Bangor University

DOCTOR OF PHILOSOPHY

High temperature effects on growth, physiology and nitrogen fixation in soybean

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Award date:
1996

Awarding institution:
University College of North Wales, Bangor

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HIGH TEMPERATURE EFFECTS ON GROWTH, PHYSIOLOGY AND NITROGEN FIXATION IN SOYBEAN

A thesis submitted to the University of Wales

by

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M.Sc. (Hons.) Agri.

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MY FATHER

(May His Soul Rest In Peace)

who did his best to uplift me

to the Heights of an ideal life

and to my respected

MOTHER

whose affections inspired

me to love mankind.

ABSTRACT

This thesis compares the physiological and morphological differences between several soybean (*Glycine max* L.) cultivars in response to high temperature stresses applied to either the leaves or the shoots, and the ability of the leaves to heat harden. In addition, the effects of heat stress on nitrogen fixation and on the growth of *Bradyrhizobium japonicum* and its ability to heat harden were investigated.

The optimum temperature for the germination of all cultivars was between 25 and 35°C, while the temperature range 40 to 42.5°C was the upper limit for germination and root growth. No growth of any cultivar occurred at 45°C. Pre-germinating the seeds at lower temperatures before subjecting them to heat stress resulted in better germination and growth at higher temperatures.

Leaf chlorophyll fluorescence analysis measured in terms of Fv/Fm ratio was used to detect differences between the heat sensitivities of different cultivars. After heat stress at 40°C, the Fv/Fm ratio decreased in all cultivars tested and after stress at 42.5°C there was a 50-70% decrease in the ratio. The main difference between cultivars was in their ability to recover after heat stress. Williams-82 and Sable were better in this respect than cultivars Bragg, Davis, Mago-80 or Hardee. There was no recovery from heat stress treatments at 45°C. Using the cultivar Williams-82 it was possible to show that heat-acclimation (hardening) treatments had a significant effect, increasing tolerance to high temperature stress.

Experiments showed that nitrogen fixation in root nodules was little affected by root temperatures up to 35°C. At 40°C, small differences were detectable between cultivars and at 45°C nitrogen fixation was severely inhibited in all cultivars. Nitrogen fixation was also reduced by heat stress applied to the leaves but this response was considerably slower.

In pure culture, *B.rhizobium japonicum* grew well up to 40°C, but growth was very slow at 45°C. Heat-hardening treatments were apparently effective in permitting faster growth at high temperatures.

Overall it is concluded that there is a only limited genetic variability in heat tolerance among the soybean cultivars examined in this study.

Key words:- SOYBEAN, HEAT STRESS, CHLOROPHYLL FLUORESCENCE, NITROGEN FIXATION.

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ACKNOWLEDGEMENTS

I wish to express my deep gratitude to my supervisor Dr. J.M. Wilson for his helpful supervision, enthusiasm and friendship during the past three years. I also thank Dr. David Laidman for the time he has spent giving helpful advice, criticism of this manuscript and his patience, continuous encouragement and friendly help during the study here. I am also indebted to Dr. David Wright for his extreme patience whilst providing many hours of advice on statistical procedures. Thanks are also due to Dr. Sheila Gunn for her help with the statistics.

I am grateful to the Vice chancellor, Sindh Agriculture University, Tandojam, for the grant of study leave. Also, my heartiest thanks go to Professor Dr. Rahim Bux Mirbahar, Co-ordinator, Tandojam-Bangor Bilateral Link, who selected me for one of the available scholarship in the Link programme and his great inspiration and continuous encouragement during my stay here.

I am also thankful to the Head of the School of Biological Sciences, University of Wales, Bangor, for providing me facilities for my study programme. My thanks also go to all the staff of the School of Biological Sciences and Pen-y-ffridd Field Station for their co-operation during my study. I would particularly like to thank Mr. Gareth Williams for his help in the laboratory and Dr. Dan Wu for his help with the computer. I am grateful to Dr. F.R. Minchin, Institute of Grassland and Environmental Research

(IGER), Aberystwyth, for providing the strains of *Rhizobium*.

I acknowledge the support and unceasing generosity to my brothers, sisters, my wife, daughters, son and other relatives for keeping me nearer to them by letters, telephone calls and for their thoughts throughout my study period at Bangor. I would also like to say thanks to all my close friends Messrs. Karim Bux Laghari, H. Islamudin Majeedano, Samiullah Agha, Noor Mohammed Soomro, Jaweed Akhtar Memon, Mr. Gwyn Morris, Mrs. Wendy Grail, Mrs. Susan Plumpton and other colleagues especially from Sindh Agriculture University Tandojam, for making my time in Bangor so enjoyable; they will all be sadly missed.

Thanks are extended to the Directors, Oil Seed Agriculture Research Institute, Tandojam, the Agriculture Research Institute, Peshawar, and the Pakistan Agricultural Research Council, Islamabad, Pakistan, for providing the soybean seeds of various cultivars.

Finally, I am thankful to Sindh Agriculture University, Tandojam, for the grant of study leave and to the Ministry of Education, Government of Pakistan, for nomination to this scholarship. Thanks are also due to Overseas Development Administration who funded my scholarship as part of the Bilateral Link programme. Their concern for the staff development at Sindh Agriculture University is sincerely appreciated.

LIST OF ABBREVIATIONS

ARA	acetylene reduction assay
ATP	adenosine triphosphate
$^{\circ}C$	degrees centigrade
^{14}C	radio labelled carbon
CFA	chlorophyll fluorescence analysis
CO_2	carbon dioxide
cm	centimetre
cm^3	cubic centimetre
cv	cultivar
fwt	fresh weight
g	gram
GC	gas chromatography
h	hour
HSP	heat shock protein
K_2HPO_4	dipotassium hydrogen orthophosphate
KH_2HPO_4	potassium dihydrogen orthophosphate
mm	millimetre
mg	milligram
$MgSO_4$	magnesium sulphate
min	minute
μmoles	micromoles
nm	nanometre (10^{-9} m)
N_2	nitrogen
NO^{-3}	nitrate ion
NH_4^+	ammonium ion
NADH	nicotinamide adenine dinucleotide (reduced)
NaCl	sodium chloride

O_2	oxygen
P	probability
psi	pounds per square inch
ppm	parts per million
po_2	partial pressure of oxygen
PS I	photosystem I
PS II	photosystem II
S.E	standard error
sec	second
UV	ultra violet
USDA-FAS	United States Department of Agriculture Foreign Agricultural Services
%	percent

CHAPTER ONE

Chapter One

General Introduction.

1.1 Historical background.

Nagata (1960) has concluded that the origin and date of soybean culture still remain obscure, although the available information indicates that the origin was in China, especially in northern and central China. Soybean has been cultivated in China since before the times of written records, of which the first is in 2838 BC. Singh and Rachie (1987) have also reported that soybean was first grown by Chinese farmers more than 5000 years ago and that they probably made substantial improvements to the crop. The soybean *Glycine max* (L) Merrill probably first emerged as a domesticated species in the north China plains around the 11th century BC and later spread into Manchuria (Hymowitz, 1970). Eventually, it spread throughout the orient, but the Manchuria region dominated world production for many years (Piper and Morse, 1923). It was introduced into Japan via Korea and records suggest that it was introduced into Korea directly from North China sometime during the period 200 BC. The first news of soybean was brought to the western hemisphere in the writings of Englebert Kaempfer in 1712 (Bening, 1951). Later, it was introduced into the Jardin des Plants, Paris, in 1740, and the Royal Botanic Gardens, Kew, in 1790, but little interest was shown in the crop until the first shipment of soybean to Europe was made in 1908.

Morse (1950) has presented a detailed account of the modern history of soybean, noting that it was introduced into the U.S.A. in 1804. According to him, not more than eight varieties of soybean were grown in the U.S.A. prior to the numerous introductions by the U.S. Department of Agriculture beginning in 1898. World attention towards soybean was stimulated after its production became widespread in the U.S.A. At present, it has become a crop of great commercial importance. Other countries producing soybean are Indonesia, Russia, Brazil, and Canada, as well as other areas throughout the world. The major credit for bringing the crop into such prominence in the 20th century must, however, go to a small groups of scientists who began using modern methods of plant improvement during the last 50 years, especially breeders in the U.S.A., Canada, Brazil, China and Australia.

Basically, soybean is a tropical or sub-tropical plant, but it has adapted to temperate zones with humid warm summers. It is an annual plant commercially grown primarily for oil and protein production. More than 90% of the crop in western countries and perhaps 30% in eastern countries is devoted to oil extraction and soybean production accounts for 35% of the world vegetable oil market. The protein meal remaining after oil extraction is valuable and it is used extensively as livestock feed. Also, soybean is used directly for human consumption. Small flat black varieties of seeds are used for vegetable dishes in southern China

and small yellow seeds are used for natto in Japan (first described in about AD 1000). The yellow seeds are also used to make tofu, a low caloric white curd, in Japan. Tofu was, in fact, described first in Chinese history more than 2000 years ago. A large black kind of seed is used to make a "New Yorks" dish in Japan (Watanabe and Kishi, 1984).

Brar and Carter, (1992), Smith and Huyser (1987) and the USDA-FAS, (1991) have reported that, throughout the world, 50 million hectares of soybean are grown. Paschal (1993, 1995) has reported that world soybean production during 1993-94 was forecast at 112.5 Mtonnes, which is 4% less than the previous year. Last year, production in the U.S.A suffered from heavy rains and flooding in important growing regions. However, production from other countries reached 61 Mtonnes, which is 7% more than the previous year. The total world production for 1994/95 was forecast at 137.9 Mtonnes, an increase of 17% or 20.4 Mtonnes as compared with 1993-94. The production from other major producing countries, Argentina, China, Brazil and Paraguay, is forecast at 68.3 Mtonnes, up 3% or 1.7 Mtonnes. Pakistan remains a relatively unimportant producer, but interest is increasing steadily.

1.2 Chemical composition of the seed.

The composition of the mature soybean seed varies with the cultivar, climatic conditions and the soil type. The black seed varieties are rich in protein, but they have low oil

content. The yellow kind of seeds have a higher oil content and they are lower in proteins. The typical chemical composition has been described by Purseglove (1974) and it is presented in Table 1.1.

1.3 Taxonomy and morphology of the plant.

It is commonly believed that soybean was domesticated from its weedy relatives *Glycine soja* Sieb. and Zucc, also known as wild soybean (Hymowitz and Bernard, 1991). The soybeans belong to the family *Leguminosae*, sub-family *Papilionoideae* and the genus *Glycine* L. The genus *Glycine* is divided into three main sub-genera, *G. max*, *G. ussuriensis* and *G. gracilis*. Another sub-genus, *Soja*, is grown mainly in China, Taiwan, Japan and Korea. Two other sub-genera exist, one indigenous in Australia and the other in Africa and India (Cobely and Steele, 1976). The botanical classification of the cultivated soybean has been controversial, however, and the multiplicity of names applied to it has created confusion. Ricker and Morse (1948) have contended that, according to international botanical rules, the correct name is *Glycine max* (L) Merrill, a viewpoint shared by most taxonomists.

Even though morphological diversity exists, the soybean generally grows from 90 to 120 cm in height (Carlson, 1973). Most cultivars are erect and bushy with tawny or grey hairs on the stem (Purseglove, 1974). In fact, all above ground vegetative parts are covered with many, small

Table 1.1 Chemical composition of soybean seed.

Component	Percentage
Water	5-9
Protein	29.6-50.3
Fat	13.5-24.2
Carbohydrates	14.0-23.9
Fibre	2.8-6.3
Ash	3.3-6.4

From Purseglove (1974).

hairs. The first two leaves are simple and opposite, while all the other leaves are alternate and trifoliate. The radicle develops into a tap root approximately 150 cm long, but most of the roots are in the top 30-60 cm of soil. Modern cultivars usually have few primary branches and no secondaries, but prostrate freely branching forms do occur. The branches may develop from buds in the lower leaf axils and flowers may develop in all leaf axils. The flowers give rise to 0-5 pods with 1-5 seeds per pod, but 2-3 seeds are normal in most cultivars. Twenty to eighty percent of the flowers drop off without forming pods (Purseglove, 1974). The developmental morphology of the soybean has been extensively described by Carlson (1973).

1.4 Germination and growth.

Usually, the seedling appears above ground 5 to 7 days after sowing. Germination is epigeal, the bent epicotyl emerging first. The epicotyl then straightens out and elongates, pulling the cotyledons from the soil. Temperature effects on germination have been studied extensively. Tyagi and Tripathi (1983) reported that the temperature range for soybean germination is 24 to 39°C. LaFavre and Eaglesham (1986), however, found that the highest temperature for the growth of soybean plants was approximately 41°C at least for short periods of up to 6 hours. Delouche (1953) reported that maximum germination in the shortest time occurred at a constant temperature of 30°C. Inouye (1953), using Japanese varieties, found that

the optimum germination temperature was 34 to 36°C, while the minimum temperature was 2 to 4°C and the maximum was 42 to 44°C. In a field study, a day-time temperature of 25°C and a nocturnal temperature of 15°C appeared to be optimum for soybean growth and yield (Mederski, 1983). In general, the minimum and maximum temperatures for germination are 5 and 40°C respectively and the optimum temperature is about 30°C. Seedling emergence also depends upon the depth of planting, the cultivar, as well as soil temperature.

Growth rate and yield are influenced by many internal and external factors, such as photosynthesis, translocation, partitioning of assimilate, each of which is affected by different environmental factors. Brown (1960) reported that the rate of growth after seedling emergence of soybean was zero at 10°C and maximum at 30°C, above which the rate declined. The height of soybean plants increases with increasing soil temperature from 2°C to about 17°C. Only small differences in height were found between 17 and 27°C and plant height decreased rapidly at a soil temperature of 37°C (Earley and Cartter, 1945). Lindemann and Ham (1979) also found that the greatest plant height occurred at 25°C soil temperature and this was not influenced by the *Rhizobium japonicum* strain infecting the roots of the plant or by the variety of the soybean.

1.5 Heat stress and leaf chlorophyll fluorescence.

High temperature is an important factor determining the

survival of plants in hot climates. Heat stress involves many physiological processes, such as photosynthesis, translocation, respiration and membrane permeability (Bjorkman, 1980). Among these processes, photosynthesis appears to be the most heat sensitive (Bjorkman *et al.*, 1980; Nash *et al.*, 1985). In particular, heat damage to the photosynthetic apparatus occurs before other symptoms of high temperature injury can be detected (Bjorkman, 1975). The optimum temperature for photosynthesis in soybean is 25 to 30°C, but it is variable between varieties. The optimum temperature for photosynthesis in leaves of the soybean cultivar Lee, for example, is as high as 35°C (Hofstra and Hesketh, 1969). In the absence of oxygen, the maximum temperature for photosynthesis is as high as 40°C. Carbon dioxide assimilation by canopies is reduced by 20% when the canopy temperature is increased from 30 to 40°C. The translocation rate within the plant is also affected by temperature.

Chlorophyll fluorescence characteristics are greatly altered by high temperatures (Schreiber and Berry, 1977). Chlorophyll can therefore be used as an intrinsic fluorescence probe of the thylakoid membrane, responding to the same changes which cause denaturation of photosynthetic systems in plants (Schreiber and Berry, 1977). The thylakoid membrane is the first component of the photosynthetic apparatus to be damaged by heat (Santarius, 1974). Berry and Raison (1981), working on macrophytes, and

Bilger *et al.* (1987) working with *Arbutus unedo*, reported that superoptimal temperatures above 40°C caused thermal disruption of the thylakoid membranes and the subsequent impairment of photosynthesis then occurs. One parameter of the fluorescence properties of chlorophyll, the Fv/Fm ratio, can be used to quantify the thylakoid membrane damage for a variety of reasons. In particular, there is a close relationship between the Fv/Fm ratio and the photochemical efficiency of PS II (Somersalo and Krause, 1989, 1990), and the ratio is directly proportional to the quantum yield of oxygen evolution (Oquist and Wass, 1988).

High temperature injury in soybean appears as necrotic lesions, particularly on the hypocotyl and stems, or as chlorosis of the leaves. Necrotic lesions also occur on fruits. The induction of heat shock protein (HSP) synthesis has been shown to be a universal response to thermal stress in a wide range of organisms including plants (Ashburner and Bonner, 1979; McAlister and Finkelstein, 1980; Key *et al.*, 1981; Baszcynski *et al.*, 1982; Schlesinger *et al.*, 1982). When the growth temperature of soybean seedlings is shifted from 28 to 40°C the pattern of protein synthesis changes rapidly. Normal protein synthesis is dramatically decreased and a new set of heat shock proteins are produced (Key *et al.*, 1981). Also, Lin *et al.* (1984) found in soybean that a 10 min exposure to 45°C followed by incubation at 28°C resulted in the synthesis of HSPs. Prolonged incubations of 1-2 h at 45°C resulted in greatly

impaired protein synthesis of all kinds and seedling death. Kimple and Key (1985) also reported that, in soybean, some heat-shock proteins were synthesised by plants exposed to drying soil and high temperature.

1.6 Heat stress and nitrogen fixation.

The combination of the high protein and high oil contents of soybean seed makes the assimilation of nitrogen important to the attainment of maximum yields. The soybean plant, like most other legumes and a few non-legumes, has two different systems of nitrogen assimilation. They can absorb fixed or chemically combined forms of nitrogen from the soil through their roots. This nitrogen, usually takes the form of nitrate ions (NO^{-3}), which are translocated through the xylem to the leaves. There, energy derived from photosynthesis (NADH) is utilized to reduce the nitrate to ammonium ions (NH_4^+) which is then incorporated into amino acids and proteins. In addition to this system, soybean can assimilate molecular nitrogen gas (N_2) in root nodules. Plate 1.1 shows these nodules as they are developed on the roots of soybean. The nodules constitute a symbiotic association between the root cell (the host) and a soil bacterium *R.japonicum*. The nitrogen diffusing from the soil into the nodule is reduced to amino nitrogen utilizing energy derived from the respiration of photosynthates translocated to the nodules from the leaves. The fixed nitrogen is then transported via the xylem, primarily as asparagine, to the leaves where it is converted to other

Plate 1.1 Root system of 8-week soybean plant (*Glycine max* L., cv. Mago-80) showing nodules (n = nodule).

amino acids.

There are many environmental factors affecting the rate of nitrogen fixation, such as temperature, moisture, salinity and available combined nitrogen. Generally, high temperatures reduce photosynthetic rates and at the same time increase respiration rates, so that the amount of photosythate for assimilatory processes (such as nitrogen fixation) is reduced. With regard to the direct effect of temperature, Pankhurst and Sprent (1976) found a broad range between 15 and 30°C for optimum nitrogen fixation by the nodules of soybean. This optimum temperature range narrowed with water stress. Both nodulation and nitrogen fixation in soybean are greatly reduced by soil temperatures in excess of 33°C. At 27°C, nodule formation, nodule development, and nitrogen fixation in soybean were found to be most rapid (Dart *et al.*, 1975). In another soybean study, soil temperatures between 30 and 33°C caused little change in the fixation rate, but temperatures above 34°C had a negative effect (Sinclair and Weisz, 1985). Munevar and Wollum (1981) also found high rates of fixation at 28°C in soybean and very low rates at 38°C. Similarly, Waughman (1977) found that fixation rates of detached soybean nodules increased to a maximum at 30°C and then decreased rapidly at 35°C. For soybean, soil temperatures between 30 to 35°C have been reported to interfere with the development and function of root nodules compared with lower temperatures (Munevar and Wollum, 1981, 1982). High

root temperatures have been reported to cause decreases in the number and weight of nodules formed, the nitrogenase activity, plant nitrogen content and dry matter production (Diatloff, 1970). Lindemann and Ham (1979) reported that, soil temperatures of 30 to 35°C are likely to interfere with the development and function of root nodules in soybean. According to Jones and Tisdale (1921), however, soybean root nodule number and weight were greatly modified only by more extreme temperatures. Greatest nodule weight and nitrogen fixation occurred at a soil temperature of 24°C. Galletti *et al.* (1971) reported that soybean varieties and the nodule *Rhizobium japonicum* strain varied in their tolerance to excessively high temperatures. In the variety Lee, the total nodule number, nodule weight, and nodule to root ratio increased from 10 to 30°C, but nodule size was greater at lower temperatures (Weber and Miller, 1972).

1.7 Root nodule bacteria.

The bacteria capable of infecting the roots of legumes and stimulating nodule formation belong to several strains of the species *Rhizobium*. Each strain is only able to infect the roots of a specific group of legumes or only one species. *Rhizobium* belongs to the family *Rhizobiacae* (Conn 1938) which consists of rod-shaped cells without endospores. They are gram negative, aerobic and they are motile, having either one polar or sub-polar flagellum or two to six peritrichous flagellae (Buchanan and Gibbons,

1974). Elkan (1981) pointed out that the *Rhizobiacae* contain two genera, *Agrobacterium*, all species of which (with the exception of *A.radiobacter*) incite cortical hypertrophies on plant roots and *Rhizobium*, species of which form true nodules on the roots of *Leguminosae*. *Agrobacterium* and *Rhizobium* are often collectively referred to as rhizobia. As described by Jordan and Allen (1974), the genus *Rhizobium* consists of two main groups of species plus a miscellaneous grouping. The taxonomic position of *Rhizobium* is more controversial than this however. A grouping of six species based upon host infection patterns coupled with biochemical test and distributed between the two main groups of Jordan and Allen (1974). The taxonomy of the genus *Rhizobium* has also been reviewed by Graham (1976), Vincent (1977), Elkan (9181) and Trinick (1982). More recent classifications described three genera of rhizobia which have been well characterized, *Rhizobium*, *Bradyrhizobium*, (Jordan, 1982), and *Azorhizobium* (Dreyfus *et al.*, 1988). In 1988, two further genera were described, *Sinorhizobium* (Chen *et al.*, 1988) and *Photorhizobium* (Hungria, personal communication). In fast-growing rhizobia, a new specie, *R.loti*, has been introduced by Jarvis *et al.* (1982). It is possible that more will be added as a wider range of legumes are studied.

The species *Rhizobium* has been divided into two groups as described by Lohins and Hansen (1921). According to Allen and Allen (1950) there are "fast growers", consisting

mostly of rhizobia associated with alfalfa, clover, beans and peas, and "slow growers" associated with for example soybean, cowpea and lupin. Several workers have reported that the major differences between the fast and slow-growing rhizobia are based on their carbohydrate nutrition. Fast growers appear to be more efficient users of carbohydrates as an energy source. Allen and Allen (1950) summarized that the slow-growing rhizobia are more specific in their carbohydrate requirement in every respect. Also, Graham (1964) found similar differences between the fast and slow-growers and concluded that carbohydrate growth tests are clearly valid criteria for the sub-division of *Rhizobium*. Graham (1976) has summarized the cross-inoculation concept for the classification of *Rhizobium* and it is presented in Table 1.2.

Many studies have reported that the growth and survival of *Rhizobium* and *Bradyrhizobium* in soils are adversely affected by high soil temperatures (Munevar and Wollum 1981, 1982; Osa-Afiana and Alexander, 1982; Kluson *et al.*, 1986; Kennedy and Wollum, 1988). The evaluation of the temperature responses of rhizobia in pure culture may, therefore, be useful in the search for *R.japonicum* strains better suited to environments in which high soil temperatures are present. Munevar and Wollum (1981) found only one *Bradyrhizobium* strain able to survive in liquid culture at 49°C, but it was not effective in nitrogen fixation. Hafeez *et al.* (1991) reported that two

Table 1.2 Classification of *Rhizobium*.

	Species	Host
<u>Group 1. (fast growers):</u>		
	Rhizobium trifolii	*Trifolium*
	Rhizobium leguminosarum	*Pisum, Lens, Lathyrus, Vicia*
	Rhizobium phaseoli	*Phaselous vulgaris*
	Rhizobium meliloti	*Melilotus, Medicago Trigonella*
<u>Group 2. (slow growers):</u>		
	Rhizobium japonicum	*Glycine max*
	Rhizobium lupini	*Lupinus, ornithopus*
	Rhizobium spp.	*Vigna, Desmodium, Centrosema, Arachis, Stylosanthes, etc*

From Graham (1976).

Bradyrhizobium strains Vm1 and Vr16 were able to survive and grow at 48°C on agar plates.

1.8 Objectives of the present study

The research described in this thesis was carried out with the following questions in mind:-

1, Do the germination rates of different soybean cultivars vary with temperature? In particular, do cultivars from Pakistan germinate well at higher temperatures?

2, Do different pre-soaking periods applied to the seeds affect the germination rate?

3, At what temperatures are germination and root growth inhibited and how does this vary between the cultivars?

4, How do different growing media and temperatures affect plant growth?

5, Can chlorophyll fluorescence analysis detect differences between soybean cultivars with respect to their heat sensitivities?

6, Can chlorophyll fluorescence analysis be used to detect and quantify heat-hardening ability in soybean cultivars?

7, At what temperatures is *Bradyrhizobium* growth

inhibited when cultured in isolation from the host plant? Are the bacteria capable of heat hardening?

8, How does the rate of nitrogen fixation change with the age of the soybean plant?

9, Since acetylene is used as the substrate in nitrogen fixation assays, to what extent does adding acetylene to the root environment decrease the rate of nitrogen fixation by root nodules? Does repeated exposure to acetylene inhibit nitrogen fixation?

11, How does high temperature affect nitrogen fixation in nodules? Does this vary between cultivars?

12, Can nitrogen fixation rates recover when the plants are returned to ambient growing temperatures following heat stress? Are some cultivars able to recover more quickly than others? Can soybean plants acclimate to high temperatures?

13, Are the roots of soybean more heat sensitive than the leaves?

CHAPTER TWO

Chapter Two

Materials and Methods.

The materials and methods described in this chapter are the main ones used in the thesis. Other methods which were used occasionally, are described in the relevant experimental chapters.

2.1 Seed source and storage.

The seed of soybean cultivars Bragg, Century-84, Bossier and Steele 5/1 were supplied by Sindh Agriculture Research Institute, Oil Seed Section, Tandojam. Cultivars Williams-82 and Davis were supplied by Pakistan Agricultural Research Council (PARC), Islamabad, and cultivars Mago-80, Sable and Hardee were supplied by Pakistan Agricultural Research Institute, Peshawar, Pakistan. Before shipping from Pakistan, the seeds were treated with insecticide. During the period of study, they were kept at 5°C in large glass jars covered with a black polythene bag.

2.2 Source of gases.

2.2.1 Acetylene.

Pure acetylene gas (100%) was supplied by BOC Ltd. Worsley, Manchester, U.K. Before use, it was transferred to a 1000 cm^3 volumetric flask through a valve system.

2.2.2 Ethylene.

Pure (98.8%) ethylene gas was available commercially in

small cylinders supplied by BDH Ltd., Poole, U.K. The pure ethylene was transferred to volumetric flasks by a water displacement method. It was then diluted to known concentrations in air, using a syringe to transfer measured volumes of the gas to 100 cm^3 volumetric flasks fitted with rubber Suba Seals.

2.3 Source of bacteria.

The bacterium *Bradyrhizobium japonicum* RCR3407 was supplied by Dr. F.R. Minchin, Institute of Grassland and Environmental Research (IGER), Aberystwyth, U.K.

2.4 Glassware.

All glassware was washed in Decon detergent (Philip Harris Scientific England), rinsed in distilled water and dried for 48h in an oven at 50°C. The glassware to be used for *B.rhizobium* culture was also autoclaved before use. Other materials (threads and wooden sticks) were washed in Decon, rinsed and dried for 48h in an oven at 50°C.

2.5 Seed pre-soaking.

Experiments were conducted to check the germination of soybean cultivars after different pre-soaking periods. Seeds of the cultivars Bragg, Century-84, Bossier, and Steele 5/1 were soaked in distilled water at room temperature for 0, 24, 48 or 72h. They were then used for germination tests which involved either wrapping them in wet chromatography paper or putting them on wet

chromatography paper in Petri dishes (see Sections 2.6 and 2.7 below).

2.6 Germination of seeds wrapped in chromatography paper.

Seeds were placed on a (23 x 57 cm) filter paper sheet (Whatman No.1, Whatman International Ltd., Maidstone, England) in 10 rows with 5 seeds in each row. The filter paper had been sterilized by UV light for 30 min and moistened with 30 cm^3 of distilled water for 0h soaking treatments or with 20 cm^3 for other soaking times. After placing the seeds on the filter paper sheet, it was rolled up around a wooden stick (32 x 0.5 cm) and placed in a polythene bag. The top of the bag was folded and tied lightly to allow air exchange. Next, the bag and its contents was stood upright in a beaker and incubated in an incubator (Vindon Scientific Ltd., Oldham, U.K.) in the dark at either 10, 15, 20, or 25°C. After 96h of incubation, germinated (emergence of radicle by 1-2 mm) seeds were counted.

2.7 Germination of seeds in Petri dishes.

Germination was tested in Petri dishes as well as in paper rolls. Seeds were placed on filter paper in a Petri dish previously sterilized and moistened with 10 cm^3 of distilled water. Ten soaked seeds were placed in each dish and incubated at 10, 15, 20 or 25°C as described above. After 96h of incubation, germinated seeds were counted.

2.8 Effects of high temperature on germination, root growth and root fresh and dry weights.

Soybean cultivars (Bragg, Mago-80, Sable, Williams-82, Davis and Hardee) were used and the seeds were germinated by the rolled filter paper method described in Section 2.6. They were incubated in the dark for 48h at 25, 30, 35, 40, 42.5, or 45°C. After 24h, the bag was gently shaken to re-moisten the paper with water which had accumulated at the bottom of the bag. After 48h, the percentage germination (emergence of radicle by 1-2 mm) was recorded. After separating the roots from the cotyledons, root lengths and root fresh weights were determined. The roots were then placed in an oven (Leec Ltd., Nottingham, U.K.) at 65°C for 48h and their dry weight was measured.

2.9 Effects of high temperature on root growth and root fresh and dry weights following germination at 25°C.

Using the same method as above (Section 2.8), other batches of the same cultivars were used. The seeds were germinated for 24h at 25°C and then transferred to 30, 35, 40, 42.5, or 45°C for a further 24h to complete 48h of root growth. After that, the germination percentage, root lengths, root fresh weights and root dry weights were recorded as described in Section 2.8.

2.10 Effects of temperature and growth media on the growth and physiology of soybean.

This experiment was conducted in the glass house at the

Pen-y-ffridd Field Station. Seeds of Bragg and Century-84 cultivars were grown in pots (12 x 13 cm) filled with either soil, sand, vermiculite (Vermiperl Horticultural Grade, Sinclair Horticulture & Leisure Ltd., Lincoln U.K.) or compost (John Innes No.1) at 18, 20, or 25°C with a 15h photoperiod. The source of light was 400 watt sodium halide discharge lamps together with natural sun light. Five seeds of each cultivar were sown at a depth of 1-1.5 cm. The pots were irrigated with tap water and always kept moist. Seedlings emerged at up to 11 days after planting, and the plants were then thinned to one plant per pot. Plant height was recorded every 4th day.

The experiment was terminated 42 days after planting and the total number of trifoliate leaves, nodes, branches, hypocotyl lengths and shoot fresh weights were recorded. Also, the rooting system was washed carefully and the fresh weight was recorded. The shoot and root materials were then separated and placed in separate bags, dried for 48h in an oven at 70°C and their dry weights recorded.

In addition, chlorophyll fluorescence (as the Fv/Fm ratio) was measured (see Section 2.11 below) at 15, 22, 29, and 36 days after planting using a leaf fluorometer (Morgan Model CF-1000, Morgan Scientific Inc., Andover, U.S.A.). For these measurements, the first trifoliate leaf was clipped between the dark acclimation clips and fluorescence was measured 30 min later at 200 μmols m^{-2} s^{-1} light intensity,

after inserting the probe of the fluorometer into the acclimation clip.

2.11 Effect of heat stress on chlorophyll fluorescence in leaves.

Soybean cultivars (Mago-80, Sable, Bragg, Williams-82, Davis, and Hardee) were grown in a growth room in sterilized pots (8, 10 cm diameter) filled with pre-washed vermiculite (Vermiperl Horticultural Grade). The room was maintained at 24°C throughout with a 16h photoperiod (light intensity 82-85 μmols m^{-2} s^{-1}).

The pots were irrigated with half-strength (nitrogen-free) Long Ashton nutrient solution (Hewitt, 1966) every other day for 4 weeks. There were two batches of pots. One batch was inoculated with 3 cm^3 per plant of a culture of *B.rhizobium japonicum* RCR3407 (see Section 2.13.2) 5 days after germination. The other batch remained un-inoculated as a control.

After 4 weeks, the first trifoliate leaf was used to study the effects of high temperature on the leaves using the chlorophyll fluorescence method (Fv/Fm ratio). For this analysis, detached leaves from 14 nodulated plants as well as leaves from non-nodulated plants were placed into a sample holder (see Plate 2.1). A constant temperature plate was placed under the sample holder and connected to a circulating water bath to maintain a constant required

Plate 2.1 The Morgan CF-1000 chlorophyll fluorometer and leaf sample holder.

temperature. The sample holder consisted of an aluminum plate, covered with two layers of filter paper (Whatman No.1), moistened with 30 cm^3 of distilled water. Leaves in the sample holder were completely moistened, because any water stress may have altered the severity of the effects of temperature on the chloroplast thylakoids (Wilson, 1976). Thus, the leaves were placed onto the filter paper and covered by a layer of plastic film (Cling Film, local Safeways Supermarket), which was permeable to air but not to water. Finally, a plastic grid plate with 3.3 mm diameter holes was placed on top of the film, (The probe head of the fluorometer could be placed into these holes for the fluorescence measurements). The sample holder and the constant temperature plate were put into a black photographic bag for 30 min dark acclimation and the control chlorophyll fluorescence (Fv/Fm ratio) was then measured. The temperature of the sample holder was monitored by inserting the probe of an electronic thermometer under one of the leaves.

Heat stress treatments of 2 and 4h at 35, 40, 42.5, or 45°C were then given to the leaves by moving the sample plates and their contents (under a green safe light) to another constant temperature plate at the higher temperature. The chlorophyll fluorescence (Fv/Fm ratio) was recorded before the heat stress and after 2 and 4h of heat-stress treatment. After the heat stress, the plates were returned to the initial temperature (24°C) for recovery and

chlorophyll fluorescence was measured at 12, 24, and 48h into the recovery period.

A typical chlorophyll fluorescence induction curve is presented in Fig. 2.1. It shows a rapid almost instantaneous rise (Fo) to level O. There then follows a slower rise in fluorescence (Fv, variable fluorescence) to a peak at P (Fm). After the peak at P, fluorescence decreases towards a minimum at S. As a consequence of the relatively slow response time of the recording method used, a small peak which should appear at O was not distinguished in the present experiments with soybean leaves (see Fig. 2.1). Instead, irradiation of dark-adapted soybean leaves was seen as a rise directly from O to the maximal at P. The initial level O is reached very rapidly and represents the constant yield component (Fo), which is insensitive to any stress-induced damage that takes place in thylakoid membrane. The magnitude of the rise from O to P represents the stress-sensitive, variable fluorescence Fv (Fm-Fo).

In the present investigation, the ratio of Fv to Fm has been used to quantify thylakoid membrane damage for a variety of reasons. In particular, Kitajima and Butler (1975) and Somersalo and Krause (1989, 1990) reported that there is a close relationship between the Fv/Fm ratio and the photochemical efficiency of PS II. It is further stated that this ratio is directly proportional to the quantum yield of oxygen evolution (Oqiust and Wass, 1988).

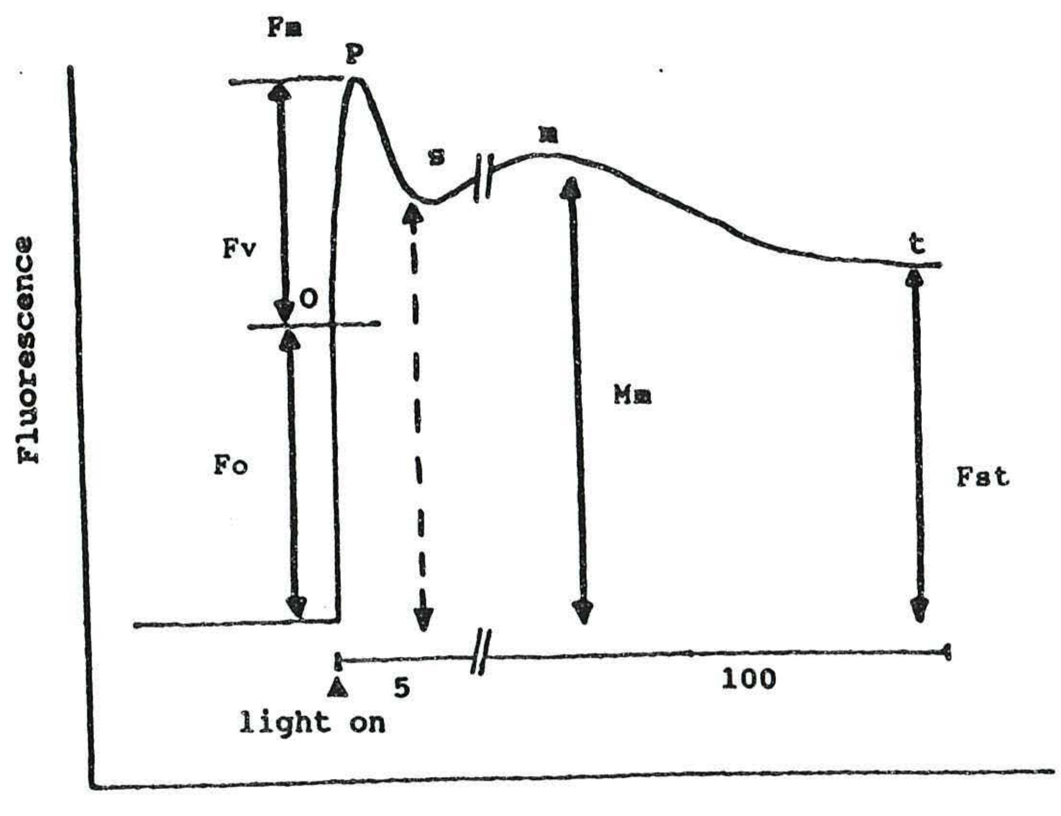


Figure 2.1 A typical fluorescence induction curve for a soybean leaf. Fo presents the initial increase in fluorescence to O. Fv is variable fluorescence between O and P. Fm is the maximal fluorescence level reached at P. Mmax is the maximum level of secondary fluorescence at m, and Fst is the steady state fluorescence at time t.

2.12 High temperature acclimation of leaves.

Seeds of the cultivar Williams-82 were grown in (10 x 5 cm) pots filled with vermiculite pre-washed with distilled water, similar to the method described in Section 2.11. In this experiment, however, the pots were incubated in a growth room (Vindon Scientific Ltd., Diggle, Oldham England) which was run with a 27/20°C ± 0.5°C day/night temperature regime and with a 16h photoperiod. The light intensity was 135-150 µmols m^{-2} s^{-1} and the humidity was 65-67%.

Leaves were detached from the plants and four types of treatments were then given to them as follows:

In the first treatment, three-week-old unifoliate leaves and first trifoliate leaves were detached and placed in a fluorometer sample holder at 27°C as described in Section 2.11 and placed into the black photographic bag. The control chlorophyll fluorescence (Fv/Fm ratio) was measured after 30 min dark acclimation. A heat-hardening treatment for 10 min at various temperatures was then given to the leaves and they were then returned to recover for 2h at 27°C. Two further 10 min hardening treatments were applied, each separated by 2h of recovery at 27°C. After the last heat hardening, a final 30 min stress was applied. The heat-hardening and stress temperatures were 40, 42.5, 45 or 47.5°C.

In the second treatment, 30 min of heat stress was applied directly to the leaves after measuring the control (Fv/Fm ratio) at 27°C. The same heat stress temperatures and a 2h recovery period at 27°C were applied as in the first treatment above.

In a third experiment, three 10 min hardening treatments were applied to the leaves at 42.5 or 45°C, separated by a 2h recovery period at 27°C. After the last hardening treatment, a further 30 min stress was applied at 47.5°C.

In the fourth experiment, four 10 min heat-hardening treatments were applied at 45°C, returning them to recover for 2h at 27°C between treatments. Finally, a 30 min stress was applied at 47.5°C.

2.13 Production of nodules in the soybean roots.

No nodulation occurred in the soybean cultivars which originated from Pakistan unless they were inoculated with *B.rhizobium japonicum*. It was therefore necessary to make a liquid culture of the bacterium for use as an inoculum.

2.13.1 Preparation of the *B.rhizobium japonicum* inoculum.

The liquid culture medium contained 10 g mannitol, 1 cm^3 of 10% K_2HPO_4, 4 cm^3 of 10% KH_2PO_4, 2 cm^3 of 10% $MgSO_4$, 1 cm^3 of 10% NaCl, and 0.4 g of yeast extract in 1 litre of distilled water, pH 7.0, contained in 2 X 500 cm^3 bottles. The bottles and their contents were autoclaved for 20 min

at 15 psi. After cooling, the medium was inoculated with *B.rhizobium japonicum* RCR3407 (Approximately 1g of stock sample to each bottle) and kept in an incubator for 10 days at 25°C.

2.13.2 Plant inoculation.

Two methods were used for inoculation. In the first method, seeds were soaked for 30 min in *B.rhizobium japonicum* inoculum and then grown in prewashed vermiculite in pots at 24°C. After 5 days of germination, 3 cm^3 of inoculum was applied directly around the roots of each plant. Plants were checked for nodulation 4 weeks after this second inoculation.

In the second method, seeds were sown without the pre-soaking in the rhizobial inoculum. The seeds were planted directly into vermiculite under the same conditions as described above and 3 cm^3 of inoculum were applied around the roots 5 days after seedling emergence.

2.14 Effects of high temperature on the growth of *B.rhizobium japonicum* RCR3407.

In the first experiment, culture medium was prepared and autoclaved as described in Section 2.13.1. Then 10 cm^3 volumes of the medium were transferred to McCartney bottles and again autoclaved. The pH was checked and readjusted to pH 7.0 if necessary. After cooling, the bottles were inoculated with 0.1 cm^3 of a 6-week-old stock culture and

transferred to water baths at 25, 35, 40 and 45°C for the heat treatments. Un-inoculated media (controls) were kept for the same time at each temperature. At 12, 24, 36 and 48h after inoculation, the growth (as optical density) was determined at 600 nm using a spectrophotometer (Jenway Model 6100, Philip Harris Scientific, England). At the same time, the pH was measured.

In a second experiment, the culture media were made up, autoclaved, inoculated and incubated as described above. At 1, 2, 3, 4, 5, 6 and 7 days after inoculation, the bacterial growth (measured as optical density) and pH were measured.

A further experiment was carried out to study the possible heat hardening of *B.rhizobium japonicum*. Cultures of *B.rhizobium japonicum*, which had been grown at 40°C or 45°C, were used to inoculate new media (0.1 cm^3 to 10 cm^3 of fresh medium). These media were then incubated at 40°C or 45°C in a water bath. Growth (as optical density) and pH were measured after 12, 24, 36 and 48h of incubation.

2.15. Determination of nitrogen fixation.

2.15.1 Plant material.

Some preliminary experiments were conducted to determine nitrogen fixation by the root nodules of soybean plants. Seeds of the cultivar Sable were soaked for 30 min in *B.rhizobium japonicum* inoculum and germinated in (10 x 5

cm) pots (2 seeds per pot) filled with vermiculite that had been pre-washed with distilled water. The pots and their contents were incubated in the growth room. The growth room was set at 24°C ± 0.5°C with a 16h photoperiod. The light intensity in the room was 82-85 μmols m^{-2} s^{-1}. Five days after sowing, the seedlings were thinned to one per pot. Plants were irrigated with half-strength (nitrogen free) Long Ashton nutrient solution (Hewitt, 1966). One-week-old plants were inoculated with 3 cm^3 of *B.rhizobium japonicum* inoculum as described in Section 2.13.2.

2.15.2 Acetylene reduction assay.

To determine nitrogen fixation rates, the acetylene reduction method was used (Hardy, *el at.*, 1968; Habte, 1983; Masterson and Murphy, 1980). The acetylene reduction method is cheap, easy and non-destructive to use. In this method, acetylene is reduced to ethylene by the nitrogenase complex in the root nodules and the ethylene product is measured. It involved injecting 30 cm^3 of pure acetylene gas into the pot contained in a closed container fitted with two Suba Seals (Plate 2.2) to give a concentration of approximately 10% acetylene inside the pot. (The pot size was 10 cm high by 5 cm diameter, and its inside capacity was 350 cm^3). Before injecting the gas, a syringe needle was put into Suba Seal No. 1 to allow the extra pressure to escape when 30 cm^3 of acetylene was added. When all the acetylene had been injected, both the injection needle and the vent needle were withdrawn leaving a sealed pot. At 10,

Plate 2.2 The method used to seal and add acetylene into pots.

20 and 30 min after acetylene injection, 1 cm^3 samples were taken from the pot using an airtight syringe and analysed for their ethylene content by gas chromatography (GC) (see Plate 2.3). The GC machine (Sveriges, Swedish University of Agri. Sciences, Stencil, Sweden) was fitted with a Dura-pack column and run with 50 cm^3/min of fresh air as the carrier gas supplied by a small air pump. The oven temperature was 45°C. The apparatus was calibrated with 4 X injections of 0.5 cm^3 of a 100 ppm ethylene standard before unknown samples were analyzed. The ethylene peaks were recorded by a chart recorder (Kipp & Zonen, Talbot Scientific Ltd., Alderley Edge, U.K.) connected to the GC. After every 20 injections, the silicon septum of the GC was changed.

After measuring its nitrogen fixation rate, the rooting system was removed from the pot and washed carefully. All root nodules were separated from the roots and counted. Their fresh weight was then determined and recorded. Next, the nodules were transferred into small paper bags and kept in the drying oven at 70°C for 48h before measuring their dry weight.

2.15.3 Effect of heat stress on nitrogen fixation in root nodules.

An experiment was conducted to investigate the effects of heat stress on nitrogen fixation by nodulated roots. Six-week-old plants of the cultivar Sable, grown and inoculated

Plate 2.3 Portable gas chromatograph for determination of nitrogen fixation rates.

as described in Section 2.15.1, were used. Heat stress at 40°C for 0, (control) 2, 4 and 6h was applied in a shaking water bath (Grant, Instrument Ltd., Cambridge, U.K.). For these treatments, the pots were covered by a plastic lid which had two holes, one for the plant shoot and the other for injecting and withdrawing gas samples. The pots were sealed with electrician water-proofing compound (Centaure MFG, Brass Division, Park Farm Ind. Est. Redditch, Worcs, England) and transferred to the water bath for the heat stress treatment. The start of the heat stress was measured 30 min after transfer to the water bath, because it took this period for the required temperature to be reached inside the pots. Temperatures inside the pot were monitored by inserting a thermocouple probe into the vermiculite. After heat treatment, the pots were removed from the water bath and nitrogen fixation was measured.

In another experiment, seeds of five cultivars (Mago-80, Sable, Bragg, Davis and Williams-82) were grown in a growth cabinet as described in Section 2.12. The seeds were soaked for 30 min in a *B.rhizobium japonicum* inoculum (Section 2.13.2). Two seeds were sown in each pot. Seven days after sowing, the seedlings were thinned to one per pot and inoculated again with 3 cm^3 of *B.rhizobium japonicum* inoculum. All the pots were irrigated with half-strength Long Ashton nutrient (nitrogen-free) solution.

To investigate the effects of heat stress applied directly

to the roots, six-week-old plants were used. Heat stress was applied for 2, 3 or 4h at 35, 40 or 45°C in the water bath as described above. Immediately after the heat stress treatments and after 72h into recovery at 27°C, the nitrogen fixation rate and fresh and dry weights were determined as described in Section 2.15.2.

2.15.4 Effects of heat stress applied to the leaves.

An experiment was also conducted to investigate the effects of heat stress applied to the leaves on nitrogen fixation by the root nodules. Soybean cultivars (Mago-80 and Bragg) were grown and inoculated as described in Sections 2.12, 2.13.2. Six-week-old plants were used. Heat stress was applied at 35, 40 or 45°C for 2, 3 or 4h in the water bath. For this, the pots were covered and sealed (Section 2.15.3) and the shoots were dipped into the water bath. Leaves reached the temperature of the water within 3 min of immersion. After the stress, the shoots were removed from the water bath and kept for 5 min at room temperature. Determinations of nitrogen fixation and fresh and dry weights were then carried out as described in Section 2.15.2.

CHAPTER THREE

Chapter Three

Germination of Soybean Cultivars.

3.1 Introduction.

This chapter evaluates the effects of different constant and changing high temperatures and soaking periods on the germination, root growth and root fresh and dry weights of several soybean cultivars from Pakistan. Soybean seeds germinate under favourable conditions of water supply, temperature, soil aeration and soil type. If these factors are not optimum they will have adverse effects on germination, growth and yield. Soybeans have generally been found to have the fastest rate of germination and emergence between 25 and 35°C (Edwards, 1934; Delouche, 1953; Hatfield and Egli, 1974). For example, Shupert (1971) found a higher germination percentage in a growth chamber at 27/21°C (day/night) temperature (14h photoperiod) than at 21/16°C or 16/10°C. Inouye (1953) reported a maximum temperature for germination of 34 to 36°C and a minimum temperature for germination of 6 to 7°C was reported by Enken (1959). Other factors can also affect germination. Differences among genotypes in the effects of temperature on germination have been reported by Emerson and Minor (1979). Also, Keigley and Mullen (1986) found that high temperatures (32/28°C day/night) during seed filling in well-watered plants grown in a growth chamber reduced subsequent seed germination. The linear response they measured indicated that the number of days of high

temperature, even under well-watered conditions, is important in determining the extent of high temperature injury to seed germination and subsequent seedling vigour.

Maximum soybean seed yield depends to a large extent upon an extensive, well-nodulated rooting system, the development of which is enhanced by an ample supply of water and nutrients from the soil and energy from respired assimilates (Hicks, 1978). Earley and Cartter (1945) observed that the greatest root weights of green-house, gravel-grown, soybeans occurred at temperatures between 27 to 32°C, although differences were small for temperature variation between 12 and 37°C. A change in root temperature affects the reaction rate of metabolic functions within the root (Nielsen, 1974).

In the field, soybeans deplete soil water to the same depth as root penetration during the early part of the growing season, while later in the season water can be depleted to a depth 15 cm greater than root penetration (Stone, *et al.*, 1976). A small portion of the root system may be responsible for much of the water uptake (Reicosky *et al.*, 1972). The dry weights of shoots and roots and the fresh weight of the root nodules all decreased as root temperature increased above 28°C (Munevar and Wollum II, 1982).

3.2 Methods.

3.2.1 Effects of different soaking periods on seed germination.

Soybean seeds of cultivars Bragg, Century-84, Bossier and Steele 5/1 were soaked in distilled water as described in Section 2.5. They were then germinated in rolled filter paper in the dark at 10, 15, 20 or 25°C as described in Section 2.6.

Another experiment on the above cultivars and using the same soaking periods (Section 2.5) was conducted using glass Petri dishes for germination, as described in Section 2.7. These were also incubated in the incubator at 10, 15, 20 or 25°C.

3.2.2 Effects of temperature on germination, root growth and root fresh and dry weights.

Unsoaked seeds of soybean cultivars Bragg, Mago-80, Sable, Williams-82 and Hardee were wrapped in wet chromatography paper as described in Section 2.6. The rolls were transferred to incubators at 25, 30, 35, 40, 42.5 or 45°C for germination. At 48h from the beginning of incubation, the germination percentage, root length and root fresh and dry weights were recorded. For details see Section 2.8.

3.2.3 Effects of temperature on germination, root growth and root fresh and dry weights of pre-germinated seeds.

Using the same cultivars as above and the method described

in Section 2.6, seeds were germinated for 24h at 25°C and then transferred directly to 30, 35, 40, 42.5 or 45°C for a further 24h. The germination, root length and root fresh and dry weights were recorded as described in Section 2.8.

3.2.4 Statistical analysis.

All means, standard deviation and standard error values were determined using a pocket scientific calculator (Sharp Model EL-531P) and checked using a personal computer (Mitac) with the Minitab statistical package (version 10.2). The analysis of variance (ANOVA) was also done by Minitab. The figures were prepared using the Systat/Sygraph software package (version 5.03, Systat Inc., Evanston, IL., U.S.A). In all figures vertical bars show the standard errors of the means.

3.3 Results.

3.3.1 Effects of different soaking periods on seed germination in rolled filter paper.

The effects of different soaking periods and temperatures on the germination of soybean cultivars were tested. The results for the cultivar Bragg, presented in Fig. 3.1, show that, after 96h in the filter paper roll, there was a high (93-95%) germination at 15, 20 and 25°C with 0h soaking. Zero germination was initially recorded at 10°C, but it increased with increasing soaking periods of up to 72h. In general, however, germination increased with increasing temperatures but decreased with longer soaking periods.

This is demonstrated best by the cultivar Century-84 (Fig. 3.2) which had rapid germination at high temperatures, but it decreased at all temperatures with increasing soaking periods. The germination in cultivar Steele 5/1 (Fig. 3.3) followed a similar pattern to that of Century-84 and, in general, germination in this cultivar was even more inhibited than in cultivars Century-84 and Bragg by longer periods of soaking. The poorest germination was found in the cultivar Bossier (Fig. 3.4), in which zero germination was observed after 96h at 10°C with all soaking periods, while it was highest (10%) at 15°C. Germination in Bossier was severely affected at all temperatures by increasing soaking periods of 24, 48 and 72h.

3.3.2 Effects of different soaking periods on seed germination in Petri dishes.

The germination of the same four cultivars as above was also tested in Petri dishes. The results for cultivar Bragg are presented in Fig. 3.1. The data show that the highest germination (96%) at 25°C was observed after 48h following 0 and 24h pre-soaking periods. Also, rapid (47 and 57%) germination was found after 24h at 20 or 25°C following a 0h pre-soaking period (data not shown). The best germination was observed at 15°C with all soaking periods, but it decreased with increasing soaking periods at high temperatures. The germination of cultivar Century-84 was best at 15, 20 and 25°C after 0h soaking and decreased for all germination temperatures with increasing soaking

periods (Fig. 3.2). Cultivar Steele 5/1 (Fig. 3.3) showed poorer germination at all temperatures and soaking periods compared to Bragg and Century-84. There was no germination at 10°C following 24, 48 and 72h pre-soaking periods. The poorest germination was shown by cultivar Bossier (Fig. 3.4), which showed very low values at all temperatures and soaking periods.

Statistical analysis by ANOVA showed that germination was strongly dependent ($P<0.05$) upon the cultivar, temperature, soaking period, and the choice of environment (Petri dish or paper roll method).

3.3.3 Effects of constant temperatures on germination and root growth.

Laboratory experiments were conducted to investigate the effects of high temperatures on the germination and root growth of six cultivars of soybean in wetted filter paper rolls (this method gave straighter roots than the Petri dish method and they were therefore easier to measure). Pre-soaking treatments were not employed. In the first experiment, seeds were germinated for 48h at different constant temperatures between 25 and 45°C and the germination percentage, root length, and root fresh/dry weights were recorded. In the second experiment, after germination for 24h at 25°C the seeds were transferred to 30, 35, 40, 42.5, or 45°C for a further 24h, after which the same parameters as above were recorded.

3.3.3.1 Germination.

The results show that, in the cultivar Williams-82 (Fig. 3.5a), 100% germination was recorded at 30°C and slightly less at 35 and 40°C, but at 42.5°C there was a sharp decrease to only 22% germination. Complete (100%) germination was found in cultivar Sable at 25°C (Fig. 3.6a). Germination in this cultivar decreased with increasing temperatures at approximately the same rate as for cultivar Hardee (cf. Fig. 3.10a). Cultivar Mago-80 (Fig. 3.7a) had greater than 95% germination at 25, 30, and 35°C and maintained 88% germination at 40°C but it fell to 13% at 42.5°C. Highest germination in cultivar Bragg was recorded at 25°C (Fig. 3.8a) and this decreased with increasing temperatures and it had the lowest germination (43%) of all six cultivars at 40°C. In cultivar Davis, similar germination to Williams-82 was found at 25, 30, and 35°C, but it decreased more than Williams-82 at 40°C, and at 42.5°C no germination was recorded (Fig. 3.9a). Finally, in cultivar Hardee similar germination rates occurred at 25 and 30°C, declining at high temperatures although 68% germination was still observed at 40°C (Fig. 3.10a). There was thus a sharp reduction in germination at 42.5°C in all cultivars with Davis, Bragg, and Hardee showing the greatest decrease. Zero germination was observed in all cultivars at 45°C.

The data were analysed using the ANOVA test, which showed that germination was strongly dependent ($P<0.05$) upon the

temperature and the cultivar.

3.3.3.2 Root length.

The effects of constant germination temperature for 48h on root growth in the cultivar Williams-82 are presented in Fig. 3.5b. Root lengths of 3.8, 4.7 and 4.2 cm were obtained at 25, 30 and 35°C respectively and this reduced sharply to 1.25 cm at 40°C. In the cultivar Sable (Fig. 3.6b), the data show that the longest root length (5.4 cm) was observed at 35°C with shorter lengths of 2.8 and 4.2 cm found at 25 and 30°C respectively. It declined sharply to 1.1 cm at 40°C. In the cultivar Mago-80, the shortest (1.2 cm) and longest (5.4 cm) root lengths were observed at 40 and 30°C respectively (Fig. 3.7b). A similar pattern was found in cultivar Bragg (Fig. 3.8b), where the longest (6.0 cm) root length was recorded at 30°C, reducing sharply to 1.0 cm at 40°C. The data in Fig. 3.9b show that, in cultivar Davis, root length increased with increasing temperatures of 25, 30 and 35°C, followed by a sharp reduction at 40°C. The root length data for cultivar Hardee gave a similar pattern to Mago-80. The longest root length (4.8 cm) was recorded at 30°C, decreasing at other temperatures especially at 40°C (Fig. 3.10b). Because there was zero germination in all cultivars at 42.5 and 45°C, no root lengths could be measured for these temperatures.

Statistically there was no significant difference ($P>0.05$) between the root length of the different cultivars, but

root length was dependent ($P<0.05$) upon temperature in all cases.

3.3.3.3 Root fresh and dry weights.

The data for root fresh and dry weights of cultivar Williams-82 are presented in Fig. 3.5c. These results show that the optimum temperature for root growth was between 30 and 35°C. A sharp reduction occurred at 40°C. The same pattern was found in the cultivar Sable (Fig. 3.6c). Figure 3.7c shows that the roots of cultivar Mago-80 had a high (140 mg) fresh weight at 30 and 35°C, but it was greatly reduced at 40°C. In cultivar Bragg (Fig. 3.8c), a similar pattern was found in root fresh weight, agreeing well with the changes in root length. The root fresh weight of cultivar Davis increased with increasing temperatures, but it was greatly reduced at 40°C (Fig. 3.9c). In cultivar Hardee (Fig. 3.10c), the highest fresh weight (120 mg) was obtained at 30 to 35°C and the lowest (23 mg) was observed at 40°C. There were no values for fresh and dry weights at 42.5 and 45°C due to zero germination.

The same patterns as those for root fresh weights were observed in root dry weight in all cultivars (Fig 3.5d - 3.10d).

Both the fresh weights and dry weights were strongly dependent ($P<0.05$) upon both the cultivar and the temperature.

3.3.4 Effects of germination temperature on pre-germinated seeds.

In this experiment, seeds were germinated at 25°C for 24h and then transferred to higher temperatures of 30, 35, 40, 42.5, or 45°C for a further 24h. Germination, root length, and root fresh and dry weight were then recorded.

3.3.4.1 Germination.

The results in Fig. 3.5a show that the cultivar Williams-82 gave 100% germination at 30, 35, and 40°C and 90% germination at 42.5 and 45°C. This contrasts markedly with the last experiment where only 22% germination occurred at a constant 42.5°C and zero germination was obtained at 45°C. In the cultivar Sable, 100% germination occurred at 25°C and this decreased slightly (by 2-3%) at 35 and 40°C. A further reduction to 87% was found at 42.5 and 45°C (Fig. 3.6a). The cultivar Mago-80 (Fig. 3.7a) showed 97 to 100% germination at 25, 30, 35 and 40°C. It declined to 75% at 42.5°C, but it increased again to 85% at 45°C. The cultivar Bragg gave 97% germination at 25 and 35°C, and decreased to 83% at higher temperatures (Fig. 3.8a). The highest germination (97%) was at 25°C and lowest (65%) was recorded at 45°C in the cultivar Davis (Fig. 3.9a). The germination of cultivar Hardee (Fig. 3.10a) decreased from 95 to 67% with increasing temperatures from 25 to 45°C.

The data were analysed using the ANOVA test, which showed that germination was strongly dependent ($P<0.05$) upon both

the cultivar and the temperature.

3.3.4.2 Root length.

Root growth of the cultivar Williams-82 seedlings germinated at 25°C for 24h and then transferred to high temperatures is presented in Fig.3.5b. The data show that the longest root length (4.95 cm) was obtained at 30°C and it declined (down to 1.3 cm) with increasing temperatures. Similar patterns were found in the other cultivars (Bragg, Davis, Mago-80, Sable and Hardee), which also gave the best root lengths at 30°C and declined at higher temperatures (Figs. 3.6b - 3.10b). The longest root length (5.2 cm) was recorded in cultivar Bragg at 30°C and the shortest (0.89 cm) was in cultivar Hardee at 45°C.

