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The environmental basis of the resistance of wheat (Triticum aestivum L. cv. Brigadier) to Stagonospora nodorum Berk

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The environmental basis of the resistance of wheat (*Triticum aestivum* L. cv. Brigadier) to *Stagonospora nodorum* Berk.

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Summary

The primary aim of the thesis was to identify mechanisms by which the nutritional status or environmental conditions of wheat influence horizontal resistance to *S. nodorum,* and to answer the question: how do changes to the crop's environment affect the nutrients available to fungal pathogens? The broader aim of the thesis was to find ways to maximise the expression of horizontal resistance, or to allow plant breeders to select for traits that improve horizontal resistance, which may result in a reduction in fungicide requirements. I postulated the hypothesis that resistance of wheat to *Stagonospora nodorum* depends on both nitrogen and carbon supply.

The hypothesis was tested by carrying out experiments under controlled conditions whereby the carbon and nitrogen supply to wheat seedlings (*Triticum aestivum* L. cv Brigadier) was manipulated. C supply was altered by shading, and N supply was altered by growing plants in hydroponics and adjusting the N form and concentration of the nutrient solution. The growth and reproduction of the fungus in response to forms of N *in vitro* was also investigated. An attempt was made to identify the physiological reasons for reduction of disease.

Susceptibility of wheat to *S. nodorum* was increased by supplying moderate and high concentrations of N compared to low N. Susceptibility was also increased when N was supplied as ammonium (NH_4^+) compared to nitrate (NO_3^-). Leaf physiology was affected by N supply, and of particular interest was the increased concentration of total amino acids, proteins and high amounts of asparagine in plants supplied with NH_4^+. Pycnidial production by *S. nodorum in vitro* was greatest when N was supplied as asparagine. In shading experiments, disease was generally reduced when light intensity was lowered between 2 and 4 days after inoculation (dai).

I concluded that the nutritional status of wheat plants is an important factor in modifying resistance to *S. nodorum*, and it is perhaps this, rather than the presence of defence compounds, that determines the success of the pathogen *in planta.*

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Abbreviations

ANOVA	- analysis of variance
AS	- asparagine synthetase
asn	- asparagine
asp	- aspartate
CZD	- Czapek-Dox agar
CZD-V8	- Czapek-Dox agar supplemented with Campbell's V8 juice
d	- days
dai	- days after inoculation
dH_2O	- distilled water
DW	- dry weight
gln	- glutamine
glu	- glutamate
gly	- glycine
GS	- glutamine synthetase
GOGAT	- glutamate synthase
h	- hours
HPLC	- high performance liquid chromatography
LSD	- least significant difference
MW	- molecular weight
NH_4^+	- ammonium
NO_3^-	- nitrate
NR	- nitrate reductase
p	- probability
r	- coefficient of correlation
r^2	- coefficient of determination
RGR	- relative growth rate
RH	- relative humidity
S	- seconds
SE	- standard error
ser	- serine
SLA	- specific leaf area (leaf area divided by leaf dry weight)
S: R	- shoot to root ratio

thr	- threonine
WUE	- water use efficiency (ratio of net photosynthesis to transpiration)

Contents

Chapter 1 General introduction

Disease resistance

Resistance or susceptibility to disease occurs at a range of levels, and is the result of complex interactions between host, pathogen and the environment. Resistance is ultimately under genetic control, and may involve one or a few major genes, or several minor genes (Parry, 1990). Resistance can involve structural and chemical barriers, and can be passive (non-inducible), including cuticles, cell walls and phenolic compounds; or active and inducible mechanisms, such as deposition of callose, lignification and production of phytoalexins (Parry, 1990). A resistant plant may exhibit a range of symptoms according to the degree of resistance possessed. Where there is complete susceptibility the pathogen will be able to successfully cause infection and carry out its life cycle; with increasing resistance there are fewer successful penetrations, fewer lesions and perhaps inhibition of sporulation (Huber and Watson, 1974). Resistance of the pathogen to fungicides and the rapid mutation rates producing new races suggest that an integrated approach to pathogen control is required and nutritional or environmental resistance mechanisms may form part of this management. Examples of the effects of altered carbon and nitrogen supply on environmental resistance are given below.

Environmental resistance

Environmental resistance offers a partial level of resistance to all races of a particular pathogen. It is governed by a number of genes and is also called horizontal, partial or durable resistance. Environmental resistance is governed by many genes that each play a minor role in the overall resistance of a plant to a pathogen (Talukder *et al.,* 2004). Expression of partial resistance results in inhibition of pathogen growth and reproduction, but does not completely prevent infection (Talukder *et al.,* 2004). It is mediated by environmental conditions, so alteration of the crop's environment in a way that increases environmental resistance is one way to tackle disease as part of an integrated control strategy. External factors such as temperature, wind, light intensity and relative humidity (RH) will affect the life cycle of a plant pathogen, as will internal plant factors such as defence mechanisms and the availability of substrates for fungal growth (Agrios, 1997).

Expression of resistance

Resistance of wheat to pathogens may be expressed as increased incubation and latent periods, reduced infection frequency, reduced lesion numbers, reduced lesion size and rate of growth, reduced area of infected leaf and reduced sporulation. These are termed 'components of partial resistance' (Jeger *et al.,* 1983). In studies investigating environmental resistance there are various techniques that may be employed to address the issue of how and when resistance is expressed. If partial resistance is observed under particular conditions, it may be pertinent to consider the stage of infection that resistance is expressed. The techniques below have been used to assess plant-pathogen interactions and aspects of environmental resistance.

Histological techniques

Determining at which stage of infection resistance is expressed may be possible using microscopy. Bird and Ride (1981) used light microscopy and transmission electron microscopy (TEM) to investigate the resistance of wheat to *Stagonospora nodorum* Berk., in relation to host lignification. Resistance had little influence on the pre-penetration growth of *S. nodorum*, in terms of germination, number of germ tubes per conidium and number of appressoria per germ tube, but the growth of germ tubes was slower on resistant varieties (Bird and Ride, 1981). Resistance mechanisms such as papillae formation occur after the hypha has penetrated the cuticle. Papillae (lignified deposits in the host cell wall) were found in wheat leaves where penetration attempts by *S. nodorum* failed. The use of fluorescence microscopy, histochemical tests and enzymic maceration tests provided information about the lignification of cells (Bird and Ride, 1981). After a failed penetration attempt the germ tube can continue to grow past the appressorium to try again. Reaction sites are formed at sites of failed penetration - these consist of a papilla in the cell wall beneath the penetration site, and a 'halo' caused by alteration of the cell wall around the penetration site and these reaction sites autofluoresce. Papilla formation occurs in response to either physical damage to the cuticle or a chemical stimulus from the fungus (Jørgensen and Smedegaard-Petersen, 1999).

Partial or complete lignification of epidermal and mesophyll cells occurs after infection in both resistant and susceptible varieties, which (it has been suggested) slows but does not completely stop fungal growth (Bird and Ride, 1981). The extent of lignification tended to be more closely related to the extent of fungal colonisation rather than the degree of resistance (Bird and Ride, 1981). Light and fluorescence microscopy were used to investigate resistance of wheat to *Septoria tritici* (Rob in Desm.) (Cohen and Eyal, 1993). As with *S. nodorum* (Bird and Ride, 1981), the germination and appressorium formation of *S. tritici* was no different on resistant and susceptible cultivars. Mature pycnidia never formed in the resistant cultivars, but arrested immature pycnidia were observed. Autofluorescence (due to lignification) was observed in resistant cultivars from 48 h after inoculation, initially in tissues around the infection sites (stomata and epidermal cell walls) then in the mesophyll cells associated with substomatal cavities which is the site of pycnidial formation. Little autofluorescence was found in susceptible cultivars (Cohen and Eyal, 1993).

Radioactive labelling techniques

Pathogens alter the C metabolism of plants and may cause accumulation of soluble or storage carbohydrates in infected leaves or regions of infected leaves and they may also reduce the rate of export of assimilates from infected leaves (Chapters 2 and 5). Pulse-chase experiments using $^{14}CO_2$ and measuring the subsequent export of ^{14}C from fed leaves may show differences between heavily infected, less infected and healthy plants. Analysis of the carbohydrate profiles of healthy and infected leaves may also provide insight into the C status of leaves prior to infection, and how the pathogen alters C metabolism. Autoradiography or phosphor-imaging of infected leaves fed $^{14}CO_2$ for a short period of time may well show accumulation of the ^{14}C in fungal colonies. To study Nitrogen (N) metabolism in infected leaves, N compounds labelled with ^{14}C or ^{15}N can be supplied to the plant and accumulation of label in plants can be visualised and / or quantified using phosphor-imaging, autoradiography or scintillation counting (^{14}C) or by mass spectrometry (^{15}N).

N metabolism could also be studied by using ^{14}C-labelled amino acids. This may be useful for assessing the preferred N source of the fungus and differences in uptake of amino acids between healthy and diseased leaves. *Stagonospora nodorum* does not alter the plant's uptake of N, but has been reported to cause a significant change in the host's amino acid metabolism with increased concentrations of glutamic acid and arginine in infected leaves (Verreet and Hoffmann, 1990). The effect of infection on N metabolism (and vice versa) is discussed in Chapter 3, and further in Chapter 4.

Assessment of fungal biomass

Assays for ergosterol and / or chitin using high performance liquid chromatography (HPLC) may be useful measures of resistance as a way of quantifying fungal biomass within leaf tissue. Chitin, a polymer of *N*-acetyl-D-glucosamine, is a cell wall component of fungi and it is the monomer that is analysed during quantification of chitin by HPLC. Ergosterol is only found in fungi and has been used to assess resistance in plants and as a measure of fungal biomass. There have been many methods published for these assays and the extraction techniques and the success of the techniques vary widely (Ekblad *et al.,* 1998; Ekblad and Nashölm, 1996; Whipps *et al.,* 1980; Mayama *et al.,* 1975; Gunnarsson *et al.,* 1996; Seitz *et al.,* 1979; Tothill *et al.,* 1992; Griffiths and Jones, 1985). A critical assessment of the validity of ergosterol as an indicator of fungal biomass (Bermingham *et al.,* 1995) assessed fungal biomass in nine species of aquatic hyphomycetes, of which only three showed a significant relationship between mycelial ergosterol and fungal biomass. The authors concluded that the ergosterol assay is a poor measure of fungal biomass. Ergosterol is believed to be correlated with metabolically active fungal biomass, thus measuring chitin and ergosterol contents of infected tissues and calculating the chitin: ergosterol ratios gives an approximate measure of total and living biomass. This method was adopted by Ekblad *et al.* (1998) to measure the fungal biomass of ectomycorrhizae in the roots of Scots pine (*Pinus sylvestris* L.) and Grey alder (*Alnus incana* (L) Moench). Ageing of the mycelium reduced both chitin and ergosterol, but was more marked for ergosterol. The ratio of chitin: ergosterol was higher in 7-month than 4 month old root tips. There was a positive correlation between chitin and mycorrhizal score (Ekblad *et al.,* 1998)

Ergosterol was used as a bioassay for predicting resistance of wheat leaves to *S. nodorum* (Griffiths and Jones, 1985). Ergosterol was extracted from segments of infected wheat leaves and saponified prior to analysis by HPLC, thin layer chromatography (TLC) and gas-liquid chromatography (GC). No ergosterol was found in healthy tissue, but in disease leaves ergosterol content increased with increasing disease severity, which led the authors to conclude that the ergosterol assay was useful for evaluating resistance of wheat to *S. nodorum* and perhaps other pathogens (Griffiths and Jones, 1980).

Resistance compounds such as polyphenols or phenylpropanoids can be measured as biochemical markers of resistance. The commonest phenolics found in wheat are the hydroxycinnamic acids (HCAs) ferulic acid and p-coumaric acid (Sander and Heitefuss, 1998; Mckeehen *et al.,* 1999), and tyrosine and caffeic acid have also been found in wheat (Gogoi *et al.,* 2001). These substances have been positively correlated with disease resistance (Sander and Heitefuss, 1998, Mckeehen *et al.,* 1999, Moerschbacher *et al.,* 1989; Kofalvi and Nassuth, 1995), but may only accumulate in small amounts in response to infection. Phenols accumulate at infection sites and restrict, but do not completely halt, the growth of the fungus (Gogoi *et al.,* 2001).

Other defence responses may include ion fluxes, oxidative reactions, hypersensitive cell death, production of phytoalexins, chitinases etc (Nicholson and Hammerschmidt, 1992). In recent years there has been research into molecular techniques for pre-symptomatic diagnosis of disease and for quantification of disease. Two important methods are enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR). This technology requires DNA primers to be identified – regions of DNA specific to the fungus that can be used as a genetic marker in the detection of the pathogen. Primers for *Septoria spp.* have been based on polymorphic sequences of internal transcribed spacer (ITS) regions of ribosomal DNA (Hollomon *et al.,* 1999). PCR amplifies the fungal DNA fragments, which are then analysed using agarose gel electrophoresis and quantified using ELISA or a fluorometric microtitre plate-formatted PicoGreen assay. PicoGreen is a cyanide dye, which fluoresces on binding to specific DNA fragments (Fraaije *et al.,* 1999). A PCR assay using the β tubulin gene as a target has been developed for *S. tritici*

(Fraaije *et al.*, 1999). The researchers amplified a 496 bp DNA fragment using PCR, then used the fluorometric microtitre plate-formatted PicoGreen assay to rapidly quantify the PCR products. The assay was sensitive enough to detect 10 pg of fungal DNA from 200 ng of wheat leaf DNA (Fraaije *et al.*, 1999). The primers used in PCR must be highly specific, otherwise there is the risk of obtaining 'false-positive' results due to detection of the wrong DNA. Clearly more research is needed on these methods in order to have accurate information about disease severity. This information will also aid the study of host-pathogen relationships.

Plant C and N metabolism

The role of N in agriculture

N supply is the main non-climatic factor controlling crop growth and yield of winter cereals in northern Europe (Olesen *et al.*, 2002). Annual inputs of N into agricultural soils are huge, and growing, with 742,000 tonnes of inorganic N applied to UK soils (FMA, 1998). Of the N applied to wheat (*Triticum aestivum* L.) crops a maximum of 80 % is recovered by the crop. The remainder may be lost in gaseous form due to denitrification and nitrification, and by leaching (King *et al.*, 2001). It was concluded that the un-recovered N was temporarily immobilised during the rapid period of crop growth (King *et al.*, 2001). Application of N Fertilisers improve yields of wheat by up to 50 %; yields achieved by fertilized crops average 7 to 8 t ha^{-1}, but in experimental field trials on soil left unfertilized yields have been less than half this (Sylvester-Bradley *et al.*, 2001).

N availability in soils

N is the mineral nutrient most commonly limiting to plant growth (Grindlay, 1997). N deficiency results in reduced growth and can seriously damage crop yield, whilst excessive application of N can depress yield (Marschner, 1995). High concentrations of N can be directly toxic or can induce deficiency of other nutrients (Marschner, 1995).

Most N is taken up as nitrate (NO_3^-) rather than ammonium (NH_4^+) (Takei *et al.*, 2002), as NH_4^+ is readily oxidised in the soil to NO_3^- during the process of nitrification, which is rapid in well-aerated soils (Crawford and Forde, 2002). The availability of NO_3^- in

agricultural soils is far greater than the availability of NH_4^+. The range of NO_3^- in soil can be 0.5 to 10 mM, but the concentration of NH_4^+ in soils tends to be less than millimolar under average conditions (von Wiren *et al.,* 1997). The uptake of NO_3^- and NH_4^+ is partly dependent on temperature, as in cold soils more ammonification than nitrification takes place, making more NH_4^+ and less NO_3^- available (von Wiren *et al.,* 1997). There is evidence that where agricultural soils receive organic N-amendments such as manure, more NH_4^+ is available (Sørensen, 2001).

Uptake and assimilation of NO_3^- and NH_4^+

NO_3^- is taken up by roots by active transport and can be assimilated, transported and stored as NO_3^-, or reduced via nitrate reductase (NR) to nitrite (NO_2^-) then to NH_4^+ via NO_2^- reductase (NiR) prior to assimilation into organic N compounds (Crawford and Forde, 2002). NH_4^+ however can be toxic to plants, particularly when it is the sole N source (Britto *et al.,* 2001; Britto and Kronzucker, 2002), NH_4^+ is assimilated via the glutamine synthetase / glutamate synthase (GS / GOGAT) pathway in the roots before transport and storage (Frechilla *et al.,* 2002). GOGAT catalyses the transfer of the amide group from glutamate to oxoglutarate to yield glutamate, using a reductant (mainly reduced ferredoxin, but sometimes NADPH), and GS combines glutamate with NH_4^+ using adenosine triphosphate (ATP) to generate glutamine, adenosine diphosphate (ADP) and Pi. NH_4^+ is generated within plant tissues during the reduction of NO_3^-, photorespiration, phenylpropanoid metabolism, and amide and protein catabolism (Joy, 1988).

Recent evidence suggests that NH_4^+ may not always be immediately assimilated in roots prior to translocation, but NH_4^+ may be translocated from root to shoot in the xylem (Schjoerring *et al.,* 2002). NH_4^+ has been found in the xylem sap, apoplast and leaf tissue of tomato (*Lycopersicon esculentum* L.) and oilseed rape (*Brassica napus* L.) grown both on NO_3^- and NH_4^+ (Husted *et al.,* 2000). In oilseed rape grown on NO_3^- then switched to a nutrient solution containing NH_4^+ there was a linear increase in the concentration of NH_4^+ in the xylem in relation to external NH_4^+ concentration and time of exposure (Husted *et al.,* 2000). A similar experiment with ryegrass (*Lolium perenne* L.) revealed a

tripling of apoplastic NH_4^+ concentration accompanied by a decrease in apoplastic pH, within 3 h of switching the plants from nutrient solutions containing NO_3^- to those containing NH_4^+ (Schjoerring *et al.*, 2002). Both high and low affinity NH_4^+ transporters have been identified in leaves (Schjoerring *et al.*, 2002). NO_3^- can be assimilated in the roots or shoots, and the predominant site of assimilation depends on the NO_3^- external concentration (Andrews *et al.*, 2001). There are two high affinity transport systems (HATS) for NO_3^- in the roots of higher plants (Glass *et al.*, 2002), and a low affinity transport system (LATS) (von Wiren *et al.*, 1997). Uptake of NO_3^- is increased when NO_3^- is supplied after a period of N deficiency (Crawford and Forde, 2002; von Wiren *et al.*, 1997).

NO_3^- uptake can be depressed by the presence of NO_3^-, NH_4^+ and amino acids (von Wiren *et al.*, 1997). NO_3^- is an important signal molecule, both for the regulation of NO_3^- uptake and for the expression of genes involved in NO_3^- metabolism (Stitt, 1999; Takei *et al.*, 2002). Genes that are specifically NO_3^--responsive include those for NO_3^- uptake and reduction, NH_4^+ assimilation, supply of redox equivalent, cofactor biosynthesis, supply of C skeletons for N assimilation, and root architecture. Other genes that are broadly responsive to N are those involved in amino acid metabolism, storage protein, photosynthesis, cell cycle, transcription / translation machinery and signal transduction (Takei *et al.*, 2002).

Plants, like fungi, have amino acid transporters or permeases for transporting amino acids into cells. These are classified as neutral and acidic amino acid transporters or general amino acid transporters, and within these groups the different transporters may have differing affinities for different types of amino acids, e.g. hydrophobic amino acids. Plants tend to use general amino acid transporters (Fischer *et al.*, 1998). The amino acid concentration and composition of plant tissues is affected by the form of N supplied. In wheat plants, supplying NH_4^+ to the roots results in an accumulation of asparagine and glutamine (Ikeda *et al.*, 2004; and see further detail and references in Chapter 3). Maize plants were fed ^{14}C-labelled $NaHCO_3$ and supplied N as NH_4^+ or NO_3^-. The incorporation of ^{14}C into amino acids (particularly asparagine) was greatest in plants supplied NH_4^+,

whilst plants supplied NO_3^- incorporated more ^{14}C into organic acids (Cramer *et al.*, 1993). Incorporation of ^{14}C into amino acids was found to be at the expense of allocation to carbohydrates in the roots and shoots of wheat supplied NH_4^+ compared to NO_3^- (Cramer and Lewis, 1993*b*).

Relationship between N and C metabolism

There is a very close relationship between N and C metabolism in plants (Coruzzi and Bush, 2001). Both N and C regulate genes associated with their own and each other's metabolism (Takei *et al.*, 2002, Coruzzi and Bush, 2001). For example, NO_3^- regulates (among others) genes for NO_3^- uptake and reduction, NH_4^+ assimilation and supply of C skeletons for N assimilation (see Takei *et al.*, 2002 for references), and C metabolites regulate genes involved in N acquisition and metabolism and photosynthesis (Coruzzi and Bush, 2001). There is a positive correlation between N supply and photosynthesis (Gastal and Lemaire, 2002; Lawlor *et al.*, 1989) and between leaf N content and net photosynthesis under given environmental conditions (Grindlay, 1997). Up to 75 % of organic N in green leaf cells is located in chloroplasts, as enzymes and as structural proteins (Marschner, 1995), and up to 30 % of the N and 50 % of the soluble protein in wheat leaves can be attributed to Rubisco (Lawlor *et al.*, 1989). Correspondingly, photosynthesis provides C skeletons and reductant for N uptake and assimilation into amino acids (Foyer *et al.*, 2003; Lawlor, 2002) and light and carbohydrates (as well as NO_3^- and glutamine) govern NO_3^- reductase activity (Foyer *et al.*, 2003).

Fungal requirements for C and N

Fungal requirements for C

Biotrophic and necrotrophic pathogens have differing modes of nutrition. Biotrophs colonise plant tissues intercellularly, only penetrating cell walls with haustoria, which are specialised hyphae that absorb nutrients from the plant cell (Isaac, 1992). Necrotrophs grow intracellularly, degrading cell walls by means of cell-wall degrading enzymes and mobile toxins, before absorbing the breakdown products. A biotroph can be thought of as a sink in its requirement for C. In the mesophyll cells of cereals only one fifth of sugar is

stored in the cytosol where the haustoria are, with the rest being stored in the vacuole, and the nutrient pool available to the fungus is insufficient for it to maintain growth (Farrar, 1995). Although cellular C pools are small, the fluxes through and around infected cells are high, and the fungus may acquire sugars from passive leakage or active unloading from the symplast (Farrar, 1995). Fluorescence microscopy has shown that in barley (*Hordeum vulgare* L) infected with brown rust *(Puccinia hordei* Otth.), although there are no fungal haustoria within vascular tissue, colonies can be located on the veins (Kneale and Farrar, 1985). This has been confirmed by feeding ^{14}C to leaf tips and following its path, which showed that immediately after feeding, the ^{14}C was found in the veins and two hours after feeding the pustules had accumulated much of the ^{14}C. This confirms that the fungus can unload sugars from the phloem (Farrar, personal communication).

Fungal requirements for N

The general N nutrition of fungi is discussed in Chapter 4, but here more detail about the uptake of amino acids by fungi is presented.

Several fungal amino acid transporters have been identified in recent years (Wipf *et al.,* 2002). These transport a broad range of amino acids and have overlapping specificities (Struck *et al.,* 2004). An amino acid permease was identified for the rust fungus *Uromyces fabae* which preferentially transports leucine, methionine and cysteine, and these were found in very low concentrations in the apoplast, suggesting that the transporter has high affinity for amino acids that are scarce in plant tissues. In contrast, asparagine was found at high concentrations in the apoplast, and transport activity for asparagine was low (investigated by injecting the cRNA of the transporter into *Xenopus* oocytes and measuring the depolarization of the plasma membrane potential using amino acids as substrates). The transporter may function in a proton-dependent manner, as pH dependent changes in oocyte membrane potential were revealed in response to various amino acids (Struck *et al.,* 2004). Yeast has been used as a model for the investigation of amino acid transporters. The amino acid transporters characterized from yeast belong to the APC ('amino acid-polyamine-choline facilitator') superfamily, which is subdivided further into families, which include Gap1 ('general amino acid permease')-related amino

acid transporters and more specific permeases, all similar to permeases found in bacteria and other fungi (Fischer *et al.,* 1998). Leucine transporters in yeast are pH-sensitive and the efficiency of the HATS is reduced with rising pH in the growth media, and with the presence of NH_4^+ in the growth media (Karagiannis *et al.,* 1999). NH_4^+ regulates N metabolism in fungi (Carlile and Watkinson, 1994) and may negatively regulate permeases for certain amino acids (Karagiannis *et al.,* 1999). The presence of NH_4^+ in the growth medium causes acidification of the medium (Carlile and Watkinson, 1994). In yeast, amino acid transport is regulated by the control of transcription of the genes encoding them, and many permease genes are subject to N repression, where transcription is down-regulated in the presence of preferred N source Fischer *et al.,* 1998). In *Saccharomyces cerevisiae* there is a specific permease with high-affinity for L-aspartate and L-glutamate, in addition to the general amino acid permease. Uptake of L-aspartate and L-glutamate was reduced by the presence of NH_4^+ in the growth media, suggesting N catabolite repression of the gene that encodes for the permease (Regenberg *et al.,* 1998).

Effects of altered C and N on infection

Effects of elevated CO_2 on infection

Hibberd *et al.* (1996*a,b,c,d*) investigated the effect of elevated CO_2 on infection of barley by *Erysiphe graminis*. Barley plants were grown in hydroponics at 350 μmol mol^{-1} CO_2 or 700 μmol mol^{-1} CO_2, and inoculated with *E. graminis*. Although there was no difference in conidial germination between the two CO_2 concentrations, at 700 μmol mol^{-1} CO_2 there were fewer conidia producing hyphae, so development was arrested at the appressorial stage. This was not directly attributable to elevated CO_2, as when conidia were placed on epidermal peels, elevated CO_2 did not affect hyphal production. At the sites of failed penetration, papillae had formed, and silicon had accumulated, indicating that high CO_2 concentration had increased photosynthetic C fixation and therefore the amount of assimilates available for plant resistance mechanisms. Conidia that did successfully colonise plants at elevated CO_2 produced colonies that grew faster than those at ambient CO_2, and the higher rate of photosynthesis may have caused accumulation of carbohydrates in leaves (Hibberd *et al.,* 1996*a*). Elevated CO_2 did cause accumulation of carbohydrate in the second leaves of barley plants, but the concentration of soluble

carbohydrates was reduced six days after inoculation with *E. graminis* (Hibberd *et al.*, 1996*b*). The second leaves of barley had an increased net photosynthetic rate at elevated CO_2, but the magnitude of this increase lessened with plant age. At both ambient and elevated CO_2 concentrations, infection with powdery mildew decreased net photosynthetic rate due to a reduction in carboxylation efficiency (Hibberd *et al.*, 1996*d*).

Dry matter production is reduced in plants infected by biotrophs, as in addition to normal plant metabolism photoassimilates are used in resistance pathways and to support pathogen growth. Infection at elevated CO_2 did not affect partitioning of dry matter between root and shoot, but dry mass of barley was reduced with respect to control plants by 63 % eight days after inoculation. In ambient CO_2 dry matter was only reduced by 25 % six days after inoculation (Hibberd *et al.*, 1996*c*).

Effects of light intensity on disease resistance

Van der Plank (1984) reviewed the concept of high-sugar and low-sugar disease processes. As a general rule, high sugar concentrations in leaves retard leaf senescence and inhibit the enzymatic degradation of plant tissues by pathogens. This provides partial resistance to infection by necrotrophs (Van der Plank, 1984). Reduction of leaf sugar by shading increased the susceptibility of Kentucky bluegrass (*Poa pratensis* L.) to *Helminthosporium sorokinianum* Sacc. (Van der Plank, 1984). Leaf sugar content of *P. pratensis* was also lowered by the application of both NH_4SO_4 and NH_4NO_3 (Van der Plank, 1984). For more examples of cases where infection has been increased by shading see Chapter 5.

Effects of N on infection

N nutrition plays an important role in the overall vigour of a plant, which influences its susceptibility or resistance to disease. Compounds involved in a plant's defence mechanisms may be affected by the plants nutritional status, for example the biosynthesis of phenols is impaired by high concentrations of N (Marschner, 1995). One of the consequences of infection by fungal pathogens for the plant is that the concentrations of

N and C in leaves can be altered, often with an increase in carbohydrate: N ratios (Hatcher 1995).

Both the form and concentration of N supplied to a crop can affect its susceptibility to infection. Application of high concentrations of N fertiliser tends to increase severity of disease caused by biotrophs. Barley plants were more susceptible to infection by *Erysiphe graminis* f. sp. *hordei* (a biotroph) when high concentrations of N were applied, and spore production and density were enhanced by N application (Jensen and Munk, 1997). In experiments to investigate the effect of host nutrition on yellow rust epidemics on wheat, applications of high concentrations of N fertiliser increased severity of rust infection and decreased yield (Ash and Brown, 1991, Danial and Parlevliet, 1995). Yield, in terms of total grain weight, was least affected at the highest N concentration, and although inoculation with the pathogen did not affect the N content of leaves, it did reduce the total N content of grain (Ash and Brown, 1991). The effects of N supply on infection of wheat by Fusarium head blight (*Fusarium* spp.) was investigated by applying five different types of N fertiliser at five different rates to wheat crops in Austria. Disease severity increased with increasing N supply, but there was no effect of the type of fertiliser. The presence of mycotoxins in the grain also increased with increasing N supply and it was concluded that management of Fusarium head blight by altered fertiliser applications was not feasible (Lemmens *et al.*, 2004).

Modified fertiliser use may help to reduce incidence and severity of disease (Jordan and Hutcheon, 1999). Under conventional farming systems, a total of approximately 200 kg N ha^{-1} is applied to a wheat crop, whereas a less intensive (integrated) crop will only use a total of approximately 140 kg N ha^{-1}, and the timing of application may vary. The canopy of a conventional crop is denser than the integrated crop, and more conducive for disease development. Comparison of the two systems revealed that in 1995, incidence of *S. tritici* occurred later and was less severe in the integrated crop, despite the application of fungicide in May and June to the conventional crop. The number of fungicide applications in integrated systems was reduced by 42 %, and the amount of active

ingredient applied per hectare was reduced by 79 % due to altered cultural practices (Jordan and Hutcheon, 1999).

The timing of N application may also influence severity of disease. Infection of winter wheat by mildew was more severe if N was applied in the early stages of crop growth (Darwinkel, 1980). Application of high concentrations of N to wheat seedlings increases their susceptibility to infection by *S. nodorum* (Shipton *et al.,* 1971). See references describing the response of *S. tritici* infection to increased N supply in field-grown wheat in Chapter 6.

The form of N available to the host or pathogen may be more important in determination of disease severity than the amount of N available (Huber and Watson 1974). For example, *Fusarium* spp. tend to produce most severe symptoms when host plants are supplied with NH_4^+ (Agrios, 1997). The severity of rust diseases is generally greater when NO_3^- is supplied as opposed to NH_4^+, and NH_4^+ fertilisers suppress rust disease (Huber and Watson, 1974). Daly (1949) studied the effect of N source on disease development of *Puccinia graminis tritici* on wheat. On moderately susceptible varieties, application of NO_3^- fertiliser increased susceptibility and application of NH_4^+ fertiliser increased resistance. Intermediate levels of disease occurred with application of NH_4NO_3 (Daly, 1949). Many fungi can use NO_3^-, and most can use NH_4^+, which can sometimes be taken up in preference to NO_3^- (Isaac, 1992). Uptake of NO_3^- by the fungus consumes more energy than uptake of NH_4^+, as NO_3^- must first be reduced to NO_2^- by NR, then the NO_2^- must be reduced to NH_4^+ before being assimilated into organic N compounds (Moore, 1998).

The form of N taken up by a plant will affect its metabolic pathways, and these affect growth and plant constituents or exudates (Huber and Watson, 1974). Supply of high N concentrations may inhibit structural resistance mechanisms, such as lignification. High N decreases the activity of some of the key enzymes involved in phenol metabolism and decreases the content of phenol (a precursor to lignin) and silicon, which is also used in resistance (Marschner, 1995).

The relationship between N and disease can be difficult to study in the field, due to the complex processes that occur in soil, such as mineralization and nitrification by microorganisms, which may alter the form of N available to plants (Huber and Watson 1974). The effects of N supply on infection are discussed further in Chapter 2.

Environmental resistance of wheat to *Stagonospora nodorum*

As part of a project funded by DEFRA, the environmental resistance of wheat to fungal pathogens was investigated. Researchers at ADAS Boxworth, Cambridge carried out field experiments to test the hypothesis that biotrophs are predominantly limited by N supply, and necrotrophs are predominantly limited by C supply. When field-grown wheat (cv. Cadenza) was shaded from growth stage 31-37 using mobile shades, prior to inoculation with *S. nodorum,* the epidemic was delayed compared with unshaded wheat (Beed *et al.,* 2004, in press). Shading had no effect on infection by yellow rust (*Puccinia striiformis* Westend.). To complement the field studies and investigate the mechanisms of the environmental resistance of wheat to a necrotroph (*Stagonospora nodorum*), experiments using wheat grown in controlled environments were carried out and the results are presented in this thesis.

