leaf sap Na^+ concentration on subsequent exposure to 300 mol m^{-3} NaCl as compared to plants pretreated at control conditions. Expression of Na^+ concentrations on a dry weight basis slightly diminished the difference between plants pretreated at 120 mol m^{-3} NaCl and control conditions,

indicating that it was due in part to the increase in leaf succulence, which occurred in plants pretreated at 120 mol m^{-3} NaCl during pretreatment. However, a large extent of the difference remained, thus suggesting that other mechanisms contributed to the observed reduction in leaf sap Na^+ accumulation in plants pretreated at 120 mol m^{-3} NaCl.

Amzallag *et al.* (1990) reported a similar effect of pretreatment at moderate salinity on accumulation of Na^+ in shoots of *Sorghum bicolor* in response to subsequent treatment at high salinity, using an experimental design, which closely resembled that of the present study with respect to levels as well as rates of salinity imposition. These authors assessed the effect of pretreatment of 8-day old plants at either 75 or 150 mol m^{-3} NaCl on plant response to subsequent treatment at 300 mol m^{-3} NaCl. Pretreatment was imposed by addition of appropriate amounts of NaCl to the culture (half-strength Hoagland) solution in daily increments of 25 mol m^{-3} NaCl. For subsequent treatment NaCl solution was increased in daily increments of 75 mol m^{-3} NaCl, and nutrient levels were adjusted to a full-strength Hoagland medium at the final salinity level. Supplemental Ca^{2+} was added in a ratio of 1:30 to Na^+. Salt-pretreatment, as compared to pretreatment at control conditions, was shown to reduce the extent of increase in concentrations of Na^+ and Cl^- in bulk shoot water on exposure to 300 mol m^{-3} NaCl. However, the results obtained by Amzallag *et al.* (1990) differ from those of the present study with respect to other physiological processes, which were protected on exposure to high salinity as a result of pre-exposure to moderate salinity. Amzallag *et al.* (1990) demonstrated that pretreatment of *Sorghum bicolor* at 75 or 150 mol m^{-3} NaCl for 20 days increased plant resistance to treatment at 300 mol m^{-3} NaCl with respect to shoot dry matter accumulation. Control-pretreated plants exhibited a permanent cessation of shoot growth on exposure to 300 mol m^{-3} NaCl. In contrast, plants pretreated at 75 or 150 mol m^{-3} NaCl showed an only transient inhibition for 3 days and 2 weeks, respectively, and then continued to grow at rates comparable to those of control plants. This stands in marked contrast to the present study, in which pretreatment of *Gossypium hirsutum* at 120 mol m^{-3} NaCl did not significantly modify shoot height increase and dry matter production in resonse to treatment at 300 mol m^{-3} NaCl. However, Umezawa *et al.* (2000) reported a phenomenon of acclimation to salinity in *Glycine max*

of similar characteristics to that observed in the present study. Pretreatment of 8-day old soil-grown plants at 34 mol m^{-3} NaCl for 23 days reduced the extent of increase in Na^+ concentration on a dry weight basis in response to subsequent treatment at 137 mol m^{-3} NaCl in roots and leaves by 27 and 71%, respectively. This salinity pretreatment had no effect on plant dry matter and leaf area production in response to subsequent treatment at 137 mol m^{-3} NaCl. However, these authors observed an increase in plant survival on exposure to 137 mol m^{-3} NaCl in plants pretreated at 34 mol m^{-3} NaCl, with plant survival rates being 60 and 93% for plants pretreated at control conditions and at 34 mol m^{-3} NaCl, respectively. In experiment 1 of the present study treatment at 300 mol m^{-3} NaCl was imposed over a period of 31 days. This resulted in severe reductions in shoot dry matter production, but did not cause plant death in either control- or salt-pretreated plants. Thus possible beneficial effects of pretreatment at 120 mol m^{-3} NaCl on plant survival on exposure to 300 mol m^{-3} NaCl may have remained undetected in experiment 1 of the present study due to insufficient length of the period of treatment at 300 mol m^{-3} NaCl.

(2) Pretreatment at 80 mol m^{-3} NaCl for 26 days, initiated, when plants were 23 days old, modified plant responses to treatment at 300 mol m^{-3} NaCl for 31 days as follows (experiment 1). (a) It significantly enhanced the extent of increase in water content relative to the experimental control level in response to treatment at 300 mol m^{-3} NaCl. Relative increases amounted to 36 and 20% in plants pretreated at 80 mol m^{-3} NaCl and at control-conditions, respectively. The increase in water content observed in plants previously treated at 80 mol m^{-3} NaCl was not due to a net increase on exposure to 300 mol m^{-3} NaCl, but mainly to the significant increase which occurred during pretreatment at 80 mol m^{-3} NaCl. (b) It prevented the increase in K^+ concentration, whether expressed on a leaf sap or dry weight basis, which occurred in control-pretreated plants on exposure to 300 mol m^{-3} NaCl. (c) It significantly reduced the extent of increase in stem dry matter production and significantly increased the extent of increase in dry matter production of reproductive organs relative to the pretreatment level as compared to control-pretreated plants. Dry matter production of stems and reproductive organs increased 2.1- and 97-fold relative to the level after pretreatment in plants pretreated at 80 mol m^{-3}

NaCl, whereas the respective relative increases in control-pretreated plants were 2.6- and 48-fold.

The reduced extent of stem dry matter production in plants pretreated at 80 mol m^{-3} NaCl as compared to control conditions on exposure to 300 mol m^{-3} NaCl suggests that pretreatment at this level of moderate salinity enhanced the adverse effects of subsequent high salinity treatment on plant growth. In addition, the increase in dry matter production of reproductive organs in plants pretreated at 80 mol m^{-3} NaCl as compared to control conditions may indicate that initiation of the reproductive phase was brought forward in plants pretreated at this level of moderate salinity. Accelerated initiation of the reproductive phase may occur in the time-course of salinity exposure, when the rate of leaf death exceeds the rate of production of new leaves, thus decreasing total plant capacity to produce photosynthate (Munns, 2002). Although pretreatment at 80 mol m^{-3} NaCl as compared to control conditions enhanced the extent of increase in leaf succulence, this was not associated with a reduction in the extent of increase in leaf sap Na^+ concentration on exposure to 300 mol m^{-3} NaCl. Collectively, these data suggest that pretreatment at moderate salinity of 80 mol m^{-3} NaCl reduced plant resistance to high salinity of 300 mol m^{-3} NaCl.

The reduction of the extent of increase in leaf Na^+ concentrations on plant exposure to 300 mol m^{-3} NaCl by pre-exposure to 120, but not 80 mol m^{-3} NaCl, indicates a crucial role of the level of pretreatment salinity on the activation of mechanisms involved in control of leaf Na^+ concentrations. This is in general agreement with the study by Amzallag *et al.* (1990), which showed a relationship between the level of pretreatment salinity and the degree of increase in salinity resistance with respect to shoot growth and shoot Na^+ accumulation in *Sorghum bicolor*. Both, pretreatment at 75 and at 150 mol m^{-3} NaCl for 20 days, allowed resumption of shoot growth at rates comparable to those of control plants on exposure to 300 mol m^{-3} NaCl, whereas pretreatment at control conditions resulted in a permanent cessation of growth. However, plants pretreated at the two levels of moderate salinity differed with respect to the length of the period of transient inhibition of growth preceding growth resumption, which was 3 days and 2 weeks in plants pretreated at 150 and 75 mol m^{-3} NaCl, respectively. Pretreatment at both

levels of salinity reduced the degree of increase in concentrations of Na^+ and Cl^- in bulk shoot water on exposure to 300 mol m^{-3} NaCl, but this effect was more pronounced in plants pretreated at 150 as compared to 75 mol m^{-3} NaCl. Further characterisation of this phenomenon of salinity acclimation in *Sorghum bicolor* revealed that pretreatment at salinity levels above a threshold of 30 mol m^{-3} NaCl could induce increased salinity resistance (Amzallag *et al.*, 1993). The relationship between pretreatment salinity level and potential for induction of increased salinity resistance observed in the present study on *Gossyium hirsutum* extended that observed by Amzallag *et al.* (1990, 1993) in that the two levels of moderate salinity had actually contrasting effects on plant reponse to high salinity treatment. Only the higher salinity level of 120 mol m^{-3} NaCl resulted in a reduced extent of increase in leaf Na^+ concentration, whereas pretreatment at 80 mol m^{-3} NaCl did not have a significant effect on leaf Na^+ concentrations and enhanced adverse effects of salinity on plant growth during exposure to 300 mol m^{-3} NaCl.