The data for root length were highly significant ($P<0.05$) for both the cultivar and the temperature.

3.3.4.3 Root fresh and dry weights.

Similar patterns to those for root lengths were found in both root fresh and root dry weights. The data are presented in Figs. 3.5c,d - 3.10c,d. The root fresh weight was maximum at 30 to 35°C and lower at 25°C. The highest root fresh weight (140 mg) was observed at 30°C in Bragg and Sable cultivars, while the lowest (97.0 mg) was in Davis. These values decreased by up to 40% at 40°C and a further reduction was found with increasing temperatures in all cultivars. Also, similar patterns were found in the

root dry weights of all the cultivars, the dry weight being approximately 9 to 11 times less than the fresh weight values in all cases. The greatest root dry weight (13.0 mg) was recorded in cultivars Williams-82 and Bragg at 30°C.

Both the root fresh and dry weights were strongly dependent ($P>0.05$) upon the cultivar and the temperature.

3.4 Summary and Discussion.

The germination of soybean cultivars was compared at various temperatures by the rolled paper and Petri dish methods, and with different pre-soaking periods. The results showed that the best germination occurred in the cultivar Bragg at all temperatures, whether in the rolled paper or Petri dish, and the most rapid germination was obtained at 25°C. However, there was no significant effect of the soaking period on the final germination percentage of cultivar Bragg, although it declined with prolonged (72h) pre-soaking. Seeds did not germinate in the paper roll after 24h of incubation, while some germination occurred in the Petri dish. Hatfield and Egli (1974) and Edwards (1934) have reported that the seeds of each plant species appear to have their own defined temperature requirement for germination. Soybean will germinate in the range of 10-40°C, but maximum rates of emergence occur at 25-30°C. Also in agreement with the present results, Matthews and Hayes (1982) reported that both germination and emergence in soybean were very slow at 10°C and below.

In soybean, seed vigour is influenced by temperature. As vigour, defined as the speed of germination, increases, the minimum temperature for germination decreases (Jordan, 1975). At sub-optimal temperatures, the rates of germination and hypocotyl-radicle elongation are reduced (Delouche, 1953) and the prolonged pre-emergence phase may predispose the seed to attack by soil pathogens (Went, 1961; Hegarty, 1972), reducing emergence percentage. Also, Bharati *el al.* (1983) reported that, for soybean under controlled laboratory conditions, pre-soaking at 31°C hastens the rate of and the synchronization of germination at 10°C and in less vigorous seed lots it increases the final germination percentage. These vigour differences may arise from differences in membrane integrity which determine whether the seed deteriorates or activates repair mechanisms at different hydration levels (Hegarty, 1978). Also, Holmberg (1973) and Littlejohns and Tanner (1976) have reported that differences have been found to exist between cultivars with regard to the rate of seedling emergence at low temperature.

In addition to temperature, moisture and aeration play an important role in seed germination. However, with an adequate supply of soil moisture and oxygen, the water uptake and the rate and percentage of germination of viable seeds is mainly dependent on soil temperature. The poorest germination was found in cultivars Steele 5/1 and Bossier, which were more severely affected by the length of pre-

soaking periods rather than by low temperature. The germination of these cultivars was slower at low temperatures compared to those of Bragg and Century-84. It is possible, therefore, that the germination of Steele and Bossier might be affected by poor seed vigour and possibly by the degree of maturity of the seed. In general, differences were also found between the paper roll and Petri dish methods for testing seed germination. Mason *et al.* (1982) suggested that increasing levels of mechanical damage significantly decreased the percent laboratory germination of the seed and generally decreased field emergence. Mederski (1983) has reported that the first process to occur prior to germination is the absorption of water by the seed followed by increased metabolic activity and finally the emergence of the radicle. In his studies, soybean, corn, sugar beet and rice were germinated in a range of soil moisture conditions and he found that the minimum moisture content for germination was 30% for corn, sugar beet and rice and 50% for soybean. Also, Hunter and Erickson (1952) reported that, at soil moisture contents too low to ensure germination, the seed may imbibe some water making them susceptible to fungal attack and eventual decay in several species.

The results in this chapter reveal that germination, root growth and root fresh and dry weights were affected by constant high temperatures up to 48h. All the cultivars gave their best germination after 48h at 25, 30 or 35°C.

Germination was significantly inhibited at 40°C, however, in all cultivars and it was severely affected at 42.5°C. No seed germinated at 45°C. The poorest germination at 42.5°C was found in Williams-82, Sable, Mago-80 and Bragg cultivars, while the cultivar Davis did not germinate at all at this temperature. This was probably due to heat shock damage to the tissues. These results are supported by Wallace (1988) who found that seedling emergence in soybean decreased with increasing temperatures above 37°C, with virtually no emergence at 40°C. Similar results to ours have been obtained by Aquino and Bekendam (1969) and Tyagi and Tripathi (1983), who reported that most soybean cultivars germinate rapidly between 25 and 35°C, while some cultivars don't germinate at 40°C (Hatfield and Egli, 1974). High temperature causes a change in the structure of the seed coats, thus causing changes in permeability (Mayer and Mayber, 1975). In this content, Emerson and Minor (1979) have reported that some soybean genotypes have variable responses to high germination temperatures. They also reported that delayed harvest influences the tolerance of the resulting seeds to this stress, presumably by negative effects of the delayed harvest on seed quality and vigour. It is important to note that, in agricultural practices, heat stress is often suspected when poor seedling establishment occurs in late-planted crops. This is thought to be due to rising soil temperatures, which are known to occur as the season progresses.

Germination was not so severely affected at high temperature in seeds pre-germinated at 25°C. The final germination of cultivars Williams-82, Sable and Mago-80 was even better at 45°C, although it declined in Bragg and Hardee with increasing temperatures. These results show that soybeans germinated at optimal temperatures can then be grown at higher temperatures. The effects of high temperatures during germination are manifest as sharply decreased hypocotyl elongation (Hatfield and Egli, 1974), increased seedling abnormalities (Aquino and Bekendam, 1969) and finally failure to germinate. At 40°C, seedling abnormalities include abnormal development of the primary leaves and a scarcity of secondary roots. Arndt (1945) has reported that heat damage to the seed cells prevents germination in cotton at high temperatures. The fact that a delay in harvesting soybean results in a loss of viability has been previously substantiated by Ellis and Sinclair (1976) and Wilcox *et at*, (1974). Their results agree with those of Tyagi and Tripathi (1983). Presumably, these various forms of damage are reduced in the seeds pre-germinated at 25°C. In general agreement with these findings Emerson and Minor (1979) reported that, in a controlled cabinet, the soybean percentage germination decreased with increased duration of the high temperature exposure.

The results reported in this chapter also reveal that root growth is affected by high temperatures. Root growth in all

the cultivars was maximal at 30°C and it was not significantly changed at a constant 35°C. Root growth was severely affected at 40°C however. Although some seeds of Williams-82, Bragg, Sable and Mago-80 cultivars germinated at 42.5°C their roots were severely damaged, while in cultivar Davis there was no germination. The root lengths were generally shorter in seedlings subjected to a change in temperatures than in seeds germinated at constant temperature. At 30°C in cultivar Williams-82 and Sable, however, root growth was better under the changing temperature regime than at constant temperatures. It is clear that high temperature has a marked effect on root growth. Up to 35°C, each cultivar showed an increase in root length with increasing temperature. There was no further increase in length when temperature stress was raised to 45°C. At 42.5 or 45°C the roots became yellowish and root tips were damaged or became necrotic in all cultivars. This was probably due to severe damage and even the death of cells due to loss of turgor and the resulting contraction of tissue. Root length following transfer from 25 to 30 or 35°C was less than in seedlings incubated at a constant 30 or 35°C. However, the seeds transferred from 25°C to temperatures between 40 and 45°C showed very significant root growth, whereas those germinated at a constant 42.5 and 45°C showed no root growth at all as they failed to germinate at these high temperatures.

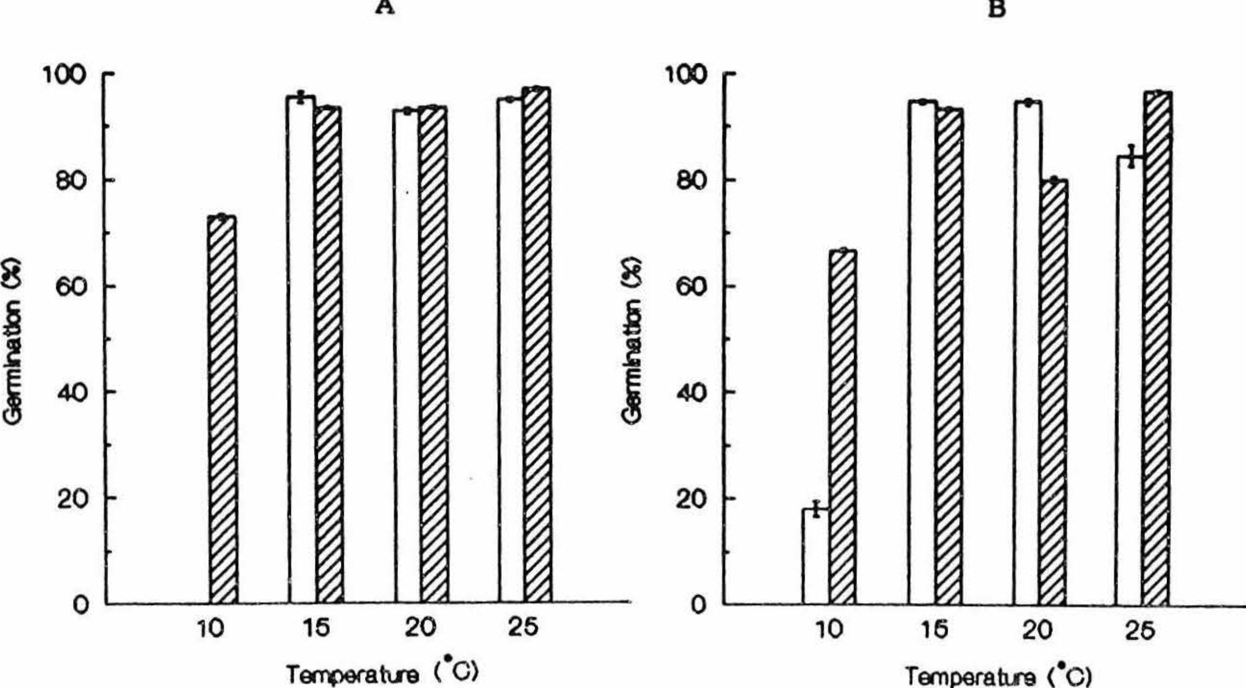
Root growth is obviously affected by environmental factors

such as temperature, moisture conditions, aeration and supply of nutrients. Also, poor aeration limits plant growth if the supply of oxygen to the root system is less than that required for respiration. Even short periods of anaerobic conditions in the soil irreparably damage the root system of many plants (Letey *et al*, 1961). Cannon (1925) provided evidence that the critical oxygen concentration for root growth is dependent on temperature. He found normal root growth for a large number of plant species at 10% oxygen when the temperature was 18°C, but at 30°C 10% oxygen was not sufficient to maintain normal root growth. These results are supported by Abdul Majid and Osman (1977), who reported that the germination and root growth of cotton ceased below or above the range of 15-45°C.

Similar patterns to those for root length were also observed in the root fresh and dry weight data for all the cultivars. Thus, root fresh weights were higher where longer root lengths were recorded either at constant or changing temperatures. Thus, fresh and dry weights were low at 25°C and higher at 30 or 35°C. Root lengths were reduced by high temperatures, so root fresh and dry weights were reduced with increasing temperatures above 35°C. Our findings support those of Salim and Saxena (1991) on rice and Adelusi and Lawanson (1978) on maize and cowpeas. In agreements with these findings Earley and Cartter (1945) reported that the greatest root fresh/dry weight in green-

house gravel-grown soybean occurred at temperatures of 27 or 32°C, although the fresh weight differences were small for temperatures between 12 and 37°C, and there was only a slight effect on dry weight. It has also been recorded that root growth in soybean is restricted by high temperatures (Munevar and Wollum, 1981, 1982).

In conclusion, the optimal temperature for the germination of soybean is between 25 and 30°C. Germination and seedling development are inhibited at temperatures above 40°C. Pre-germination for 24h at 25°C allows germination and root development to proceed at 42.5 and 45°C in the six cultivars tested, presumably by allowing temperature-sensitive events during the early stages of imbibition to proceed. For example, the formation and reorganisation of membranes during imbibition could be prevented by high temperatures and lead to the delay or failure of germination. Adaptation during germination at 25°C would allow this membrane development to take place so that the seedlings becomes more heat tolerant.



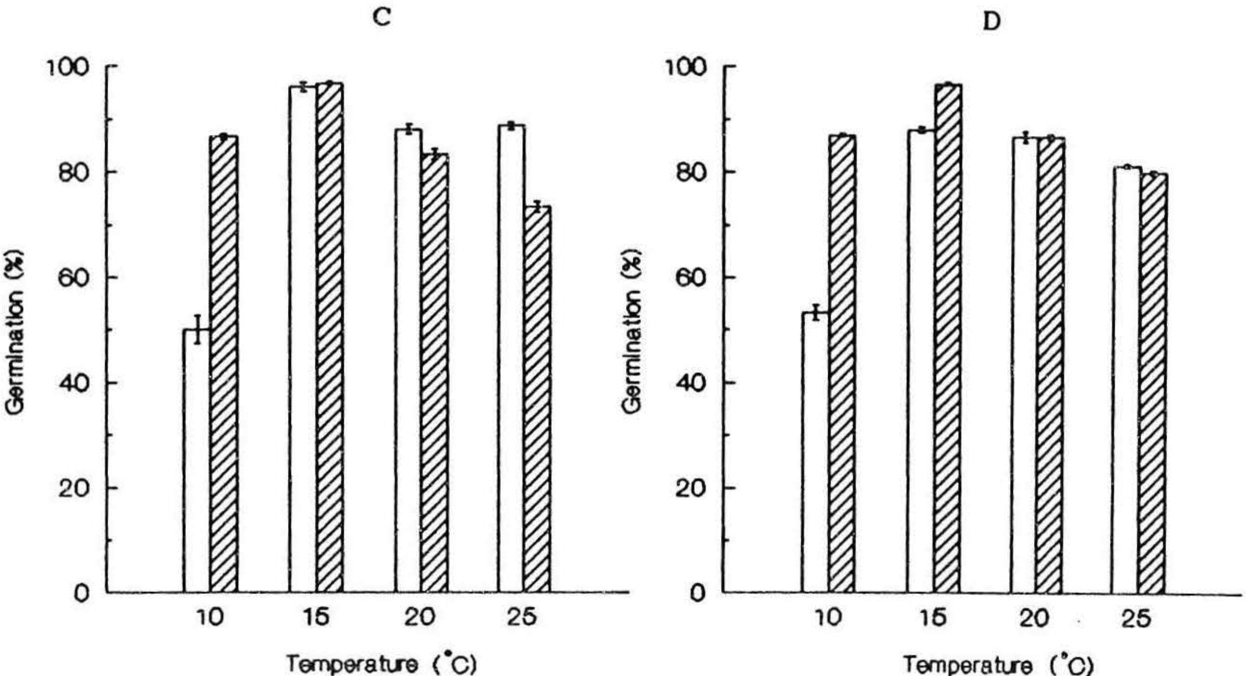
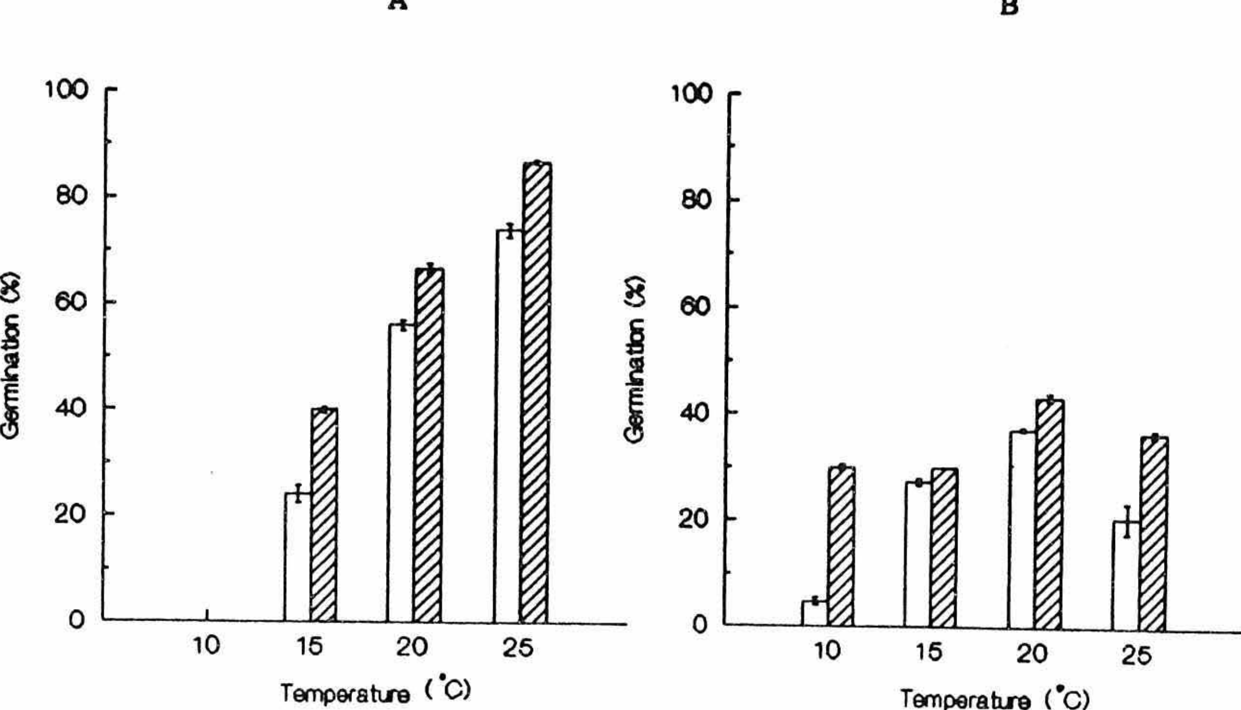


Figure 3.1 Effects of temperature and pre-soaking period on the germination at 96h of cv. Bragg. A, 0h; B, 24h; C, 48h; D, 72h pre-soaking; ▭, in filter paper; ▨, in Petri dishes.



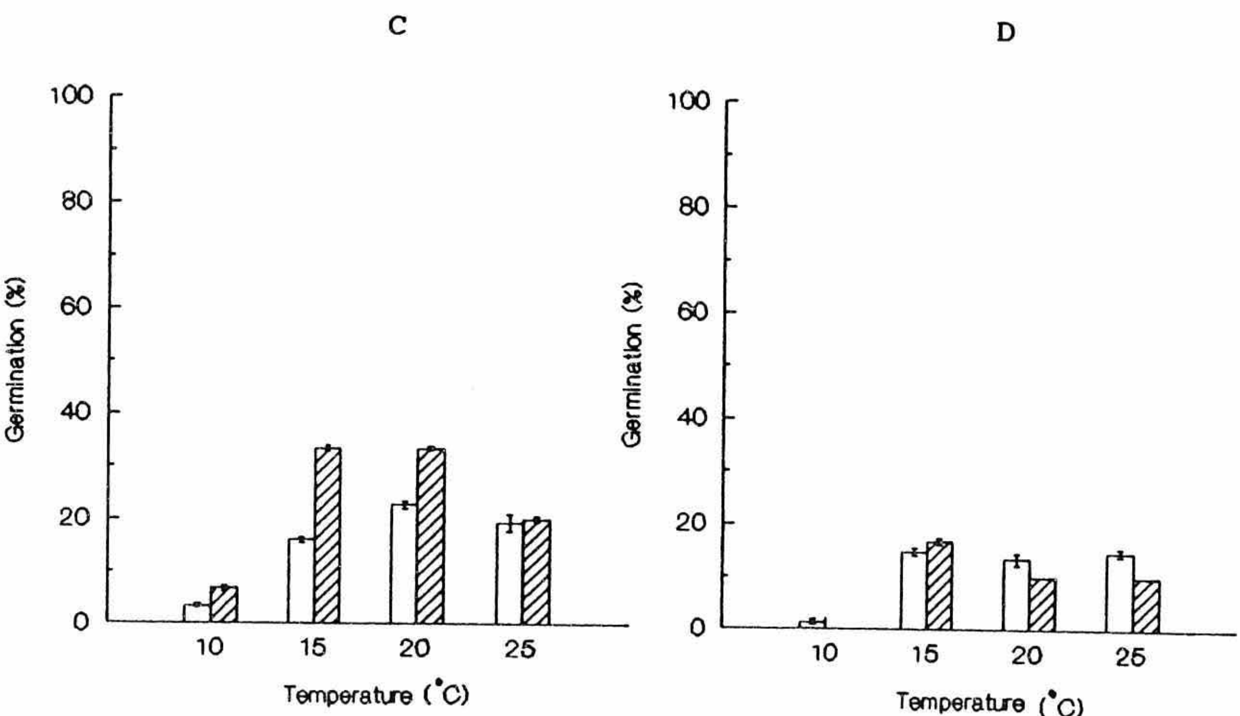
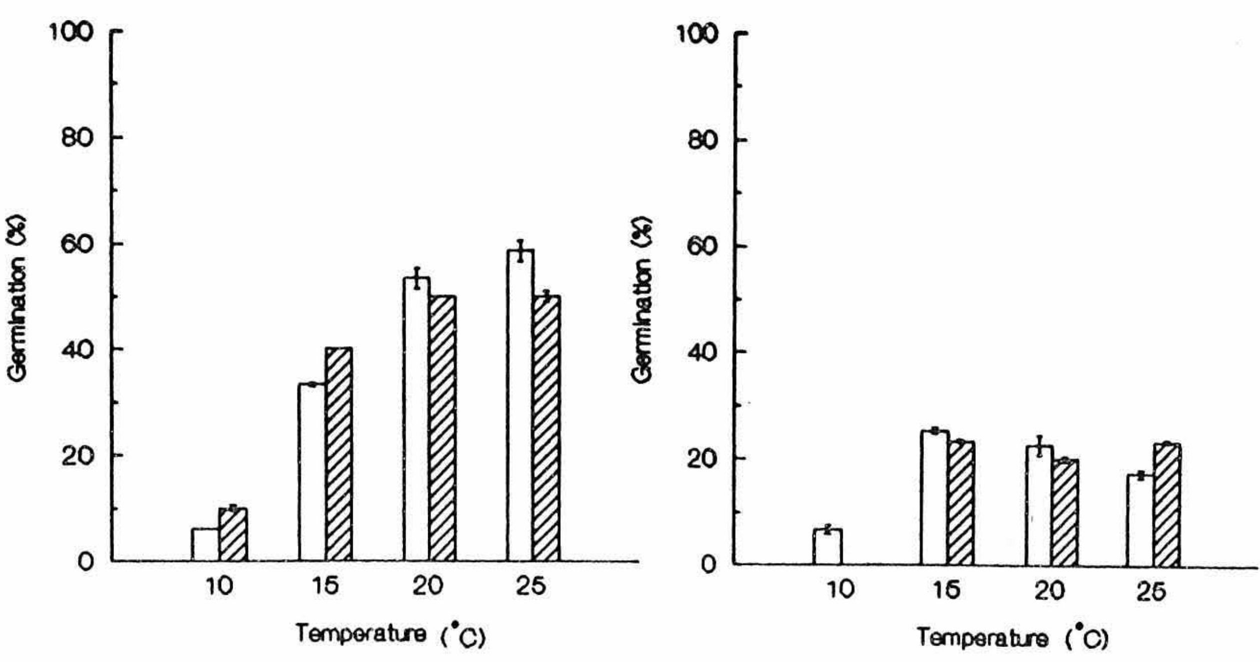


Figure 3.2 Effects of temperature and pre-soaking period on the germination at 96h of cv. Century-84. A, 0h; B, 24h; C, 48h; D, 72h pre-soaking; ▭, in filter paper; ▨, in Petri dishes.



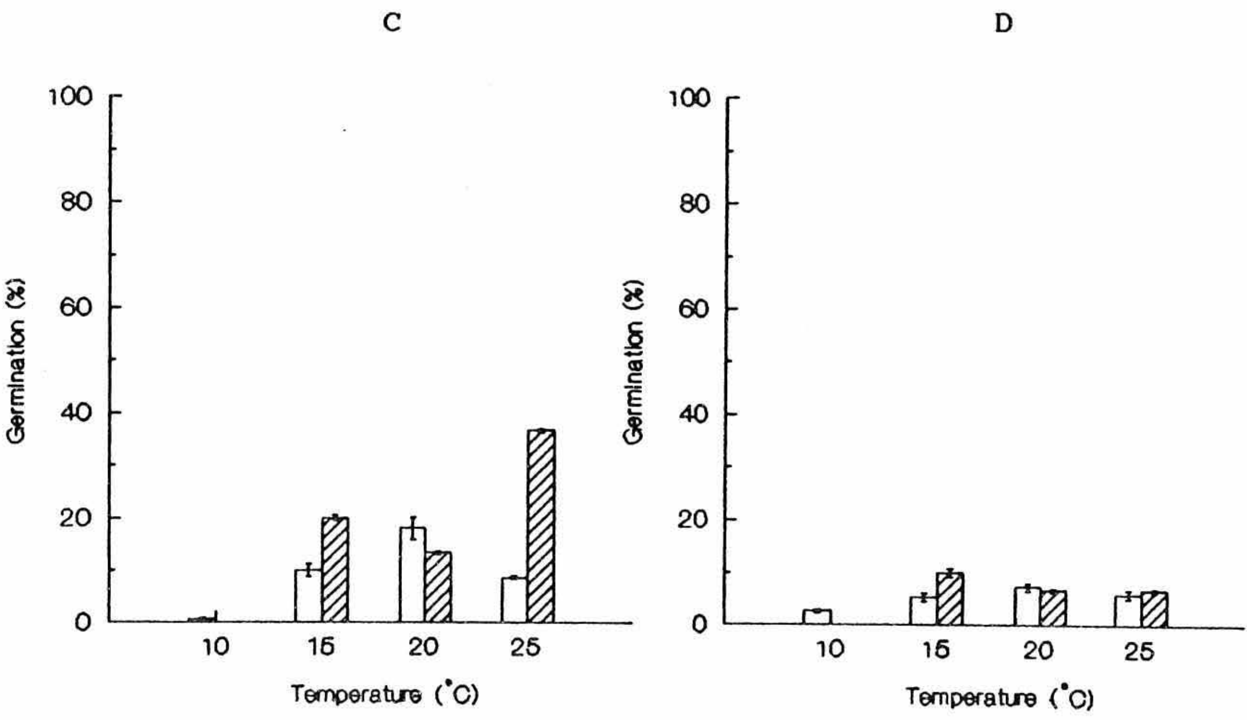
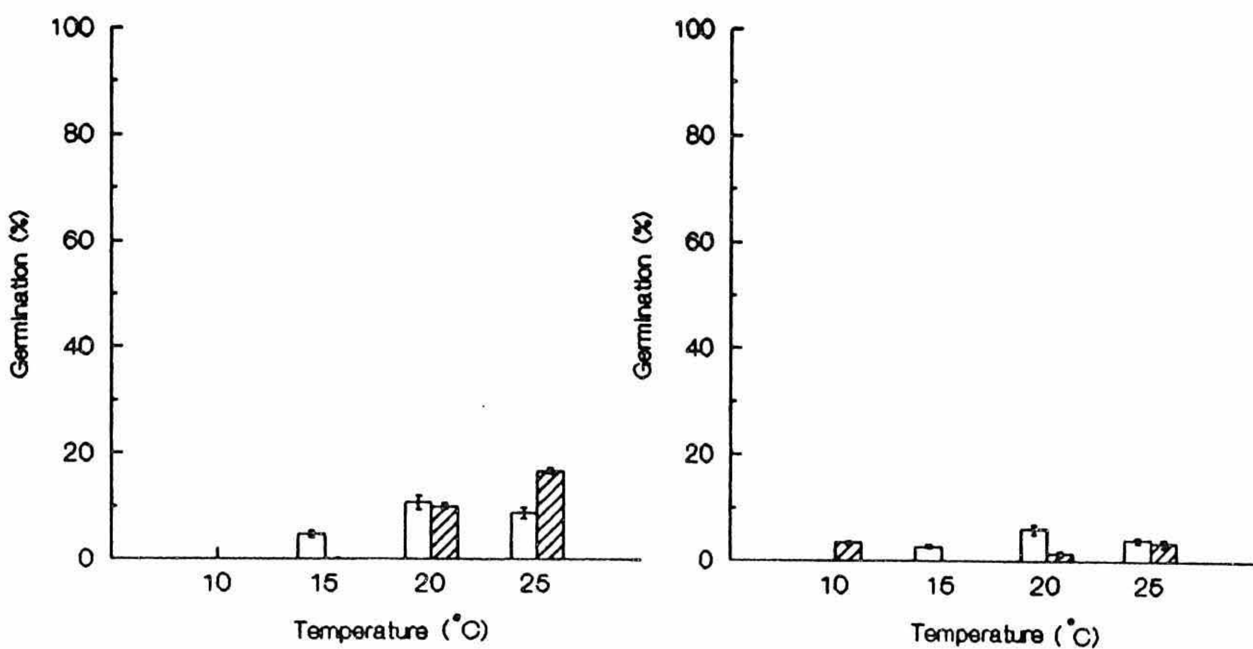


Figure 3.3 Effects of temperature and pre-soaking period on the germination at 96h of cv. Steele 5/1. A, 0h; B, 24h; C, 48h; D, 72h pre-soaking; □, in filter paper; ▨, in Petri dishes.



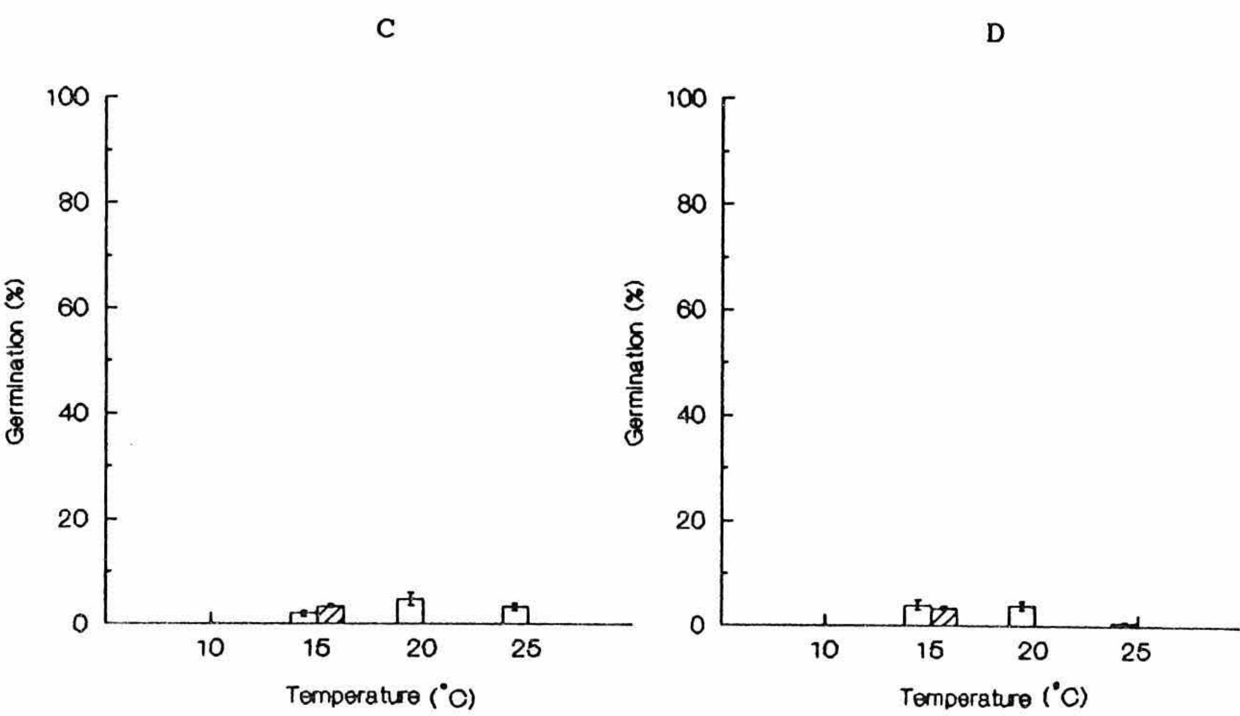
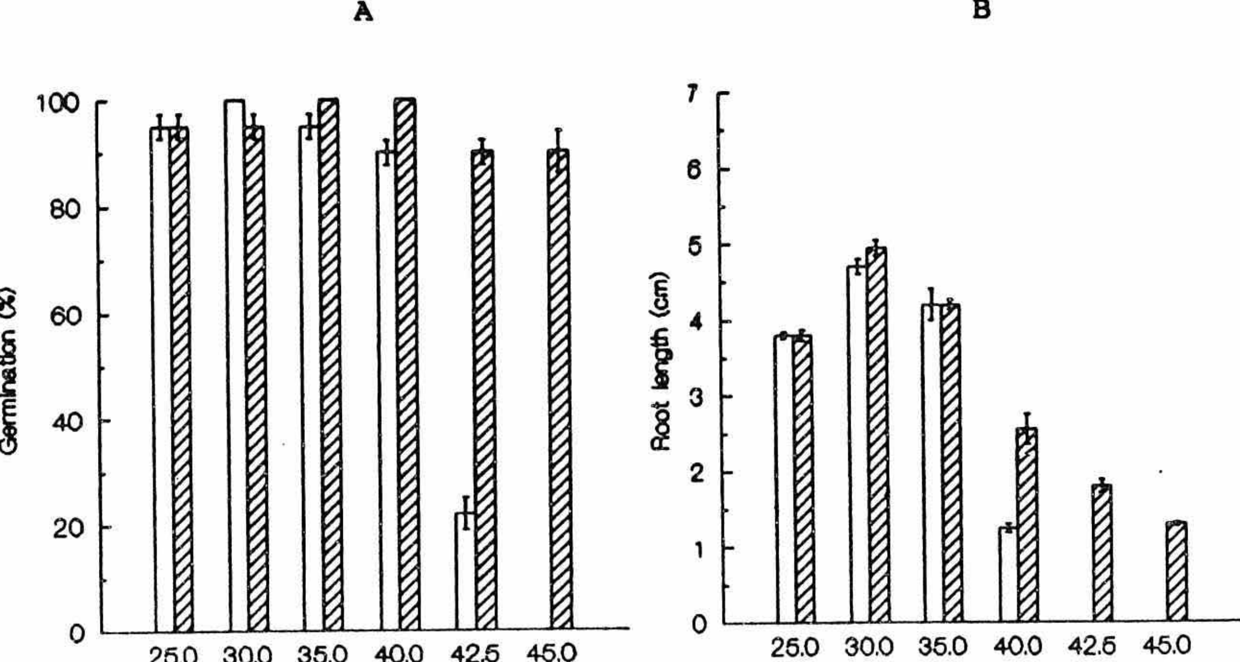


Figure 3.4 Effects of temperature and pre-soaking period on the germination at 96h of cv. Bossier. A, 0h; B, 24h; C, 48h; D, 72h pre-soaking; [open bar], in filter paper; [hatched bar], in Petri dishes.



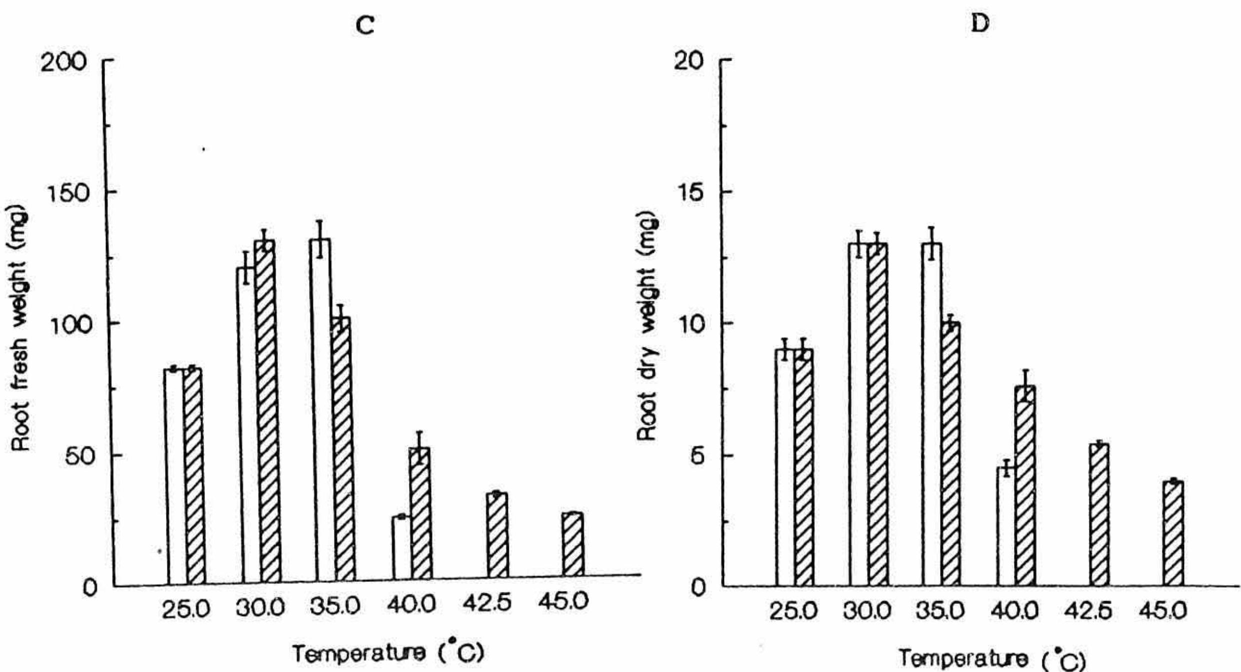
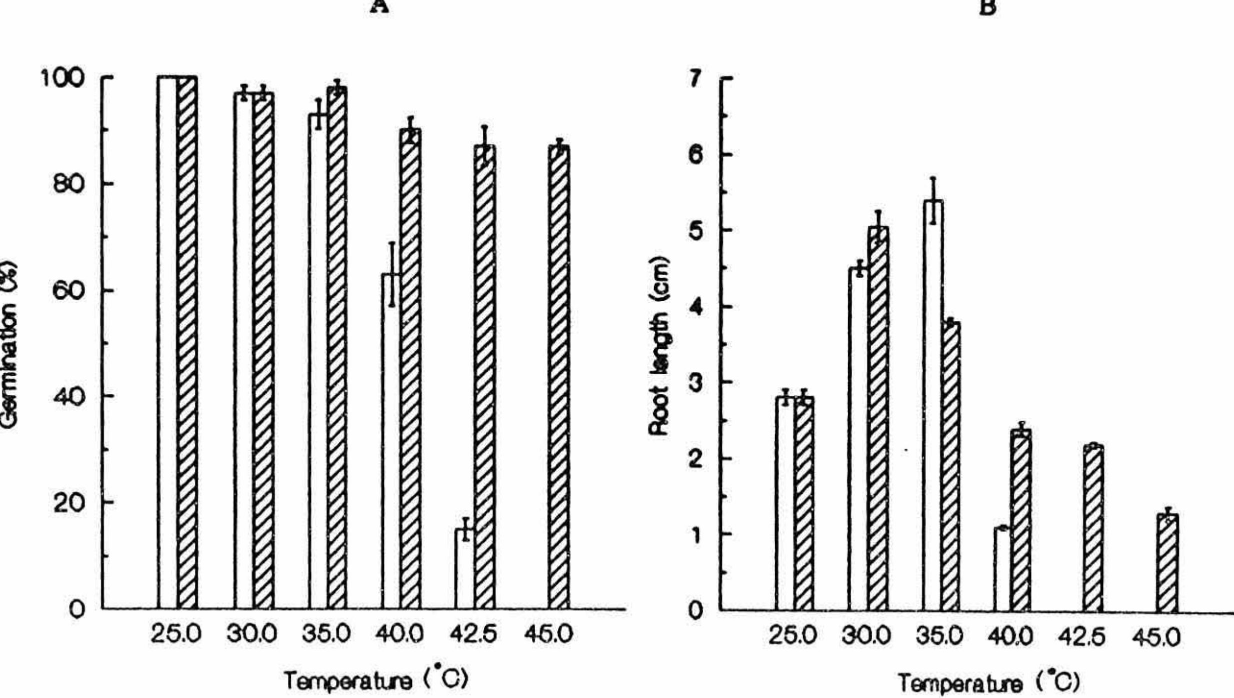


Figure 3.5 Effect of temperature on A, germination; B, root length; C, root fresh weight; D, root dry weight of cv. Williams-82. [open bar], 48h at same temperature; [hatched bar], 24h at 25°C followed by 24h at higher temperature.



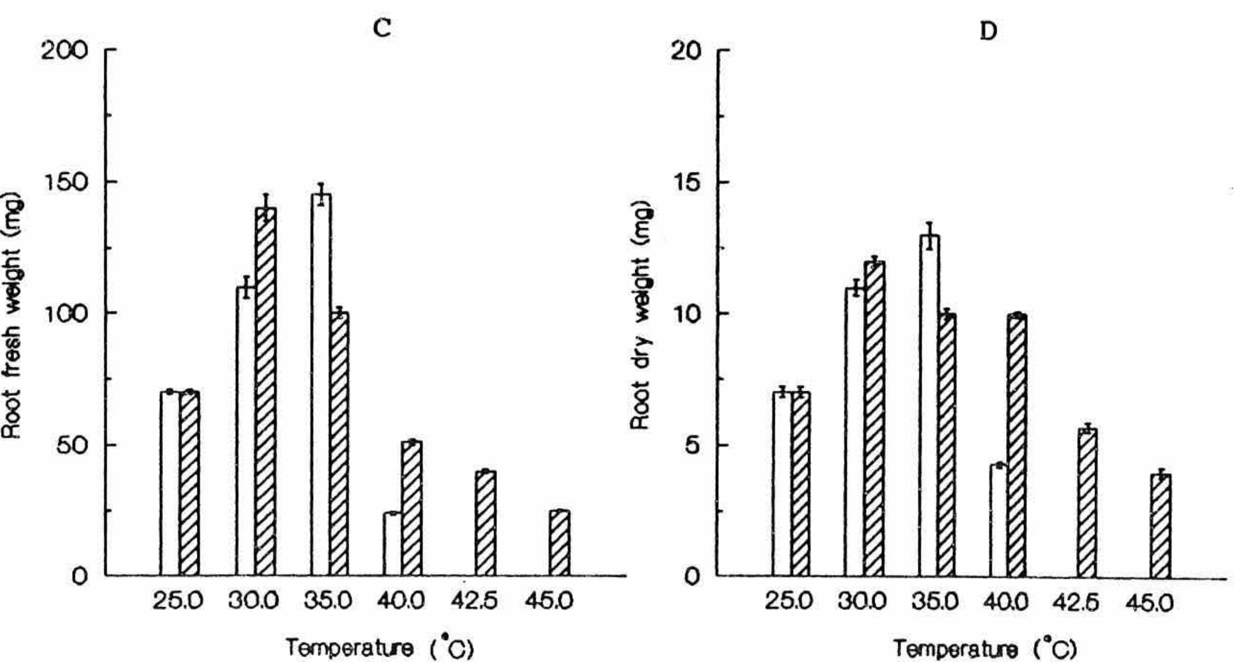
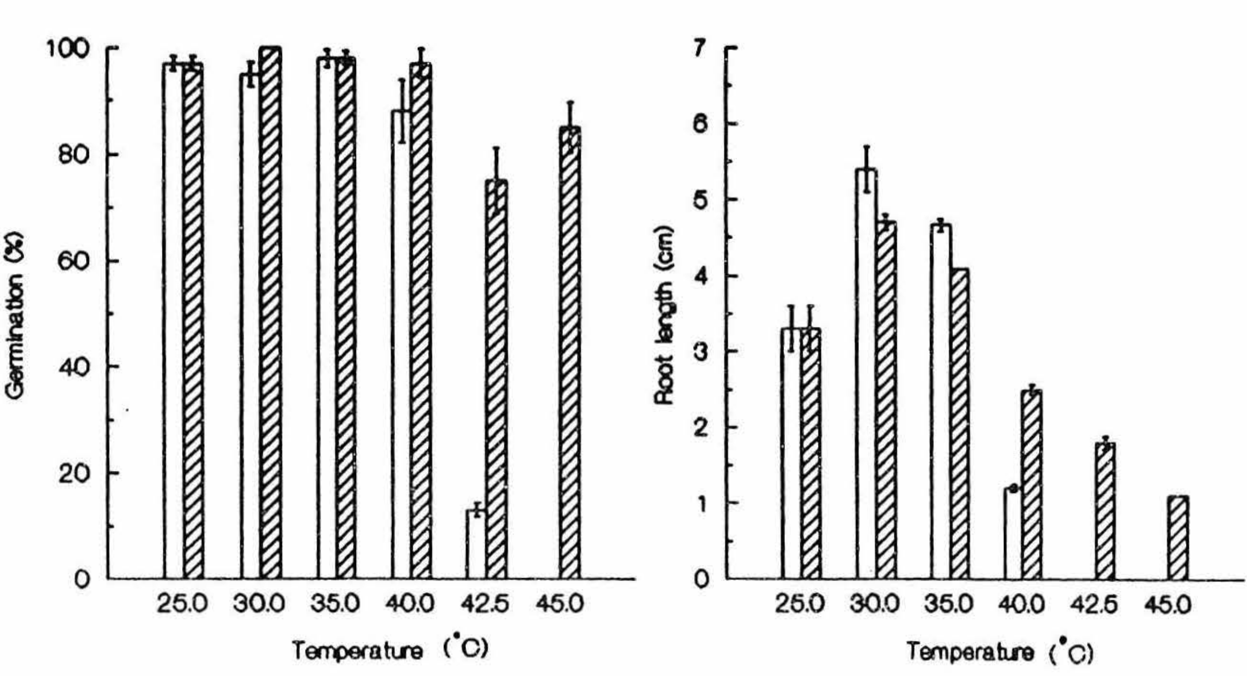


Figure 3.6 Effect of temperature on A, germination; B, root length; C, root fresh weight; D, root dry weight of cv. Sable. ☐, 48h at same temperature; ▨, 24h at 25°C followed by 24h at higher temperature.



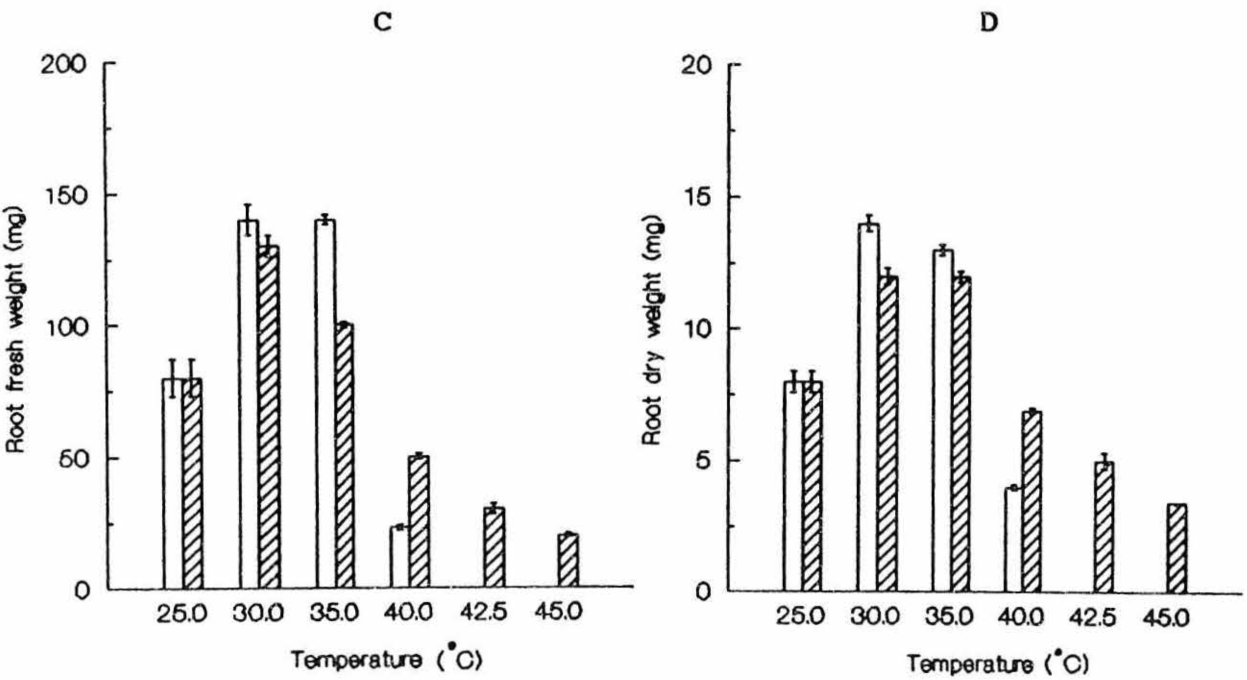
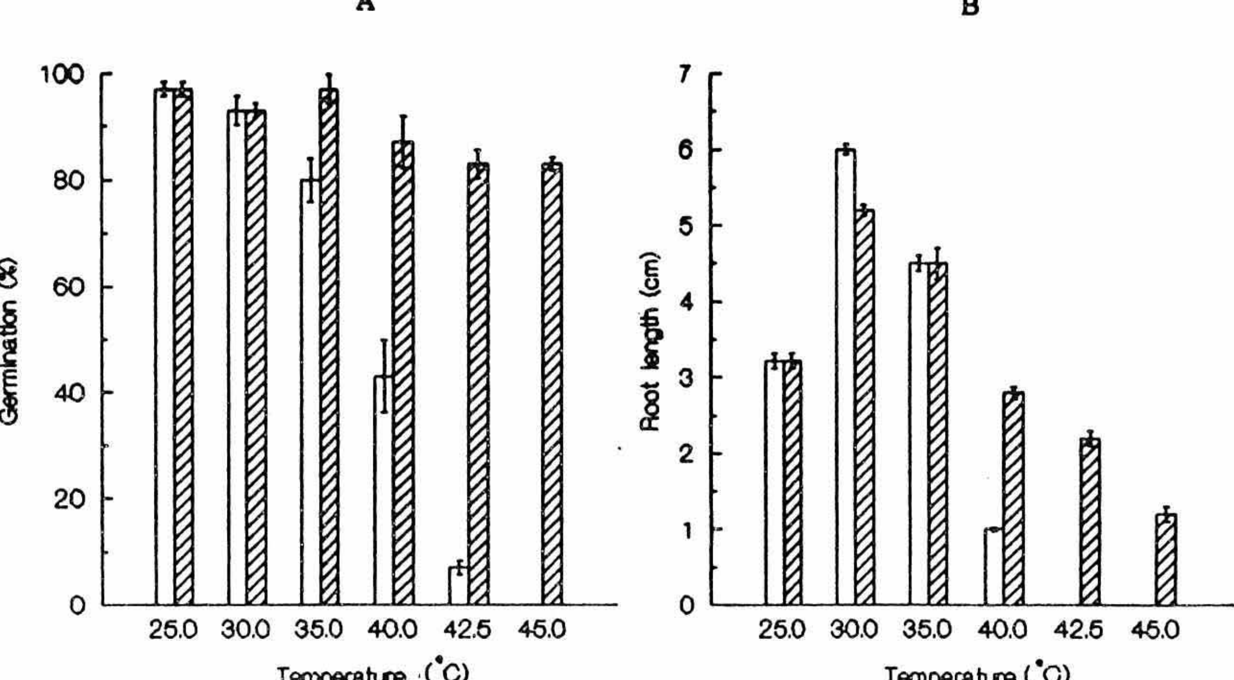


Figure 3.7 Effect of temperature on A, germination; B, root length; C, root fresh weight; D, root dry weight of cv. Mago-80. [ ], 48h at same temperature; [hatched], 24h at 25°C followed by 24h at higher temperature.



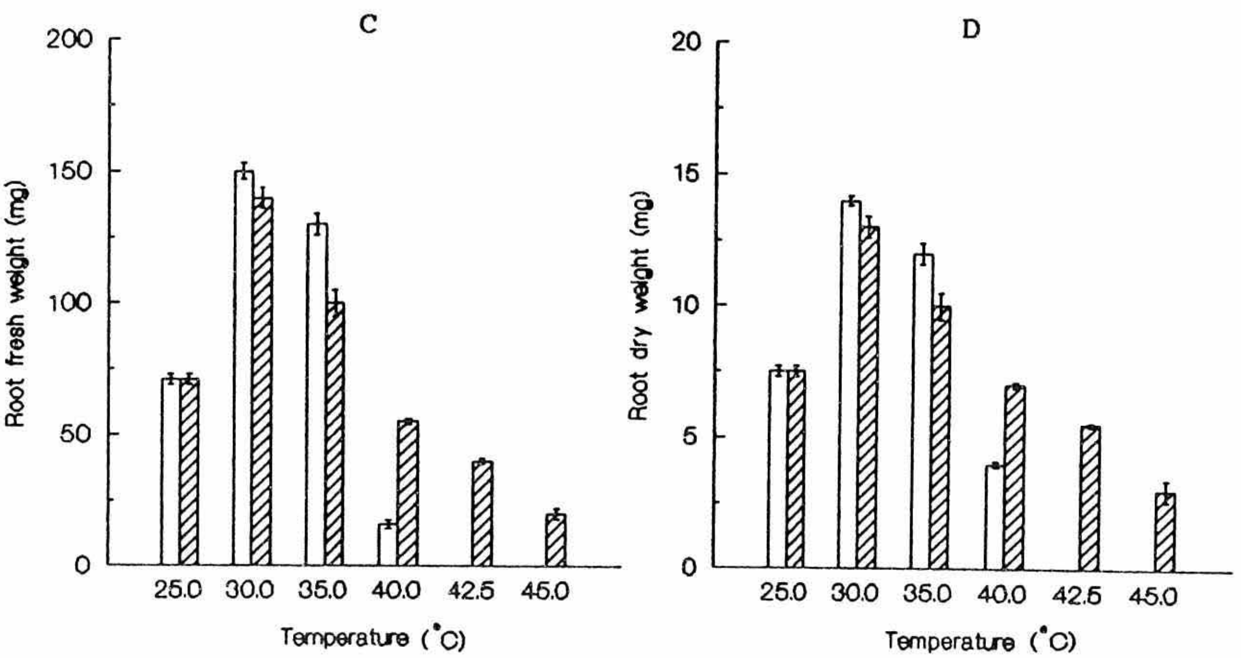
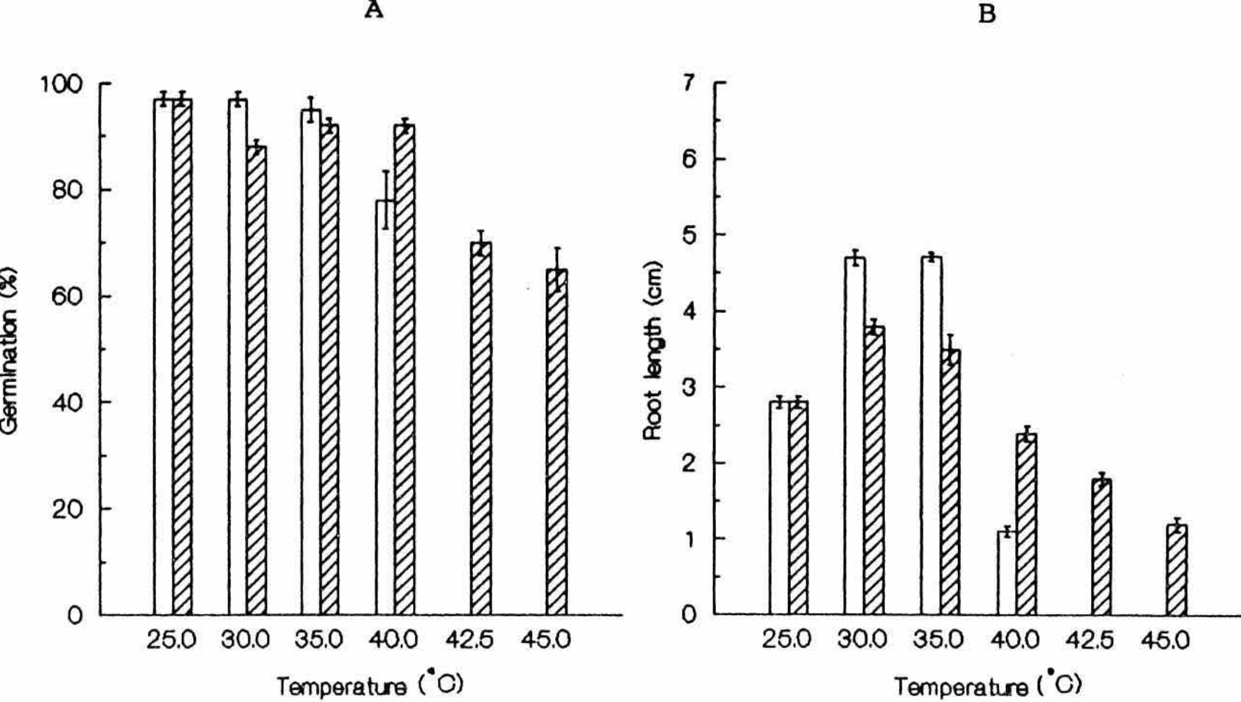


Figure 3.8 Effect of temperature on A, germination; B, root length; C, root fresh weight; D, root dry weight of cv. Bragg. [open bar], 48h at same temperature; [hatched bar], 24h at 25°C followed by 24h at higher temperature.



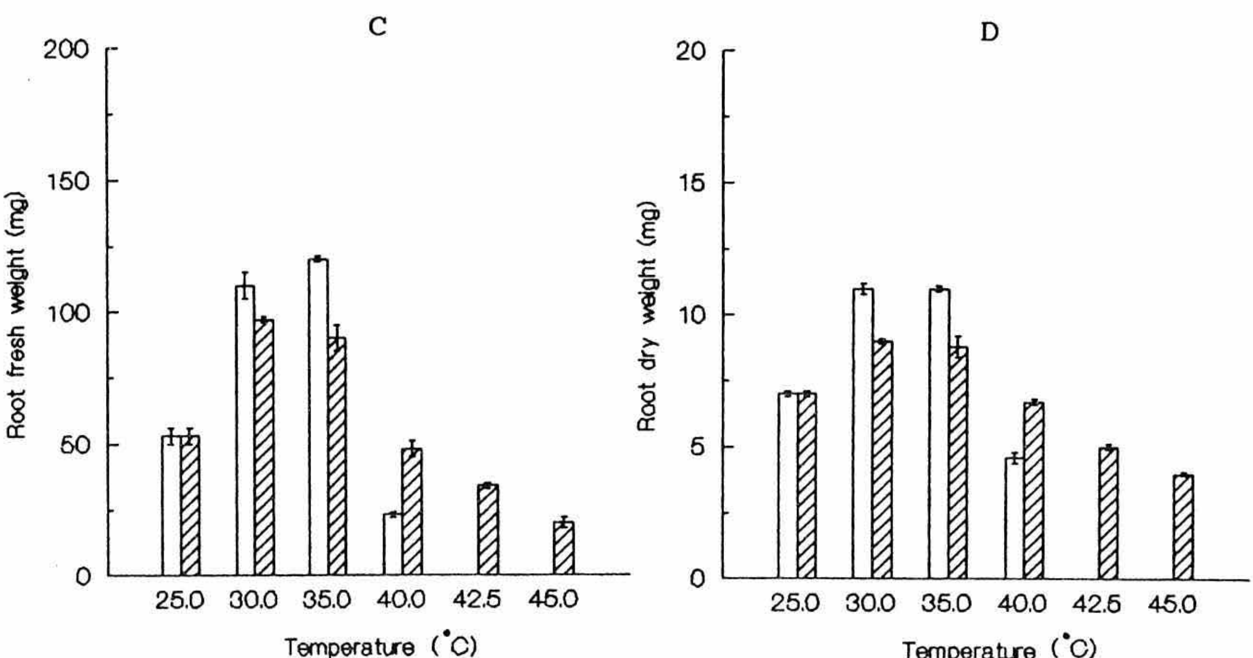
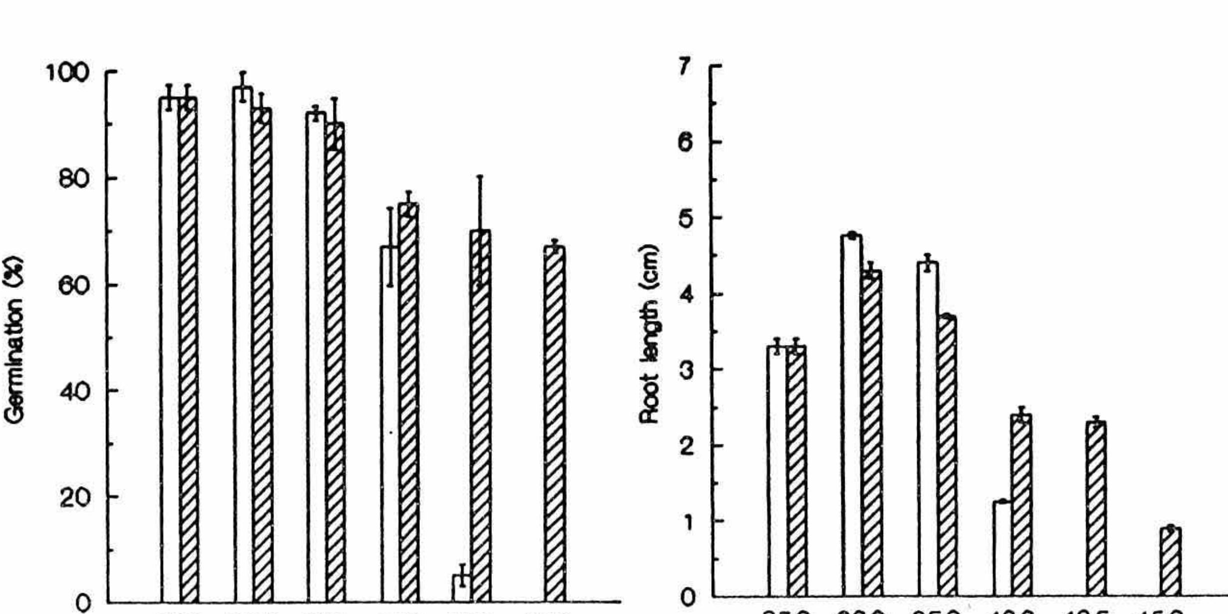


Figure 3.9 Effect of temperature on A, germination; B, root length; C, root fresh weight; D, root dry weight of cv. Davis. [open bar], 48h at same temperature; [hatched bar], 24h at 25°C followed by 24h at higher temperature.