Economic importance of wheat

Wheat is probably the most important cereal crop grown in the world. It has been domesticated for around 12,000 years, and is grown in most parts of the world (Gooding and Davies, 1997). Of the total grain yield, 65 % is used directly as human food, 21 % is used as livestock feed, 8 % provides seed and 6 % has other uses e.g. industrial raw materials (Orth and Shellenberger, 1988 cited in Gooding and Davies, 1997). In the UK the area under wheat production in 2002 was approximately 1,891,000 ha and in that year the total cost of controlling disease was £135.5 m, with an additional £37 m lost due to disease (Hardwick *et al.,* 2002).

Economic importance of *Stagonospora nodorum*

Stagonospora nodorum (Berk.) Castellani and E.G. Germano, (teleomorph *Phaeosphaeria nodorum* (E. Müller) Hedjaroude, syn. *Leptosphaeria nodorum* E. Müller) syn., *Septoria nodorum* (Berk.), the causative organism of leaf and glume blotch of wheat used to be one of the most serious cereal pathogens in the UK, but has been overtaken in importance by *Septoria tritici* Rob. and Desm. [teleomorph *Mycosphaerella graminicola* (Fuckel)], causative organism of septoria leaf blotch. This is in part due to the introduction of wheat varieties with greater susceptibility to *S. tritici* (Bayles, 1990), and partly due to unknown factors (Hardwick *et al.,* 2002). Since 1989 more than 50 % of wheat cultivars each year in England and Wales were susceptible to *S. tritici*, but on average less than 50 % of wheat cultivars were susceptible to *S. nodorum* in the same period. *S. nodorum* was found in progressively fewer crops between 2000 and 2002, where it was found in 48 % and 12.5 % of UK wheat crops respectively (Hardwick *et al.,* 2002). The switch in dominance from *S. nodorum* to *S. tritici* appeared to happen between 1982 and 1985 (Hardwick *et al.,* 2001). *S. tritici* has been the most severe foliar disease of wheat in the UK for 12 consecutive years, but severity is variable between years (Hardwick *et al.,* 2002). This pathogen is a hemibiotroph, which means it has both a necrotrophic phase and a biotrophic phase.

Annual UK yield losses caused by *S. nodorum* in previous years were not usually greater than 10 % (Parry, 1990) but can be as high as 50 % under appropriate conditions (Eyal *et al.,* 1987). In the years 1989 to 1998 yield losses due to *S. nodorum* were less than 1 % (Hardwick *et al.,* 2001). *S. nodorum* occurs worldwide, being prevalent in North America and Western Europe, affecting wheat and occasionally barley, rye (Parry, 1990) and triticale (Oettler and Schmid, 2000). Disease progression is dependent on environmental conditions. Occurrence of the disease increases linearly with increasing distance from the equator, and the amount of precipitation received during growing seasons (Leath *et al.,* 1993). Disease is most severe when it occurs late in the season affecting the flag leaf or glumes (Holmes and Colhoun, 1974) and during periods of high rainfall (Gilbert *et al.,* 1998). *S. nodorum* can infect young wheat but usually infects mature plants (Parry, 1990). The growth stage at the time of inoculation with *S. nodorum* can significantly

affect disease progression, with greater disease severity occurring when plants are inoculated at later growth stages (Wainshilbaum and Lipps, 1991).

Stagonospora nodorum *biology and infection process*

Stagonospora nodorum is a necrotrophic fungal plant pathogen. The teleomorph, (perfect or sexual state) *P. nodorum*, syn. *L. nodorum* does not commonly occur, but has been found in regions of north and south America, Australia and western Europe (Leath *et al.*, 1993) and can occur in the UK (Gooding and Davies, 1997). *P. nodorum* is an ascomycete, which produces pseudoperithecia containing asci and ascospores (Zadoks, 1988). Ascospores are not required for infection to be initiated, but they may appear in countries that do not normally see the teleomorph as they can be blown over long distances (Sanderson and Scott, 1988).

Ascospores, or more usually pycnidiospores, are transported by wind and rain splash to plant tissues. Germination of the spore can occur within 2 hours after inoculation (Jørgensen and Smedegaard-Peterson, 1999) but it requires high RH (Jones and Odebunmi, 1971, Holmes and Colhoun, 1974, Eyal *et al.*, 1977, Jeger *et al.*, 1981) or leaf wetness (Jeger *et al.*, 1981). The duration of high RH required is variable with plant age, cultivar and temperature (Holmes and Colhoun, 1974), with the minimum period required for infection ranging from one (Shipton *et al.*, 1971) to 50 hours (Holmes and Colhoun, 1974). In the 24 h period after germination, RH should not drop below 60% for longer than four hours in order for infection to become established (Jeger *et al.*, 1981). In an experiment to determine the length of RH required for infection to occur, wheat plants were inoculated with *S. nodorum* and subjected to periods of high RH ranging from 0 - 100 hours. Resistant cultivars were found to exhibit symptoms of infection after long periods of high RH, and susceptible cultivars showed a linear increase in severity of symptoms with increasing periods of high RH (Eyal *et al.*, 1977). In contradiction to these findings, an experiment to determine the effects of RH and cirrhus (the gelatinous mass of spores exuded from pycnidia) extract on spore germination, it was found that spores rapidly lost viability at RH greater than 80 % (Griffiths and Peverett, 1980). The microclimate of the crop is important in determining resistance to *S. nodorum*. Tall

varieties of wheat are more resistant than short varieties as the distance the spores have to travel between leaf layers is greater, and the microclimate of the crop is more open, with lower RH than in shorter crops. Disease escape may also be a factor, as short wheat varieties may mature earlier than tall varieties, plus taller varieties may have inherited vigour (Scott *et al.*, 1985).

The spore may penetrate the leaf directly through the cuticle (Karjalainen and Lounatmaa, 1986) or through stomata (Shipton *et al.*, 1971, Ahmad, 1981), and penetration can occur as soon as 10 hours after inoculation (Bird and Ride 1981) but penetration events can still occur several days after inoculation (Karjalainen and Lounatmaa, 1986). An appressorium and penetration peg is produced (Karjalainen and Lounatmaa, 1986), which releases extracellular enzymes to break down plant cell walls (Jørgensen and Smedegaard-Peterson, 1999). After 24 hours the hyphae grow through intercellular spaces, branching and remaining in close association with cell walls, but hyphae are not seen in the mesophyll until 9 days after inoculation (Karjalainen and Lounatmaa, 1986). Eventually cell walls are penetrated; after extensive intracellular invasion by the fungus the host cells collapse (Ahmad, 1981) and chloroplasts collapse (Karjalainen and Lounatmaa, 1986). Visual symptoms may occur 48 hours after inoculation, with small areas of chlorosis or brown spots surrounded by halos of chlorosis (Shipton *et al.*, 1971, Parry, 1990). The lesions are usually oval or lens shaped, but may merge into irregular blotches. Pycnidia appear as brown specks in the centre of the lesions (Sanderson and Scott, 1988). Pycnidia formation can occur within 10-21 days after inoculation (Shipton *et al.*, 1971) and may be induced by a 24 hour period of high RH (Jeger *et al.*, 1981). Pycnidia are formed in sub-stomatal cavities with their ostioles positioned directly under the stomatal pore (Shipton *et al.*, 1971). Towards the end of the growing season when infection has spread to culms, glumes, nodes and leaf sheaths, the fungus becomes saprophytic, and pseudothecia may form in dead plant tissue (Sanderson and Scott, 1988, Parry, 1990). Infected plant parts have lowered rates of photosynthesis and altered N status, with N being diverted from grain production to the fungal colonies. Amino acid metabolism may also be altered (Verreet and Hoffmann, 1986 and 1990, cited by Jørgensen and Smedegaard-Peterson, 1999).

S. nodorum can degrade plant tissues by means of toxins and extracellular enzymes. A trypsin-like protease encoded by the *SNP1* gene has been isolated from *S. nodorum* which is secreted whilst the pathogen is growing on the leaf and during penetration (Bindschedler *et al.*, 2003). Because of the extensive surface growth on the leaf by *S. nodorum*, and its lack of defined infection structures, it is difficult to determine when *S. nodorum* penetrates the host, but expression of *SNP1* appears to be induced by the leaf surface shortly after spore germination (Bindschedler *et al.*, 2003). *SNP1* is an alkaline protease capable of releasing hydroxyproline from cell walls. It has been isolated from infected leaves and found in liquid cultures containing host cell walls as the only N source (Carlile *et al.*, 2000). *SNP1* is not essential for pathogenicity alone, but acts in conjunction with a range of proteases (Bindschedler *et al.*, 2003). The release of proteases by *S. nodorum* suggests that peptides are a source of N for the fungus. A gene (*Ptr2*) for the transport of di / tripeptides in *S. nodorum* has been characterised and was strongly expressed during N starvation, but not during N and C starvation. Growth of mutants lacking *Ptr2* was extremely poor when supplied the dipeptides arginine-leucine (Arg-Leu) and glutamate-glutamate (Glu-Glu), but its growth on NO_3^- was not affected by the lack of *Ptr2*. The activity of *Ptr2* was upregulated during the initial phase of infection, but the pathogenicity of mutants was not compromised, suggesting that di / tri peptide uptake by *S. nodorum* is not required for pathogenicity (Solomon *et al.*, 2003*a*).

Production of cell wall degrading enzymes by the perfect stage of *S. nodorum*, *Phaeosphaeria nodorum* was investigated (Halama *et al.*, 1999) by *in vitro* growth of the pathogen and enzyme assays on filtrates from the cultures. Pathogenicity tests were also carried out. The fungus secreted several cell-wall-degrading enzymes, and four of them (xylanase, cellulase, polygalacturonase and butyrate-esterase) were positively correlated with isolate aggressiveness (Halama *et al.*, 1999). Phytotoxins have been isolated from *S. nodorum*. These generally belong to two families: the septorin family (*N*-methoxyseptorine and *N*-methoxyseptorinol) and the mellein family (= ochracin, -4-hydroxymellein, -5-hydroxymellein and -7-hydroxymellein) and these uncouple the mitochondria in wheat coleoptiles, but their direct involvement in pathogenicity is not yet understood (Eyal, 1999).

S. nodorum has a gene (*NIA1*) which encodes for NR, and the use of this as a potential target for fungicides was investigated. There was no loss of pathogenicity by NO_3^- non-utilising mutants however, so it was concluded that NR is not a useful target for fungicides (Howard *et al.*, 1999).

Culturing of S. nodorum in vitro

Methods for producing cultures of *S. nodorum in vitro* have been well documented (Cooke and Jones, 1970; Scharen and Krupinsky, 1970; Dhingra, 1985). In carrying out experiments to determine the best method of producing sporulating colonies, it was found that a Czapek Dox-V8 media (Czapek Dox agar supplemented with 20% Campbell's V8 vegetable juice) supported growth of the fungus and placing the growing colonies under continuous near UV (NUV) light at 20 °C (after an initial dark incubation at 22 °C) provided the best conditions for sporulation (Cooke and Jones, 1970). Frequent subculturing of the fungus can cause it to lose pathogenicity, but it is most likely to remain viable if it is alternately subcultured onto low and high-nutrient media (Dhingra, 1985). See Chapter 4 for a discussion of the response of *S. nodorum* to N supply *in vitro*.

Disease control

Disease control in agriculture is a continuous struggle. Fungicides have been in use since the late 19th century, when a mixture of copper sulphate and lime was used to prevent grapes from becoming infected with downy mildew (*Plasmopara viticola*) (Agrios 1997). Today, chemical fungicide use is widespread, but this can be damaging to the environment and human health (Jordan and Hutcheon, 1999). Fungicides are used for controlling *S. nodorum*, both in the form of seed treatments which control seed-borne infection and provide some form of protection for seedlings, and as foliar sprays when the crop is maturing (Cook, 1999), normally in two applications (Hardwick *et al.*, 2002). Controlling disease on the top two or three leaves is most important as they provide most of the grain-filling capacity and thus infection of these leaves causes the biggest reduction in yield (Fraaije *et al.*, 1999). *Septoria* diseases are currently controlled using both triazole and strobilurin fungicides, often supplemented with chlorothalonil (Clark and Paveley, 2004). Strobilurins, which inhibit mitochondrial respiration have become

increasingly popular (Godwin et al., 1999), with 96 % of all treated wheat crops in 2002 having at least one application (Hardwick et al. 2002). However, resistance to strobilurins in recent years has occurred and is increasing (Clark and Paveley, 2004).

Plant breeding programmes and genetic engineering have produced many varieties of wheat (and other crops) that are resistant to one or several plant pathogens. Resistant varieties can be highly effective, and greatly reduce the need for fungicide inputs. However, as pathogens have such rapid life cycles compared to plants, genetic mutations can create whole new races of a pathogen, which can overcome the plant's resistance mechanisms in short periods of time. There is currently no evidence for immunity or a gene-for-gene interaction in the wheat-*S. nodorum* pathosystems (Schnurbusch *et al.*, 2003). Resistance in the spike and in the leaves are independent of each other (Eyal, 1999) and resistance of wheat to *S. nodorum* is generally thought to be partial and a quantitative trait (Schnurbusch *et al.*, 2003). Quantitative trait loci (QTLs) have been identified for resistance to *S. nodorum* that are strongly correlated with QTLs for plant height (Schnurbusch *et al.*, 2003), suggesting that tall plants are more resistant to *S. nodorum*, which is supported by the evidence for canopy architecture affecting resistance (Lovell *et al.*, 2004)

It is now been believed that to maximise crop protection and minimise input of fungicides there is a need for integrated pest management (IPM), which uses a combination of resistant cultivars, fungicides, biological control and cultural practices aimed at controlling or eradicating pests and pathogens whilst maintaining productivity (Lucas, 1998). Cultural practices such as removal of infected stubble can reduce the amount of primary inoculum (Eyal, 1999).

A potential biological control agent for *S. nodorum* and *S. tritici* has been identified (Nolan and Cooke, 2000). Resistant (cv. Hussar) and susceptible (cv. Brigadier) cultivars of wheat were inoculated with the non-host pathogen *Drechslera teres* prior to inoculation with *S. nodorum* and *S. tritici*. No symptoms were caused by *D. teres* but severity of infection by both wheat pathogens (alone or together) was reduced on cv.

Hussar. In the more susceptible cultivar, severity of *S. nodorum* was initially increased by prior inoculation with *D. teres*, there was no effect on infection by *S. tritici*, but when plants were inoculated with a mixture of *S. tritici* and *S. nodorum* disease symptoms were reduced by pre-treatment with *D. teres*. Yield (1000 grain weight) was increased by pre-treatment with *D. teres* on cv. Hussar, but there was no effect of *D. teres* on yields of wheat. It was pointed out that differences between cultivars are important when assessing pathogen interactions, and the paper concluded that *D. teres* (and perhaps other non-host pathogens) shows potential as an agent of biological control for *S. nodorum* and *S. tritici* (Nolan and Cooke, 2000).

Researchers at IACR-Long Ashton Research Station near Bristol have been running a project called Less Intensive Farming and Environment. The project aims to compare conventional farming systems with less intensive, integrated systems that remain profitable whilst having lower inputs of agrochemicals and a generally more environmentally friendly approach (Jordan and Hutcheon, 1999). Disease forecasting systems may also be useful in quantifying the risk of disease resistance and to inform fungicide programs. These have been based on the movement of spores by rainfall within the crop canopy, but recent research suggests that canopy architecture is also an important factor in determining disease spread (Lovell *et al.*, 2004).

Chapter 2 N affects resistance of wheat (*Triticum aestivum* cv. Brigadier) to *Stagonospora nodorum* (Berk.)

Introduction

N supply to wheat crops

The yield of winter cereals is largely regulated by N supply (Olesen *et al.*, 2002). Under conventional farming systems there are substantial inputs of N fertiliser (Lawlor, 2002) - a total of approximately 200 kg N ha^{-1} is applied to a wheat crop (Jordan and Hutcheon, 1999). The N content required for maximal growth varies according to species and age (Grindlay, 1997), but is generally between 2 and 5 % of dry weight (Marschner, 1995). Uptake of N by a crop depends on availability of N within the soil and the crop growth rate, and there is a non-linear relationship between crop N content and crop biomass (Gastal and Lemaire, 2002). The critical N concentration (the minimum crop N concentration in shoots required to achieve maximum above ground dry matter) has been determined statistically for many crops including wheat (Justes *et al.*, 1994) and this can be used to determine whether N supply is deficient, optimal or supra-optimal (Gastal and Lemaire, 2002). The equation describing the critical N dilution curve for wheat (when shoot biomass is between 1.55 and 12 t ha^{-1}) is:

$$N_{ct} = 5.35\ DM^{-0.442}$$

Where N_{ct} is the critical N concentration corresponding to the amount of dry matter produced and *DM* is accumulated crop biomass dry matter (t ha^{-1}) (Justes *et al.*, 1994).

Nitrate (NO_3^-) is generally the most abundant form of available N in agricultural soils, but if both NO_3 and ammonium (NH_4^+) are available then NH_4^+ is taken up preferentially by many species (von Wiren *et al.*, 1997). Most plants grow better on NO_3^- than NH_4^+ (unless they are specifically adapted to acid soils) (Pilbeam and Kirkby 1992) and the highest growth rates and yields of crop plants are generally achieved using a combination of both (Marschner, 1999). It is metabolically more costly for a plant to take up and assimilate NO_3^- than it is to take up and assimilate NH_4^+, as NO_3^- needs to be reduced to ammonia (NH_3) via NO_3^- and nitrite (NO_2) reductases prior to assimilation into organic N compounds (e.g. amino acids) (Pilbeam

and Kirkby 1992; Marschner, 1999; Zerihun, 1997). NH_4^+ must be assimilated via the glutamine synthetase / glutamate synthase (GS / GOGAT) pathway in the roots, but NO_3^- can be assimilated in the roots or shoots, and the predominant site of assimilation depends on the NO_3^- concentration available (Andrews *et al.*, 2001).

Effect of N supply on plant biomass, dry matter partitioning and photosynthesis

Increasing N supply increases dry matter production (Marschner 1995) and shoot: root (S: R) ratio (Andrews *et al.*, 2001; Marschner, 1995; Zerihun *et al.*, 1998). Root growth depends on N supply and where N is limiting there will be greater partitioning of assimilates to roots to maximise N capture (Lawlor, 2002). Increased N supply increases the concentration of amino acids and proteins in tissues, and diverts more of the products of photosynthesis from structural growth to assimilation (Zerihun *et al.*, 1998). Increased N supply also increases photosynthesis (Gastal and Lemaire, 2002; Hirose and Werger, 1987; Lawlor *et al.*, 1989), so more assimilates will be available for N assimilation and structural growth and therefore increase in biomass. S: R ratios are positively correlated with leaf N content and leaf protein content (Andrews *et al.*, 2001).

Increasing supply of N generally increases photosynthesis (Gastal and Lemaire, 2002; Lawlor *et al.*, 1989). There is a roughly linear relationship between the increasing N content of a leaf and increasing net photosynthesis under given environmental conditions (Grindlay, 1997). Up to 75 % of organic N in green leaf cells is located in chloroplasts, as enzymes and as structural proteins (Marschner, 1995), and up to 30 % of the N and 50 % of the soluble protein in wheat leaves can be attributed to Rubisco (Lawlor *et al.*, 1989). Concentrations of chlorophyll and Rubisco can be decreased by N deficiency, which leads to reduced photosynthetic capacity and carboxylation efficiency (Delgado *et al.*, 1994; Lawlor *et al.*, 1989).

The form of N supplied can also affect photosynthesis, dry matter production (Cramer and Lewis, 1993*a*) and partitioning (Andrews *et al.*, 1999; Cramer and Lewis, 1993*a*). Wheat plants supplied NH_4^+ (4 or 12 mmol dm^{-3}) had lower dry weights and higher S: R ratios than those supplied NO_3^-. Differences were greater at the higher concentration, but there was no difference in dry weights or S: R ratio between plants supplied 4 and 12 mmol dm^{-3} N (Cramer and Lewis, 1993*a*). Plants supplied NH_4^+

sometimes had higher S:R ratios than plants supplied NO_3^- of similar dry weights. In these cases the leaf N contents of the plants supplied NH_4^+ were higher than those of plants supplied NO_3^- (Andrews *et al.,* 2001). Total dry weights of hydroponically-grown tomato (*Lycopersicon esculentum* L.) plants and fruits were lower when plants were supplied NH_4^+ compared with NO_3^- but S: R ratio was not affected by N form (Claussen, 2002). Wheat plants supplied 12 mmol dm^{-3} NH_4^+ had significantly lower photosynthetic rates, stomatal conductance, and intercellular CO_2 concentrations than plants supplied 12 mmol dm^{-3} NO_3^-. There was little difference in these parameters in plants supplied 4 mmol dm^{-3} N. Stomatal conductance was very sensitive to N form (Cramer and Lewis, 1993*b*).

N supply and disease resistance

The nutritional status of a plant, especially N nutrition, plays an important role in its overall vigour, which in turn influences its resistance to disease. Plants deficient in N are weak, slow growing, have reduced synthesis of soluble proteins and increased structural proteins and senesce quickly (Lawlor, 2002), so may be more susceptible to certain pathogens (Snoeijers *et al.,* 2000). Plants supplied with optimal or luxury amounts of N have a higher proportion of young, green tissues and more vigorous growth so may be more resistant to certain pathogens (Marschner, 1995).

Increasing N supply can increase the incidence of many pathogens including *Erysiphe graminis* f. sp. *hordei* (Oerke and Schonbeck, 1990), *Fusarium* spp. (Lemmens *et al.,* 2004), *Oidium lycopersicum* (Hoffland *et al.,* 2000), *Puccinia striiformis* (Danial and Parlevliet, 1995), *Septoria tritici* (Leitch and Jenkins, 1995; Lovell *et al.,* 1997; Olesen *et al.,* 2003; Simon *et al.,* 2003), but has no effect on incidence of *Fusarium oxysporum* (Hoffland *et al.,* 2000) and reduces the incidence of *Botrytis cinerea* (Hoffland *et al.,* 1999a). As plants grown with high N supply will have high amounts of N in tissues they may be nutritionally favourable to pathogens. This relationship is not, however, straightforward. The concentration of compounds involved in a plant's defence mechanisms may be altered by its nutritional status, for example high N status may inhibit structural resistance mechanisms, such as lignification. High N decreases the activity of some of the key enzymes involved in phenol metabolism e.g. phenylalanine-ammonia lyase (PAL) (Matsuyama and Dimond, 1973) and decreases the content of phenol (a precursor to lignin) and silicon, which is also used in

resistance. There is a lower proportion of phenols and high proportion of young tissue (therefore less lignin) in plants supplied high N, and the content of phenolics is often higher in N deficient plants (Marschner 1995).

N supply also affects the canopy structure within a crop, which affects the crop microclimate, and consequently affects both epidemiology and resistance to *S. nodorum* (and other pathogens) (Jensen and Munk, 1997; Jordan and Hutcheon, 1999; Olesen *et al.*, 2003; Scott *et al.*, 1985; Shaw and Royle, 1993). With high N supply the canopy may be dense, resulting in increased relative humidity (RH) which favours spore germination (Shaw and Royle, 1993).

Many fungi can use NO_3^-, and most can use NH_4^+, which can sometimes be taken up in preference to NO_3^- (Isaac, 1992). As is the case for plants, it is energetically more costly for the fungus to take up NO_3 [which must first be reduced by NO_3^- and NO_2^- reductases (NR and NiR)] than to directly take up the amino acids assimilated from NH_4^+ (Moore, 1998). Despite there being a wealth of research on the effect of N concentration on plant disease (see above), including some on *S. nodorum* (Leitch and Jenkins, 1995), little has addressed the problem of how infection responds to different forms of N.

The form of N applied to plants, as well as the concentration, can significantly affect disease progression, and usually (but not always) NO_3^- tends to decrease the incidence of disease caused by necrotrophs, whereas NH_4^+ increases the incidence of disease caused by necrotrophs (Huber and Watson, 1974). For example, the severity of rust diseases (biotrophs) is generally greater when NO_3^- is supplied as opposed to NH_4^+, and fertilisers containing NH_4^+ suppress rust disease. On moderately susceptible varieties of wheat, application of NO_3^- fertiliser increased susceptibility to *Puccinia graminis* and application of NH_4^+ fertiliser increased resistance. Intermediate levels of disease occurred with application of NH_4NO_3 (Daly, 1949). Among the necrotrophs, *Fusarium, Rhizoctonia* and *Sclerotinium* on citrus, wheat, cotton, tomato and sugar beet are stimulated by NH_4^+, but *Pythium, Phymatotrichum* and *Pseudomonas* causing corn and pea root rots, cotton root rot and tobacco and tomato wilts respectively are favoured by NO_3^- (Snoeijers *et al.*, 2000)

Effect of N supply and infection on C export

Investigations into the effect of nutrient status on export of photosynthetically-fixed C have been carried out: leaves of *Poa alpina* L. had higher photosynthetic rates and slightly higher rates of carbohydrate export when nutrient supply (N and P) was high (2.5 mmol dm^{-3} N and 0.5 mmol dm^{-3} P) (Baxter and Farrar, 1999), but there has been no work on the effect of N form on C export.

Wheat leaves infected by *S. nodorum* retain assimilates (Scharen and Taylor, 1968; Wafford and Whitbread, 1976), a common feature of many diseases (Doodson *et al.*, 1964; Hale and Whitbread, 1973; Hibberd *et al.*, 1996*b*; Lucas, 1998). In spring wheat, there was no difference in ^{14}C assimilation between healthy and infected leaves, but export of assimilated ^{14}C was reduced from 69 to 60 % when the leaf fed $^{14}CO_2$ was infected with *Septoria nodorum* (Wafford and Whitbread, 1974).When a pathogen successfully colonises plant tissues, less C is available from the nutrient pool (containing soluble translocates and metabolites and insoluble storage carbohydrates) for storage and translocation (Ayres *et al.*, 1996).

Hypotheses

In this Chapter I aim to investigate the effects of N supply on resistance of wheat to *S. nodorum*, by supplying wheat plants with N as NH_4^+ or NO_3^- at three different concentrations to test the hypotheses that N form and concentration both affect aspects of plant growth and partitioning, which in turn affects resistance of wheat to *S. nodorum*, specifically:

1. Plants supplied high (8 mmol dm^{-3}) concentrations of N will have the highest dry weights and S: R ratios, and plants supplied moderate (2 mmol dm^{-3}) concentrations of N will have higher dry weights and S: R ratios than plants supplied low (0.04 mmol dm^{-3}) concentrations of N.

2. Plants supplied high (8 mmol dm^{-3}) concentrations of N will have higher rates of photosynthesis and greater amounts of chlorophyll than plants supplied moderate (2 mmol dm^{-3}) concentrations of N.

3. Plants supplied NO_3^- will have higher dry weights and lower S: R ratios than plants supplied NH_4^+.

4. Plants supplied NO_3^- will have higher rates of photosynthesis, higher stomatal conductance and higher internal CO_2 concentrations than plants supplied NH_4^+.

5. Plants supplied NO_3^- will show greater ^{14}C efflux than plants supplied NH_4^+.

6. Healthy plants will show greater ^{14}C efflux than diseased plants.

7. Plants supplied moderate and high (2 or 8 mmol dm^{-3}) concentrations of N will be more susceptible to *S. nodorum.*

8. Plants supplied with NO_3^- will be less susceptible to *S. nodorum* than plants fed with NH_4^+.

Methods

This Chapter describes results from several experiments of similar design.

Growth of plants

Long Ashton solution was modified to allow N form and concentration to be manipulated, by omitting the N containing compounds KNO_3 and $Ca(NO_3)_2.4H_2O$ and replacing the lost calcium and potassium with $CaCl_2$ and KH_2PO_4. All non-N nutrients were available at concentrations equivalent to those in ½ strength Long Ashton solution. Nitrate ($NaNO_3$) and ammonium $(NH_4^+)_2\ SO_4$ solutions were added to solutions separately. Six treatments were as follows: low NO_3^- or NH_4^+ (0.04 mmol dm^{-3}), moderate NO_3^- or NH_4^+ (2 mmol dm^{-3}), high NO_3^- or NH_4^+ (8 mmol dm^{-3}).

Thirty 4-d old seedlings were planted in each of 4 7 dm^3 troughs containing nutrient solution. Plants assigned to the low N treatment were germinated 2 d before those receiving moderate and high N, to enable them to be at the same developmental stage when inoculation was carried out. Nutrient solutions were changed every 3 d, and aerated at a rate of 1 $dm^3\ min^{-1}$. Plants were grown in controlled environment cabinets (Sanyo-Gallenkamp, Fi-totron PG660/C/RO/HQI, Loughborough, UK) at a light photon flux density of 450 µmol photons $m^{-2}\ s^{-1}$ for 14 h d^{-1}, 70 % RH, and ambient CO_2 concentration (350 ppm) provided by drawing air from above the roof of the building at a rate giving 4 air changes h^{-1}. Plants were moved between two different cabinets each time nutrient solutions were changed.

Production of inoculum and inoculation procedure

Initially, cultures of *S. nodorum* (supplied by ADAS Boxworth, Cambridge, UK) were maintained on Czapek-Dox (CZD) agar supplemented with Campbell's V8 juice (CZD-V8). To maintain virulence, colonies were sub-cultured every 4 weeks onto high (100 % CZD) or low (25 % CZD) nutrient media alternately. Plates (9 cm diameter Petri dishes) were kept in the dark at 22 °C for 48 h then placed under near UV (continuous) and white light (14 h photoperiod) to encourage sporulation. After 2 weeks the plates were stored at 4 °C. Periodically, the pathogen was re-isolated from infected plants. For longer term storage and to maintain virulence, pycnidiospores were harvested from actively sporulating colonies and added to moist, autoclaved

wheat grains (*Triticum aestivum* L. cv. Brigadier) in McCartney (Universal) bottles. The bottles were sealed with screw caps and Parafilm and stored at 4 °C. To produce a colony, a few wheat grains were shaken out of the bottle onto a 9 cm Petri dish containing CZD-V8 and incubated in the dark at 22 °C for 48 h then under near UV (continuous) and white light (14 h photoperiod) to encourage sporulation. Two weeks later, the Petri dishes were stored at 4 °C. Periodically, the pathogen was re-isolated from infected plants and stored on sterile wheat grains as above.

Spore suspensions were always made from 14 d old cultures, by flooding the plate with sterile, deionised water and agitating the surface of the colony with a sterile scalpel. After 20 min the spore concentration was measured using a haemocytometer, and adjusted to 10^6 or 5 x 10^5 spores cm^{-3} if necessary. One drop of Tween 20 was added per 10 cm^3 of suspension. Plants were inoculated with *S. nodorum* when the second seedling leaf was fully expanded, using a squirrel hair paintbrush dipped in the suspension. Control plants were brushed with sterile deionised water containing Tween 20. After inoculation, plants were returned to growth cabinets. A humid atmosphere (required for spore germination) was maintained for the 48 h period after inoculation by enclosing troughs in Perspex boxes that had been sprayed inside with distilled water.

Analysis of plant material

Plants were harvested on two or three occasions after inoculation, with the first harvest being 1, 2 or 7 d after inoculation (dai), and the final harvest being 10 or 14 dai, depending on the analyses being carried out (**Table 1**, this Chapter and Chapter 3). At each harvest three to six diseased and healthy plants per treatment were selected at random. Each plant was separated into roots, shoots, and blade of leaf 2. Leaf 2 of each plant was assessed for disease (number of lesions and percentage area of leaf covered by lesions and associated chlorosis), chlorophyll content (Chlorophyll meter SPAD-502, Konica-Minolta Co., Ltd., Japan) and leaf area (flatbed scanner with computer software, Delta T Devices, Cambridge, UK). Blades of second leaves were dried prior to weighing, or frozen or freeze dried prior to further analysis (Chapter 3). The remaining shoot material and roots of each plant were then dried to constant weight at 60 °C.

Table 1 N treatments and analyses for each of the experiments. For each analysis the number of days after inoculation (dai) that the plant material was harvested or non-destructive assessments were carried out is given. N.B. throughout this Chapter, dai is used as a measure of time for both inoculated and uninoculated plants.

Analysis	N concentrations supplied		
	0.04 mmol dm^{-3}	**2 mmol dm^{-3}**	**8 mmol dm^{-3}**
Disease assessment	7, 10 dai	7, 10 dai 7, 9, 12, 14 dai	7, 9, 12, 14 dai
Specific leaf area	7 dai	7 dai	
Chlorophyll content (using SPAD meter)	7, 10 dai	7, 10 dai	7, 10 dai
Photosynthesis		10 dai	11 dai
Efflux of $^{14}CO_2$		8 dai	
Dry weights	7, 10 dai	7, 10 dai 7, 14 dai	7, 14 dai (no leaf 2 dry wts)
Shoot: root ratios and allometry	7, 10 dai	7, 10 dai 7, 14 dai	7, 14 dai

Measurement of photosynthesis

Photosynthesis was measured in growth cabinets (conditions described previously) 10 or 11 d after inoculation. Measurements began approximately 3 h and ended approximately 7 h into the photoperiod. The rates of photosynthesis, stomatal conductance, internal CO_2 concentration and transpiration were determined for the second fully expanded leaf by means of an infra red gas analyser (IRGA) with a leaf chamber designed for grass and cereal leaves (CIRAS, PP Systems, Hitchin, Herts., UK). Leaves from each treatment were randomly selected to reduce any effects of time in the photoperiod on measurements. The width of the middle part of the leaf blade to be analysed was measured in order to calculate the leaf area, then the middle part of the leaf was placed in the cuvette. Measurements were taken when a steady state of CO_2 uptake was attained (usually after the leaf had been in the cuvette for a minimum of 10 min). Five readings were recorded at 30 s intervals for each replicate.