(3) Pretreatment at 100 mol m^{-3} NaCl for 18 days, initiated, when plants were 47 days old, had the following effects on plant response to treatment at 300 mol m^{-3} NaCl over a period of 14 days (experiment 2). (a) It significantly reduced the extent of reductions in net rate of CO_2 uptake, transpiration, and stomatal conductance, determined on day 7 of treatment at 300 mol m^{-3} NaCl, relative to the experimental control level. In plants pretreated at 100 mol m^{-3} NaCl relative reductions in transpiration, stomatal conductance, and net rate of CO_2 uptake amounted to 73, 82, and 65%, respectively, whereas the respective values for control-pretreated plants were 81, 88, and 83%. In addition, plants pretreated at 100 mol m^{-3} NaCl exhibited significantly lower values of intercellular CO_2 concentration, when determined on day 7 of treatment at 300 mol m^{-3} NaCl as compared to control-pretreated plants. Thus pretreatment at 100 mol m^{-3} NaCl appeared to partially protect gas exchange parameters, and in particular net rate of CO_2 uptake, from salinity-induced reductions. However, this protective effect was only apparent in the initial phase of treatment at 300 mol m^{-3} NaCl, as determination of gas exchange parameters on day 11 of treatment showed no significant difference between plants pretreated at 100 mol m^{-3} NaCl and at control conditions.

Responses similar to those on day 11 were observed on days 13 and 14 of treatment at 300 mol m^{-3} NaCl (data not shown). (b) It significantly enhanced the extent of increase in leaf sap Na^+ concentration relative to the experimental control level determined in response to treatment at 300 mol m^{-3} NaCl. Plants pretreated at 100 mol m^{-3} NaCl exhibited a 14-fold, control-pretreated plants only a 6-fold increase in leaf sap Na^+ concentration. Calculations of absolute increases in leaf Na^+ concentrations (on a dry weight basis) since pretreatment revealed that the relatively higher absolute values of leaf Na^+ concentration observed in plants pretreated at 100 mol m^{-3} NaCl after exposure to 300 mol m^{-3} NaCl could be explained by Na^+ levels previously accumulated during moderate salinity pretreatment. (c) It prevented the increase in leaf K^+ concentration, whether expressed on a leaf sap or dry weight basis, which occurred in control-pretreated plants on exposure to 300 mol m^{-3} NaCl. (d) It significantly reduced the extent of increase in leaf Ca^{2+} and Mg^{2+} concentration, expressed on a dry weight basis, which occurred in control-pretreated plants on exposure to 300 mol m^{-3} NaCl.

Pretreatment at 100 mol m^{-3} NaCl for 18 days thus increased plant salinity resistance with respect to net rate of CO_2 uptake, and, to a lesser degree, transpiration and stomatal conductance in the intial phase of subsequent treatment at 300 mol m^{-3} NaCl. This was associated with lower levels of intercellular CO_2 concentration in plants pretreated at 100 mol m^{-3} NaCl as compared to control conditions on exposure to 300 mol m^{-3} NaCl, indicating a higher activity of CO_2 assimilation in the former. Plaut & Federman (1991) observed a similar phenomenon of acquirement of increased resistance to salinity with respect to CO_2 assimilation during salinity acclimation in *Gossypium hirsutum*. 25-day old plants, growing hydroponically in half-strength Hoagland solution, were subjected to 3 levels of osmotic potential, -0.3, -0.6, and -0.9 MPa, obtained by adding appropriate amounts of NaCl to the culture medium. Salinity levels were imposed gradually over a period of 5-8 days (acclimating treatment) or rapidly within 1-2 days (non-acclimating treatment). Salinity-acclimated plants exhibited a reduced extent of decrease in net rate of CO_2 uptake, but not transpiration, as compared to non-acclimated plants at all three salinity levels. Determination of response curves of net rates

of CO_2 uptake to increasing intercellular CO_2 concentrations (A/c_i curves) revealed increased slopes of the linear non-saturating part of response curves in acclimated, but not non-acclimated plants, exposed to osmotic potentials of -0.3 and -0.6 MPa relative to the control level. The increase in slope was interpreted as indication of increased enzyme levels or activity of Ribulose bisphosphate carboxylase.

Pretreatment of 47-day old plants at 100 mol m^{-3} NaCl for 18 days differed from pretreatment at 120 mol m^{-3} NaCl for 26 days, initiated at a plant age of 23 days, with respect to effects on three major aspects of leaf cation relations. Firstly, pretreatment at 100 mol m^{-3} NaCl resulted in substantially higher extents of increase in leaf Na^+ concentrations in the course of the pretreatment period as compared to pretreatment at 120 mol m^{-3} NaCl. Secondly, pretreatment at 100 mol m^{-3} NaCl and subsequent treatment at 300 mol m^{-3} NaCl appeared to increase leaf Na^+ concentrations in an additive manner, as indicated by similar extents of absolute increases in leaf Na^+ concentration since pretreatment, *i.e.* during the period of treatment at 300 mol m^{-3} NaCl, in salt- and control-pretreated plants. In contrast, pretreatment at 120 mol m^{-3} NaCl as compared to control conditions reduced the extent of increase in leaf Na^+ concentration on plant exposure to 300 mol m^{-3} NaCl. Thirdly, pretreatment at 100 mol m^{-3} NaCl caused a reduction in extent of increase in Ca^{2+} and Mg^{2+} concentrations relative to the experimental control level on exposure to 300 mol m^{-3} NaCl, an effect which did not occur in response to pretreatment at 120 mol m^{-3} NaCl.

The differential effects of these two pretreatment regimes on leaf cation relations observed in the present study may be explained based on a study by Amzallag *et al.* (1993) who further characterised the phenomenon of salinity acclimation in *Sorghum bicolor* first described by Amzallag *et al.* (1990). These authors carried out a detailed investigation addressing the influence of plant age at the time of initiation of salinity pretreatment at a constant salinity level and duration of 150 mol m^{-3} NaCl and 20 days, respectively, on the induction of increased salinity resistance in *Sorghum bicolor*. This revealed a decrease in the ability of pretreatment to induce increased salinity resistance with respect to shoot growth with increasing plant age at initiation of pretreatment. Initiation of salinity pretreatment at a plant age of 5 or 8 days

allowed survival and resumption of growth, whereas initiation at an age of 12, 15, or 21 days resulted in plant death on exposure to 300 mol m^{-3} NaCl. However, plants first exposed to pretreatment, when 12, 15, or 21 days old, differed in the length of the period of growth which preceded plant death. Initiation of pretreatment at an age of 12 days allowed continued growth for 1 week, whereas initiation at an age of 21 days resulted in immediate death on exposure to 300 mol m^{-3} NaCl. Plants first pretreated at moderate salinity at an age of 5 or 8 days exhibited a much lower extent of increase in Na^+ concentration in bulk shoot water as compared to plants exposed to pretreatment at a later stage. It thus appears likely that the differences between the two pretreatment regimes involving salinity levels of 120 and 100 mol m^{-3} NaCl in the present study with respect to their effects on leaf Na^+ concentrations on exposure to 300 mol m^{-3} NaCl were due to the differences in plant age at initiation of pretreatment. The observation by Amzallag *et al.* (1993) that pretreatment regimes which did not allow continuation of shoot growth at control rates still resulted in differences in plant survival on exposure to 300 mol m^{-3} NaCl suggests that such differences may have existed between differently pretreated plants in the present study, but remained undetected, as the treatment periods at 300 mol m^{-3} NaCl used in the present study did not cause plant death in plants from any pretreatment.