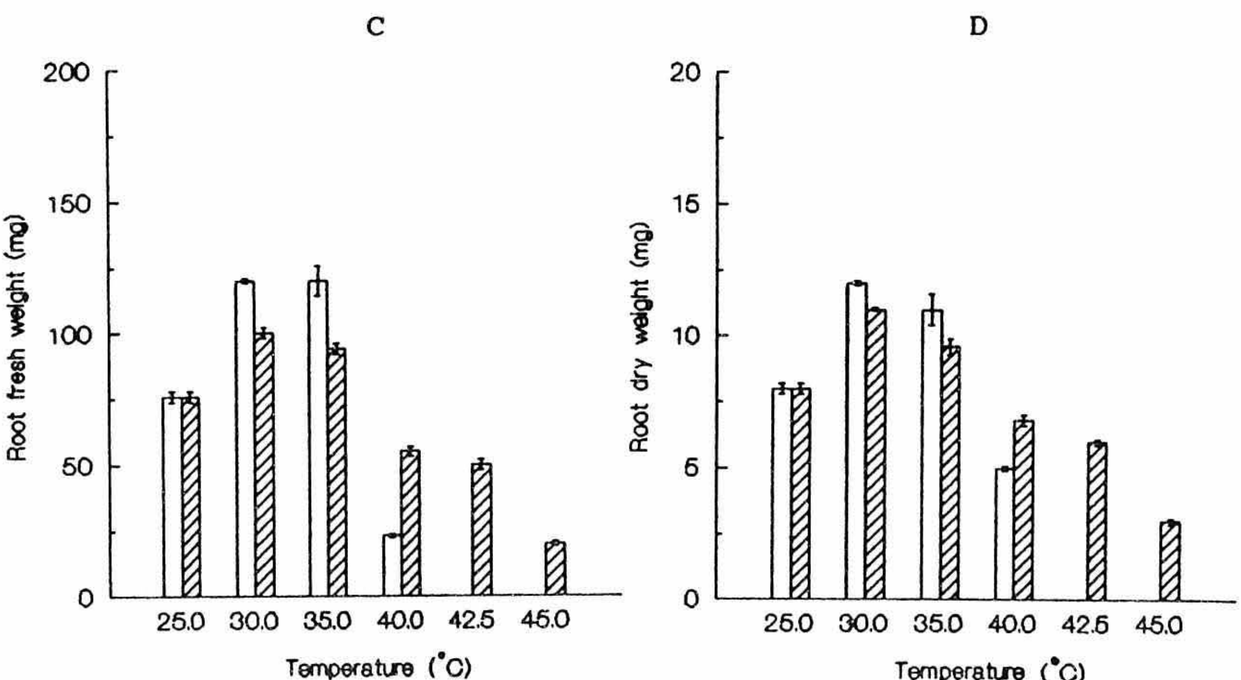


Figure 3.10 Effect of temperature on A, germination; B, root length; C, root fresh weight; D, root dry weight of cv. Hardee. [open bar], 48h at same temperature; [hatched bar], 24h at 25°C followed by 24h at higher temperature.

CHAPTER FOUR

Chapter Four

Effects of Temperature and Growing Medium on the Physiology and Growth of Soybean Cultivars.

4.1 Introduction.

Like other plants, soybean germination and development is affected by environmental factors such as temperature, water, light, salinity, waterlogging, air composition, nutrient supply etc. Temperature is probably the most important factor and the optimum for most cultivars is a 30 to 35°C day temperature or 30 to 32°C on a mean 24h basis. The maximum speed of development usually occurs close to 30°C.

High temperatures during seed formation or storage affect seed viability and vigour. Periods of high temperature in the last 45 days of seed maturation adversely affect these characteristics in some cultivars resulting in low emergence, poor growth and reduced yields. Green *et al.* (1965) reported that later dates of planting produced small-sized seeds in soybean. These small seeds exhibit a higher laboratory germination as well as higher field emergence than large seeds do. The optimum temperature for growth also varies through the life of the plant and, during certain periods, night temperature has a significant effect on development. It is also axiomatic that root growth and activity are influenced by environmental variables. Temperature, moisture condition, aeration and

the supply of nutrients are factors of obvious importance (Howell, 1963).

The soybean plant responds strongly to day length and temperature in its phasic development. The day length responses vary greatly between cultivars. Development of the soybean cultivars Clark and Midwest were delayed at long day length, but that development was more rapid as temperature increased from 16 to 27°C (Van Schaik and Probst, 1958). Soybean has a juvenile stage after seedling emergence, however, when it is primarily sensitive to temperature and insensitive to day length (Major and Johnson, 1977; Borthwick and Parker, 1938). The experiments described in this chapter were conducted to investigate the morphological and physiological differences between two soybean cultivars. The two cultivars were tested in four growth media at three temperatures.

4.2 Methods.

4.2.1 Effects of temperature and growing media on the physiology and growth of soybean cultivars.

Experiments were conducted in the glass-house at the Pen-y-ffridd University Field Station. Two soybean cultivars, Bragg and Century-84 were investigated. The experiments were performed with 4 replications of each cultivar between November, 1992 and January, 1993. The cultivars were grown in four different media (soil, sand, vermiculite and compost) in 12x13 pots cm at 18, 20 or 25°C. The

experiments were terminated after 42 days. Germination percentage, shoot length, hypocotyl length, leaf chlorophyll fluorescence, trifoliate leaf number, shoot branch number, total node number, shoot and root fresh and dry weights were recorded. For details see Section 2.10.

4.2.2 Statistical analysis.

Means, standard deviations and standard errors were calculated using a pocket scientific calculator (Sharp Model EL-531P). They were checked with a personal computer (Mitac) using the Minitab statistical package. The statistical analysis of variance (ANOVA) was done with Systat (version 5.03) and Minitab (version 10.2). The figures were plotted using Systat/Sygraph (version 5.03). In all figures, vertical bars show the standard errors of the means.

4.3 Results.

4.3.1 Germination.

The data for cultivar Bragg (Fig. 4.1) show that, at 18°C, 45% germination was recorded five days after planting in soil, but only 5, 10, and 15% in vermiculite, sand, and compost respectively. Germination in vermiculite increased to 95% after 8 days however. The germination in sand was slower than in soil or vermiculite and the slowest germination occurred in compost. Germination in soil was slower at 20°C than at 18°C. However, germination was faster in compost and soil at 20°C compared with 18°C. The

highest rate of germination at 25°C was recorded in vermiculite, but these high rates were only found after the 6th day, whereas they had occurred after the 5th day at 18°C. The rates of germination were faster in all the growing media at 25°C compared to 18 or 20°C. The final germination percentage were now approximately the same for all the media (80 to 85% by the 5th day).

The results for Century-84 were different from those of Bragg. The data in Fig. 4.2 shows that, at 18°C, only 5% germination was found in soil or sand after 11 days. The highest germination was recorded in vermiculite (60% after 10 days). There was no germination in compost at 18°C. At 20°C, germination increased to 20% in soil, to 50% in sand and to 75% in vermiculite after 9 days. Thirty percent germination now occurred in compost after 9 days, whereas none had occurred in this medium at 18°C. In contrast to Bragg, the germination of Century-84 decreased significantly at 25°C in soil, sand, and compost compared with 20°C. Only in the vermiculite was a high rate of germination maintained at this temperature. Although the germination of Century-84 in vermiculite was best at 25°C, it was still only 40% germination at the 5th day. This is half the rate of germination of Bragg (85%) under the same conditions (cf. Fig. 4.1).

The data were analysed using the ANOVA test, which showed that germination was strongly dependent ($P<0.05$) upon the

cultivar, temperature and the growing medium, but there were no significant ($P>0.05$) interactions between cultivar, temperature and growing medium.

4.3.2 Shoot length.

The total shoot lengths of the plants were recorded every fourth day. The value was affected by temperature in both cultivars. The data from cultivar Bragg in Fig. 4.3a show that, at 18°C, the shoot length increased progressively with increasing growing period. The greatest lengths were observed in compost and soil and smaller ones were recorded for vermiculite. In sand, the shoots stopped growing three weeks after germination. At 20°C, (Fig. 4.3b) the shoot lengths were higher than at 18°C and they increased progressively with increasing growing period in soil, sand and compost. The slowest shoot growth now occurred in vermiculite. Similar comparative shoot lengths were recorded at 25°C, although all the plants grew somewhat faster at 25°C than at 20°C (Fig. 4.3c).

The data in Fig. 4.4a show that shoot lengths were lower in cultivar Century-84 than in cultivar Bragg. At 18°C, the lowest shoot lengths were recorded in soil and sand, but they were a little better in vermiculite. Due to zero germination, there were no plants to measure in the compost. The shoot lengths increased with increasing growing period in all the media at 20°C (Fig. 4.4b). After 42 days, the shoots were 30 to 40 cm long in all the media

at this temperature. At 25°C, the longest shoot lengths were recorded in soil and shortest occurred in sand (Fig. 4.4c). The shoot lengths in compost and sand were smaller than those in the same media at 20°C.

Shoot length was significantly ($P<0.05$) dependent upon the cultivar, temperature and growing medium. No significant interactions ($P>0.05$) were observed between the cultivar, temperature and the growing medium.

4.3.3 Hypocotyl length.

The hypocotyl length was recorded at the time of harvesting after 42 days of growth. The data for the cultivar Bragg in Fig. 4.5 shows that, at 18°C, approximately the same hypocotyl length (7-7.3 cm) was recorded in compost, sand and vermiculite. The value was slightly greater (8 cm) when the plants were grown in soil. At 20°C, an 11 cm hypocotyl length was found in sand and a 10 cm value in soil. Only an 8 cm hypocotyl length was recorded in compost and an even smaller value was observed in vermiculite. The hypocotyl length declined in all media after 42 days when 5-6 cm long hypocotyls were recorded for all media.

The data in Fig. 4.6 show that, in the cultivar Century-84 at 18°C, the highest hypocotyl length (6 cm) was recorded in vermiculite and lower values were observed in soil and sand. Due to zero germination, there were no plants in the compost. At 20°C, 6 to 7 cm hypocotyl lengths were recorded

in all media. However, these values were reduced at 25°C. At this temperature, the hypocotyl length was 3 and 4 cm in soil and vermiculite respectively and 1 cm in compost and sand.

Statistical analysis by ANOVA showed that hypocotyl lengths were strongly dependent ($P<0.05$) upon the cultivar, temperature and growing medium. There were no significant ($P>0.05$) interactions between cultivar, temperature and growing medium.

4.3.4 Leaf chlorophyll fluorescence during growth at different temperatures.

Chlorophyll fluorescence analysis (CFA) was used to determine the photosynthetic efficiency of the leaves at the different temperatures and in the different growth media (see Section 2.10). Measurements were taken in the morning 4h after the start of the day-light photoperiod and they were carried out from the 2nd to 5th week after sowing. An exciting light intensity of 200 μmols m^{-2} s^{1} was used for the measurements and Fv/Fm ratios are presented.

The data for the cultivar Bragg grown at 18°C are presented in Fig. 4.7. They show that, at the 2nd week of growth, similar Fv/Fm ratios were recorded for all the growth media. The value tended to increase slightly up to the 5th week of growth especially in the case of plants grown in soil. In sand, compost, and vermiculite, however, the Fv/Fm

decreased slightly by the end of the 5th week of growth. At 20°C, the Fv/Fm ratio increased slightly in plants grown in soil and compost, but it decreased slightly with age in vermiculite. It similarly increased slightly up to the 4th week in sand, but then it decreased in the 5th week. At 25°C, the Fv/Fm ratio remained high in soil and compost grown plants up to the 3rd week, but thereafter it declined. In sand at 25°C, the Fv/Fm decreased all the way from the 2nd to the 5th week, while a smaller decrease occurred in vermiculite-grown plants.

In the case of the cultivar Century-84 grown at 18°C, there were no fully opened leaves in any of the media at two week after planting. No Fv/Fm values could be recorded therefore. The Fv/Fv ratios increased slightly between the 3rd and the 4th week, and decreased again in the 5th week in plants grown in soil, sand or vermiculite. Due to zero germination in compost at 18°C, there were no plants available for measurement in this medium. At 20°C, there were no fully opened leaves by the 2nd week after planting in soil, and hence no Fv/Fm values are shown. In the 3rd, 4th, and 5th weeks, the Fv/Fm ratio increased slightly in these soil-grown plants. In sand, compost, and vermiculite at 20°C no clear trends were discernable. At 25°C, however, a fairly clear decrease in Fv/Fm ratio was observed from the 2nd to the 5th week in plants grown in all growth media.

The data were analysed using the ANOVA test, which showed that chlorophyll fluorescence was not dependent ($P>0.05$) upon cultivar or growing period. It was, however, dependent ($P<0.05$) upon the temperature and the growing medium.

4.3.5 Trifoliate leaf number.

The total trifoliate leaf numbers were counted at the time of experiment termination (42 days). The data in Fig. 4.9 show that the trifoliate leaf number in the cultivar Bragg generally increased with increasing temperature. At 18°C, approximately twice as many (3.7 and 4) trifoliate leaves were present on compost and soil-grown plants than in sand and vermiculite-grown plants (1.5 and 1.7) after 42 days. At 20°C, there were only small changes in the numbers of trifoliate leaves compared with the numbers at 18°C, except in the case of sand-grown plants where the number nearly doubled from 1.7 at 18°C to 3.0 at 20°C. At 25°C, there was a more marked increase to approximately 8.0 leaves on plants grown in soil or compost. The increase in the numbers of leaves on plants grown in sand and vermiculite at 25°C was similar.

The data for cultivar Century-84 are presented in Fig. 4.10. In this cultivar, fewer trifoliate leaves occurred at all temperatures and in all growth media in comparison with cultivar Bragg (cf. Fig. 4.9). At 18°C, more trifoliate leaves were recorded in sand and vermiculite-grown plants than in plants grown in soil. The compost medium gave no

germination at 18°C, so that leaf number could not be counted. The number of trifoliate leaves in both sand and vermiculite was very low (0.75) at this temperature. At 20°C, the number of trifoliate leaves increased dramatically for all the media used to values of between 2 and 3.5 leaves. At 25°C, the number increased further to 5.2 in soil and the value nearly doubled in vermiculite. Leaf number decreased in sand and compost, however, compared with 20°C.

The trifoliate leaf number was strongly dependent ($P<0.05$) upon the cultivar, temperature and growing medium, but there were no significant ($P>0.05$) interactions between cultivar, temperature and growing medium.

4.3.6 Shoot branch number.

The shoot branch number was also counted at the time of the experiment termination. The data for both cultivars, Bragg and Century-84 are presented in Table 4.1. The data for Bragg show that the mean number was 1.2 and 1.5 branches in soil and compost respectively, but no branching occurred in sand or vermiculite after 42 days at 18°C. At 20°C, the same number of branches occurred in soil as at 18°C (1.2 branches) while no branching was observed in the other media. At 25°C, the branch number increased to 4.5 branches in soil and 3.0 branches in compost. Again, there was no branching in sand or vermiculite after 42 days.

Table 4.1 Total branch number in soybean cultivars grown in four different media at 18, 20 and 25°C.

Medium	Temperature°C		
	18	20	25
Cv. Bragg			
Soil	1.2±0.6	1.2±0.2	4.5±0.2
Sand	0.0	0.0	0.0
Vermiculite	0.0	0.0	0.0
Compost	1.5±0.2	0.0	3.0±0.5
Cv. Century-84			
Soil	0.5±0.4	0.2±0.2	4.7±1.4
Sand	0.3±0.2	0.0	0.0
Vermiculite	0.0	0.0	0.0
Compost	0.0	0.8±0.4	1.0±0.8

Each value is the mean ± S.E from four replicates.

In the case of Century-84, the total number of branches recorded at 18°C in soil and sand was 0.5 branches and 0.3 respectively. In this cultivar, there was no branching in the vermiculite and compost-grown plants at 18°C following poor germination. At 20°C, branching only occurred in soil (0.2 branches) and in compost (0.8 branch). No branching was observed in the plants grown in sand or vermiculite. At 25°C, a high degree of branching occurred in soil-grown plants (4.7 branches) and to a lesser extent in plants grown in compost (1.0 branch). No branches were recorded in plants grown in sand or vermiculite at 25°C after 42 days.

Statistically there were no differences ($P>0.05$) between the two cultivars with respect to shoot branch number, but shoot branch number was dependent ($P<0.05$) upon the temperature and the growing medium. Also, there were no significant ($P>0.05$) interactions between the cultivar, temperature, and growing medium.

4.3.7 Node number.

The data for total node number for both cultivars are presented in Table 4.2. In the cultivar Bragg, 1.2, 2.5, 4.7 and 5.0 nodes were recorded at 18°C in plants grown in sand, vermiculite, compost and soil respectively. The numbers increased with increasing temperatures of 20 or 25°C. The numbers at 25°C were about double those at 18°C. In cultivar Century-84, 0.3, 1.0 and 1.7 nodes occurred at 18°C in soil, sand and vermiculite-grown plants

Table 4.2 Total node number in soybean cultivars grown in four different media at 18, 20 and 25°C.

Medium	Temperature °C		
	18	20	25
Cv. Bragg			
Soil	5.0±0.2	6.2±0.2	10.0±0.0
Sand	1.2±0.2	4.0±0.0	6.2±0.2
Vermiculite	2.5±0.4	3.2±0.6	4.7±0.5
Compost	4.7±0.0	5.0±0.8	10.0±0.0
Cv. Century-84			
Soil	0.3±0.2	4.5±0.2	6.7±1.9
Sand	1.0±0.5	4.0±0.0	1.5±1.2
Vermiculite	1.7±0.5	3.0±0.0	5.2±0.2
Compost	0.0	4.2±0.6	2.2±1.9

Each value is the mean ± S.E from four replicates.

respectively, while no nodes occurred in compost. At 20°C, node number increased to 4.5 and 4.2 in soil and compost while 4 nodes occurred in sand. This time, 3 nodes were recorded in vermiculite. A sharp increase in node number (6.7 and 5.2 nodes) occurred in soil and vermiculite at 25°C and a big decrease was obtained in sand and compost-grown plants.

The node number was strongly dependent ($P<0.05$) upon the cultivar, temperature and the growing medium, but there were no significant ($P>0.05$) interactions between the cultivar, temperature, and the growing medium.

4.3.8 Shoot fresh and dry weights.

When the experiment was terminated 42 days after germination, and shoot fresh weights were recorded and dry weights were obtained after 48h of drying at 70°C. The data for cultivar Bragg, presented in Fig. 4.11, show that higher shoot fresh weights always occurred in plants grown in soil and compost compared with sand and vermiculite. At 18°C, the values were 10.4 and 8.8 g fresh weight for the plants grown in soil and compost respectively. Slightly higher values were recorded for the plants grown in soil at 20°C, but about the same fresh weights were now observed in sand, compost and vermiculite compared with the values at 18°C. At 25°C, the shoot fresh weights for soil and compost-grown plants were much higher (49.2 and 37.3 g respectively) and also there were small increases in sand

and vermiculite-grown plants. Indeed, the value was 5 times greater at 25°C in the soil and compost-grown plants than at any other temperature or in any other growing medium. There were small increases in the values for sand and vermiculite-grown plants at 25°C compared with the lower temperatures. The dry weights followed similar patterns to the fresh weight values.

The data for cultivar Century-84 in Fig. 4.12 show that, at 18°C, very low fresh shoot weights (0.3 to 1.0 g) were recorded for all the growth media. Due to zero germination in the compost there were no plants to be weighed. At 20°C, sharply higher shoot fresh weights occurred in all plants. The highest weight (5.2 g) was recorded in compost-grown plants and soil-grown plants were about as good. At 25°C, the highest fresh weight was observed in plants grown in soil and the lowest values were recorded in compost and vermiculite-grown plants. As expected, the shoot dry weights followed the same pattern as the fresh weights. The fresh and dry weights were generally lower in Century-84 in comparison to Bragg under the same conditions (cf. Fig. 4.11).

The data were analysed using the ANOVA test. This showed that shoot fresh weights and shoot dry weights were strongly dependent ($P<0.05$) upon the cultivar, temperature and growing medium. There were no significant ($P>0.05$) interactions between the cultivar, temperature and growing

medium.

4.3.9 Root fresh and dry weights.

The root fresh and dry weight patterns were similar to the shoot fresh and dry weights at different temperatures and in the different growing media. The results in Fig. 4.13 show that, in Bragg grown at 18°C, the highest root fresh weight (3.7 g) was recorded in soil and compost-grown plants. At 20°C, a little further increase was found in the fresh weights of plants grown in all the growth media. The fresh weights sharply increased to (26.0 and 16.2 g) in soil and compost-grown plants at 25°C. Also, small increases were found in plants grown in compost and vermiculite. Similar patterns to the root fresh weights were found for the root dry weights.

The data for Century-84 in Fig. 4.14 show that very much smaller root fresh weights were obtained in all the growing media at 18°C compared with Bragg. Due to zero germination in compost, no plants were weighed at this temperature. At 20°C, the fresh weights increased similarly in all growing media. Finally, at 25°C, a higher in fresh weight (18.2 g) occurred in the plants grown in soil. Small increases in fresh weight were also found in plants grown in compost and vermiculite at this temperature. Similar patterns to the fresh weights were found in the dry weight values.

Statistically, the root fresh and dry weights were

dependent ($P<0.05$) upon the cultivar, temperature and the growing medium. There were no interactions ($P>0.05$) between cultivar, temperature and the growing medium.

4.4 Summary and Discussion.

The two soybean cultivars Bragg and Century-84 were compared for their morphological and physiological responses to different temperatures in four different growing media. The results show that, at 18°C, germination in the cultivar Bragg was high in vermiculite and low in compost. A similar trend was obtained for the cultivar Century-84 in all the media but the overall germination percentages were much lower compared with cultivar Bragg. No Century-84 seeds germinated in the compost. At 20°C, both cultivars germinated best in vermiculite. In cultivar Bragg, approximately the same germination occurred in sand, soil and vermiculite at 20°C. In cultivar Century-84, however, relatively poor germination was found in soil and compost at 20°C. At 25°C in the cultivar Bragg, good germination was found at 25°C in sand, soil and compost, but it remained a little higher in vermiculite. In cultivar Century-84 at 25°C, germination was very poor in compost and sand, but better in soil. Germination was good in vermiculite. Overall, the cultivar Century-84 germination percentage remained much lower than that of Bragg. These results are in general agreement with results reported by Bharati *et al.* (1983), who found that the optimal temperatures for the germination of soybean lies between 27

and 31°C. Delouche (1953) similarly reported that maximum germination occurs in the shortest time at a constant temperature of 30°C. Variations in germination can have many causes, including differences in the vigour of different seed lots used. It has already been mentioned that smaller soybean seeds often have faster germination (Edwards and Hartwig, 1971) and higher total laboratory germination and field emergence (Green *et al.*, 1965) than large ones. Stucky (1976) reported that, in soybean, the percentage field emergence decreases with increasing planting depths of 5, 7.5 and 10 cm. Field emergence percentage also decreases as temperature is increased from 16 to 32°C. The latter results are in agreement with those of Hooper *et al.* (1979) who reported that, again for soybean, the time required for 50% germination decreased from 18.8 to 4.0 days as the temperature was increased from 10 to 30°C.

The present results also reveal that shoot length gradually increases as the temperature is raised to 25°C. At 18°C in cultivar Bragg, the shoot lengths were higher in plants grown in soil and compost and shorter lengths were obtained in sand and vermiculite. In the cultivar Century-84, however, the smallest shoot lengths were observed in soil and sand and slightly longer shoots were found in vermiculite. At 20°C, the shoot lengths of Bragg increased for all media. Now, approximately the same lengths were found in sand and vermiculite, while the shoots were only

a little longer in soil and compost. In cultivar Century-84 at this temperature, the maximum shoot length was found in soil medium. The shoot lengths were somewhat smaller in the compost and sand, but smaller still in vermiculite. The shoot lengths were generally lower than in Bragg. At 25°C in the cultivar Bragg, shoot lengths rapidly increased with increasing growth periods compared with 20°C. The highest shoot lengths still occurred in soil and compost and lower lengths were found in sand and vermiculite. At 25°C, Century-84 grew much better in soil, with vermiculite a good second. The shoot growth in compost and sand was poor at this temperature. In general, the growth of Century-84 was slower at all temperatures in comparison to Bragg.

The observed effects of temperature on shoot growth agree with those of Lindemann and Ham (1979), who reported that the highest shoot lengths in soybean occurred at 25°C. Matthews and Hayes (1982) found that soybean growth steadily improved as the temperature increased up to 25°C. Kvien and Ham (1985) similarly reported that high shoot lengths were observed at 30°C. These and others reports show that the optimum temperature for shoot development is approximately 25 to 30°C in most cultivars including Bragg. It is lower in other cultivars, where the temperature optimum is approximately 20 to 25°C depending on the growth medium. Our results indicate that Bragg belongs to the first group while Century-84 belongs to the second group of cultivars. Of the two cultivars tested in the present

studies, Century-84 seems to be the most sensitive to high temperatures. Nielsen and Humphries (1966) and Cooper (1973) found that root temperature also affects plant shoot growth, they reported that, not only do species differ in their response to root temperature but cultivars within a species may also differ.

It is clear that hypocotyl length was suppressed at 25°C compared with 18 or 20°C. In the cultivar Bragg, hypocotyl length was not greatly affected by the growing medium. In the case of Century-84, however, the longest length occurred in vermiculite and shorter lengths were found in compost, sand and soil, at 18 and 25°C at least. At 20°C, the hypocotyl lengths of Century-84 were approximately the same for all the growth media. The hypocotyl length of both cultivars was reduced at 25°C compared with 20°C irrespective of the growth medium. Gilman *et al.* (1973) have shown that the rate of hypocotyl elongation of soybean increases as the temperature is increased from 20 to 30°C, although some cultivars exhibit a decrease in growth rate at 25°C. He reported that genotypes with inhibited hypocotyl elongation at 25°C are known to have inferior emergence potential, which may also be compounded by soil mechanical resistance. Also, Grabe and Metzer (1969) found that hypocotyl growth in the soybean cultivar Ford was strongly suppressed at 25°C. Bragg and Century-84 presumably belongs to this group of cultivars. These results may be explained by the reports of Burris and

Knittle (1975), who suggested that some inhibitor or inhibitor-precursor may be located in the cotyledons of the short hypocotyl cultivars and that this inhibitor reduces hypocotyl elongation of soybean.

The data reported in this chapter reveal that leaf chlorophyll fluorescence is not significantly affected by either growth medium or by the age of the plant, in plants grown at 18 or 20°C at least. The Fv/Fm ratios in Bragg was reduced by the end of the 5th week at 25°C in plants grown in all the growth media however. The reduction was greater in sand-grown plants than in the plants grown in the other media. In Century-84 grown at 25°C, the Fv/Fm ratio was markedly reduced by the 5th week in vermiculite-grown plants, with less reduction in sand and soil. There was relatively little reduction in the ratio in compost grown plants. In general, however, there were no significant differences between the cultivars. These results indicate that the leaves of plants grown for 5 weeks at 25°C have passed their peak photosynthetic efficiency and may be ageing. Leaves at 18 or 20°C, on the other hand, have a slower rate of ageing and they are still at peak photosynthetic efficiency even at week 5. Alternatively, plants grown in some media at 25°C were suffering from environmental stress by the fifth week.

Chlorophyll fluorescence analysis measurement on intact leaves provides a rapid assessment of the photosynthetic

condition of plants. The measurement takes only few seconds and it can be used in screening for genotypical variation in stress tolerance or in measuring plant product quality. Adams *et al.* (1990) working on soybean, sunflower and cotton, suggested that the Fv/Fm can be used as a probe to monitor the activity of photosynthetic carbon assimilation in the field. Rosenqvist *et al.* (1991) found that, when the air temperature was lowered from 25 to 15°C, photoinhibition was accelerated in willow leaves. They suggested that this temperature effect is mediated by a decrease in the rate of energy dissipation through photosynthesis. This was indicated by an increase in the number of closed PS II reaction centres. Decreasing temperatures into the chilling and freezing range also decrease the rate at which the various stomatal enzymes of the C_3 cycle operate, thereby lowering the overall rate at which photosynthesis proceeds (Berry and Bjorkman, 1980; Baker, 1994). Kao and Forseth (1992) found that the greatest reduction in Fv/Fm occurred in horizontally restrained leaves of nitrogen-deficient and drought-stressed soybean plants at midday.

The trifoliate leaf number was counted at the time of termination of the experiment. In the cultivar Bragg, the highest number of trifoliate leaves were produced by plants grown in soil, but only a slightly lower number were present on plants grown in compost. The sand and vermiculite-grown plants produced lower numbers however. Cultivar Century-84 produced only a few trifoliate leaves

in any of the media at 18°C. At 20°C and 25°C, cultivar Bragg again produced most trifoliate leaves in soil and compost, with the lower numbers in sand and vermiculite. Increasing temperatures thus increase the number of trifoliate leaves in Bragg. The response of Century-84 to temperature was less uniform although clear-cut temperature effects were noted in soil and vermiculite-grown plants. It is known that soybean leaf development is closely related to temperature and it increases as the temperature increases throughout the range of 18 to 30°C (Ciha and Brun, 1975). Hesketh *et al.* (1973) working on soybean, have reported, however, that, in green-house plants grown in midsummer, the trifoliate leaf number on the main stem was fairly constant between 12 and 30°C. At 30°C, the rate of trifoliate leaf growth was much less in late fall than in early spring, because the supply of photosynthetic assimilates was limiting in the late fall. Also, Musser *et al.* (1983) found that higher rates of leaf elongation in soybean plants were obtained at 25°C than in plants grown at 10°C. Matthews and Hayes (1982b) reported that the number of leaves, branches and internodes were reduced at 10°C in soybean, whereas the size increased steadily at 25°C.

It is clear that the maximum number of branches occurred at 25°C in the present experiment. In the cultivar Bragg grown in soil, for example, similar branch numbers were found at 18 and 20°C and they increased at 25°C. The growth medium

also influenced the degree of branching, so that more complex pictures were obtained with some media for example in compost. In the cultivar Century-84, the branch number was lower in soil at 18 and 20°C compared with Bragg, but a similar branch number was found at 25°C. Again, there were marked interaction between the effects of the growth medium and the temperature. Nevertheless, the results show that temperatures below 25°C can be regarded as sub-optimal for the development of both branches and trifoliate leaves in both cultivars used in this study.

The literature on the control of branching in soybean is limited. Duke *et al.* (1979) found that, in soybean, maximum branch number occurred at 20°C, but no branches were found at 13°C. Huxley *et al.* (1976) reported that taller, more branched and later-flowering plants were produced in longer days as compared with shorter daylengths. Final soybean plant dry weight was increased by 50% in the longer days.

The total node number increased as temperature was raised to 25°C. In the cultivar Bragg, the number increased with increasing temperatures in all growing media. The maximum number was found in soil and compost at 25°C, while the lowest number occurred in sand and vermiculite at 18°C. In the cultivar Century-84, the node number increased in soil and vermiculite as the temperature was raised to 25°C. In sand and compost, however, the maximum number was found at 20°C and the number was smaller at 25°C. Our results are

supported by those of Duke *et al.* (1979), who found that more nodes were obtained at 20°C than at 13°C. Sionit *et al.* (1987) reported that main stem length and the number of nodes and leaves increase slightly in soybean with increasing temperatures and also with increasing CO_2 concentrations.

The results in this chapter revealed that shoot fresh and dry weights were higher at 25°C than at 18 or 20°C. In the cultivar Bragg, the highest weights at 18°C were found in plants grown in soil and compost, while the lowest weights were obtained in sand and vermiculite. Similar results were observed at 20°C. At 25°C, the fresh and dry weights were significantly increased in soil and compost, but very little increase was found in sand and compost. The weights in soil and compost at 25°C were five times higher than at 18 or 20°C. The cultivar Century-84 grown at 18°C produced low shoot weights in soil, sand and vermiculite and no plants grew in the compost. At 20°C, the weights were increased in all media compared to 18°C, the highest values being in soil and compost-grown plants. Greater increases were found especially in soil, at 25°C. However, a small decrease was found in sand compared with the corresponding 20°C value. In both cultivars, the fresh and dry weights followed similar patterns.

At 18°C in the cultivar Bragg, the best root fresh and dry weights were observed using soil and compost and the lowest

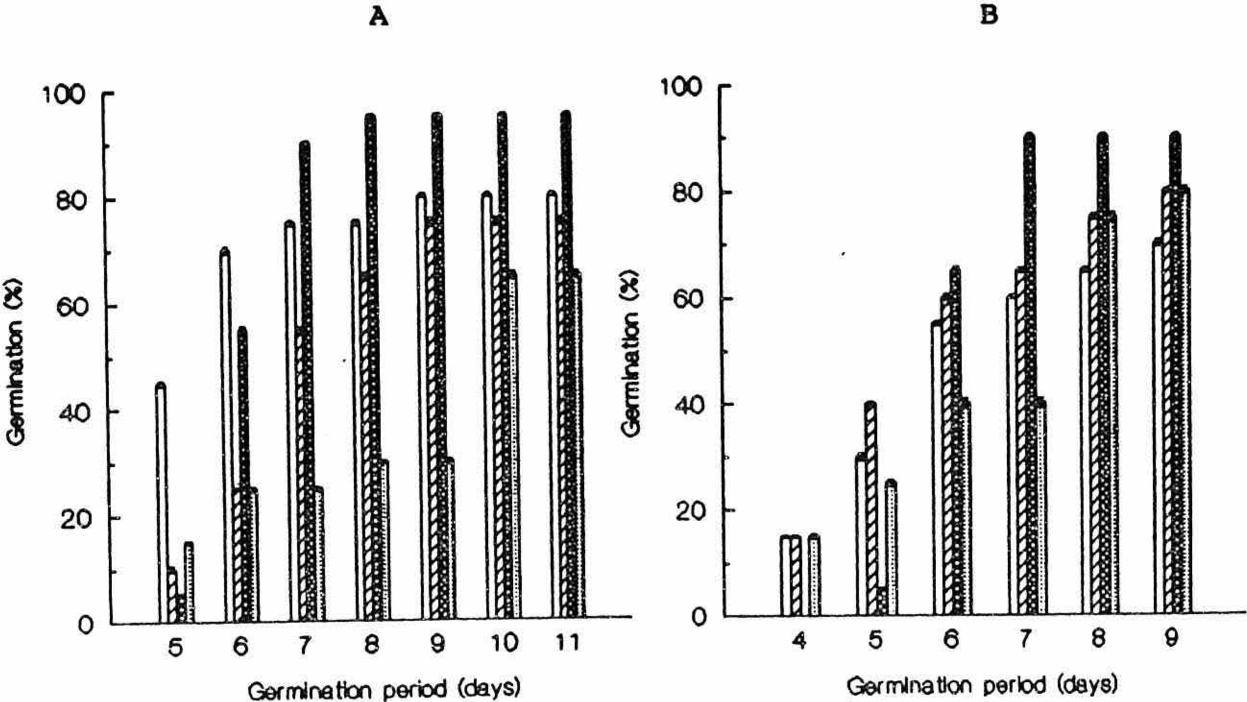
weights were found in sand and vermiculite. Small increases were found at 20°C in sand, soil and vermiculite media, while the weights remained the same in compost-grown plants. At 25°C, fresh and dry weights were higher in all media, the greatest increase being in soil and compost. In the cultivar Century-84, the root fresh and dry weights were very low in all media. In compost, no seeds germinated. The weights increased in all media at 20°C. When the temperature was raised to 25°C, a marked increase in weights was found in soil-grown plants, while smaller increases occurred in compost and vermiculite. A small decrease was found in the sand medium at 25°C compared with 20°C. As with the shoots, the root fresh and dry weights followed similar patterns in both cultivars.

In general, the present results for shoot and root weights agree with those of earlier workers. Earley and Cartter (1945), reported that the greatest root fresh and dry weights are produced at between 27 and 32°C in soybean. Warrington *et al.* (1977) reported that the soybean shoot dry weight was highest with a day/night temperature regime of 29/17°C. In conclusion, the optimum temperature is 25°C or slightly higher. Our results are also supported by those of Munevar and Wollum (1982), who also found the highest dry weight at 25°C. Kvein and Ham (1985) also reported that plant dry weight increased with increasing temperature, maximum values occurring at 25°C. Jones and Tisdale (1921) similarly found that the greatest shoot dry weight occurred

at between 24 and 35°C and Patterson and Flint (1979) reported that higher weights of leaves and stems were observed at higher temperature. Trang and Giddens (1980) found that shoot and root dry weight and nitrogen content of soybean plants declined with increasing amounts of shading. Matthews and Hayes (1982b) found that soybean plant growth, root and shoot dry weight, leaf area and plant height all increased over a range of root temperatures from 10 to 25°C.

Duke *et al.* (1979) reported that root weights of plants grown at 13°C were only 12% of the values for plants grown at 20°C. Also, Letey *et al.* (1961) and Stolzy *et al.* (1961) found that root growth generally decreased with decreasing oxygen content above the soil surface, root elongation being clearly related to the diffusion of oxygen into the soil. Rufty *et al.* (1981) reported that, under standard conditions, soybean root growth decreased at 18 and 30°C compared with 24°C. Our results are more closely supported by Trang and Giddens (1980), who found that there was a four-fold increase in soybean plant dry weight between 15 and 20°C and an additional 50% increase between 20 and 25°C.

In conclusion, the cultivar Bragg gave better responses in terms of the germination, shoot growth, branching and fresh and dry weights compared with the cultivar Century-84.



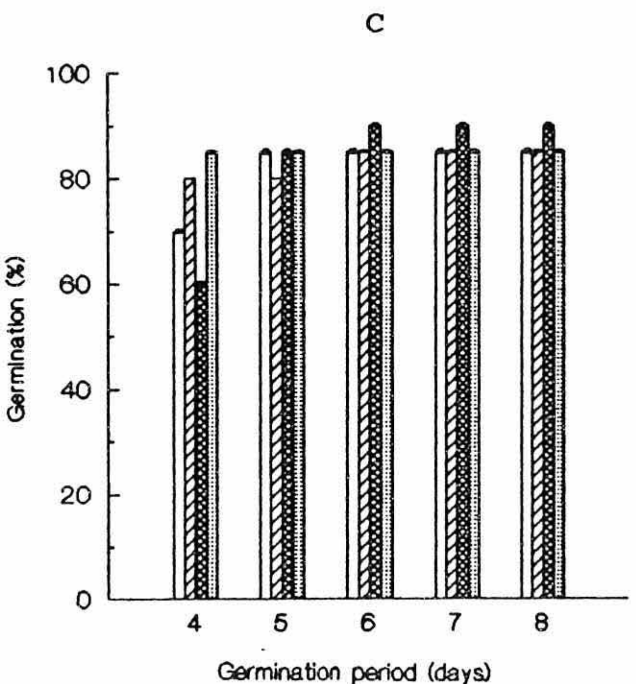
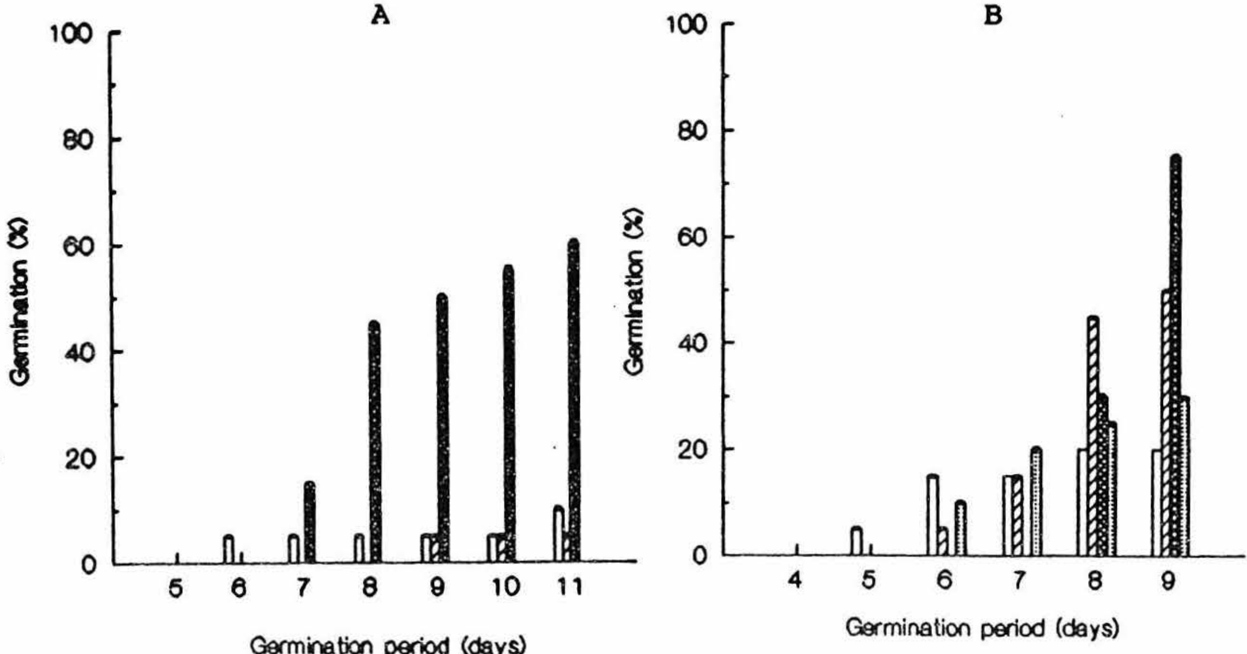


Figure 4.1 Effects of temperature and growth medium on the germination of cv. Bragg. A, 18°C; B, 20°C; C, 25°C; [white bar], soil; [hatched bar], sand; [dark stippled bar], vermiculite; [light stippled bar], compost.



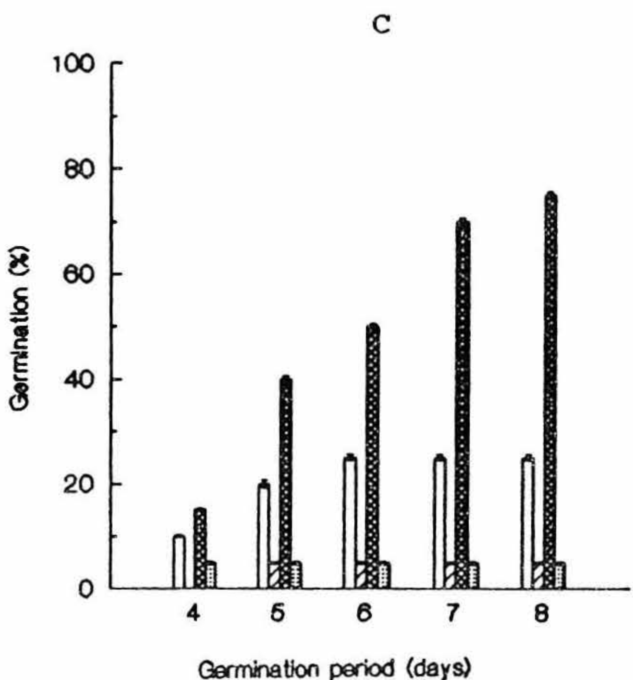
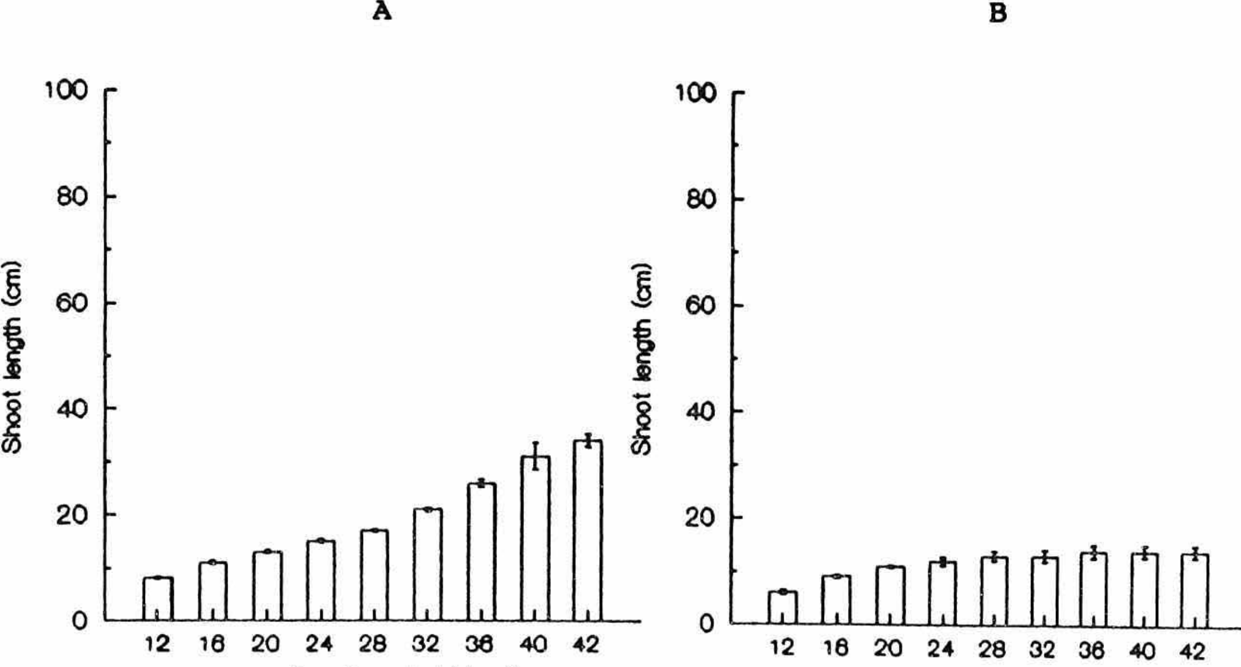


Figure 4.2 Effects of temperature and growth medium on the germination of cv. Century-84. A, 18°C; B, 20°C; C, 25°C; [open box], soil; [hatched box], sand; [dark stippled box], vermiculite; [light stippled box], compost.



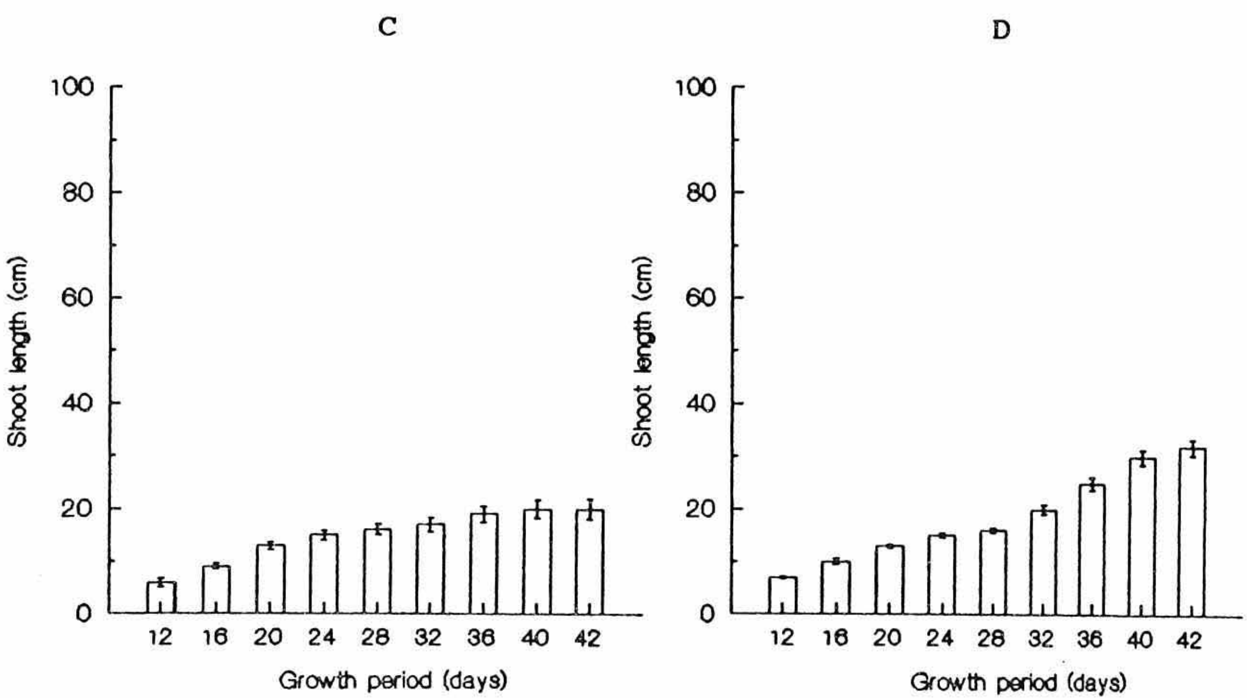
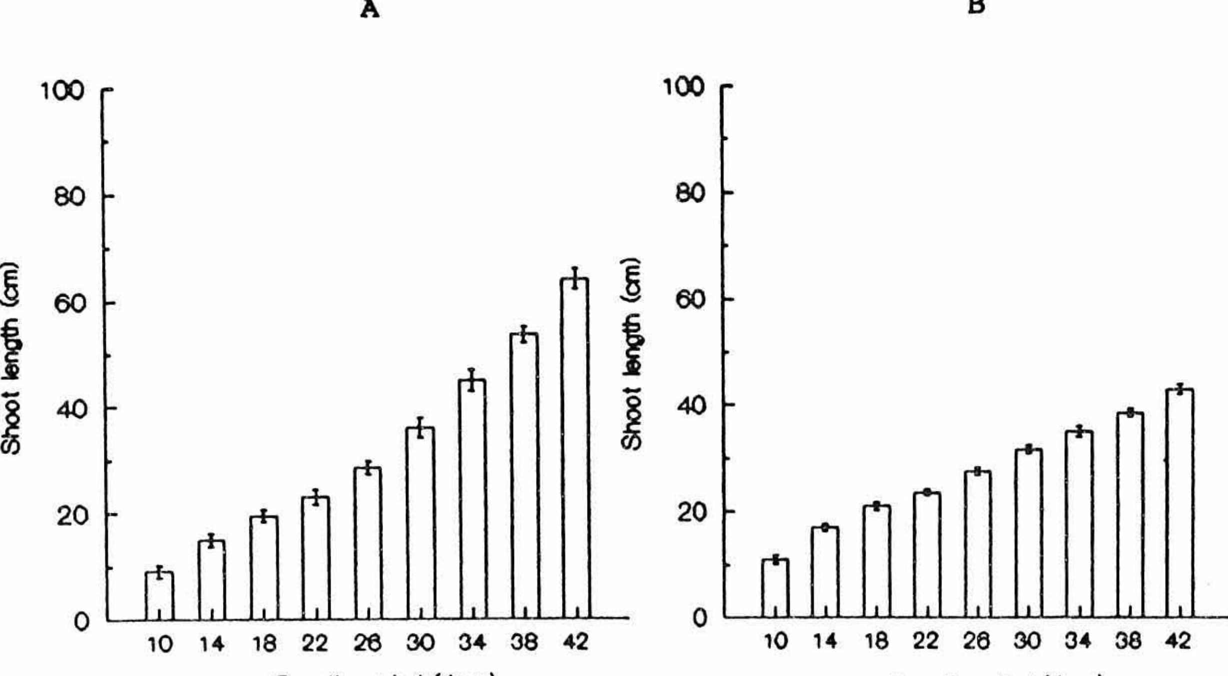


Figure 4.3a Effects of growth medium on shoot growth of cv. Bragg at 18°C. A, soil; B, sand; C, vermiculite; D, compost.



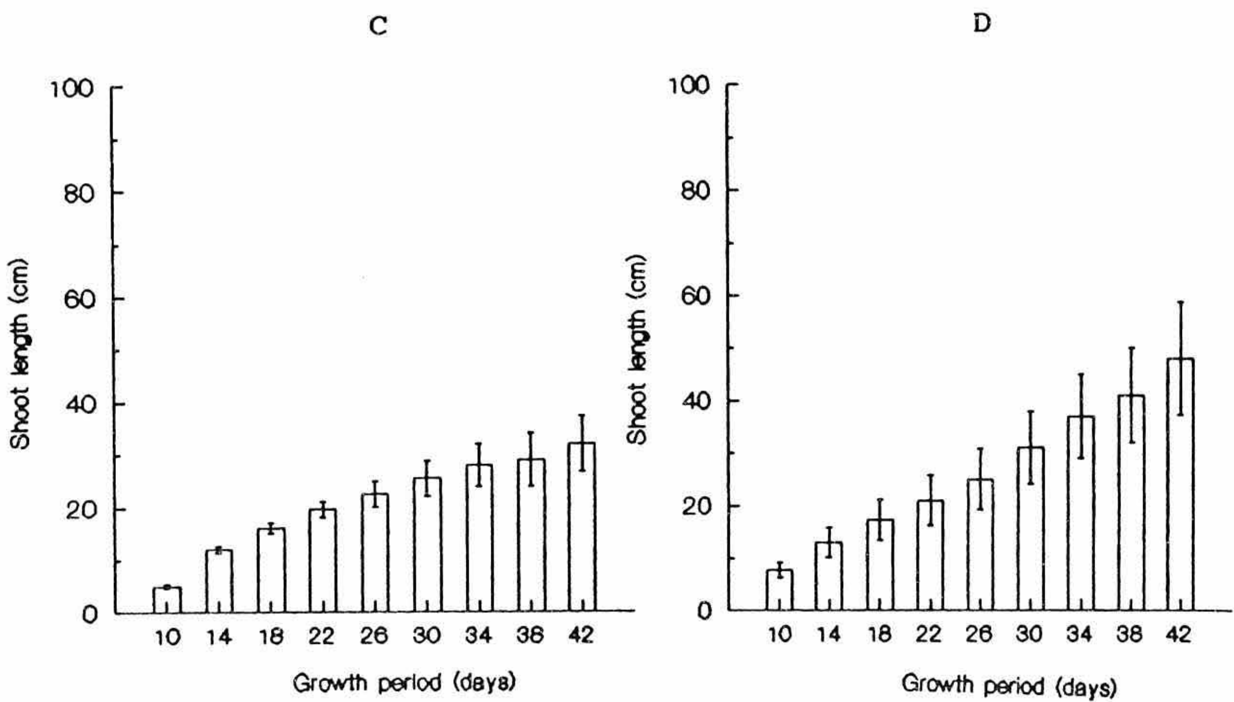
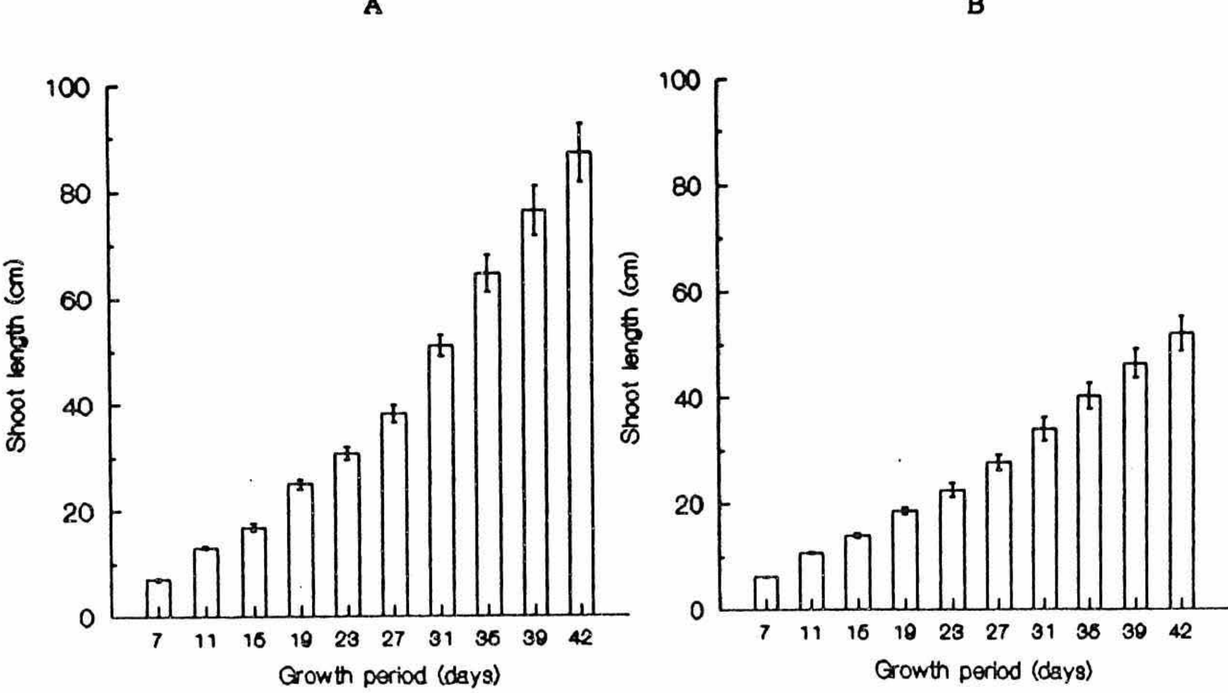


Figure 4.3b Effects of growth medium on shoot growth of cv. Bragg at 20°C. A, soil; B, sand; C, vermiculite; D, compost.