Measurement of translocation by ^{14}C efflux

^{14}C efflux was measured in second leaves of diseased and healthy plants supplied with 2 mmol dm^{-3} NO_3^- or NH_4^+, using a custom-made air-stirred cuvette with two built-in Geiger-Müller tubes. Due to equipment constraints efflux could only be measured on two plants at a time. In order for each replicate pair of plants from each N treatment

(healthy and diseased) to be at the same developmental stage at the time of ^{14}C feeding, plants were grown and inoculated as previously described, except that sowing and inoculation of individual plants from both treatments was staggered. Measurements were made 8 dai. Plants were placed in beakers containing the nutrient solution they had been grown in, and the solution was aerated using an aquarium pump and air stone. The leaf to be fed was placed in the feeding chamber over a Geiger Muller tube and clamped into place. A fan was on continuously in the feeding chamber. Air flow rate was maintained at 0.8 dm^3 min^{-1}. The feeding apparatus was set up under a light bank so the leaves were illuminated with a 16 h photoperiod. Leaves were fed $^{14}CO_2$ 4 h into the photoperiod after equilibrating in the chambers for approximately 1.5 h. To prepare the feed, 200 µl of 100 µCi (3.7 Bq) Na H $[^{14}C]O_3$ was mixed with 20 cm^3 sterile buffered distilled water (dH_20) in a beaker and drawn into a perfusor syringe. The injection speed on the perfusor pump was set at 60 cm^3 h^{-1}. $^{14}CO_2$ was created by perfusing the Na $H[^{14}C]O_3$ into 5 cm^3 lactic acid through which air was bubbled. This was carried via tubing to the leaves in the feeding chamber and the radioactive counts were recorded by a rate meter attached to a chart recorder. During the feed, counts were measured every 60 s. After the fourth reading after the end of the feed this was altered to every 120 s for approximately 3 h when it was changed to every 300 s. After the feed, plants were left for 20 h before being freeze-dried then used for autoradiography.

Autoradiography

Prior to freeze-drying, leaves fed ^{14}C were sandwiched between four sheets of chromatography paper and two brass plates secured with bulldog clips, then transferred to a freeze drier for 36 h. After freeze-drying, leaves were affixed to rectangular strips of card using spray glue and covered in a smooth layer of cling film. Each strip of card was placed leaf-side up on top of a rubber-coated brass plate (slotted into a metal press) and in total darkness a strip of photographic film (Ilford FP4) was placed emulsion side down on top of the leaf. A second rubber-coated brass plate was placed rubber-side down on top of the film to hold it in place. This was repeated with each sample to form layers of plates, sample and film and the whole was placed in a press. The top of the press was then screwed tightly in place. The whole press was wrapped in black cloth, followed by a black refuse sack. To develop the autoradiograph, after 5 d exposure the lid of the press was unscrewed (in complete

darkness) and one at a time each layer of film was carefully removed and fed into a spool. The spool was immersed in a circular tank containing developing fluid (Ilford Ilfotec LC29) and agitated every minute. After 3 min the spool was removed and washed in two changes of dH_20 before immersing in a circular tank containing fixer (Ilford Ilfofix II) for 5 min. All solutions were kept at 20 °C in a water bath. After fixing the spool was rinsed under running water for 10 min, and then film strips were dried.

Data analysis

Data were compared using one-way ANOVA with post-hoc tests of least significant difference (SPSS 9.0, SPSS Inc, Chicago, USA. Differences were considered statistically significant at $P < 0.05$. Data from efflux of $^{14}CO_2$ chart recordings were fitted to double exponential decay curves using Sigmaplot (Sigmaplot 8.0, SPSS Inc, Chicago, USA). From curves of net counts per second (cps), data were used to plot curves of the percentage of the total assimilation which was retained in the leaf with time.

Results

Dry weights of leaf 2

Effect of N form

Form of N did not significantly affect dry weights of leaf 2 (**Fig 1**), with the exception that at 7 dai second leaves from plants supplied 2 mmol dm^{-3} NH_4^+ had significantly higher dry weights than second leaves from plants supplied 2 mmol NO_3^- ($P < 0.01$ in diseased plants and $P < 0.05$ in healthy plants). Second leaves of plants supplied 8 mmol dm^{-3} NH_4 had higher dry weights than those of plants supplied 8 mmol dm^{-3} NO_3^- but the difference was not significant (not shown).

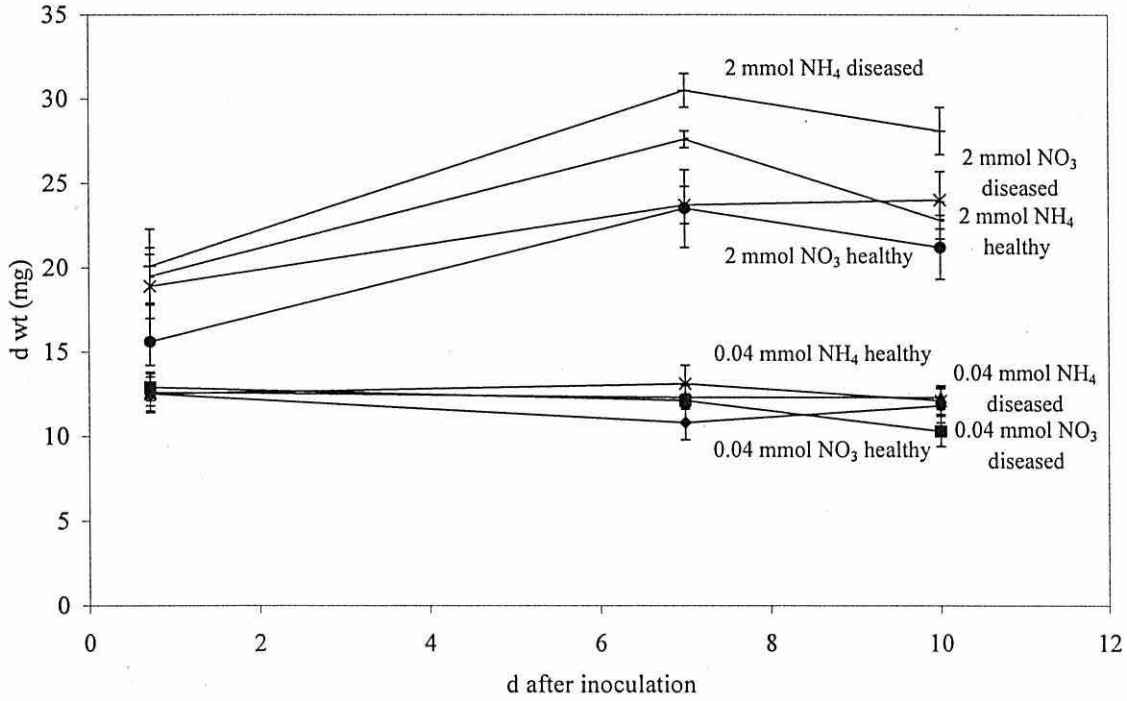

Figure 1 Dry weights (mean ± SE) (mg) of second leaves of wheat from plants that were supplied NO_3^- or NH_4^+ at 0.04 mmol dm^{-3} (labelled as 0.04 mmol on graph) or 2 mmol dm^{-3} (labelled as 2 mmol on graph). Second leaves of plants were inoculated when plants were 14 d old. n = 4 (1 and 7 dai), or n = 6 (10 dai).

Effect of N concentration

Second leaves of plants supplied 2 mmol dm^{-3} N had significantly higher dry weights than those of plants supplied 0.04 mmol N ($P < 0.01$). There was no significant difference in the dry weights of second leaves between plants supplied 2 mmol dm^{-3} N and those supplied 8 mmol dm^{-3} N (not shown).

Effect of disease

Dry weights of diseased second leaves were not significantly different to dry weights of healthy second leaves.

Specific leaf area

Effect of N form

Specific leaf areas (SLA) of plants supplied 0.04 mmol dm^{-3} NO_3^- were significantly higher than those of plants supplied 0.04 mmol dm^{-3} NH_4^+ ($P < 0.05$) (**Fig. 2**). There was no significant difference in specific leaf area (SLA) between plants supplied 2 mmol dm^{-3} NO_3^- and plants supplied 2 mmol dm^{-3} NH_4^+ (**Fig. 3**).

Effect of N concentration

Plants supplied 0.04 mmol dm^{-3} N had significantly higher SLAs than plants supplied 2 mmol dm^{-3} N ($P < 0.001$), with the exception of diseased plants supplied NO_3^- where there was no significant difference in SLA between plants supplied low and high N.

Effect of disease

There was no significant difference in SLA between diseased and healthy plants.

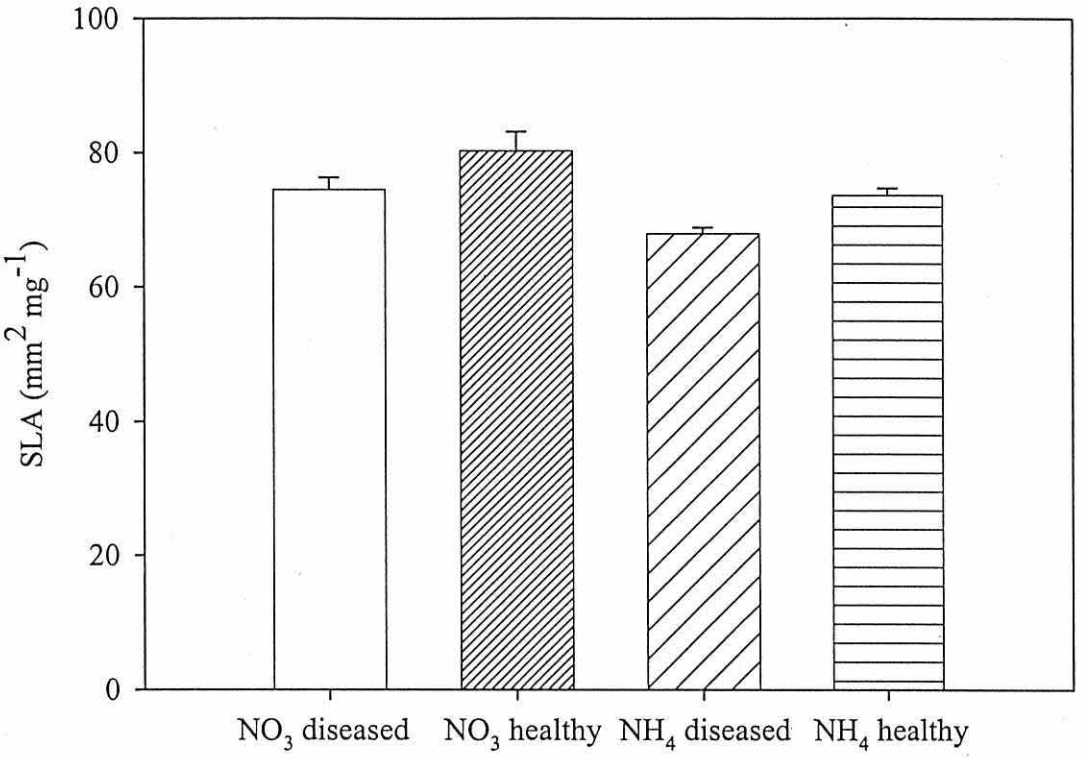

Figure 2 Specific leaf area (SLA; mean ± SE) 7 dai, of second leaves of wheat from plants that were supplied N as 0.04 mmol dm^{-3} $NaNO_3$ or $(NH_4)_2$ SO_4. Second leaves of plants were inoculated when plants were 14 d old. n = 3

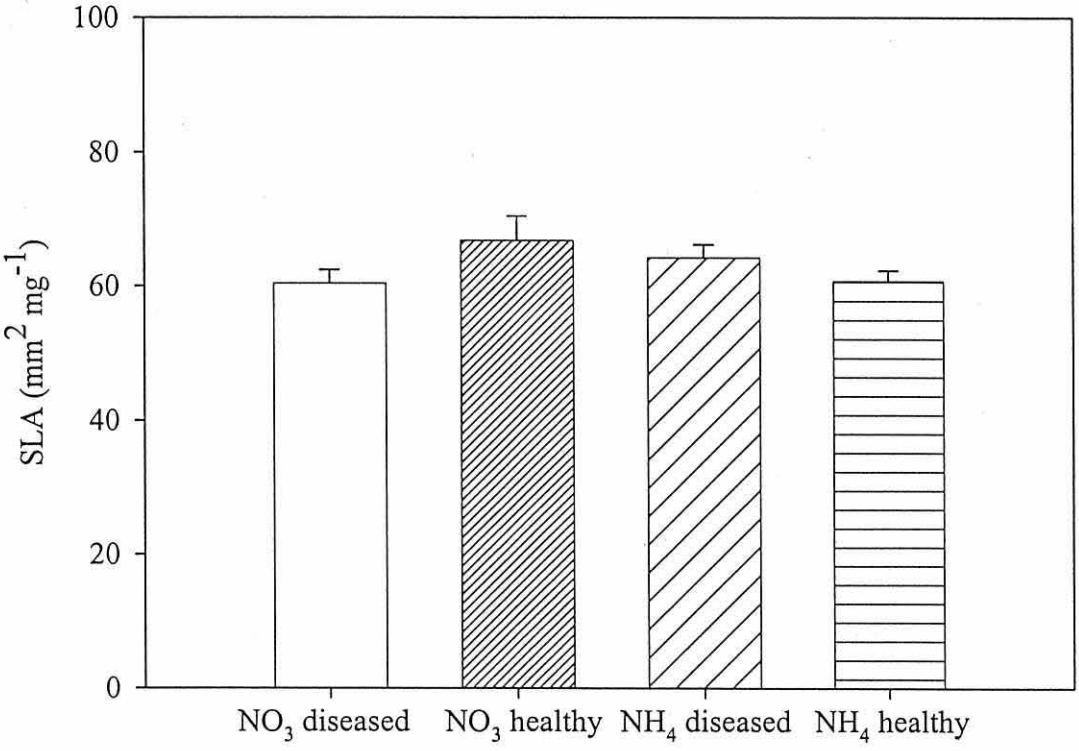

Figure 3 Specific leaf area (SLA; mean ± SE) 7 dai, of second leaves of wheat from plants that were supplied N as 2 mmol dm^{-3} $NaNO_3$ or $(NH_4)_2$ SO_4. Second leaves of plants were inoculated when plants were 14 d old. n = 3

Chlorophyll contents of leaf 2

Effect of N form

The effect of N form on chlorophyll was only significant in plants supplied 2 mmol dm^{-3} N (**Table 2**), whereas at 7 dai plants supplied NH_4^+ had 14 % higher chlorophyll contents than plants supplied NO_3 ($P < 0.01$), but by 10 dai, plants supplied NO_3^- had 25 % higher chlorophyll contents than plants supplied NH_4^+ ($P < 0.05$).

Table 2 Chlorophyll contents (SPAD meter readings; mean ± SE) of second leaves of wheat from plants that were supplied N as 0.04 mmol dm^{-3} NO_3^- or NH_4^+, 2 mmol dm^{-3} NO_3^- or NH_4^+, or 8 mmol dm^{-3} NO_3^- or NH_4^+. Second leaves were inoculated with *S. nodorum* the day after full expansion, when plants were 14 d old. N.B. to convert SPAD readings to mg chlorophyll m^{-2} multiply value by 6.2. n = 3

N form	N concentration (mmol)	7 dai		10 dai	
		diseased	healthy	diseased	healthy
NO_3^-	0.04	15 ± 1.4	14 ± 1.0	11 ± 1.9	10 ± 1.1
NH_4^+	0.04	17 ± 0.9	17 ± 0.9	14 ± 1.2	10 ± 1.6
NO_3^-	2	42 ± 2.5	46 ± 1.3	32 ± 2.8	28 ± 2.0
NH_4^+	2	52 ± 0.9	52 ± 1.4	25 ± 2.6	20 ± 2.6
NO_3^-	8	45 ± 1.8	47 ± 1.0	45 ± 0.8	43 ± 2.1
NH_4^+	8	52 ± 1.2	49 ± 2.8	49 ± 2.2	48 ± 1.6

Effect of N concentration

Plants supplied the lowest concentration of N had the lowest chlorophyll contents. At 7 dai second leaves of plants supplied 0.04 mmol dm^{-3} N had an average of 67 % lower chlorophyll contents than leaves from plants supplied both 2 mmol dm^{-3} N and 8 mmol dm^{-3} ($P < 0.001$). By 10 dai second leaves of plants supplied 0.04 mmol dm^{-3} N had an average of 54 % lower chlorophyll contents than leaves from plants supplied 2 mmol dm^{-3} N and 75 % lower chlorophyll contents than leaves from plants supplied 8 mmol dm^{-3} ($P < 0.01$). At this second harvest plants supplied 8 mmol dm^{-3} N also had significantly higher chlorophyll contents (by 43 %) than plants supplied 2 mmol dm^{-3} N ($P < 0.05$).

Effect of disease

The only significant difference in chlorophyll content between diseased and healthy plants was in plants supplied 2 mmol dm^{-3} NH_4^+ at 10 dai, where healthy plants had 22 % higher chlorophyll contents than diseased plants ($P < 0.05$).

Photosynthesis

Net photosynthesis (CO_2 assimilation)

Effect of N form

Net photosynthesis was significantly lower in plants supplied NH_4^+ than in plants supplied NO_3^-, (healthy plants only in the case of plants supplied 2 mmol dm^{-3} N) (**Table 3**). Differences in photosynthesis attributable to N form were more significant in healthy plants ($P < 0.01$) than diseased plants ($P < 0.05$).

Table 3 CO_2 assimilation rate (mean ± SE) of second leaves of wheat from plants that were supplied N as either 2 mmol dm^{-3} $NaNO_3$ or $(NH_4)_2$ SO_4 or 8 mmol dm^{-3} $NaNO_3$ or $(NH_4)_2$ SO_4. Second leaves of plants were inoculated when plants were 14 d old. Photosynthesis was measured 10 dai (2 mmol dm^{-3} N) or 11 dai (8 mmol dm^{-3} N). n = 3.

	2 mmol dm^{-3} N 10 dai		8 mmol dm^{-3} N 11 dai	
	Healthy	Diseased	Healthy	Diseased
NO_3^-	9.8 ± 0.2	9.7 ± 0.1	10.2 ± 0.3	8.8 ± 0.3
NH_4^+	8.8 ± 0.2	9.1 ± 0.1	9.0 ± 0.2	7.9 ± 0.4

Effect of N concentration

N concentration only had a significant effect on photosynthesis in diseased plants. Diseased plants supplied 8 mmol dm^{-3} N had significantly lower CO_2 assimilation rates than diseased plants supplied 2 mmol dm^{-3} N ($P < 0.01$).

Effect of disease

Differences between diseased and healthy plants were only observed in plants supplied the higher concentration of N (8 mmol dm^{-3}) where assimilation rates were significantly higher in healthy plants than in diseased plants ($P < 0.001$ NO_3^- and $P < 0.01$ NH_4^+).

Sub-stomatal CO_2 concentration

Effect of N form

Plants supplied NO_3^- generally had higher sub-stomatal CO_2 concentrations than plants supplied NH_4^+ ($P < 0.01$), but there was no significant difference between healthy plants supplied 8 mmol dm^{-3} NO_3^- and NH_4^+ (**Table 4**).

Table 4 Sub stomatal CO_2 concentration (ppm; mean ± SE) of second leaves of wheat from plants that were supplied N as either 2 mmol dm^{-3} $NaNO_3$ or $(NH_4)_2$ SO_4 or 8 mmol dm^{-3} $NaNO_3$ or $(NH_4)_2$ SO_4. Second leaves of plants were inoculated when plants were 14 d old. Photosynthesis was measured 10 dai (2 mmol dm^{-3} N) or 11 dai (8 mmol dm^{-3} N). n = 3

	2 mmol dm^{-3} N 10 dai		8 mmol dm^{-3} N 11 dai	
	Healthy	Diseased	Healthy	Diseased
NO_3^-	292 ± 2.1	290 ± 1.6	276 ± 2.3	280 ± 2.1
NH_4^+	266 ± 3.6	259 ± 3.9	271 ± 2.9	268 ± 2.3

Effect of N concentration

Sub-stomatal CO_2 concentrations were lower in diseased and healthy plants supplied 8 mmol NO_3^- than in diseased and healthy plants supplied 2 mmol dm^{-3} NO_3^- ($P < 0.01$), and were higher in diseased plants supplied 8 mmol dm^{-3} NH_4^+ than in diseased plants supplied 2 mmol dm^{-3} NH_4^+ ($P < 0.05$).

Effect of disease

There was no significant difference in sub-stomatal CO_2 concentration between diseased and healthy plants.

Transpiration rate

Effect of N form

Plants supplied NO_3^- had significantly higher transpiration rates than plants supplied NH_4^+ ($P < 0.001$) (**Fig. 4**).

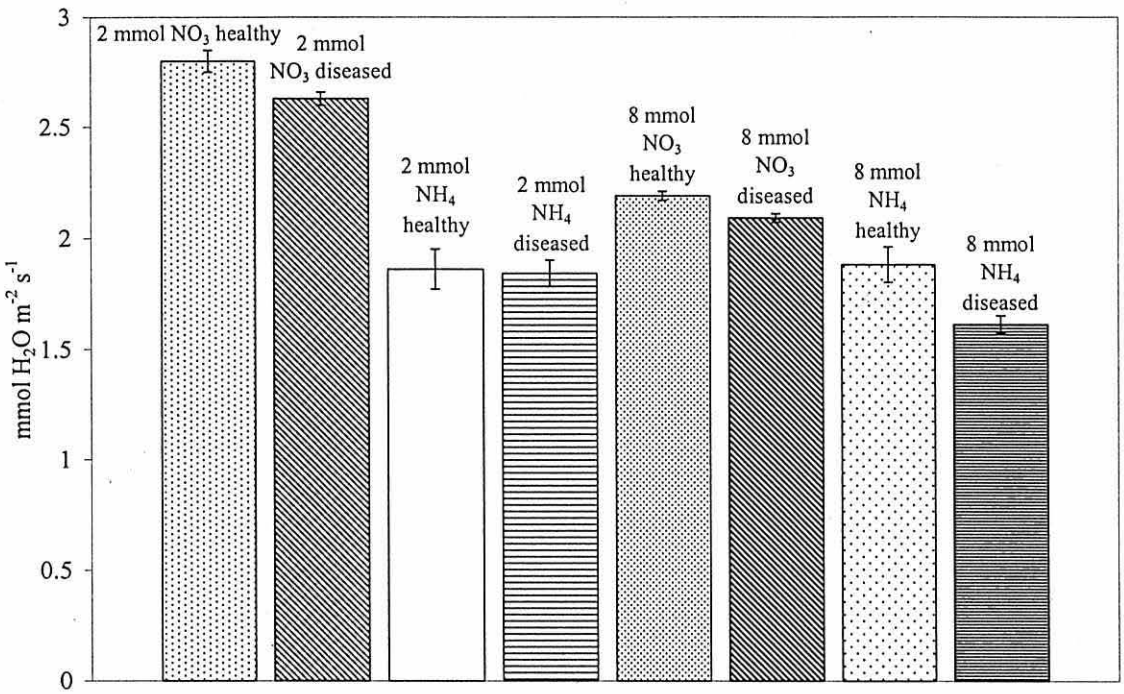

Figure 4 Transpiration rates (mean ± SE) of second leaves of wheat from plants that were supplied N as either 2 mmol dm^{-3} (labelled as 2 mmol on graph) $NaNO_3$ or $(NH_4)_2$ SO_4, or 8 mmol dm^{-3} (labelled as 8 mmol on graph) $NaNO_3$ or $(NH_4)_2$ SO_4. Second leaves of plants were inoculated when plants were 14 d old. Photosynthesis was measured 10 dai (2 mmol dm^{-3} N) or 11 dai (8 mmol dm^{-3} N). n = 3

Effect of N concentration

Plants supplied 2 mmol dm^{-3} N had significantly higher transpiration rates than plants supplied 8 mmol dm^{-3} N ($P < 0.01$) with the exception that there was no significant difference between healthy plants supplied 2 mmol dm^{-3} and 8 mmol dm^{-3} NH_4^+.

Effect of disease

Healthy plants had higher transpiration rates than diseased plants, but differences were only significant between healthy and diseased plants supplied 2 mmol dm^{-3} NO_3^- ($P < 0.05$) and between healthy and diseased plants supplied 8 mmol dm^{-3} NH_4^+ ($P < 0.001$).

Water use efficiency (WUE)

Effect of N form

N form only affected WUE (**Fig. 5**) in diseased plants supplied 8 mmol dm^{-3} N, where plants supplied NO_3^- used 15 % more water per mol of CO_2 fixed than plants supplied NH_4^+, and therefore had higher WUE (but were less efficient).

Effect of N concentration

Plants supplied 2 mmol dm^{-3} NO_3^- had significantly lower WUE than plants supplied 8 mmol dm^{-3} NO_3^- (24 % more water per mol of CO_2 fixed was used by healthy plants and 11 % more water was used by diseased plants supplied 2 mmol dm^{-3} N) ($P < 0.05$).

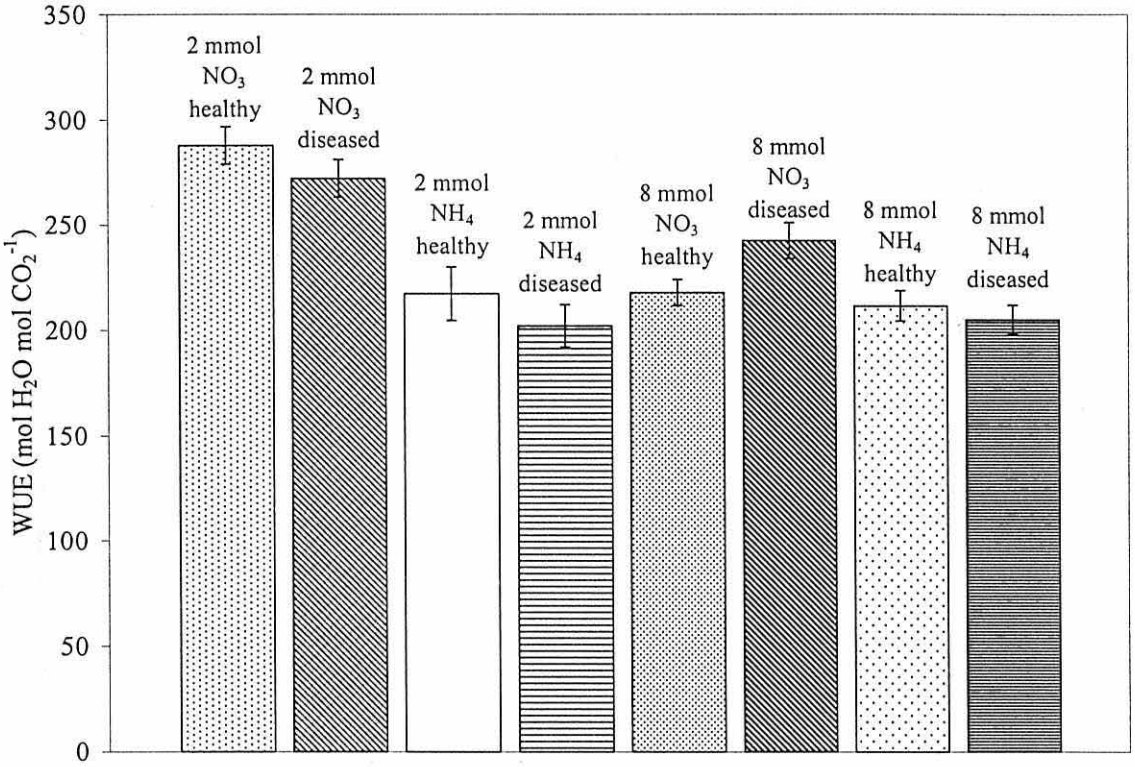

Figure 5 Water use efficiency (WUE; mean ± SE) of second leaves of wheat from plants that were supplied N as either 2 mmol dm^{-3} (labelled as 2 mmol on graph) $NaNO_3$ or $(NH_4)_2$ SO_4 or 8 mmol dm^{-3} (labelled as 8 mmol on graph) $NaNO_3$ or $(NH_4)_2$ SO_4. Second leaves of plants were inoculated when plants were 14 d old. Photosynthesis was measured 10 dai (2 mmol dm^{-3} N) or 11 dai (8 mmol dm^{-3} N). n = 3.

Effect of disease

There was no significant difference in WUE between diseased and healthy plants.

Leaf surface conductance

Effect of N form

Leaf surface conductance (**Fig. 6**) was significantly higher in plants supplied NO_3^- than plants supplied NH_4^+ ($P < 0.001$).

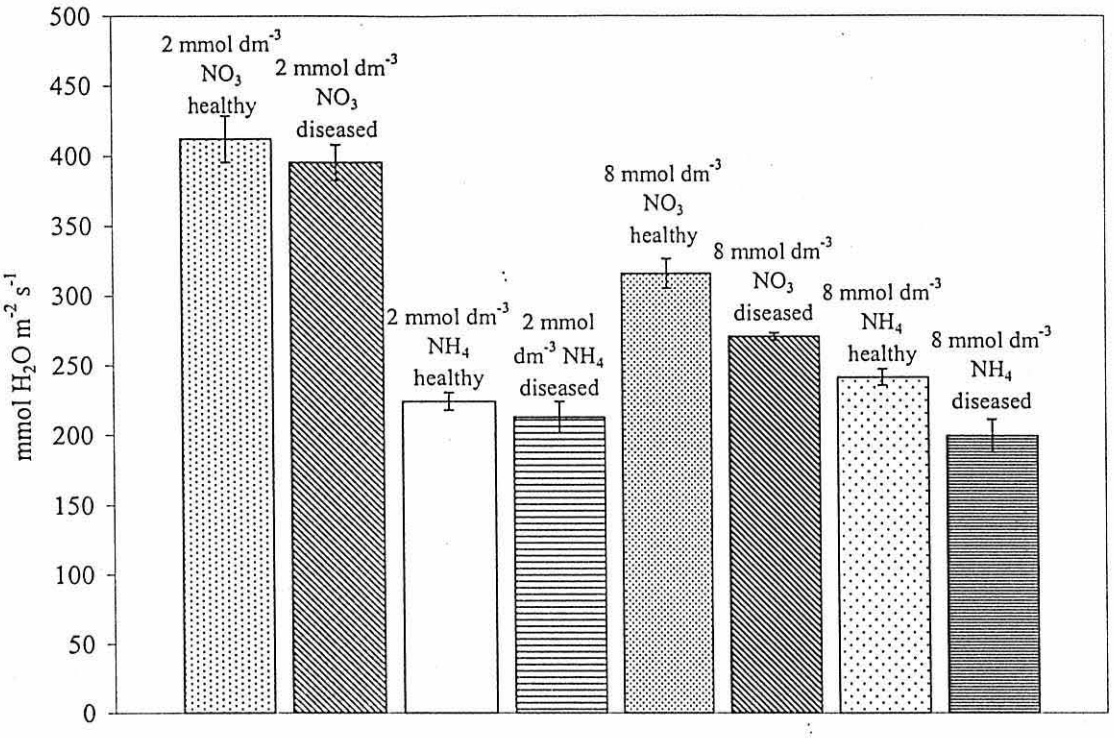

Figure 6 Leaf surface conductance (mean ± SE) of second leaves of wheat from plants that were supplied N as either 2 mmol dm^{-3} $NaNO_3$ or $(NH_4)_2$ SO_4 or 8 mmol dm^{-3} $NaNO_3$ or $(NH_4)_2$ SO_4. Second leaves of plants were inoculated when plants were 14 d old. Photosynthesis was measured 10 dai (2 mmol dm^{-3} N) or 11 dai (8 mmol dm^{-3} N). n = 3

Effect of N concentration

Leaf surface conductance was significantly higher in plants supplied 2 mmol dm^{-3} NO_3^- than plants supplied 8 mmol dm^{-3} NO_3^- ($P < 0.001$).

Effect of disease

There was no significant difference in leaf surface conductance between diseased and healthy plants supplied 2 mmol dm^{-3} N. In plants supplied 8 mmol dm^{-3} N, leaf surface conductance was significantly higher in healthy plants than in diseased plants ($P < 0.01$).

Disease symptoms on leaf 2

Symptoms first appeared 3-4 dai as very small, yellow spots on the leaves, changing to pale brown spots by 6-7 dai. Lesions were initially fairly uniform in size; they expanded in length (to, on average, 1-2 mm long but some were larger) more than width, and became dark brown and oval surrounded by pale green–yellow tissue as

time progressed. Lesion numbers appeared to increase with time – this may be due to early lesions being missed during counting, as they were difficult to see, rather than new infection sites appearing. As lesions grew larger they coalesced, so lesion numbers appeared to decrease late in infection in some cases. Disease severity on leaf 2 changed over time, with differences between treatments becoming smaller as the leaf senesced. It must be remembered however that disease severity becomes harder to assess as the leaf ages, when it becomes difficult to distinguish between disease symptoms and senescence. Total lesion numbers and necrotic area varied between experiments, but the trends were the same, so data described below are from two representative experiments.