5.3.3.3 Mechanism of acclimation to salinity

In the present study acquirement of increased resistance to high salinity during pretreatment at moderate salinity in *Gossypium hirsutum* was demonstrated at two levels.

(a) Pretreatment at 120 mol m^{-3} NaCl reduced the extent of increase in leaf sap Na^+ concentration on subsequent exposure to 300 mol m^{-3} NaCl, and thus appeared to influence the control of Na^+ concentration in leaves. This was in part due to the increase in leaf succulence, which occurred during pretreatment. Leaf succulence results in dilution of salts accumulated in the vacuole, thus increasing vacuolar capacity for further salt storage (Marschner, 1995, Glenn *et al.*, 1997). However, when Na^+ concentrations were expressed on a dry weight basis, thus eliminating effects of changes in leaf water content, a large extent of the difference between plants pretreated at 120 mol

m^{-3} NaCl and control conditions remained (although this was not statistically significant anymore). This indicates that other mechanisms are likely to have contributed to the observed reduction in leaf sap Na^+ accumulation on exposure to 300 mol m^{-3} NaCl as a result of pretreatment at 120 mol m^{-3} NaCl. Cotton is classified as a glycophyte (Glenn *et al.*, 1997), and more specifically as a crop of moderate salinity resistance (Brugnoli & Björkman, 1992, Ashraf, 2002). Glycophytes may have more or less efficient mechanisms of salt exclusion, but exhibit a lower ability to compartmentalise salt in leaves as compared to halophytes (Munns, 2002). Although glycophytes thus rely to a higher degree on mechanisms of salt exclusion than compartmentalisation as compared to halophytes, high salinity resistance among glycophyte species is generally related to the capacity for salt compartmentalisation in leaves as well as efficient exclusion at the root level (Glenn *et al.*, 1997). Leidi & Saiz (1997) compared two cultivars of *Gossypium hirsutum*, Z407 and P792, differing in salt resistance with respect to plant growth, and demonstrated a positive correlation between salinity resistance and Na^+ accumulation in leaves. Cultivar Z407, which exhibited lower extents of reductions in leaf and root fresh weight production, leaf area, specific leaf area, and total leaf number, showed higher levels of leaf water content as well as higher Na^+ and lower K^+ concentrations in leaves, whereas the less resistant cultivar P792 exhibited higher Na^+, and lower K^+ concentrations in stems. However, the majority of comparative physiological studies on cotton cultivars differing in salinity resistance with respect to growth have shown a relationship between salinity resistance and partial exclusion of Na^+, selective accumulation of K^+ in leaves/shoots, as indicated by high K^+/Na^+ ratios, and in some cases partial exclusion of Cl^- (Ashraf, 2002). On a molecuolar level Na^+ exclusion and compartmentalisation are mediated by energy-dependent Na^+/H^+ antiporters at the plasma membrane and the tonoplast, which are involved in Na^+ efflux and Na^+ sequestration in the vacuole, respectively. ATP-driven H^+ pumps at the plasma membrane (PM-ATPase) and the tonoplast (V-ATPase) generate the electrochemical potential which forms the drving force for secondary energy-dependent transport (Hasegawa *et al.*, 2000). Lin *et al.* (1997) investigated the responsiveness of PM-ATPase and V-ATPase in roots of young seedlings of a line of *Gossypium hirsutum*, selected for salt-sensitivity during germination

and emergence, to treatment at 75 mol m^{-3} NaCl or KCl for 72 hours. Exposure to 75 mol m^{-3} NaCl, but not KCl, induced an increase in specific activity of PM-ATPase, and this was not associated with increased concentrations of this transport protein. In contrast, the specific activity of V-ATPase was not responsive to treatment at 75 mol m^{-3} NaCl, but increased slightly on exposure to 75 mol m^{-3} KCl. The responsiveness of PM-ATPase, but not V-ATPase, in roots of young seedlings of this salt-sensitive line of cotton to Na^+ lends further support to the general consensus that salinity resistance in cotton is mainly based on Na^+ exclusion, and not vacuolar compartmentalisation. The reduced extent of increase in leaf Na^+ concentrations on a dry weight basis in *Gossypium hirsutum* plants pretreated at 120 mol m^{-3} NaCl as compared to control conditions on subsequent exposure to 300 mol m^{-3} NaCl in the present study may thus indicate that this salinity pretreatment induced an increase in activity or NaCl-responsiveness of ion transport systems involved in Na^+ exclusion.

(b) Pretreatment of 47-day old plants at 100 mol m^{-3} NaCl for 18 days partially protected photosynthetic CO_2 assimilation in the first few days of treatment at 300 mol m^{-3} NaCl. The restriction of this protective effect to the initial phase of treatment at 300 mol m^{-3} NaCl suggests that it reflected acclimation to the osmotic component of salinity stress. Plant responses to salinity exposure on a time-scale of days are generally attributed to salinity-induced reductions in osmotic potential in the rooting medium (Munns, 2002). In the study by Plaut & Federman (1991) mentioned above maintenance of higher values of photosynthetic CO_2 assimilation in salinity-acclimated as compared to non-acclimated plants of *Gossypium hirsutum* was associated with greater extents of reductions in leaf osmotic potential, indicating osmotic adjustment, in acclimated plants. Reductions in osmotic potential in acclimated as well as non-acclimated plants could be accounted for by increases in leaf concentrations of Na^+, Cl^-, and organic solutes, and the extent of these increases was greater in acclimated as compared to non-acclimated plants. It was hypothesised that osmotic adjustment may have contributed to maintenance of chloroplast volume and function during salinity exposure. In the present study pretreatment at 100 mol m^{-3} NaCl for 18 days induced an 8-fold increase in leaf sap Na^+ concentration which may have reduced leaf

osmotic potential, as suggested by the significant decrease in relative water content, associated with a slight increase in leaf water content. Pretreatment at 100 mol m^{-3} NaCl may thus have increased plant resistance with respect to net rate of CO_2 uptake to the osmotic stress component of salinity in the initial phase of treatment at 300 mol m^{-3} NaCl via higher levels of osmotic adjustment.

6 General Discussion

In the present study cotton (*Gossypium hirsutum*) was assessed for its potential to acclimate to abiotic environmental stresses by pre-exposure to the same or different stresses. The choice of cotton is of particular interest due to its economical importance. Cotton supplies the world's most important textile fibre and the second most important meal seed. *Gossypium hirsutum* in particular is grown in tropical and temperate latitudes from 47° North in the Ukraine and 37° South in the USA to 32° South in South America and Australia and supplies 90% of the world's cotton production (Wendel, 1995)

Mechanisms of plant environmental resistance fall into two broad categories: adaptation and acclimation. The current use of terminology defines adaptations as constitutively expressed and thus genotypically determined traits for stress resistance resulting from evolutionary selection for environmental fitness of a population of organisms, and acclimation as adjustment of individual organisms in response to changes in environmental conditions (Buchanan *et al.*, 2000). However, the ability to acclimate is also genetically determined and referred to as acclimation potential of a genotype (Berry & Björkman, 1980). In this sense acclimation has been defined as expression of the genetic potential for stress resistance under inductive conditions (Pearce, 1999).