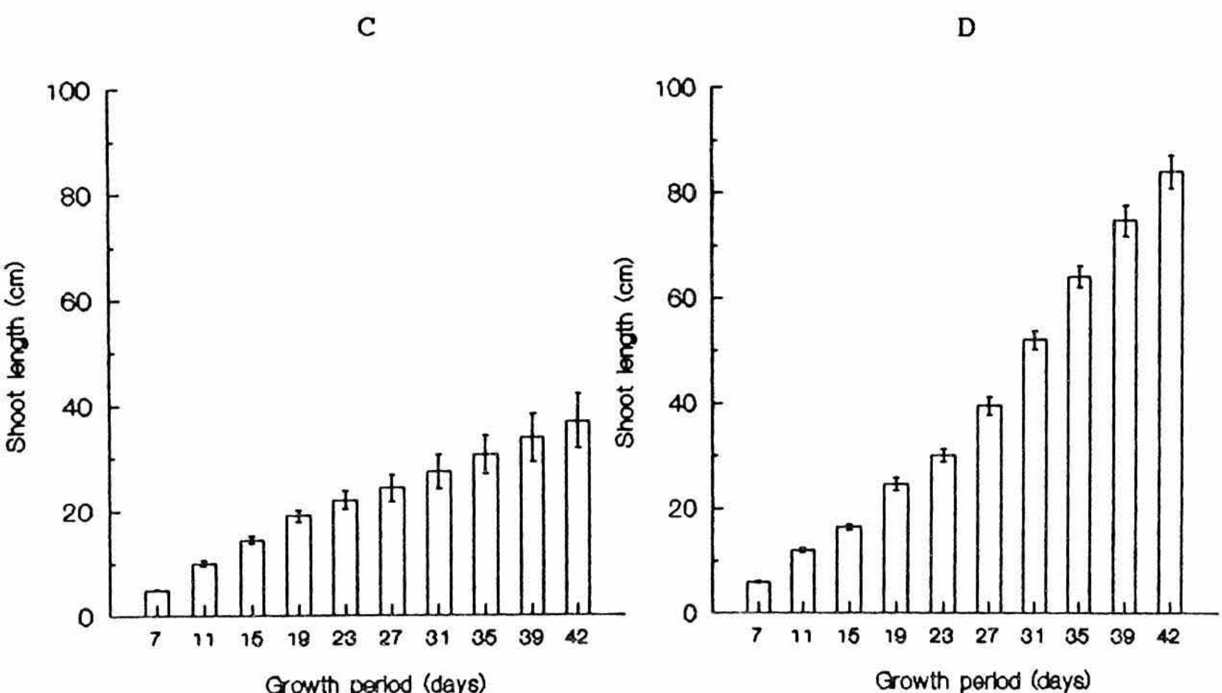
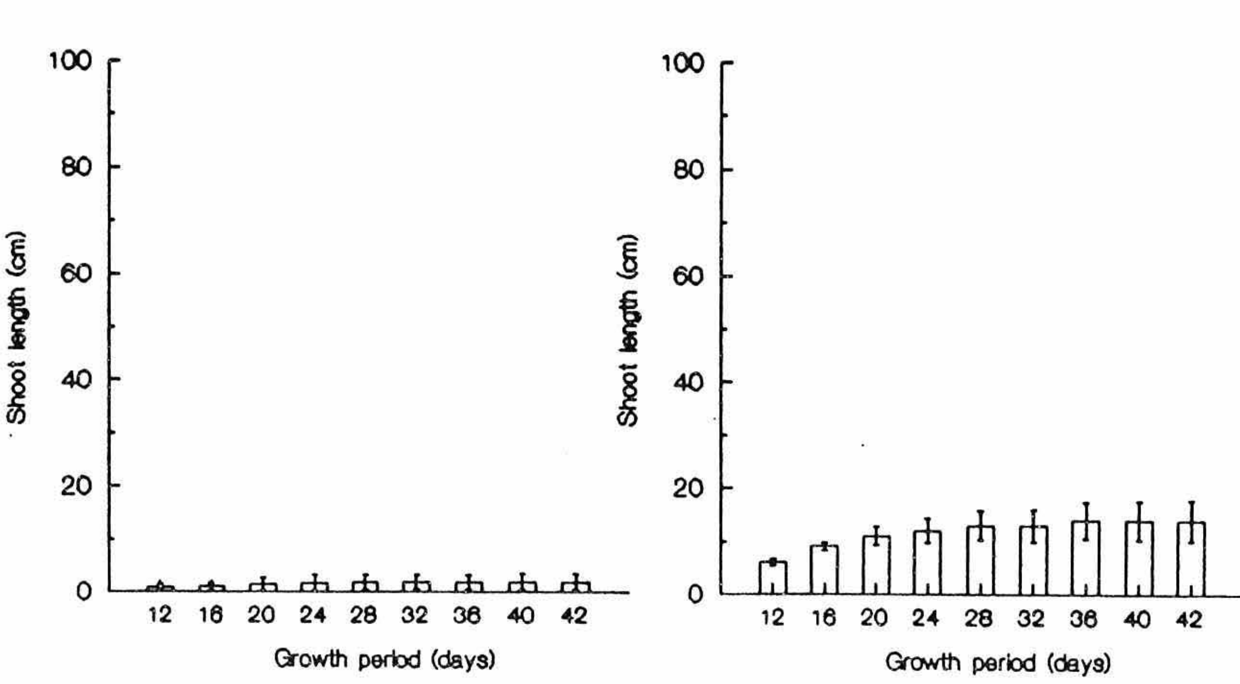


Figure 4.3c Effects of growth medium on shoot growth of cv. Bragg at 25°C. A, soil; B, sand; C, vermiculite; D, compost.



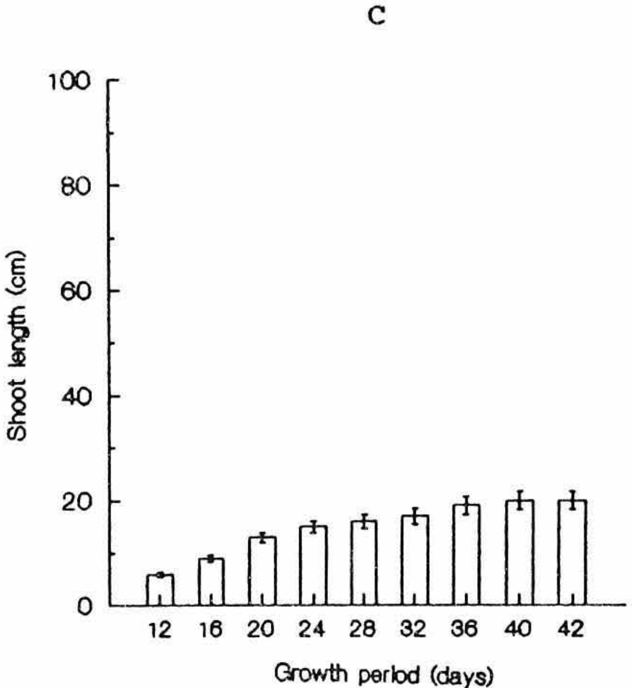
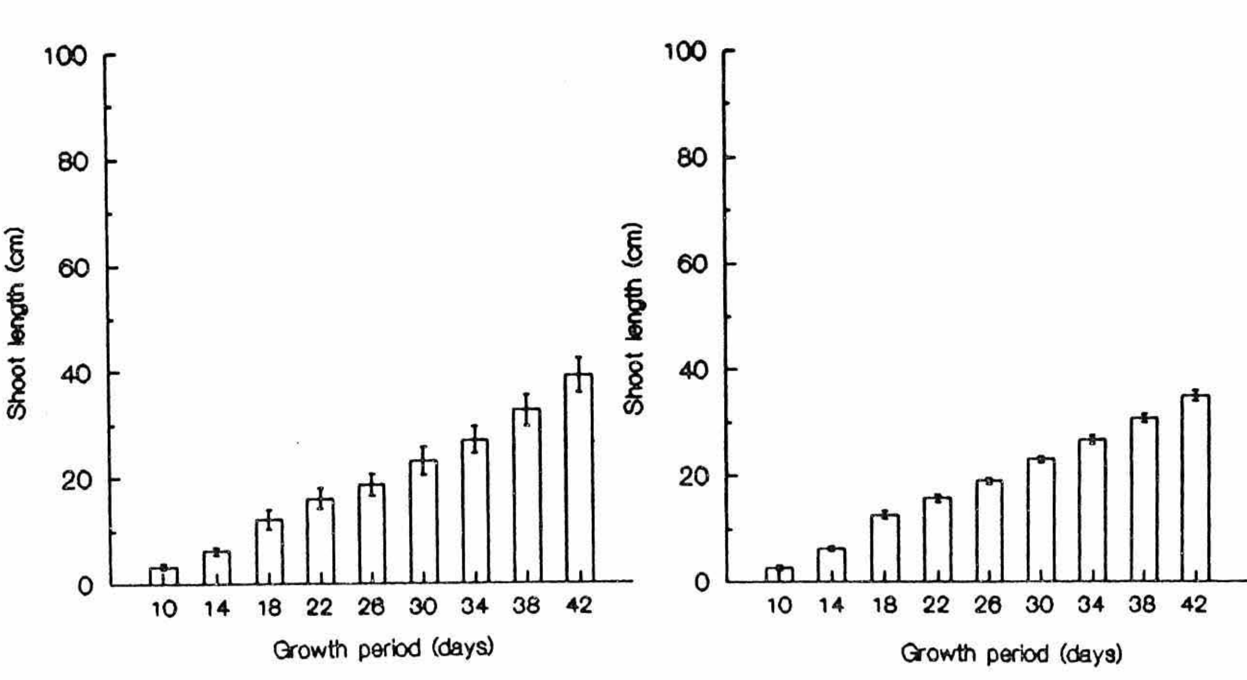


Figure 4.4a Effects of growth medium on shoot growth of cv. Century-84 at 18°C. A, soil; B, sand; C, vermiculite.



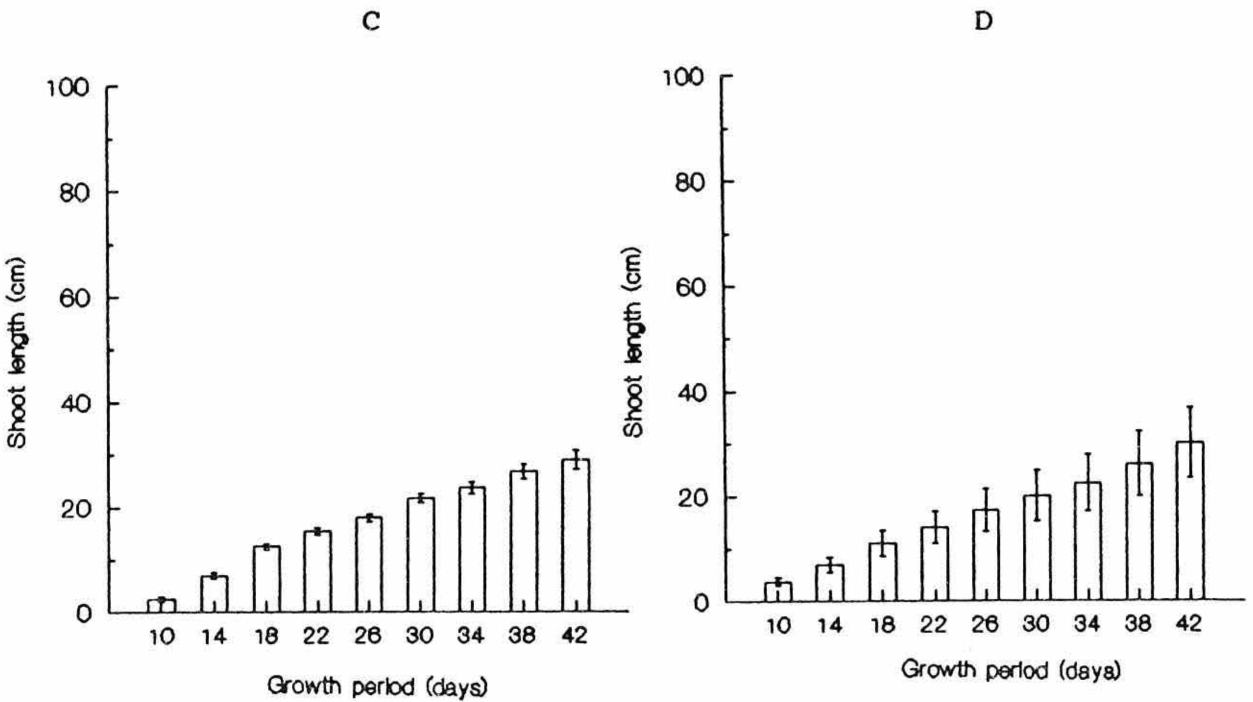
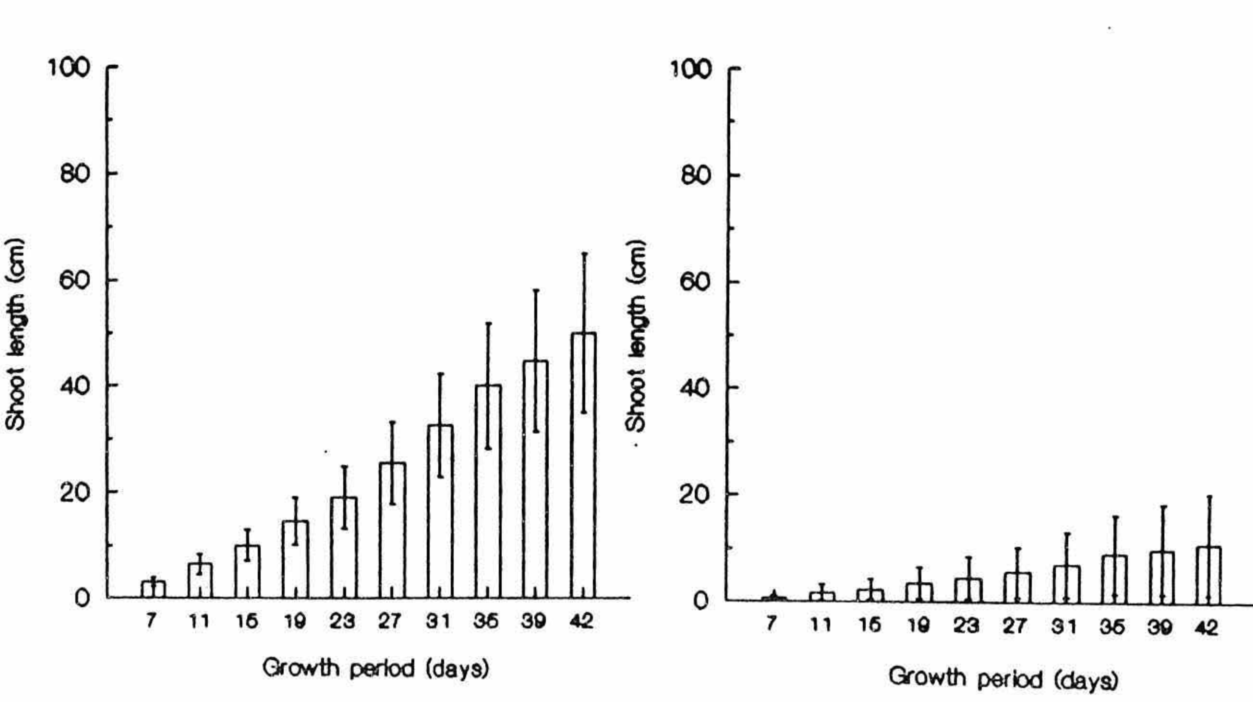


Figure 4.4b Effects of growth medium on shoot growth of cv. Century-84 at 20°C. A, soil; B, sand; C, vermiculite; D, compost.



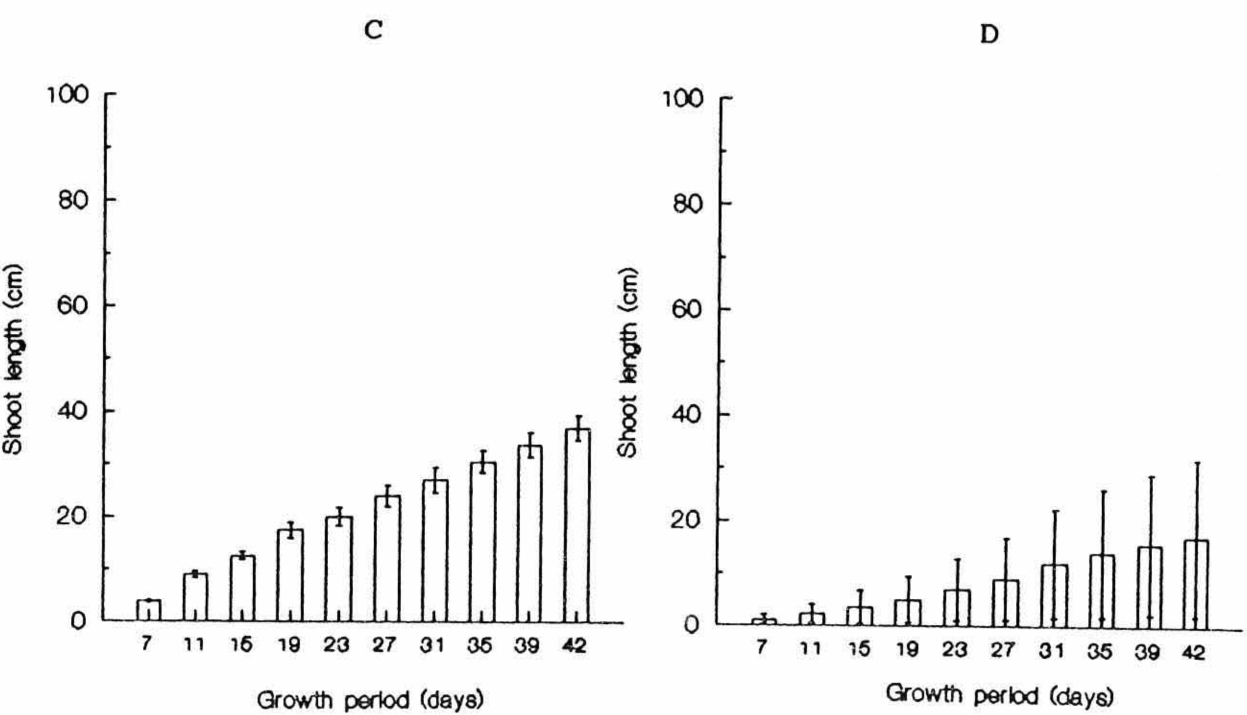
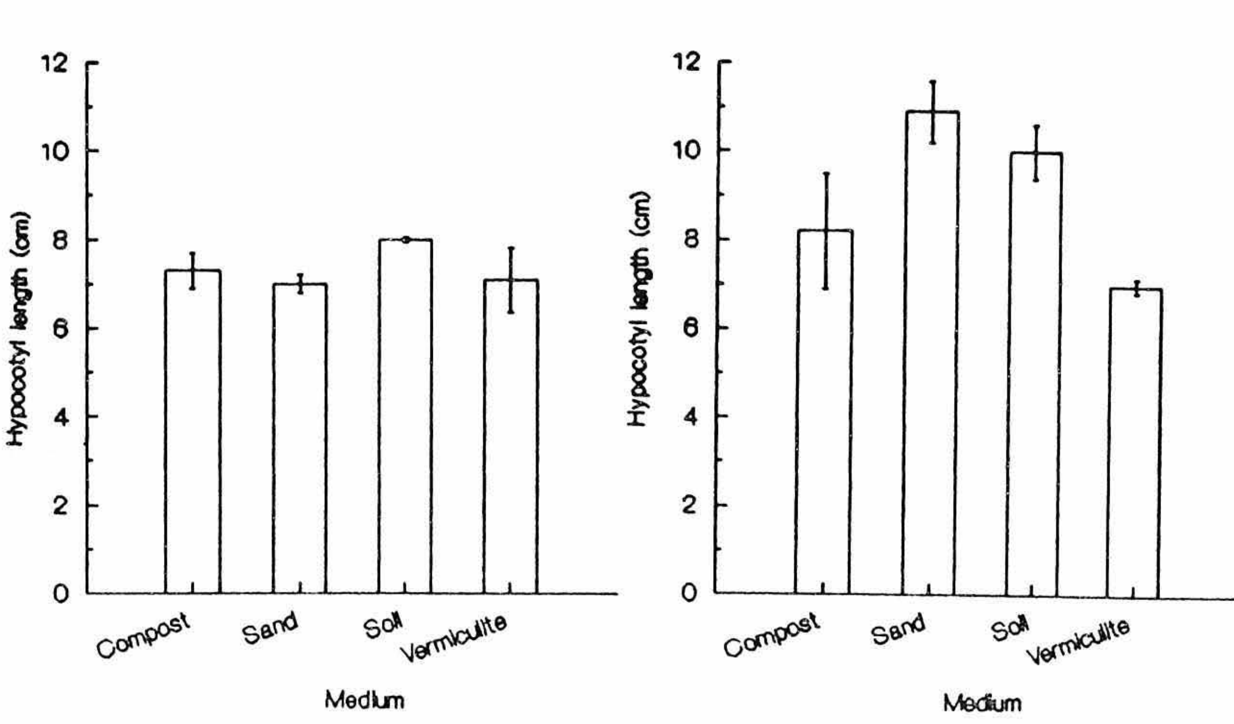


Figure 4.4c Effects of growth medium on shoot growth of cv. Century-84 at 25°C. A, soil; B, sand; C, vermiculite; D, compost.



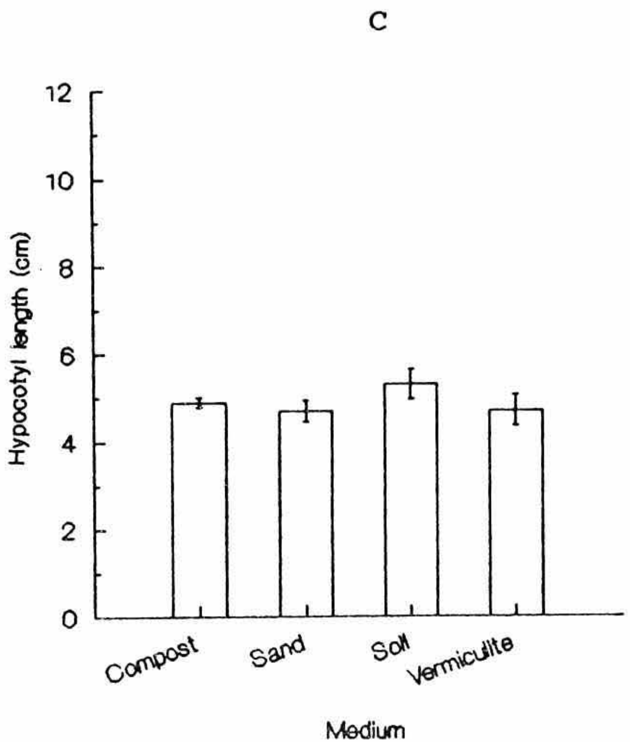
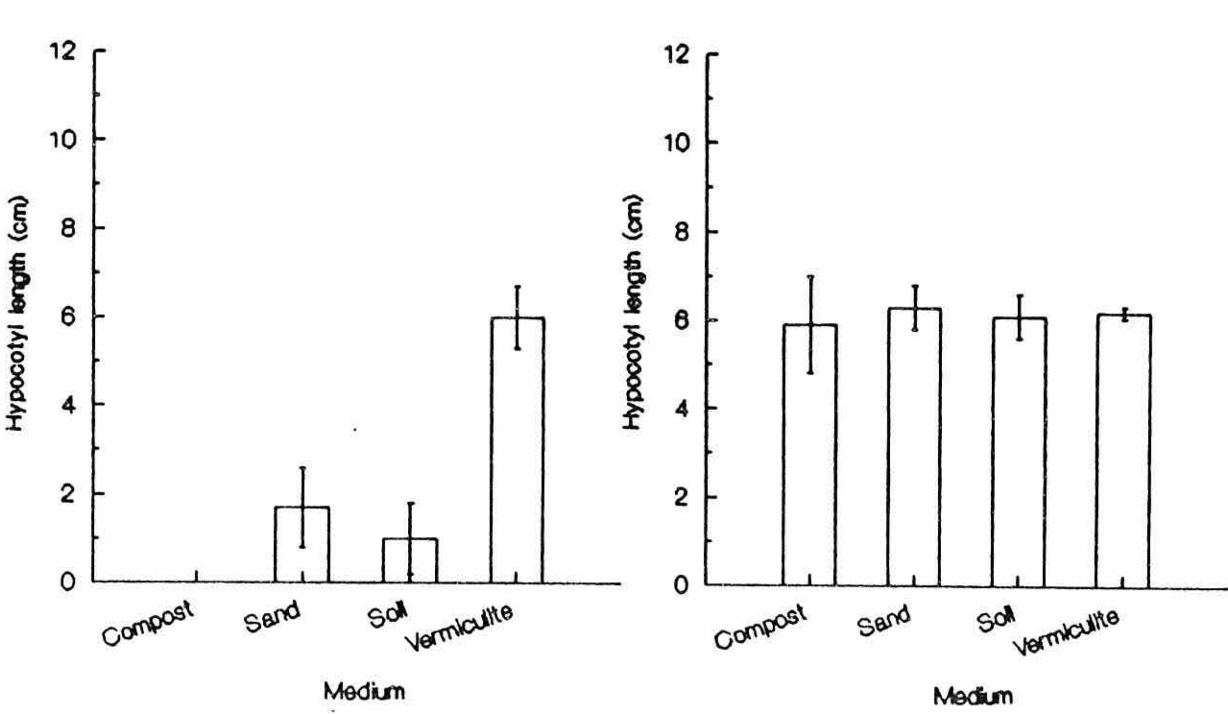


Figure 4.5 Effects of temperature and growth medium on the hypocotyl length of cv. Bragg. A, 18°C; B, 20°C; C, 25°C.

A



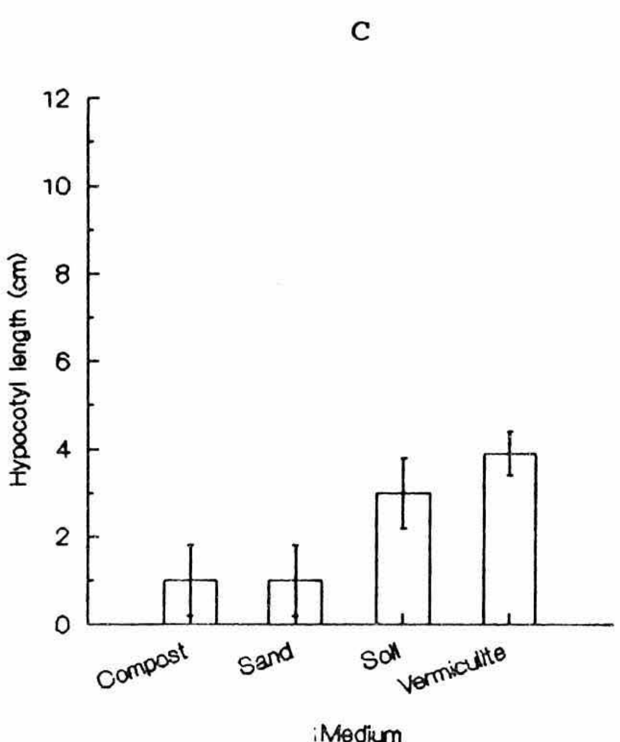
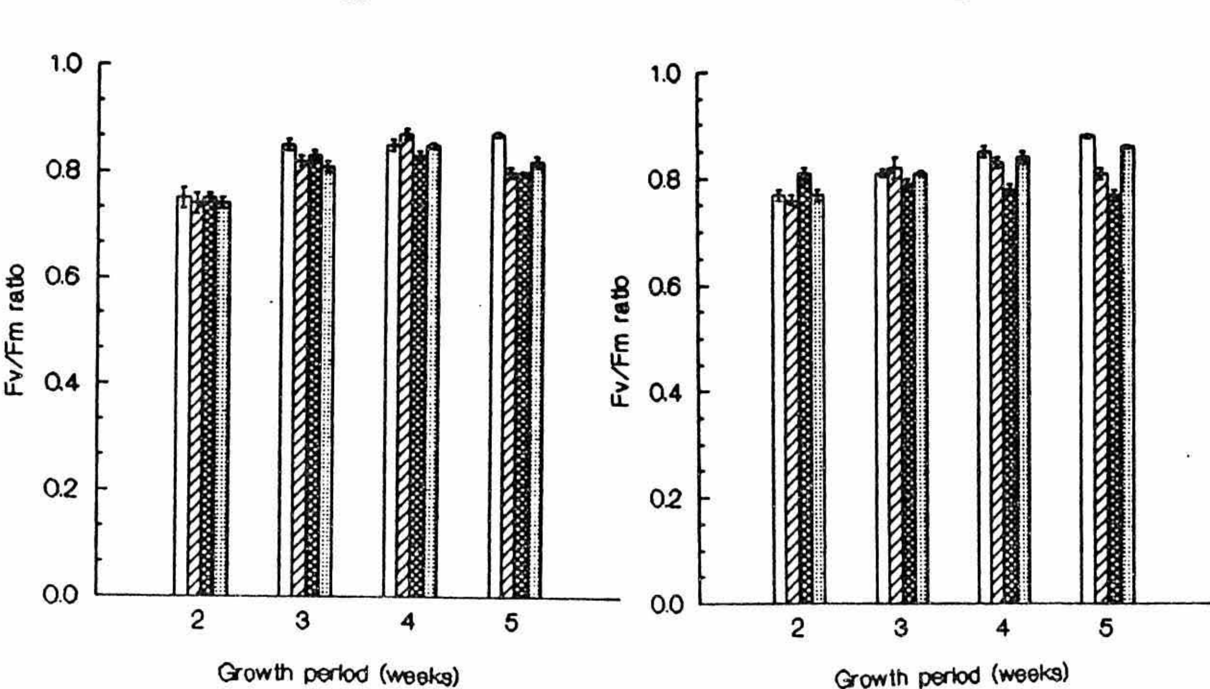


Figure 4.6 Effects of temperature and growth medium on the hypocotyl length of cv. Century-84. A, 18°C; B, 20°C; C, 25°C.



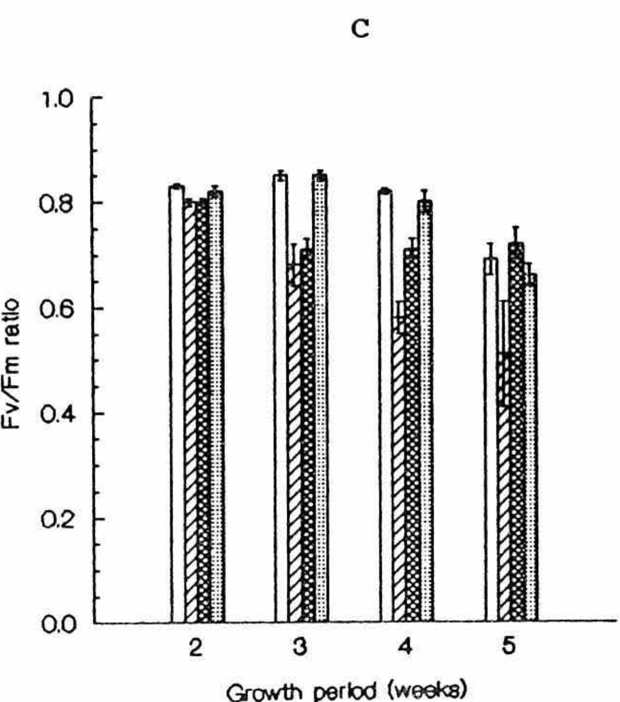
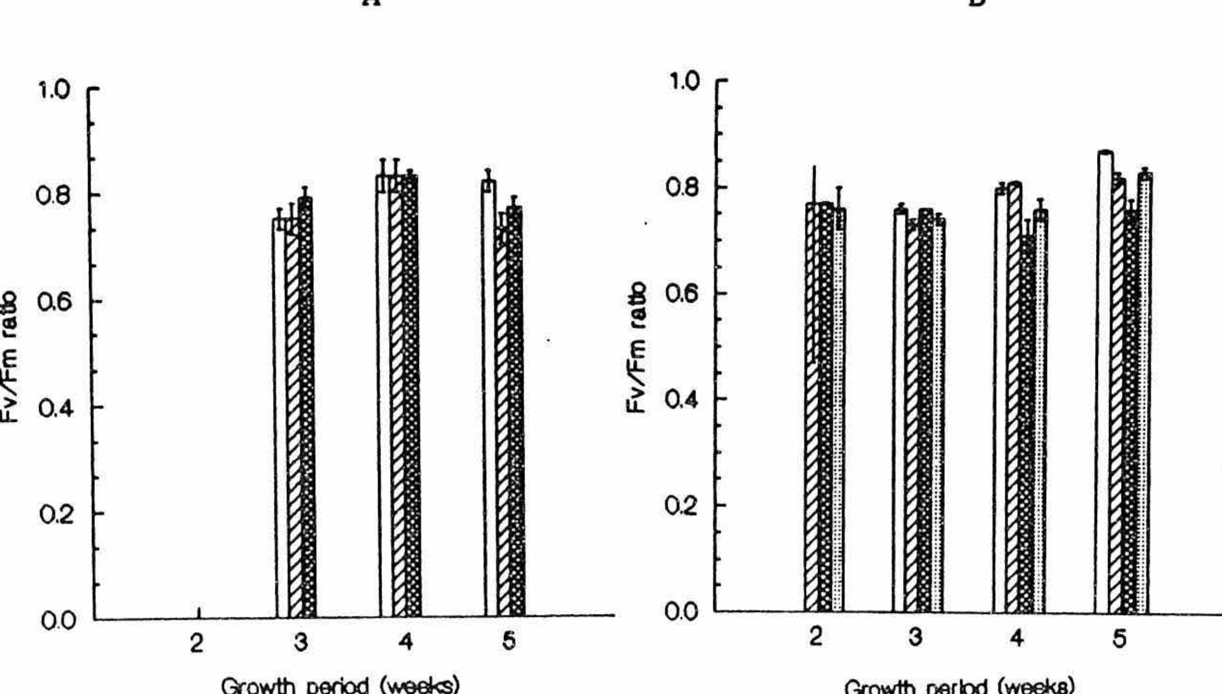


Figure 4.7 Effects of temperature and growth medium on leaf chlorophyll fluorescence in cv. Bragg. A, 18°C; B, 20°C; C, 25°C; ▭ , soil; ▨ , sand; ▩ , vermiculite; ▦, compost.



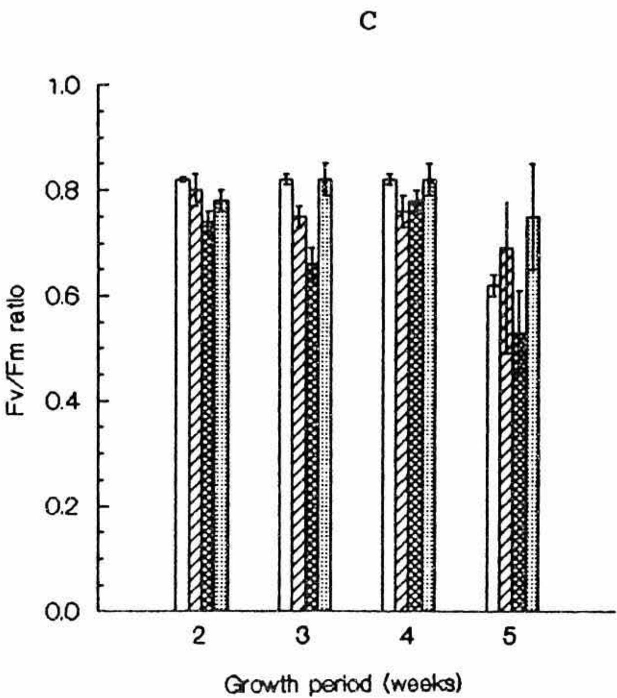
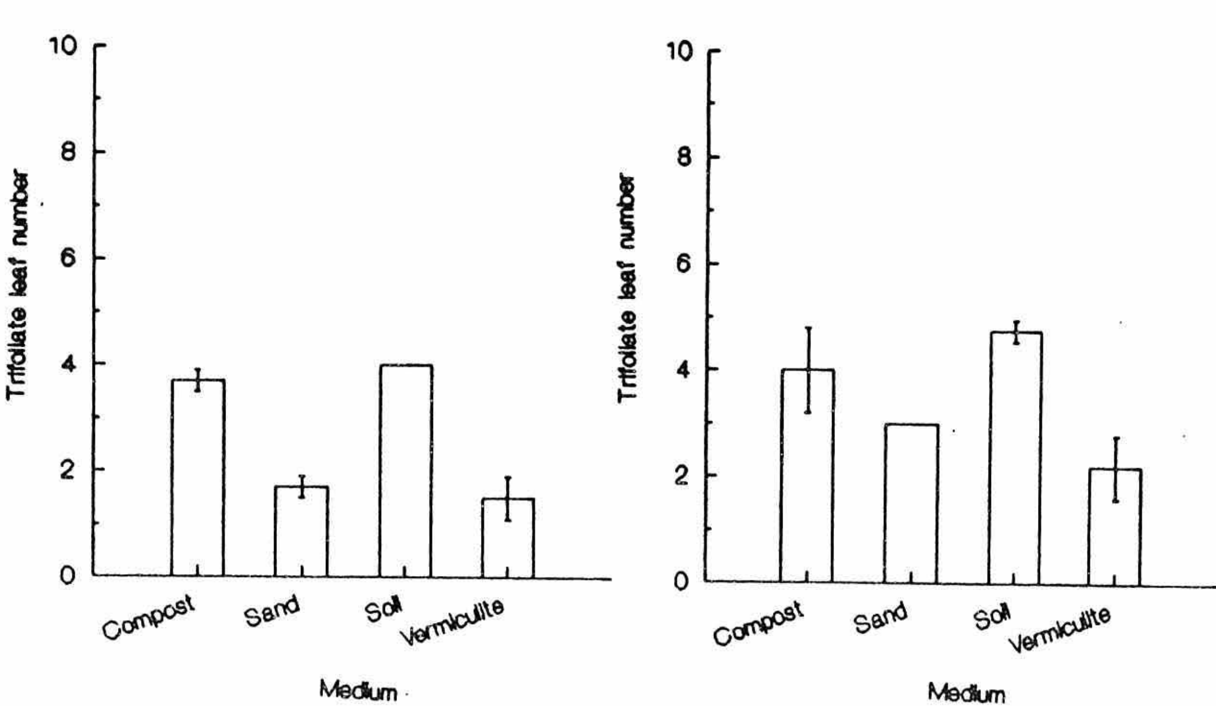


Figure 4.8 Effects of temperature and growth medium on leaf chlorophyll fluorescence in cv. Century-84. A, 18°C; B, 20°C; C, 25°C; [open bar], soil; [hatched bar], sand; [cross-hatched bar], vermiculite; [dotted bar], compost.



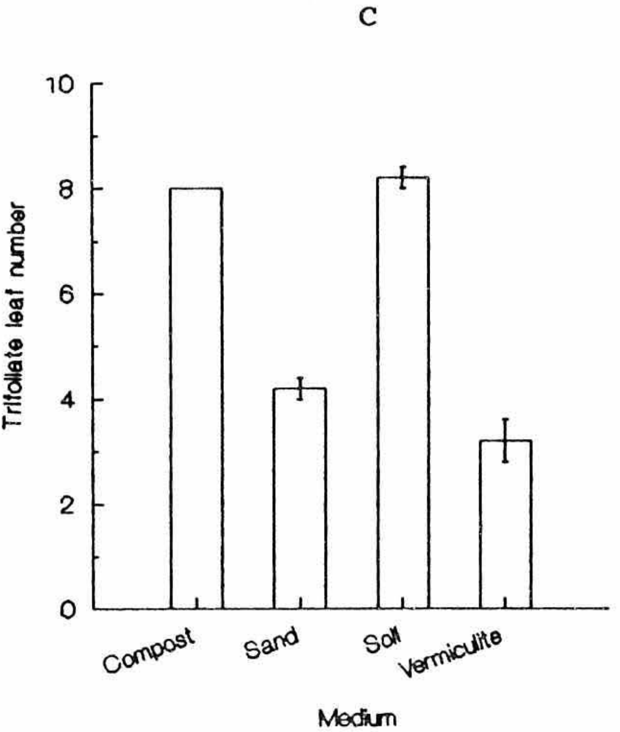
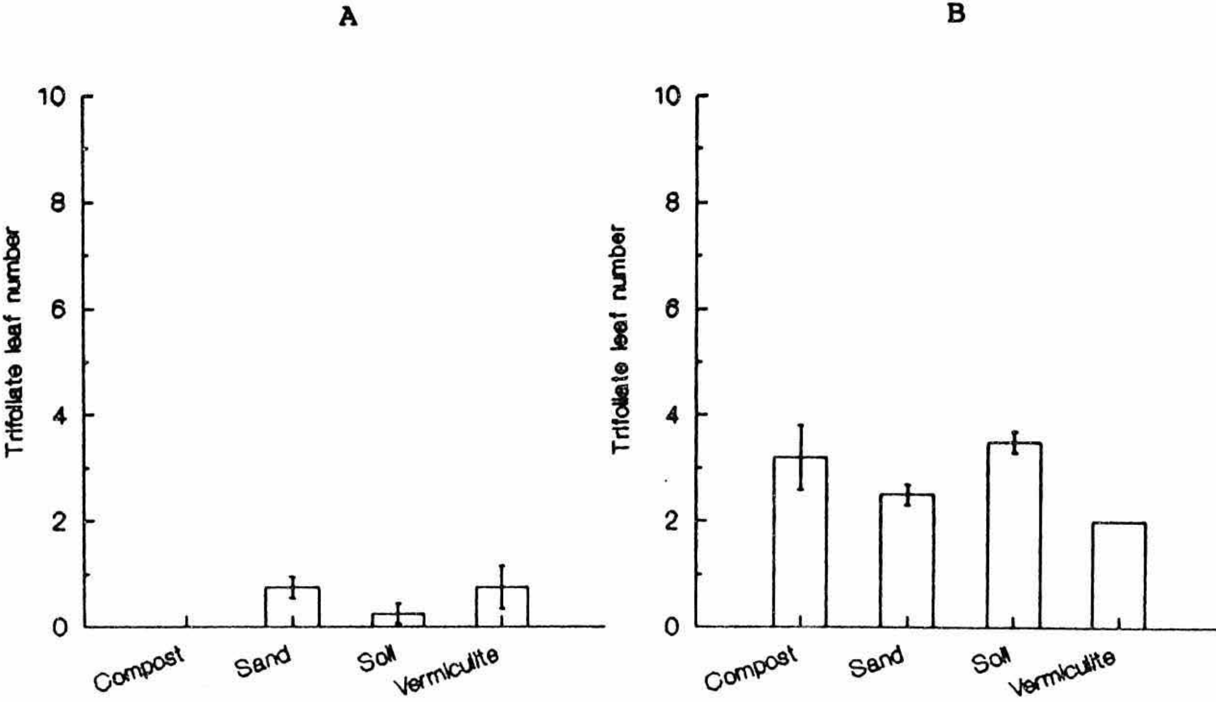


Figure 4.9 Effects of temperature and growth medium on trifoliate leaf number of cv. Bragg. A, 18°C; B, 20°C; C, 25°C.



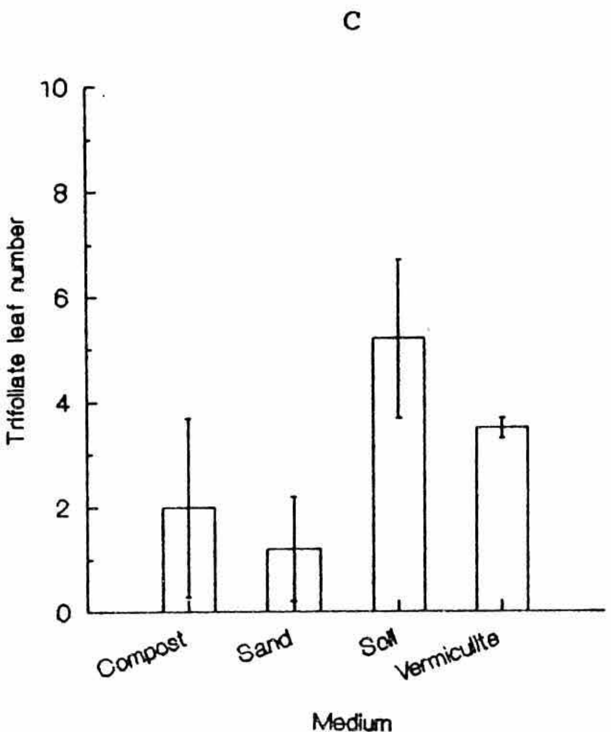
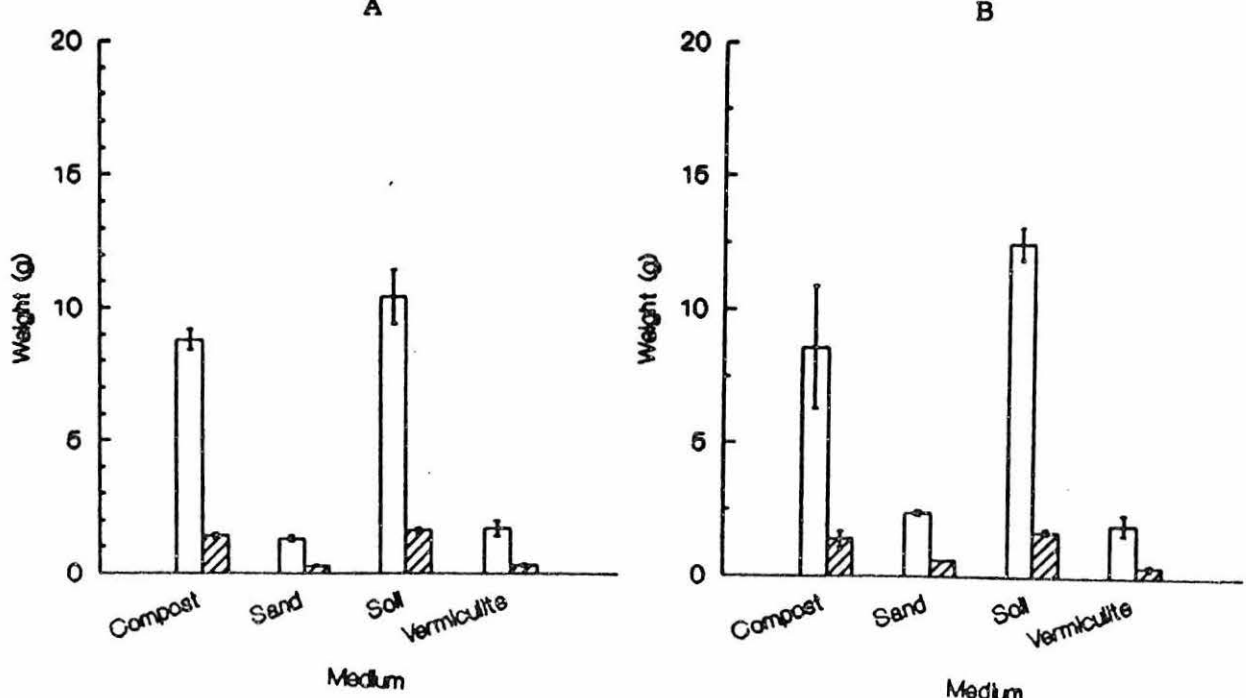


Figure 4.10 Effects of temperature and growth medium on trifoliate leaf number of cv. Century-84. A, 18°C; B, 20°C; C, 25°C.



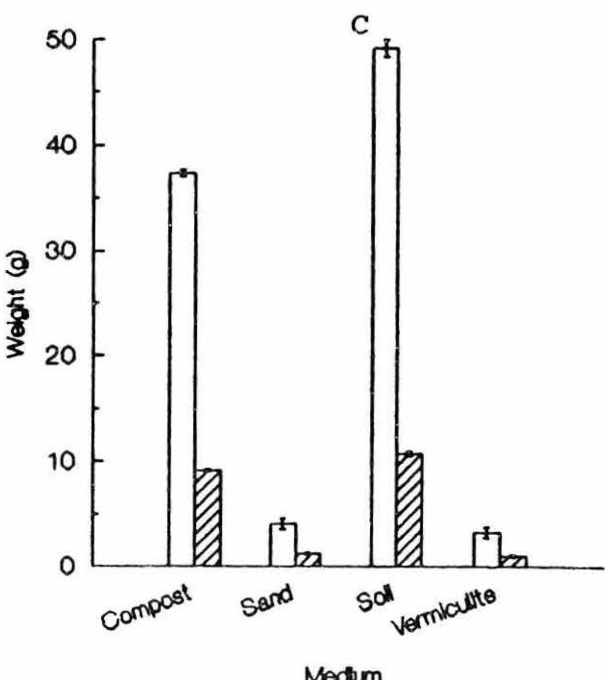
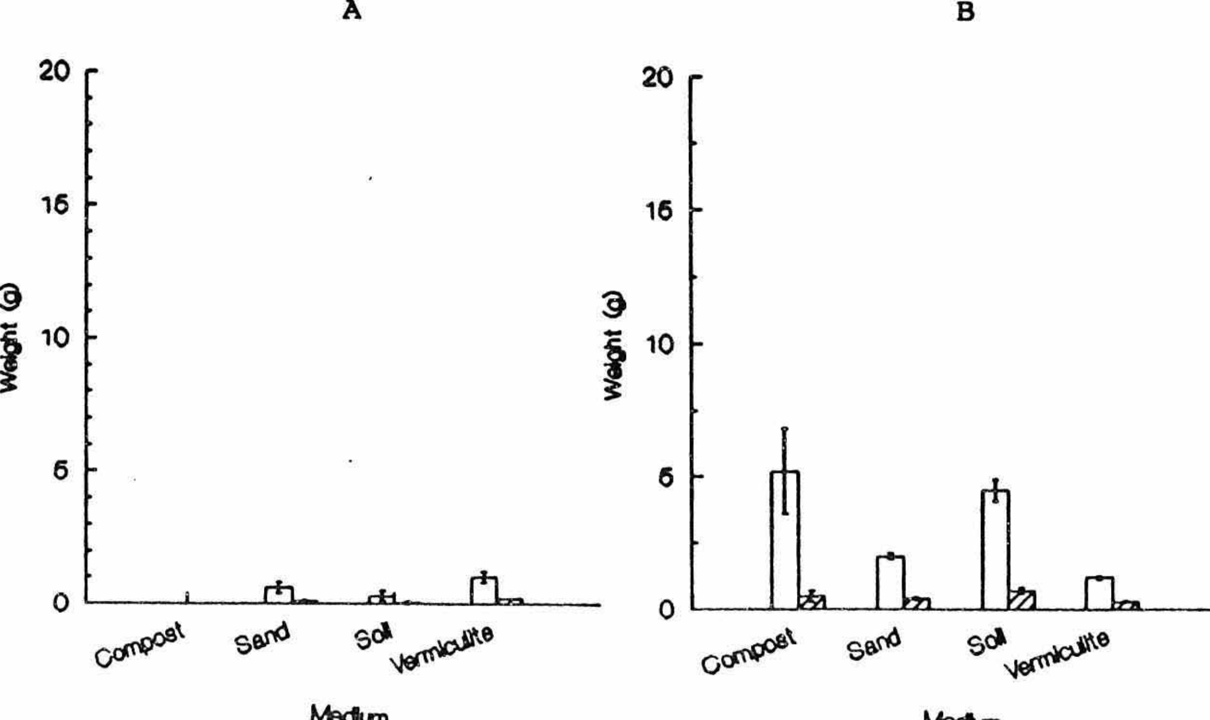


Figure 4.11 Effects of temperature and growth medium on shoot weight in cv. Bragg. A, 18°C; B, 20°C; C, 25°C; ▭ , fresh weight; ▨ , dry weight.



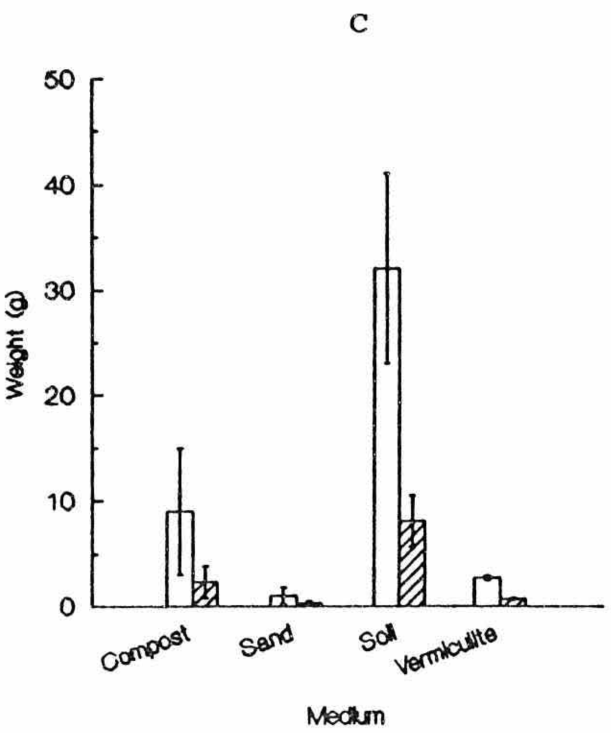
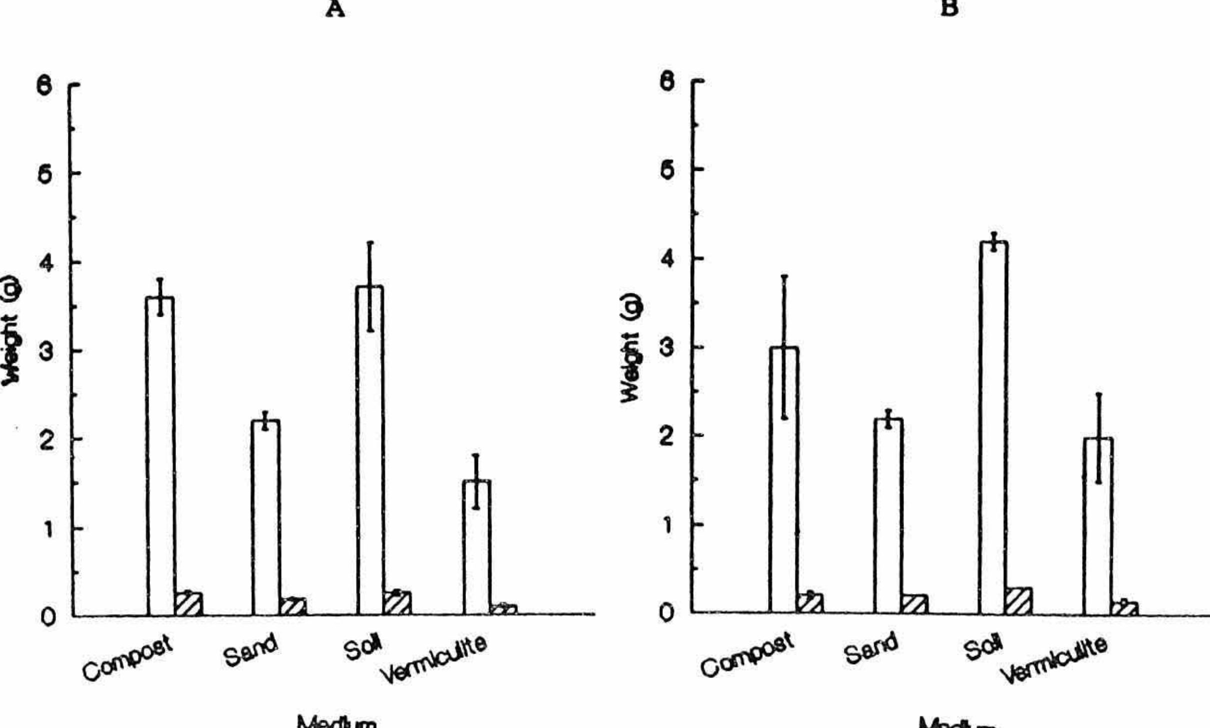


Figure 4.12 Effects of temperature and growth medium on shoot weight in cv. Century-84. A, 18°C; B, 20°C; C, 25°C; [ ] ; fresh weight; [hatched] , dry weight.



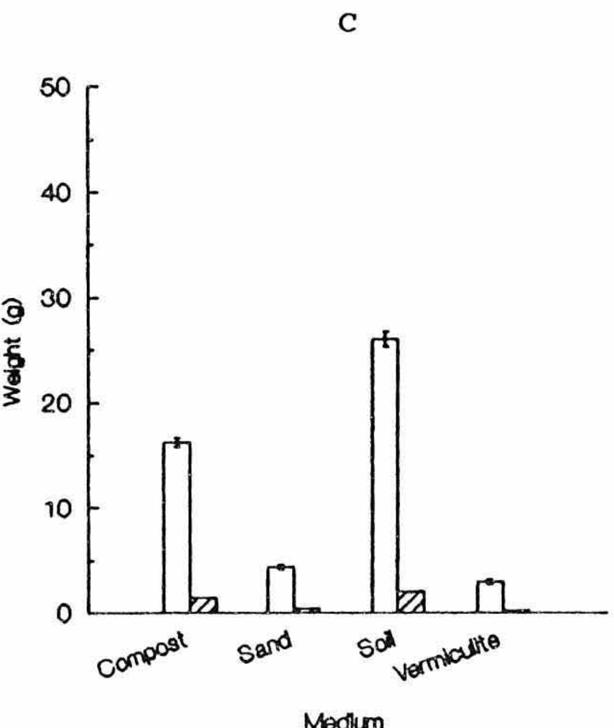
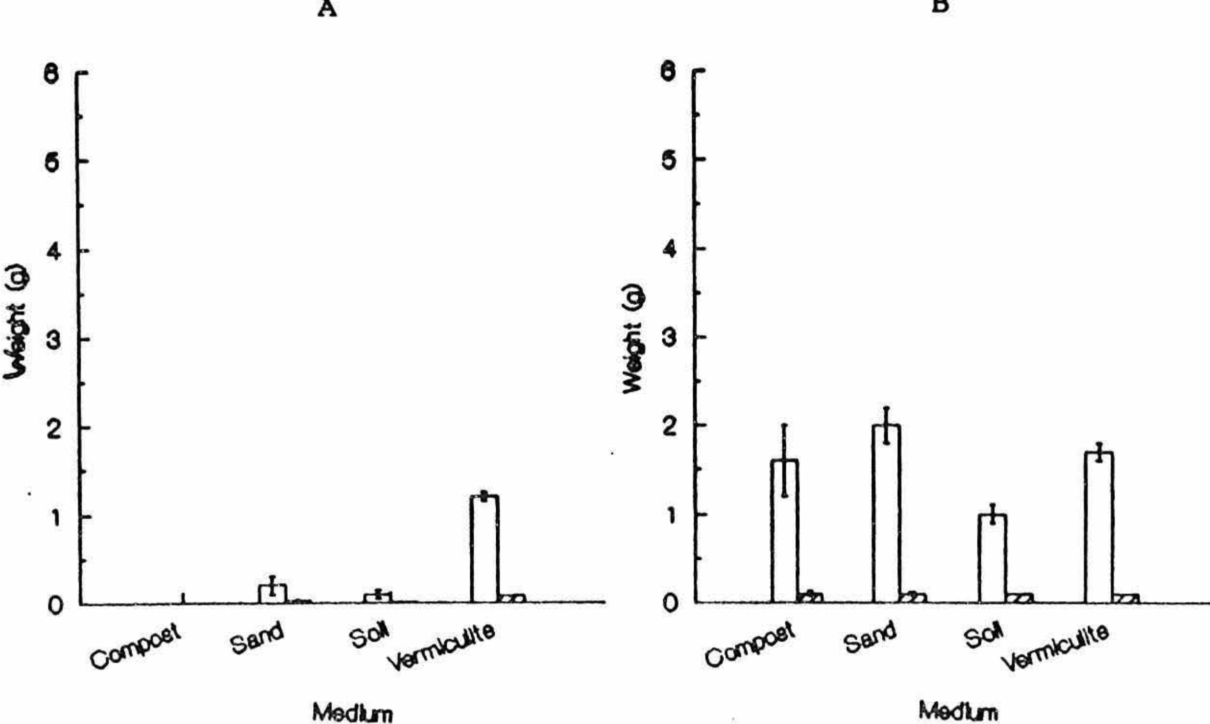


Figure 4.13 Effects of temperature and growth medium on root weight in cv. Bragg. A, 18°C; B, 20°C; C, 25°C; ▭ , fresh weight; ▨, dry weight.



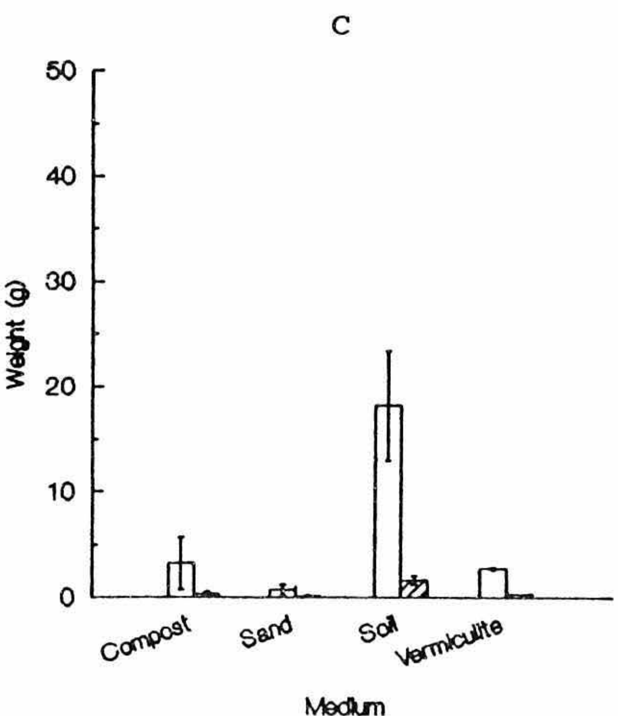
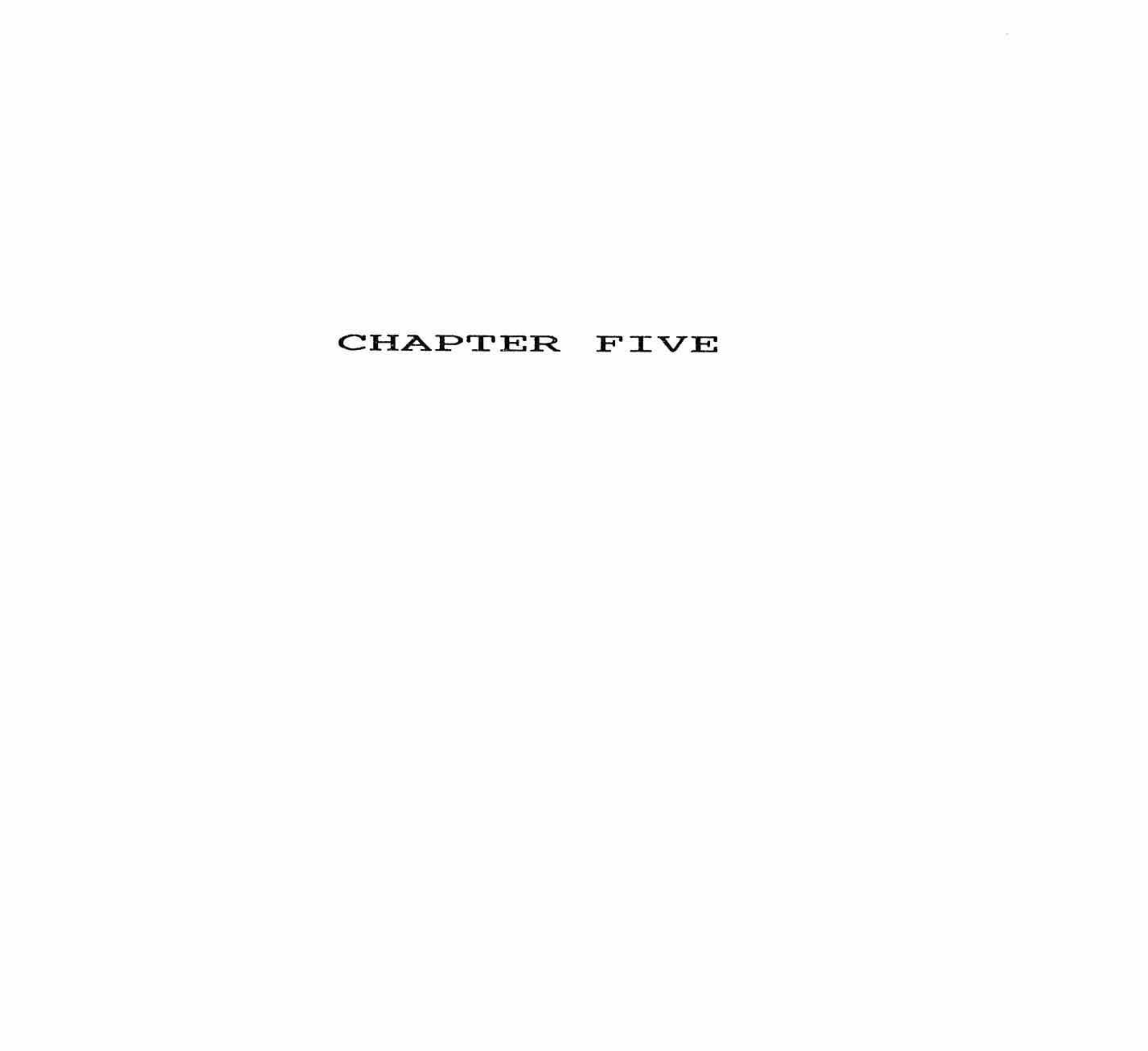


Figure 4.14 Effects of temperature and growth medium on root weight in cv. Century-84. A, 18°C; B, 20°C; C, 25°C; [open box]; fresh weight; [hatched box], dry weight.