Effect of N form

Plants supplied NO_3^- had significantly fewer lesions and less diseased area on infected leaves than plants supplied NH_4^+ (**Figs 7 and 8**). Daily increase in lesion numbers (calculated as lesions $leaf^{-1}$ d^{-1}, not shown) was greatest in plants supplied NH_4^+. Lesion size was variable on all leaves but lesions were sometimes larger on plants supplied NH_4^+ (not shown).

Effect of concentration

There were negligible disease symptoms on plants supplied 0.04 mmol dm^{-3} N (either form) (**Fig. 7**) and most disease on plants grown in 2 and 8 mmol dm^{-3} NH_4^+ (**Fig. 8**). There was no significant difference in disease symptoms between plants supplied 2 mmol dm^{-3} N and plants supplied 8 mmol dm^{-3} N.

In the experiment comparing plants supplied 0.04 mmol dm^{-3} N and 2 mmol dm^{-3} N, daily increase in lesion numbers (calculated as lesions $leaf^{-1}$ d^{-1}) was greatest in plants supplied 2 mmol dm^{-3} N [24 and 11 lesions per d between 7 and 10 dai (NH_4^+ and NO_3^- respectively)]. In the experiment comparing plants supplied 2 and 8 mmol dm^{-3} N the daily increase in lesions was much smaller, but very similar between the two concentrations (average 6 lesions d^{-1} at both concentrations).

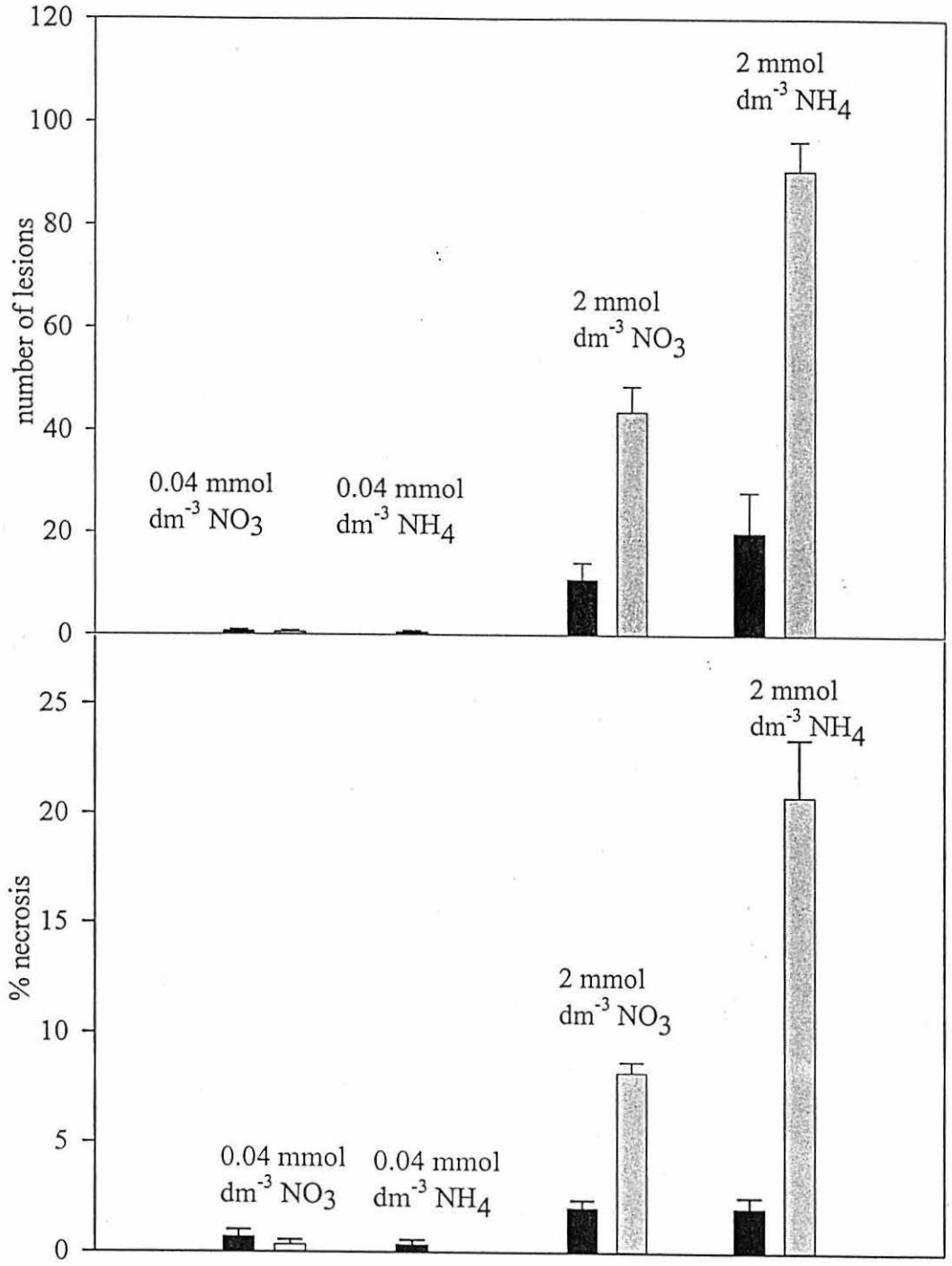

Figure 7 Lesion numbers (mean ± SE) and necrotic area (mean ± SE) 7 dai (black bars) and 10 dai (grey bars) on second leaves of wheat from plants that were supplied N as either 0.04 mmol dm^{-3} $NaNO_3$ or $(NH_4)_2$ SO_4 or 2 mmol dm^{-3} $NaNO_3$ or $(NH_4)_2$ SO_4. Second leaves of plants were inoculated when plants were 14 d old. n = 6

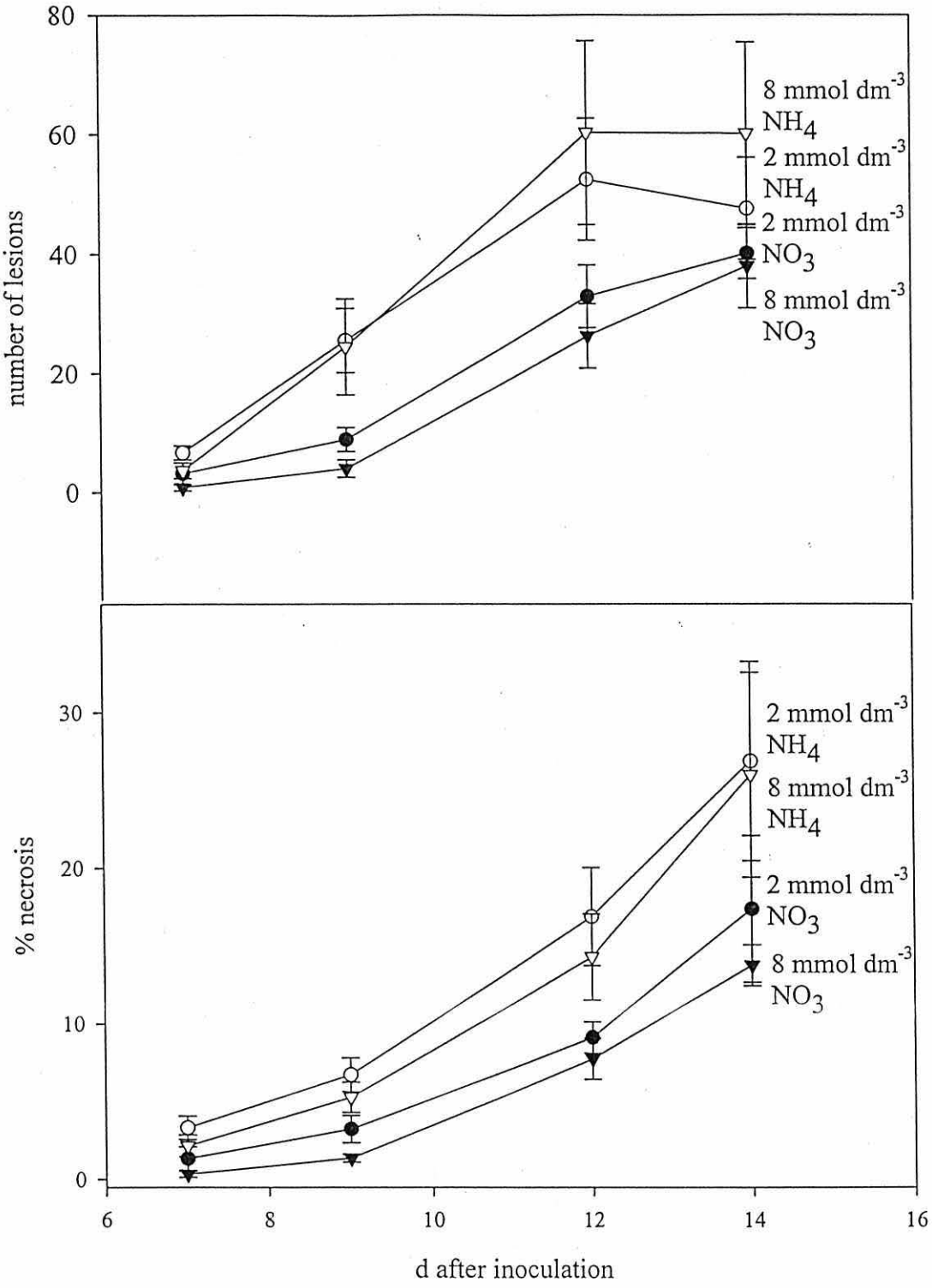

Figure 8 Lesion numbers (mean ± SE) and necrotic area (mean ± SE) on second leaves of wheat from plants that were supplied N as either 2 mmol dm^{-3} $NaNO_3$ or $(NH_4)_2$ SO_4 or 8 mmol dm^{-3} $NaNO_3$ or $(NH_4)_2$ SO_4. Second leaves of plants were inoculated when plants were 14 d old. n = 8

Efflux of ^{14}C

Substantial efflux of ^{14}C from mature leaves occurred over a 20 h period (almost 100 % of the fixed ^{14}C was exported in most cases). The loss of ^{14}C was described by a double exponential decay curve (describing a two phase exponential loss; an example of this is given in figure 9; mean values from all data are given in table 5).The double exponential decay curves of ^{14}C efflux had 4 parameters: A (representing ^{14}C partitioned into a readily transported pool of C from which rapid export occurs), B (the exponential coefficient of A), C (^{14}C partitioned into a temporary storage pool in the vacuole from which slow exponential export occurs) and D (the exponential coefficient of C). **Table 5** shows the mean values of the parameters obtained from the curves that showed ^{14}C efflux as a percentage of the maximum taken up by the leaf.

Table 5 Values (mean ± SE) of the four parameters of double exponential decay curves representing loss of $^{14}CO_2$ from fed leaves 8 d after inoculation with *S. nodorum*. Leaves were from plants that were either supplied N as 2 mmol dm^{-3} NO_3^- or NH_4^+. Diseased plants were inoculated with *S. nodorum* at 14 dai. n = 3

	A	B	C	D
NO_3^- healthy	52.7 ± 1.8	2.5 ± 0.4	47.3 ± 1.8	0.05 ± 0.00
NO_3^- diseased	60.8 ± 5.5	1.1 ± 0.2	39.2 ± 5.7	0.04 ± 0.00
NH_4^+ healthy	53.1 ± 9.9	1.1 ± 0.2	46.9 ± 9.9	0.06 ± 0.00
NH_4^+ diseased	62.6 ± 8.6	0.8 ± 0.2	37.4 ± 8.6	0.05 ± 0.01

Effect of N form

There was no significant difference in ^{14}C export from leaves of plants supplied NH_4^+ and NO_3^-, with the exception that leaves from healthy plants supplied NO_3^- had significantly higher exponential coefficients for the first phase (parameter B) than all other leaves.

Effect of disease

There were differences (although not statistically significant) between healthy and diseased leaves. Values for the initial uptake of ^{14}C (parameter A) were higher in diseased leaves than in healthy leaves, and values for the export of ^{14}C (parameter C), and the exponential coefficient of C (parameter D) were higher in healthy leaves than diseased leaves.

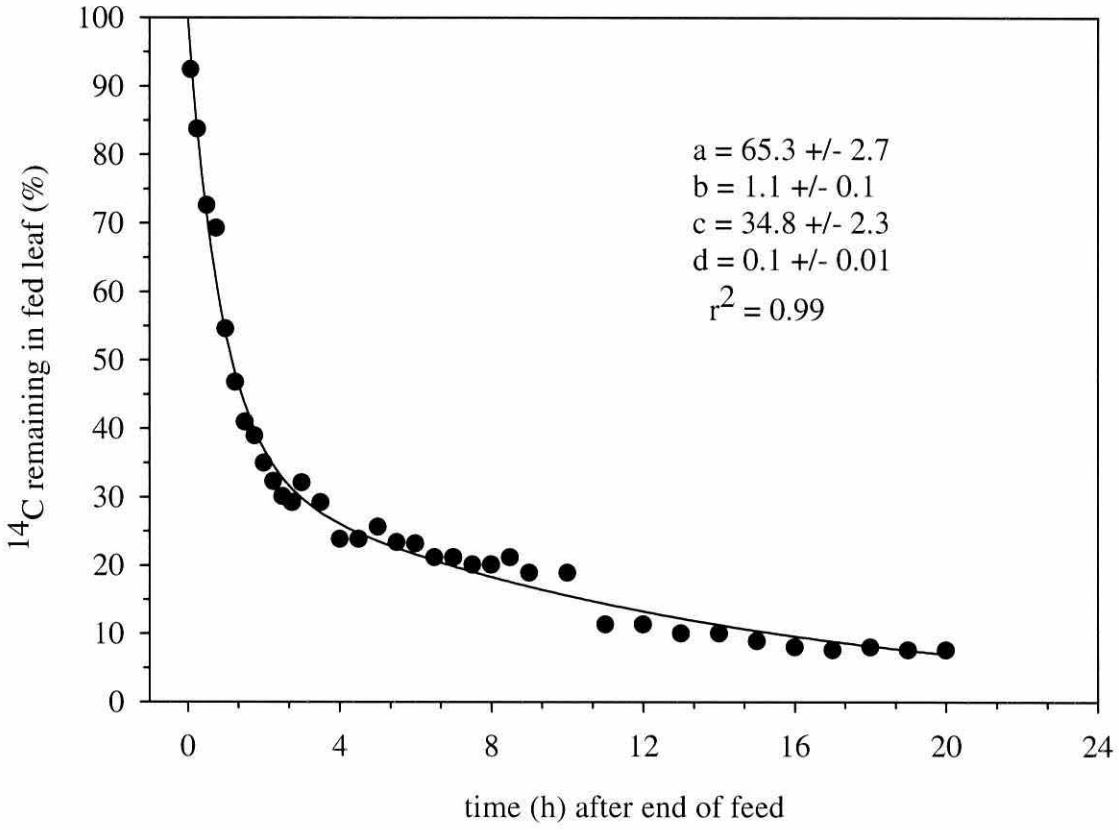

Figure 9 Example of a double exponential decay curve showing loss of ^{14}C from leaf 2 of a wheat plant during the 20 h period after feeding $^{14}CO_2$. The leaf was fed $^{14}CO_2$ for c. 0.3 h 8 d after inoculation with *S. nodorum*.

Autoradiography of leaves that had been fed $^{14}CO_2$ showed accumulation of fixed ^{14}C around lesion sites (**Plate 1**) 20 h after supplying the ^{14}C.

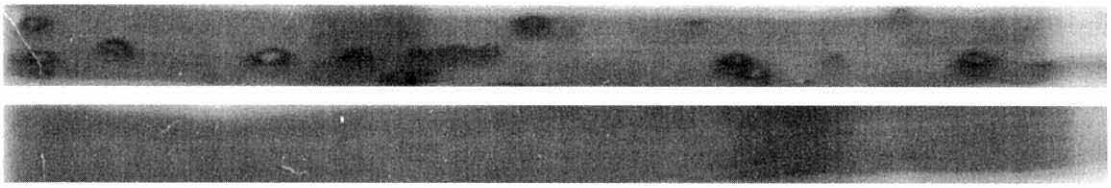

Plate 1 Autoradiographs of an infected (top) and healthy (bottom) wheat leaf 20 h after feeding with $^{14}CO_2$, showing accumulation of ^{14}C around lesion sites (dark regions are ^{14}C). Leaves were inoculated when plants were 14 d old and leaves were fed $^{14}CO_2$ 8 dai.

Whole plant dry weights

Effect of N form

There was no significant difference in whole plant dry weights between plants supplied NO_3^- and NH_4^+. The only exception to this was at 14 dai (**Table 7**) when

plants supplied 2 mmol dm^{-3} NO_3^- had significantly higher dry weights than plants supplied 2 mmol dm^{-3} NH_4^+ ($P < 0.05$).

Effect of N concentration

Plants supplied 2 mmol dm^{-3} N had significantly higher dry weights than plants supplied 0.04 mmol dm^{-3} N ($P < 0.01$) (**Table 6**). Plants supplied 0.04 mmol dm-3 N were visibly much smaller than plants supplied 2 mmol dm^{-3} N (**Plate 2**). There was no significant difference between plants supplied 2 mmol dm^{-3} N and plants supplied 8 mmol dm^{-3} N (**Table 7**), with the exception that healthy plants supplied 8 mmol dm^{-3} NH_4^+ had significantly higher dry weights than healthy plants supplied 2 mmol dm^{-3} NH_4^+, 14 dai ($P < 0.05$).

Plate 2 Wheat plants (24 d old) grown in hydroponics in a controlled environment cabinet. Plants were supplied N as (from left) 2 mmol dm^{-3} NO_3^-, 0.04 mmol dm_{-3} NO_3^-, 2 mmol dm^{-3} NH_4^+, 0.04 mmol dm^{-3} NH_4^+.

Effect of disease

Diseased plants had significantly lower dry weights than healthy plants at the two higher N concentrations 10 and 14 dai ($P < 0.01$) (**Tables 6 and 7**). There was no significant difference between diseased and healthy plants supplied 0.04 mmol dm^{-3} N (**Table 6**).

Table 6 Dry weights (g; mean ± SE) of whole plants (roots and shoots) from an experiment comparing the effects of NO_3^- and NH_4^+ at concentrations of 0.04 mmol dm^{-3} and 2 mmol dm^{-3}. Second leaves were inoculated with *S. nodorum* the day after full expansion, when plants were 14 d old. n = 4 (7 dai), or n = 6 (10 dai).

Treatment	7 dai		10 dai	
	Healthy	Diseased	Healthy	Diseased
0.04 mmol NO_3^-	135 ± 5	123 ± 20	141 ± 19	144 ± 15
0.04 mmol NH_4^+	119 ± 23	134 ± 22	117 ± 14	144 ± 16
2 mmol NO_3^-	343 ± 71	263 ± 19	601± 45	347 ± 65
2 mmol NH_4^+	357 ± 24	245 ± 11	571 ± 28	322 ± 62

Table 7 Dry weights (g; mean ± SE) of whole plants (roots and shoots) from an experiment comparing the effects of NO_3^- and NH_4^+ at concentrations of 2 mmol dm^{-3} and 8 mmol dm^{-3}. (Note different final harvest in this experiment). Second leaves were inoculated with *S. nodorum* the day after full expansion, when plants were 14 d old. n = 6 (7 dai), n = 8 (14 dai).

Treatment	7 dai		14 dai	
	Healthy	Diseased	Healthy	Diseased
2 mmol NO_3^-	367 ± 31	313 ± 46	1728 ± 98	1025 ± 149
2 mmol NH_4^+	318 ± 30	265 ± 20	1350 ± 57	905 ± 142
8 mmol NO_3^-	361 ± 43	305 ± 22	1665± 79	872 ± 136
8 mmol NH_4^+	332 ± 34	310 ± 32	1698 ± 52	911 ± 89

Relative growth rates (RGR)

Effect of N form

Plants supplied 0.04 mmol dm^{-3} NO_3^- had significantly higher RGRs over the period between 7 to 10 dai (when plants were 21-24 d old) than plants supplied 0.04 mmol dm^{-3} NH_4^+ (RGRs of healthy plants were 94 % higher and RGRs of diseased plants were 56 % higher in plants supplied NO_3^- than plants supplied NH_4^+) (**Fig.10**). There was no significant difference in RGR over the period between 7 to 14 dai (when plants were 21-28 d old) between plants supplied NO_3^- and NH_4^+ at the two higher concentrations (**Fig. 11**).

Effect of N concentration

Plants with the lowest RGRs were those supplied 0.04 mmol dm^{-3} N, with a maximum RGR being 0.18 mg mg^{-1} d^{-1}. RGRs of plants supplied 2 and 8 mmol dm^{-3} N were not significantly different from each other, but were much higher than those of plants supplied 0.04 mmol dm^{-3} N, with values between 0.3 and 0.6 mg mg^{-1} d^{-1}. The plants

grown at the two higher concentrations had a later final harvest, so plants would have been bigger due to the later harvest as well as the higher concentrations of N.

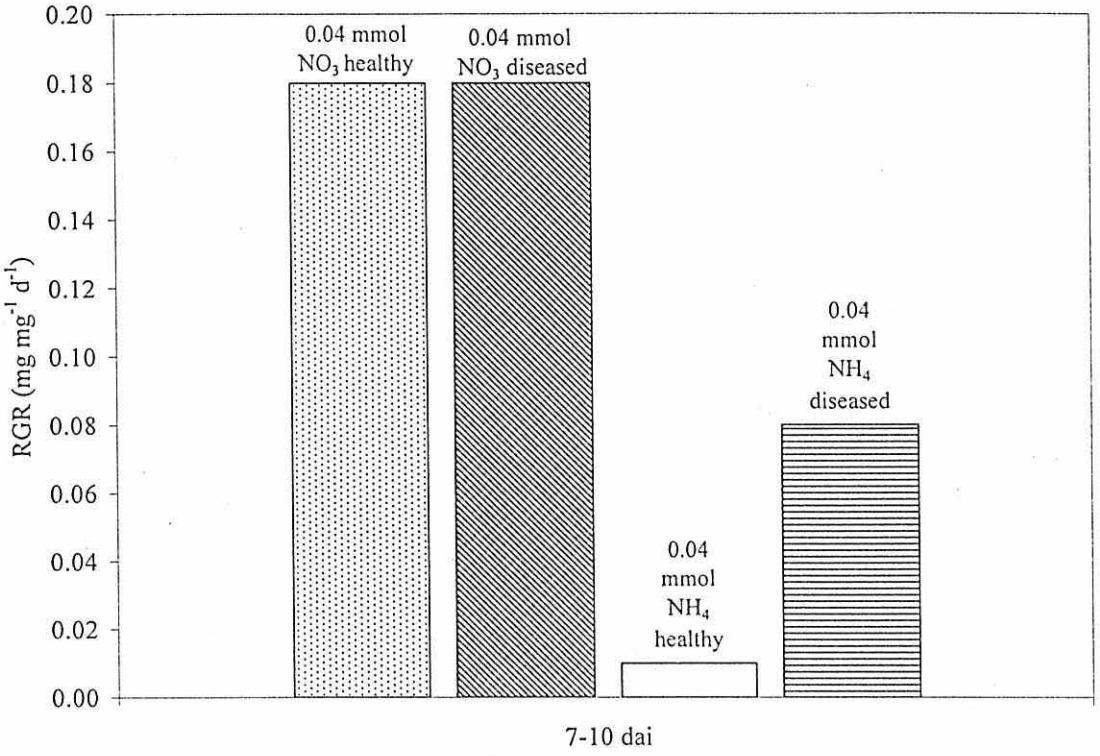

Figure 10 Relative growth rates (RGR) of wheat plants that were supplied N as either $NaNO_3$ or $(NH_4)_2 SO_4$ at 0.04 mmol dm^{-3} (labelled as 0.04 mmol on graph). Second leaves of plants were inoculated when plants were 14 d old.

Effect of disease

In plants supplied 0.04 mmol dm^{-3} NH_4^+, diseased plants had higher RGRs than healthy plants. In plants supplied 2 and 8 mmol dm^{-3} NO_3^- and NH_4^+, healthy plants had significantly higher RGRs than diseased plants.

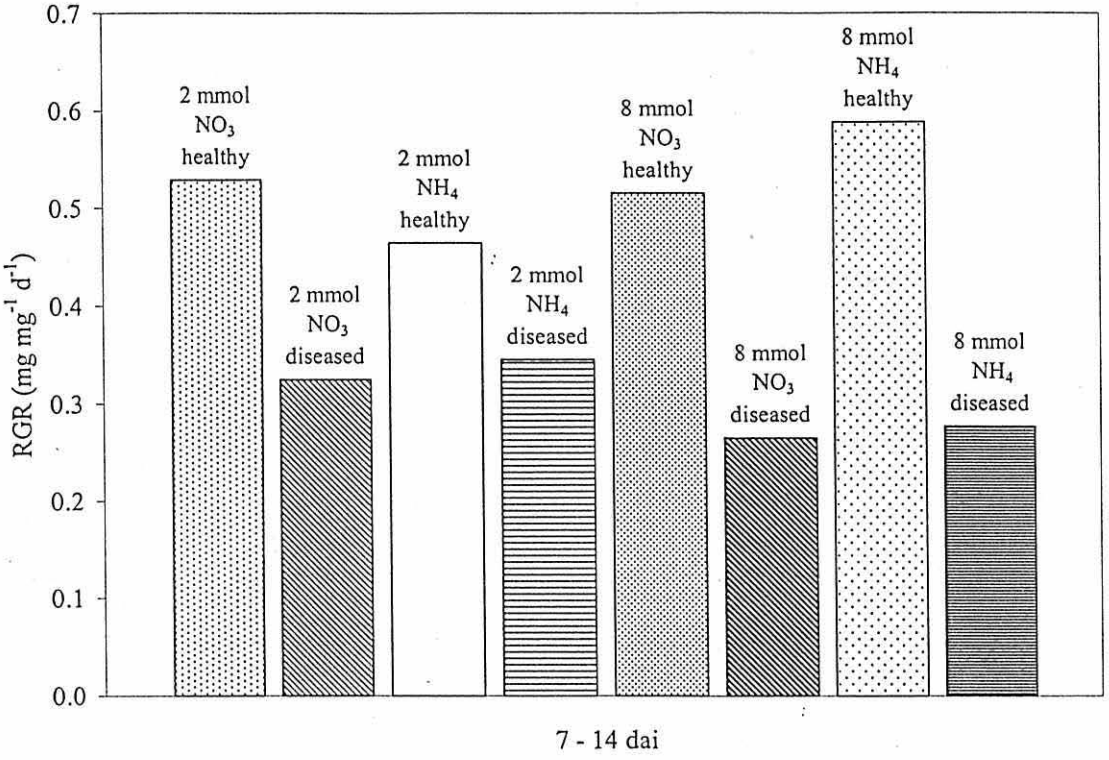

Figure 11 Relative growth rates (RGR) of wheat plants that were supplied N as either 2 mmol dm^{-3} (labelled as 2 mmol on graph) $NaNO_3$ or $(NH_4)_2 SO_4$ or 8 mmol dm^{-3} (labelled as 8 mmol on graph) $NaNO_3$ or $(NH_4)_2 SO_4$. Second leaves of plants were inoculated when plants were 14 d old.

Shoot: root partitioning of dry weight

Effect of N form

Shoot: root ratios appeared to be inconsistent between experiments. In one experiment (**Table 8**) plants supplied 2 mmol dm^{-3} NH_4^+ had higher shoot: root ratios than plants supplied 2 mmol dm^{-3} NO_3^- 7 and 10 dai ($P < 0.01$), mainly due to higher shoot dry weights in plants supplied NH_4^+. In another experiment there was no significant difference between plants supplied NO_3^- and NH_4^+ at 7 dai (**Table 9**), but at 14 dai healthy plants supplied 2 mmol dm^{-3} NO_3^- had higher S: R ratios than healthy plants supplied 2 mmol dm^{-3} NH_4^+ ($P < 0.001$). This was attributable to plants supplied NO_3^- having higher shoot weights than plants supplied NH_4^+. Diseased plants supplied 8 mmol dm^{-3} NH_4^+ had higher S: R ratios than diseased plants supplied 8 mmol dm^{-3} NO_3^- ($P < 0.01$). This was attributable to higher shoot weights and lower root weights in plants supplied 8 mmol dm^{-3} NH_4.

Effect of N concentration

Plants supplied 2 mmol dm^{-3} N had significantly higher S: R ratios than plants supplied 0.04 mmol N 7 and 10 dai ($P < 0.001$), and plants supplied 8 mmol dm^{-3} NH_4^+ had significantly higher S: R ratios than plants supplied 0.04 mmol dm^{-3} or 2 mmol dm^{-3} N as NH_4^+ (14 dai only, $P < 0.01$).

Effect of disease

The effect of disease on S: R ratio was also inconsistent. In one experiment there were no significant differences between diseased and healthy plants (**Table 8**), yet in another (**Table 9**), healthy plants supplied 2 mmol dm^{-3} N had significantly higher S: R ratios than diseased plants supplied 2 mmol dm^{-3} N, 7 dai ($P < 0.05$). Diseased plants supplied 8 mmol dm^{-3} N had higher S: R ratios than healthy plants supplied 8 mmol dm^{-3} N 14 dai ($P < 0.01$).

Table 8 Shoot: root ratios (mean ± SE) of wheat plants from an experiment comparing the effects of NO_3^- and NH_4^+ at concentrations of 0.04 mmol dm^{-3} and 2 mmol dm^{-3}. Second leaves were inoculated with *S. nodorum* the day after full expansion, when plants were 14 d old. n = 4 (7 dai), or n = 6 (10 dai).

Treatment	7 dai		10 dai	
	Healthy	Diseased	Healthy	Diseased
0.04 mmol NO_3^-	0.6 ± 0.0	0.6 ± 0.0	0.5 ± 0.1	0.5 ± 0.1
0.04 mmol NH_4^+	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	0.5 ± 0.0
2 mmol NO_3^-	2.2 ± 0.2	1.9 ± 0.3	2.1 ± 0.1	1.9 ± 0.1
2 mmol NH_4^+	2.5 ± 0.1	2.5 ± 0.1	2.2 ± 0.0	2.3 ± 0.1

Table 9 Shoot: root ratios (mean ± SE) of plants from an experiment comparing the effects of NO_3^- and NH_4^+ at concentrations of 2 mmol dm^{-3} and 8 mmol dm^{-3}. (Note different final harvest in this experiment). Second leaves were inoculated with *S. nodorum* the day after full expansion, when plants were 14 d old. n = 6 (7 dai), n = 8 (14 dai).

Treatment	7 dai		14 dai	
	Healthy	Diseased	Healthy	Diseased
2 mmol NO_3^-	2.5 ± 0.3	1.9 ± 0.3	2.3 ± 0.1	2.4 ± 0.1
2 mmol NH_4^+	2.3 ± 0.2	2.0 ± 0.2	1.6 ± 0.1	1.9 ± 0.2
8 mmol NO_3^-	2.5 ± 0.2	2.6 ± 0.2	2.3 ± 0.1	2.9 ± 0.2
8 mmol NH_4^+	2.8 ± 0.1	2.6 ± 0.2	2.5 ± 0.1	3.9 ± 0.4

The apparent inconsistencies between experiments disappeared when data were plotted allometrically (**Fig. 12**). It became clear that neither N form nor disease affect partitioning between shoot and root, but N concentration does have an effect by increasing both root and shoot weights, and increasing partitioning of biomass to the shoot at low N supply.

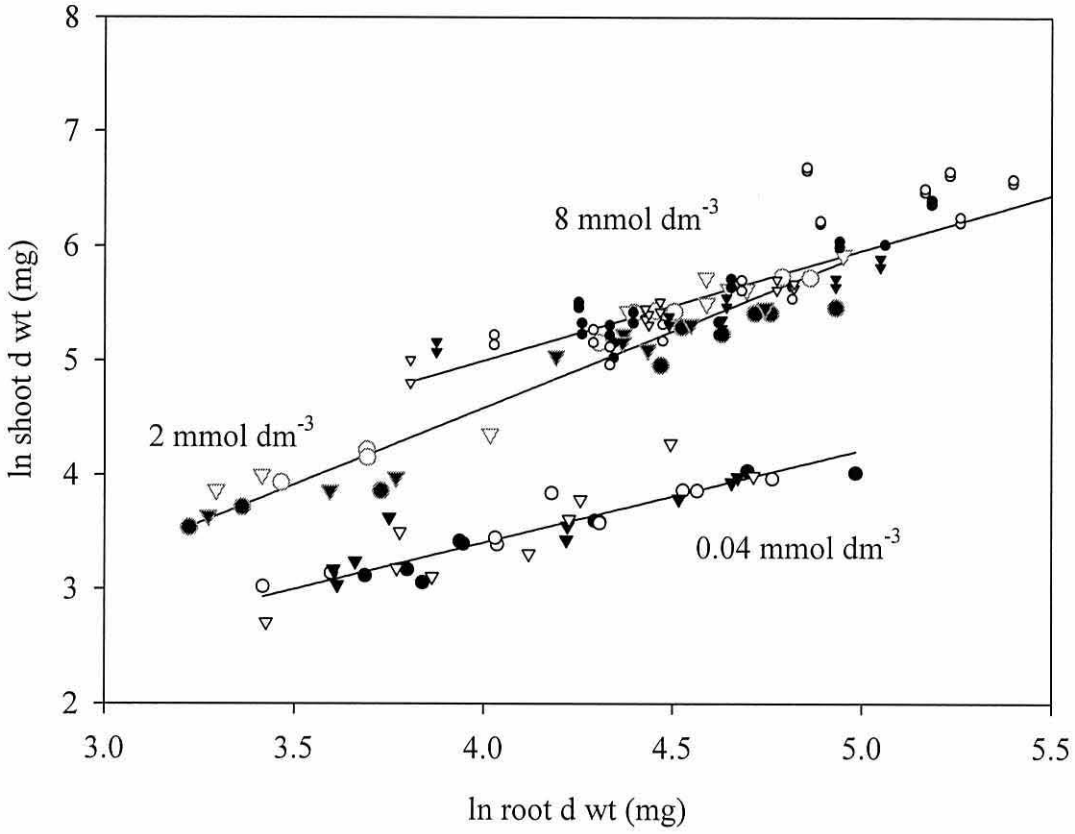

Figure 12 The allometric relationship between shoot and root dry weight for wheat plants. Plants were supplied N as $NaNO_3$ (closed symbols) or $(NH_4)_2$ SO_4 (open symbols) at 0.04 mmol dm^{-3} (*r* 0.91), 2 mmol dm^{-3} (*r* 0.97) or 8 mmol dm^{-3} (*r* 0.96). Second leaves of plants were inoculated with *S. nodorum* when plants were 14 d old. (diseased ●,○,○; healthy ▼, ▽, ▽)

Table 10 Summary of main findings. NSD = no significant difference.