In crop species genetically determined mechanisms of adaptation and acclimation potential may not only be the result of evolutionary, but also human selection in the course of domestication and breeding of modern cultivars. The modern cotton cultivars grown today are the product of a long history of selection processes for high yield, improved fibre quality, day-length neutrality and annual growth habit from originally tropical perennial ancestors with relatively short sparse fibre. Development of the four categories of modern cultivars of *Gossypium hirsutum* (Acala, Delta, Plains, and Eastern) in the south eastern USA involved additional selection criteria of early maturity (to reduce susceptibility to the boll weevil), and adaptation to environmental conditions in the cultivation area (Wendel, 1995). In addition, modern cultivar development today may locally focus on improvement of resistance to cold, drought and high soil salinity (Rehm & Espig, 1991). The importance of environmentally inducible mechanisms of stress resistance in the process of human selection of crop plants for improved

stress resistance is illustrated by comparative physiological studies of crop cultivars differing in heat resistance, based on field observations. Chen *et al.* (1982) assessed two genotypes each of tomato and bean differing in heat resistance, based on yield and fruit set in the field, with respect to their constitutive and inducible heat resistance, based on tissue ion leakage in response to treatment at 50°C. Heat resistance as observed in the field was shown to be related to inducible and not constitutive heat resistance in physiological terms. A similar comparison of two potato varieties showed that the variety with the higher heat resistance, based on field observations, displayed lower levels of constitutive resistance, determined as thermotolerance of photosystem II (PSII), but a higher increase in PSII thermotolerance in response to pretreatment at moderately elevated temperature (Havaux, 1993).

Domestication and breeding involved in the development of crop plants may have changed or obscured mechanisms of environmental stress resistance originally present in the wild species (Chen *et al.*, 1982). Selection for specific agronomical traits involved in the development of modern cultivars of *Gossypium hirsutum* apparently resulted in severe reductions in genetic variation (Wendel, 1995). The present study demonstrates that *Gossypium hirsutum* CIM 443 has a genetic potential to acquire increased resistance to high levels of heat, chilling and salinity in response to suitable pretreatments at moderate levels of the respective stress factors. This suggests that a large genetic potential for inducible resistance to environmental stress factors appears to have been preserved in cotton throughout the long history of human selection processes involved in the development of modern cultivars of this crop.

In the present study assessment of a variety of pretreatment regimes at moderate levels of high temperatures, chilling temperatures and soil salinity for their potential to induce increased resistance to the same stress allowed the identification of some general criteria defining the suitability of a stress pretreatment to induce acclimation. Suggested criteria are outlined in the following.

The results of the present study indicate that acclimation occurs only in an optimum range of stress dose, *i.e.* the product of stress intensity and duration, above and below which the plant ability to acquire increased stress resistance appears to decline. Comparison of two pretreatment regimes at high temperatures,

40°C and 45°C, each applied over a period of 7 days, showed that the former prevented rapid dehydration of all main stem leaves, the latter of only 62% of main stem leaves per plant. In the case of acclimation to chilling temperatures pretreatment at temperatures decreasing gradually from 18°C to 13°C by 1°C per day followed by 1 day of recovery in control temperature reduced the extent of photoinhibition and leaf death in response to subsequent treatment at 11°C. Pretreatment at a similar gradual decrease in temperature, but reaching a minimum temperature of 12°C followed by 8 days of recovery in control temperature, reduced the extent of photoinhibition only at later stages of treatment at 8°C and did not alleviate low temperature-induced plant injury. Comparison of salinity pretreatment regimes differing in the NaCl concentration applied revealed even opposite effects of different stress levels on the state of stress resistance. Pretreatment at 80 mol m^{-3} NaCl actually decreased the extent of stem dry matter production relative to the pretreatment level, and did not reduce the degree of Na^+ accumulation in leaves on exposure to treatment at high salinity of 300 mol m^{-3} NaCl. In contrast, pretreatment at 120 mol m^{-3} NaCl significantly reduced the extent of increase in leaf sap Na^+ concentration on exposure to the high salinity level. In the case of salinity acclimation, not only the level of salinity, but also the developmental stage at initiation of salinity pretreatment appeared to be crucial for the induction of acclimative processes. Treatment of 23-day old plants at 120 mol m^{-3} NaCl significantly reduced the extent of increase in leaf sap Na^+ concentration on exposure to high salinity at 300 mol m^{-3} NaCl, whereas pretreatment of 47-day old plants at 100 mol m^{-3} NaCl increased leaf Na^+ concentration in an additive manner with the high salinity treatment.

The lower stress threshold, which has to be exceeded to result in induction of acclimation processes, appears to reflect the level of the respective environmental factor inflicting a sufficient degree of plant stress, which in turn induces acclimation to an extent, which allows growth or survival even at severe stress levels. This threshold level thus depends on the specific constitutive resistance of a species to the respective environmental factor. Regarding its constitutive temperature and salinity resistance, cotton as the subject of the present study has been described as chilling-sensitive (Kornyeyev *et al.*, 2002), and chilling generally refers to non-freezing temperatures of 0 to 12°C (Allen & Ort, 2001), and as moderately salinity resistant (Brugnoli & Björkman, 1992).

Studies on the phenomenon of acquired thermotolerance, *i.e.* the short-term development of tolerance to otherwise lethal high temperature treatment by pre-exposure to non-lethal high temperatures, have led to a clear definition of the minimum stress threshold required for induction of increased thermotolerance based on the constitutive resistance of a species. A number of studies have demonstrated a correlation between development of thermotolerance of seedling growth and the kinetics of heat shock protein (HSP) synthesis. Synthesis of HSPs is induced by pretreatment of seedlings at temperatures 5°C or more above the optimal growth temperature (Vierling, 1991). The optimal thermal range of a species may be determined based on whole-plant growth or on enzyme function (Mahan *et al.*, 1997). Thermal kinetic windows (TKWs) of optimal enzyme function are defined as temperature range over which the apparent Michaelis-Menten constant (K_m) is within 200% of the minimum apparent K_m of that enzyme (Burke, 1995). In cotton the TKW for optimal enzyme function has been shown to lie in the temperature range of 23.5 - 32.0°C (Lu *et al.*, 1997). In the present study on cotton the minimum stress levels used for heat and chilling pretreatment were 40 and 18°C to 13°C, respectively. These temperature levels clearly exceed the optimal thermal range of this genus by several degrees, thus indicating their potential to induce plant stress. *Amzallag et al.* (1993) who are among the few who demonstrated acclimation to salinity on a whole plant level identified a minimum threshold salinity level of 30 mol m^{-3} NaCl which was required for induction of increased resistance to high salinity at 300 mol m^{-3} NaCl in *Sorghum bicolor*. The actual enhancement of, and not protection from, adverse effects of high salinity at 300 mol m^{-3} NaCl by pre-exposure to 80 mol m^{-3} NaCl suggests that the minimum threshold salinity may be higher in *Gossypium* as compared to *Sorghum*.