CHAPTER FIVE

Chapter Five

Effects of Temperature on leaf Chlorophyll Fluorescence.

5.1 Introduction.

Chlorophyll fluorescence is now routinely used as a probe of thylakoid membrane activity in many aspects of photosynthesis research. The chlorophyll fluorescence technique is also frequently used to assess the effects of environmental stress on thylakoid metabolism. Reactions of the plant thylakoid membrane are considered to be the first processes to be damaged by heat, (Smillie, 1979; and Santarius, 1975).

Several fields of plant stress physiology have used chlorophyll fluorescence as a tool to assess the degree of environmental injury incurred by plants and genotypic differences in response to stress exposure. These include high temperature stress (Bilger *et al.*, 1987; Moffat *et al.*, 1990), chilling (Hetherington and Oquist, 1988; Neuner and Larcher, 1990), freezing (Greaves and Wilson, 1987), water stress (Schapendonk, 1987; Havaux and Lannoye, 1983), light (Chritchley and Smillie, 1981), and salinity (Larcher *et al.*, 1990). Heat damage, for example, is reflected in drastic changes of the light-induced fluorescence characteristics (Schreiber, 1971; Pearcey *et al.*, 1977; and Berry *et al.*, 1975). The thermal environment in which a plant is grown also markedly affects the temperature dependency of photosynthesis (Bjorkman *et al.*, 1975). These

heat stress effects can be assessed rapidly by the chlorophyll fluorescence technique. It is a fast and cheap method that can be used *in vivo* or *in vitro* (Larcher, 1980). Alternative methods are often more costly and time consuming.

Thus, chlorophyll fluorescence has been widely used to study the activity of the photosynthetic machinery. In particular, the linear relationship between quantum yield and the ratio of variable fluorescence to maximum fluorescence (Fv/Fm) (Adam *et al.*, 1990) has proved especially useful. The Fv/Fm ratio must be measured in dark-adapted photosynthetic systems and with an actinic light bright enough to saturate all the electron acceptors of photosystem II (PS II) at the P-peak. The dark adaptation usually takes 5-10 min to ensure that all energy-dependent quenching is relaxed (Krasue and Weis, 1984). Further details of the technique are given in Section 2.11. The present experiments were conducted to assess the utility of the chlorophyll fluorescence technique for our purposes and to evaluate soybean cultivars for high temperature tolerance.

5.2 Methods.

The work described in this chapter consisted of two major experiments.

5.2.1 Effects of high temperature stress on chlorophyll fluorescence.

Seeds of soybean cultivars, Mago-80, Sable, Davis, Bragg, Hardee and Williams-82 were germinated and grown at 24°C in the growth room as described in Section 2.11. The plants were inoculated with *B.rhizobium japonicum* RCR4307 five days after germination (Section 2.13.2). Plants without root nodules were grown without inoculation with *B.rhizobium.* One four-week-old first trifoliate leaf from each nodulated and non-nodulated plant was used to determine the effects of high temperature on chlorophyll fluorescence (measured as the Fv/Fm ratio) in the excised leaves (see Section 2.11). Fourteen replicates plants were used for each treatment.

Heat stress treatments of 2 and 4h were applied to the leaves at 35, 40, 42.5 or 45°C. The chlorophyll fluorescence (Fv/Fm ratio) was recorded before the application of the heat stress (control) and after the 2 and 4h treatments. Then, recovery was checked by again determining the Fv/Fm ratio after returning the plants to the growing temperature of 24°C for 12, 24 or 48h.

5.2.2 High temperature acclimation in soybean leaves.

Seeds of soybean cultivar Williams-82 were germinated and grown in a 27/20°C day/night temperature regime in the plant growth room (see Section 2.15.3). The plants were not inoculated with *B.rhizobium japonicum.* Three weeks after

germination, one unifoliate leaf and one (the first) trifoliate leaf from each of ten replicate plants were excised and used for each treatment. Four experiments were carried out on the excised leaves as follows (see Section 2.12):-

In the first experiment, the chlorophyll fluorescence (Fv/Fm ratio) was recorded to obtain a control value before applying three successive 10 min acclimation (heat-hardening) treatments at one of four different temperatures (40, 42.5, 45 or 47.5°C). Each heat-hardening treatment was separated by a two hour recovery at 27°C. After the final 10 min hardening treatment, the leaves were directly subjected to a further 30 min heat stress at the same temperature (40, 42.5, 45 or 47.5°C).

In the second experiment, the chlorophyll fluorescence (Fv/Fm ratio) was again recorded to obtain a control value before applying a single 30 min heat stress treatment at 40, 42.5, 45 or 47.5°C. Recovery was checked after returning the leaves to 27°C for 2h.

In the third experiment, heat-hardening treatment was applied at 42.5 or 45°C for three 10 min periods with 2h recoveries at 27°C following each of the first two periods of hardening. A further 30 min heat stress was then applied at 47.5°C directly following the last hardening treatment.

In the fourth experiment, four 10 min heat-hardening treatments were applied at 45°C. Each of the first three treatments were followed by 2h recovery at 27°C. A final 30 min heat stress was then applied at 47.5°C directly following the last hardening at 45°C.

5.2.3 Statistical analysis.

All means, standard deviation and standard error values were calculated using a pocket scientific calculator (Sharp Model EL-531P) and confirmed using a personal computer (Mitac). Statistical analysis of variance (ANOVA) was done using Minitab for Windows (version 10.2). All the figures were plotted using the Systat/sygraph (version 5.03). In all figures vertical bars show the standard errors of the mean values.

5.3 Results.

5.3.1 Effects of high temperature stress on chlorophyll fluorescence.

Experiments were conducted to investigate the effects of high temperatures on chlorophyll fluorescence (Fv/Fm ratio) in leaves of six soybean cultivars (see Section 5.2.1).

The data for cultivar Williams-82 presented in Fig. 5.1 show that, before heat stress, the Fv/Fm ratio was similar in control leaves from non-nodulated and nodulated plants. The value was 0.87 in both cases. Two or 4h heat stress treatments at 35°C did not significantly affect the ratio

in either group of leaves, and there were insignificant changes in the ratio through 12, 24, and 48h of recovery at 24°C. Also, there were no differences between the nodulated and non-nodulated plant leaves. Heat stress at 40°C for 2 and 4h caused a significant decrease (up to 50%) in the Fv/Fm ratio in the non-nodulated plant leaves. There were noticeably smaller decreases in the nodulated plants following the same treatments. After a 12h recovery period, the Fv/Fm ratio was fully recovered in the leaves of the nodulated plant and there was little further change with increased recovery time up to 48h. In the non-nodulated plant leaves, the Fv/Fm ratio recovered more slowly than in the nodulated plants. After 2h heat stress at 42.5°C, there was a 30% reduction in the Fv/Fm ratio in nodulated plants and a 37% reduction in the non-nodulated plant leaves. In both cases a further (20%) reduction was recorded after 4h of stress. Both the nodulated and non-nodulated plant leaves recovered their Fv/Fm ratio at approximately the same rate through 12, 24, and 48h. Neither of them fully reached the value of the control however. The most dramatic effect on chlorophyll fluorescence was observed at 45°C. After 2h of heat stress, the Fv/Fm ratio was reduced by 78% in the leaves from the nodulated plants and by 85% in non-nodulated plants. There was little additional effect of 4h heat stress in either group of plant. Most strikingly, there was no recovery in the Fv/Fm ratio in any of the plant leaves following recovery at 24°C.

The data in Fig. 5.2 shows that, in the cultivar Sable, the control Fv/Fm ratio was similar to the value for Williams-82 and it was the same in nodulated and non-nodulated plant leaves. Indeed, the control Fv/Fm ratios were very similar for all nodulated and non-nodulated plants in all of the cultivars studied (cf. Figs. 5.1 - 5.6). These control values will, therefore, not be mentioned again. After 2 and 4h heat stress at 35°C the ratio did not change significantly. Similar Fv/Fm ratios were also observed through the period of recovery. Two hours of heat stress at 40°C caused the ratio to decreased slightly, the effect being greater in the non-nodulated than in the nodulated plant leaves. A 40-45% reduction was observed after 4h heat stress in both groups of leaves. After 12h of recovery, the Fv/Fm ratio was fully recovered in the leaves of nodulated plants. A somewhat slower recovery was found in the leaves of the non-nodulated plants, where the value did not quite reach the control value. The Fv/Fm ratio was decreased by 15 to 20% after 2h heat stress at 42.5°C in both groups of plants. Little further change was observed after 4h heat stress in the leaves of the nodulated plants, but an approximately 40% reduction was recorded in the non-nodulated plants. After 12h of recovery at 24°C, the Fv/Fm ratio decreased further in leaves from nodulated plants, while a 20% recovery was found in the leaves from the non-nodulated plants. Further recoveries were observed after 24h and 48h. The Fv/Fm ratio was severely affected (80% decrease) after 2h heat stress at 45°C in both groups of

plants. Surprisingly, smaller reductions were observed after 4h heat stress. After a 12h recovery period, a very small recovery was found in the leaves from nodulated plants, but there was no recovery in the non-nodulated plants. This effect was short-lived, as zero recovery was recorded in both group of plants after 24 and 48h recovery.

The data in Fig. 5.3 show that the cultivar Mago-80 was not affected by heat stress at 35°C. A small effect following 2 and 4h heat stress at 40°C was observed in leaves of nodulated plants and a slightly greater effect was seen in the non-nodulated plants. Slow recoveries were evident in the both groups of plants, but the Fv/Fm ratios did not reach the control value in either case. Following heat stress at 42.5°C, the Fv/Fm ratio was reduced by 45% in the leaves of nodulated plants and by approximately 70% in leaves from non-nodulated plants. These values collapsed to very low levels during the first 12h recovery at 24°C. Recovery in the leaves from non-nodulated plants was very slow, amounting to only 5%. A somewhat better recovery took place in the leaves of the nodulated plants, where the Fv/Fm ratio recovered to about 35% of the control after 48h. At 45°C, a sharp reduction of 78% in the ratio was observed after 2h of heat stress in the leaves from nodulated plants and a 72% reduction was recorded in those from non-nodulated plants. A further 15-20% decrease was found after 4h heat stress in both groups of leaves. There was no recovery from these very low values.

The Fv/Fm ratios for cultivar Bragg (Fig. 5.4) show that there were no significant effects of 2 or 4h heat stress at 35°C. At 40°C, small reductions were found after 2 and 4h of stress in both groups of plants. Through 48h of the recovery period following this treatment at 40°C, the Fv/Fm ratio continued to decrease in the leaves from nodulated plants. A little recovery was observed in the leaves of the non-nodulated plants however. At 42.5°C, the Fv/Fm ratio decreased by 55% after 2h of heat stress and a further 10-15% reduction was observed after 4h heat stress in both groups of leaves. The ratios collapsed totally to zero by the end of the first 12h of the following recovery period at 27°C. A small improvement was found after 24h in the leaves from nodulated plants, while at 48h there was approximately 30% increase in the Fv/Fm ratio in these leaves. There was a 15% increase in the value for the leaves from the non-nodulated plants. A sharp reduction of 70% in the Fv/Fm ratio was recorded after 2h heat stress at 45°C and a further 10% reduction took place after 4h of heat stress in both groups of leaves. These ratios fell to zero after 12h in recovery and they did not change from this values.

The data in Fig. 5.5 show that the Fv/Fm ratio in cultivar Davis gave similar initial results to the other cultivars. There was little change in the value following heat stress at 35°C and subsequent recovery at 24°C. The only exception to this was a small 15% reduction after 12h of recovery in

the leaves from non-nodulated plants. This was probably a spurious result. At 40°C, the Fv/Fm ratio was reduced by about 7-12% after 2h of heat stress in both groups of leaves and a further 12% reduction was observed after 4h of stress in nodulated plant leaves. Small recoveries in the ratios were obtained after 12h of recovery at 24°C in both groups of plants, after which no further recoveries were observed. After 2h heat stress at 42.5°C, a 30% decrease in the Fv/Fm ratio occurred in leaves from nodulated plants and 20% reduction in those from non-nodulated plants. There were further small reductions after 4h of heat stress. After 12 and 24h of recovery treatment at 24°C, the Fv/Fm ratios recovered very slowly, if at all, in the leaves from both groups of plants. They then decreased by about 10-15% after 48h of recovery. Following heat stress at 45°C, the Fv/Fm ratio underwent an 85% reduction after 2h in both types of leaves, and these values did not change after 4h heat stress. Zero values for the Fv/Fm ratio were recorded after 12, 24 and 48h into the recovery period in both groups of leaves.

The results for the last cultivar Hardee are presented in Fig. 5.6. They show that the Fv/Fm ratio was not affected by 2 or 4h heat stress at 35°C. After 12h of the recovery period, however, the Fv/Fm ratio was reduced by about 13%, but it fully recovered after 24h in the leaves from both groups of plants. At 40°C, a 10% decrease in the Fv/Fm ratio occurred after 2h of heat stress and no further

reduction was observed after 4h of stress in either group of plants. After 12h into the recovery period following treatment at 40°C, the ratio showed only a small recovery and it then remained the same at 24 and 48h into the recovery period. The Fv/Fm ratio decreased by 28-31% after 2h heat stress at 42.5°C and a little further reduction was obtained after 4h of stress in the leaves from both groups of plants. After 12h of recovery at 24°C, small increases amounting to about 15% of the control value were noted in each group of leaves. The Fv/Fm ratio then remained the same up to 24h of recovery, but another reduction was observed after 48h. At 45°C, heat stress produced large changes in the Fv/Fm ratio. After 2h of stress, the Fv/Fm ratio was decreased by 80-85% in both group of leaves and remained the same after 4h of heat stress. A very small improvement was observed after 12h of recovery at 24°C, but the ratio fell back to zero after that.

The data were analysed using the ANOVA test, which showed that the Fv/Fm ratio was strongly dependent ($P<0.05$) upon the cultivar, temperature, stress period and the recovery period. There was also a statistically significant ($P<0.05$) different between the nodulated and non-nodulated plants with regard to their overall responses to the applied temperature regimes. Also there was significant interaction ($P<0.05$) between the cultivar, temperature, and the nodulated and non-nodulated plants.

5.3.2 High temperature acclimation in soybean leaves.

In this experiment, different types of heat-hardening treatments were applied to the first unifoliate leaves and the first trifoliate leaves from the soybean cultivar Williams-82 (see Section 5.2.2.).

The results from the first of the four experiments are presented in Figs. 5.7 - 5.10. The data in Fig. 5.7 show that, before applying heat-hardening treatments, the Fv/Fm ratio was the same in the first unifoliate leaves and first trifoliate leaves. The values in each case were 0.85 and 0.87 respectively. There were no significant effects of three 10 min hardening treatments at 40°C on the ratio in either type of leaf (Fig. 5.7). Furthermore, the Fv/Fm ratio did not change significantly during the recovery period between each 10 min hardening treatment. The Fv/Fm ratio was also not affected during the final 30 min of heat stress at 40°C.

The acclimation (heat-hardening) treatment at 42.5°C caused only small changes to take place in the Fv/Fm ratio (Fig. 5.8). A 10% decrease in the ratio occurred after the first 10 min treatment in both the unifoliate leaf and the trifoliate leaf. The ratio recovered fully after the subsequent 2h recovery at 27°C in both cases. Small (8-10%) reductions were observed after the second 10 min hardening treatment, but the ratio recovered fully again in both cases during the subsequent recovery period. After the

third 10 min hardening, an 8-10% decrease occurred in both leaf types. A final 30 min heat stress at 42.5°C produced no further reduction in the case of unifoliate leaf or the trifoliate leaf. There were only small differences between the unifoliate and the trifoliate leaves at any point through the sequence of treatments.

At 45°C (Fig. 5.9), a 23% decrease in the Fv/Fm ratio occurred after the first 10 min heat-hardening treatment in unifoliate leaves and a 30% reduction was observed in trifoliate leaves. After 2h of recovery at 27°C, the ratio recovered to within 20-23% of the control in both types of leaves. Then, it decreased by approximately 18-20% after the second 10 min of hardening and in both type of leaves it decreased further by about 25% during the following 2h recovery. After the third 10 min heat-hardening treatment, increases to within about 15-20% of the controls occurred in both types of leaves. There was no further increase or reduction in the ratio following the final 30 min of heat stress in the unifoliate leaves, but a 40% reduction was observed in the trifoliate leaves. At this stage, there appeared to be a considerable difference between the two types of leaves.

The data in Fig. 5.10 shows that the first heat-hardening treatment at 47.5°C caused the Fv/Fm ratio to decrease by approximately 55 and 75% in unifoliate leaves and trifoliate leaves respectively. During recovery from this

treatment, 30% of the control Fv/Fm ratio was regained in unifoliate leaves and 40% recovery was observed in trifoliate leaves. Following the second 10 min hardening, the ratio decreased again to 65% of the control values in unifoliate leaves and to 80% of the control value in trifoliate leaves. After the second 2h recovery, a smaller recovery in the Fv/Fm ratio occurred in both types of leaves. A decrease, of 30-35% then took place after the third heat-hardening treatment in both types of leaves. There were no further changes during the final 30 min heat stress. At this temperature there were apparently considerable significant differences between the trifoliate and unifoliate leaves.

Statistically, the Fv/Fm ratio was dependent ($P<0.05$) upon the temperature regime applied. There were, however, no overall statistically significant differences between the responses of the unifoliate and the trifoliate leaves to the applied temperature regimes. This was true despite the apparent differences mentioned above.

In the second acclimation experiment, 30 min heat stress treatments were applied at 40, 42.5, 45 or 47.5°C after measuring the control values at 27°C. The results for the experiment are presented in Figs. 5.11 - 5.14. Following a single treatment at 40°C, there was no convincing change in the Fv/Fm ratio in either type of leaf and no change was found after subsequent recovery at 27°C (Fig. 5.11). At

42.5°C (Fig. 5.12), a very small (10%) and probably insignificant decrease in the ratio was found after 30 min heat stress in both types of leaves. In both cases, the ratio then fully recovered during the recovery period at 27°C. The data in Fig. 5.13 shows that heat stress at 45°C caused a 25% decrease in the Fv/Fm ratio in both types of leaves. There was a further reduction of 7% after 2h recovery in the unifoliate leaf, but a further 30% decrease occurred in the trifoliate leaves. Finally, Fig. 5.14 shows that a 75% decrease occurred in the Fv/Fm ratio after 30 min heat stress at 47.5°C in both types of leaves and that the ratio increased to within about 20% of the control value after recovery at 27°C.

Statistically, there were no differences ($P>0.05$) between the responses of the unifoliate leaf and the trifoliate leaf, but the Fv/Fm ratio was strongly dependent ($P<0.05$) upon the temperature.

In the third regime of acclimation treatment, three 10 min heat-hardening treatments were applied at 42.5 or 45°C with a 2h recovery between each treatment. A further 30 min heat stress was then applied at 47.5°C directly following the last hardening treatment. The data in Fig. 5.15 show that, before applying heat stress, the Fv/Fm ratio was the same in both types of leaves. After the first 10 min of heat hardening at 42.5°C, a very small (6-10%) reduction in the ratio was observed, also in both types of leaves. Then, it

fully recovered after 2h recovery at 27°C in both types of leaves. Approximately 9-12% decreases in the ratio were found after the second hardening treatment. In this case, recoveries to within 3-5% of the control value were observed. By the end of the third hardening treatment, the ratio had declined again to just below that recorded at the end of the second hardening treatment. The final 30 min heat stress at 47.5°C produced a dramatic 50% reduction in the Fv/Fm ratio in the unifoliate leaves and approximately 70% reduction in trifoliate leaves compared with the control value.

At 45°C (Fig. 5.16), the first heat-hardening treatment caused a 43% decrease in the Fv/Fm ratio in the unifoliate leaves and a 58% decrease was found in trifoliate leaves. During the following 27°C recovery period, there was no increase in the value for the unifoliate leaves, but an increase was observed in trifoliate leaves up to about 65% of the control. After the second heat hardening, the Fv/Fm ratio in unifoliate leaves fell to 62% and the trifoliate leaf value fell to 77% of the control value. The ratio did not increase again during the next recovery period in either type of leaf. After the third heat hardening, a very small (5%) increase occurred in unifoliate leaves and an approximately 30% increase was obtained in trifoliate leaves. The final 30 min heat stress at 47.5°C produced a small (5-8%) reduction in the Fv/Fm ratio in both types of leaves.

The data for the 42.5 and the 45°C treatments were analysed together using the ANOVA test. This showed that the Fv/Fm ratio was strongly dependent ($P<0.05$) upon the temperature, but it was not dependent ($P>0.05$) upon the leaf types.

In the fourth acclimation experiment, four 10 min heat-hardening treatments were applied at 45°C with 2h recovery at 27°C between each one. A final 30 min of heat stress was then applied at 47.5°C. The results are presented in Fig. 5.17. In the control, the Fv/Fm ratio was again the same in both types of leaves and they agreed with the control values from earlier experiments. In both types of leaves, a 50% reduction was observed after the first hardening treatment. After 2h of recovery, both ratios regained to within about 25% of the control value. Another reduction to about 55% of the control value was observed after the second heat-hardening treatment in unifoliate leaves and a decrease to about 69% of the control occurred in trifoliate leaves. Another recovery similar to the first one took place during the next recovery period. The Fv/Fm ratios were reduced again after the third hardening treatment, but this time the value remained slightly higher in comparison with those at the end of the second hardening treatment. Again, the ratio increased during the following recovery period. Further small reductions followed the fourth heat-hardening treatment in both types of leaves. Both values remained a little higher than those following the third hardening treatment however. Finally, the 30 min heat

stress applied at 47.5°C caused approximately 10% decrease in the Fv/Fm ratio in the unifoliate leaves, but no reduction occurred in the trifoliate leaves. Interestingly, the extent of the recoveries decreased progressively following each heat-hardening treatment. There was a suggestion, however, that both the first unifoliate leaves and the trifoliate leaves became more tolerant to the heat stress itself after the second treatment.

The Fv/Fm ratio was significantly affected ($P<0.05$) by the hardening treatment, but there were no significant differences between the unifoliate leaves and the trifoliate leaves.

5.4 Summary and Discussion.

There is very little published work on the effects of high temperature on soybean. Most research on the species has been done on low temperature effects. In the first experiment conducted in the present study, the first trifoliate leaves of soybean from nodulated and non-nodulated plants were used to determine the effects of high temperatures on the chlorophyll fluorescence. The comparison was confined to fully expanded healthy leaves of similar age, because immature or senescent leaves might give excessively thermolabile responses (Bilger *et al.*, 1987; Moffat *et al.*, 1990).

The results from this first experiment reveal that the same

value of Fv/Fm ratio (approximately 0.87) was recorded in the controls of all the cultivars. Leaves from nodulated and non-nodulated plants also gave the same value. Furthermore, no changes were found in the ratio when leaves were stressed for 2 or 4h at 35°C or following subsequent recovery at 24°C. After 4h stress at 40°C, however, there was an approximately 50% decrease in the Fv/Fm ratio of Williams-82 and Sable cultivars. Smaller decreases occurred in Bragg, Davis, Mago-80 and Hardee cultivars. The Fv/Fm ratio recovered fully in all the cultivars following stress at 40°C, although slower recovery appeared to take place in leaves from non-nodulated plants compared with those from the nodulated plants. The Fv/Fm ratio was more severely affected by heat stress at 42.5°C. At this temperature, the effects were greater in cultivars Mago-80 and Bragg compared with the other cultivars. Again, leaves from non-nodulated plants appeared to be affected more than those from nodulated plants in Mago-80 and Sable, but in the cultivar Davis the leaves from nodulated plants seemed to be more sensitive to temperature stress. The Fv/Fm ratio recovered fully from the 42.5°C treatments in Williams-82 and Sable. Slower recoveries were found in Davis and Hardee cultivars. The leaves of Bragg showed only a poor ability to recover from the treatments. Even slower recovery was found in cultivar Mago-80. In all the cultivars except Davis and Hardee, the leaves from nodulated plants apparently recovered better than those from non-nodulated plants. It is clear that chlorophyll fluorescence was

severely affected in all the cultivars when the temperature was raised to 45°C. There were approximately 80% decreases in the Fv/Fm ratios after 2h stress at this temperature. Further reductions were found after 4h stress in Mago-80 and Bragg, but no further changes were found in Williams-82, Davis, Sable or Hardee. After this heat stress, many leaves were visibly very badly damaged.

The present results are in general agreement with those of Sethar (1993) working on cotton, who found that the chlorophyll fluorescence values in several cultivars dramatically decreased at 45°C with no recovery at all during subsequent 24h recovery at 30°C. It is generally agreed that reductions in chlorophyll fluorescence with respect to temperature stress are indicative of chloroplast thylakoid damage. Either chilling or heat stress, when applied to leaves, elicits decreases in induced chlorophyll fluorescence in several species (Schreiber and Berry, 1977; Smillie and Gibbons, 1981; Potvin, 1985).

Yucel *et al.* (1992) have reported that PS II chlorophyll fluorescence transients in wheat seedlings were inhibited by 40-50% following 30 min heat stress at 37°C. Continued heat treatment of the seedlings for up to 5h inhibited PS II fluorescence by around 80%. Complete recovery of PS II variable fluorescence was found after the seedlings were returned to 22°C for 24h. Kislyuk (1979) has found that, when photosynthesis is not totally inhibited by heat stress

on cucumber and tradescantia leaves, recovery of the photosynthetic apparatus after removal of the heat stress is faster in light than in the dark. Martineau *et al.* (1979 a, b) have indicated that, in soybean, a significant genotypic component exists for heat tolerance, a result that is not unexpected.

Protein dissociation and denaturation is the most common explanation for heat injury in biological systems. Christiansen (1978) has suggested that, in plants, the proteins of two major kinds, enzymes and membranes proteins, are potentially the most vulnerable to high temperatures. Heat-induced blocking of PS II reaction centres, combined with dissociation of the light-harvesting chlorophyll a/b protein complex, has been postulated to occur at temperatures corresponding to those affecting fluorescence decrease in *Larrea divaricta* leaves (Schreiber and Armond 1978; Armond *et al.*, 1978). Aoki (1990) has reported that chlorophyll fluorescence values in detached cucumber leaves decreased with increasing temperature and duration of the heat treatment. Membrane disruption in plants may alter water, ion and organic solute movement, as well as photosynthesis and respiration (Christiansen, 1978). It has already been pointed out that, in the present study, leaves from nodulated plants generally appeared to recover better than those from non-nodulated plants. One possible and obvious reason for this may be a better nitrogen supply in the leaves of the nodulated plants.

Nitrogen supply for protein synthesis is required for the recovery of PS II from photochemical damage in bean and spinach leaves (Greer *et al.*, 1986; Samuelsson *et al.*, 1987).

Havaux *et al.* (1991) have reported that, in pea leaves, heat stress in darkness increases the capacity for cyclic electron flow around PS I, while heat stress in the light reduces PS I-driven cyclic electron transport. Heat stress in darkness results in the progressive closure of PS I reaction centres, whereas PS II centres under steady illumination remain open, reflecting adjustment of the photochemical efficiency of un-damaged PS I to the reduced activity in PS II. At supraoptimal temperatures above 40°C, thermal alterations of the thylakoid membranes (Berry and Raison, 1981) and the related impairment of photosynthesis (Bilger *et al.*, 1987) become apparent.

These and our own results on heat stress can be partly explained by the *in vitro* studies of Gounaris *et al.* (1984), working on *Vicia faba* and broad bean. They reported that granal attachment sites in isolated chloroplasts were stable for up to 5 min when incubated at 45°C but not beyond. Havaux and Lannoye (1983) have also reported that exposure of maize to drought caused injury to the thylakoid structure affecting photosynthetic electron transport. Changes in chlorophyll fluorescence *in vivo* also reflect underlying changes in pigment composition and electron

transport through PS II (Papageorgiou, 1975). Cleland *et al.* (1990) reported that the Fv/Fm ratio also depends on photon flux densities and that high light intensities decreased the Fv/Fm ratio in soybean leaves. Also, Nauner and Larcher (1990) reported that, in soybean, the chlorophyll fluorescence ratio decreased at high temperatures and also at chilling temperatures. Clearly there are complex interactions between several environmental factors, including heat, with regard to the stability of PS II.

The data in this chapter reveal that chlorophyll fluorescence was affected by various heat-acclimation (hardening) treatments. In these experiments, the treatments were applied to the detached first unifoliate leaves and the first trifoliate leaves. The resulting data show that similar values for the Fv/Fm ratio were recorded in both kinds of control leaves. In the first of the acclimation experiments, no significant changes in the Fv/Fm ratio were found after three 10 min heat-hardening treatments at 40°C or following recovery at 27°C. Also, no effects were found following a further 30 min stress at the same temperature. Hardening treatments of the leaves at 42.5°C also produced little change in the Fv/Fm ratio either during the hardening or during subsequent recovery. Even after a final 30 min of stress at 42.5°C, the Fv/Fm ratio remained at 0.80, indicative of healthy undamaged leaves. At 45°C, however, the Fv/Fm ratio was decreased by

approximately 25% after the first heat-hardening treatment and it did not fully recover to the control value afterwards when the leaves were returned to 27°C for 2h. The same reduction in Fv/Fm ratio was found after the second hardening treatment. On this occasion, however, the Fv/Fm ratio decreased further during the following recovery period, indicating that more severe damage had been inflicted. Interestingly, the Fv/Fm ratio increased during the next hardening treatment. The increase in ratio following this third heat-hardening treatment may have been due to acclimation perhaps involving the production of heat-shock proteins. The final heat stress at 45°C produced no further changes in the first unifoliate leaves, but a sharp decline was found in trifoliate leaves. The Fv/Fm ratio was decreased by 50-70% following heat-hardening at 47.5°C, although the effect appeared greater on trifoliate leaves compared with unifoliate leaves. Although recovery took place after heat-hardening at 47.5°C, the extent of the recovery became smaller with each subsequent treatment. These reductions in chlorophyll fluorescence presumably reflect progressive and irreparable injury to the thylakoid structure. Berry and Raison (1981) have indicated that, at supraoptimal temperatures above 40°C, thermal alterations to the thylakoid membranes becomes apparent.

In the second acclimation experiment, involving single heat stress treatments at 40, 42.5, 45 or 47.5°C followed by 2h of recovery at 27°C, the Fv/Fm ratio was decreased by

temperatures of 45°C or above, especially at 47.5°C. There was a small recovery from the 45°C treatment, but not from the treatment at 47.5°C.

The results from the third acclimation experiment show that repetitive 10 min heat-hardening treatments at 42.5°C led to small but accumulative decreases in the Fv/Fm ratio with equally small increases during the following recovery periods. There were no significant differences between the first true leaves and first trifoliate leaves. The final 30 min heat stress at 47.5°C still caused a 50% decrease in the Fv/Fm value despite the preceding hardening treatments. The effect of this final stress treatment was apparently greater on the unifoliate leaves than on the trifoliate leaves. The heat-hardening treatments at 45°C produced markedly stronger effects than the ones at 42.5°C. There was now little evidence of recovery during the intervening recovery periods at 27°C, although surprisingly there was evidence of a small recovery during the final treatment at 45°C. The final 30 min heat stress at 47.5°C now caused little further change in the Fv/Fm ratio. This latter situation was taken to indicate a small degree of acclimation, at least in the trifoliate leaves.

In the final (fourth) experiment on acclimation, in which four 10 min heat-hardening treatments were applied at 45°C, more evidence of effective heat hardening was obtained. This was evident in two respects. Firstly, the effect of

each exposure to 45°C became progressively less after the second exposure, although the recovery values continued to decline through the successive treatments. Secondly, the final exposure to 47.5°C did not reduce the Fv/Fm ratio much further below the level that it had reached at the end of the heat-hardening treatments. Indeed, the final stress at 47.5°C had no effect on the trifoliate leaves.

The designs of the third and fourth acclimation experiments are open to an important criticism. They did not contain internal controls consisting of leaves subjected to heat stress at 47.5°C without prior hardening treatments at lower temperatures. Such treatments were present, however, in the first and second acclimation experiments and they can be used as stop-gap controls. Taking unifoliate leaves first, hardening at 42.5°C followed by heat stress at 47.5°C produced a final Fv/Fm ratio of 0.36 (Fig. 5.15). Hardening at 45°C followed by heat stress at 47.5°C produced final Fv/Fm ratios of 0.30 (Fig. 5.16) or 0.36 (Fig. 5.17). Corresponding control values for unifoliate leaves subjected directly to 47.5°C are either 0.27 (Fig. 5.10) or 0.22 (Fig. 5.14). This makes the situation inconclusive as far as the unifoliate are concerned. The data for the trifoliate leaves are more convincing however. Heat-hardening at 42.5°C followed by heat stress at 47.5°C produced an Fv/Fm ratio of 0.25 (Fig. 5.15). Hardening at 45°C followed by stress at 47.5°C produced values of 0.36 (Fig. 5.16) and 0.36 (Fig. 5.17). These values compare with

control values of 0.16 (Fig. 5.10) and 0.24 (Fig. 5.14). Thus, the hardening treatments at 45°C appear to have produced a positive acclimation response in the case of trifoliate leaves. This argument is made somewhat more convincing by the fact that the controls were subjected to 47.5°C for only 10 min, whereas the heat-hardened leaves were subjected to stress at that temperature for 30 min. If more time had been available, the whole of the fourth acclimation experiment would have been repeated with its own internal control. A more detailed statistical analysis of the resulting data would also be essential. This might involve making comparisons between individual sets of data, for example between the control and the final Fv/Fm ratios discussed above. In the present study, statistical analyses were carried out only on the total body of data. These unfortunately found no differences between the unifoliate and the trifoliate leaves and they could not confirm the existence of a heat-hardening response.

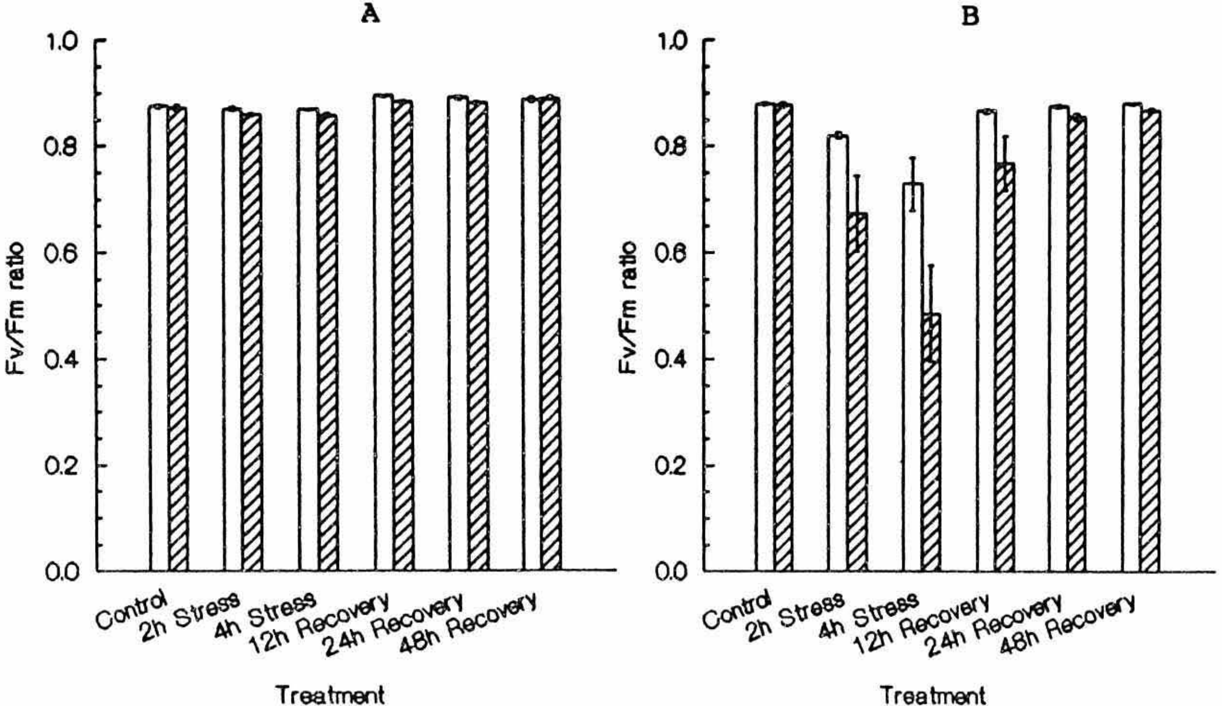
Possible explanations for the increases in the Fv/Fm ratio after heat acclimation include the regeneration or replacement of damaged proteins and other biomolecules or the production of protective substances. These ideas are supported by several workers (Berger *et al.*, 1946; Feldman, 1962; Oku and Tomita, 1971; Krause and Santarius, 1975, and others), who have suggested that the heat inactivation of bio-membranes may be prevented by the synthesis and accumulation of protective compounds surrounding the

membranes or by biochemical and ultrastructural changes within the membranes themselves. It has been shown that various water-soluble compounds including sugars and proteins are able to protect sensitive cell structures against heat inactivation *in vitro* (Berger *et al.*, 1946; Feldman, 1962; Oku and Tomita, 1971; Krause and Santarius, 1975, and others).

Alexandrov (1964) has pointed out that the ability of plants to acclimate at moderately high temperatures, thereby reducing heat injury and increasing the ability to recover from heat stress, are important factors in determining crop performance in high temperature environments. The exact nature of this process is unknown. Alexandrov (1964) has also reported that plant cells do not change their heat tolerance immediately when moving over a wide range of temperature. Instead, they begin to respond by increasing their tolerance when the temperature approaches the injurious zone. The acclimation is a specific reaction of the cells toward the injurious action of heat.

Different crop species show different degrees of tolerance to high temperature. From the limited literature, it appears that soybean is more heat tolerant than cowpea. Thus, Larcher *et al.* (1990) concluded from fluorescence quenching kinetics that temperatures around 40°C are supraoptimal for cowpea. This contrasts with the higher

temperatures recorded for soybean in the present study. Aoki (1990) found that cucumber seedlings grown under a high temperature regime were more resistant to heat and showed less pronounced decreases in chlorophyll fluorescence values compared with seedlings grown under lower temperature conditions. High temperature pretreatment increased PS I activity, while PS II activity was inhibited. In the dark, high temperature inhibited photosynthetic oxygen evolution (by approximately 80%), as well as photochemical energy storage, in pea and this is correlated with a marked loss of PS II chlorophyll fluorescence (Havaux *et al.*, 1991).



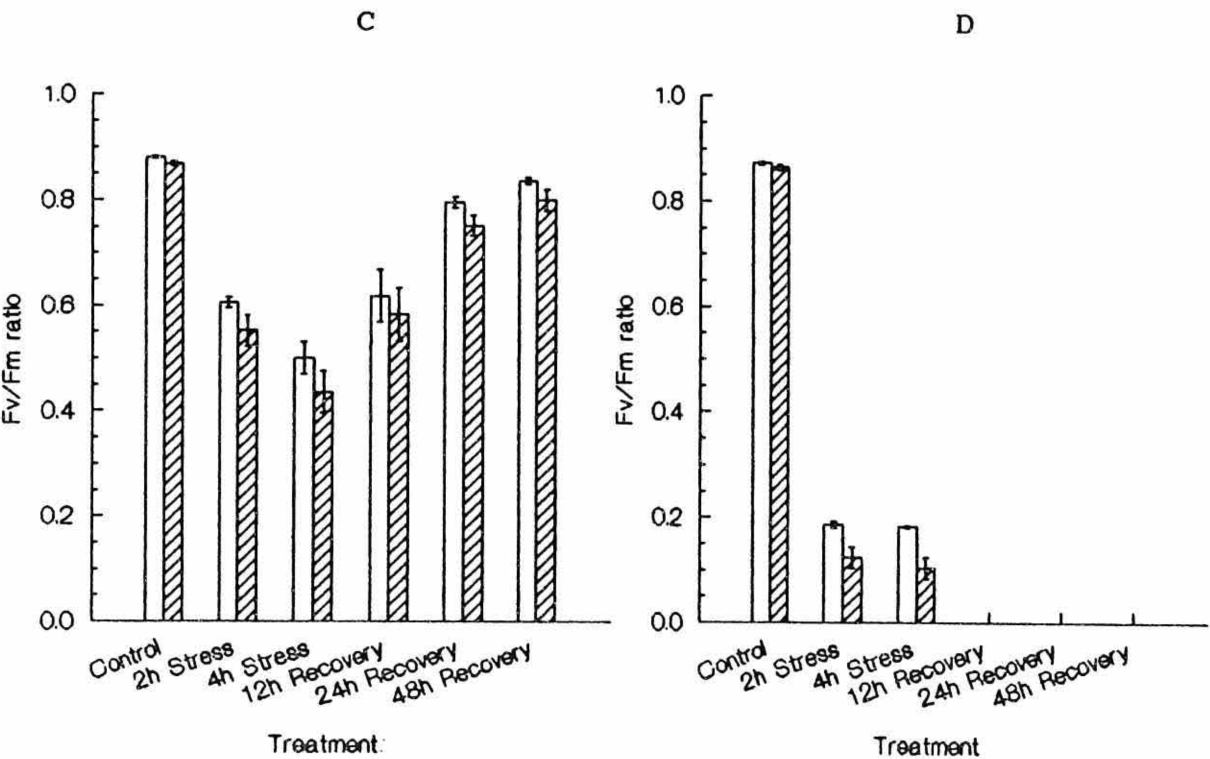
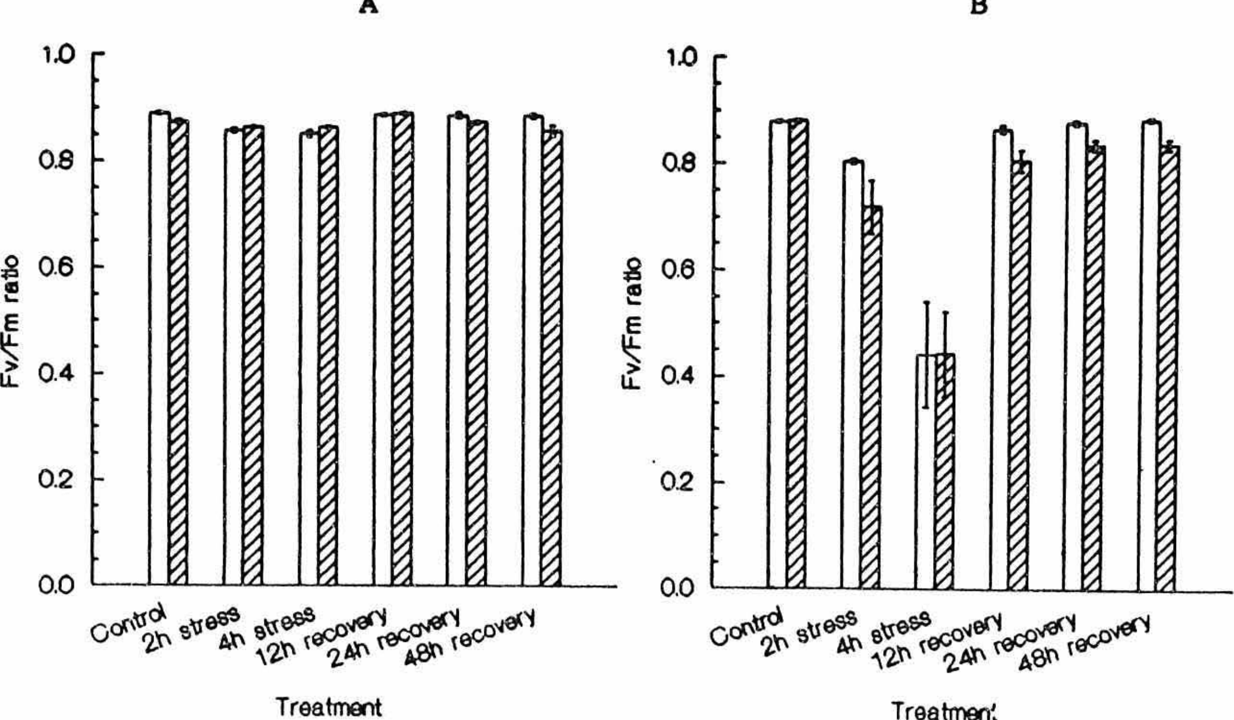


Figure 5.1 Effect of heat stress on leaf chlorophyll fluorescence in cv. Williams-82. A, 35°C; B, 40°C; C, 42.5°C; D, 45°C; [open box], nodulated plants; [hatched box], non-nodulated plants.



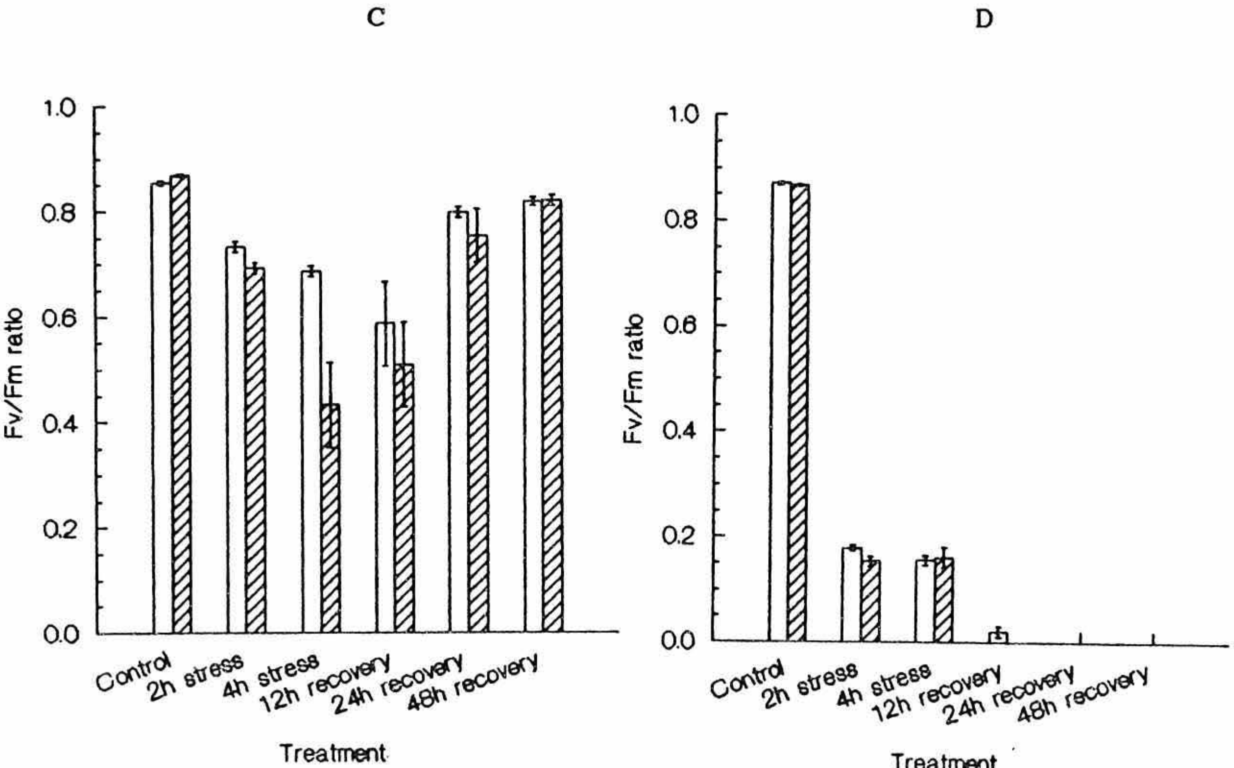
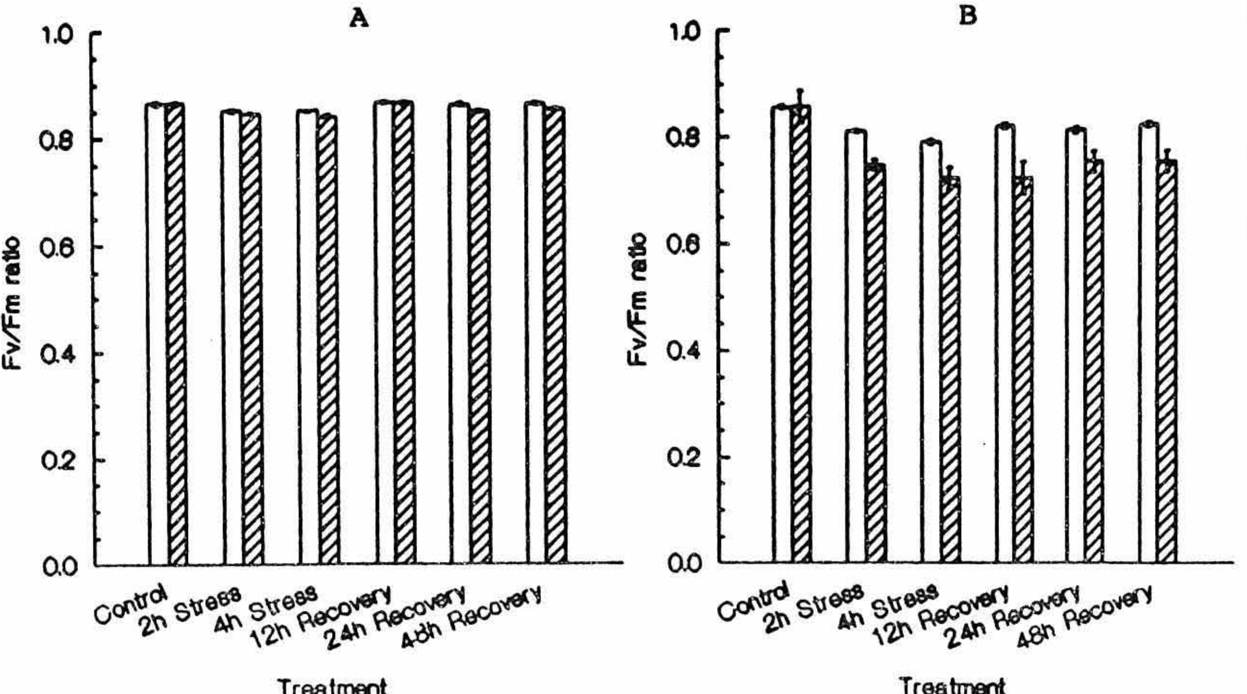


Figure 5.2 Effect of heat stress on leaf chlorophyll fluorescence in cv. Sable. A, 35°C; B, 40°C; C, 42.5°C; D, 45°C; [open bar], nodulated plants; [hatched bar], non-nodulated plants.



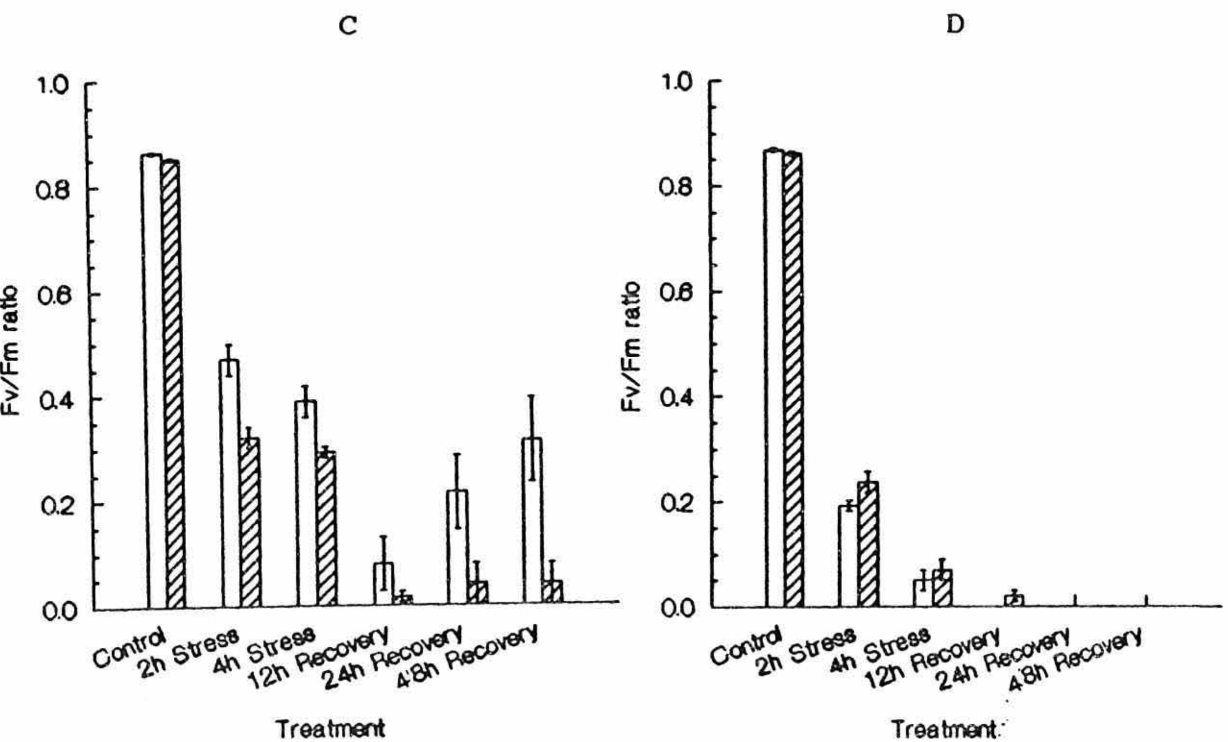
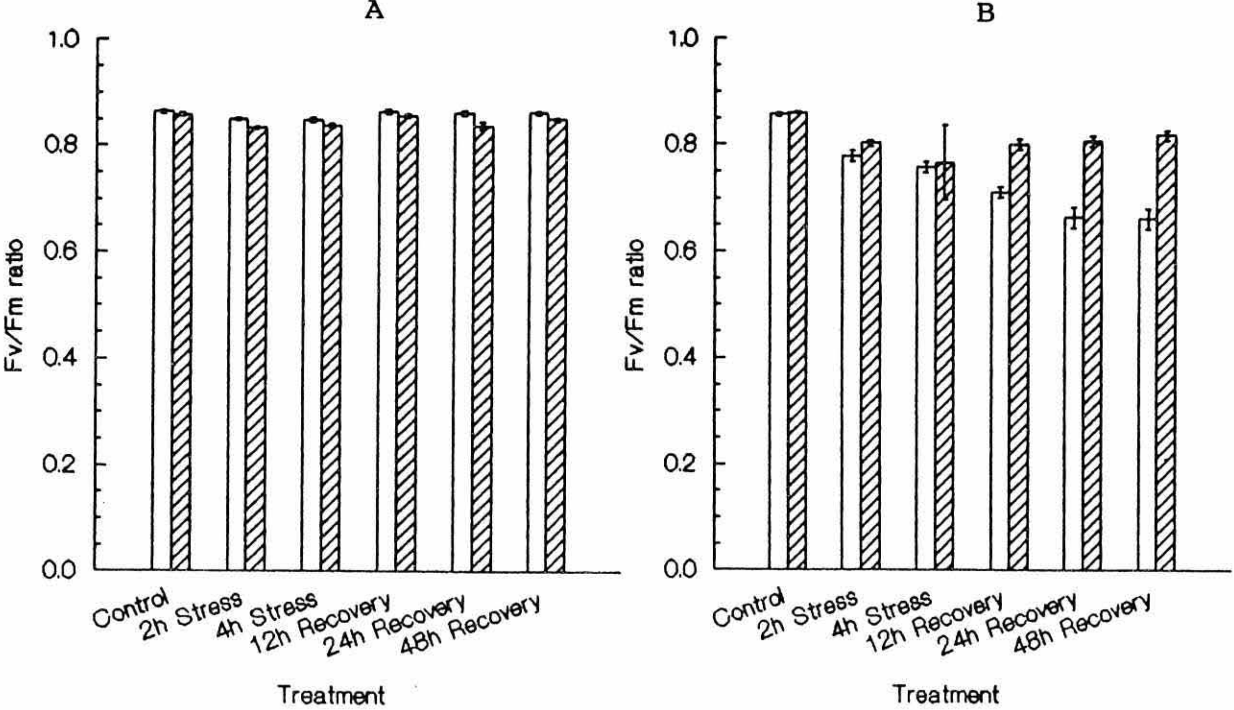


Figure 5.3 Effect of heat stress on leaf chlorophyll fluorescence in cv. Mago-80. A, 35°C; B, 40°C; C, 42.5°C; D, 45°C; [ ] , nodulated plants; [hatched] , non-nodulated plants.