Analysis	Effect of N form	Effect of N concentration	Effect of disease
Dry weight of leaf 2	$NH_4^+ > NO_3^-$	2 & 8 mmol dm^{-3} > 0.04 mmol dm^{-3}	NSD
Specific leaf area	$NO_3^- > NH_4^+$ 0.04 mmol dm^{-3} NSD 2 mmol dm^{-3} N/A 8 mmol dm^{-3}	0.04 mmol dm^{-3} > 2 mmol dm^{-3} N/A 8 mmol dm^{-3}	NSD
Chlorophyll content (using SPAD meter)	NSD except 2 mmol dm^{-3} $NO_3^- < NH_4^+$ 7 dai $NO_3^- > NH_4^+$ 10 dai	8 mmol dm^{-3} > 2 mmol dm^{-3} > 0.04 mmol dm^{-3}	NSD except 2 mmol dm^{-3} NH_4^+ healthy > diseased
Net photosynthesis	$NO_3^- > NH_4^+$	8 mmol dm^{-3} NO_3^- > 2 mmol dm^{-3} NO_3^- 8 mmol dm^{-3} NH_4^+ < 2 mmol dm^{-3} NH_4^+ (diseased only)	Healthy > diseased (8 mmol dm^{-3} only)
Sub-stomatal CO_2 concentration (Ci)	$NO_3^- > NH_4^+$	8 mmol dm^{-3} NO_3^- < 2 mmol dm^{-3} NO_3^- 8 mmol dm^{-3} NH_4^+ > 2 mmol dm^{-3}	NSD
Transpiration	$NO_3^- > NH_4^+$	2 mmol dm^{-3} > 8 mmol dm^{-3}	Healthy > diseased (2 mmol dm^{-3} NO_3^-, 8 mmol dm^{-3} NH_4^+)
WUE	8 mmol dm^{-3} diseased NO_3^- less efficient than NH_4^+	2 mmol dm^{-3} NO_3^- less efficient than 8 mmol dm^{-3} NO_3^-	NSD
Leaf surface conductance	$NO_3^- > NH_4^+$	2 mmol dm^{-3} >8 mmol dm^{-3}	Healthy > diseased (8 mmol dm^{-3})
Disease symptoms	$NO_3^- < NH_4^+$	2 & 8 mmol dm^{-3} > 0.04 mmol dm^{-3}	N / A
Efflux of $^{14}CO_2$	NSD	N / A	NSD
Dry weights	14 dai 2 mmol dm^{-3} $NO_3^- > NH_4^+$	2 & 8 mmol dm^{-3} > 0.04 mmol dm^{-3}	Healthy > diseased
Shoot: root ratios and allometry	NSD	8 > 2 > 0.04 mmol dm^{-3}	NSD

Discussion

Both N form and concentration regulated the infection process and affected plant growth and partitioning. NH_4^+ and high concentrations of N favoured infection.

Effect of N supply and disease on plant biomass, dry matter partitioning, photosynthesis and photosynthetic C export

The hypothesis that plants supplied high (8 mmol dm^{-3}) concentrations of N will have the highest dry weights and shoot: root (S: R) ratios, and plants supplied moderate (2 mmol dm^{-3}) concentrations of N will have higher dry weights and S: R ratios than plants supplied low (0.04 mmol dm^{-3}) concentrations of N is, at least partially, accepted. Plants supplied 8 mmol dm^{-3} N and 2 mmol dm^{-3} N did have significantly higher dry weights than plants supplied 0.04 mmol dm^{-3} N but there was no significant difference in dry weights between them. Dry weights of leaf 2 were also significantly higher in plants supplied 2 mmol dm^{-3} N than plants supplied 0.04 mmol dm^{-3} N. S: R ratios were generally in agreement with the hypothesis although data were somewhat inconsistent; however plotting shoot and root dry weights allometrically revealed that whilst there were small differences attributed to N form and disease, only N concentration consistently affected partitioning of biomass to shoots and roots. Partitioning to the shoot was highest in plants supplied 8 mmol dm^{-3} N and lowest in plants supplied 0.04 mmol dm^{-3} N. Increasing N supply tends to increase the growth of both roots and shoots, but shoot growth increases more than root growth, causing an increase in S: R ratio. The surface area of roots may increase with high N supply as branching of the roots tends to increase (Marschner, 1995) but root growth is favoured more than shoot growth when N supply is deficient, in order to maximise uptake of N (Lawlor, 2002). See later for further discussion on the effects of N supply on S: R partitioning.

The hypothesis that plants supplied high (8 mmol dm^{-3}) concentrations of N will have higher rates of photosynthesis and greater amounts of chlorophyll than plants supplied moderate (2 mmol dm^{-3}) concentrations of N is partially accepted. N concentration only affected photosynthesis in diseased plants and the effect of N concentration on photosynthesis was linked to N form, where the effect on plants supplied NO_3^- at a particular concentration was generally opposite to the effect on plants supplied NH_4^+. In diseased plants supplied NO_3^- the hypothesis that plants supplied 8 mmol dm^{-3} N

would have higher rates of photosynthesis than plants supplied 2 mmol dm^{-3} N was supported, but diseased plants supplied NH_4^+ had higher photosynthetic rates at the lower concentration of N (2 mmol dm^{-3}). Sub-stomatal CO_2 concentrations were higher in plants supplied 8 mmol dm^{-3} NO_3 than in plants supplied 2 mmol dm^{-3} NO_3, but transpiration rates and stomatal conductances were higher in plants supplied 2 mmol dm^{-3} N than plants supplied 8 mmol dm^{-3} N. Plants supplied 8 mmol dm^{-3} N used less water per mole of C fixed, therefore they had greater water use efficiency (WUE).

Chlorophyll contents were significantly reduced in plants supplied 0.04 mmol dm^{-3} N, and they were highest in plants supplied 8 mmol dm^{-3}, as predicted by the hypothesis. Chlorophyll content may be correlated with net photosynthesis (Grindlay, 1997) Measurements of photosynthesis were not carried out on plants supplied 0.04 mmol dm^{-3} due to time constraints, but I suggest their photosynthetic rates would have been severely depressed in comparison with plants supplied N at higher concentrations. Net photosynthesis under given environmental conditions is correlated with leaf N content (Grindlay, 1997), and net photosynthesis (under saturating photon flux density) tends to increase in a roughly linear fashion with increasing N per unit leaf area (Field and Mooney, 1986; Evans, 1989a). The photosynthetic rate of cocksfoot (*Dactylis glomerata* L). was depressed when NO_3^- was supplied at low concentrations (0.6 mmol dm-3) in comparison with higher concentrations of NO_3 (0.6, 1.5 and 6 mmol dm-3). N contents and Rubisco contents of leaves were decreased significantly when the concentration of N supplied was below 0.15 mmol dm^{-3} (Harmens *et al.*, 2000). In field-grown winter wheat supplied no N or 200 kg ha^{-1} N fertiliser, and spring wheat grown in sand culture supplied 0.1, 1 or 20 mmol dm^{-3} NO_3^-, both net photosynthesis and carboxylation efficiency increased with increasing N supply. Correspondingly the amount and activity of Rubisco protein increased with increasing N supply. Rubisco accounted for approximately 50 % of the total soluble proteins in the flag leaf, and Rubisco content decreased with age (and according to N supply) in parallel to soluble protein contents (Lawlor *et al.*, 1989).

The hypothesis that plants supplied NO_3^- will have higher dry weights and lower S: R ratios than plants supplied NH_4^+ is rejected. On the whole, dry weight data did not agree with the hypothesis as there was no significant difference in dry mass between

plants supplied NO_3^- and NH_4^+, but at the latest harvest (14 dai) in one experiment plants supplied 2 mmol dm^{-3} NO_3^- did have higher dry weights than plants supplied 2 mmol NH_4^+, as predicted by the hypothesis. Other researchers have found that plants supplied NH_4^+ have lower dry weights than plants supplied NO_3^- (Claussen, 2002; Cramer and Lewis, 1993*a*).

S: R ratio results were inconsistent, but in some cases S: R ratios were lower in plants supplied NO_3^- than plants supplied NH_4^+ which agreed with the hypothesis. Plotting shoot dry weights against root dry weights allometrically did not reveal a consistent difference in partitioning of biomass between roots and shoots between plants supplied NO_3^- and NH_4^+. In plants supplied NO_3^- and NH_4^+ S: R ratios increase with increasing N concentration, and in some species plants of similar dry weights can have higher S: R ratios in plants supplied NH_4^+ than those supplied NO_3^- (Andrews *et al*; 2001). Investigations into the mechanisms governing partitioning of dry matter to shoot and root have revealed that there is a strong positive correlation between leaf soluble protein content and plant dry weight that explain most of the variation in S: R ratios. Leaf N concentration was also correlated with dry weight, but leaf soluble protein content is more important in determining S: R ratios (Andrews *et al.,* 1999; Andrews *et al.,* 2001). Here, although results were inconsistent, in one experiment where plants supplied NH_4^+ had higher S: R ratios than plants supplied NO_3^- the soluble protein content of second leaves was measured, and plants supplied NH_4^+ did, at least initially, have higher soluble protein contents than plants supplied NO_3^- (Chapter 3), which supports the findings of Andrews *et al.* (1999). NH_4^+ tends to have a negative effect on root growth (Cramer and Lewis, 1993*a*) and as NH_4^+ assimilation takes place in the roots and requires large amounts of C skeletons, this may divert assimilates away from root growth (Andrews *et al.,* 2001; Marschner, 1995). The use of C skeletons in the N assimilation process is not thought to have a significant effect on S: R ratios however (Zerihun *et al.,* 1998).

The hypothesis that plants supplied NO_3^- will have higher rates of photosynthesis, higher leaf surface conductance and higher sub-stomatal CO_2 concentrations than plants supplied NH_4^+ is accepted. With the exception of diseased plants supplied 2 mmol dm^{-3} N where there was no significant difference, plants supplied NO_3^- had higher net photosynthesis than plants supplied NH_4^+. Plants supplied NO_3^- had higher

sub-stomatal CO_2 concentrations than plants supplied NH_4^+, with the exception of healthy plants supplied 8 mmol dm^{-3} N where there was no significant difference. Transpiration rates and leaf surface conductance were both higher in plants supplied NO_3^- than plants supplied NH_4^+. Plants supplied NH_4^+ had higher mean water use efficiencies than plants supplied NO_3, particularly in plants supplied 2 mmol dm^{-3} N. This is in agreement with results reported by Yin and Raven (1998), where wheat plants supplied 2 mmol dm^{-3} NH_4^+ had significantly higher WUE than plants supplied 2 mmol NO_3^- or NH_4NO_3 (Yin and Raven, 1998); however there was no difference in WUE between wheat plants supplied NO_3^- and NH_4^+ at 4 mmol dm^{-3} or 12 mmol dm^{-3} (Cramer and Lewis, 1993*a*).

Chlorophyll content was affected by N form in plants supplied 2 mmol dm^{-3} N. Results only agreed with the hypothesis 10 dai, as prior to this harvest plants supplied NH_4^+ had the highest chlorophyll content. Chlorophyll content decreased over time in all treatments, but it probably decreased to a greater extent in plants supplied NH_4^+ due to their greater amounts of infection and associated chlorosis.

The hypothesis that plants supplied NO_3^- will have higher rates of ^{14}C efflux than plants supplied NH_4^+ is rejected as there was no significant difference in efflux between plants supplied NO_3^- and NH_4^+. Rapid exponential export of fixed ^{14}C from fed leaves was observed in all treatments in the first 2 h after feeding, followed by a slower exponential phase between 2 and 20 h after feeding. The exponential coefficient of the first phase (parameter B) was significantly higher in leaves from healthy plants fed NO_3^- than in all other leaves (Table 5). This was the only statistically significant difference. The values for the other parameters are clearly similar between plants supplied NO_3^- and NH_4^+. There are however non- significant but nevertheless interesting differences between diseased and healthy plants. During the first phase of export approximately 10 % more ^{14}C was exported from leaves of diseased plants than from healthy plants, suggesting that the labile (transport) pool of C was larger in infected leaves. Consequently, during the second phase of efflux, leaves from diseased plants had less ^{14}C available for export than healthy plants, suggesting smaller storage pools of C. Interestingly, the total amount of ^{14}C taken up by the leaves (not shown) was consistently less in diseased plants than in healthy plants, although differences were not significant. This would be expected if

photosynthesis was depressed by the presence of disease. Net photosynthesis was not measured on the plants that were fed ^{14}C, but in plants supplied 2 mmol dm^{-3} N from another experiment where photosynthesis was measured there was no significant difference in net photosynthesis between diseased and healthy plants at 10 dai. Efflux of ^{14}C was measured at a relatively early stage of infection (8 dai); however fed leaves from plants supplied NO_3^- had an average of 31 lesions and 10 % necrosis, and fed leaves from plants supplied NH_4^+ had an average of 43 lesions and 13 % necrosis, and the fungus had probably begun to ramify into the mesophyll tissue (Karjalainan and Lounatmaa, 1986). ^{14}C had clearly accumulated around lesions, which indicates accumulation of the ^{14}C by the fungal mycelium; perhaps there were alterations in C export from infected leaves that were too small to be detected in this analysis.

During later stages of infection lesions appeared on younger leaves that were not yet expanded when second leaves were being inoculated. As a consequence, perhaps these younger infected leaves were diverting assimilates from the infected leaves that were fed $^{14}CO_2$. Quantification of ^{14}C in the rest of the plant would have been useful to investigate the distribution of assimilates. Wafford and Whitbread (1976) investigated the effect of infection with *S. nodorum* on translocation of ^{14}C-labelled assimilates in spring wheat (cv. Kolibri). There was little difference in the total fixation of $^{14}CO_2$ between healthy and diseased leaves, regardless of whether the fed leaf was infected or a healthy leaf on an infected plant. In most cases export of ^{14}C was unaffected by infection, with the exception that at 5 dai export of ^{14}C from leaf 6 was reduced by almost 10 % in infected leaves. Analysis of ^{14}C in the rest of the plant showed a high proportion of ^{14}C had accumulated in leaf 7 (the flag leaf). At 12 dai, when infected leaves had 60 to 70 % necrosis, export of ^{14}C from leaf 6 was slightly higher in infected plants than healthy plants (Wafford and Whitbread, 1976). Here, leaves were fed ^{14}C at 8 dai and had low levels of infection, but as in the study by Wafford and Whitbread, when efflux was measured at 12 dai export from infected leaves appeared to be slightly increased (although not significantly).

The retention of assimilates by infected leaves is often higher for leaves infected by biotrophs than leaves infected by necrotrophs (Wafford and Whitbread, 1976). Hibberd *et al.*, (1996*b*) suggest that accumulation of assimilates by infected leaves may depend on the initial carbohydrate status of leaves however, in studies reporting

accumulation of sucrose by leaves infected with powdery mildew, the initial leaf sucrose contents were low (Hibberd *et al.*, 1996*b*). In barley leaves infected with powdery mildew (*Erysiphe graminis*) soluble carbohydrate and sucrose concentrations were similar in healthy and infected leaves, and there was no difference in efflux of ^{14}C from second leaves 3 and 5 dai. When compartmental analysis was used to calculate the sizes of the labile and storage pools of C within the leaf however at 5 dai infection reduced the size of the labile pool, and so reduced the export of C (Hibberd *et al.*, 1996*b*).

Effect of N supply on disease resistance

The hypothesis that high concentrations of N will increase susceptibility of wheat to *S. nodorum* is accepted. Plants supplied 0.04 mmol dm^{-3} N had very few disease symptoms, and plants supplied 2 and 8 mmol dm^{-3} N had the most disease symptoms. Plants supplied 0.04 mmol dm^{-3} N were slower to develop than plants supplied higher concentrations of N (they had to be sown 2 d before plants supplied higher concentrations of N in order for them to be at the same developmental stage when inoculating). They were very pale (with little chlorophyll), small plants which produced little biomass over the experimental period and senesced quickly. As plants supplied 0.04 mmol dm^{-3} N were likely to have been suffering from N deficiency they would probably have had higher concentrations of polyphenols and lignin (Matsuyama and Dimond, 1973, Marschner 1995), which can act as defence compounds.

That there was no significant difference in disease symptoms between plants supplied 2 mmol dm^{-3} N and 8 mmol dm^{-3} N is a little surprising, considering there is a four-fold difference in N supply; however, this extra N is likely to represent accumulation of N (as NO_3^- or reduced N compounds) due to luxury consumption, which can occur when the rate of N supply exceeds the amount of N required for maximum growth (Justes *et al.*, 1994). Plotting disease symptoms against the N content of leaves revealed a negative (but non- significant) relationship (Chapter 3). This seems odd as plants supplied high concentrations of N had significantly more N. More data points may have improved the significance of this plot, as only a sub-sample of infected leaves was analysed for total N content, and variability of symptoms between individual leaves was relatively high. In an experiment to investigate the effect of

shading on the resistance of wheat to *S. nodorum* (Chapter 5) a significant negative relationship was found between lesion numbers and leaf N content. There was a corresponding positive relationship between lesion numbers and C: N ratios of infected leaves, which was significant in the experiment described in Chapter 5, but not in the experiments described in this Chapter.

The hypothesis that plants supplied with NO_3^- as the sole N source will be less susceptible to *S. nodorum* than plants fed with NH_4^+ is accepted. Plants supplied NH_4^+ consistently had significantly more disease symptoms than plants supplied NO_3^-. Daily increase in lesion numbers and necrotic area also showed the same trend. Towards the end of each experiment the differences in symptoms between plants supplied NO_3^- and NH_4^+ lessened, suggesting that although supply of NO_3^- to plants reduces disease symptoms in comparison to plants supplied NH_4^+, symptom development may only be delayed in plants supplied NO_3^- (see fig. 8). Identifying the differences between plants supplied NO_3^- and NH_4^+ is necessary in order to investigate possible mechanisms of resistance. This issue is addressed in Chapter 3.

Lesion number and diseased area are components of partial resistance which are measured to assess environmental resistance (Jeger *et al.,* 1983; Jørgensen and Smedegaard-Petersen, 1999). Lesion number and diseased area represent infection frequency (number of lesions at the end of the incubation period) and necrosis (the percentage leaf area necrotic (and chlorotic) at the end of the latent period, discounting the area of natural senescence on healthy leaves) respectively. Other components of partial resistance which may be measured include: incubation period (the number of days between inoculation and appearance of the first symptoms); latent period (number of days from inoculation to the first appearance of pycnidia visible under low magnification); lesion size (the length of four representative lesions at the end of the latent period); lesion cover (the percentage leaf area covered by lesions at the end of the latent period, disregarding necrotic (and chlorotic) areas; and spore production (the number of conidia washed from leaves at the end of the latent period) (Jeger *et al.,* 1983). All the above are useful measures of partial resistance, but it is not always practical (or necessary) to assess all components. In this study, incubation period and lesion size were noted but not routinely measured, and attempts were made at counting pycnidia and spores but these were on the whole unsuccessful (although

pycnidia were observed in infected leaves at times), and it was generally felt that events occurring in the early stages of infection (penetration and colonisation) were more interesting or important than those occurring in the later stages. In controlled conditions (and on account of the relatively short experimental period) the plants did not always produce pycnidia; indeed pycnidial production or sporulation often needs to be induced under non-field conditions by enclosing plants in polythene bags or chambers to increase relative humidity (Jeger *et al.*, 1983).

Pycnidia were found in the lesions in this study, and production of spores was observed after incubation of heavily infected leaves on moist paper towels in Petri dishes, but after harvesting, rapid scanning followed by freezing, drying or freeze-drying of leaves prior to analysis at each harvest was necessary, and a shortage of plant material meant there were not usually any spare leaves from which to collect spores. Analysis of more of the components of partial resistance would however have been useful in further analysis of the effect of N supply on partial resistance.

Conclusions

In conclusion, increasing N supply increases net photosynthesis, chlorophyll contents, dry matter production and partitioning of dry matter to the shoot, and decreases SLA, transpiration rate and WUE. Supplying NO_3^- increases net photosynthesis, has some effect on dry matter production and decreases WUE compared to supplying NH_4^+. There is no significant difference in efflux of ^{14}C or S: R allometry, and differences in chlorophyll content vary with leaf age. The susceptibility of young wheat grown in controlled environments to *S. nodorum* is increased by supplying NH_4^+ and by supplying N at concentrations between 2 and 8 mmol dm^{-3}. This susceptibility may be due to altered leaf biochemistry, which will be discussed in Chapter 3.

Chapter 3 The physiological basis of the environmental resistance of wheat (*Triticum aestivum* cv. Brigadier) to *Stagonospora nodorum* (Berk.)

Introduction

The environmental resistance of wheat to *Stagonospora nodorum* was investigated (see Chapter 2). Wheat plants grown in hydroponics in a nutrient solution containing nitrate (NO_3^-) as the sole N source had fewer lesions and less necrosis after inoculation with *S. nodorum* compared with wheat plants supplied ammonium (NH_4^+) as the sole N source. There was negligible infection on plants supplied 0.04 mmol dm^{-3} N, and high amounts of infection on plants supplied 2 and 8 mmol dm^{-3} N. In response to these findings I investigated possible mechanisms of environmental resistance related to N supply.

Effects of N supply on contents of soluble carbohydrates and phenolics in leaves

Increasing N supply causes a decrease in the amounts of soluble and storage carbohydrates in plants. Increasing N supply to perennial ryegrass (*Lolium perenne* L.) increases leaf N and sometimes lignin content, but decreases the content of sucrose, polyfructosans and starch (Marschner, 1995). Increased N supply requires increased C skeletons for assimilation into organic N compounds, and increases the demand for assimilates to be diverted to growth (Ikeda *et al.*, 2004). Hoffland *et al* (1999) found that leaves of tomato (*Lycopersicon esculentum* L.) supplied high N had lower contents of soluble carbohydrates than those supplied low N. When N supply is deficient the concentration of amino acids and proteins in leaves is reduced and growth is decreased more than the rate of photosynthesis, resulting in an accumulation of soluble carbohydrates.

The amount and type of carbohydrates in plants can be affected by the form of N supplied to plants. Leaves of broad-leaved dock (*Rumex obtusifolius* L.) supplied 0, 1 or 5 mmol NO_3^- had lower soluble carbohydrate contents than those supplied 10 mmol NO_3^-, but plants supplied low concentrations of N (NO_3^- or NH_4^+) had higher concentrations of fructans than plants supplied high N and infected plants supplied NH_4^+ had more fructans than healthy plants supplied NH_4^+ (Hatcher and Ayres 1998). The soluble carbohydrate

contents of leaves from plants supplied NH_4^+ may be reduced compared to leaves of plants supplied NO_3^- due to the high demand for C skeletons required in the root for the assimilation of NH_4^+ into organic compounds. There is also high demand for C skeletons in the roots or leaves of plants supplied NO_3^- however, whilst NO_3^- may be taken up, transported and stored as NO_3^-, it must be oxidised to NO_2^{2-} and NH_4^+ prior to assimilation into organic N compounds (Pilbeam and Kirkby 1992; Marschner, 1999; Zerihun, 1997). Soluble carbohydrate contents of leaves from plants supplied NH_4^+ may therefore be lower, higher or no different to the soluble carbohydrate contents of leaves from plants supplied NO_3^-. There was no difference in the content of non-structural carbohydrates when hydroponically-grown *Lolium perenne* was supplied with N as NH_4^+ and NO_3^- (Gloser *et al.*, 2002).

The content of phenolics is often higher in plants with low N supply and N fertilisation reduces the content of phenolics in leaves of wheat (Kiraly, 1964 cited by Marschner, 1995) and tomato (Hoffland *et al.*, 1999*b*). The form and amount of N applied also affects the amount of phenolics in wheat to varying degrees depending on the plant organ (i.e. leaf, grain or straw) and the age of the organ (Sander and Heitefuss, 1998; Harms, 1983).

Effects of N supply on amino acid contents of leaves

The concentration of free amino acids in leaves increases with increasing N concentration (Haynes and Goh, 1978; Darral and Wareing, 1981; Barneix *et al.*, 1984, cited by Barneix and Causin, 1996) and concentrations of amino acids are higher in plants supplied NH_4^+ compared to NO_3^- (Causin *et al.*, 1992; Huber and Watson, 1974). Amino acid profiles are affected by N form, with plants supplied NH_4^+ generally containing more amides e.g. glutamine and asparagine (Barneix and Causin, 1996; Huber and Watson, 1974). Protein-N is higher in plants supplied NH_4^+ than plants supplied NO_3^- (Huber and Watson, 1974). High N availability to tomato plants increased amounts of soluble proteins in leaves (Hoffland *et al.*, 1999*b*).

Infection of plants by pathogens also affects their chemical composition; the focus of this chapter is on differences arising from the manipulation of N nutrition. Comparison of the

chemical profiles of diseased and healthy plants will provide an insight into effects that may be directly attributable to the pathogen.

Hypotheses

The aim of these experiments was to elucidate the differences between plants grown on NH_4^+ and NO_3^- at different concentrations, and hopefully to identify some of the chemical characteristics of wheat leaves. I chose to analyse substances that were known either to be altered by N nutrition (total N and C: N ratios, soluble polyphenols, soluble proteins, total amino acids and amino acid profiles) or by infection (soluble carbohydrates, total C and C: N ratio, soluble polyphenols and amino acid profiles) or both. The following hypotheses were tested:

1. Plants supplied moderate concentrations of N (2 mmol dm^{-3}) will have higher total amino acid contents and higher total N contents than plants supplied low concentrations of N (0.04 mmol dm^{-3}).

2. Plants supplied moderate and high concentrations of N (2 mmol dm^{-3} and 8 mmol dm^{-3}) will have lower soluble carbohydrate contents, lower soluble polyphenol contents and lower C: N ratios than plants supplied low concentrations of N (0.04 mmol dm^{-3}).

3. Plants supplied NH_4^+ will have higher soluble carbohydrate contents, higher total amino acid contents, higher soluble protein contents, lower soluble polyphenol contents, higher C: N ratios, and lower total N contents than plants supplied NO_3^-.

4. Plants supplied NH_4^+ will contain more amides (glutamine and asparagine) than plants supplied NO_3^-.

Methods

Growth of plants

For full details see Chapter 2, briefly:

Plants were grown in hydroponics in controlled environments. Long Ashton solution was modified to allow N form and concentration to be manipulated, and made up omitting the N, but with balancing ions re-supplied. $NaNO_3^-$ and $(NH_4^+)_2 SO_4$ solutions were made up separately. Six treatments were as follows: low NO_3^- or NH_4^+ (0.04 mmol), moderate NO_3^- or NH_4^+ (2 mmol), high NO_3^- or NH_4^+ (8 mmol). Nutrient solutions were changed twice weekly, and aerated at a rate of 1 dm^3 min^{-1}. Plants were grown in controlled environment cabinets (Sanyo-Gallenkamp, Fi-totron P G660/C/RO/HQI, Loughborough, UK) at a light intensity of 450 µmol photons m^{-2} s^{-1}, 70% RH, and ambient CO_2 concentration (350 µmol m^{-2} s^{-1}).

Inoculum production and inoculation procedure for S. nodorum

For details see previous chapter, but briefly:

Cultures were maintained on Czapek-Dox agar supplemented with Campbell's V8 juice (CZD-V8). Spore suspensions were made by flooding the culture with sterile, deionised water and agitating the surface of the colony with a sterile scalpel. The spore concentration was adjusted to 10^6 or 5 x 10^5 spores cm^{-3} if necessary. One drop of Tween 20 was added per 10 cm^3 of suspension. Plants were inoculated with *S. nodorum* when the second seedling leaf was fully expanded, using a squirrel hair paintbrush dipped in the suspension. After inoculation, plants were returned to growth cabinets. A humid atmosphere (required for spore germination) was maintained for the 48 h period after inoculation by enclosing troughs in Perspex boxes that had been sprayed inside with distilled water.

Analysis of plant material

For details see previous chapter, but briefly:

Harvests were carried out on two or three occasions after inoculation, with the first harvest being 1, 2 or 7 d after inoculation (dai), and the final harvest being 10 or 14 dai, depending on the analyses being carried out. At each harvest three to six diseased and

healthy plants per treatment were selected using random number tables. Each plant was separated into roots, shoots and blade of leaf 2. Blades of second leaves were scanned to allow measurement of leaf area then dried, frozen or freeze-dried prior to analysis (**Table 1**). The remaining shoot material and roots of each plant were then placed in labelled paper bags, dried to constant weight at 60 °C and weighed.

Table 1 N treatments and analyses carried out. For each analysis the number of days after inoculation (dai) that the plant material was harvested or non-destructive assessments were carried out is given. Blank squares indicate that the analysis was not performed on plants from the corresponding treatment. N.B. throughout this chapter, dai is used as a measure of time for both inoculated and uninoculated plants. Plants were inoculated with *S. nodorum* when 14 d old.

	N concentration supplied		
Analysis	**0.04 mmol dm-3**	**2 mmol dm-3**	**8 mmol dm-3**
Soluble carbohydrates	6 dai	7, 14 dai	7, 14 dai
Total Carbon and Nitrogen (CHN)	1, 6 dai	7, 14 dai	7, 14 dai
Total soluble polyphenols	7, 12 dai	7, 12 dai	
Total soluble proteins	1 dai	1, 7, 10 dai	
Total amino acids	1, 7, 10 dai	1, 7, 10 dai	
Amino acid profiles		1 dai (healthy) 10 dai (diseased)	

Extraction and analysis of soluble carbohydrates

(N.B. leaves from plants supplied all three concentrations of N were analysed, but leaves from plants supplied 0.04 mmol N were analysed 1 and 6 dai, whereas leaves from the other two concentrations were analysed 7 and 14 dai. Comparisons between leaves from plants supplied 0.04 mmol N and the other two concentrations are only made at 6 / 7dai). Soluble carbohydrates were extracted from the middle 3 cm of the dried leaf blade, which was cut into three portions and weighed. Soluble carbohydrates were extracted in ethanol and water as follows: 2 h at 80 °C in 2 cm^3 95% ethanol, 2 h at 60 ° C in 2 cm^3 80 % ethanol, overnight at 60 ° C in 2 cm^3 distilled water (dH_2O), 2 h at 60 ° C in 2 cm^3 dH_2O. The extracts from each sample were combined, made up to 20 cm^3 and analysed or stored frozen prior to analysis. Samples were analysed using the phenol-sulphuric acid method

(Dubois, 1956). Aliquots of 1 cm^3 of each extract were transferred to thick-walled, wide, glass test tubes. In a fume hood, 1 cm^3 of 5% phenol was added to each sample, followed by 5 cm^3 concentrated sulphuric acid, then tubes were immediately vortexed. Tubes were left for 30 min to cool before reading in a grating spectrophotometer (Cecil Instruments) at 485 nm against a blank containing water, 5% phenol and H_2SO_4. A sucrose standard curve (0 to 150 µg) was used for calibration.

CHN analysis

Leaves were chopped to pass a 1 mm sieve. Each sample was then re-weighed and sealed in a foil cup and analysed for total C, H and N content using a LECO CHN 2000 combustion analyser (Leco Corp., Missouri, USA), calibrated with EDTA.

Extraction of free amino acids

Frozen leaf discs of 4 mm diameter were ground with a pestle and mortar in 750 µl 80 % EtOH. Two tubes were filled with 750 µl 80 % EtOH but without sample, to act as blanks. Extracts and blanks were spun in a microfuge in 1.5 cm^3 eppendorf tubes at 14000 *g* for 5 min. The supernatant was transferred to fresh tubes and evaporated to dryness in a vacuum centrifuge. The residue was re-suspended in 250 µl dH_20 with a glass bead, using a vortex. Each sample was mixed with 80 µl light petroleum, b.p. 40-60 °C, which formed a layer containing pigments at the top of the sample. This was removed by pipette and discarded. Samples were dried again, then re-suspended in 1 cm^3 d H_2O immediately prior to analysis. Alanine (ala), asparagine (asn), aspartate (asn), glutamate (glu), glutamine (gln), glycine/threonine (gly/thr) and serine (ser) were determined.