The present study suggests that the upper stress level, beyond which the acclimating effect of pretreatment declines, is related to the balance between acclimation and injury processes induced by the specific stress level. This has been most clearly demonstrated in the case of low temperature pretreatment, during which responses to the individual temperature steps of the gradual decreasing temperature regime were closely monitored. A gradual decrease in low temperature from 18°C to 12° C by 1°C per day induced a photosynthetic response of predominantly acclimative nature in the initial phase from day 1 to 4, whereas

responses in the later phase from day 5 to 7 reflected the onset of more severe injury. Accordingly, pretreatment at the same sequence of gradually decreasing temperatures, but not including the last step of exposure to 12°C, decreased shoot dry matter production to a much lower extent and allowed complete recovery of PSII activity several days earlier. Similarly, comparison of plant responses to the two levels of moderate heat stress tested, 40 and 45°C, revealed that the higher temperature of 45 °C caused significant reductions in net rate of CO_2 uptake, leaf stomatal conductance, and intercellular CO_2 concentration, whereas these parameters were not affected by treatment at 40°C. In the case of salinity acclimation it appeared to be plant age at initiation of salinity pretreatment, which determined the effective stress dose, as indicated by accumulation of Na^+ in leaves. The relatively low levels of Na^+ accumulation in leaves in plants pre-treated at 120 mol m^{-3} NaCl, when 23 days old, were attributed to the developmental stage of the majority of leaves at this plant age, which were not fully expanded. Expanding tissues generally maintain low levels of Na^+ concentration as a result of dilution of salts in expanding vacuoles (Munns, 1993). In contrast, pretreatment of 47-day old plants at 100 mol m^{-3} NaCl led to a large accumulation of Na^+ in the already mature fully expanded leaves of these plants. Thus the lower ability of pretreatment at 45 as compared to 40°C, of pretreatment at gradually decreasing temperatures to a minimum temperature of 12 as compared to only 13°C, and of pretreatment of 47-day old as compared to 23-day old plants at moderate salinity, to induce increased resistance to high levels of the respective stress factors, may be explained by the higher degree of injury these pretreatment regimes induced.

An upper threshold of stress dose, above which the acclimating effect of stress pretreatment declines, has been recognised early in the context of heat acclimation of crop plants. Chen *et al.* (1982) investigated the acclimation potential of a number of species to heat stress and found that the acclimating effect of heat pretreatment decreased above a certain temperature. Similarly, Havaux (1993) investigated the effects of pretreatment of leaf discs of *Solanum tuberosum* at a range of moderately elevated temperatures on thermotolerance of photosystem II. He found that pretreatment temperatures above 30°C and up to the highest tested temperature of 41°C resulted in increased structural resistance of photosystem II to high temperatures. However, pretreatment temperatures above

35°C also induced reductions in the maximum quantum yield of photosystem II, thus indicating the dominance of injury. More generally, the balance between stress-induced acclimation and injury may be explained by the 4-phasic model of plant stress response proposed by Lichtenthaler (1996). The first (alarm) phase results in activation of stress coping mechanisms, which are put into action in the second (restitution and resistance) phase. Stress coping mechanisms may include repair as well as hardening processes, the latter leading to an increase in plant resistance. However, with further stress exposure the stress dose may exceed total plant resistance, consisting of the constitutive and the acquired component, and may thus result in a decline in metabolic activities and physiological functions (phase of exhaustion). In the present study even the low temperature regime consisting of a gradual decrease in temperature from 18°C to 13°C by 1°C per day, which caused relatively less damage as compared to pretreatment at the same gradual decrease in temperature, but reaching a minimum temperature of 12°C, resulted in large reductions in net rate of CO_2 uptake, transpiration and stomatal conductance. Thus even plants pretreated at the temperature regimes decreasing to a minimum temperature of only 13°C were transiently transferred to control temperature for 1 day to allow recovery of injury, and to shift the balance between acquired resistance and stress-induced injury towards the resistance component. The balance between acquired stress resistance and stress-induced damage during plant recovery on return to control conditions may again be illustrated by the model of plant stress responses proposed by Lichtenthaler (1996). In the regeneration phase the plant will recover, provided that the stress was removed before senescence processes became dominant, and will move to a new physiological standard between resistance minimum (constitutive resistance) and maximum (constitutive and acquired resistance), the relative stage of which depends on the extent of damage inflicted. Such a recovery phase may also allow resumption of plant cellular functions required to fully express mechanisms activated during stress pretreatment. This has been demonstrated for the well-studied phenomenon of acquired thermotolerance. Moderately elevated temperatures induce transcription as well as translation of heat shock proteins (HSPs), and result in parallel develoment of thermotolerance, whereas more severe high temperatures may result in HSP transcription, but not in development

of thermotolerance, which requires a recovery period, during which HSPs may be actually synthesised (Pollock *et al.*, 1993).

Recent research focus has shifted from investigations of plant responses to severe stress levels to closer analysis of responses to sub-lethal moderate stress levels, which are of predominately acclimative nature and thus allow elucidation of mechanisms underlying plant resistance (Grover *et al.*, 2001). This approach has allowed suggestions about the mechanisms of acquired resistance to heat, cold, and salinity in the present study. Pretreatment at 40°C for 6 days induced a significant increase in transpiration rate, and on return to control temperature for 4 days a significant increase in stomatal conductance, collectively indicating an increase in plant hydraulic conductance. This in turn suggested an increased plant capacity for transpirational cooling, a mechanism of heat avoidance. The first 4 days of low temperature pretreatment, with temperatures decreasing gradually from 18°C to 15°C, resulted in photoinhibitory quenching of a predominantly photoprotective nature and a slight increase in net rate of CO_2 uptake between days 1 and 2, suggesting photosynthetic adjustment. In addition, return of low temperature-pre-treated plants to control temperature resulted in a significant increase in F_m values above the control level, possibly suggesting increased electron sink capacity. Pretreatment at 80 and 120 mol m^{-3} NaCl resulted in similar extents of reductions in gas exchange, dark-adapted chlorophyll fluorescence, leaf water relation and leaf cation relation parameters, in spite of the difference in applied salinity concentration of 40 mol m^{-3} NaCl. The only low degree of increase in Na^+ concentration in plants exposed to 120 mol m^{-3} NaCl, when 23 days old, suggested a possible activation of mechanisms involved in control of leaf Na^+ concentration (ion transport systems), without increasing the potential for plant injury as a result of ion toxicity.

The present study also demonstrated that pre-exposure to low temperatures decreasing gradually from 18°C to 13°C by 1°C per day followed by 1 day of recovery in control temperature resulted in increased resistance of plant photosynthetic processes to subsequent treatment at 49°C.

This phenomenon of cross-resistance is in general agreement with the "general adaptation syndrome" (GAS) hypothesis, which states that pre exposure to sub-lethal levels of one stress may induce increased resistance to a different stress. This is based on the observation that different stress factors may induce

overlapping acclimative responses (Leshem & Kuiper, 1996). These authors suggest that plant exposure to diurnal and seasonal variations in environmental conditions in natural environments may have evolutionarily selected for multiple stress resistance. However, induction of specific acclimation mechanisms by certain stress factors may have the advantage of producing responses adjusted to the specific nature of the stress factor as well as reducing overall metabolic energy costs involved in the synthesis of stress proteins (Knight & Knight, 2001).

Investigations of phenomena of cross resistance are considered to be of interest not only with respect to their potential practical value, but also because they allow conclusions about links between responses to different stress factors at a cellular level (Arora *et al.*, 1998). The following links between high and low temperature stress have been suggested on a molecular level. (a) Heat shock proteins (HSPs), which are thought to function as molecular chaperones (Sun *et al.*, 2002), may not only be induced by heat, but also by a variety of other abiotic stress factors including cold (Viswanathan & Khanna-Chopra, 1996). Pretreatment at moderately elevated temperature has been shown to induce an increase in resistance to chilling-induced tissue damage in tomato fruits, hypocotyls of mung bean, and seeds and cotyledons of cucumber, and this was associated with synthesis of HSPs during heat pretreatment (Sabehat *et al.*, 1998). However, the reverse effect of acquirement of increased heat resistance during pre-exposure to chilling temperatures does not appear to have been observed. (b) Any environmental stress factor, which induces reductions in photosynthetic CO_2 assimilation, may potentially induce photoinhibition, if applied in combination with moderate light (Osmond, 1994). It has been demonstrated that treatment at high as well as low temperatures concomitantly with moderate light can induce photoinhibition and photoinactivation of catalase. This has been attributed to the fact that both temperature stresses decrease rates of protein synthesis, thus reducing plant capacity of repair of D1 and catalase (Feierabend *et al.*, 1992). Short- and long-term exposure of plants to moderately increased as well as decreased temperatures relative to the optimum growth temperature in combination with moderate light may induce increases in levels of soluble antioxidants (Badiani *et al.*, 1997). The mechanism underlying the phenomenon of partial protection of photosynthetic processes during exposure to high temperature as a result of pre-exposure to low temperatures observed in the

present study may thus involve heat shock proteins or measures of photoprotection, both of which form a potential link between high and low temperature stress. However, there appear to be no reports in the literature, which directly correlate the phenomenon of acquirement of increased resistance to heat stress by pre-exposure to chilling with these links on a cellular level.