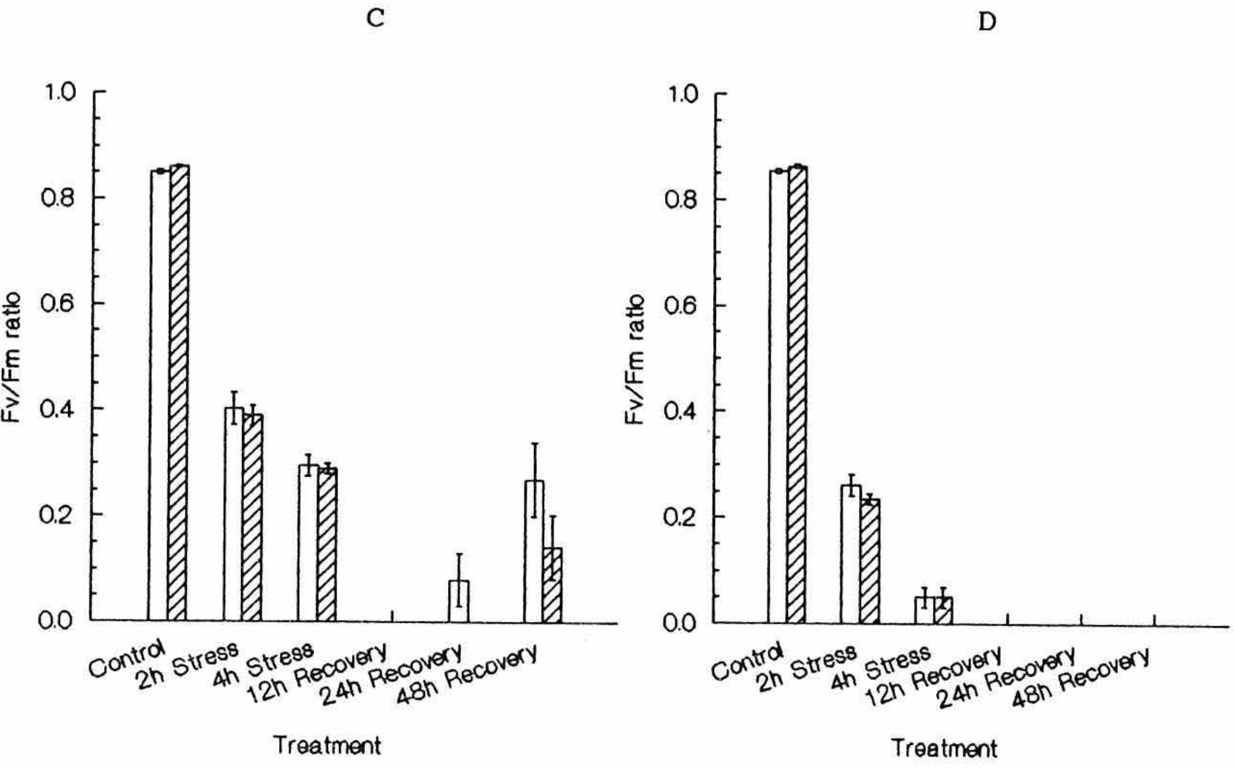
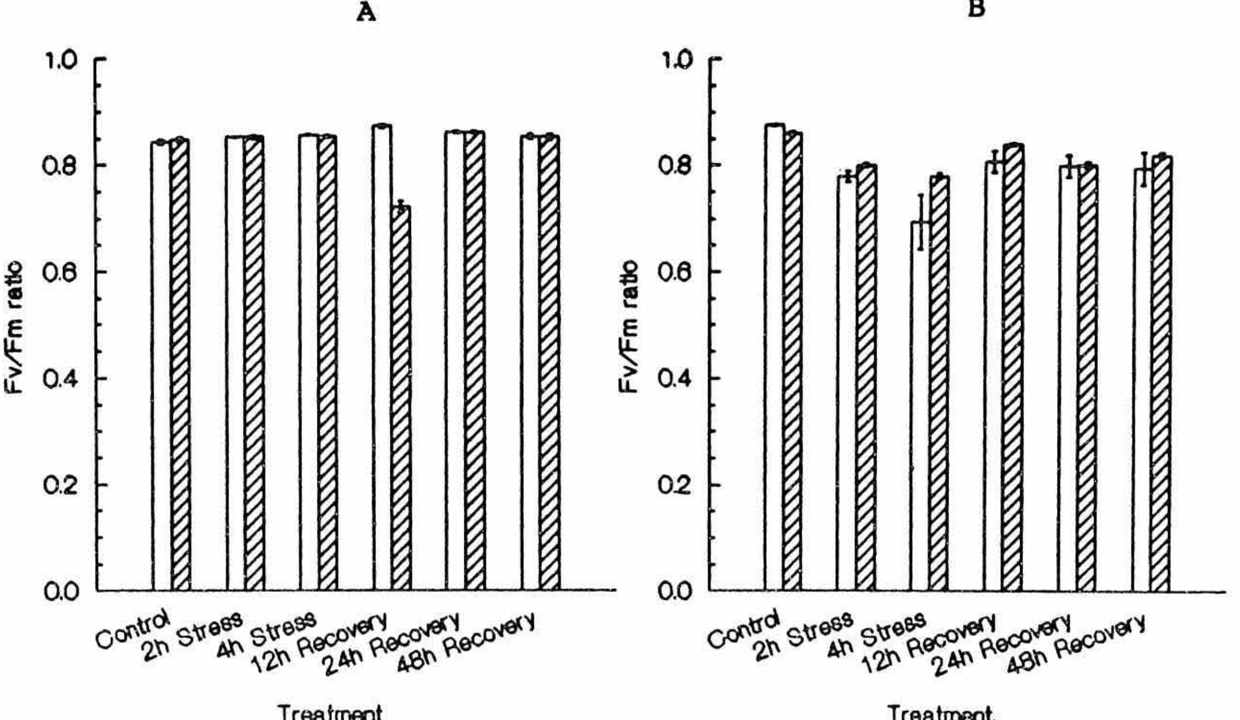


Figure 5.4 Effect of heat stress on leaf chlorophyll fluorescence in cv. Bragg. A, 35°C; B, 40°C; C, 42.5°C; D, 45°C; [open box], nodulated plants; [hatched box], non-nodulated plants.



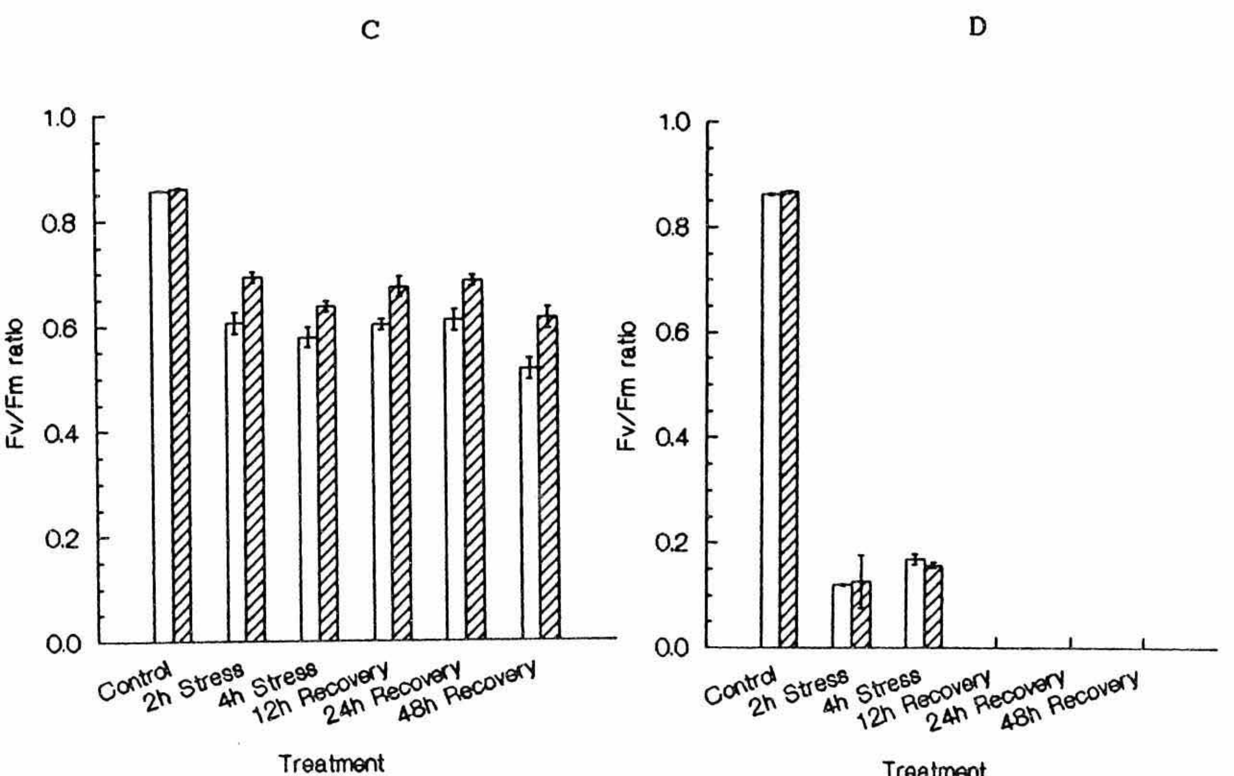
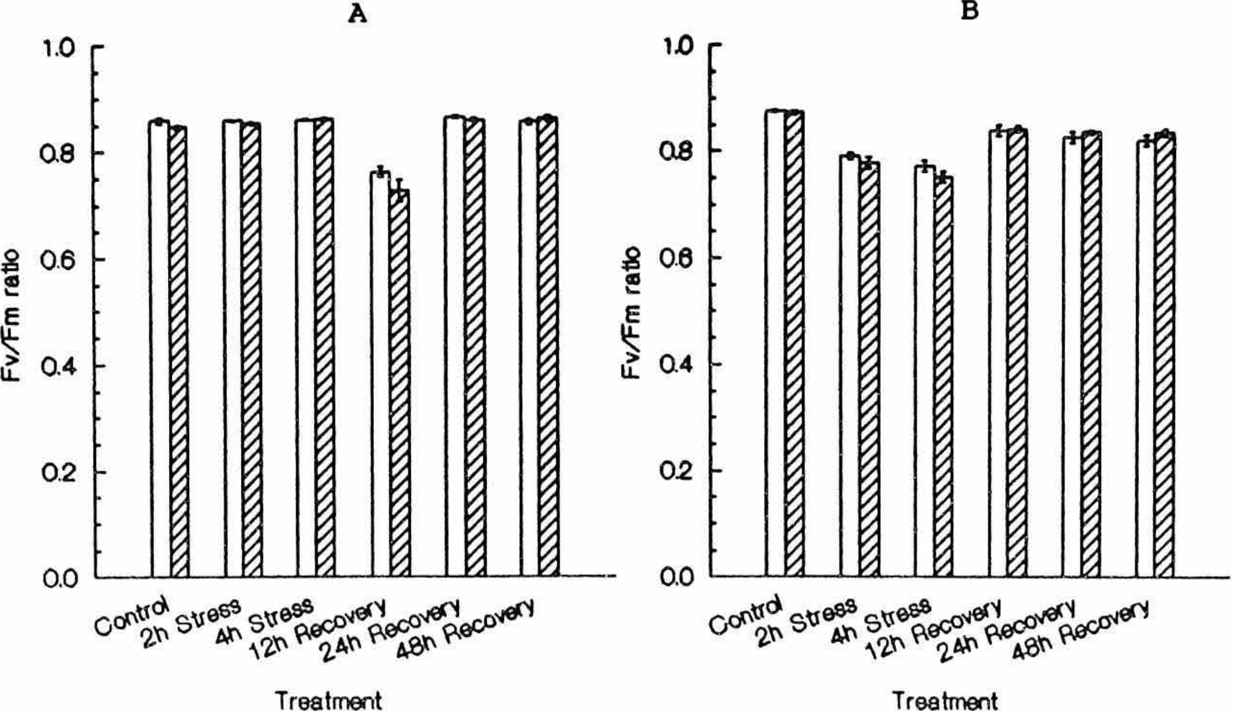


Figure 5.5 Effect of heat stress on leaf chlorophyll fluorescence in cv. Davis. A, 35°C; B, 40°C; C, 42.5°C; D, 45°C; ▭ , nodulated plants; ▨ , non-nodulated plants.



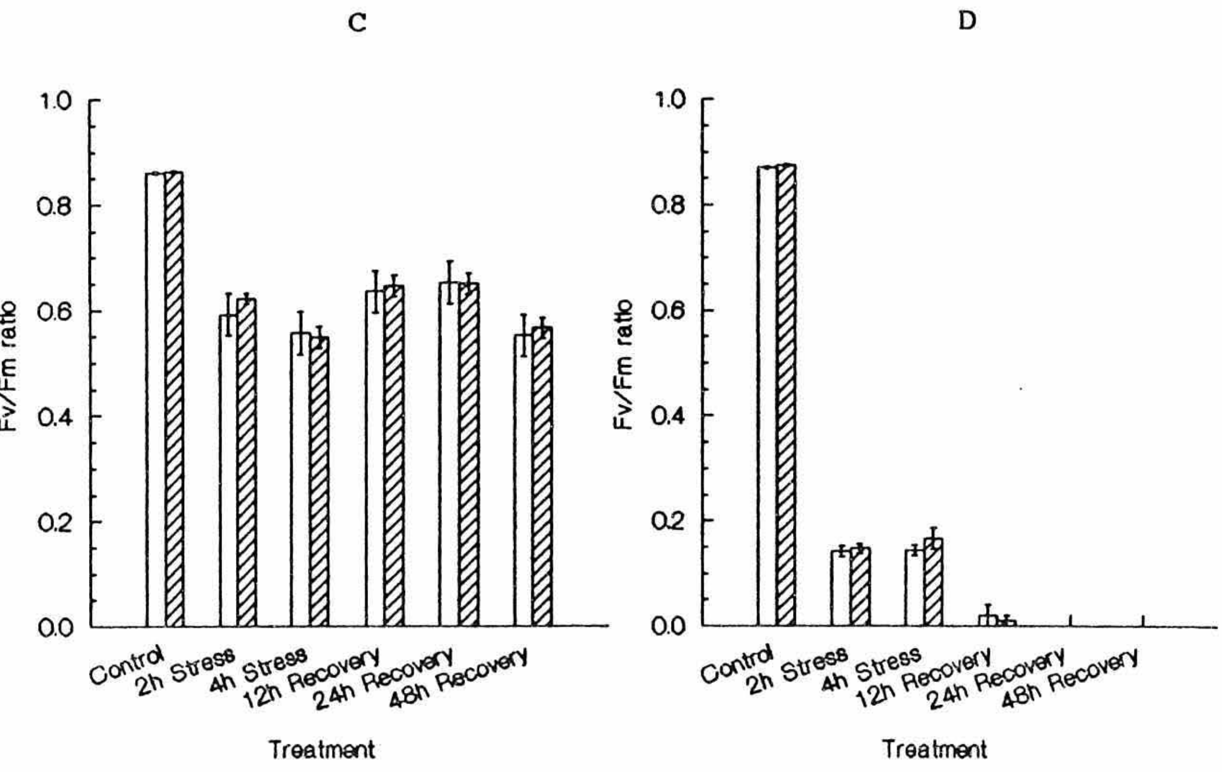


Figure 5.6 Effect of heat stress on leaf chlorophyll fluorescence in cv. Hardee. A, 35°C; B, 40°C; C, 42.5°C; D, 45°C; ▭ , nodulated plants; ▨ , non-nodulated plants.

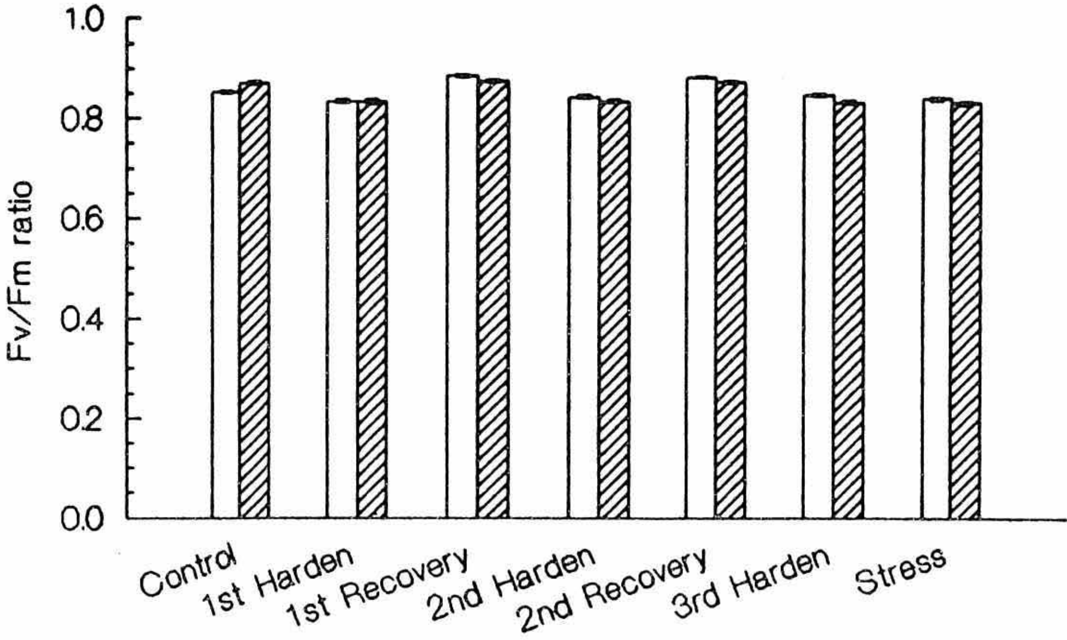


Figure 5.7 Effects of heat hardening and stress at 40°C on leaf chlorophyll fluorescence in cv. Williams-82. ▭, unifoliate leaf; ▨, trifoliate leaf.

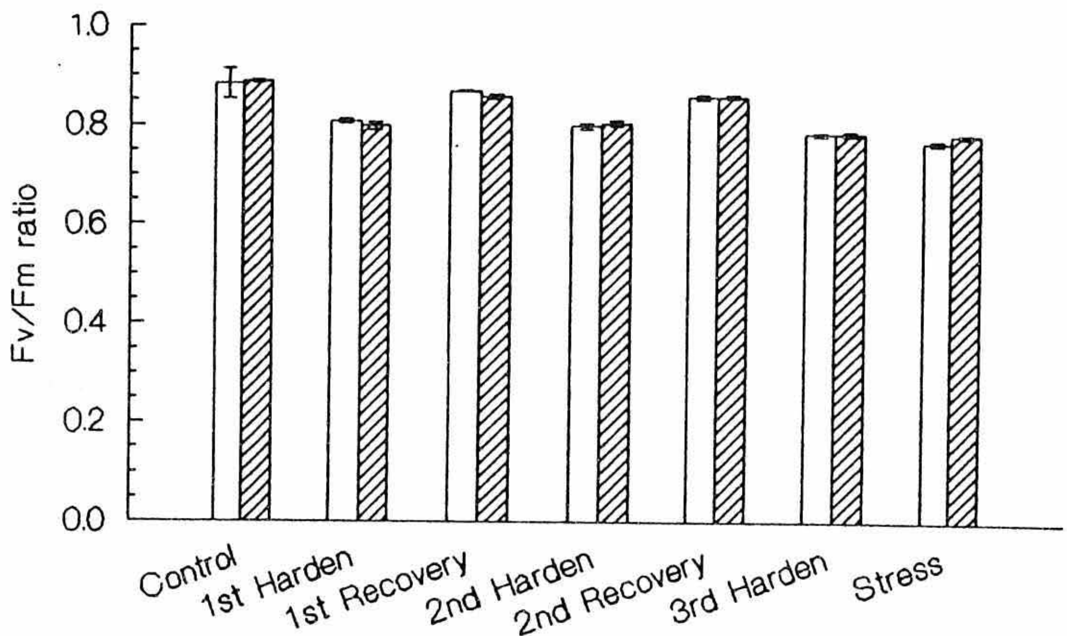


Figure 5.8 Effects of heat hardening and stress at 42.5°C on leaf chlorophyll fluorescence in cv. Williams-82. ▭, unifoliate leaf; ▨, trifoliate leaf.

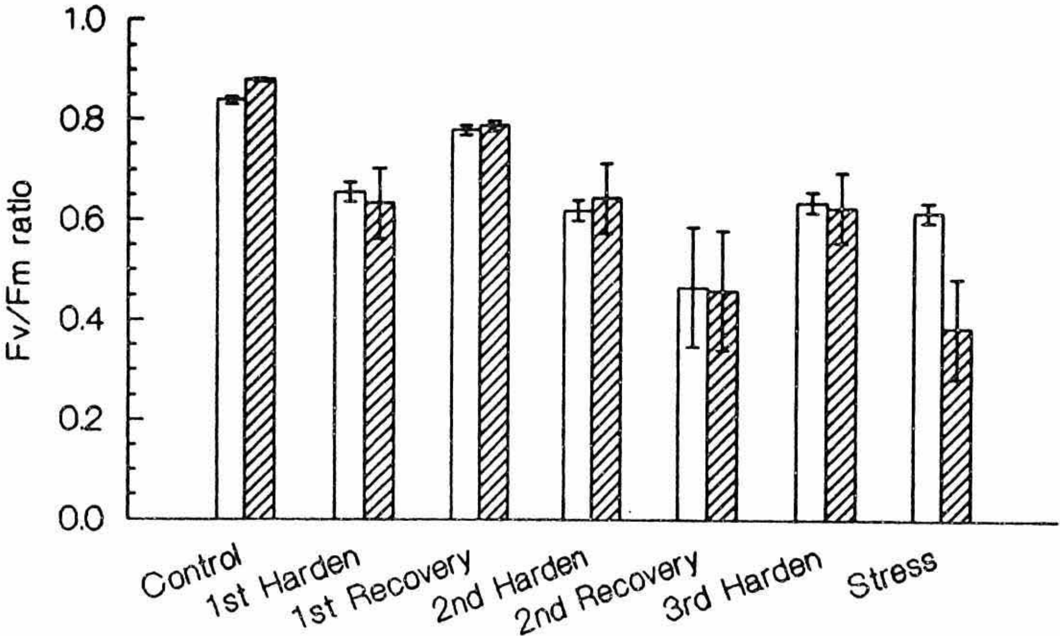


Figure 5.9 Effects of heat hardening and stress at 45°C on leaf chlorophyll fluorescence in cv. Williams-82. ▭, unifoliate leaf; ▨, trifoliate leaf.

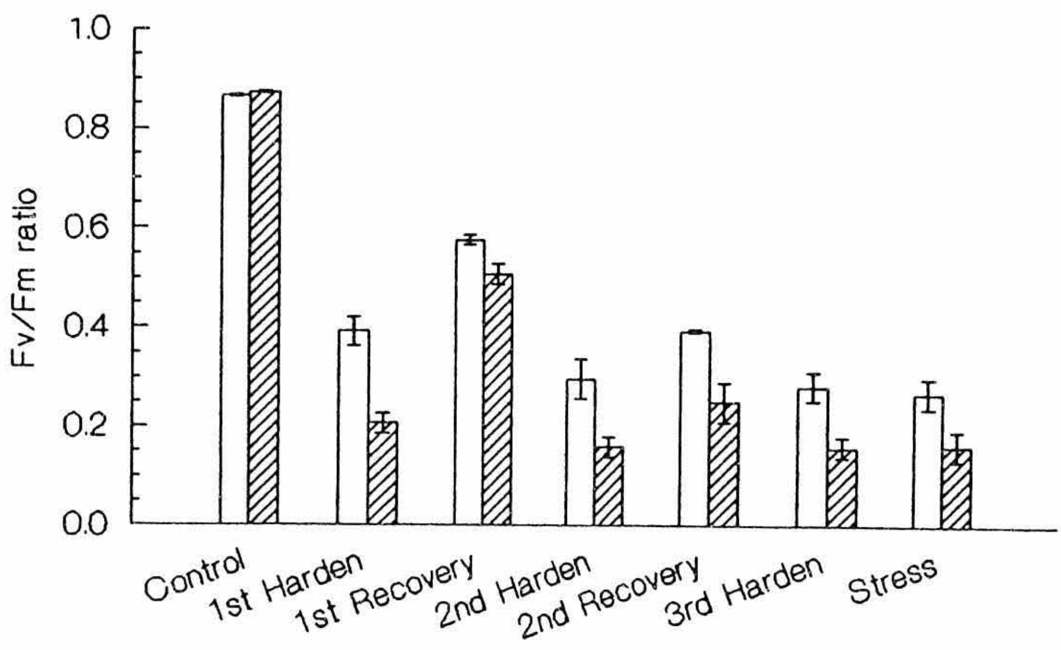


Figure 5.10 Effects of heat hardening and stress at 47.5°C on leaf chlorophyll fluorescence in cv. Williams-82. ▭, unifoliate leaf; ▨, trifoliate leaf.

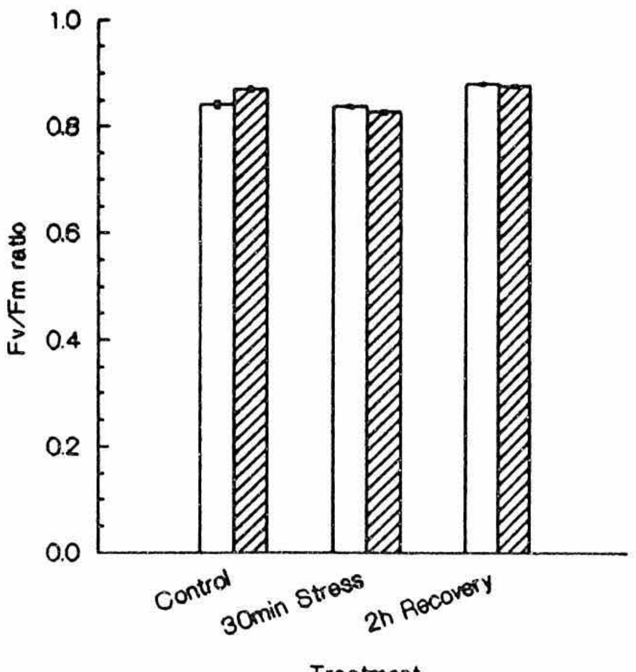


Figure 5.11 Effect of heat stress at 40°C on leaf chlorophyll fluorescence in cv. Williams-82. [hatched box], unifoliate leaf; [open box], trifoliate leaf.

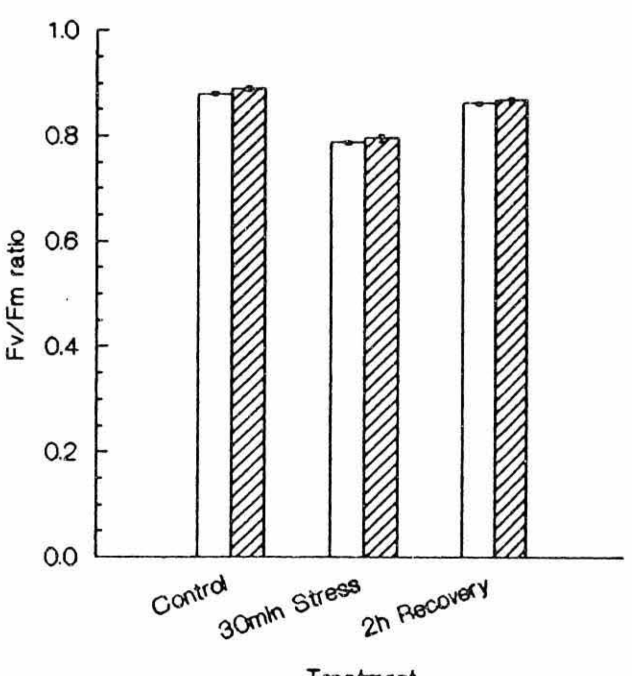


Figure 5.12 Effect of heat stress at 42.5°C on leaf chlorophyll fluorescence in cv. Williams-82. [hatched box], unifoliate leaf; [open box], trifoliate leaf.

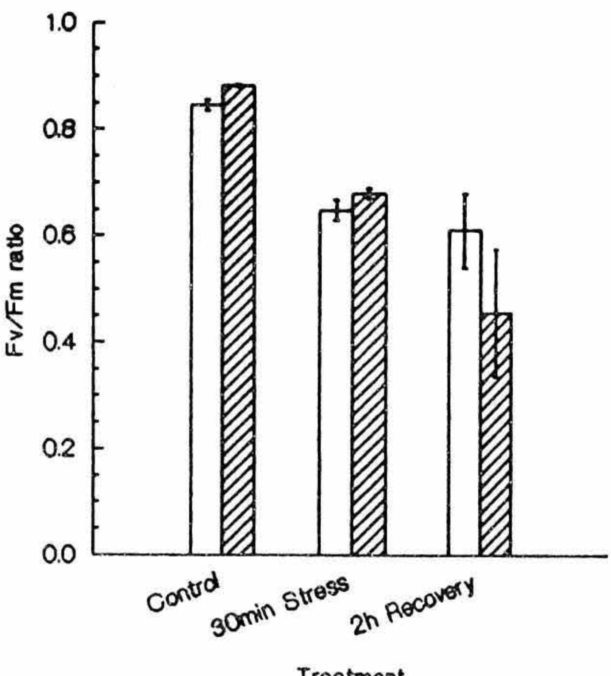


Figure 5.13 Effect of heat stress at 45°C on leaf chlorophyll fluorescence in cv. Williams-82. ▨, unifoliate leaf; □, trifoliate leaf.

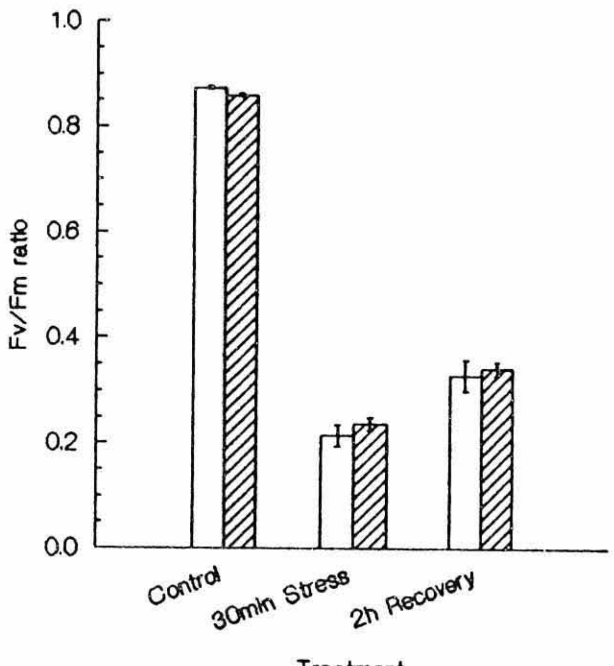


Figure 5.14 Effect of heat stress at 47.5°C on leaf chlorophyll fluorescence in cv. Williams-82. ▨, unifoliate leaf; □, trifoliate leaf.

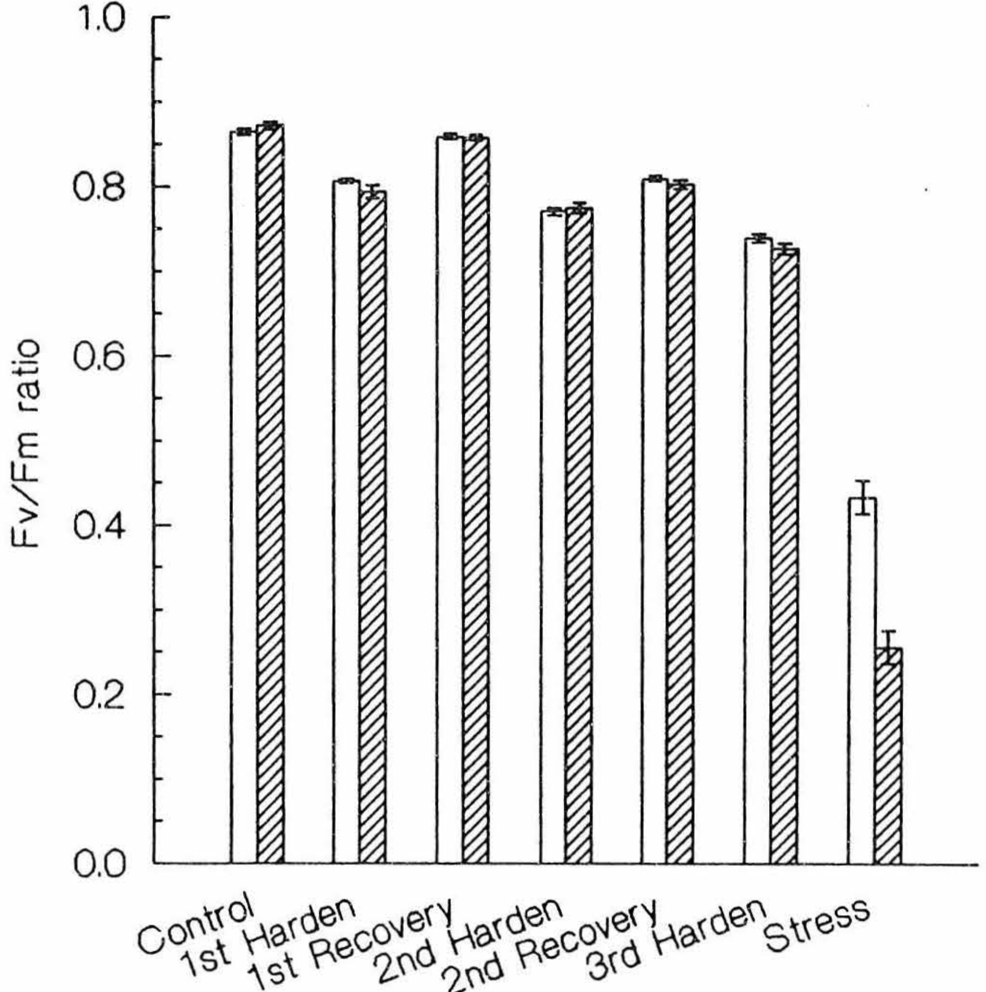


Figure 5.15 Effect of heat hardening at 42.5°C followed by heat stress at 47.5°C on leaf chlorophyll fluorescence in cv. Williams-82. ▭, unifoliate leaf; ▨, trifoliate leaf.

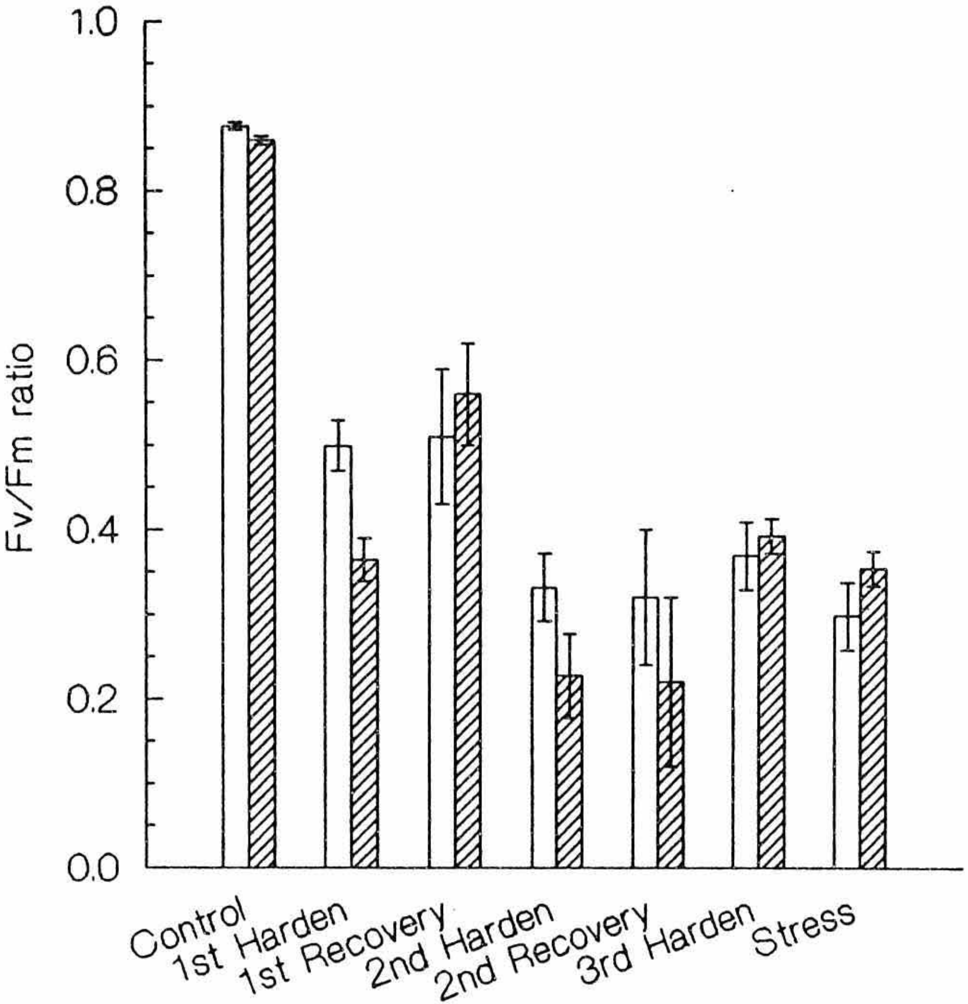


Figure 5.16 Effect of heat hardening at 45°C followed by heat stress at 47.5°C on leaf chlorophyll fluorescence in cv. Williams-82. ▭ , unifoliate leaf; ▨ , trifoliate leaf.

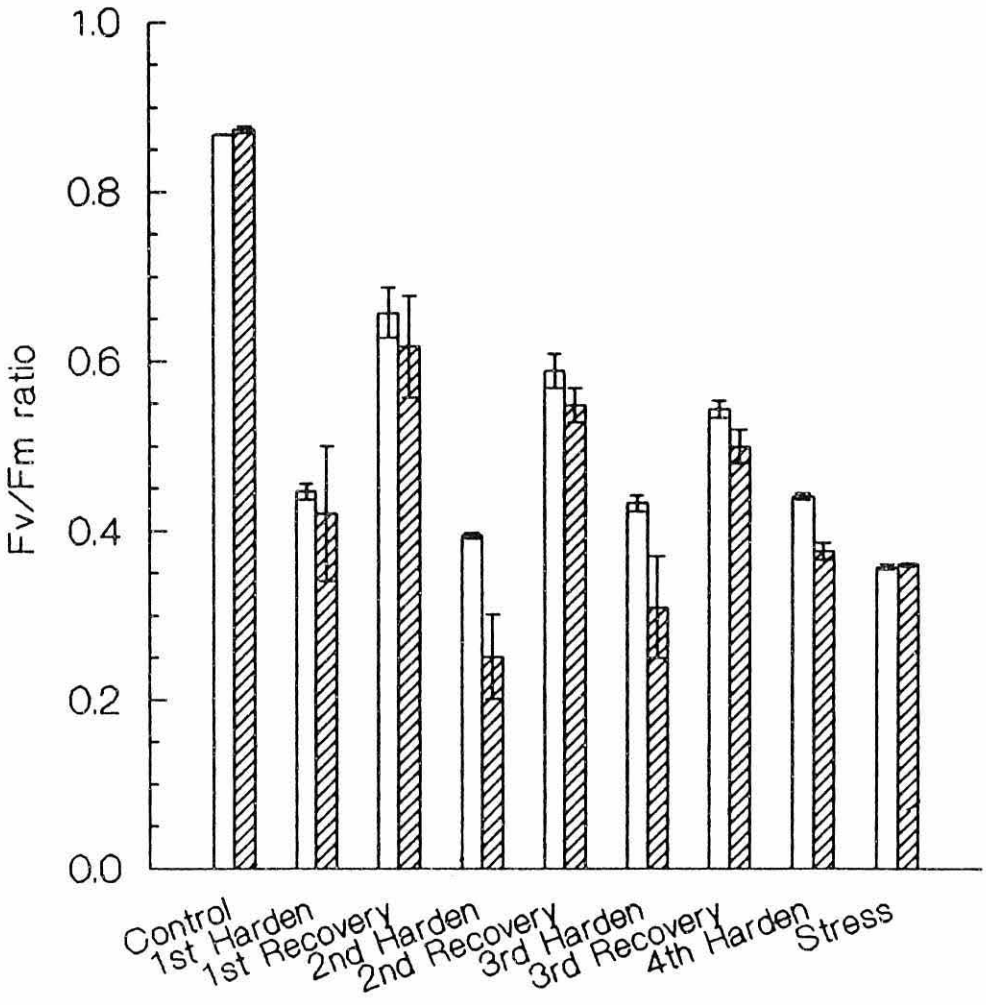


Figure 5.17 Effect of heat hardening at 45°C followed by heat stress at 47.5°C on leaf chlorophyll fluorescence in cv. Williams-82. ▭ , unifoliate leaf; ▨ , trifoliate leaf.

CHAPTER SIX

Chapter Six

Effects of High Temperature on the Growth of *B.rhizobium japonicum* RCR3407.

6.1 Introduction.

The best-known nitrogen fixing organisms are the bacteria that live in the roots of leguminous plants such as peas, beans and clover. In the roots of legumes, cortical cells are often infected with *Rhizobium*. This bacterium infects the roots through the root hairs and, once inside, forms an infection thread that permeates the root and eventually becomes a nodule. Nodulation of soybean is a developmentally complex process requiring interaction between *B.rhizobium japonicum* and the plant host, which is regulated by both genotypic and environmental factors (Long, 1989; and Quispel, 1988). The effects of high temperatures on the survival of rhizobia in soil has been reported by Willatt (1966) and by Chatel and Parker (1973), in laboratory growth media by Graham *et al.* (1963), Gillberg (1968) and Okafor and Alexander (1975) and on seeds by Herridge and Roughley (1974) and Philpotts (1977). Soybean nodule inhibition may be a mechanism by which the root of the host plant prevents excessive nodulation (Bhuvaneswari *et al.*, 1980).

Nodulation can be affected by several physiological and environmental factors, for example temperature, water stress and salinity. Galletti *et al.* (1971) have reported

that, for soybean, root temperatures of more than 30°C generally decreased nodule initiation and growth. In a mixed strain *R.japonicum* population, the ability of different strains to form nodules in soybean is influenced by the host genotype, planting date, temperature and their relative numerical strength (Weber *et at.*, 1971). This chapter reports the effects of high temperatures on the growth of *B.rhizobium japonicum* strain RCR3407 in liquid culture.

6.2 Methods.

6.2.1 Preparation of culture media.

Culture media were made up in bottles (see Section 2.13.1). After autoclaving, the bottles were inoculated with the bacterium and placed in an incubator in the dark at 25°C (see Section 2.13.1).

6.2.2 Growth of *B.rhizobium japonicum* over a 12-48h period at various temperatures.

The culture media were made up as described in Section 2.13.1. Then, liquid media were transferred to bottles and again autoclaved and the pH checked. After inoculation, (Section 2.14) they were incubated in water baths preset at 25, 35, 40 or 45°C. At the same time, other un-inoculated bottles of media were kept as control at each of the temperatures (Section 2.14). The bacterial growth (measured as optical absorbance) was determined at 12, 24, 36 and 48h after inoculation as described in Section 2.14.

6.2.3 Growth of *B.rhizobium japonicum* over a 1-7 day period at various temperatures.

In another experiment, culture media were again prepared as described in Section 2.13.1, then autoclaved and inoculated. They were then kept in the water baths at 25, 35, 40 or 45°C. The bacterial growth (measured as optical absorbance) was measured over 1-7 days after inoculation (see Section 2.14).

6.2.4 Heat-hardening treatment of *B.rhizobium japonicum*.

Cultures of *B.rhizobium japonicum*, which had been grown heat hardening at either 40°C or 45°C for 7 days, were used to inoculate new culture media as described in Section 2.14. These inoculates were incubated at either 40 or 45°C. Controls consisted of cultures grown directly at either 40°C or 45°C without prior hardening. Growth was measured at 12, 24, 36 and 48h after inoculation (for details see Section 2.14).

6.2.5 Changes in the pH of bacterial cultures.

The changes in the pH of incubated bacteria cultures were recorded at the same time as measuring optical absorbance in all the experiments described above.

6.2.6 Statistical analysis.

Means, standard deviation and standard error values were calculated using a pocket scientific calculator (Sharp Model EL-531P) and checked using a personal computer.

Further statistical analysis by analysis of variance (ANOVA) was done using a personal computer (Mitac) with Minitab for Windows (version.10.2). Figures were plotted using Systat/Sygraph software (version 5.03, Systat Inc., Evanston, IL., U.S.A). In all figures, vertical bars show the standard errors of the mean values.

6.3 Results.

6.3.1 Growth of *B.rhizobium japonicum* in un-inoculated (control) culture media.

In all experiments, the un-inoculated (control) tubes showed no change in optical absorbance at any temperature or at any time of incubation. Also, no changes in pH occurred in these un-inoculated control media.

6.3.2 Growth of *B.rhizobium japonicum* over a 12-48h period at various temperatures.

The growth of *B.rhizobium japonicum* RCR3407 was followed at 25, 35, 40 or 45°C. The results are presented in Fig. 6.1. The optical absorbance had increased to 0.32 after 12h of incubation at 25°C and it increased to 0.54 and 0.61 after 24 and 36h respectively, but a small reduction was found after 48h of incubation. The absorbance was higher (0.53 and 0.57) at 35°C than at 25°C after 12 and 24h and it declined after 36 and 48h of incubation. At 40°C, the growth was slower than at 35°C, but it increased with time up to 36h. A similar reduction was observed at 48h of incubation however. At 45°C, slower growth (0.05, 0.18 and

0.27 at 12, 24 and 36h respectively) was observed. There was no growth after 36h at 45°C.

The data were analysed using the ANOVA test, which showed that growth was strongly dependent ($P<0.05$) upon temperature and time (incubation period). There was also a significant ($P<0.05$) interaction between temperature and time.

6.3.3 Changes in pH of the growth media over the 12-48h growth period.

The pH was monitored at the same time as measuring the growth at 12, 24, 36 and 48h of incubation at 25, 35, 40 or 45°C (Fig. 6.2). Before incubation, the pH of media was 6.8-7.0. This decreased to 5.2 after 12h at 25°C and further reductions to 5.0, 4.9 and 4.8 were recorded after 24, 36 and 48h of incubation. At 35°C, the pH decreased to 4.7 after 12h. It declined further to 4.3 and 4.0 with increasing time periods of 24 and 36h, but no more reduction was recorded after 36h of incubation. The fastest decrease in pH occurred at 40°C. It decreased from 6.8 to 4.2 after 12h. There was a further small decrease to pH 4.0 after 24h, but little further change occurred after that. At 45°C, there was no significant reduction in pH after 12, 24, 36 or 48h of incubation.

Statistically, the pH was highly dependent ($P<0.05$) upon temperature and time (incubation period). Also, there was

significant interaction (P<0.05) between temperature and time.

6.3.4 Growth of *B.rhizobium japonicum* over a 1-7 day period at various temperatures.

In this experiment, growth was recorded during 1-7 days of incubation at the same constant temperatures as those reported in Section 6.2.3. The data presented in Fig. 6.3 show that the highest growth (0.66) occurred at 25°C after 1-2 days of incubation and the optical density gradually decreased with increasing periods of incubation after that. Growth at 35°C reached its maximum value after 24h of incubation and the optical density then remained constant for the next 6 days. Slower initial growth was recorded at 40°C (0.41 - 0.47) during the first 1-2 days of incubation compared with that at 25 and 35°C. By the fourth day at 40°C, growth had reached a maximum of 0.6 and then slowly declined to 0.57 at the 7th day of incubation. Growth was inhibited more at 45°C. It was very slow during the first day (0.17), but a sharp increase to 0.37 was found by the 2nd day of incubation. Optical absorbance then declined slowly with increasing periods of incubation, reaching 0.23 at the 7th day.

The growth was strongly dependent (P<0.05) upon temperature and time (incubation period). Significant interactions were also obtained between temperature and time.

6.3.5 Changes in pH of the growth media over the 1-7 day growth period.

The data presented in Fig. 6.4 show that, at 25°C, the pH decreased from 7.0 to 4.9 after the 1st day of incubation and it declined further to 3.8 with increasing incubation up to 7 days. At 35°C, a greater reduction to 4.2 was observed after the first day of incubation. The pH then remained at approximately 3.8 for the next 6 days. The pH changes at 40°C were similar to those at 25 and 35°C. A sharp reduction to 4.0 was observed after the first day of incubation and it then remained unchanged up to the 7th day of incubation. At 45°C, the earlier large decrease in pH was not observed after 24h of incubation. At the end of the first day it had declined to only pH 6.0 and it stayed approximately at that value up to the seventh day of incubation.

The data were analysed using the ANOVA test, which showed that pH was dependent ($P<0.05$) upon temperature and time (incubation period). Significant ($P<0.05$) interaction were also found between temperature and time.

6.3.6 Heat-hardening treatment of *B.rhizobium japonicum*.

Heat-hardening treatments at 40 or 45°C were given to *B.rhizobium japonicum* and growth was then observed after 12, 24, 36 and 48h of incubation at either 40 or 45°C.

In the first treatment, the bacteria were heat hardened for

7 days at 40°C, re-inoculated into new culture media and kept at either 40 or 45°C for 48h. Fig. 6.5a shows that the growth of pre-hardened cultures was initially (up to 24h) greater at 40°C than it was at 45°C. At 36h and 48h, however, the two cultures had similar optical densities. More importantly, the growth of the pre-hardened cultures after 24h at both 40°C and 45°C was greater than their corresponding control. This suggest that heat hardening had taken place.

In the second treatment, the *B.rhizobium japonicum* was heat hardened for 7 days at 45°C, then re-inoculated into new culture media and grown at either 40 or 45°C. The results from this experiment were very similar to those from the first experiment and they provide more clear evidence for heat hardening (Fig. 6.5b). The pre-hardened cultures again grew faster at 40°C than at 45°C, at least up to 24h. After 24h, their optical densities were similar. The growth of the heat-hardened cultures after 24h was clearly greater than the corresponding controls.

Statistically the bacterial growth was strongly dependent ($P<0.05$) upon temperature and the time (incubation period). There was no interaction ($P>0.05$) between temperature and time.

6.3.7 Changes in pH of the growth media of the heat-hardened *B.rhizobium japonicum*.

For the first treatment (heat-hardening at 40°C), the data in Fig. 6.6a show that the pH of the 40°C culture media declined to 4.0 with increasing time of incubation up to 36h and it then slightly increased again to 4.3 at 48h. There was no change in pH after 12h in the 45°C culture and only a very small reduction in pH was observed at 48h of incubation. A similar pattern of pH change was observed in the second treatment using the 45°C-hardened bacteria (Fig. 6.6b). A striking feature of the results was the absence of any difference between the pH of these heat-hardened cultures and the pH of their corresponding controls. This was in contrast to the differences observed between the optical densities of the cultures (cf. Fig. 6.5).

The pH in cultures grown at 40°C was strongly dependent ($P<0.05$) upon the incubation period, while the pH of the 45°C cultures were not influenced ($P>0.05$) by the incubation period. These significances were true for both the control and heat-hardened cultures. The pH was also significantly ($P<0.05$) affected by temperature. There were no interactions ($P>0.05$) between the temperature and time.

6.4 Summary and Discussion.

The growth of *B.rhizobium japonicum* RCR3407 was evaluated at 25, 35, 40 and 45°C after 12, 24, 36 and 48h of incubation. The results show that the optimal growth

temperature for this strain of *B.rhizobium japonicum* is 25 to 35°C. The growth was slower after 12h of incubation at 25°C than at 35°C, but growth at 25°C continued with increasing growth periods up to 36h. At 35°C, growth was maximal after 12h incubation and there was little further change for up to 48h. At 40°C, growth was slower (approximately 50% less) than at 35°C, but it progressed slowly up to 36h of incubation. At 45°C, however, the growth was very slow up to 48h compared with 25 and 35°C.

Before and during the incubations, the pH was recorded in all the growth tubes. The data show that the pH declined with increasing bacterial growth at optimal temperatures. A sharp decline from pH 6.8 to 5.2 was found after 12h of incubation at 25°C. A greater decrease was obtained at 35 and 40°C compared with that at 25°C (from 6.8 to 4.2). There was no further reduction in pH with increasing incubation periods, although the growth rate remained higher at these temperatures than at 25°C. At 45°C, the pH was reduced only slightly at 12h and it did not decreased further after that. This was despite the fact that there was further growth after 12h. It is particularly striking that, although growth continued with increasing incubation periods of 24, 36 and 48h at 25, 35 and 40°C, there was no further reduction in pH.

The experiments reported in this chapter also revealed that the cell density of *B.rhizobium japonicum* cultures slowly

declined at higher temperatures (45°C) during prolonged incubation periods of up to 7 days. Very slow growth was found at 45°C up to the 2nd day, but this was followed by a decline in cell numbers. At 25°C, maximum growth was found during the 1st day of incubation and the cell density then remained stable up to the 3rd day. A slow decline was found, however, with further periods of incubation. At 35°C, rapid growth also occurred for 1 day, then the cell density remained constant up to the 7th day of incubation. At 40°C, a slower growth was found for the first 2 days then the cell density became stable.

The pH measurements in this experiment showed that a large decrease in pH from 7.0 to 4.0 occurred at 25, 35 or 40°C after the 1st day of incubation and it was then stable up to the 7th day. Little change in pH was found at 45°C compared to other temperatures. Clearly, rhizobial cultures change pH during growth. If peptone were the principal organic nutrient of *Rhizobium*, it is known that the pH rises due to ammonia formed from the deamination of amino acids (Meynell and Meynell, 1965). In contrast, if fermentable sugars are present(as in these experiments), the pH falls due to acid production. At pH 6.8, some glucose is also converted to other sugars, including gentiobiose (Khan and Walker, 1958).

Generally, the results show that pH declined with increasing growth at 25, 35 and 40°C but not at 45°C. There

was no close quantitative correlation between the bacterial growth and the pH change however. This may be explained by differences in metabolism at low and high temperatures. The picture obtained from the scientific literature is confusing however. Generally, fast growing rhizobia can be expected to utilize a wider range of carbon compounds than slow growing rhizobia (Graham and Parker, 1964; Stowers, 1985). Most *Rhizobium* strains can produce strongly ureolytic enzymes. Many of the slow-growing strains, however, fail to reduce nitrate or reduce it at extremely slow rates (Graham and Parker, 1964). In contrast, Neal and Walker (1935) have suggested rapid nitrate utilization by slow-growing root nodule bacteria. It is also known that oxygen can play an important role in the growth of *Rhizobium*. Growth at low oxygen tension appears to be essential for the development of nitrogenase activity in free-living rhizobia. The exclusion of oxygen inhibits both growth and enzymic activity, whereas high levels of oxygen stimulate growth but the bacteria are devoid of nitrogenase activity (Evans and Keister, 1976). Postgate (1971) has reported that, with the aerobic nitrogen fixing bacteria, nitrogen fixation is most effective at hypobaric oxygen pressures and this also seems to be the case of symbiotic rhizobia. Differences in symbiotic responses due to high temperatures and differences between strains seem to be related to the different temperature characteristics as measured in legume plant studies (Munevar, 1981).

Rhizobia present in air-dry soils survive higher temperatures than would be experienced under natural conditions and in moist soils the tolerance of medic rhizobia to higher temperature is much lower (Wilkins, 1967). Bowen and Kennedy (1959), Vyas and Prasad (1960) and Marshall (1964) have all reported that different rhizobia strains differ in their resistance to high temperatures and that strains of *R.trifolii* of different climatic origins vary in resistance to high temperatures in moist soil. Marshall (1964), for example, found that *R.lupini* and *R.japonicum* were more able to survive heat treatments than *R.meliloti*. Also he suggested that, in sandy soils, the ability of rhizobia to withstand high temperatures was influenced by the soil physical properties. Medic and Psoralea strains of rhizobia survived a daily temperature regime of 45°C for 8h alternating with 16h at 30°C for up to 10 days (Wilkins, 1967). The optimum temperature for the growth of *B.rhizobium* was found to vary between 27.7 and 35.2°C by Munevar and Wollum (1981b). La Favre and Eaglesham (1986) found that strains of cowpea rhizobia tolerated up to 43°C, and Karanja and Wood (1988) found strains of *R.leguminosarium* biovar *phaseoli* capable of multiplying at 47°C. Nutman (1975), Dart (1977), and Pate (1961) reported that survival and growth of rhizobia in soil and their symbiotic association with leguminous plants are severely affected by high soil temperatures. Also, similar results were found by Brockwell and Phillips (1965), who reported that survival of rhizobia in inoculant

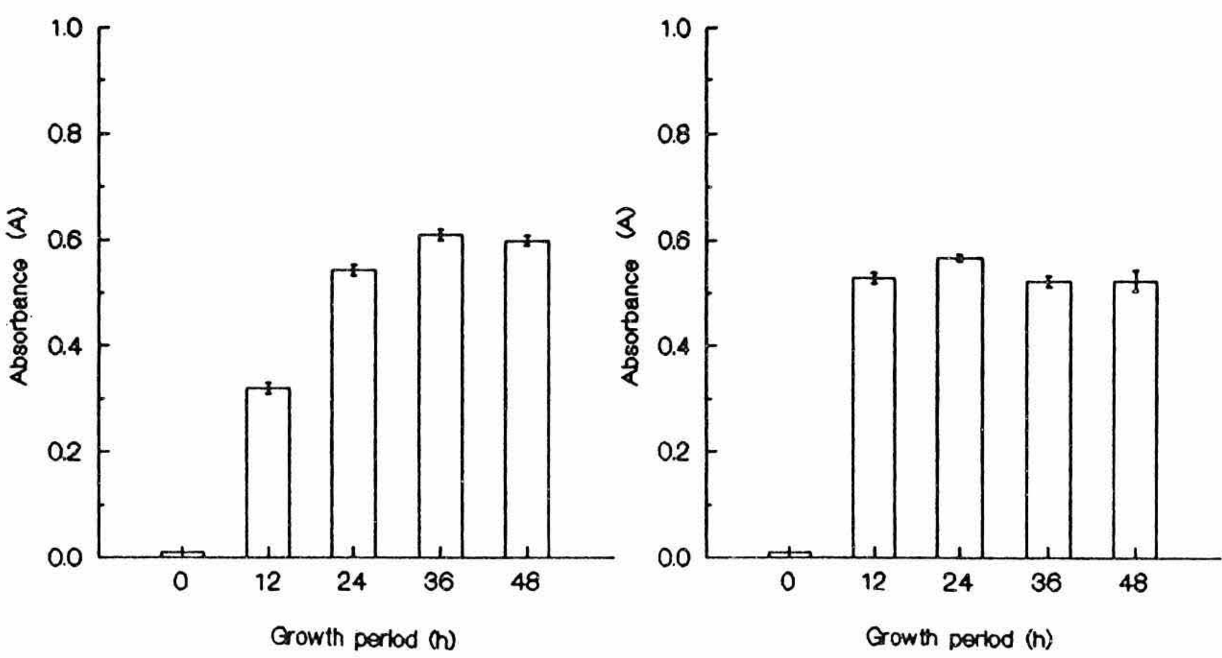
materials was adversely affected by high temperatures. Munevar and Wollum (1981) have suggested three characteristic temperature ranges for rhizobia. An optimum temperature range from 27.4 to 35.2°C, a maximum permissible growth temperature of 29.8 to 38°C and, finally, a maximum survival temperature from 33.7 to 48.7°C (depending on strain). Thus, temperature is probably the most important environmental factor influencing bacterial growth and also survival in many natural environments.

In general, the results from the heat-hardening experiments indicate that *B.rhizobium japonicum* grew better at high temperatures after heat-hardening treatments. During the first 12h of incubation after hardening at 40°C, the rhizobia sometimes grew less well than the controls, but growth then increased more quickly up to 36h of incubation compared with the controls. A small decrease in cell population was measured after 48h, however, similar to that in the control. At 45°C, the heat-hardened rhizobia grew similar to the controls during the first 12h, then a sharp increase was found after 36h. Growth was then much better in the hardened cultures than in the controls. These results present clear evidence for heat hardening taking place in the RCR3407 strain of *B.rhizobium japonicum*.

Results similar to the earlier experiments were observed with respect to the pH measurements in cultures hardened at 40°C and incubated at 40 or 45°C. In cultures incubated at

40°C, the pH declined sharply after 12h and remained stable on further incubation. However, the pH of the culture which was incubated at 45°C did not decline during the first 24h of incubation and only a very slow decline was found after that. Similar results were found in the cultures which had been hardened at 45°C. In cultures which were incubated at 40°C, the pH decreased only slightly after 12h of incubation and became stable on further incubation. At 45°C, no decrease in the pH was found up to 24h and then a small decline took place with increasing incubation periods. These findings confirm that the earlier observation that there is not a strict quantitative correlation between bacterial growth and the pH change. The literature on heat-hardening of *B.rhizobium japonicum* is very limited. Graham and Parker (1964) reported that some rhizobial and Agro-bacterium strains survive heating at 50°C for 10 min, while Day *et al.* (1978) reported that the viability of rhizobia strain 5000 present on seeds declined rapidly following three daily exposures of 5h at 39 and 42°C, while at 45°C few bacteria survived. Also, other rhizobia of the cowpea group vary greatly in their tolerance to high temperatures (Day *et al.*, 1978). Some survived well at 45°C, whereas others behaved like strain 5000. The viability of a number of strains declined rapidly at 42°C and after 3 days at 45°C fewer than three rhizobia per seed survived. Cowpea group strains also varied greatly in their tolerance of high temperatures. Some even survived well at 45°C (Day *et al.*, 1978). Elsheikh and Wood (1989)

have found that several strains of soybean and chickpea rhizobia all grow at 25°C but *B.rhizobium* growth was slower than strains of *Rhizobium*. In all the strains, growth at 37°C was reduced compared with that at 25°C. None of the strains grew at 45°C. This variability in heat tolerance is significant, since soil surface temperatures between 40 and 60°C have been reported in tropical and sub-tropical climates (Chatel and Parker, 1973; Day *et al.*, 1978 and Philpotts, 1967). A particularly interesting observation in the present study was the finding that the pH changes in heat-hardened *B.rhizobium* cultures grown at 40°C and 45°C was similar to the pH changes in non-hardened cultures although the bacterial growth rates were different in the two cases. This indicates that the metabolism of the hardened bacteria is different from that of the unhardened bacteria.