Determination of total amino acids using ninhydrin

Standards were prepared by dissolving 0.5362 g glycine in 100 cm^3 dH_2O to give a solution of 1000 µg cm^{-3} glycine-N. Standards of 0, 1, 2.5, 5 and 7.5 µg cm^{-3} were made by adding 0.1, 0.25, 0.5 and 0.75 cm^3 of stock solution to 80 % ethanol and making up to 100 cm^3. Citrate buffer was made by adding 21 g citric acid and 8 g NaOH to 450 cm^3 dH_2O, adjusting the pH to 5.0 with 10 M NaOH and making up to 500 cm^3 with dH_2O. 50 cm^3 of dH_2O was mixed with 50 cm^3 Industrial methylated spirits (IMS) to make the

dilution solution. 0.3 cm^3 extract or standard was added to a glass test tube, followed by 0.7 cm^3 citrate buffer. 0.5 cm^3 ninhydrin reagent (Sigma) was added slowly before mixing on a vortex. Glass marbles were placed on the tubes which were then placed in a boiling water bath in a fume cupboard for 25 min. Tubes were removed from the water bath and allowed to cool before adding 2 cm^3 dilution solution and vortexing. An aliquot of 200 µl of each sample and standard was pipetted into a 96 well plate and absorbance was read against a blank at 570 nm in a microplate reader.

Analysis of amino acid profiles

Very few samples could be analysed, so of plants supplied 2 mmol dm^{-3} NO_3 and NH_4, one sample each from healthy leaves and two samples each from diseased leaves were analysed. Healthy leaves were harvested at 15 d old (equivalent to 1 dai) and diseased leaves were harvested at 24 d old and 10 dai. Amino acids were extracted and dried as above, then re-suspended in 0.2 M borate buffer pH 8.2. Aliquots were analysed by o-pthaldialdehyde (OPA) pre-column derivatisation. HPLC of derivatives was done using a Dynamax Microsorb 5 µm C18 column, 4.6 · 150 mm. The HPLC equipment comprised a Phillips PU 4000 system, Rheodyne 7152 injector, Gilson 210 fraction collection and Perkin Elmer LS1 fluorescence detector, excitation filter, 340 nm, emission 420 nm, and a Spectra Physics SP 4270 integrator (Webster, 1991). Analysis was performed by Simon G. Webster, University of Wales, Bangor.

Total soluble proteins

Soluble proteins were extracted from frozen leaf tissue (3 leaf discs of 4 mm diameter per replicate) by rapidly grinding in 1 cm^3 cold extraction buffer (100 mmol NaCl in phosphate buffer, pH 7) at 4 °C. Samples were centrifuged at 4 °C for 20 min at 3500 *g*. Total soluble protein was determined following Bradford (1976). 3 cm^3 of Coomassie Brilliant Blue G250 was added to 100 µl of the supernatant and vortexed; the mixture was left in the dark for 10 min before being read in a spectrophotometer at 595 nm against a blank (NaCl / buffer / reagent). Soluble protein concentrations were determined using a calibration curve prepared from 1 mg cm^{-3} bovine serum albumen (BSA). Aliquots were

removed from the stock solution to give the following amounts of protein: 0, 1, 2, 5, 10, 25 and 50 μg BSA then made up to 100 μl with buffer.

Determination of soluble polyphenols

Soluble polyphenols were determined in leaves from plants supplied 0.04 mmol N and 2 mmol N and harvested 7 and 12 dai using Folin-Ciocalteau reagent after Constantinides and Fownes (1994). Oven-dried leaves weighing approximately 20 mg were chopped into 1x1 mm pieces, placed in tubes and weighed. To each sample 2 cm^3 of extractant (50 % methanol with 0.1 mg cm^{-3} ascorbic acid) was added. A glass marble was placed on top of each tube then tubes were heated to 75 °C for 1 h in a heating block. Extracts were filtered through Whatman No. 1 filter paper and made up to 5 cm^3 with dH_2O. 100 μl of each sample was added to 10 cm^3 volumetric flasks, followed by 6 cm^3 dH_2O, then 0.5 cm^3 Folin-Ciocalteau reagent After 2 min but before 8 min 1.5 cm^3 saturated Na_2CO_3 was added. Samples were made up to volume with dH_2O and incubated at room temperature for 2 h. A tannic acid standard curve using 0, 100, 200, 300, 400, 500 and 600 μl of a 0.1 mg cm^{-3} tannic acid solution was prepared using the above method. 200 μl of each sample and standard was pipetted into a 96 well plate and absorbance was read against a blank at 760 nm in a microplate reader.

Data analysis

Data were compared using one-way ANOVA with post-hoc tests of least significant difference (SPSS 9.0, SPSS Inc, Chicago, USA). Differences were considered statistically significant at $P<0.05$. Linear regression was used in some cases (Sigmaplot 8.0, SPSS Inc, Chicago, USA).

Results

Soluble carbohydrates

Effect of N form

Second leaves from healthy plants supplied 0.04 mmol dm^{-3} NO_3^- had higher soluble carbohydrate contents than second leaves from healthy plants supplied 0.04 mmol dm^{-3}. NH_4^+. There was no significant difference in soluble carbohydrate contents of second leaves from diseased plants supplied 0.04 mmol dm^{-3} NO_3^- or NH_4^+ (**Fig. 1**), but At 14 dai second leaves of plants supplied 2 and 8 mmol dm^{-3} NH_4^+ had significantly higher soluble carbohydrate contents than those supplied 2 and 8 mmol dm^{-3} NO_3^- (**Fig. 2**), although at the higher concentration the difference was only significant in diseased plants.

Effect of N concentration

Leaves from plants supplied 0.04 mmol dm^{-3} had the highest soluble carbohydrate contents (soluble carbohydrate contents at 6 dai of leaves from plants supplied 0.04 mmol dm^{-3} were compared with soluble carbohydrate contents at 7 dai of leaves from plants supplied the two higher N concentrations as harvest times were different). There were no significant differences in soluble carbohydrate content between plants supplied 2 mmol dm^{-3} N 8 mmol dm^{-3} N.

Effect of disease

There was no significant difference between healthy and diseased plants supplied 0.04 mmol dm^{-3} N. In plants supplied the higher concentrations of N, leaves of diseased plants had significantly higher soluble carbohydrate contents than second leaves of healthy plants 14 dai, except in the case of plants supplied 8 mmol NO_3^- where there was no significant difference between diseased and healthy plants. Soluble carbohydrate contents of diseased leaves all increased between 7 and 14 dai, with the exception of leaves from plants supplied 8 mmol NO_3^- where the mean soluble carbohydrate content decreased by 14 %. Soluble carbohydrate contents of all healthy leaves decreased between 7 and 14 dai, with the exception of leaves from plants supplied 8 mmol NH_4^+ where there was no change in the mean value.

Linear regression (**Fig. 3**) showed that there was a linear relationship between the lesion numbers and soluble carbohydrate contents of diseased leaves ($r^2 = 0.706$, $P < 0.05$), suggesting that the sucrose content of leaves influences susceptibility to infection.

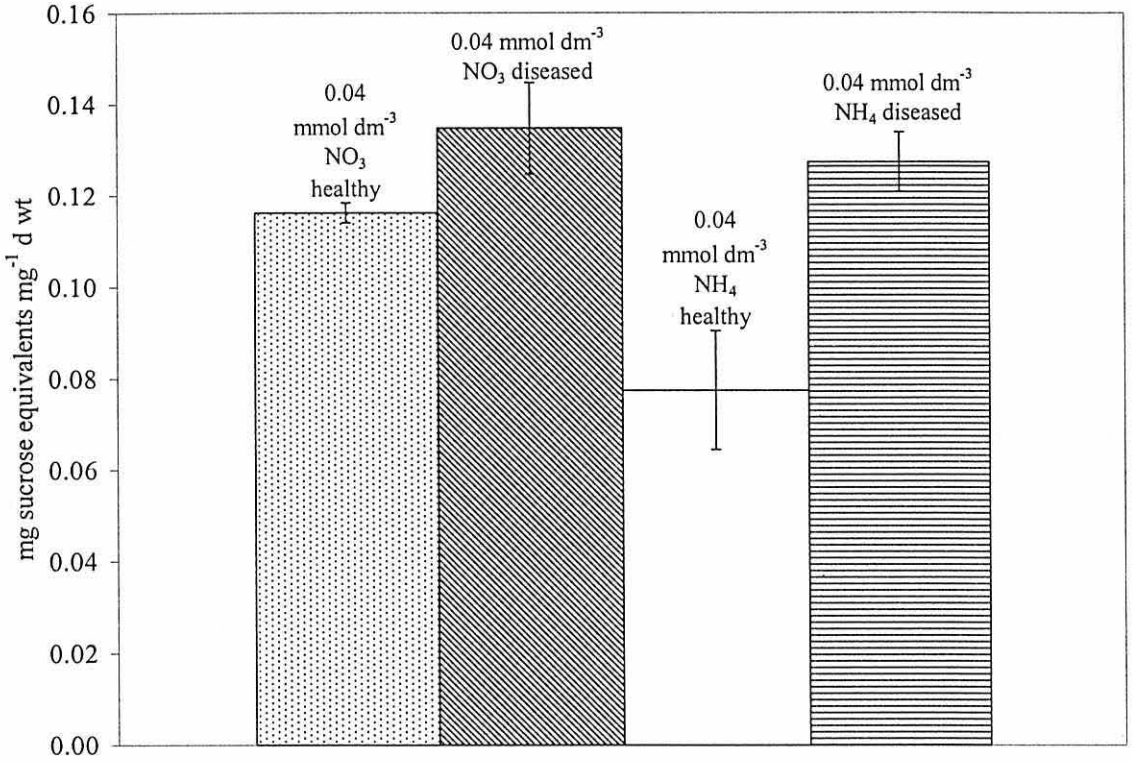

Figure 1 Soluble carbohydrate contents (mean ± SE) of second leaves of wheat plants 6 dai. Plants were supplied N as 0.04 mmol dm^{-3} NO_3^- or NH_4^+ and were inoculated with *S. nodorum* at 14 d old.

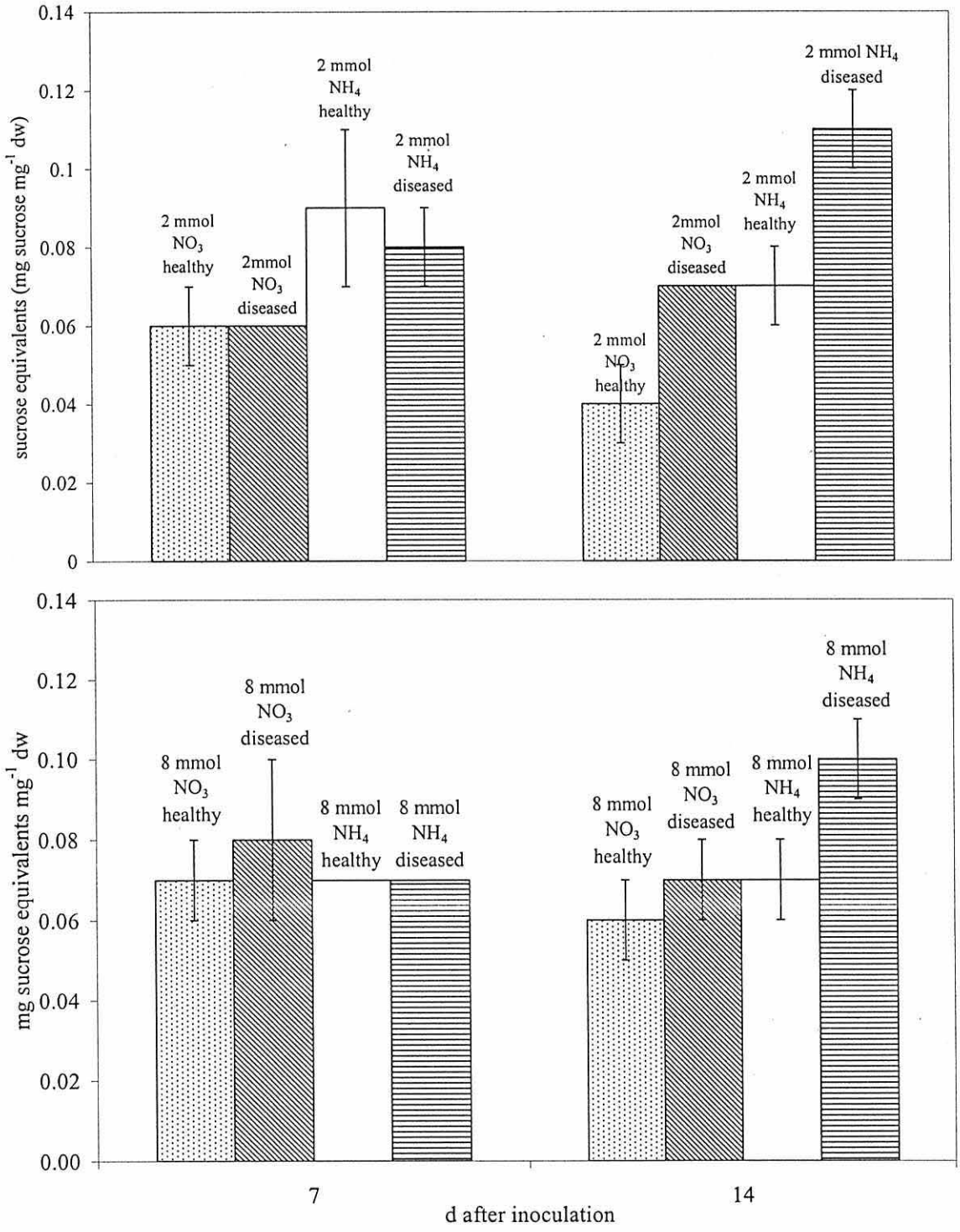

Figure 2 Soluble carbohydrate contents (mean ± SE) of second leaves of wheat plants. Plants were supplied N as 2 mmol dm^{-3} NO_3^- or NH_4^+ (top) or 8 mmol dm^{-3} NO_3^- or NH_4^+ (bottom) and were inoculated with *S. nodorum* at 14 d old. n = 3

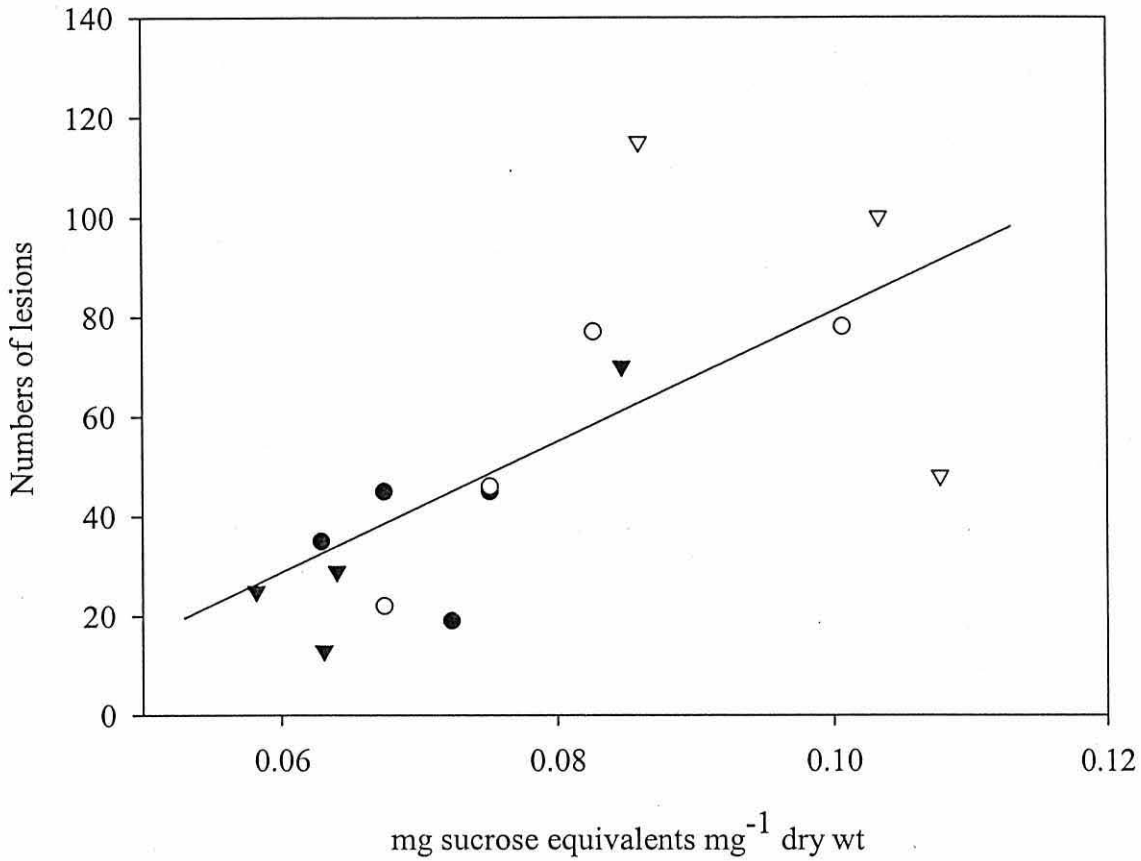

Figure 3 Linear regression of lesion numbers against sucrose contents at 14 dai, of leaves from plants that were supplied N as 2 mmol dm^{-3} NO_3^- (●), 2 mmol dm^{-3} NH_4^+ (○), 8 mmol dm^{-3} NO_3^- (▼) or 8 mmol dm^{-3} NH_4^+ (▽) 14 d after inoculation with *S. nodorum.* Leaves were inoculated at 14 d old.

Total C

Approximately 40 % of leaf dry weight was represented by C. Disease and N form and concentration all had visible effects on leaf C content.

Effect of N form

The form of N supplied affected C content at 2 mmol dm^{-3} and second harvest (14 dai) only (**Table 2**). Plants supplied 2 mmol dm^{-3} NH_4^+ had significantly lower C contents had than plants supplied 2 mmol dm^{-3} NO_3^-.

Table 2 C contents (% dry weight; mean ± SE) of second leaves of wheat from plants that were supplied N as 0.04 mmol dm^{-3} NO_3^- or NH_4^+, 2 mmol dm^{-3} NO_3^- or NH_4^+, or 8 mmol dm^{-3} NO_3^- or NH_4^+. Second leaves were inoculated with *S. nodorum* the day after full expansion, when plants were 14 d old. n = 3

N form	N concentration (mmol)	1 dai		6 dai	
		diseased	healthy	diseased	healthy
NO_3^-	0.04	39.0 ± 0.2	36.7 ± 0.6	38.5 ± 0.2	39.1 ± 0.4
NH_4^+	0.04	38.8 ± 0.3	39.8 ± 0.5	38.2 ± 0.2	38.3 ± 0.7
		7 dai		14 dai	
		diseased	healthy	diseased	healthy
NO_3^-	2	39.6 ± 0.2	38.3 ± 0.09	37.3 ± 0.2	36.6 ± 0.3
NH_4^+	2	40.0 ± 0.4	38.7 ± 0.4	35.0 ± 0.3	33.3 ± 0.4
NO_3^-	8	40.2 ± 0.2	38.5 ± 0.6	38.2 ± 0.2	37.1 ± 0.9
NH_4^+	8	41.6 ± 0.3	39.3 ± 0.6	38.4 ± 0.9	37.1 ± 0.2

Effect of N concentration

The concentration of N supplied to plants had some effect on C contents of second leaves: Plants supplied 0.04 mmol N had similar C contents to plants supplied 2 mmol N (except healthy plants supplied 2 mmol NH_4^+ had higher C contents at 7 dai than healthy plants supplied 0.04 mmol NH_4^+ at 6 dai). Plants supplied 8 mmol NO_3^- or NH_4^+ generally had higher C contents than plants supplied 2 mmol NO_3^- or NH_4^+ 14 dai, but the only significant differences were in plants supplied NH_4^+. C contents decreased slightly between 7 and 14 dai.

Effect of disease

Diseased plants (2 and 8 mmol) had significantly higher C contents than healthy plants 7 dai but the differences were not significant 14 dai (with the exception of plants supplied 2 mmol dm^{-3} NH_4^+).

Total N

Effect of N form

There was no significant difference in N content between plants supplied 0.04 mmol dm^{-3} NO_3^- and NH_4^+ (data not shown). In plants supplied the two higher concentrations of N (**Figs 4 and 5**), second leaves of plants supplied NO_3^- had higher N contents than plants supplied NH_4^+. Differences in N content between plants supplied NO_3^- and NH_4^+ were

significant for both higher concentrations at 7 dai, but by 14 dai differences were only significant in plants supplied 2 mmol dm^{-3} N (**Fig. 4**).

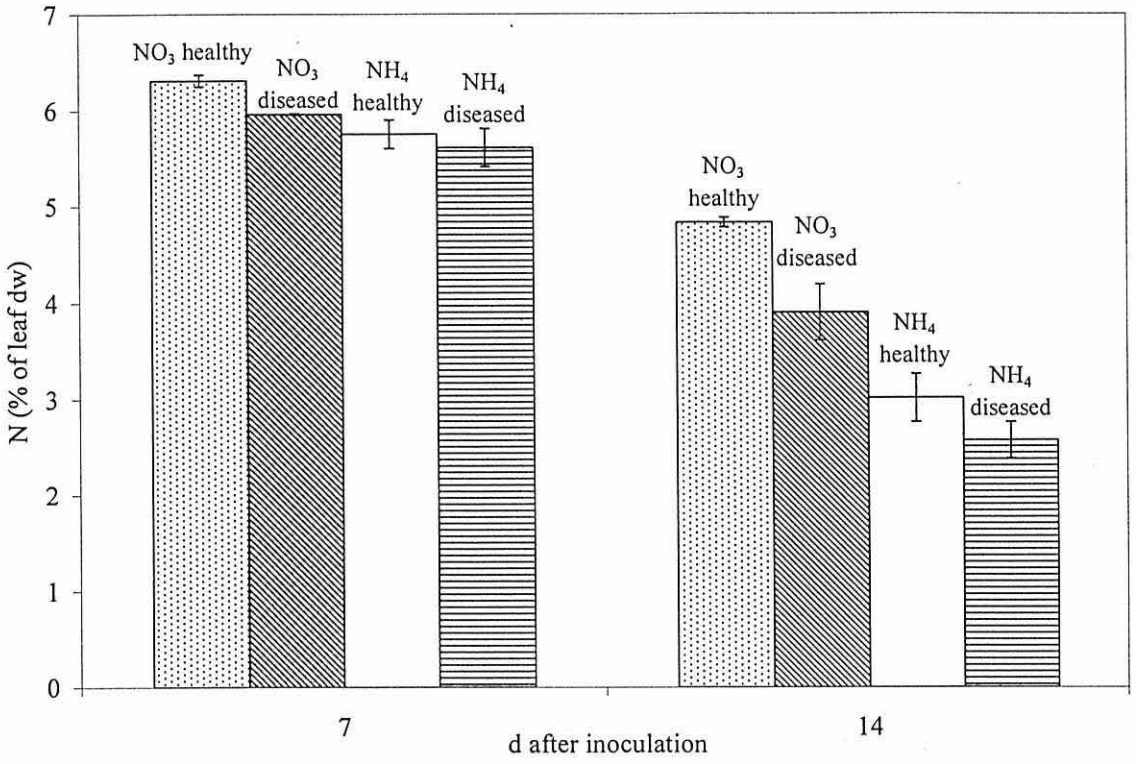

Figure 4 N contents (% of leaf dry weight; mean ± SE) of second leaves of wheat plants. Plants were supplied N as 2 mmol dm^{-3} NO_3^- or NH_4^+ and were inoculated with *S. nodorum* at 14 d old

Effect of N concentration

Second leaves of plants supplied 0.04 mmol dm^{-3} N (data not shown) had the lowest N contents (less than 2 % of dw 6 dai) and these were between 66 % and 73 % lower than the N contents of plants supplied 2 and 8 mmol dm^{-3} N (where N accounted for 5.6 to 6.5 % of leaf DW 7 dai). There was no significant difference in N content between plants supplied 8 mmol dm^{-3} N and 2 mmol dm^{-3} N, except 14 dai when plants supplied 8 mmol dm^{-3} NH_4^+ had significantly higher N contents than plants supplied 2 mmol dm^{-3} NH_4^+.

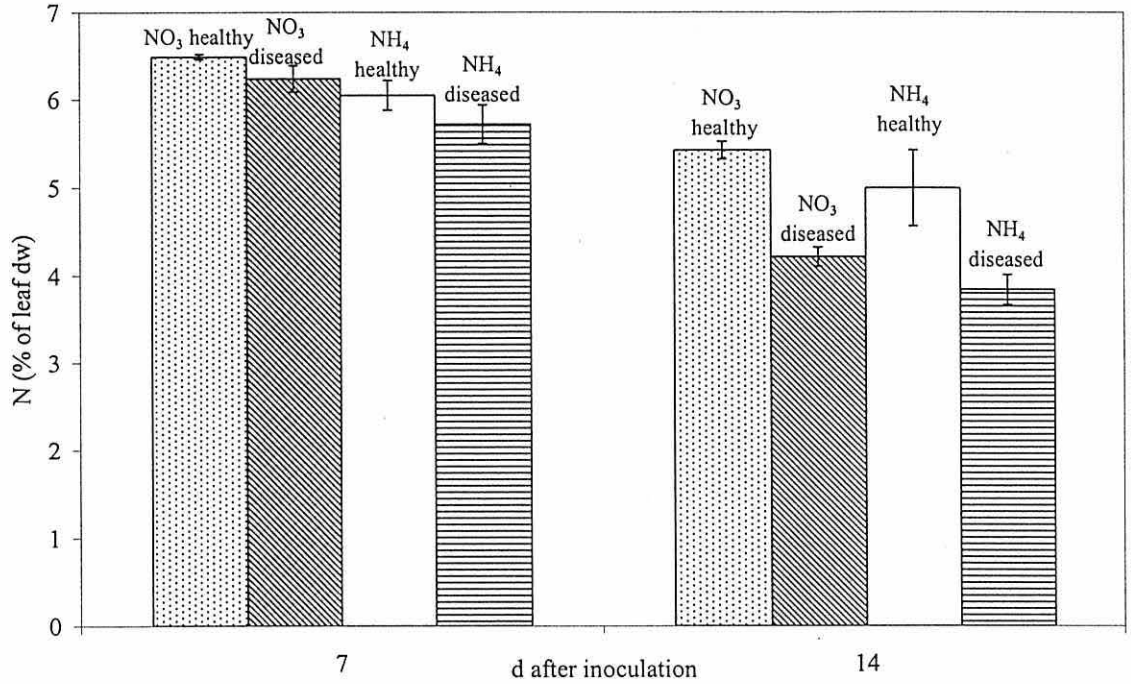

Figure 5 N contents (% of leaf dry weight; mean ± SE) of second leaves of wheat plants. Plants were supplied N as 8 mmol dm^{-3} NO_3^- or NH_4^+ and were inoculated with *S. nodorum* at 14 d old

Effect of disease

There was no significant difference in N content between diseased and healthy plants supplied 0.04 mmol N (data not shown). N contents of second leaves of plants supplied 2 mmol dm^{-3} and 8 mmol dm^{-3} N were lower in diseased plants than healthy plants. The differences were only significant 14 dai, and there was no significant difference between diseased and healthy plants supplied 2 mmol dm^{-3} NH_4^+.

Plotting disease symptoms against N content of diseased leaves (data not shown) revealed a non-significant negative correlation ($P > 0.05$).

C: N ratios

Effect of N form

Leaves from plants supplied NH_4^+ had significantly higher C: N ratios than leaves from plants supplied NO_3^- (**Figs.** 7 and 8) 7 dai only (2 and 8 mmol dm^{-3} N only).

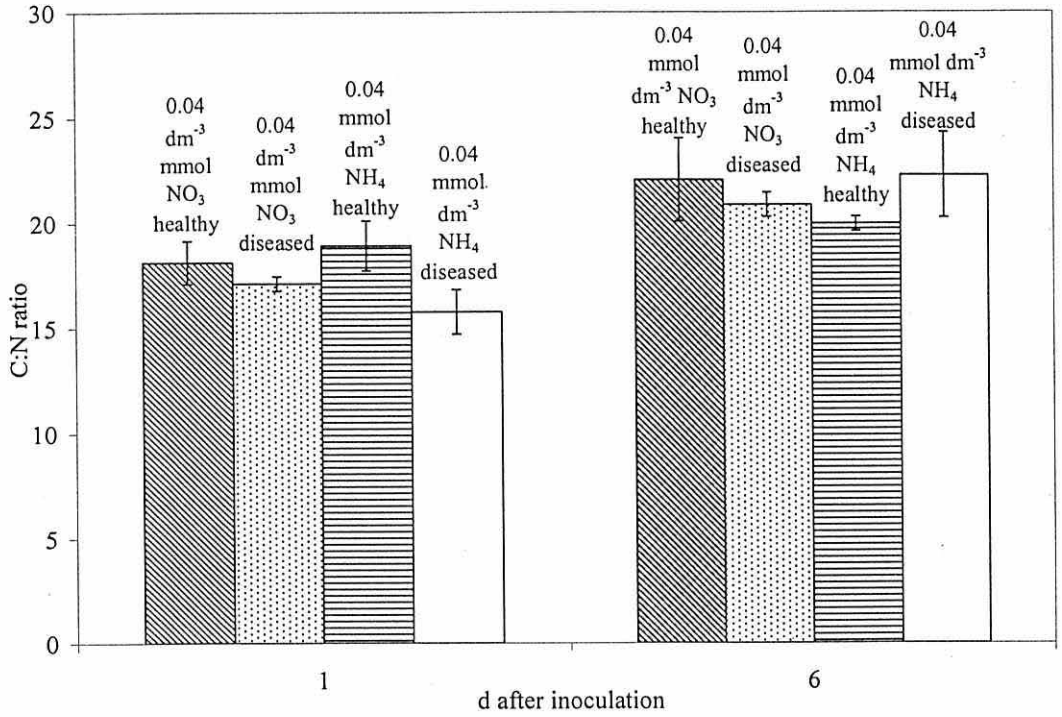

Figure 6 C: N ratios (mean ± SE) of second leaves of wheat plants. Plants were supplied N as 0.04 mmol dm^{-3} NO_3^- or NH_4^+ and were inoculated with *S. nodorum* at 14 d old

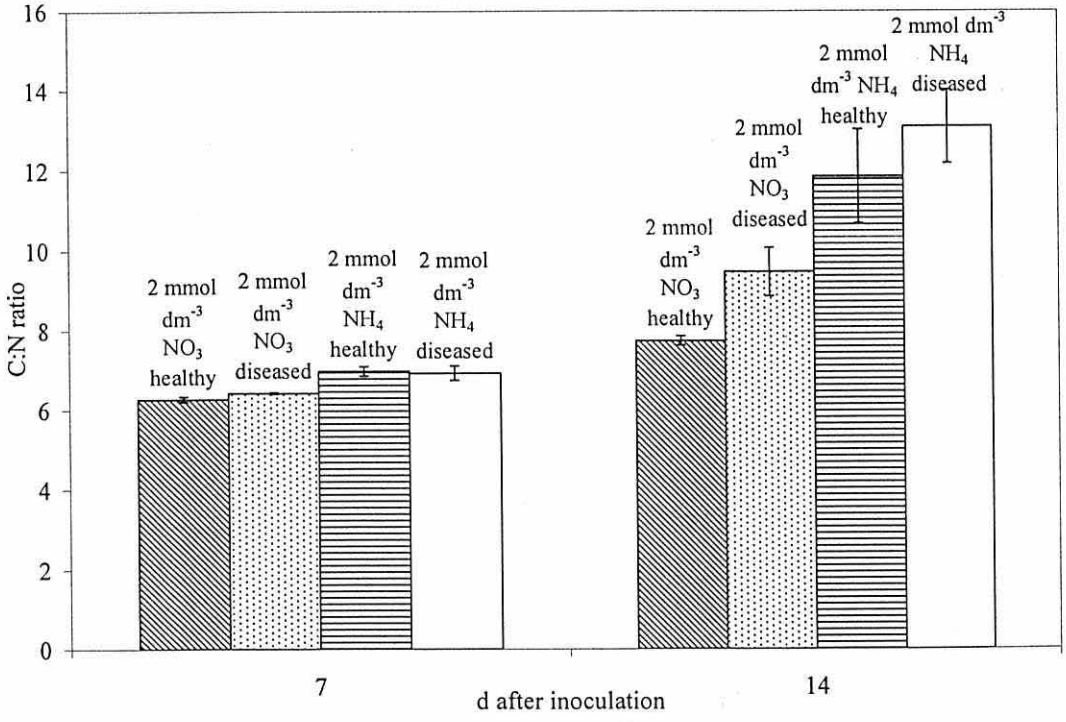

Figure 7 C: N ratios (mean ± SE) of second leaves of wheat plants. Plants were supplied N as 2 mmol dm^{-3} NO_3^- or NH_4^+ and were inoculated with *S. nodorum* at 14 d old.