The phenomenon of acquired heat resistance with respect to photosynthesis as a result of pre-exposure to low temperature observed in the present study is particularly intriguing, as it is apparently unparalleled in natural environments. Plants growing in habitats with large seasonal variations in temperature have been shown to shift their temperature optimum of photosynthesis in parallel to changes in growth temperature, while maintaining relatively constant rates of photosynthesis. Thus acclimation of photosynthesis to seasonal changes in temperature is by necessity associated with decreased photosynthetic performance at the respective other end of the temperature scale (Berry & Björkman, 1980). This may also apply to diurnal changes in photosynthetic thermotolerance, as Havaux & Tardy (1996) demonstrated that heat pretreatment of potatoe leaves for only two hours resulted in increased values of actual quantum yield of PSII photochemistry on subsequent exposure to high, but reduced values on exposure to low temperatures. The fact that this type of acquirement of multiple stress resistance of photosynthesis to high and low temperatures does not appear to have been observed in natural environments indicates that it may not be a common plant response to temperature and may be restricted to specific species. It further suggests that this phenomenon may not necessarily be based on an overlapping response on a biochemical and molecular level induced by both stress factors. Collectively, these observations suggest the accumulation of glycinebetaine as potential mechanism underlying the phenomenon of cross resistance observed in the present study. Glycinebetaine has been shown to accumulate in response to long-term exposure to salinity and drought stress, and less frequently in response to low temperature stress in species which are natural accumulators of this compound (Rhodes & Hanson, 1993). The majority of species of Malvaceae including *Gossypium* have been reported to be natural accumulators of glycine betaine (Gorham, 1996, Blunden *et al.*, 2001). In the present study cold-pretreatment of *Gossypium hirsutum* at gradually decreasing temperatures from 18 to 13°C by 1°C per day induced a large

significant increase in leaf glycine betaine levels. A number of studies have demonstrated a protective effect of glycine betaine on photosynthetic reactions in isolated thylakoid membranes and PSII reaction centre complexes *in vitro* (Allakhverdieva *et al.*, 1999, Allakhverdiev *et al.*, 2003). It is thus tempting to speculate that accumulation of glycine betaine in response to cold-pretreatment may have contributed to the observed increase in heat resistance with respect to photosynthesis.

In the present study cold-pretreatment at gradually decreasing temperatures from 18 to 13°C by 1°C per day also appeared to increase plant resistance to drought stress. This was apparent from reduced extents of reductions in net rate of CO_2 uptake, transpiration, and stomatal conductance and maintenance of higher values of water use efficiency of photosynthesis in cold- as compared to control-pretreated plants. However, cold-pretreatment itself induced significant reductions in shoot height and dry matter production. Statistical removal of effects of plant size on responses of gas exchange parameters to progressive soil drying revealed that these differences in plant size could fully account for the observed phenomenon of protection. The phenomenon of increased drought resistance apparently solely as a result of reduction in plant size in the present study clearly pointed out the importance of addressing effects of stress pretreatment on plant growth and development. Particularly studies investigating the effects of long-term growth at low temperatures or soil water deficit on resistance to subsequent exposure to the respective stress factor have addressed this problem by introducing adjustments in plant age to allow comparison of plants of similar developmental stage. Thus Hurry & Huner (1992) compared 75-day old wheat plants cold-hardened at 5°C over a period of 68 days with 25-day old non-hardened plants. Streb *et al.* (1999) assessed effects of cold-hardening of rye plants at 4°C for 5 weeks by comparison with non-hardened plants grown in control temperature for 6 days. Matthews & Boyer (1984) exposed sunflower plants to pretreatment with soil water deficit over a period of several weeks and compared their responses to subsequent soil drying with those of control-pretreated plants of the same age (age control) as well as of the same developmental stage (developmental control). Comparisons of plants of the same age, but different plant sizes may be particularly problematic in connection with stresses involving resource supply such as soil drying (Osório *et al.*, 1998) and in

experiments involving long periods of pretreatment. However, future studies investigating effects of stress pretreatment on plant response to subsequent exposure to the same or different stresses should generally include a developmental control balancing reductions in plant growth as a result of stress pretreatment.

7 Conclusions & Future Perspectives

7.1 Conclusions

In the present study a number of pretreatment regimes at moderate levels of heat, chilling, and salinity stress were screened for their potential to activate stress coping mechanisms and thus increase plant resistance to subsequent treatment at high levels of the same stress factors in cotton (*Gossypium hirsutum*). These experiments revealed a large potential in cotton to acclimate to these stresses.

- Pretreatment at 40°C and a relative humidity of > 80% for 6 days partially protected photosynthetic gas exchange and increased transpiration on exposure to 49°C. Monitoring of plant responses to heat-pretreatment itself showed an increase in transpiration during exposure to moderate heat and an increase in stomatal conductance on subsequent return to control conditions, collectively indicating an increase in plant hydraulic conductance. Thus the heat acclimation phenomenon in the present study appears to be based on activation of mechanisms to increase transpirational cooling.
- Pretreatment at low temperature gradually decreasing from 18 to 13°C by 1°C per day reduced the extent of irreversible leaf damage and photoinhibition of photosynthesis, which occurred in control-pretreated plants in response to treatment at 11°C. The chilling-pretreatment regime itself induced a two-phase response pattern. The initial phase reflected mainly acclimation, showing some recovery of photosynthetic gas exchange and photoinhibitory quenching of predominantly photoprotective nature. The second phase was dominated by the onset of more severe injury, as evidenced by a steep decline in photosynthetic gas exchange, associated with a sudden steep drop in stomatal conductance, and photoinhibitory quenching reflecting photon damage. Chilling-pretreatment was also associated with a 3-fold rise in leaf sap concentration of glycinebetaine.
- Pretreatment of 23-day old plants at moderate salinity of 120 mol m^{-3} NaCl for 26 days reduced the extent of increase in leaf sap Na^+ concentration on exposure to 300 mol m^{-3} NaCl. This effect was partly due to increased leaf succulence, and possibly also to an increase in activity or NaCl-responsiveness

of ion transport systems involved in Na^+ exclusion, as suggested by the fact that some extent of the difference in leaf Na^+ concentration between salinity- and control-pretreated plants remained, when values were expressed on a dry weight basis. The salinity-pretreatment regime itself induced only a 2-fold increase in leaf sap Na^+ concentration, whereas pretreatment of 47-day old plants at 100 mol m^{-3} NaCl for 18 days induced an 8-fold increase in this parameter. The latter pretreatment regime did not restrict Na^+ accumulation in leaves, but appeared to increase leaf Na^+ concentration in an additive manner on exposure to 300 mol m^{-3} NaCl. However, this pretreatment regime partially protected photosynthetic gas exchange during the initial phase of treatment at 300 mol m^{-3} NaCl.