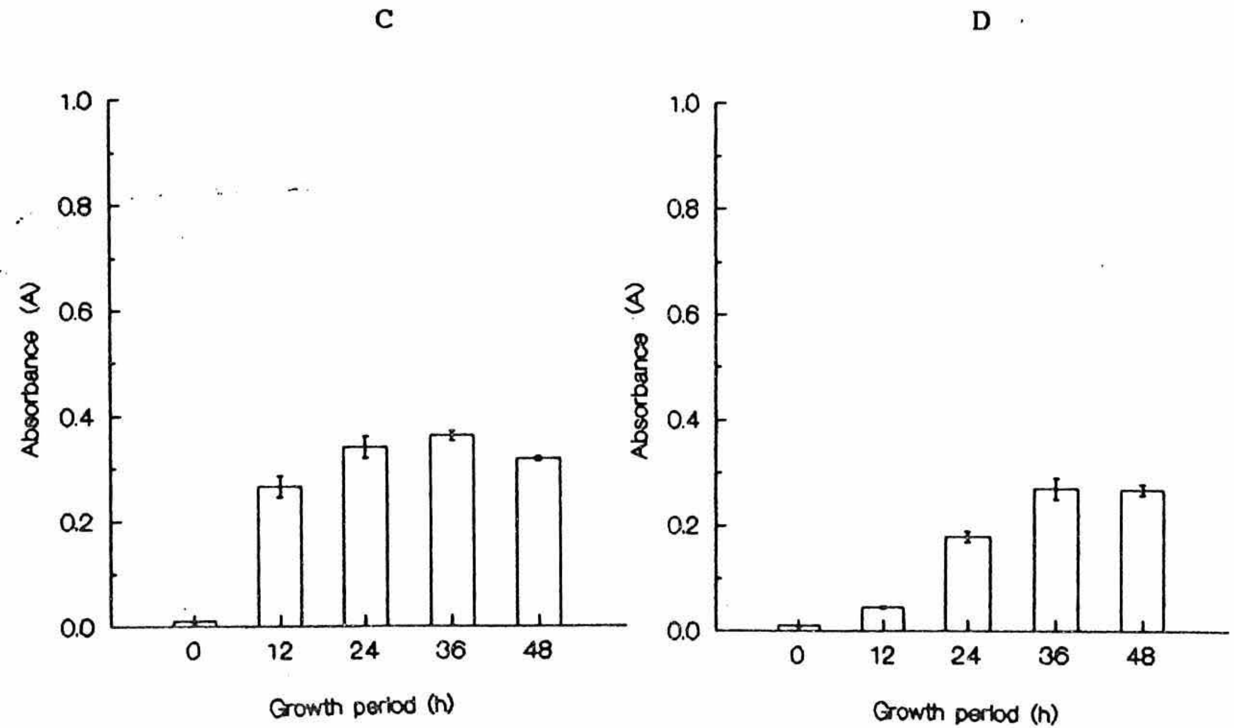
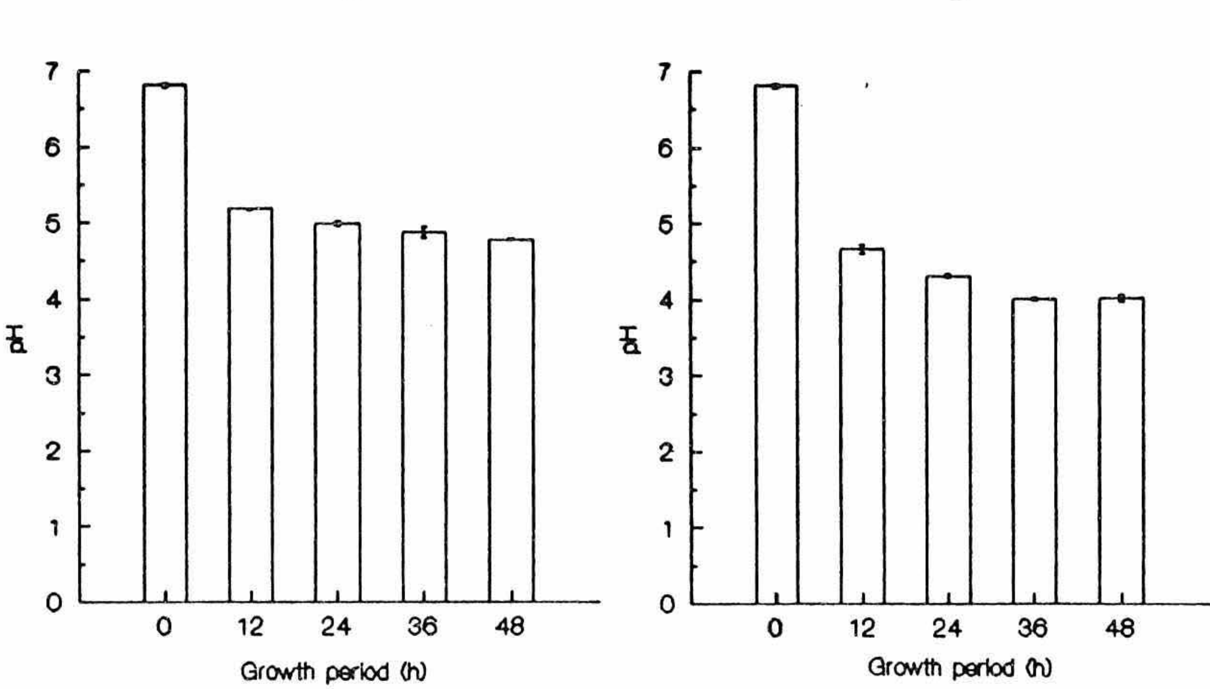


Figure 6.1 Growth of *B.rhizobium japonicum* at A, 25°C; B, 35°C; C, 40°C; D, 45°C.



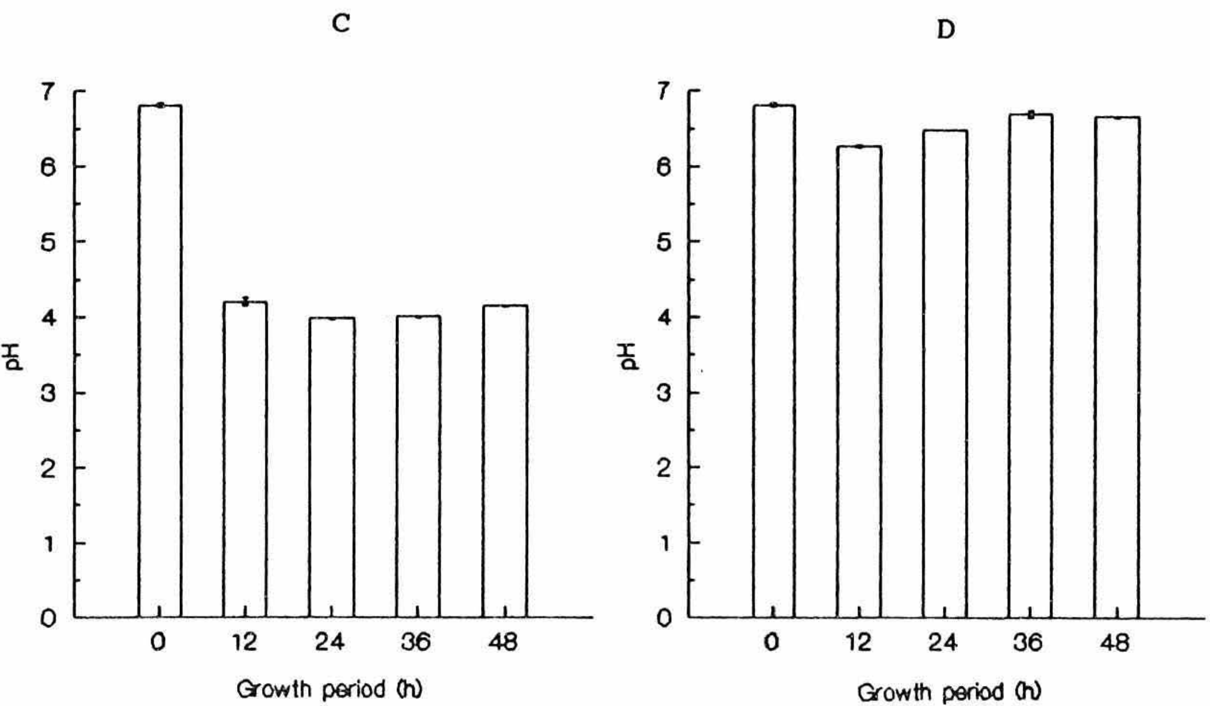
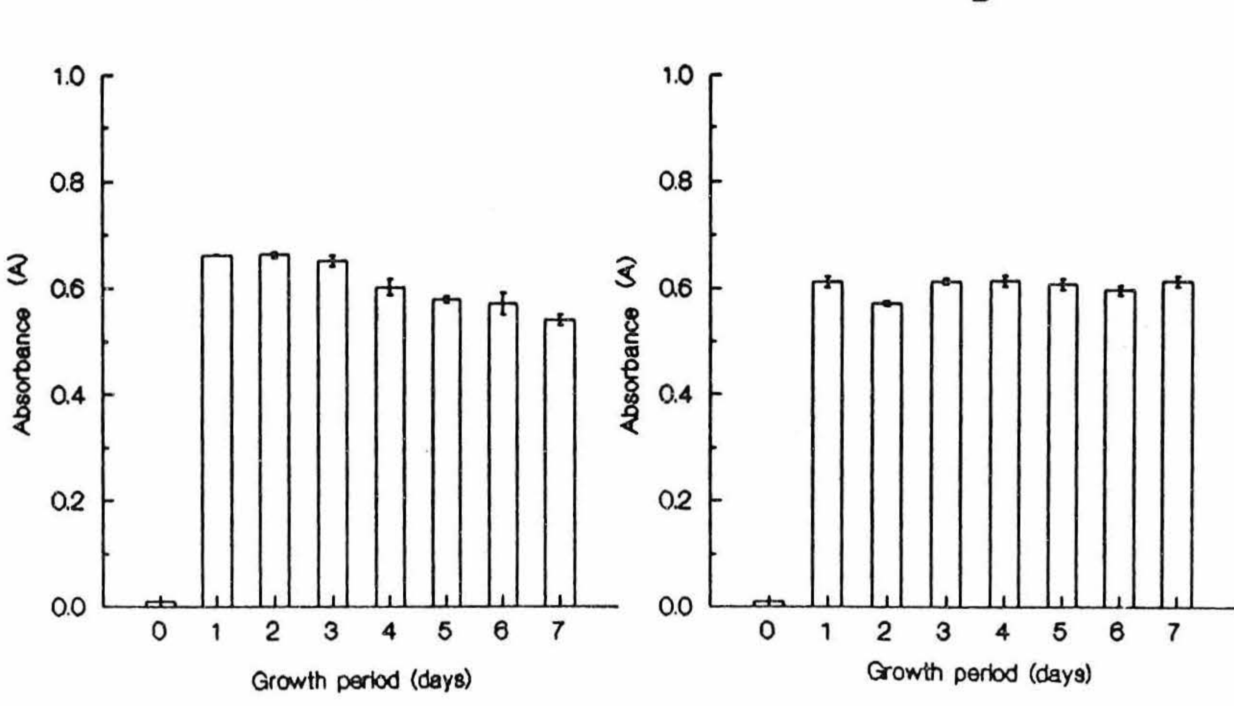


Figure 6.2 Changes in pH of *B.rhizobium japonicum* cultures at A, 25°C; B, 35°C; C, 40°C; D, 45°C.



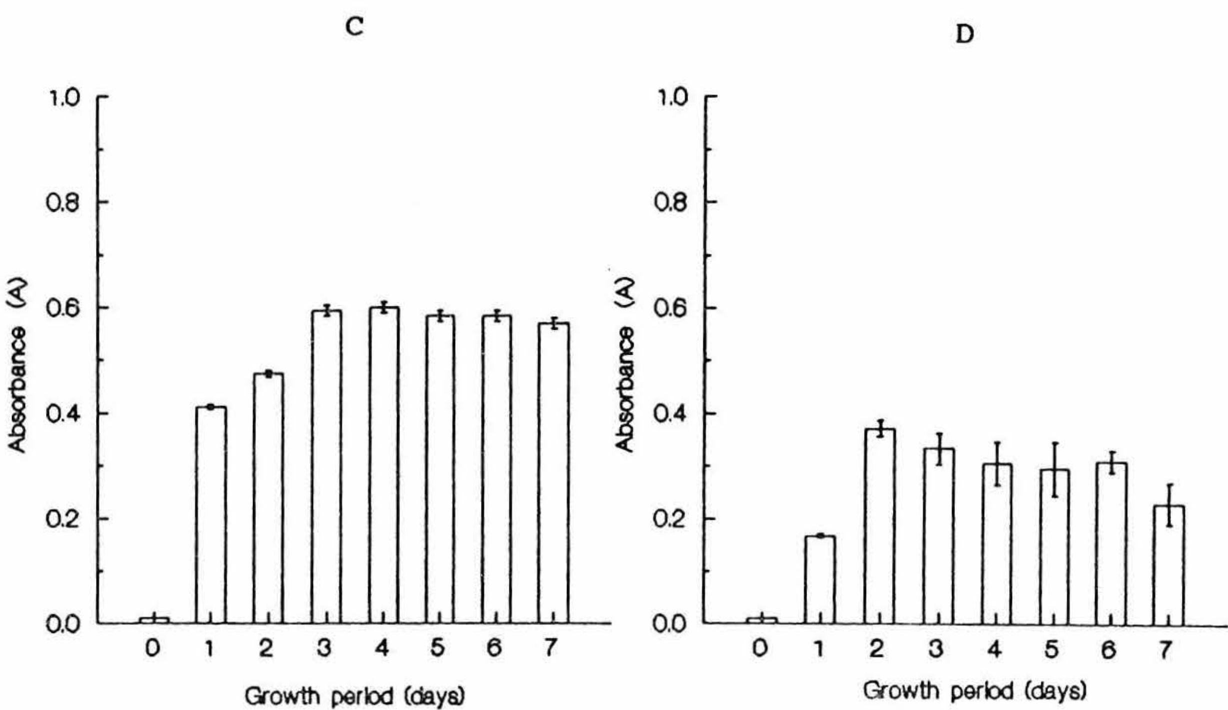
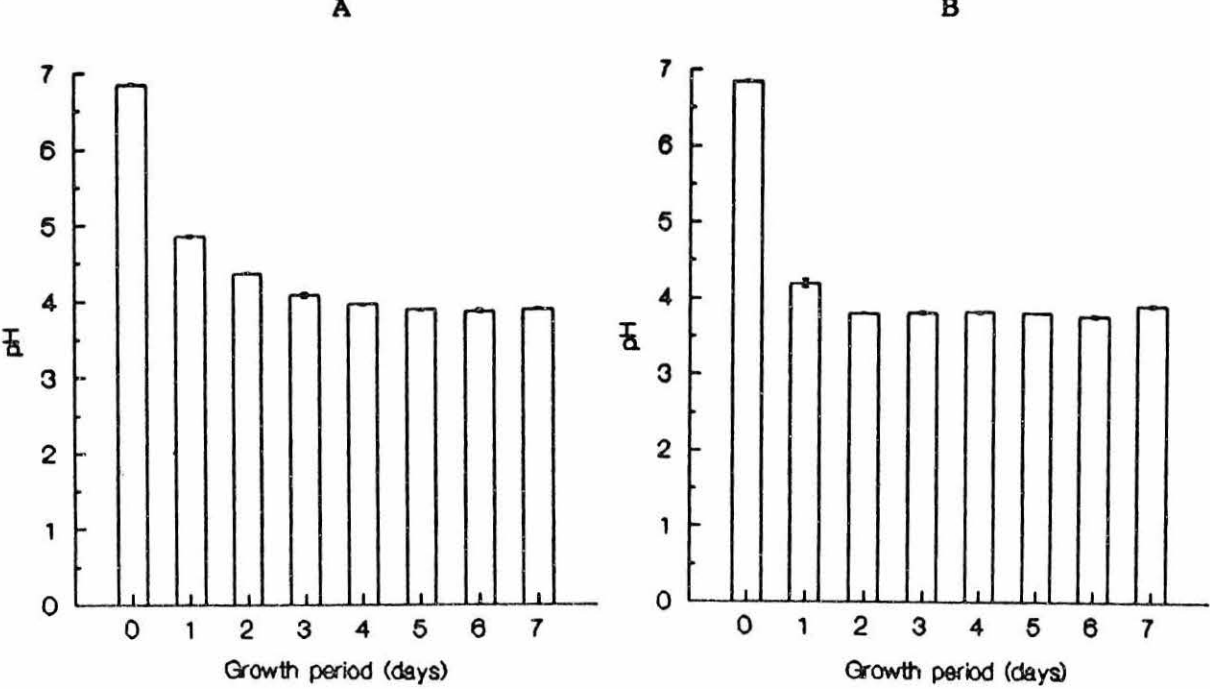


Figure 6.3 Growth of *B.rhizobium japonicum* at A, 25°C; B, 35°C; C, 40°C; D, 45°C.



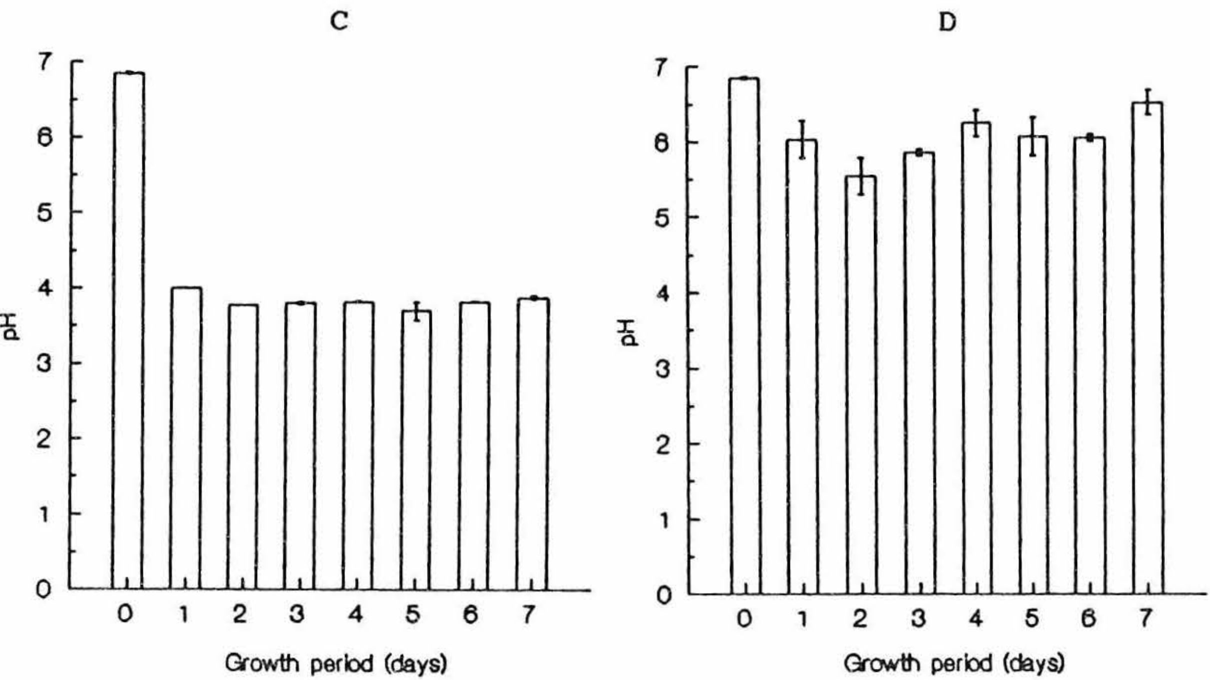


Figure 6.4 Changes in pH of *B.rhizobium japonicum* cultures at A, 25°C; B, 35°C; C, 40°C; D, 45°C.

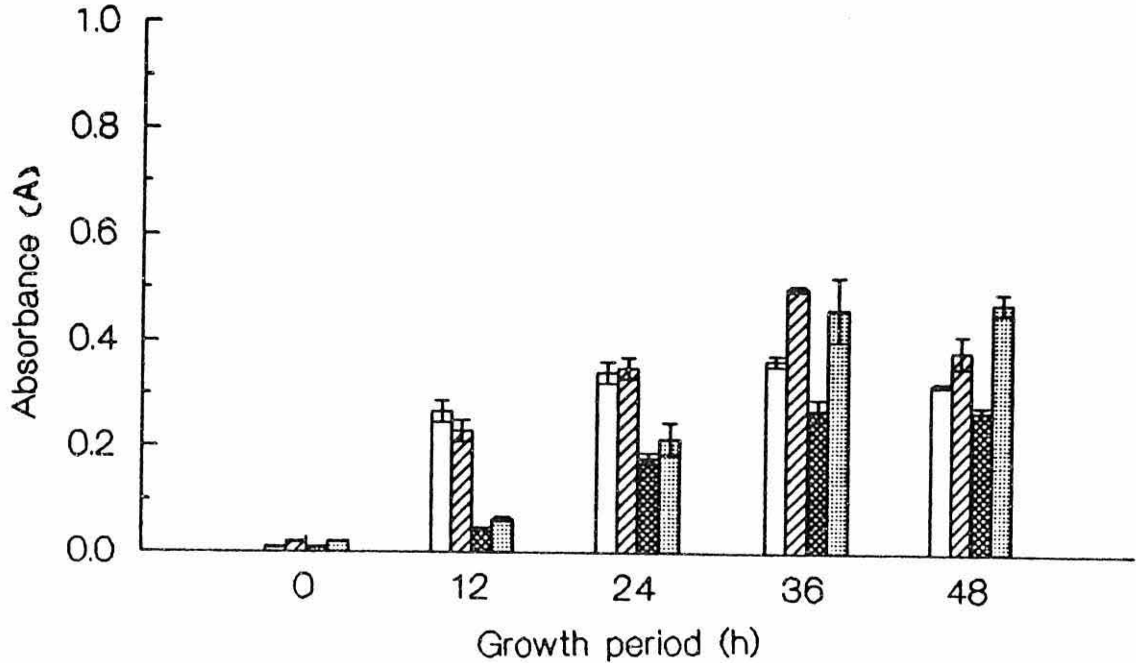


Figure 6.5a Effect of heat-hardening on growth of *B.rhizobium japonicum.* [ ] , control grown at 40°C; [hatched] , pre-hardened at 40°C then grown at 40°C; [cross-hatched] , control grown at 45°C; [dotted] , pre-hardened at 40°C then grown at 45°C.

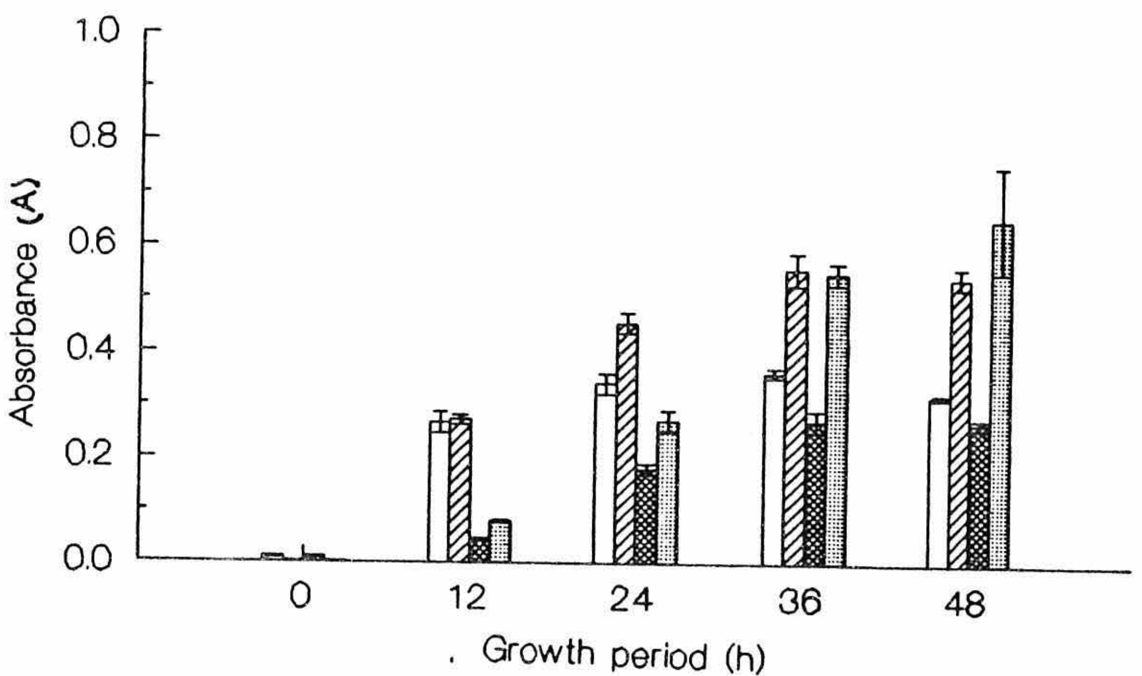


Figure 6.5b Effect of heat-hardening on growth of *B.rhizobium japonicum.* [ ] , control grown at 40°C; [hatched] , pre-hardened at 45°C then grown at 40°C; [cross-hatched] , control grown at 45°C; [dotted] , pre-hardened at 45°C then grown at 45°C.

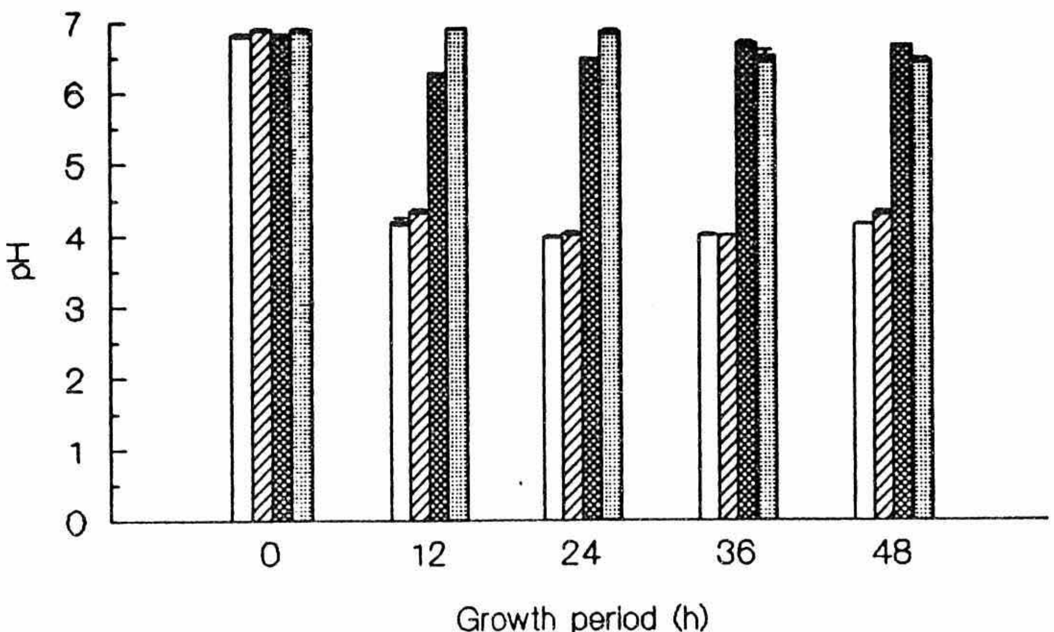


Figure 6.6a Changes in pH of *B.rhizobium japonicum* cultures following heat-hardening. ▭, control grown at 40°C; ▨, pre-hardened at 40°C then grown at 40°C; ▩, control grown at 45°C; ▤, pre-hardened at 40°C then grown at 45°C.

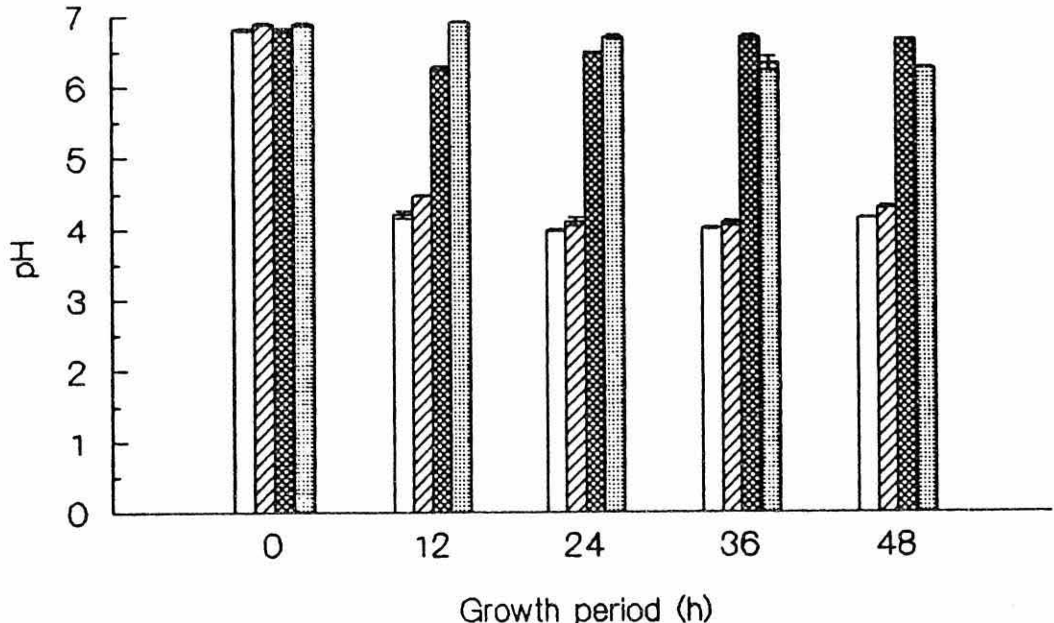
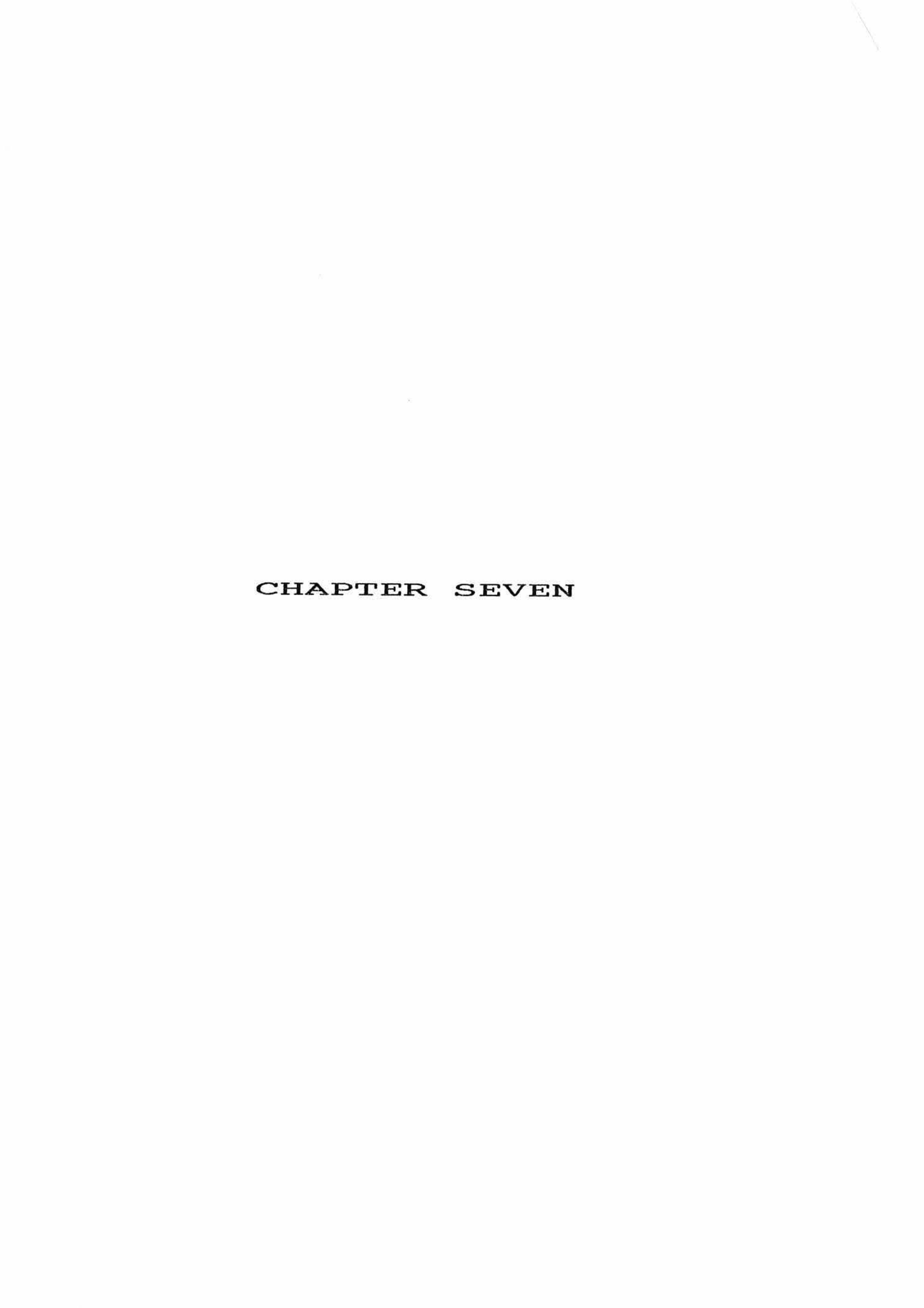


Figure 6.6b Changes in pH of *B.rhizobium japonicum* cultures following heat-hardening. ▭, control grown at 40°C; ▨, pre-hardened at 45°C then grown at 40°C; ▩, control grown at 45°C; ▤, pre-hardened at 45°C then grown at 45°C.



CHAPTER SEVEN

Chapter Seven.

Effects of High Temperatures on Nitrogen Fixation.

7.1 Introduction.

Most of the nitrogen in plants and indirectly in animals comes from the soil in the form of inorganic ions absorbed by plant roots. Some nitrogen, however, is also fixed from the air. It is converted to ammonia and other nitrogenous substances by either free living or symbiotic nitrogen-fixing bacteria.

There are many different physiological and environmental factors affecting the rate of nitrogen fixation in legume root nodules, such as temperature, waterlogging, water stress, salinity, combined nitrogen levels, pH, nutrients etc. Research on these factors in different legumes has been performed in relation to temperature by Sinclair and Weisz (1985), Kuo and Boersma (1971) and Pankhurst and Sprent (1976), water stress by Sprent (1971a,b, 1972a), Sprent and Gallacher (1976) and Ray (1987), soil dehydration by Sall and Sinclair (1991), Weiz *et al.* (1985), Sinclair *et al.* (1987) and Durand *et al.* (1987), and on nitrate application by Wu and Harper (1990), Streeter (1988) and Riguad *et al.* (1973). Many of these factors, including temperature, affect many aspects of nitrogen fixation and assimilation, as well as factors such as respiratory activity, gaseous diffusion and the solubility of dissolved gasses which ultimately affect

plant growth.

Some techniques used to study legume nitrogen fixation are destructive to the whole plant. For example, the Kjeldhal and ^{15}N-tracer methods usually involve the complete harvesting of the plant for these analyses. However, the acetylene reduction method is not destructive. It has been widely used to assay nitrogen fixation in intact plants and also in excised root systems (Hardy *et al.*, 1968; Lawn and Brun, 1974). Acetylene reduction activity (ARA) has been widely used to estimate comparative rates of nitrogen fixation in soybean at different temperatures. Low soil temperatures have been shown to adversely affect nodule formation and hence the rate of nitrogen fixation by nodulated legume roots (Dart and Day, 1971; Waughman, 1977). The deleterious effects of high root temperatures on nitrogen fixation have been shown for numerous legumes (Gibson, 1977; Sprent and Minchin, 1984) and biological nitrogen fixation is often affected more by temperature than the general growth and photosynthesis of the plant is affected (Granhall, 1981). Steward (1966) has also suggested that nitrogen fixation is often especially inhibited by temperature extremes which have less effect on plant growth.

Experiments are described in this chapter to evaluate the effects of high temperature stress applied to the roots and leaves of soybean plants on the rate of nitrogen fixation

during the stress and during subsequent recovery. Preliminary experiments were carried out to investigate nitrogen fixation at different growth stages of the soybean plant, the effects of acetylene on nitrogen fixation and the effects of decapitation.

7.2 Methods.

7.2.1 Preliminary experiments.

Several preliminary experiments were carried out as detailed below. In all cases, the plants were germinated and grown at 24°C in pots in plant growth rooms (see Section 2.15.1).

7.2.1.1 Preparation of inoculation media and plant inoculation.

Liquid inoculation medium containing *B.rhizobium japonicum* strain RCR3407 was made up as described in Section 2.13.1. Soybean plants were inoculated with 3 cm^3 of the medium applied around the main roots of the plants 7 days after seedling emergence as described in the Section 2.15.1. The formation of root nodules was observed after 28 days and nodule number was recorded. The experiment was conducted in triplicate using the cultivar Sable.

7.2.1.2 Measurement of nitrogen fixation at different stages of nodule growth.

An experiment was conducted to determine nitrogen fixation at different stages of nodule growth. Seeds of the cultivar

Mago-80 were germinated and inoculated one week after seedling emergence as described in Section 2.15.3. The nitrogen fixation measurements, made according to Section 2.15.2, were started 2 weeks after inoculation and ended after the seventh week. The nitrogen fixation assays were carried out 10 min after the addition of acetylene to the pots. At the end of the experiment, nodule count and nodule fresh and dry weights were recorded as described in Section 2.15.2. Five replicate plants were used throughout the experiment.

7.2.1.3 Effects of repeated daily injections of acetylene on nitrogen fixation.

Using the cultivar Sable and growing conditions as described in Section 2.15.1, nitrogen fixation was measured (Section 2.15.2) for six days following the daily injection of acetylene into the pots. The experiment was started five weeks after inoculation. The pots were vented after each daily experiment was complete. At the end of the experiment, nodules were counted and weighed as described in Section 2.15.2. The experiment was conducted with three replicates.

7.2.1.4 Effects of plant decapitation on nitrogen fixation.

The cultivar Sable was grown and inoculated as described in Sections 2.13.2 and 2.15.1. Six weeks after planting, the plants were decapitated at the soil surface. Then,

acetylene was injected and fixation was measured (Section 2.15.2) at 10, 20, 30, 40, 50 and 60 min after injection. At the end of the experiment, nodules were counted and their fresh and dry weights were recorded as above. Three replicate plants were used for this experiment.

7.2.1.5 Effects of heat stress on nitrogen fixation.

A preliminary experiment was conducted to investigate the effects on nitrogen fixation of heat stress applied to the roots. Seeds of cultivar Sable were germinated and grown in pots (see Section 2.15.1) and the plants were inoculated 7 days after sowing as described in Section 2.13.2. The plants were used when they were five weeks past-inoculation. Heat stress was applied to the roots for 2, 4 or 6h (2h daily) at 40°C in a water bath (Section 2.15.3). To determine the nitrogen fixation rate, the acetylene reduction method was used (Section 2.15.2). Nitrogen fixation was measured at 10, 20 and 30 min after injecting the acetylene. The nodules were counted at the end of the experiment and their fresh and dry weights were determined. The experiments was carried out with 3 replicates for each treatment.

7.2.1.6 Effects of high-temperature stress and subsequent recovery.

Seeds of the cultivar Sable were grown and inoculated as described in Sections 2.13.2, and 2.15.1. The plants were used for the experiment five weeks after the inoculation.

Using the same method as described in Section 2.15.3, heat stresses of 4 and 6h duration at 40°C were applied to the roots (Section 2.15.3) and nitrogen fixation was measured immediately after the stress and after 24, 48, 72 and 144h of recovery at the normal (24°C) growing temperature. In each case, nitrogen fixation was measured at 10, 20 and 30 min after the injection of acetylene into the pots. At the end of the experiment, nodules were counted and their fresh and dry weights were recorded as mentioned above. The experiment was conducted with 3 replicates for each treatment.

7.2.2 Effects of root heat stress and recovery on nitrogen fixation.

A more complete experiment was conducted to determine the effects of heat stress and recovery on nitrogen fixation. Seeds of five cultivars (Mago-80, Sable, Bragg, Davis and Williams-82) were germinated, grown and inoculated at 27°C as described in Section 2.15.3. The plants were used for the heat-stress treatments five weeks after inoculation. Heat stresses of 2, 3 or 4h were applied at 35, 40 or 45°C in the water bath. After the heat stress treatments nitrogen fixation was measured and the plants were then allowed to recover for 72h at 27°C. Then again, nitrogen fixation was measured. Nodule number and their fresh and dry weights were taken by the same method as described in Section 2.15.2. The experiment was conducted with five replicates.

7.2.3 Effects of shoot heat stress and recovery on nitrogen fixation.

An experiment was conducted to investigate the effects of heat stress on nitrogen fixation by applying the heat stress to the leaves. Soybean cultivars Mago-80 and Bragg were grown at 27°C and inoculated as described in Section 2.15.3. The experiment was started five weeks after inoculation. Heat stresses of 2, 3 or 4h were applied at 35, 40 or 45°C by immersing the leaves in the water bath (see Section 2.15.4). After the stress, the plants were allowed to recover for 72h at 27°C. Determinations of nitrogen fixation were made after the heat stresses and after 72h of recovery. Nodule number and their fresh and dry weights were determined as described in Section 2.15.2. Five replications were made for the each treatment.

7.2.4 Statistical analysis.

Means, standard deviations and standard errors were determined using a pocket scientific calculator (Sharp Model EL-531P) and they were checked by personal computer. Statistical analysis by analysis of variance (ANOVA) was done using the personal computer (Mitac) with the Minitab statistical package (version 10.2) or Genstat. All the figures were plotted using Systat/Sygraph software (version 5.03, Systat Inc. Evanston, IL. USA). In all figures, vertical bars show the standard errors of the mean values.

7.3 Results.

7.3.1 Development of nodules in soybean roots at 24°C.

The results from the first experiment, show that, without rhizobial inoculation, nodulation did not occur in two soybean cultivars, Bragg and Century-84, at 18, 20 or 25°C in either soil, sand, vermiculite or compost media (data not presented). Two methods were then used to infect the roots with rhizobia (see Section 2.13.2). By both methods, approximately 10 to 15 nodules (80% pink) per plant were recorded after four weeks of growth and these occurred on and close to the main part of the tap root. The first method described in Section 2.13.2 was chosen for all subsequent experiments.

The nodule number and nodule fresh and dry weights for the cultivar Sable used in this first preliminary experiment are presented in Table 7.1. The minimum (8.0) and the maximum (13.3) number of nodules were recorded respectively in the control plants grown at 24°C and plants heat shocked for 6h or given repeated acetylene applications. The highest nodule fresh weight was 223 mg in plants that had received repeated treatments with acetylene and the lowest was 98 mg in the plants heat stressed for 6h (2h daily). Dry weights followed a similar pattern to the fresh weights. Statistical analysis of the data in this experiment was not carried out.

Table 7.1 Nodule number and nodule fresh and dry weights (mg) in soybean roots (cv.Sable).

Treatment	N.No	F.W	D.W
Control	8.00 ±0.47	102.4 ±6.45	20.37 ±1.30
2h stress at 40°C (2h daily)	10.00 ±1.90	132.8 ±25.5	28.03 ±5.61
4h " "	11.67 ±1.65	117.8 ±13.4	24.57 ±2.32
6h " "	13.33 ±0.72	97.83 ±20.1	20.80 ±4.83
4h continuous stress at 40°C	12.33 ±1.10	132.5 ±19.8	30.10 ±5.00
6h " "	12.66 ±2.88	150.0 ±29.6	36.50 ±7.04
Shoot decapitation	11.30 ±1.18	116.3 ±3.24	22.27 ±1.25
Repeated acetylene injection	13.33 ±2.37	222.9 ±74.9	57.10 ±20.0

Each value is the mean ± S.E. from three replicates. N.No = nodule number; F.W = fresh weight; D.W = dry weight.

7.3.2.1 Measurement of nitrogen fixation at different stages of nodule growth.

In this preliminary experiment (see Section 7.2.1.2), using the soybean cultivar Mago-80 grown at 27/20°C, nitrogen fixation measurements were started 2 weeks after inoculation.

The data in Fig. 7.1a show that 5.1 μmoles (100 mg fwt)$^{-1}$ h^{-1} ethylene were produced 2 weeks after plant inoculation. At 3 weeks after inoculation, this activity had increased three-fold and a further 20% increase took it to its maximum by 4 weeks after inoculation. A small decrease in activity occurred by the 5th week, however, and further reductions were recorded by the 6th and 7th weeks. Ethylene production was statistically dependent ($P<0.05$) upon the growing period.

The data in Fig. 7.1b show that 16.4 nodules were obtained 2 weeks after inoculation and this gradually increased with increasing growth period. A sharp increase in nodule number was observed between the 6th and 7th week after inoculation. The data in Fig. 7.1c show that 73.3 mg nodule fresh weight was recorded after 2 weeks, but there was no significant change at 3 weeks. Then, it rapidly increased with increasing growth of the plants. The highest fresh weight (490.7 mg) occurred at the 7th week after inoculation. Similar results to the fresh weights were obtained for nodule dry weights (Fig. 7.1c). The nodule

number, and both the nodule fresh and dry weights were strongly dependent ($P<0.05$) upon the periods of growth.

7.3.2.2 Effects of repeated daily injections of acetylene on nitrogen fixation.

This experiment was conducted to evaluate the effects of repeated daily injections of acetylene on nitrogen fixation (see Section 7.2.1.3). Five weeks after inoculation, acetylene was injected to the pots and nitrogen fixation was measured 10 min later. The treatment was repeated daily up to 6 days. Each day, the pots were vented after the fixation assay and returned to the growth room.

The data in Fig. 7.2 show that 5.3 μmoles (100 mg fwt)$^{-1}$ h^{-1} of ethylene were produced on the 1st day of acetylene treatment and this increased by approximately 90% at the 2nd day. However, by Day 3 it had decreased to the Day 1 value. On the 4th day a 100% increase was observed, but on the 5th day the rate was even a little lower than the Day 1 value. On the final (6th day) an approximately 75% increase was obtained over the five day value. Thus, activity appeared to follow a cyclic pattern, increasing and falling on alternate days. Significant differences were observed ($P<0.05$) between the values.

7.3.2.3 Effects of plant decapitation on nitrogen fixation.

In this preliminary experiment, the shoot was decapitated

at the soil surface 5 weeks after inoculation with *B.rhizobium japonicum* (see Section 7.2.1.4). Nitrogen fixation was measured at several times from 10 to 60 min after adding acetylene to the pots.

The results in Fig. 7.3 show that 29.1 μmoles (100 mg fwt)$^{-1}$ h^{-1} of ethylene were produced 10 min after acetylene injection, but this increased to 31.6 μmoles (100 mg fwt)$^{-1}$ h^{-1} at 30 min and it was reduced to 28.8 μmoles (100 mg fwt)$^{-1}$ h^{-1} at 40 min. After 40 min following acetylene injection, the value decreased still further. Statistically, the rate of ethylene production was strongly dependent ($P<0.05$) upon the time period after shoot decapitation.

7.3.2.4 Effects of heat stress on nitrogen fixation.
Heat stress treatments of 2, 4 or 6h (2h daily) were applied at 40°C to the roots and nitrogen fixation was measured 10, 20 and 30 min after injecting acetylene into the pots (see Section 7.2.1.5).

The data in Fig. 7.4 show that 15.1 μmoles (100 mg fwt)$^{-1}$ h^{-1} of ethylene were produced 10 min after acetylene addition to the control plants (0h, no heat stress treatment). The rate then decreased with increasing incubation periods of 20 and 30 min after the acetylene addition. Approximately 25% reduction in the rate of ethylene production occurred as a result of a single 2h heat stress treatment. A further 50% reduction was observed after 4h heat stress. Finally,

another small decrease was found after 6h of heat stress. Similar patterns of declining activity were observed at 10, 20 and 30 min after acetylene injection in all cases. The data also show that both heat stress and the injection of acetylene had adverse effects on ethylene production.

The data were analysed statistically using the ANOVA test, which showed that, the ethylene production rate was significantly ($P<0.05$) dependent upon the length of the stress period and the timing of the acetylene production assay. There were no significant interaction between the stress period and assay timing.

7.3.2.5 Effects of high-temperature and subsequent recovery.

This experiment (see Section 7.2.1.6) was conducted to determine the effects of high root temperatures on nitrogen fixation and to assess the speed of recovery. Heat stresses of 4 or 6h duration were applied at 40°C and recovery was checked after 24, 48, 72 and 144h.

The data in Fig. 7.5a show that ethylene production measured 10 min after acetylene injection decreased by 90% after 4h heat stress compared with the control. After 24h of recovery, however, the rate had increased again to approximately 40% of the control value. The ethylene production rate continued to increase with increasing recovery periods of 48 and 72h. A slight decrease was

measured after 144h of recovery however. Similar results were recorded for assays carried out at 20 and 30 min after acetylene addition.

After 6h of heat stress (Fig. 7.5b), there was an approximately 80% decrease in activity, followed by a pattern of recovery similar to that seen in the 4h heat-shocked plants. The rate of ethylene production similarly declined from 10 to 30 min following acetylene injection at all points in the experiment. Analysis by ANOVA showed that both the heat stress treatments and the recovery treatments had significant ($P<0.05$) effects on the ethylene production.

7.3.3 Effects of root heat stress and recovery on nitrogen fixation.

This was the first full-scale experiment (see Section 7.2.2). Five cultivars (Williams-82, Sable, Mago-80, Bragg and Davis) were studied. Heat stresses of 35, 40 or 45°C were given for 2, 3 or 4h to five replicate plants 5 week after inoculation with *B.rhizobium japonicum*. The nitrogen fixation was measured before applying heat stress (controls) and immediately after applying the heat stresses. Recovery was then checked after 72h at the normal growing temperature of 27°C.

Data for nodule numbers, fresh and dry weight were also recorded in this experiment. The nodule number was variable

between cultivars and between individual plants (Table 7.2). The maximum number of nodules (35.5 and 34.0) occurred in the cultivars Williams-82 and Mago-80 with fewer in Bragg and Davis cultivars. The lowest nodule number was found in the cultivar Sable. Due to variable nodule numbers per plant, variation was also found in nodule fresh weights per plant. Williams-82 and Davis cultivars had the highest values, while the lowest value was in Sable. A similar pattern to nodule fresh weight was recorded for nodule dry weights. The fresh weight of the nodules decreased by approximately 80% after 48h of drying at 70°C. The data for the nodule number, nodule fresh and dry weights were not analysed statistically .

The data for cultivar Williams-82 in Fig. 7.6 show that 2h stress at 35°C caused approximately 60% reduction in nitrogen fixation (ethylene production) and that little recovery was observed after 72h recovery at 27°C. The control value was 14.1 μmoles (100 mg fwt)$^{-1}$ h^{-1} and the activity after 2h at 35°C was 5.6 μmoles (100 mg fwt)$^{-1}$ h^{-1}. Three hours heat stress at 35°C caused approximately 95% reduction in activity, but there was recovery to approximately 47% of the controls after 72h at 27°C. A smaller (77%) reduction in activity occurred after 4h heat stress at 35°C and this recovered to a level of about 65% of the controls. At 40°C, the rate of ethylene production decreased by 80% after 2h heat stress. However, there was a recovery to approximately 75% of the control value after

Table 7.2 Nodule number and nodule fresh and dry weights (mg) in soybean roots.

Cultivar	N.No	F.W	D.W
Heat stress to roots.			
Williams-82	35.50 ±2.17	243.13 ±28.84	49.02 ±6.00
Sable	12.60 ±1.86	127.10 ±19.40	25.60 ±3.85
Mago-80	34.00 ±3.20	207.00 ±24.33	43.18 ±5.20
Bragg	28.94 ±1.55	152.45 ±11.72	32.41 ±2.64
Davis	25.64 ±2.50	215.00 ±32.82	51.03 ±9.02
Heat stress to leaves.			
Mago-80	18.56 ±2.74	221.25 ±40.82	43.84 ±8.20
Bragg	13.53 ±1.48	125.14 ±29.52	26.47 ±6.62

Each value is the mean ± S.E. from at least five replicates. N.No = nodule number; F.W = fresh weight; D.W = dry weight.

72h at 27°C. A similar reduction was found after 3h heat stress, but a smaller recovery was obtained in this case. A smaller (50%) reduction in activity was recorded after 4h stress at 40°C and recovery to about 80% of the control was observed. Finally, at 45°C, no activity was detected after 2, 3 or 4h heat stresses. After 72h recovery at 27°C, however, approximately 60% of the control rate was regained in the 2h-stressed plants and 15-20% recovery was observed in the 3 and 4h-stressed plants.

The data for the cultivar Sable are presented in Fig. 7.7. The nitrogen fixation of the control plants (measured as ethylene production) was 3 times higher than that in cultivar Williams-82. Heat stress at 35°C caused a small decrease in activity after 2h and this increased to about 25% over the control value after 72h of recovery. A 65% reduction in nitrogen fixation was found after 3 or 4h heat stress at 35°C. Relatively little recovery was observed in the 3h-stressed plants, but recovery was better in the 4h-stressed plants. Following heat stress at 40°C, approximately 85% of the control activity was lost after 2h of stress, but 75% of the control rate was recovered after 72h at 27°C. A 45% decline was found after 3h heat stress with a similar recovery level compared with the 2h stress value. Approximately 75% of the control capacity was lost after 4h of heat stress and a recovery to 70% was observed after 72h at 27°C. Finally, at 45°C, 95% reductions in the rate of ethylene production were found after 2, 3 or 4h

heat stress and only 10-15% of the control rate was regained after 72h of recovery.

In Mago-80 (Fig. 7.8), the control rate of ethylene production was similar to that for cultivar Williams-82. Following 2h of heat shock at 35°C, there was an approximately 45% decrease in activity and no regeneration was obtained after 72h recovery at 27°C. Three hours heat stress at 35°C resulted in an approximately 70% decrease, but now there was recovery to a level 30% higher than the control. No decrease in activity was found after 4h heat stress and this activity remained the same after 72h of recovery. Following heat stress at 40°C, only a 10-15% decrease in the ethylene production rate occurred after 2, 3 or 4h of heat stress. Moreover, the subsequent recovery of activity at 27°C was to a level more than double the control value in the 2h-stressed plants. Also, an increase to 20-40% above the control values occurred in the 3 and 4h-stressed plants. At 45°C, 95-98% of the control activity was lost after 2, 3 or 4h of stress. After 72h recovery at 27°C, however, the activity was greater than the control in the 2 and 3h-stressed plants. Only a 50% recovery was observed in the 4h-stressed plants.

Fig. 7.9 contains the data for cultivar Bragg. They show that the rate of ethylene production in control plants, 17.1 μmoles (100 mg fwt)$^{-1}$ h^{-1}, was approximately the same as for cultivars Williams-82 and Mago-80. When subjected to

heat stress at 35°C, a 50-65% decrease in activity occurred after 2, 3 or 4h and in each case a very small recovery was found after 72h recovery at 27°C. At 40°C, approximately 70, 80 and 90% reductions occurred after 2, 3 or 4h of heat stress respectively. This recovered to a level higher than the control value in the 2h-stressed plants, however, and it fully recovered in the 3h-stressed plants. Activity did not reach the control value in the 4h-stressed plants after recovery. Following heat stress at 45°C, approximately 90-95% reductions in the ethylene production rate were found after 2, 3 or 4h and 80, 70 and 50% of the control value was regained in the 2, 3 and 4h stress plants respectively after 72h of recovery.

Figure 7.10 contains the data for the cultivar Davis. The control fixation rate was only second to that of Sable at 24.7 μmoles (100 mg fwt)$^{-1}$ h^{-1} ethylene produced. Two or 4h of heat stress at 35°C did not affect this rate. In addition, 40 and 10% increases were found in the 2 and 4h-stressed plants after 72h of recovery. However, a 44% decrease in activity was observed after 3h of heat stress and this was followed by a moderate increase during recovery. Heat stress at 40°C produced 65-70% reductions in the rate of ethylene production after 2, 3 or 4h, but 70-85% of the control rate was regained after 72h of recovery. Finally, 85-90% of ethylene production capacity was lost after 2, 3 or 4h heat stress at 45°C. A 50% recovery occurred in the 2h-stressed plants but only 15% recoveries

were found in the 3 and 4h-stressed plants.

The results were analysed statistically using the ANOVA test, which showed that, ethylene production was strongly dependent ($P<0.05$) upon the cultivar, temperature and stress period. There were also significant interactions between the cultivar, temperature and stress period.

7.3.4 Effects of shoot heat stress and recovery on nitrogen fixation.

This experiment was conducted to evaluate the effects of high temperatures applied to the leaves on nitrogen fixation in the roots (see Section 7.2.3). Two cultivars, Mago-80 and Bragg, were employed. Stresses at 35, 40 or 45°C for 2, 3 or 4h were applied to the leaves.

Data for cultivars Mago-80 and Bragg in Table 7.2 show that, in this experiment where the heat stress was applied to the leaves, both cultivars produced smaller numbers of nodules than in the experiment where heat stress was applied to the roots (cf. Section 7.2.2). The cultivar Mago-80 still had more nodules than Bragg however.

The data for cultivar Mago-80 in Fig. 7.11 show that a rather low rate of 5.8 μmoles (100 mg fwt)$^{-1}$ h^{-1} ethylene production was measured in control plants. This rate was not affected by either 2 or 4h heat stress at 35°C, but following 3h of stress the value was actually 75% higher

than the control. After 72h of recovery at 27°C, a 60% increase in activity over the control occurred in the 2h-stressed plants and a 180% increase over the control was observed in the 3h-stressed plants. Surprisingly, a 40% decline in the fixation rate occurred in the 4h-stressed plants during recovery. Following heat stress at 40°C, a 100% increase in ethylene production was found after 2h and the rate remained the same at the end of the period of recovery. After 3h stress at 40°C, a 40% increase in activity occurred compared with the control, but this was followed by a small decrease during recovery. After 4h of heat stress at 40°C, the ethylene production rate increased by 75% and it increased further to about three times the control value after recovery. Finally, at 45°C, ethylene production was more than double the control after 2h heat stress, but it decreased to just below the control level after recovery. A small reduction in activity was observed after 3h heat stress and a further 60% decrease was measured after the period of recovery. After 4h heat stress, a 50% decrease in activity occurred and a further 50% decrease was measured after the recovery.

The corresponding data for cultivar Bragg are presented in Fig. 7.12. They show that 2h of heat stress at 35°C did not affect ethylene production, but a 150% increase above the control value occurred after recovery. An approximately 45% reduction was observed after either 3 or 4h heat stress, while increases to 30% above the control value occurred in

both cases after recovery. At 40°C, a 25% decrease in capacity occurred after 2h of heat stress, while no effect resulted from 3h of stress. An increase to 40% over the control was found in the 2h-stressed plants after recovery. The 3h-stressed plants showed a small recovery in activity. Approximately 50% of the control capacity was lost after 4h of heat stress, but an increase to 10% above the control value was found after the 72h period of recovery. Finally at 45°C, 50-75% decreases in the ethylene production rate occurred after 2, 3 or 4h heat stress. The rates remained the same in the 2 and 4h-stressed plants after recovery. In the 3h stressed plants, however, 50% of the control rate was regained after the recovery period.

Despite the large differences observed between the mean values for the various treatments, statistics analysis by ANOVA failed to show any significant dependence ($P>0.05$) of the ethylene production rate upon the cultivar, stress temperature or stress period. This is probably due to the large standard errors associated with the mean values.

7.4 Summary and Discussion.

It is known that nitrogen fixation in legume nodules is affected by a wide range of adverse environmental and physiological conditions such as temperature, salinity, drought, chemicals etc. This chapter reports experiments conducted to evaluate the effects of high temperatures on nodule nitrogen fixation in different soybean cultivars.

The cultivar Sable, used in the first preliminary experiment, produced fewer nodules compared with other cultivars which were included in later experiments. The data from the later preliminary experiments show that the highest nodule numbers were found in Williams-82 and Mago-80 cultivars. In the cultivar Sable, the nodule number was only one third those in Williams-82 or Mago-80. The cultivars Bragg and Davis each produced similar nodule numbers, but less than cultivars Williams-82 and Mago-80. Similar patterns were found in the nodule fresh and dry weights. In later experiments, cultivars Mago-80 and Bragg were used, although they then produced lower nodule numbers than in their first experiment. This was probably due to the use of an old inoculum which may have lost some of its viability.

Our results are in general agreement with many reports from other researchers. Thus, some *Rhizobium* strains produce a large mass of nodules with low specific activity, while others can fix the same amount of nitrogen with a smaller nodule mass (Dobereiner *et al.*, 1970; Duque *et al.*, 1982; Rosendahl, 1984). Pulver *et al.* (1982) also found that genotypic variation exists in soybean with respect to its ability to establish an effective symbiosis with local populations of *Rhizobium spp.* Keyser *et al.* (1982) found big variations in nodule fresh weights and nitrogenase activities between soybean and cowpea cultivars. Rai (1988) observed that, in cheena, nitrogenase activities and root

dry weights increased after 30 days growth following inoculation treatments compared with un-inoculated plants, similar to our observations. The mechanisms which control nodulation are not fully understood. Nodule number in soybean appears to be regulated by the plant, however, through a variety of mechanisms (Caetano-Anolles and Gresshoff, 1993), the major one being inhibition of further nodule formation by existing or developing nodules.

In one of the preliminary experiments, nitrogen fixation was measured in cultivar Mago-80 from the 2nd to the 7th week after inoculation with *B.rhizobium japonicum*. The results show a low rate of nitrogen fixation by the end of the 2nd week, in spite of the fact that nodule number was quite high. Presumably, the nodules were still immature at this time and they did not start to fix significant amounts of nitrogen until the end of the 3rd week. The maximum rate of fixation was found during the 4th week after inoculation and it then declined slightly with increasing growth period. This decline in the rate of nitrogen fixation was surprising, as the nodule number continued to increase up to the 6th week after inoculation and it increased rapidly during the 7th week. Also, the fresh and dry weights of the nodules increased during weeks 5, 6 and 7. The supply of photosynthate from the leaves may have been limiting during these weeks restricting the rate of nitrogen fixation. Weber and Miller (1972) have similarly reported, that, in soybean, nodule number and mass increased as the plants

developed. They also found that the greatest nodule number and fresh weight occurred at 30°C. Cohen *et al.* (1980) found that, in *Setaria italica* the rate of nitrogen fixation is dependent upon a variety of plant factors, including the stage of development, plant genotype and temperature. Francisco and Harper (1995) found that nodule number in the cultivars Williams-82 and *NOD1-3* varied with plant age at the time of inoculation and delayed inoculation led to more profuse nodulation.