Effect of N concentration

Leaves from plants supplied 0.04 mmol dm^{-3} N (**Fig. 6**) had the highest C: N ratios, which were significantly higher (2.9 to 3.6 times higher depending on treatment) than the C: N ratios of plants supplied 2 (**Fig. 7**) or 8 mmol dm^{-3} N (**Fig. 8**). Leaves from plants supplied 8 mmol N had the lowest C: N ratios. Leaves from plants supplied 8 mmol dm^{-3} NH_4^+ had significantly lower C: N ratios than leaves from plants supplied 2 mmol dm^{-3} NH_4^+ 14 dai.

Effect of disease

C: N ratios were generally higher in healthy plants than diseased plants 7 dai, but were lower in healthy plants than diseased plants 14 dai The only significant difference however was between healthy and diseased plants supplied 8 mmol dm^{-3} NH_4^+ 14 dai. Lesion numbers were positively correlated with C: N ratios (not shown) and revealed a positive linear relationship; the relationship is not significant however ($P > 0.05$).

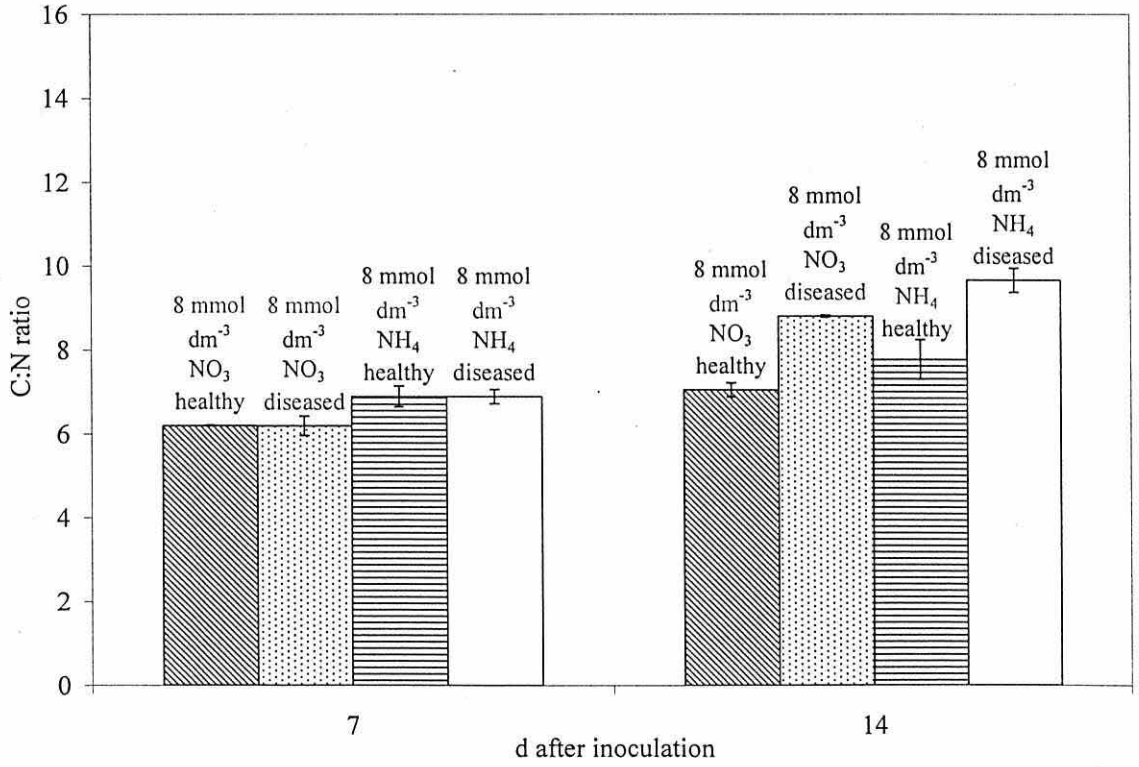

Figure 8 C: N ratios (mean ± SE) of second leaves of wheat plants. Plants were supplied N as 8 mmol dm^{-3} NO_3^- or NH_4^+ and were inoculated with *S. nodorum* at 14 d old.

Total soluble proteins

There was a decrease in the soluble protein contents of second leaves of plants supplied 2 mmol dm^{-3} N between 1 and 7 dai in all treatments, from between 6 - 10 g m^{-2} to between 2 - 6 g m^{-2}. Soluble protein contents were over 50 % lower at the second harvest than the first. Between 7 and 10 dai protein contents rose again to between 4 - 8 g m^{-2} and were all higher at the final harvest than at the second (except second leaves of healthy plants supplied NH_4^+, which had their lowest soluble protein contents at the final harvest).

Effect of N form

Soluble proteins were 25 % (diseased leaves) and 36 % (healthy leaves) higher in leaves from plants supplied NH_4^+ than plants supplied NO_3^- 1 dai ($P < 0.01$) and 30 % (diseased leaves) and 61 % (healthy leaves) higher in leaves from plants supplied NH_4^+ than plants supplied NO_3^- 7 dai ($P < 0.05$) (**Fig. 9**). There was no significant difference 10 dai.

Effect of N concentration

Proteins were only analysed from plants supplied 0.04 mmol dm-3 at 1 dai but at this time they were significantly lower (82 % in plants supplied NO_3^- and 78 % in plants supplied NH_4^+) than in second leaves of plants supplied 2 mmol dm^{-3} N (data not shown).

Effect of disease

There was no significant difference in soluble protein contents between healthy and diseased leaves of plants supplied 2 mmol dm^{-3} NH_4^+. Leaves from diseased plants supplied 2 mmol dm^{-3} NO_3^- had significantly higher soluble protein contents than healthy plants supplied 2 mmol dm^{-3} NO_3^- 1 dai, but there was no significant difference 7 or 10 dai.

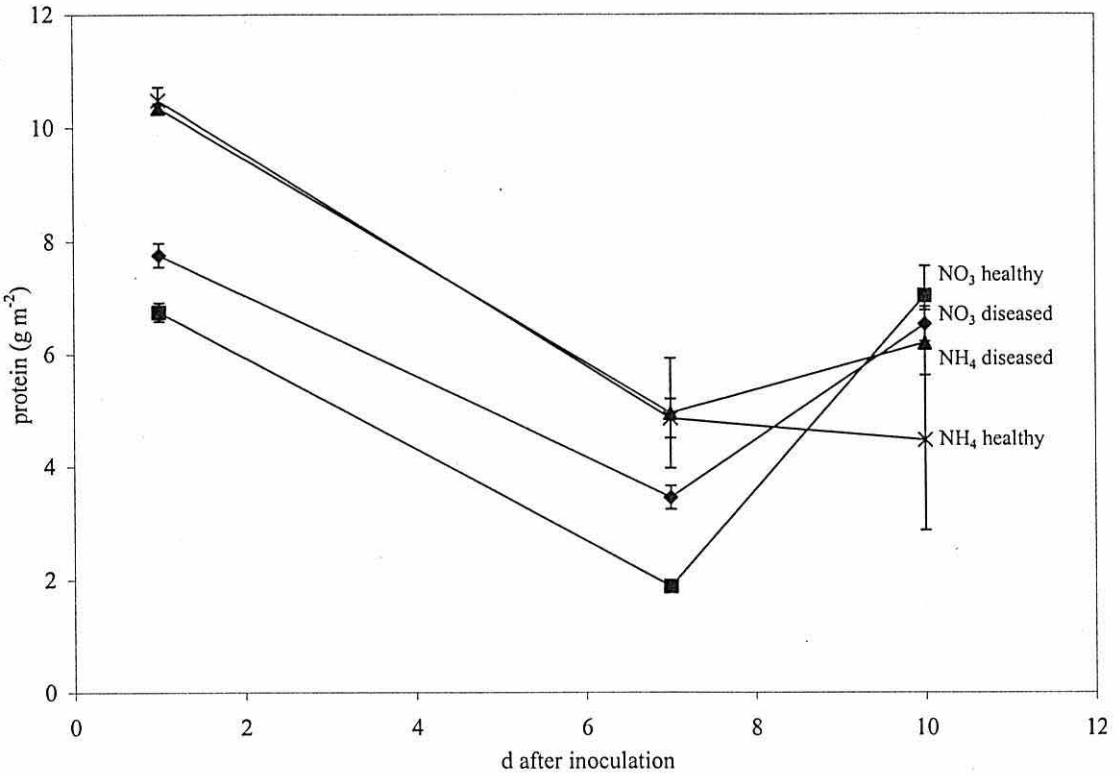

Figure 9 Total soluble protein contents (mean ± SE) of second leaves of wheat plants. Plants were supplied N as 2 mmol dm^{-3} NO_3^- or NH_4^+ and were inoculated with *S. nodorum* at 14 d old.

Total amino acids

Amino acid contents increased between 1 and 7 dai, then decreased between 7 and 10 dai to slightly higher than they had been at 1 dai.

Effect of N form

Differences in amino acid contents of plants supplied 0.04 mmol dm^{-3} NH_4^+ and 0.04 mmol dm^{-3} NO_3^- were not significant (**Fig. 10**). At 1 dai and 10 dai second leaves from diseased plants supplied 2 mmol dm^{-3} NH_4^+ had significantly higher amino acid contents than second leaves from diseased plants supplied 2 mmol dm^{-3} NO_3^- (**Fig. 11**). At 7 dai second leaves of healthy plants supplied 2 mmol dm^{-3} NH_4^+ had significantly higher amino acid contents than second leaves of healthy plants supplied 2 mmol dm^{-3} NO_3^-. Amino acid contents of second leaves from plants supplied 2 mmol dm^{-3} NH_4^+ were on

average 52 % higher (diseased plants) and 58 % higher (healthy plants) than plants supplied 2 mmol dm^{-3} NO_3^-.

Effect of concentration

Plants supplied 0.04 mmol N had low amino acid contents (generally less than 0.5 g m^{-2}), compared with plants supplied 2 mmol N (between >0.5 and 4 g m^{-2}). At 1, 7 and 10 dai amino acid contents of plants supplied 0.04 mmol dm^{-3} N (both forms) were significantly lower than plants supplied 2 mmol dm^{-3} NH_4^+ (diseased and healthy; in most cases $P <$ 0.01).

Effect of disease

There was no significant difference in amino acid contents between diseased plants and healthy plants. Plotting lesion numbers against amino acid contents of leaves did not reveal a significant relationship.

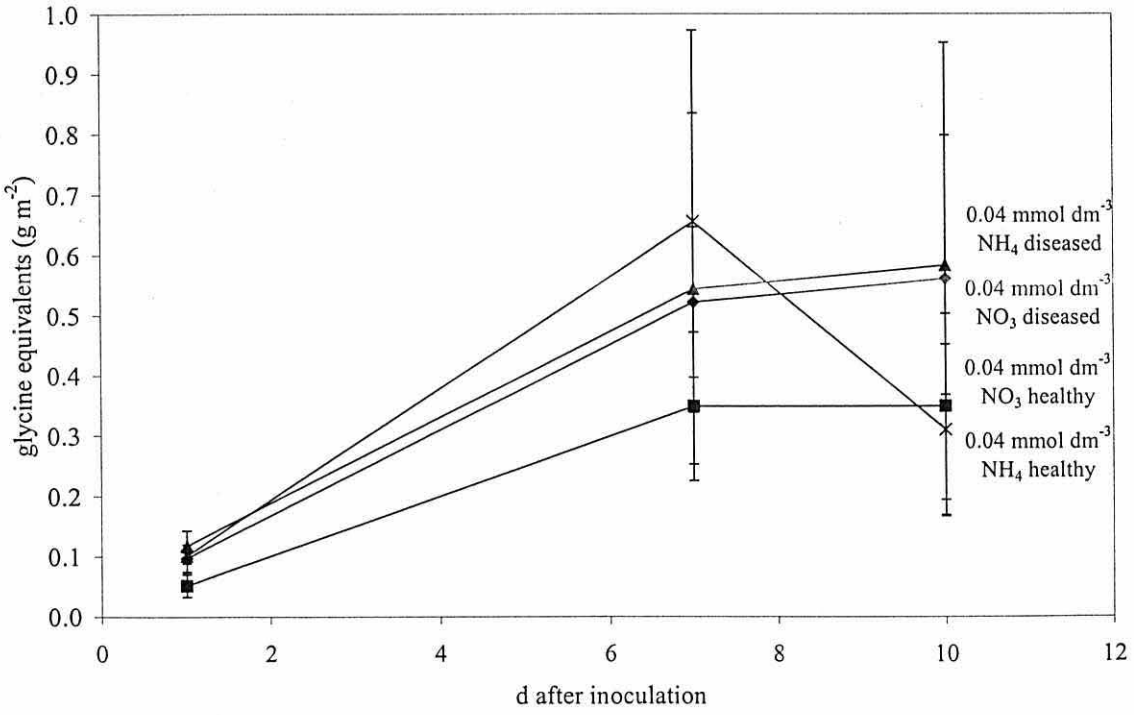

Figure 10 Mean (± SE) total amino acid contents of second leaves of wheat plants. Plants were supplied N as 0.04 mmol dm^{-3} NO_3^- or NH_4^+ and were inoculated with *S. nodorum* at 14 d old.

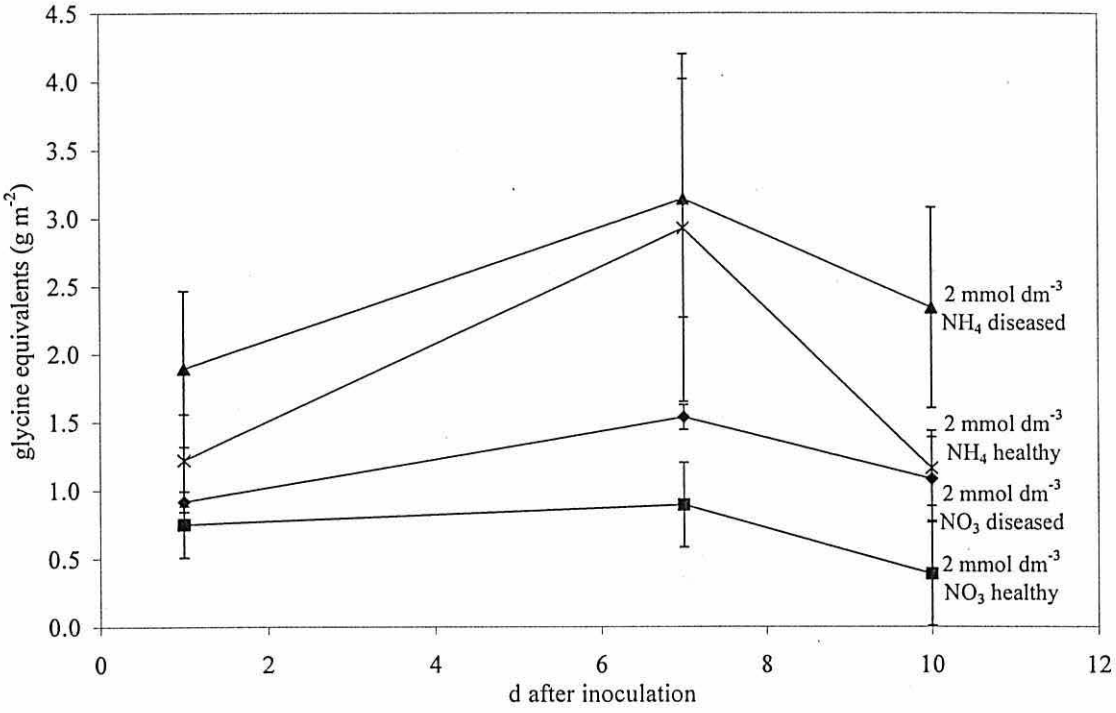

Figure 11 Total amino acid contents (mean ± SE) of second leaves of wheat plants. Plants were supplied N as 2 mmol dm^{-3} NO_3^- or NH_4^+ and were inoculated with *S. nodorum* at 14 d old.

Amino acid profiles

At 15 d old, leaves from healthy plants supplied N as NH_4^+ or NO_3^- had strikingly different amino acid profiles (**Fig. 12**). Leaves from plants supplied NH_4^+ had more of all the amino acids and amides analysed for except asp. In particular, second leaves of plants supplied NO_3^- had no glu or asn, whereas in leaves from plants supplied NH_4^+, 7 % of the total amino acid content was glu and 72 % of the total was asn. Second leaves of plants supplied NH_4^+ had 86 % more glu and 76 % more gly/thr than plants supplied NO_3^-, and had more ser and ala. Second leaves of plants supplied NO_3^- had 78 % more asp than second leaves of plants supplied NH_4^+. Asp made up 52 % of the amino acid profile of plants supplied NO_3^-, but only 2 % of the profile of plants supplied NH_4^+. The total concentration of amino acids and amides combined in leaves from plants supplied NO_3^- was 85 % lower than that of leaves from plants supplied NH_4^+, which is mainly due to the unusually high levels of asn in leaves from plants supplied NH_4^+. As the sample number was only one for healthy leaves, the high asn content may be anomalous; however, even

if asn is discounted the total amino acid concentration in leaves from plants supplied NH_4^+ is still 46 % higher than the concentration in leaves from plants supplied NO_3^-, which is comparable to the differences in total amino acids found in leaves from plants supplied 2 mmol dm^{-3} NO_3^- and NH_4^+ (**Fig. 12**).

At 24 d old (10 dai) the amino acid profiles were still different, but the differences were less marked. Total concentrations of amino acids were lower than in the 15 d old samples, and there was less of a difference in concentration between plants supplied NH_4^+ and NO_3^-. Plants supplied NH_4^+ had 87 % more asn and 57 % more gln than plants supplied NO_3^-, but significantly less asp and gln than second leaves of plants supplied NO_3^-.

Leaves from diseased plants supplied NO_3^- had more ala, asn, glu, gln, gly/thr and ser, but less asp, than leaves from healthy plants supplied NO_3^-. Leaves from diseased plants supplied NH_4^+ had more ala, gln and ser, but less asn, asp, gln and gly/thr than leaves from healthy plants supplied NH_4^+.

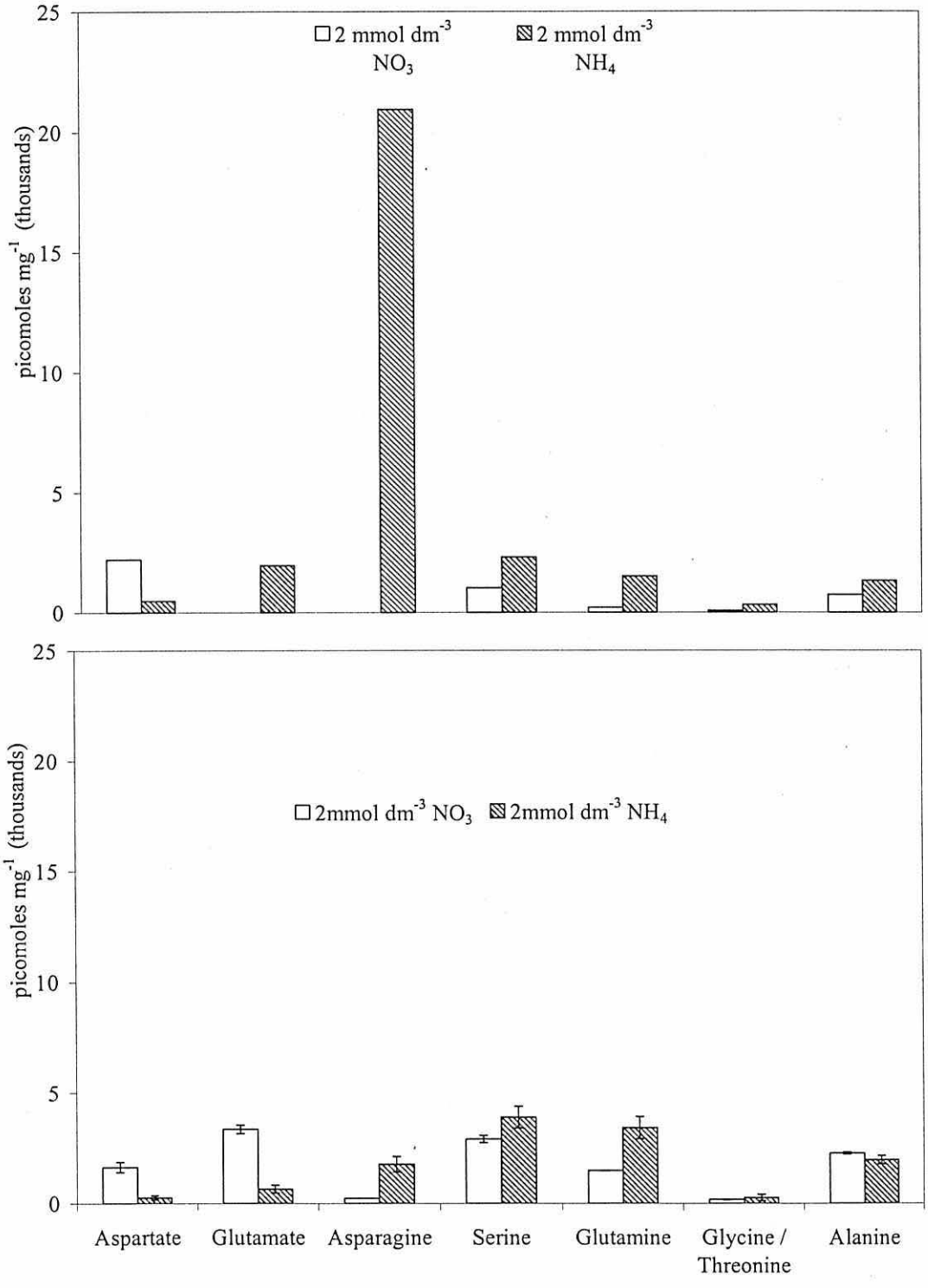

Figure 12 Amino acid profiles of second leaves of wheat plants. Plants were supplied N as 2 mmol dm^{-3} NO_3^- or NH_4^+, and were inoculated with *S. nodorum* at 14 d old. Leaves from healthy plants were analysed at 15 d old (top) and inoculated leaves were analysed at 24 d old (10 dai), (bottom). n = 1 (top) or n = 2 (bottom).

Soluble polyphenols

Effect of N form

There was no significant difference in soluble polyphenol contents between plants supplied 0.04 mmol dm^{-3} NH_4^+ and NO_3^- (**Fig. 13**). At 7 dai, soluble polyphenol contents were slightly higher in leaves from plants supplied NH_4^+ compared with plants supplied NO_3^-. This was reversed at 12 dai, when leaves from plants supplied NO_3^-. had slightly higher soluble polyphenol contents than leaves from plants supplied NH_4^+. At 12 dai there was a significant difference in soluble polyphenol contents between leaves from diseased plants supplied either 2 mmol dm^{-3} NO_3^- or NH_4^+, where diseased leaves from plants supplied NO_3^- had 54 % more soluble polyphenols than diseased plants supplied NH_4^+.

Effect of N concentration

Leaves from plants supplied 0.04 mmol dm^{-3} N (both forms) had significantly higher soluble polyphenol contents than leaves from plants supplied 2 mmol dm^{-3} N harvested 7 or 12 dai. Soluble polyphenol contents were generally slightly higher 12 dai compared to 7 dai. There was a negative (but non-significant) relationship between soluble polyphenol contents of leaves and leaf N content (not shown).

Effect of disease

In general, leaves from healthy plants had higher soluble polyphenol contents than leaves from diseased plants however this difference was not statistically significant. The only case where there was a statistically significant difference in soluble polyphenol contents between diseased and healthy plants was in leaves from plants supplied 2 mmol dm^{-3} NO_3^-, 12 dai, where diseased plants had 36 % higher soluble polyphenol contents than healthy plants.

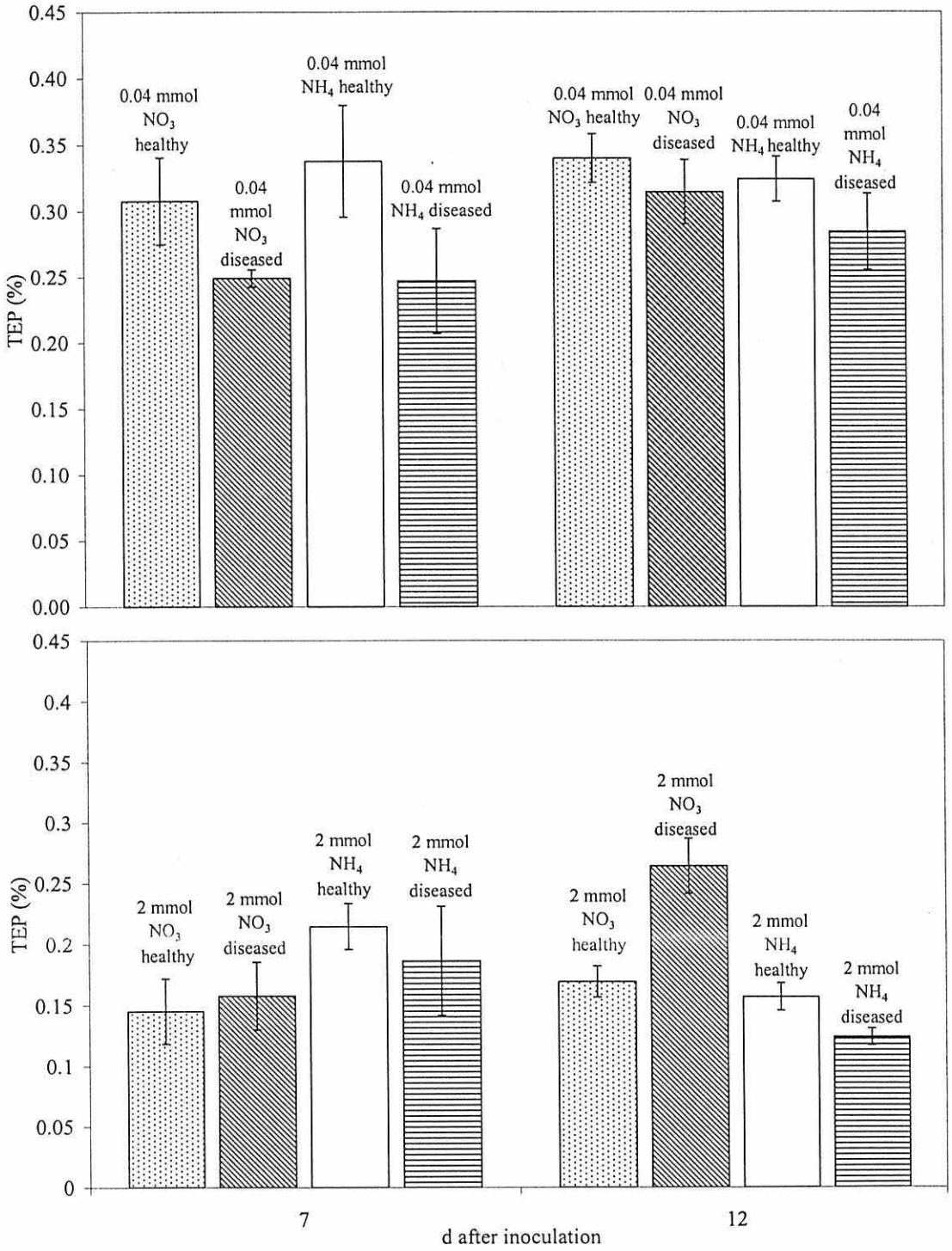

Figure 13 Soluble polyphenol contents [total exchangeable polyphenols (TEP) as a % of leaf dry weight; mean ± SE] of second leaves of wheat plants. Plants were supplied N as 0.04 mmol dm^{-3} (labelled as 0.04 mmol on graph) NO_3^- or NH_4^+ (top) or 2 mmol dm^{-3} (labelled as 0.04 mmol on graph) NO_3^- or NH_4^+ (bottom) and were inoculated with *S. nodorum* at 14 d old.

Table 3 Summary of main findings. NSD = no significant difference.

Analysis	Effect of N form	Effect of N concentration	Effect of disease
Soluble carbohydrates	$NH_4^+ < NO_3^-$ (0.04 mmol dm^{-3}, healthy plants only) $NH_4^+ > NO_3^-$ (2 and 8 mmol dm^{-3} 14 dai)	0.04 mmol dm^{-3} > 2 and 8 mmol dm^{-3}. NSD between plants supplied 2 and 8 mmol dm^{-3}	Diseased > healthy Positive linear relationship between disease and carbohydrates.
Total C	14 dai NO_3^- > NH_4^+ (2 mmol dm^{-3})	14 dai 8 mmol dm^{-3} NH_4^+ > 2 mmol dm^{-3}	7 dai diseased < healthy
Total N	NSD between plants supplied 0.04 mmol dm^{-3} NO_3^- and NH_4^+. NO_3^- > NH_4^+ (2 and 8 mmol dm^{-3})	N content lowest in plants supplied 0.04 mmol dm^{-3} N. NSD between plants supplied 2 and 8 mmol dm^{-3} N (except 14 dai 8 mmol dm^{-3} NH_4^+ >2 mmol dm^{-3} NH_4^+)	NSD between diseased and healthy plants supplied 0.04 mmol dm^{-3} N. Generally diseased leaves < healthy leaves (2 and 8 mmol dm^{-3})
C: N ratios	7 dai NH_4^+ > NO_3^- (2 and 8 mmol dm^{-3})	0.04 > 2 > 8 mmol dm^{-3}	14 dai diseased NH_4^+ > healthy NH_4^+ (8 mmol dm^{-3})
Total soluble polyphenols	12 dai diseased NO_3^- > diseased NH_4^+	0.04 mmol dm^{-3} > 2 mmol dm^{-3}	12 dai diseased NO_3^- (2 mmol dm^{-3}) > healthy NO_3^- (2 mmol dm^{-3}).
Total soluble proteins	NH_4^+ > NO_3^- 1 and 7 dai	2 mmol dm^{-3} > 0.04 mmol dm^{-3}	1 dai diseased NO_3^- > healthy NO_3^-
Total amino acids	NSD between plants supplied 0.04 mmol dm^{-3} NO_3^- and NH_4^+. 1 and 10 dai (2 mmol dm^{-3}) diseased NH_4^+ > diseased NO_3^-; 7 dai healthy NH_4^+ > healthy NO_3^-	0.04 mmol dm^{-3} NO_3^- and NH_4^+ < 2 mmol dm^{-3} NH_4^+	NSD

Discussion

The form and concentration of N supplied to wheat had a significant effect on the physiological components of second leaves.

The effects of N concentration on leaf N status

The data support the hypothesis that plants supplied moderate (2 mmol dm^{-3}) and high (8 mmol dm^{-3}) N have higher total amino acid contents and higher total N contents than plants supplied low (0.04 mmol dm^{-3}) N. There was no significant difference in N content between plants supplied 2 mmol dm^{-3} N and 8 mmol dm^{-3} N however.

It was expected that plants supplied high amounts of N would have higher total N contents and total amino acid contents than plants supplied low amounts (Marschner, 1995, Zerihun *et al.*, 1998, Kirkman and Miflin, 1979 cited by Lea and Miflin 1980). It was surprising that there was no significant difference in N content between plants supplied 2 mmol dm^{-3} N and plants supplied 8 mmol dm^{-3} N. The N content required for optimal growth varies between 2 and 5 % of plant dry weight, depending on growth stage, species and organ (Marschner, 1995). As second leaves of plants supplied 2 mmol dm^{-3} N contained at least 5 % N at 21 d old, perhaps this amount of N supply is adequate for growth and 8 mmol dm^{-3} represented supra-optimal N availability. Indeed there were no significant differences in dry weights between plants supplied 2 mmol dm^{-3} N and 8 mmol dm^{-3} N (Chapter 2).

Plants supplied 0.04 mmol dm^{-3} N were clearly deficient in N as their growth was slow, development was delayed, and their leaves were very pale (data discussed in Chapter 2), so consequently total N and amino acid contents were expected to be much lower than in plants supplied higher concentrations of N. At 1 dai, mean amino acid contents of second leaves of plants supplied 0.04 dm^{-3} N (all treatments) were more than ten fold lower than the mean amino acid contents of plants supplied 2 mmol dm^{-3}. By 10 dai amino acid contents were approximately 3 times greater in plants supplied 2 mmol dm^{-3} N. Soluble protein contents were only measured at 1 dai in second leaves of plants supplied 0.04

mmol dm^{-3} N but at this time they were approximately 80 % lower than in plants supplied 2 mmol dm^{-3}.

Disease expression was almost nil in plants supplied 0.04 mmol dm^{-3} N compared with disease levels of up to 26 % necrosis in plants supplied 2 and 8 mmol dm^{-3} N (see chapter 2) which supports the hypothesis that in general increasing N supply will increase susceptibility to disease in certain pathosystems (Agrios 1997; Hoffland *et al.,* 1999, 2000; Snoeijers *et al.,* 2000). Researchers have studied the effects of increasing N supply to wheat crops on the severity of infection by *Septoria tritici* (Leitch and Jenkins, 1995; Olesen *et al.,* 2003; Simón *et al.,* 2003) and found that increasing N supply increases severity of infection by *S. tritici*. The area under the disease progress curve and leaf N concentration have a consistently linear relationship for *S. nodorum* on wheat (Olesen *et al.,* 2003), and it is suggested that this is principally due to changes in the crop microclimate and proximity of leaves that create more favourable conditions for germination. Crop microclimate is an important factor for fungal spore germination, but data presented here suggest that the increase in severity of infection by *S. nodorum* with increasing N supply is also associated with changes in the nutrient status of leaves.