- The present study presents what appears to be the first evidence for acquirement of cross-resistance to chilling-induced photoinhibition as well as heat-induced reductions in photosystem II activity and photosynthetic gas exchange by pretreatment at chilling temperatures. The mechanism underlying the phenomenon of acquirement of increased heat-resistance during pretreatment at chilling temperatures was not resolved. However, based on the observed accumulation of glycinebetaine in response to chilling-pretreatment in the present study and evidence from previous studies demonstrating a protective role of glycinebetaine against heat inactivation of thylakoid membranes *in vitro*, a possible role of this compound is suggested.

7.2 Future perspectives

Creative experimental designs aiming to identify pretreatment regimes suitable to induce acclimative responses, varying intensity and duration of stress-pretreatment, the developmental age of plants, and the physiological parameters assessed, may reveal the existence of large potential to acclimate to abiotic stress in a variety of species. Detailed understanding of the environmental conditions capable of inducing this "sleeping potential" may be of practical value in agriculturally important crop plants such as cotton. Firstly, it would allow incorporation of acclimative plant response patterns into models predicting changes in plant growth and physiological responses as a result of changes in environmental conditions up to the scale of global change. Secondly, stress pretreatments with a demonstrated potential to induce increased stress resistance

may be administered in the field. This may be a simple and inexpensive process in the case of stress factors involving resource supply, like soil drying, or stress factors involving excess of potentially toxic elements, like salinity. In contrast, temperature-pretreatment may be economically feasible only in crop plants, which are initially grown under greenhouse conditions and transplanted to the field at a later developmental stage, but poses substantial technical problems in crops cultivated in the field during the entire growing season. In the latter case exploitation of phenomena of cross-acclimation may be of particular practical value, as pretreatment regimes with stress factors characterised by ease of application in the field may also be suitable to induce increased resistance to a different stress.

In the present study monitoring of plant responses in the course of stress pretreatment on a physiological level has yielded some clues about the potential mechanisms underlying acclimation phenomena on a correlative basis. Further studies on a biochemical and molecular level are needed to clarify the involvement of these mechanisms in increased stress resistance. On a molecular level, analysis of changes in gene expression during stress pretreatment may allow identification of gene products contributing to increased stress resistance. However, responses may be homeostatic, compensatory, related to hardening, developmental, or even non-functional (Pearce, 1999). In a next step the question, whether the acquirement of stress resistance and the induction of specific metabolic processes or accumulation of biochemical compounds are actually causally related, may be addressed by comparing the kinetics of acclimation and de-acclimation with those of induction of the process or accumulation of the compound in question. This type of argument has been used to substantiate the involvement of heat shock proteins in the phenomenon of acquired thermotolerance of seedling growth (Vierling, 1991) and to question the contribution of increased levels of antioxidants to acquirement of increased resistance to photoinhibition during cold acclimation of winter rye (Streb & Feierabend, 1999). Finally, direct evidence for the involvement of a gene or biochemical compound in acquired stress resistance may be produced on a biochemical or molecular level in the form of biochemical essays, inhibitor studies, overexpression of genes or antisense experiments.

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9 Appendix: Quantification of the effect of plant size on plant response to drought stress

9.1 Experimental protocol

Four sets of plants were sown at intervals of 7 - 8 days from each other, on July 5th, 13th, 21st, and 28th 2000, forming 4 different age groups (age group 1 to 4). They were each transplanted to 2.5-dm^3 pots 42 to 43 days after sowing. Plants were treated with plant nutrient solution containing phostrogen at 2 g dm^{-3} and micronutrients as in half strength Hoagland's solution in intervals as follows. The first set was fed 18, 30, 38, 40, 43, and 46 days after sowing, the second set 10, 22, 30, 32, 35, and 38 days after sowing, the third set 14, 22, 24, 30, 32, 34, and 38 days after sowing, the fourth set 23, 27, and 31 days after sowing; after that plants were fed about every second day.

Plants from each age group were randomly selected for drought treatment: 2 plants each (replicates a and b) from age groups 1, 2, and 4, and 1 plant from age group 3. 4 days before start of treatment they were transplanted to 15-dm^3 pots. Severe drought was imposed by withholding water for 16 to 23 days (as specified below for different age groups). At the beginning of drought treatment plants of age groups 1 to 4 were 76, 68, 60, and 53 days old, respectively. Development of soil moisture deficit over time was investigated by measuring volumetric soil water content in the plant substrate every to every third day over a drought period of 15 days (age group 1 and replicate a of age group 2), 19 days (replicate b of age group 2 and age group 3), or 20 days (age group 4). The time course of plant response to drought treatment was assessed by measuring gas exchange parameters daily from day 11 to 15 after initiation of treatment. In addition, leaf water relation parameters (water content and relative water content) and shoot height were measured on days 15 and 16, respectively, of drought treatment. Above ground dry matter production was measured on day 16 (age group 1 and replicate a of age group 2), 19 (replicate b of age group 2), or 23 (age groups 3 and 4) of drought treatment.

9.2 Results

Four groups of plants differing in age, with age groups 1, 2, 3, and 4 being 76, 68, 60, and 53 days old, respectively, were exposed to soil drying.

The differences in plant age between age groups 1 to 4 were associated with differences in plant size. The latter was quantified based on the following growth parameters: shoot height, total shoot dry matter production and dry matter contributions of stem, leaves, and reproductive organs (Table 1). Shoot height was determined after 16 days of soil drying for plants of all age groups. Determination of shoot dry matter production included some adjustment of drought exposure time to allow equivalent degrees of drought injury (as judged from qualitative assessment of leaf wilting) to develop in plants of different sizes. Thus plants of age group 1 and replicate a of age group 2 were harvested after 16 days, replicate b of age group 2 after 19 days, and age groups 3 and 4 after 23 days of soil drying. The relative reductions in average values of shoot height of age groups 2, 3 and 4 as compared to the oldest plants (age group 1) were 5, 23, and 40%, respectively. The corresponding relative reductions in total shoot dry matter production were 18, 43, and 61%. The relative reductions in shoot dry matter production with decreasing plant age were associated with a similar pattern in reductions of stem and leaf dry matter. Dry matter production of reproductive organs showed more of a threshold response to decreasing plant age, with relative reductions in average values for age groups 2, 3 and 4 as compared to the oldest plants (age group 1) amounting to 28, 48, and 93%, respectively. Increments in age by 7 to 8 days between age groups 1, 2, 3, and 4 were thus associated with increments in shoot dry matter production of similar extent between the different age groups. In contrast, increments in shoot height with increasing age were of similar extent only between age groups 2, 3, and 4, whereas age groups 1 and 2 showed a comparatively small difference with respect to this parameter.

Table 1 Shoot height and above ground dry matter production (whole shoot and individual contributions of stem, leaves and reproductive organs) in *Gossypium hirsutum* in response to withholding water. 4 groups of plants were sown in intervals of 7 - 8 days from each other, resulting in 4 different age groups (Age 1 - 4). At the beginning of treatment plants of groups Age 1, 2, 3, and 4 were 76, 68, 60, and 53 days old, respectively. Each age group consisted of 2 replicates (rep a and b), except group Age 3, for which there was only 1 replicate. Shoot height was measured after withholding water for 16 days, above ground dry matter production after withholding water for 16 (Age1 rep a and b, Age 2 rep a), 19 (Age 2 rep b) or 23 days (Age 3, Age 4 rep a and b).