The preliminary experiment on the effects on the nitrogen fixation of short, daily exposure of nodules to acetylene in the cultivar Sable showed a very fluctuating pattern from day to day. This fluctuation is difficult to explain. In spite of the fluctuating values, however, the overall trend was a slow decline in nitrogen fixation over the 6-day period of the experiment. This may have been due to a long-term adverse effect of acetylene on nitrogen fixation. Habte (1983) has reported that repeated exposure of plants to acetylene led to a consistent decrease in ethylene production capacity. It is well known that, when acetylene is in contact with the nodules, it can produce rapid short-term decreases in nitrogen fixation in some species. For example, Minchin *et al.* (1983) found that, when the nodulated roots of some legumes were exposed to acetylene, the rate of ethylene production decreased by 40 to 60% during the first 30 min of the assay. Similarly in the present experiments (Section 7.3.2.2), it was found that

the acetylene rapidly decreased the rate of nitrogen fixation by approximately 50% during the second 10 min of incubation and by a further 30% by the end of the 30 min of the incubation of acetylene. In contrast, Mederski and Streeter (1977) suggested that, in soybean, a 60% decline in nitrogenase activity only occurred at the end of 6 days continuous exposure to an acetylene-air mixture.

Decapitation of soybean plants resulted in a very rapid decrease in the rate of acetylene reduction twenty min after the decapitation. By 20 min, the rate was only 13% of the control. This was presumably due to the cessation of the flow of carbohydrates from the shoots to the nodules and the inhibiting effects of the acetylene on nitrogen fixation. These results agree with those of Ohyama and Harper (1991), who reported that, in soybean, the rapid decline in nitrogen fixation in soybeans following decapitation was primarily due to the interruption of carbohydrate supply from the shoots. Also, Herdina and Silsbury (1990) found that removal of faba bean (*Vicia faba* L.) shoots resulted in a rapid decrease in acetylene reduction activity in both vegetative and reproductive plants, but the effect was much higher in the latter. Mederski and Streeter (1977) reported that, in soybean after shoot excision, the acetylene reduction activity in the roots began to decline within a few min and it was about 65% of the original level at the end of 2h. Similar results were also found by Huang *et al.* (1975). In

contrast, Trinick *et al.* (1976) found that decapitation of *Lupinus* spp. had little or no effect on acetylene reduction by root nodules, but removal of the nodules from the roots reduced their activity by 70 to 85% within 30 min. In *Lupinus* the roots were presumably able to serve as a nutrient source for the nodules.

The results of the preliminary experiment on heat stress applied to the roots showed that nitrogen fixation (acetylene reduction) was inhibited by heat stress of 40°C or above and fixation activity generally declined with increasing periods of heat stress from 2 to 6h. In both the 4 and 6h stresses at 40°C, the fixation rate had declined by 70%, although after 2h of stress the rate had only declined by 15%. In spite of these large decreases in the fixation rate after 4 and 6h of heat stresses, the plants were able to recover much of their fixation capacity between 24 and 144h after the end of the heat stress. The maximum recovery occurred 72h after cessation of heat stress. Fixation capacity recovered better in the 4h-stressed plants than in 6h-stressed plants. These results roughly agree with those of Dart and Day (1971) and Dart *et al.* (1975), who reported that acetylene reduction by nitrogen-fixing organisms occurred over a wide temperature range with maximum activity between 24 and 33°C, rapidly declining at higher temperatures. Sinclair and Weisz (1985) observed that acetylene reduction rates in soybean increased with soil temperatures up to 30°C. Rates then

declined slightly up to 34°C, but above this temperature they were greatly reduced. Gibson (1969) reported a linear decline in the rate of nitrogen fixation with long daily exposures to 30°C. His results indicated that the effect was transient, however, and directed towards some temperature-sensitive step or steps in the nitrogen fixation reaction.

The preliminary experiments discussed above provided sufficient experience and data to design detailed studies on the effects of heat stress and recovery on nitrogen fixation. The data from the first of these experiments revealed that nitrogen fixation declined after high temperature stress applied to the roots in all five soybean cultivars tested. The effects of heat stress at the different applied temperatures did not always follow a simple pattern however. In the case of cultivar Williams-82 at 35°C, for example, fixation was inhibited more by 3h heat stress than by 2 or 4h stress. Also, nitrogen fixation at 40°C was higher after 4h stress than after 2 or 3h stresses. This was surprising and it may be due to experimental error or other unknown changes in nodule physiology affecting its temperature sensitivity. These results may also indicate some heat-acclimation potential, but further experiments are needed to determine if this is significant. No nitrogen fixation was detected after 2, 3 or 4h heat stress at 45°C and the activity was not regained fully during subsequent recovery.

Control acetylene reduction activity in the cultivar Sable was 4-fold higher than in the cultivar Williams-82 and the effects of heat stress were different. A 2h heat stress at 35°C applied to Sable had little effect on activity. After 3 and 4h at 35°C, there was approximately 50% decrease in activity. At 40°C, the 2 and 4h stresses decreased nitrogen fixation more than the 3h stress, similar to Williams-82. Like Williams-82, Sable, was strongly affected by heat stress at 45°C. After 72h recovery, the values in plants stressed for 2h at 35°C were higher than the controls and also better recovery occurred in 4h-stressed plants than in the 3h-stressed plants. Plants which were stressed for either 2, 3 and 4h at 40°C showed the same degree of recovery of nitrogen fixation. Very slow recovery of nitrogen fixation occurred in plants heat stressed at 45°C, however, compared to the 35 or 40°C-stressed plants.

The data for cultivar Mago-80 also contain some unexpected features. They indicate that nitrogen fixation was reduced by 2 and 3h stresses at 35°C, but surprisingly no change was observed after 4h stress. Also, no significant effect was found at 40°C. At 45°C, however, a decreasing trend, similar to that seen in cultivar Williams-82, was observed. After 72h of recovery, the acetylene reduction values were higher in most treatments compared with the controls, with the exception of the 2h and 4h stressed plants at 35 and 45°C. Overall, cultivar Mago-80 appeared to recover better than the other cultivars tested here.

In cultivar Bragg, similar control values for nitrogen fixation to those of Mago-80 were measured. In general, as the temperature and stress period increased, the acetylene reduction rate decreased. Plants which were stressed at 40°C recovered better than those stressed at 35°C.

Overall, the cultivar Davis showed a similar pattern of nitrogen fixation to Sable, when exposed to stress at 35°C. Ethylene production was not inhibited by 2 or 4h stresses, but declined after a 3h stress. At 40°C, similar decreases in activity were found in all treatments. At 45°C, activity was more affected by 2h stress than by 3 or 4h stresses. After recovery, the values were higher in plants stressed for 2 or 4h at 35°C compared with controls, and fixation recovered fully in 4h-stressed plants. Also, a better recovery was found in plants stressed at 40°C than in the 35°C stressed plants, but activity did not reach control values during recovery. Although nitrogen fixation recovered in plants stressed for 2h at 45°C, no recovery was observed in the plants stressed for 3 or 4h at this temperature.

The demonstration that there were statistically significant differences between cultivars with respect to the heat treatments employed in the present study is interesting. It has already been pointed that there are several possible causes of nitrogen fixation inhibition at high temperatures. In the present study, the responses which

were observed in the different soybean cultivars were presumably due to the combined effects of high temperature on many different processes involved in the plant-rhizobium symbiotic association. These include the survival of the bacterial cell in the root nodules, nodule metabolism, changes in the physiology of the host plant and the heat sensitivity of the nitrogenase enzyme. Also, temperatures above 37°C prevent the synthesis of most nitrogen fixation polypeptides and this effect is due to the thermo-lability of the *nfi* A gene product. Thus, elevated temperatures cause all *nfi* operons except *nfi* RLA to be turned off (Rai, 1988).

In addition, the oxygen supply to nodules may be affected by high temperatures. Oxygen is essential for symbiotic nitrogen fixation, and it is necessary for the metabolism of respiratory substrates and oxidative phosphorylation which supplies the required ATP to drive nitrogen fixation. Also, the role of leghaemoglobin in delivering this oxygen to the respiratory sites without inhibiting the oxygen-sensitive nitrogenase enzyme system (Brun, 1978) may be affected by high temperatures. Bergerson (1962) reported that, when the oxygen concentration around the nodules is increased above atmospheric levels, the initial effect is to stimulate host cell activity resulting in increased supplies of reductant and/or ATP to the nitrogen-fixing bacteriods. Above a pO_2 of 50%, however, oxygen reaches the bacteriods which become aerobic and cease fixing nitrogen

in soybean. Hartwig *et al.* (1987) working on clover and Vessey *et al.* (1988) working on soybean, have suggested that nitrogenase is limited by oxygen supply (and thereby ATP availability) rather than by reductant (reduced ferredoxin) availability. Also, Sheehy *et al.* (1983) have reported that, in legume nodules, oxygen is both a respiratory substrate and a potent, irreversible inhibitor of nitrogenase. Heat stress may affect the permeability of the nodules to O_2 by interfering with the normal mechanisms which regulate diffusion of oxygen into the nodules. These aspects are at present poorly understood (Kuzma and Layzell, 1994).

The data presented in this chapter show that heat stress applied to the leaves caused only small reductions in nitrogen fixation (acetylene reduction) compared with stress applied to the roots. The data for Mago-80 show that ethylene production was not decreased by heat stresses of 35 or 40°C applied to the leaves. There was even a tendency for activity to increase in the plants as a result of the heat stress. Only after the leaves were held for 4h at 45°C was there a large (50%) decrease in activity. After 72h recovery at 27°C, ethylene production was higher in plants stressed for 2 or 3h at 35°C compared with the controls. Similar strong recoveries were found in plants stressed at 40°C. No recovery was found in plants stressed for 4h at 35°C however. Nitrogen fixation also recovered poorly in plants which were stressed at 45°C. In general, nitrogen

fixation recovered better than in the previous experiment where heat stress was applied to the roots.

Similarly, in the cultivar Bragg, acetylene reduction capacity was not significantly affected by heat stress applied at 35°C and 40°C to the leaves. There was an approximately 50 to 75% decline in activity due to stress at 45°C however. The recovery following heat stress was generally greater in Bragg than in Mago-80. It recovered to a value twice that of the controls in plants stressed for 2h at 35°C. Also, recovery values in other treatments were higher when compared with the controls. As expected, however, little or no recovery in activity was found in plants stressed for 2 and 4h at 45°C.

In the above experiment on Mago-80 and Bragg, nitrogen fixation may be affected by leaf dehydration during high-temperature stress, because this will reduce photosynthesis. Photosynthesis plays a very important role in nitrogen fixation in legumes. Nodules need a continuous supply of carbohydrates for nitrogen fixation (Burris, 1971; Evans and Russell, 1971; Hardy *et al.*, 1968; Lawn and Brun, 1974) and photosynthesis is the source of this carbohydrate (Lawn and Brun, 1974; Pate, 1962). Transpiration is also important for nitrogen fixation, since Pate *et al.* (1969) and Pate (1962) reported that 80-90% of the nitrogen fixed by nodules is transported to the upper parts of the host plant via the transpiration (xylem)

stream in several species. It is not surprising, therefore, that, Durand *et al.* (1987) found that, in soybean, a decrease in nitrogen fixation preceded the decrease in plant photosynthesis as the soil dried. Furthermore, air temperature may have as great, if not greater, effect than soil temperature on nodule activity, because of its effect on transpiration and the translocation of carbohydrates from the leaves to the nodules (Mague and Burris, 1972). The results reported in this chapter do not support this view of Mague and Burris, but that is probably because the present experiments were short term ones. Exposure of the leaves to high temperature for longer periods may be needed to find the effects observed by Mague and Burris. Clearly, the effects of temperature applied to the shoots and leaves on nitrogen fixation in the roots of soybean are complex. In particular, it is difficult to explain why the fixation rates in the present experiment should rise so much higher than the controls following the heat stress. Sall and Sinclair (1991) have reported that genetic variation exists in soybean with respect to the sensitivity of nitrogen fixation to drying soils. Our present data tend to support this.

In general, therefore, high temperature appears to be a major factor affecting symbiotic nitrogen fixation in the soybean cultivars examined in the present study. It can be seen that the cultivar Mago-80 seems to be the most tolerant to high temperatures and it also recovered from

heat stress better than the other cultivars. Davis was not as heat tolerant as Mago-80, but it was more tolerant than cultivars Williams-82, Bragg or Hardee. These last three cultivars were very similar in their sensitivities to heat stress, although the data suggest that Bragg is perhaps the more sensitive.

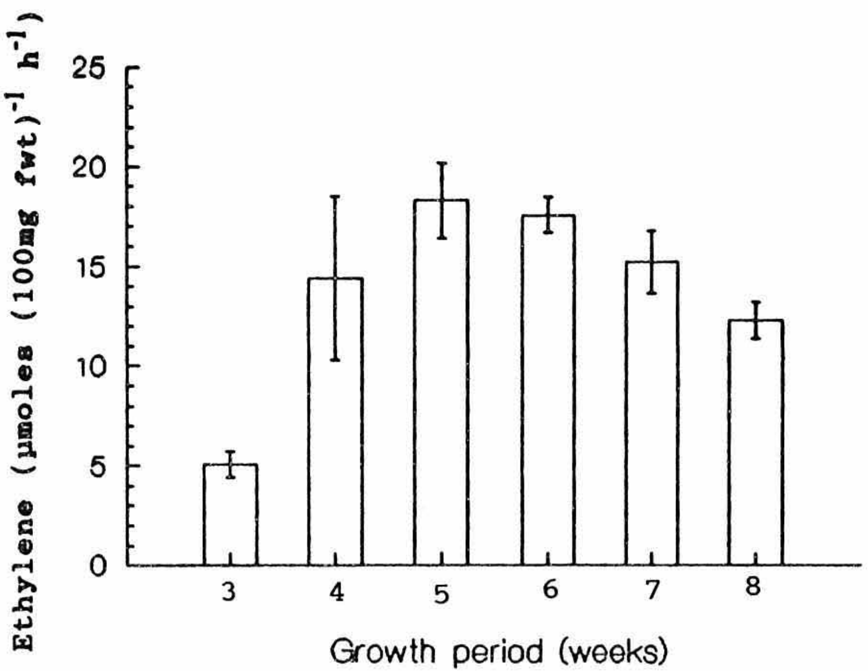


Figure 7.1a Nitrogen fixation in cv. Mago-80 inoculated with *B.rhizobium japonicum*.

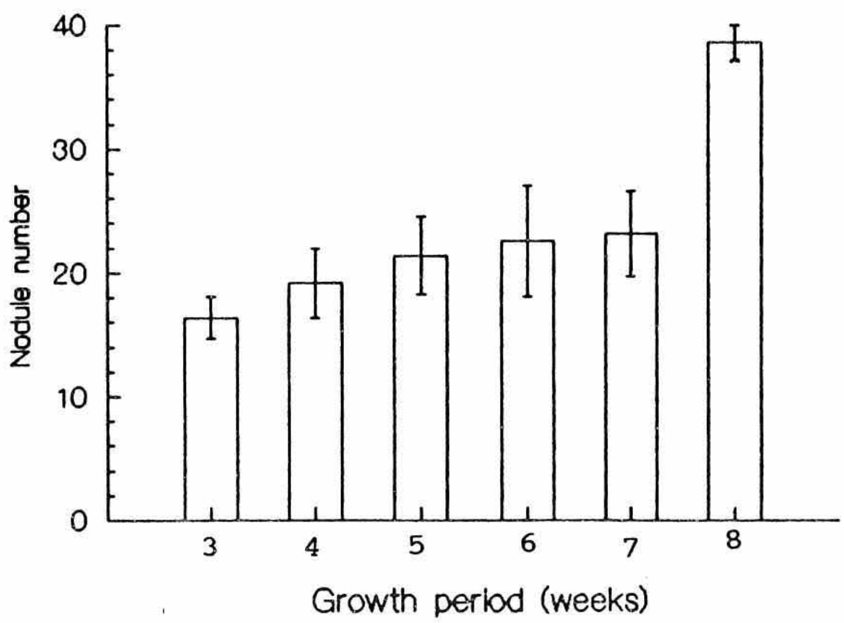


Figure 7.1b Root nodule numbers in cv. Mago-80 inoculated with *B.rhizobium japonicum*.

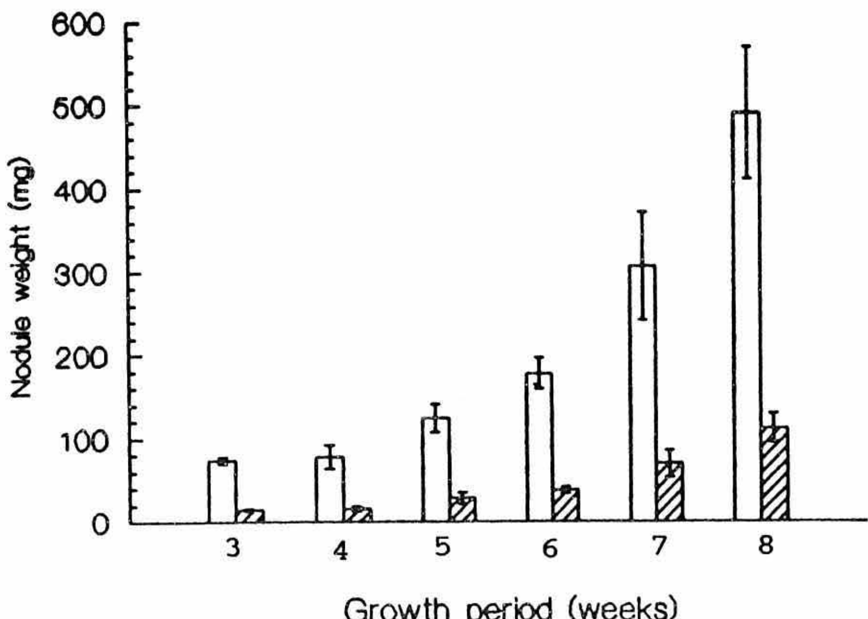


Figure 7.1c Root nodule weights in cv. Mago-80 inoculated with *B.rhizobium japonicum*. ▭, fresh weight; ▨, dry weight.

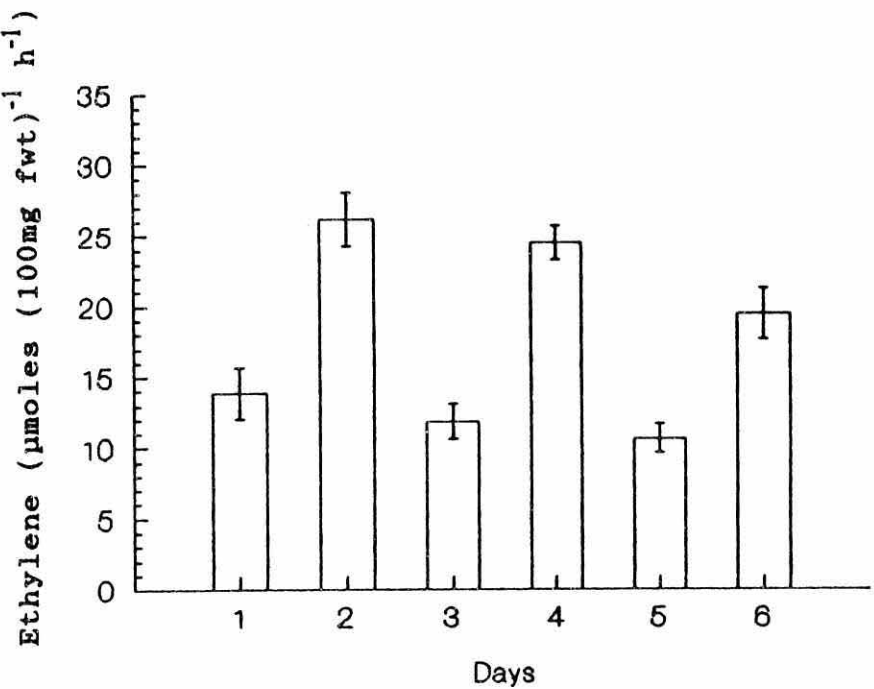


Figure 7.2 Effect of daily acetylene treatments on nitrogen fixation in soybean cv. Sable.

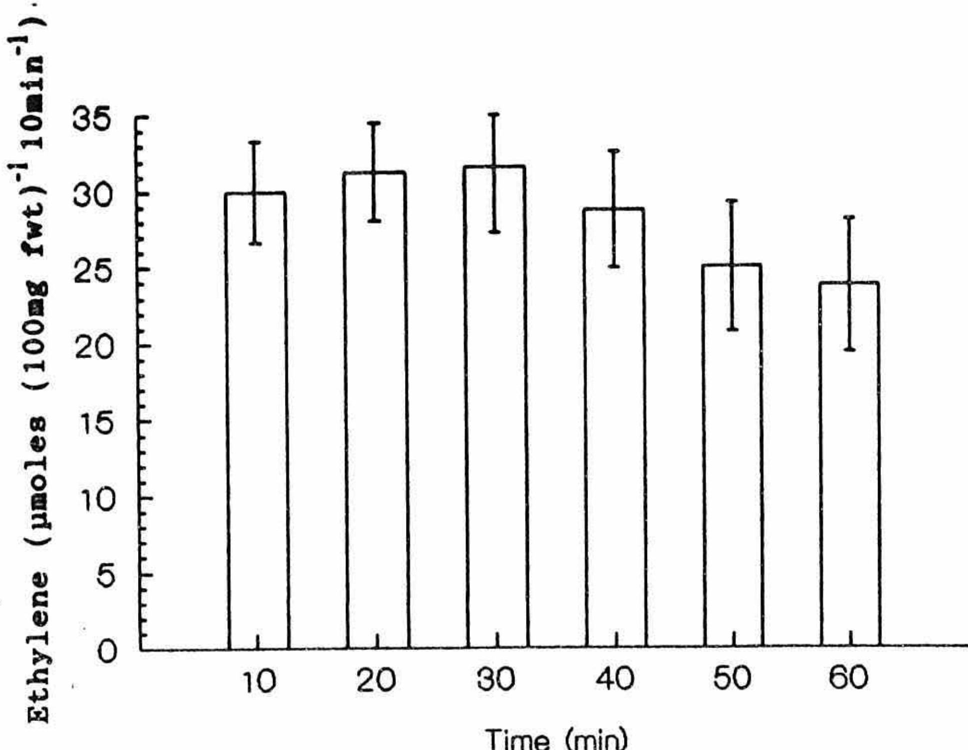


Figure 7.3 Effect of shoot decapitation on nitrogen fixation in cv. Sable.

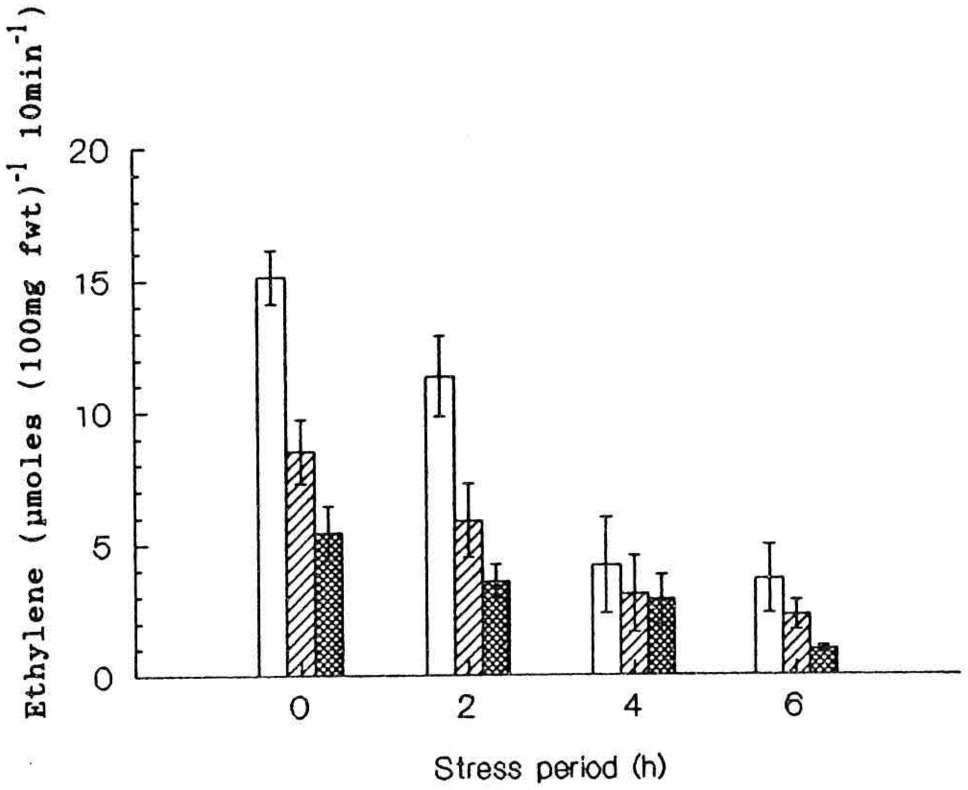


Figure 7.4 Effect of root heat stress on nitrogen fixation in soybean cv. Sable. ☐, 10 min; ▨, 20 min; ▩, 30 min. after the stress.

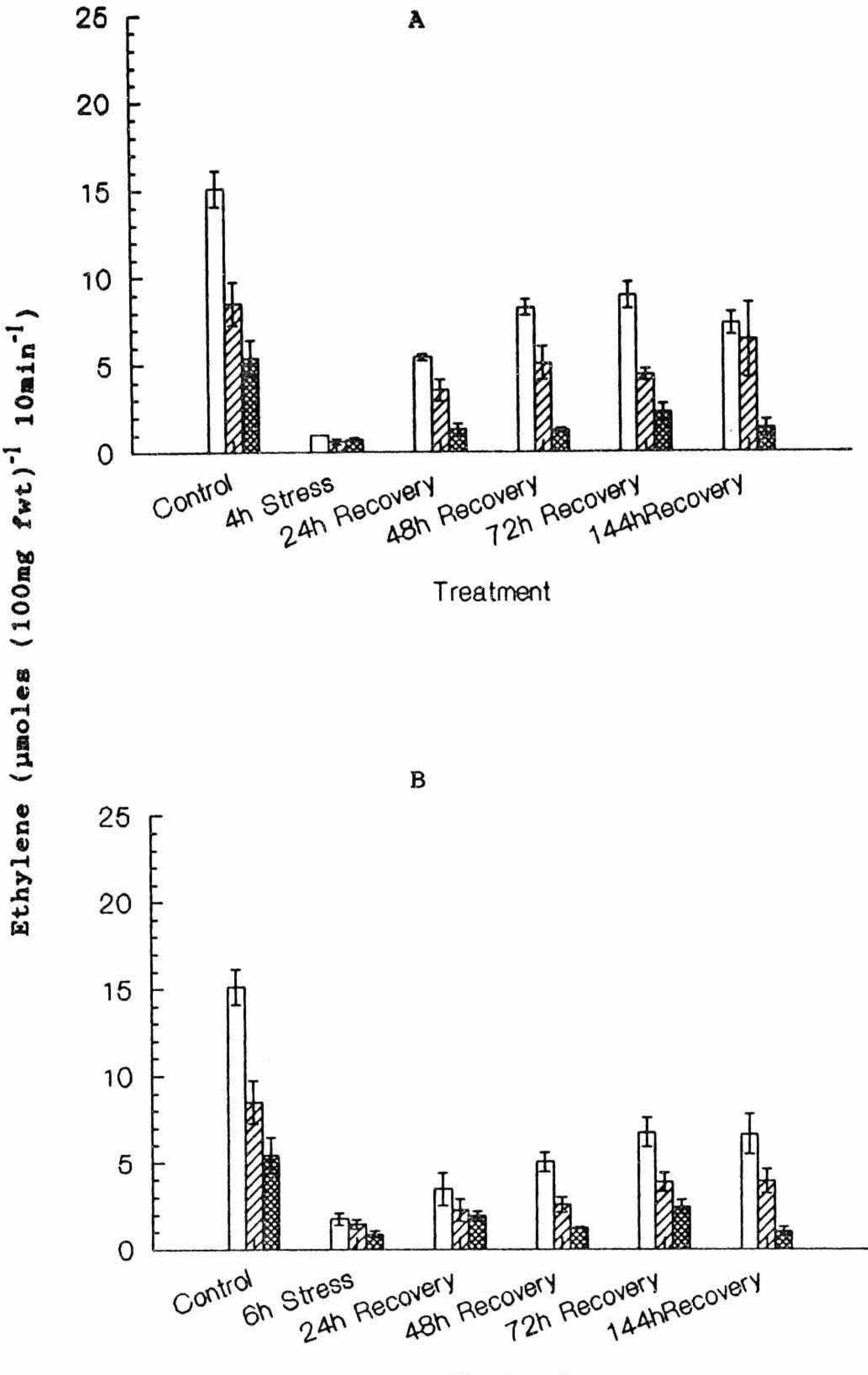
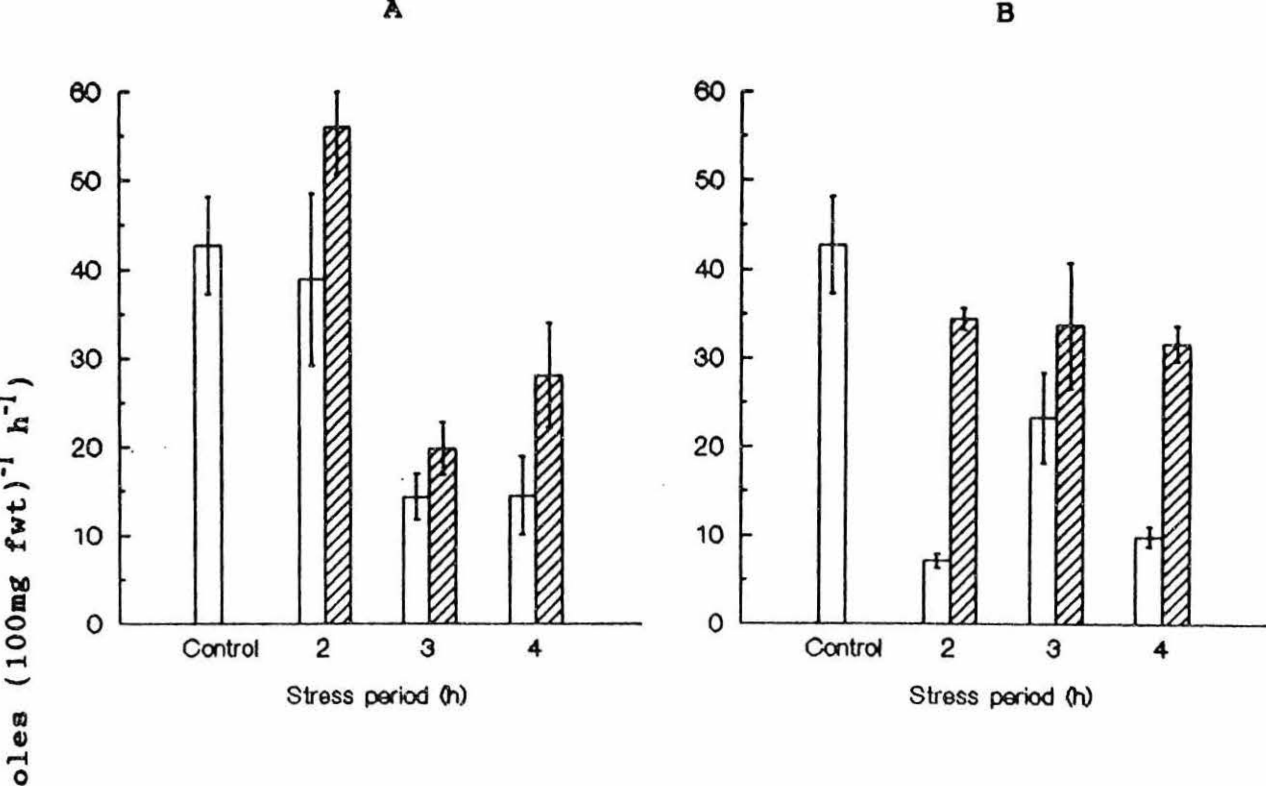


Figure 7.5 Effect of root heat stress on nitrogen fixation in soybean cv. Sable. A, 4h stress; B, 6h stress; ☐, 10 min; ▨, 20 min; ▩, 30 min. after the stress.

Figure 7.6 Effect of root heat stress on nitrogen fixation in cv. Williams-82. A, 35; B, 40; C, 45°C; [open box], at the end of heat stress period; [hatched box], following recovery for 72h.



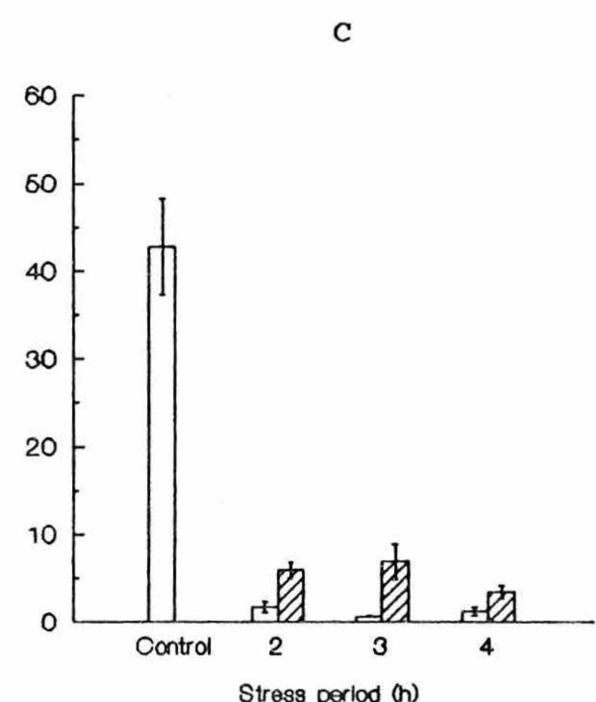
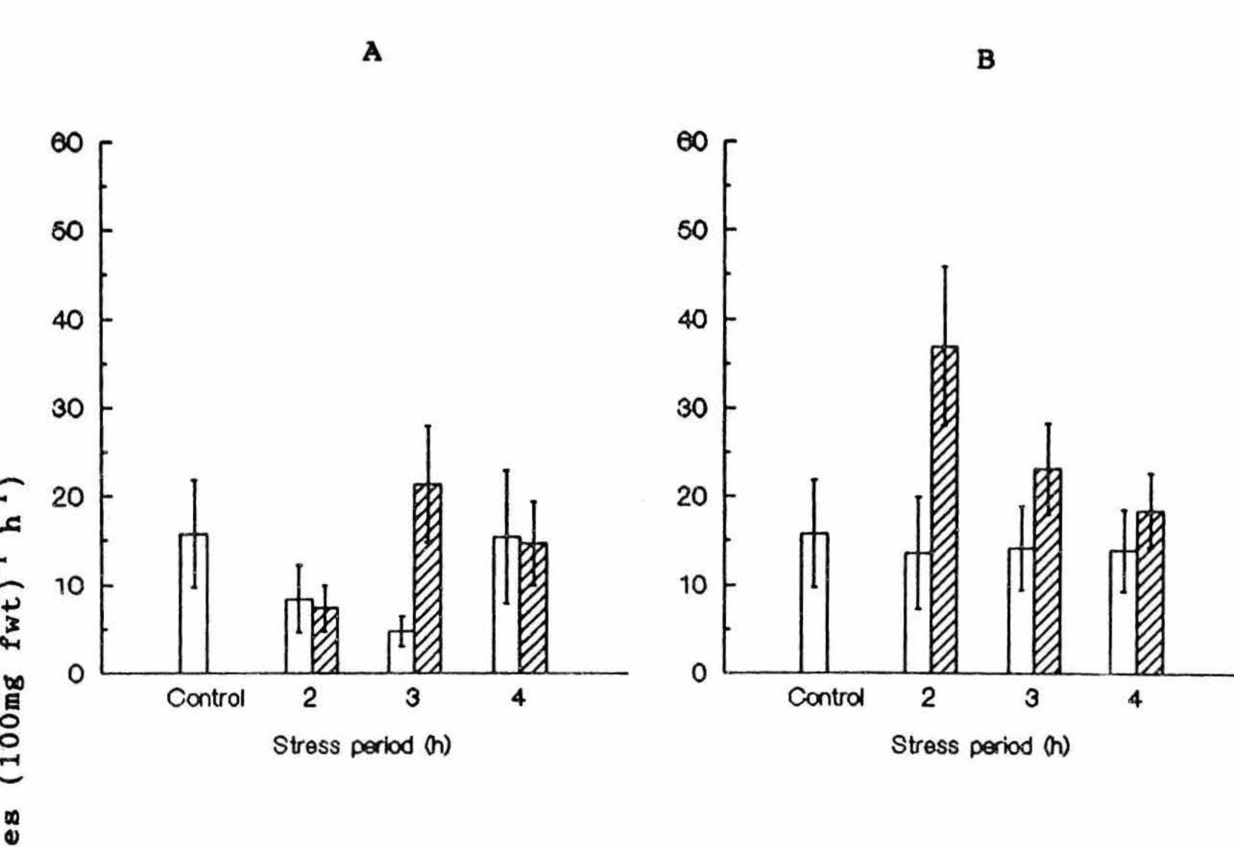


Figure 7.7 Effect of root heat stress on nitrogen fixation in cv. Sable. A, 35; B, 40; C, 45°C; [open bar], at the end of heat stress period; [hatched bar], following recovery for 72h.



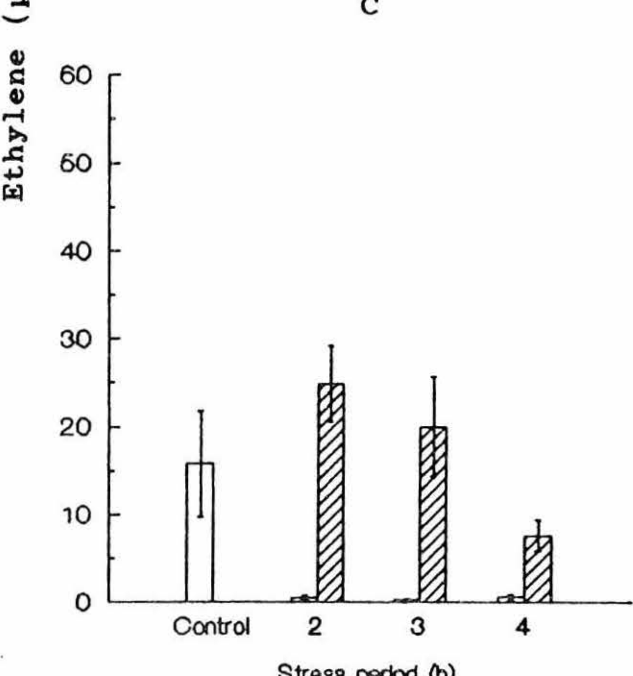
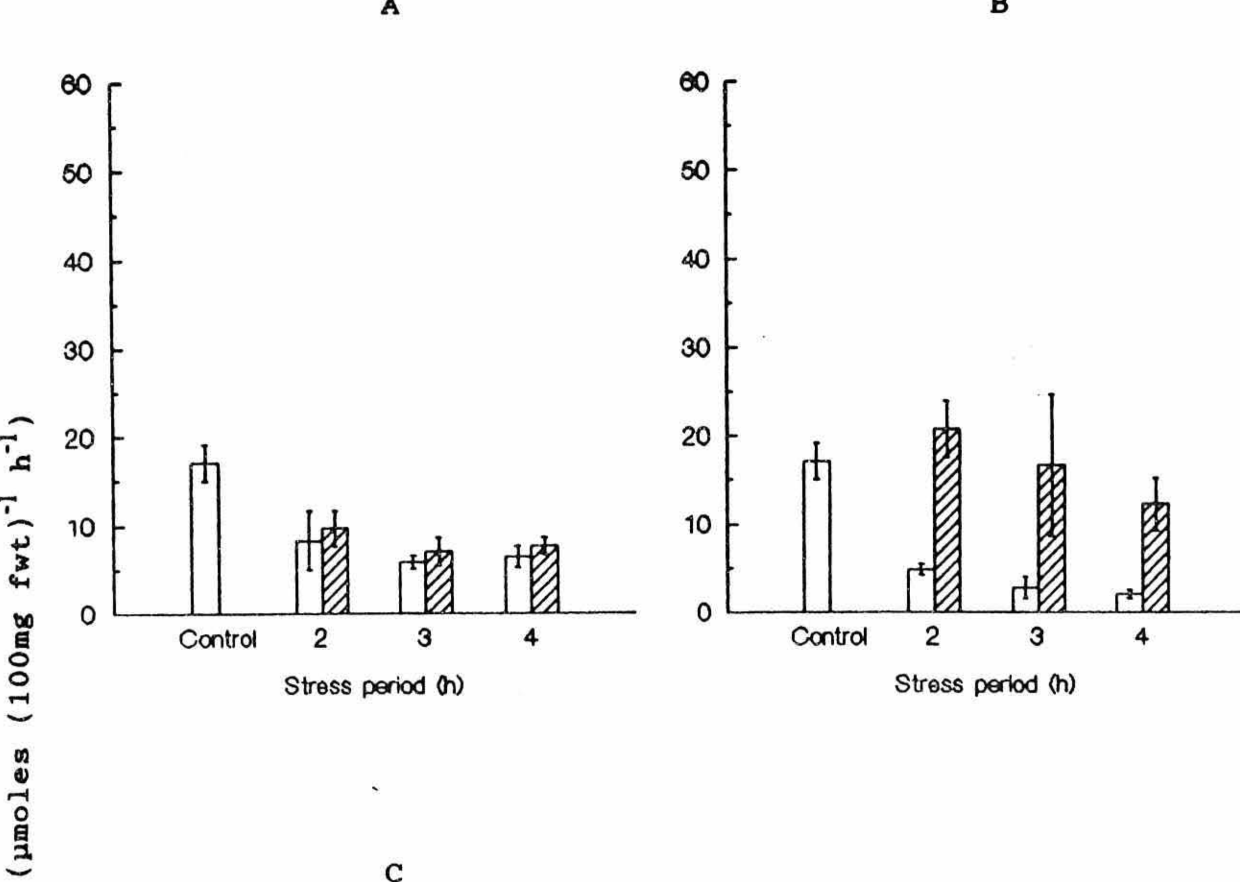


Figure 7.8 Effect of root heat stress on nitrogen fixation in cv. Mago-80. A, 35; B, 40; C, 45°C; ▭ , at the end of heat stress period; ▨ , following recovery for 72h.



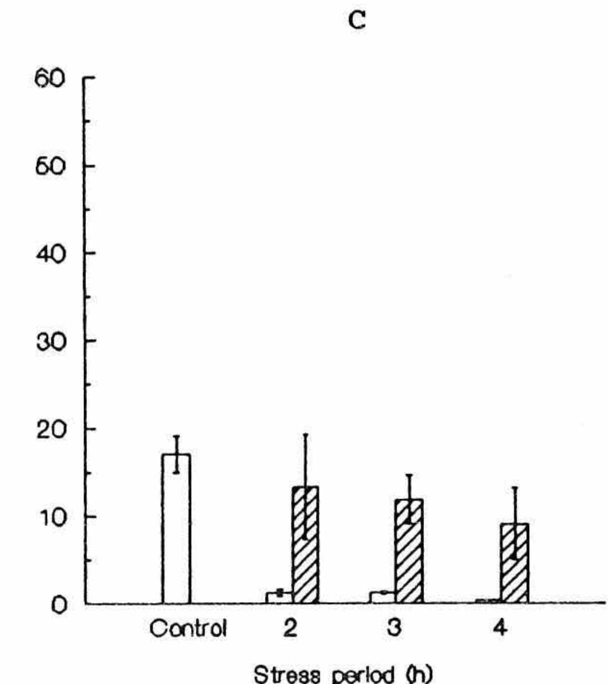
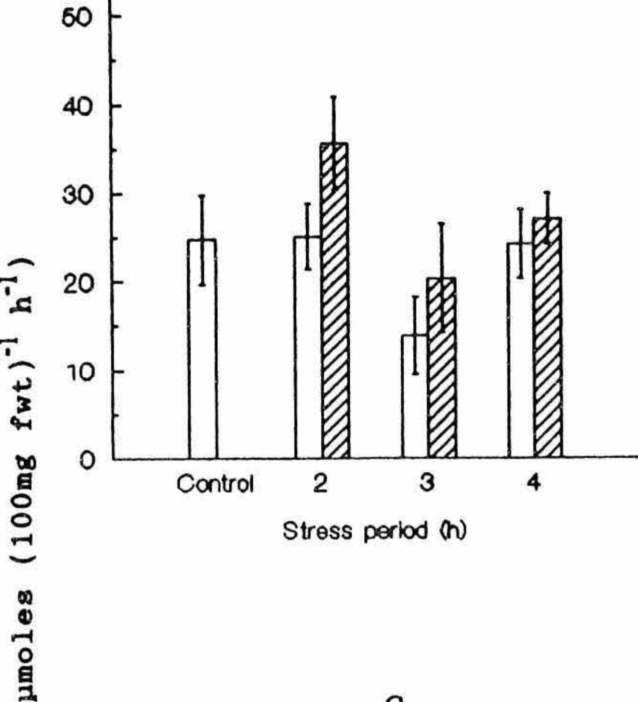


Figure 7.9 Effect of root heat stress on nitrogen fixation in cv. Bragg. A, 35; B, 40; C, 45°C; [open bar], at the end of heat stress period; [hatched bar], following recovery for 72h.



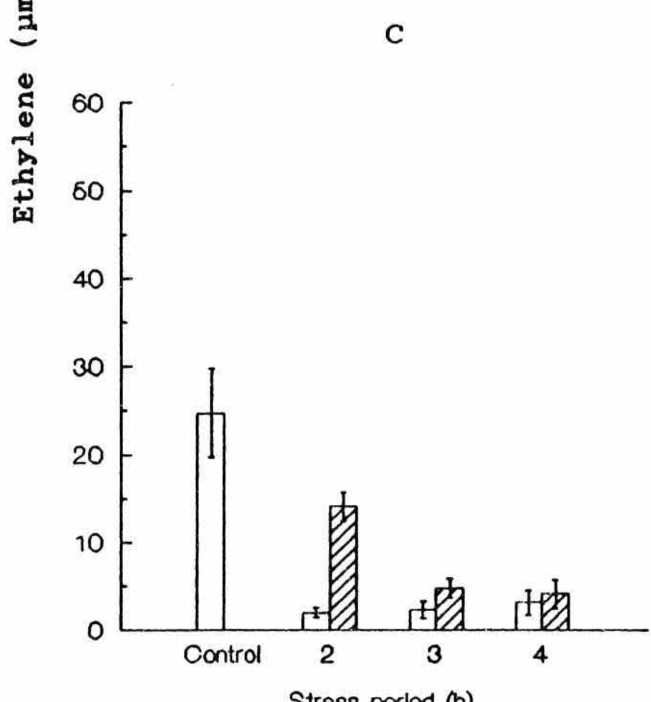


Figure 7.10 Effect of root heat stress on nitrogen fixation in cv. Davis. A, 35; B, 40; C, 45°C; ▭, at the end of heat stress period; ▨, following recovery for 72h.

Figure 7.11 Effect of leaf heat stress on nitrogen fixation in cv. Mago-80. A, 35; B, 40; C, 45°C; □, at the end of heat stress period; ▨, following recovery for 72h.

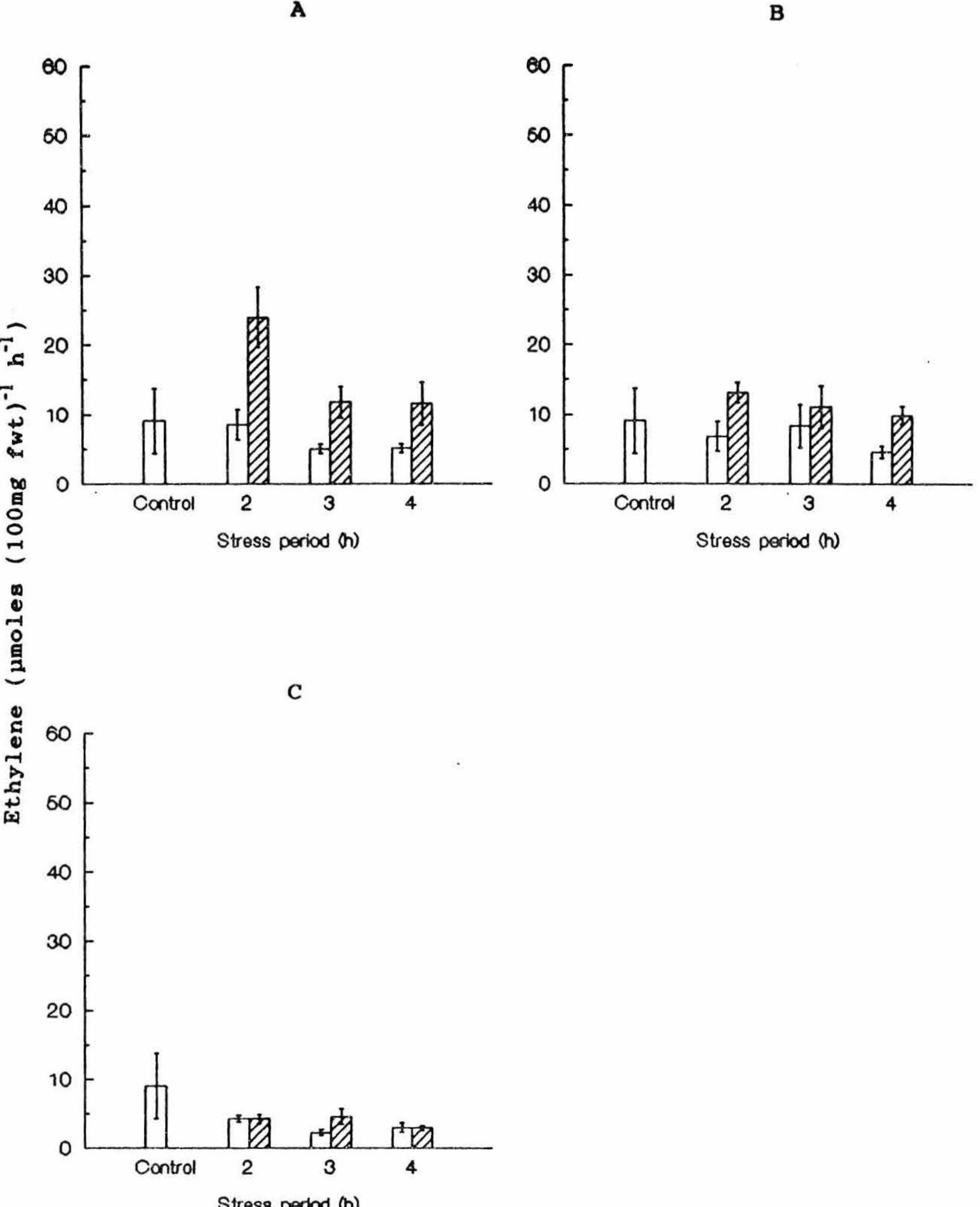


Figure 7.12 Effect of leaf heat stress on nitrogen fixation in cv. Bragg. A, 35; B, 40; C, 45°C; [open bar], at the end of heat stress period; [hatched bar], following recovery for 72h.

CHAPTER EIGHT

Chapter Eight

General Discussion.

The speed of germination of soybean seeds has been shown to depend on the length of the pre-soaking period, the germination environment (filter-paper roll or Petri dish), temperature and the soybean variety. Germination is therefore a complex process that can be affected by a variety of different factors. In general, all of the cultivars studied here germinated best at 25 to 35°C and germination declined at 42.5°C with no germination occurring at 45°C. Pre-germinating the seeds for 24h at 25°C prior to heat stress treatments allowed for better germination and root growth at high temperatures. It can therefore be concluded that pre-germination at lower temperatures allows heat-sensitive events to occur during the first 24h that would be inhibited if the early germination occurred at the higher temperatures.

Germination of cultivars Bossier, Steele 5/1 and Century-84 was severely affected with increasing soaking periods, but fast germination occurred in Bragg. This was possibly due to the seeds Bossier, Steele 5/1 and Century-84 becoming over-flooded and anaerobic. Orphanos and Heydecker (1968) reported that, in *Phaseolus vulgaris* seeds, changes occur as the seed was soaked, eventually causing the cotyledons to become flooded with excess water, so reducing the supply of oxygen to the tissues of the embryonic axis. Differences

between species and cultivars are to be expected in this respect. Simon (1984) found that broad bean seeds germinated more rapidly when they were soaked for 72h, while pea, sunflower and some other bean seeds germinated less rapidly if they were soaked for longer than 24h. Differences between seed lots within the same cultivar are also to be expected, since vigour and viability will be affected by seed harvesting and storage conditions.

Zero germination occurred in all of the soybean cultivars at 45°C, presumably due to heat damage to the tissues. On the other hand, better germination at 45°C was observed with pre-germination for 24h at 25°C. A possible reason for this is adaptation during germination at optimal temperature allowing membrane development to take place so that seedlings became more heat tolerant. Changes in temperature may also alter the duration of the cell cycle and consequently the growth of plant organs. Francis and Barlow (1988) have suggested that the cycle is prolonged at temperatures both above and below the optimal growth temperature. Thus, at extreme temperatures, the tight coupling which exists between nuclear division and cell growth may be dissociated. Similar results to the present ones for soybean have been reported for other crop species. Ashraf *et al.* (1994), for example, reported that cotton cultivars MNH-93 and B-557 had relatively higher germination at 40°C than CIM-70, NIAB-78 and S-12, but none germinated at 50°C. Nielsen and Humphries (1966) and Cooper

(1973) have also reported that not only do species differ in their response to temperature but cultivars within species may also differ.

The morphological differences between two cultivars, Bragg and Century-84, were investigated when grown under greenhouse conditions with a range of different growth media and at different temperatures. Cultivar Bragg was generally better with respect to germination and shoot growth, and it also produced more branches and leaves at 25°C than cultivar Century-84 did. It is concluded that the cultivar Bragg is probably a better variety to grow in the field than cultivar Century-84.

Chlorophyll fluorescence analysis was shown to be a valuable quantitative technique to assess the heat tolerance of the leaves of the different soybean cultivars used in this study. This could be measured as a decrease in the Fv/Fm ratio immediately after heat stress and during subsequent recovery of the plants at ambient temperatures. In general, the Fv/Fm ratio in all cultivars decreased by approximately 50 to 70% at 42.5°C and it was difficult to rank cultivars on this basis. One of the main differences between cultivars, however, was their ability to recover after heat stress. When they were returned for 72h to the ambient growth temperature following heat stress, Williams-82 and Sable appeared to recover fully, but no recovery occurred in the other cultivars. Heat stress of 45°C was

extremely damaging to all cultivars and there was no recovery of their Fv/Fm ratios when they were returned from this to lower temperatures. There were no significant differences between the leaves from nodulated and non-nodulated plants in these experiments.

Chlorophyll fluorescence was also used to try to detect differences in the abilities of leaves to acclimate to high temperatures (heat harden). Very small difference were found between non-acclimated and heat-hardened leaves when they were heat stressed at 40 or 42.5°C. Some differences were found, however, in heat-hardened plants which enabled them to survive better at temperatures of 45 and 47.5°C. More research needs to be done on the effectiveness of the temperature and the duration of different heat-hardening treatments, especially as they relate to field conditions. For Pakistan, it is important to survey a wider range of soybean cultivars for heat tolerance and acclimation potential, because the best cultivars may not yet have been found. Emphasis on the use of the chlorophyll fluorescence method in this survey will be important because of its convenience.

The results obtained in this study show that changes in chlorophyll fluorescence and in visual symptoms due to heat injury indicate that chlorophyll fluorescence is a particularly useful technique, especially as it can detect damage before any visible symptoms occur. Several

investigations have shown that the reactions of the plant thylakoid membranes are among the first process to be damaged by heat in several species (Mukohota *et al.*, 1973; Smillie, 1979). Heat stress adversely damages the water-splitting PS II activity and ATP formation in spinach leaves (Santarius and Muller, 1979). Also, Clarke and Critchley (1994) reported that, in sorghum leaves at high temperature, a dramatic decrease occurred overall in photosynthetic efficiency. In the present study the degree of leaf browning was almost equal in all cultivars after heat stress at high temperatures. Zavadkaya and Intropova (1979) found similar brown spots in two species, *Commelina africana* and *Danae recemose*, after heating them at 42 and 53°C. They speculated that this might be due to denaturation of some enzymes in the chloroplast complex, but oxidation of phenolic substances following loss of cellular integrity is a more likely explanation.

Members of the genera *Rhizobium* and *B.rhizobium* are symbiotic nitrogen-fixing bacteria which are able to invade and form nodules on the roots of leguminous plants. *B.rhizobium japonicum* strain RCR3407 showed mixed characteristics of both fast and slow growing rhizobia (Elsheikh and Wood, 1989). Experiments on the effects of temperature on the growth of strain RCR3407 in pure culture showed that they grew fast at between 25 and 35°C and much more slowly at 45°C. Heat-hardening treatments were shown to be effective in permitting better growth at high

temperatures. These experiments indicate that, in the natural environment of the soil, heat acclimation of the *B.rhizobium japonicum* may take place which would allow nitrogen fixation in soybean at high temperatures. Furthermore, it may be possible to select other strains of *B.rhizobium japonicum* which show better heat acclimation potential for use as inoculants in the field. A better understanding of the factors affecting the growth responses of rhizobia may assist in this selection. Such a possibility is supported by results from other workers. Rajakumar and Lakshmanan (1995) found that *Azotobacter chroococcum* strains 8005 and BKMB 1030 grew better at 20°C, while the tropical strain B and MKU 109 grew better at 40°C. Also, Kishinevsky *et at.* (1992) reported that strain 32HI of ground nut *B.rhizobium* was more sensitive to high temperature than strains 280A and 2209A in laboratory culture. With respect to future work on soybean in Pakistan, it will be important to find out if the roots becomes infected with rhizobia and whether they form active nodules in Pakistan soils. Pakistan soils may not have the relevant bacteria. This will be an interesting area in which to continue research involving different types of soils in the field as well as in laboratory experiments.

Further experiments were conducted to evaluate the effects of high temperature applied to either the leaves or the roots of soybean on the rate of nitrogen fixation by the root nodules. When high temperatures were applied to the

roots, nitrogen fixation was unaffected up to 35°C and only small decreases were recorded at 40°C in all cultivars. Fixation was severely inhibited at 45°C in all cultivars, especially in Williams-82. High temperatures applied to the shoots produced less rapid decreases in the nitrogen fixation rate than heat stress applied to the roots. Little or no recovery from either shoot or root heat stress occurred after heat stress at 45°C. After heat stress applied to the leaves at 35 or 40°C, however, both cultivars Mago-80 and Bragg were able to show good recovery. This was especially true in the case of cultivar Mago-80. Heat stress applied to the leaves should be an interesting future field of study, to assess a wide range of soybean cultivars the in the summer conditions of Pakistan.

Nitrogen fixation is dependent upon a number of factors which must be supplied either by the host plant or by the soil. The results from this study indicate that nitrogen fixation is sensitive to high temperatures. As mentioned before, oxygen is essential for symbiotic nitrogen fixation, because it is needed for the oxidative phosphorylation of respiratory substrates which supply required ATP. Leghaemoglobin presents this oxygen at the respiratory sites without inhibiting the oxygen-sensitive nitrogenase enzyme system (Burn, 1978). The fact that nitrogen fixation in soybean is severely inhibited at high temperatures has been reported by several investigators.

Whigham and Minor (1978) reported that nodulation and nitrogen fixation in soybean was greatly affected by soil temperature. The optimum temperature for both nodulation and nitrogen fixation is 27°C (Dart *et al.*, 1975). Nambiar and Dart (1983) reported that, compared to 25°C, temperatures of 30 and 35°C reduce nitrogenase activity in peanut root nodules. Suboptimal root temperatures also generally decrease soybean nitrogenase activity.

In the present study, the results reveal a very limited variability among the cultivars with respect to the effects of temperature on germination, root growth, chlorophyll fluorescence and nitrogen fixation. Although some cultivar differences were found with respect to chlorophyll fluorescence and nitrogen fixation activity, the differences were relatively small and not always statistically significant. Significant differences were observed, however, in germination following pre-soaking and pre-germination. Cultivar Bragg gave the best germination with soaking periods up to 72h, while Bossier and Steele 5/1 did not germinate with increasing soaking periods.

As already stated above, research should continue on the different performances of soybean cultivars in the field as well as in the laboratory. In Pakistan, summer temperatures generally reach 45-50°C. Under these conditions crops have difficulties in surviving. Among the summer crops, soybean will have difficulty in maintaining metabolic balance and

consequently growth. This future work should aim to select heat-tolerant soybean cultivars with respect to chlorophyll fluorescence and nitrogen fixation capacity. Locally grown and national cultivars as well as imported cultivars should be tested. Also, local *Rhizobium* inoculants, if they exist, should be tested on the different cultivars. In addition, the soybean crop sometimes has to face monsoon floods. Some experiments should be, therefore, carried out to determined nitrogen fixation capacity under flood conditions.

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