Age group	Replicate	Shoot height (cm plant^{-1})	Dry matter production (g plant^{-1})			
			Stem	Leaves	Flowers / fruits	Shoot
Age 1	Rep a	118.0	33.0	27.1	4.63	64.7
	Rep b	113.0	26.8	25.8	4.49	57.0
Age 2	Rep a	116.0	26.8	23.0	5.20	54.9
	Rep b	104.0	21.7	21.8	1.30	44.8
Age 3		89.5	16.7	15.6	2.41	34.7
Age 4	Rep a	70.5	10.4	11.9	0.60	22.8
	Rep b	68.5	10.7	14.0	0.00	24.7

Plants of age groups 1 to 4 showed differences in the rate at which they depleted water in the substrate in response to soil drought, as determined by reductions in volumetric soil water content (Figure 1). In age groups 1 and replicate a of age group 2 (with shoot dry weights of 64.7 to 54.9 g plant^{-1}) volumetric soil water content exhibited a steep decrease from 22 to 27% on day 1 to 9% between days 2 and 4 after cessation of watering. From then on values showed only a slight further decline, reaching 5 to 6% on day 15. Replicate b of age group 2 (with 44.8 g shoot dry matter production) showed a more gradual decline, starting at 30 % volumetric soil water content on day 1 after cessation of watering and reaching 9% between days 8 and 11, from where on values decreased only slightly, reaching 6% on day 19. In age group 3, consisting of only one replicate (with shoot dry matter production amounting to 34.7g), volumetric soil water content declined at a similar rate to that observed in replicate b of age group 2, but values remained slightly higher than those of replicate b of age group 2 through most of the drying period. In the two replicates of age group 4 (with shoot dry matter production ranging from 22.8 to 24.7 g plant^{-1}) volumetric soil water content declined at a very slow rate, reaching 9% only between days 11 and

12. The relative difference in volumetric soil water content between the oldest (age group 1) and the youngest (age group 4) plants varied with exposure time to soil drying. On day 1 after cessation of watering this difference was relatively small (the average value for age group 1 was 21% smaller than that for age group 4), it reached a maximum on day 4 (when the average value for age group 1 was only 42% of that of age group 4), and then decreased again with continuing drought exposure time.

Consistent reductions in gas exchange parameters with drought exposure time occurred from day 11 onwards, and differences between plants from age groups 1 to 4 were assessed in the period from day 11 to 15 after cessation of watering. Stomatal conductance, transpiration, and net rate of CO_2 uptake showed essentially parallel response patterns (data not shown), but differences between age groups were most pronounced with respect to the net rate of CO_2 uptake. Age group 4 showed higher values of net rate of CO_2 uptake as compared to all other age groups, and values of age group 3 were generally higher as compared to those of age groups 1 and 2 (Figure 2a). Age groups 1 and 2 were, however, not clearly distinguished with respect to the response of net rate of CO_2 uptake. Age groups 1 and 2 showed a rise in the intercellular CO_2 concentration (which is a calculated parameter, based on measured net rate of CO_2 uptake, stomatal conductance to CO_2 diffusion, and CO_2 concentration in the bulk air), when the net rate of CO_2 uptake approached zero (Figure 2b). In contrast, values of age groups 3 and 4 exhibited only minor changes in the observed period of soil drying. Similarly, water use efficiency of net photosynthesis decreased in age group 1 and 2, when the net rate of CO_2 uptake approached zero, whereas values of age groups 3 and 4 showed only minor changes (Figure 3).

Determination of leaf water relation parameters also revealed considerable differences between age groups (Table 2). Age group 4 had higher values of water content and relative water content as compared to all other groups, and values of age group 3 were higher compared to those of age groups 1 and 2. Age groups 1 and 2 were not clearly distinguished with respect to these parameters.

Table 2 Leaf water content (WC) and relative water content (RWC) in *Gossypium hirsutum* after withholding water for 15 days. Age groups as in table 1.

Age group	Replicate	WC (%)	RWC (%)
Age 1	Rep a	156	54
	Rep b	182	48
Age 2	Rep a	117	42
	Rep b	164	48
Age 3		231	61
Age 4	Rep a	321	82
	Rep b	331	83

The relationship between plant size and the response of net rate of CO_2 uptake to soil drying was quantified by calculating correlation coefficients between net rate of CO_2 uptake, measured on particular days in the soil drying period, and parameters directly describing plant size, including shoot height and stem and leaf dry matter production. All of these parameters tested showed a statistically significant correlation with net rates of CO_2 uptake determined on days 11 to 15 of the soil drying period (Table 3). Shoot height yielded the highest correlation coefficients, followed by stem, then leaf dry matter production.

It was shown earlier in this study that the rate of water depletion from the substrate, as determined by reductions in volumetric soil water content, tended to increase with plant size / plant age (Figure 1). Therefore relationships between net rate of CO_2 uptake, determined on particular days in the soil drying period, and the following parameters related to water depletion in the substrate were investigated. (a) Volumetric soil water content measured in the substrate of each plant on that particular day. (b) Volumetric soil water content measured on day 4. The latter was chosen, as it was the day with the highest relative difference in volumetric soil water content between the oldest (age group 1) and the youngest (age group 4) plants in the drying period (Figure 1). Both parameters were

significantly correlated with net rates of CO_2 uptake, determined on days 11 to 15 of the soil drying period, although correlation coefficients for these parameters tended to be slightly lower than those for shoot height and stem dry matter production (Table 3). Averaged over the drying period, volumetric soil water content measured on day 4 appeared to be slightly superior to volumetric soil water content determined on the same day as net rate of CO_2 uptake, in predicting the response of the latter to soil drying.

Corresponding correlation matrices were generated between leaf water content and relative water content, determined on day 15 of the soil drying period, on the one hand, and the above mentioned parameters related to plant size on the other hand (Table 4). Shoot height yielded the highest correlation coefficients among the parameters directly describing plant size. Of the two parameters related to depletion of water in the substrate, volumetric soil water content determined on day 4 was superior in predicting leaf water content and relative water content measured on day 15 of the drying period.

Table 3 Pearson product-moment correlations between net rate of CO_2 uptake (A) measured after withholding water (drought treatment) for 11, 12, 13, 14, and 15 days and measures **(1)** – **(4)** related to plant size in 4 age groups of *Gossypium hirsutum*. Age groups as in table 1. Parameters related to plant size: **(1)** volumetric soil water content measured on the same day as A; **(2)** volumetric soil water content measured after withholding water for 4 days; **(3)** dry matter production of stem and leaves, measured after withholding water for 16 (Age 1 rep a and b, Age 2 rep a), 19 (Age 2 rep b), and 23 days (Age 3, Age 4 rep a and b); **(4)** shoot height measured after withholding water for 16 days. Asterisks *, **, *** represent significance at P = 0.05, 0.01, and 0.001, respectively.

Day [a]		**(1) Soil wc**	**(2) Soil wc d4**	**(3) Dry matter production**		**(4) Shoot height**
				Stem	Leaves	
11	**A**	0.914 **	0.857 *	-0.928 **	-0.905 **	-0.957 **
12	**A**	0.799 *	0.865 *	-0.843 *	-0.759 *	-0.887 **
13	**A**	0.784 *	0.812 *	-0.867 *	-0.844 *	-0.892 **
14	**A**	0.952 **	0.919 **	-0.927 **	-0.894 **	-0.977 ***
15	**A**	0.760 *	0.808 *	-0.862 *	-0.858 *	-0.906 **

[a] day after initiation of drought treatment

Table 4 Pearson product-moment correlations between leaf water content (WC) and relative water content (RWC) measured after withholding water (drought treatment) for 15 days and measures **(1)** – **(4)** related to plant size in 4 age groups of *Gossypium hirsutum*. Age groups as in table 1. Parameters related to plant size as in table 3. Asterisks *, **, *** represent significance at P = 0.05, 0.01, and 0.001, respectively.

	(1) Soil wc	**(2) Soil wc d4**	**(3) Dry matter production**		**(4) Shoot height**
			Stem	Leaves	
WC	0.819 *	0.901 **	-0.899 **	-0.871 *	-0.967 ***
RWC	0.770 *	0.873 *	-0.837 *	-0.842 *	-0.939 **