The effects of N concentration on leaf C status

The hypothesis that plants supplied high concentrations of N will have lower soluble carbohydrate contents, lower soluble polyphenol contents and lower C: N ratios than plants supplied low concentrations of N is accepted. Carbohydrate contents of plants supplied 0.04 mmol dm^{-3} N were significantly higher than those of plants supplied 2 and 8 mmol dm^{-3} N. Soluble polyphenol contents and C: N ratios were significantly lower in plants supplied moderate and high concentrations of N than plants supplied low concentrations of N, and there was a negative (although non-significant) relationship between polyphenol contents and N contents of leaves. When N supply is low partitioning of growth to the roots is favoured, and although photosynthesis is decreased there is a reduced demand for C skeletons for N assimilation and structural growth of shoots so carbohydrates tend to accumulate (Lawlor, 2002), and this may account for the increased polyphenol contents of leaves from plants supplied low N (Hoffland et al., 1999).

That plants supplied 0.04 mmol dm^{-3} N have high C: N ratios in was not surprising as the total C content on these plants was high and the total N content was low. That plants supplied 0.04 mmol dm^{-3} NH_4^+ have high soluble polyphenol contents was also unsurprising as the reduction of soluble polyphenol contents with increasing N content is well documented. High N supply can reduce the activity of some key enzymes of phenol metabolism e.g. phenylalanine-ammonia-lyase (PAL) (Matsuyama and Dimond, 1973,) and the content of some phenolics (Kiraly, 1964 cited by Marschner, 1995). Applying N fertiliser to wheat plants caused a reduction in soluble phenolics in leaves, and a corresponding increase in susceptibility to stem rust (*Puccinia graminis tritici*) (Kiraly, 1964 cited by Marschner, 1995). Leaves from beech trees that had been fertilised with NH_4NO_3 had lower contents of phenolics than unfertilised trees (Pahlsson, 1992). High N reduced the exudation and accumulation of phenolics from roots of white lupin (*Lupinus albus* L.), and the inhibitory effect was greater when N was supplied as NO_3^- compared to NH_4^+ (Wojtaszek *et al.,* 1993). Here, plants supplied 0.04 mmol dm^{-3} N had little disease compared to plants supplied 2 mmol dm^{-3} N, so it is possible that polyphenol content may have been involved in resistance. However, plants supplied NH_4^+ were more susceptible to *S. nodorum* than plants supplied NO_3^-, and there was no significant difference in polyphenol content between plants supplied NH_4^+ and NO_3^- at either concentration. The synthesis of phenolic compounds in response to pathogen attack is usually restricted to localised infection sites however, so their concentration in a whole leaf or plant may well be diluted and not show up as significant in infected tissues (Hoffland *et al.,* 2000).

The effects of N form on leaf C and N status

The data generally support the hypothesis that plants supplied NH_4^+ will have higher soluble carbohydrate contents, higher total amino acid contents, higher soluble protein contents, lower soluble polyphenol contents, higher C: N ratios, and lower total N contents than plants supplied NO_3^-.

N form clearly has a significant effect on the chemical composition of leaves (**Table 3**), with plants supplied NH_4^+ being very different to plants supplied NO_3^-. Although plants supplied NH_4^+ had lower total C contents than plants supplied NO_3^- they are otherwise

(i.e. their soluble pools) relatively C rich, having high soluble carbohydrate contents, C: N ratios and amino acid contents.

Wheat plants supplied NH_4^+ were more susceptible to infection by *S. nodorum* than plants supplied NO_3^- (see Chapter 2). *S. nodorum* is a necrotrophic pathogen, using extracellular enzymes and toxins to break down host tissues prior to absorbing the breakdown products. Fungi can obtain C by breaking down plant cell walls to release the C in cellulose (Jennings and Lysek 1999), but the soluble carbohydrates available in leaf tissue are much more readily available. Soluble carbohydrate contents were highest in plants supplied NH_4^+ (where infection was greatest), and there was a positive linear relationship between disease symptoms and soluble carbohydrate contents of leaves. This must be viewed with caution however, as a correlation was found for disease symptoms and soluble carbohydrates at 14 dai. The soluble carbohydrate contents of infected leaves was higher than in healthy leaves, and generally increased between 7 and 14 dai, whereas in healthy leaves the soluble carbohydrate content decreased during this time period, suggesting soluble carbohydrates accumulate in infected leaves, possibly due to reduced export (Doodson *et al.,* 1964; Hale and Whitbread, 1973; Hibberd *et al.,* 1996b; Lucas, 1998). Whilst leaf carbohydrate status may be important for *S. nodorum*, it is likely that the presence of the pathogen caused an accumulation of soluble carbohydrates in leaves rather than a direct causal relationship between high concentrations of soluble carbohydrates and increasing susceptibility.

S. nodorum can synthesise nitrate reductase (NR), but uptake of N in the form of amino acids is energetically less costly, and NR will only be produced by the fungus if NO_3^- is the sole source of N (Moore, 1998). It has also been shown that NR is not required for pathogenicity of *S. nodorum* (Howard *et al.*, 1999). Therefore it may be that the availability of a high concentration of free amino acids (and proteins) in the leaves of plants supplied NH_4^+ provide a source of relatively accessible N (and C). This will provide the fungus with a more nutritionally favourable environment in comparison with pathogens infecting plants supplied NO_3^-. Amino acids and amino sugars are a good source of N and C compounds for fungi. The most immediate source of amino acids to a

fungus is usually by the assimilation of NH_4^+, which is converted to glutamate via glutamate dehydrogenase (GDH) (Moore, 1998). Amino acids can be taken up directly and fungi have a number of different transport systems with differing characteristics and affinities depending on the type of amino acid (i.e. whether acidic, basic or neutral). There may be preferential uptake of a particular amino acid, and a high concentration of the preferred amino acid will inhibit uptake of the others (Jennings and Lysek, 1999).

Proteins are a significant source of N and C for fungi (Moore, 1998), and many fungi can assimilate simple peptides as well as amino acids (Jennings and Lysek, 1999). Again, soluble protein contents were highest in leaves from plants supplied NH_4^+ where infection was greatest. Infection of wheat by *S. nodorum* can increase grain protein content, and research has shown that controlling *S. nodorum* with fungicides (including triazole and strobilurin fungicides) reduces the grain protein content of some varieties of wheat (Dimmock and Gooding, 2002), but interactions between grain protein content and fungicide use vary according to factors such as cultivar, N fertiliser use and weather (Dimmock and Gooding, 2002).

At 12 dai diseased leaves from plants supplied 2 mmol dm^{-3} NO_3^- had 54 % higher soluble polyphenol contents than diseased leaves from plants supplied 2 mmol dm^{-3} NH_4^+. As plants supplied NO_3^- were less susceptible to infection than plants supplied NH_4^+ this result is reassuring. It is surprising that, as polyphenol synthesis is a rapid response to infection (Nicholson and Hammerschmidt, 1992), differences were not seen until 12 dai. However, the polyphenol concentrations were very low, accounting for less than 0.5 % of leaf dry weight, so perhaps there were localised differences but that were initially diluted by the large volume of leaf (Hoffland *et al.*, 1999). At the same time that differences were found between plants supplied NH_4^+ and NO_3^-, there was a significant difference in polyphenol contents of healthy and diseased plants supplied 2 mmol dm^{-3} NO_3^-. In this case, leaves of diseased plants had 36 % higher polyphenol contents than leaves of healthy plants. In the experiment in which polyphenols were analysed (disease data not shown), at the earliest disease assessment (7 dai), second leaves of plants supplied NH_4^+ had over twice as many lesions as leaves from plants supplied NO_3^-. By 12

dai symptoms had increased on leaves from plants supplied both forms of N, and there was still 43 % difference in lesion numbers and necrotic area between the two.

In agreement with the hypothesis total N contents were highest in plants supplied NO_3^-, which had lower levels of infection than plants supplied NH_4^+. Although the total amount of N available to the fungus is important, the type of N compounds available is clearly at least as important nutritionally as the total N content. A plant with a higher N content may also produce more N-based defence compounds such as alkaloids (Hoffland *et al.*, 1999), which were not measured here. It is clear that although nitrogen supply affects resistance to disease, it is not just the nitrogen that is important, but the complex metabolic changes that arise from supply of different forms of N.

The effects of N form on the amino acid profiles of leaves

The data support the hypothesis that plants supplied NH_4^+ will contain more amides (glutamine and asparagine) than plants supplied NO_3^-. Healthy plants supplied NH_4 contained 86 % more glutamine than healthy plants supplied NO_3^-. Asparagine was not detected in healthy plants supplied NO_3^-, whereas asparagine made up 72% of the total amino acid profile in healthy plants supplied NH_4^+. Diseased plants supplied NH_4^+ contained 87 % more asparagine and 57 % more glutamine than diseased plants supplied NO_3. Comparable figures were found by Cramer and Lewis (1993*a*). The xylem sap of wheat plants that were supplied N as 4 mM NO_3^- or NH_4^+ was analysed and the concentration of amides (asparagine and glutamine) were 87 % and 42 % higher respectively in plants supplied NH_4^+ than in plants supplied NO_3^- Total amino acid concentrations were not significantly different, but plants supplied NH_4^+ had significantly higher amounts of organic N than plants supplied NO_3^- (Cramer and Lewis, 1993*b*). Lewis *et al.*, 1982 investigated the composition of xylem sap from 20 d old barley plants that had been supplied N as 2 or 8 mmol dm^{-3} NO_3^- or NH_4^+, and found that plants supplied NH_4^+ had 34 % and 65 % more asparagine in plants supplied 2 and 8 mmol d^{-3} NH_4^+ (respectively) than plants supplied 2 and 8 mmol dm^{-3} NO_3^-. Contents of glutamate, glutamine, aspartate (2 mmol dm^{-3} N only) and total organic N were all higher in plants supplied NH_4^+ than plants supplied NO_3^- (calculated from Lewis *et al.*, 1982). Other

research exists to support the data showing accumulation of asparagine in wheat plants supplied NH_4^+ (Cramer, Lewis and Lips, 1993; Ikeda *et al.*, 2004).

It is impossible to compare amino acid profiles directly between healthy and diseased leaves in this study, as (due to a very small number of samples being analysed) healthy and diseased leaves were sampled at different times. Any differences in amounts of a particular amino acid or amide may therefore be due to e.g. the age of the leaf rather than as a response to infection. However, it may be noted (with caution) that in leaves from plants supplied NO_3^-, diseased leaves had higher amounts of all the amino acids in the profile except aspartate; diseased leaves from plants supplied NH_4^+ had more serine, glutamine and alanine than healthy leaves from plants supplied NH_4^+.

Other researchers have found accumulation of certain amino acids and amides in response to N supply and infection. Pahlsson (1992) found that fertilising *Fagus sylvatica* with NH_4NO_3 increased glutamate, aspartate, glutamine and asparagine concentrations in leaves. Farkas and Kiraly (1961) inoculated wheat plants with *Puccinia graminis tritici* and found that infection caused increases in NH_3, glutamine and asparagine contents in leaves of susceptible varieties. There was no change in glutamic acid in response to infection. Addition of NH_4^+ salts to healthy tissue increased glutamine but not asparagine, and decreased levels of glutamic acid. Glutamine synthetase (GS) activity was found to be higher in infected tissues and the authors suggested that the fungus produces NH_4^+, which increases GS activity. Glutamine is important for fungal development, including the biosynthesis of purines and chitin (Farkas and Kiraly 1961), so modification of plant metabolism in such a way that increases glutamine production would be favourable for the fungus. Similar responses were found by Sadler and Scott (1974) who infected barley (*Hordeum vulgare* L. cv Prior) with powdery mildew (*Erysiphe graminis* f. sp. *hordei*). They found that infected leaves contained elevated NH_4^+ but little increase in NO_3^-, and the activities of GS and asparagine synthetase (AS) were increased. Concentrations of glutamate, aspartate and glutamine in leaves rose as soon as 1 d after inoculation, and asparagine contents began to rise 3 d after inoculation. Solomon and Oliver (2001) found a 4 to 5 fold increase in concentration of most amino acids in tomato leaves between 7

and 14 d after inoculation with *Cladosporium fulvum*. It was suggested that the fungus was using apoplastic amino acids and amides such as glutamate, glutamine, aspartate and asparagine, and possibly γ-aminobutyric acid (GABA) which was present at high concentrations (Solomon and Oliver, 2001). It has been suggested that the fungus somehow manipulates the host plant's metabolism to increase apoplastic amino acids, particularly GABA in the case of *C. fulvum* in tomato (Solomon and Oliver, 2002; Solomon *et al.*, 2003*b*).

Asparagine is rich in N; it has 4 C atoms and 2 N atoms, and is sufficiently stable for long distance transport and long term storage (Sieciechowicz *et al.*, 1988; Taiz and Zeiger 1998). Asparagine is more stable than glutamine and is mobile at physiological pH. Both asparagine and glutamine are products of protein degradation, and are synthesized and transported during leaf senescence. Asparagine and glutamine may therefore accumulate during the breakdown of plant proteins by necrotrophic plant pathogens. Amides may also act as markers of stress as they are synthesised during exposure of plants to stresses such as carbohydrate starvation (Brouquisse *et al.*, 1992). N may be stored as asparagine when there are high levels of organic N relative to C skeletons (Lam *et al.*, 1996).

It is possible that asparagine is important for the reproduction of *S. nodorum*. Experiments investigating the effects of N nutrition on the reproduction *in vitro* of *S. nodorum* (see chapter 4) found that pycnidial production and sporulation were greatest in cultures grown on asparagine. Cultures grown on glutamine did not produce pycnidia, but grew well vegetatively. Richards (1951) performed similar experiments but combined different N sources with different C sources, and also found that cultures grown on asparagine combined with various carbon sources produced abundant pycnidia.

The experiments described in this chapter highlight the differences in leaf chemistry between plants supplied different forms and concentrations of N, and indicate that these differences may account, at least partially, for the differing susceptibility of wheat plants to *S. nodorum* in response to altered N supply. As well as nutritional differences resulting from altered N supply, there are some significant differences in defence compounds,

although further investigation of these may be beneficial in order to really understand the defence mechanisms involved. The results are not clear cut, and would benefit from increased sample numbers to allow each analysis to be carried out on plants grown at all three N concentrations. In some cases performing analyses at more time points, particularly before or soon after inoculation would have been beneficial. The small sample numbers combined with variability of the data do not allow relationships between disease and the different chemical characteristics to be fully explored, as apparent correlations between disease and e.g. amino acid content or N content were not significant. Sample numbers used for amino acid profiling were very low as we were limited by the number of samples that could be analysed, but we can be fairly confident in the results after comparing them where possible with those of other researchers.

The most significant finding of these experiments was that the form of N supplied to wheat plants significantly affects their leaf chemistry, more so than the concentration of N supplied.

Conclusions

In conclusion, I suggest that the greater availability of soluble carbohydrates, proteins, amino acids and amides in leaves of wheat plants supplied NH_4^+ compared with leaves from plants supplied NO_3^- provides a more favourable environment for *S. nodorum*, rendering these plants more susceptible to infection. Plants supplied very low amounts of N (0.04 dm^{-3} mmol) are poor hosts for the fungus, probably due to a combination of low nutrient availability and high levels of phenolic defence compounds. Plants supplied N at concentrations above 2 mmol dm^{-3} are likely to be receiving luxury amounts of N, where any increased N availability does not significantly affect susceptibility to *S. nodorum*. The nutritional status of wheat plants is an important factor in modifying its susceptibility to *S. nodorum*, and it is perhaps as important as defence compounds in determining the success of the pathogen *in planta*.

Chapter 4 Development and reproduction of *Stagonospora nodorum in vitro* is sensitive to the form of nitrogen

Introduction

Fungal N metabolism

In a leaf infected by a fungal pathogen, the form of N available to the fungus will vary according to the nutritional status of the host plant, but may be nitrate (NO_3^-), ammonium (NH_4^+), proteins, amino acids or amides (Solomon *et al.*, 2003*b*). *S. nodorum* is able to reduce NO_3^- using NO_3^- reductase, but to convert one molecule of NO_3^- to NH_3, consumes the equivalent of 4 molecules of $NADPH_2$ (880 kJ), plus the additional energy required to assimilate the NH_3 (Moore, 1998). NO_3^- reductase is only synthesised by *S. nodorum* if NO_3^- is the only available source of N (Moore, 1998). NO_3^- is probably taken up via direct diffusion into hyphae down the gradient that is produced by the intracellular activities of NO_3^- and NO_2 reductases (Watkinson, 1999). Fully pathogenic NO_3^- non-utilising mutants of *S. nodorum* and other fungi have been found (Howard *et al.*, 1999), suggesting that amino acids and other reduced N sources can be used in preference to NO_3^- during infection (Solomon *et al.*, 2003*b*). NH_4^+, glutamine (gln) and glutamate (glu) are the preferred N sources for most fungi although other N compounds including purines, other amino acids and protein can be used (Marzluf, 1997).

NH_4^+ is a major regulator of N metabolism. Uptake of NH_4^+ by fungi is poorly understood, but it is likely to be taken up via active transport (Carlile and Watkinson, 1994; Watkinson, 1999) and is then assimilated using NADP-linked glutamate dehydrogenase (GDH) and 2-oxoglutarate to yield glu. Fungi take up amino acids by active transport systems (the number of which varies between species), which have differing affinities for different amino acids, and certain amino acids may be taken up in preference to others (Jennings and Lysek, 1996; Carlile and Watkinson, 1994). Amino acid transport is via permeases, which are specialised proteins that span the plasma membrane. Permeases operate by symport of amino acids with protons across the plasma membrane, and transport is in one direction only; permeases do not transport amino acids out of the cell (Watkinson, 1999).

Research by Nason *et. al.* (Chapter 3) has shown that both form and concentration of N affect resistance of wheat (*Triticum aestivum* cv. Brigadier) to *S. nodorum*. Amino acid profiles of healthy 15 d old leaves showed that they contain aspartate (asp), glutamate (glu), asparagine (asn), serine (ser), glutamine (gln), glycine (gly), threonine (thr) and alanine (ala). Amounts of glu, asn, ser and gln were greater in plants fed NH_4^+ compared with plants fed NO_3^-. Amounts of asp were greatest in plants fed NO_3^-, but amounts of the other amino acids were not significantly different. Relative amounts of amino acids in diseased plants at 24 d old followed the same trend with the exception that plants fed NO_3^- contained more glu as well as asp, than plants fed NH_4^+.

Fungal growth and sporulation

Vegetative growth of fungal colonies on a solid medium is by extension of hyphal apices and branching (Carlile and Watkinson, 1994; Isaac, 1992). Extension is linear, but due to branching, there is an exponential increase in the number of hyphal tips and the total length of the hyphal system. On solid medium there is an approximately circular region of undifferentiated mycelium growing from the point of inoculation, which is a sink for nutrients from the medium and a source of excreted metabolites. A clearly defined colony margin forms and shows a linear increase in radius with time as the colony grows at uniform rate. On solid medium growth will eventually stop due to exhaustion of nutrients or a build up of toxic metabolites or 'staling products', such as ammonia (NH_3) (Carlile and Watkinson, 1994). Mycelia in the colony are a source for nutrients assimilated from the medium, and these nutrients must be taken up in sufficient quantities to support growth and accumulation of nutrient reserves (Moore, 1998). In order for reproductive structures to be produced the colony must comprise healthy mycelia that have accumulated sufficient reserves of nutrients and are preconditioned to undergo morphogenesis. A high C: N ratio is usually required for the production of reproductive structures in fungi, as the C is required for energy. Stored N is diverted from mycelia into the fruiting bodies (Moore, 1998).

Growth and sporulation *in vitro* of *S. nodorum* is affected by growth media (Richards, 1951) in addition to conditions such as light quality and intensity and temperature (Cooke and Jones, 1970). Richards (1951) showed that pycnidial production and sporulation of *S. nodorum* was strongly influenced by the source of N in the growth media, and to a lesser extent by the source of C. Pycnidial production was greatest in cultures supplied with organic N (as amino acids) compared to inorganic N sources and gly was the best amino acid for promoting pycnidial production. Asn also favoured high levels of pycnidial production. Few pycnidia were produced on media containing potassium nitrate (KNO_3) and no pycnidia were produced when ammonium sulphate [$(NH_4)_2$ SO_4] was the N source.

To further investigate the mechanisms of the nutritional basis of resistance of wheat to *S. nodorum*, an experiment was designed based on the research of Richards (1951) to compare the *in vitro* growth and reproduction of *S. nodorum* on NO_3^-, NH_4^+, gly, asn and gln. Gln and asn are commonly used as N storage and transport compounds in plants, and are therefore likely to be widely available to the fungus *in planta*. Asn also has a high ratio of N: C relative to other amino acids. Gly was chosen as an example of an amino acid on which *S. nodorum* reproduces well. A treatment with no N was also included to investigate the effects of N-deprivation. The aim of the experiment was to test the general hypothesis that *in vitro* growth and reproduction of *S. nodorum* is sensitive to N source.

A second experiment investigated further the response of *S. nodorum* to the different amino acids present in leaves of wheat plants supplied N as NH_4^+ or NO_3^- (Chapter 3). Of the amino acids found in leaves, asn, glu, asp and ala were chosen for this experiment. As the concentration of asn had been high in leaves from plants supplied N as NH_4^+ (plants which also had highest incidence of disease) it was important to include asn. Asp was chosen because it was present in high concentrations in both healthy and diseased leaves from plants supplied NO_3^-, being 4.6 and 6.5 times greater than in healthy and diseased leaves, respectively, from plants supplied NH_4^+. The plants supplied NO_3^- also had lower incidence of disease. Glu was chosen because concentrations of glu were high in healthy leaves from plants supplied NH_4^+, but apparently absent from healthy leaves from plants

supplied NO_3^-, and diseased leaves from plants supplied NO_3^- had 5 times more glu than diseased leaves from plants supplied NH_4^+. Ala was chosen because there were similar amounts of ala in leaves of plants grown on NO_3^- and NH_4^+. As concentrations of asp, glu and asn were different in plants grown on NH_4^+ and NO_3^- the effects of supplying these amino acids to the fungus in mixtures was also explored. The aim of these simple experiments was to answer the following questions:

How is radial growth of S. nodorum *colonies affected by N source and how can we relate this to growth of the fungus* in planta*?*

Which N-compounds promote pycnidial production?

Is there evidence that some N-compounds inhibit pycnidial production?

How does in vitro *pycnidial production respond to mixtures of amino acids and how can this be related to reproduction of the fungus* in planta*?*

Methods

Production of inoculum

Maintenance of cultures is described fully in Chapter 2. 14 d old cultures were produced from inoculum stored on sterile wheat grains. To produce a colony, a few wheat grains were shaken out of the bottle onto a 9 cm Petri dish containing CZD-V8 and incubated in the dark at 22 °C for 48 h then under near UV (continuous) and white light (14 hr photoperiod) to encourage sporulation. After 2 weeks the cultures were used to provide inoculum for the experiments described in this chapter.

1. Comparison of nitrate, ammonium and amino acids as N sources for S. nodorum

Preparation of media

A basic nutrient solution containing micronutrients and carbon sources was made up following the recipe of Richards (1951). The carbon sources were sucrose and glucose in equal amounts (each providing 10 g C dm^{-3}). The N sources were ammonium ($(NH_4)_2SO_4$), nitrate ($NaNO_3$), asparagine (asn), glutamine (gln) and glycine (gly). These were added separately to 250 cm^3 Duran bottles, in sufficient quantities to provide the equivalent of 0.425 g N dm^{-3} solution (equivalent to the amount of N in 2 g asn). A control treatment with no added N was included. The basic nutrient solution was added to each bottle, and then the pH was adjusted to pH 6 using 10 M NaOH before autoclaving. Under sterile conditions, approximately 40 cm^3 of media was poured into each Petri dish (5 dishes per treatment), then allowed to set.

Experimental set up

Under sterile conditions a 4 mm diameter plug of *S. nodorum,* taken from the edge of a sporulating culture, was placed in the centre of each Petri dish. Petri dishes were incubated in the dark at 22 °C for 48 h, then under a light bank with white light (14 h photoperiod) and continuous black (near UV) light. The temperature was maintained at 20 ° C by placing the Petri dishes on metal plates containing tubing linked to a water bath. For each treatment 5 replicate dishes were included, arranged in a randomised block

design. Colony diameter was measured 1, 4, 6, 8, 10 and 14 d after inoculation. Pycnidial production was assessed by estimating the area (%) of the colony covered by pycnidia. Colour and colony morphology were noted, and photographs of the colonies were taken.

2. Comparison of amino acids, singly and in mixtures, based on amino acid profiles of leaves as N sources for S. nodorum

Media were prepared as above, but the N treatments were based on amino acids found in healthy wheat leaves from plants grown in hydroponics and supplied with NO_3^- or NH_4^+ as the sole N source (Chapter 3). Treatments were designed to provide N as amino acids, either singly or in mixtures, but to provide a total of 0.425 g N dm^{-3} (as in the previous experiment) (**Table 1**).

Table 1. Description of N sources (amino acids and amides) supplied to *S. nodorum* in solid media as treatments used to investigate the *in vitro* growth and reproduction of *S. nodorum* in response to N. Growth media also contained micronutrients and carbon.

Treatment name	Amino acids	N content (g N dm^{-3})
1 (asn)	asparagine,	0.425
2 (glu)	glutamate,	0.425
3 (asp)	aspartate,	0.425
4 (asp + glu)	aspartate + glutamate,	0.2125 + 0.2125
5 (asp + asn)	aspartate + asparagine,	0.2125 + 0.2125
6 (glu + asn)	glutamate + asparagine,	0.2125 + 0.2125
7 (glu + asp + asn)	glutamate + aspartate + asparagine,	0.1417 + 0.1417 + 0.1417
8 (ala)	alanine	0.425

In this experiment, cultures were placed in a temperature-controlled room (20 °C), and the Petri dishes were cooled as above.

Data analysis

Data were compared using one-way ANOVA with post-hoc tests of least significant difference (LSD) using (SPSS 9.0, SPSS Inc, Chicago, USA). Growth curves of colony diameter were plotted using Sigmaplot (Sigmaplot 8.0, SPSS Inc, Chicago, USA) and linear equations were fitted to the curves.

Results

1. Comparison of NO_3^-, NH_4^+ and amino acids as N sources for S. nodorum

Colony appearance

The treatments produced colonies which were strikingly different in colour, morphology and general appearance (**Plate 1**). There was little variation in appearance between the replicates of each treatment. The appearance of all colonies changed over the course of the experiment. Most colonies, once established, were pink surrounded by yellow, changing to mostly pink surrounded by a little yellow. The change in colour occurred after 8 dai in colonies growing on gly, after 10 dai in colonies growing asn, and after 14 dai in colonies growing on gln and NO_3^-. Colonies growing on NH_4^+ stayed mainly yellow with a little pink. Colonies grown on media containing no N were white to colourless. The morphology of the colonies was initially round for all treatments, but the edges of colonies grown on NH_4^+ became very irregular in shape from 6 dai. The mycelium in this treatment was visibly more branched than those in the other treatments (with the exception of No N which was also highly branched). Some cultures had 'satellites' (small round areas of growth slightly away from the edge of the colony); this was most common in colonies grown on asn.

Colony growth

Significant differences in colony diameter began to appear 4 d after inoculation of the media. Colonies grown on NH_4^+ had significantly smaller diameters ($P < 0.01$ except gln) than those from all other treatments (**Figure 1.**). Colonies with the largest diameter were those grown on gly, with a mean diameter of 56 mm 14 dai. Growth of colonies on gly was significantly higher than growth on NH_4^+ from 4 dai ($P < 0.01$); NO_3^- and gln from 6 dai, and from all other N sources after 8 dai ($P < 0.01$ or < 0.05). Colonies grown without N had the second largest diameters, with a mean value of 44 mm 14 dai. At 8 dai these colonies were significantly larger than colonies grown on NH_4^+ and NO_3^- ($P < 0.05$); at 10 dai they were larger than colonies grown on NH_4^+, NO_3^- and asn ($P < 0.05$) but were not significantly different from colonies grown on gln; and by 14 dai were significantly higher than the rest of the N treatments other than gly ($P < 0.05$). However, N-deprived

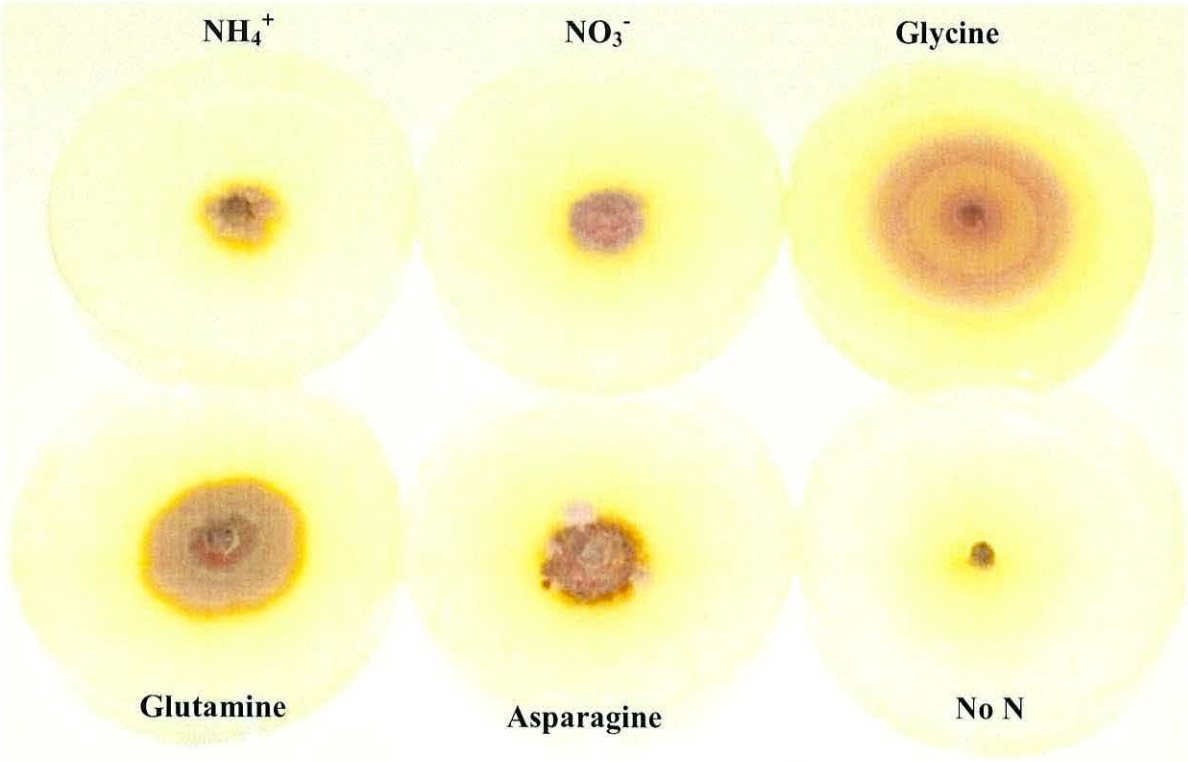

Plate 1. Photograph of 14 d old *S. nodorum* colonies grown on solid medium containing NH_4^+, NO_3^-, glycine, glutamine or asparagine as the sole N source, or no N.

colonies were almost colourless, with very sparse, mostly submerged mycelium, and bore little resemblance to a 'normal' *S. nodorum* colony. Colonies grown on gln had very similar diameters to those grown on No N until 14 dai. By 10 dai colonies grown on asn were smaller than those grown on gln (P <0.05). Colonies with the smallest diameters were those grown on NO_3^- and NH_4^+, and there was no significant difference in size between these two treatments after 8 dai.

All colonies displayed a linear increase in diameter (**Table 2**) [although for gly, goodness of fit (r^2) and significance of fit (P) were better when a single exponential equation plus constant was fitted to the curve, in this case, r^2 was 0.96 and $P < 0.0001$]. Growth rates predicted by the equation follow the same trend as diameter, with colonies grown on gly having the fastest growth rates, followed by colonies growing on no N; then colonies grown on asn and gln had similar, intermediate growth rates, and colonies grown either on NO_3^- or NH_4^+ had the slowest growth rates.

Table 2 Goodness (r^2) and significance (P) of fit of, and radial growth from, a linear equation (y = a + b*x) fitted to change in mean diameter of *S. nodorum* colonies (mm) over time (0-14 d). Colonies were grown on solid medium where N was supplied as NH_4^+, NO_3^-, glycine (gly), glutamine (gln) asparagine (asn), or no N. The total amount of N provided was 0.425 g dm^{-3} in all treatments.

Treatment	r^2	P	**fitted radial growth rate (mm d^{-1})**
gly	0.95	0.0002	2.7 ± 1.7
no N	0.97	<0.0001	2.3 ± 1.5
gln	0.98	<0.0001	2.0 ± 1.4
asn	0.99	<0.0001	2.0 ± 1.3
NO_3^-	0.98	<0.0001	1.5 ± 1.1
NH_4^+	0.99	<0.0001	1.3 ± 1.0

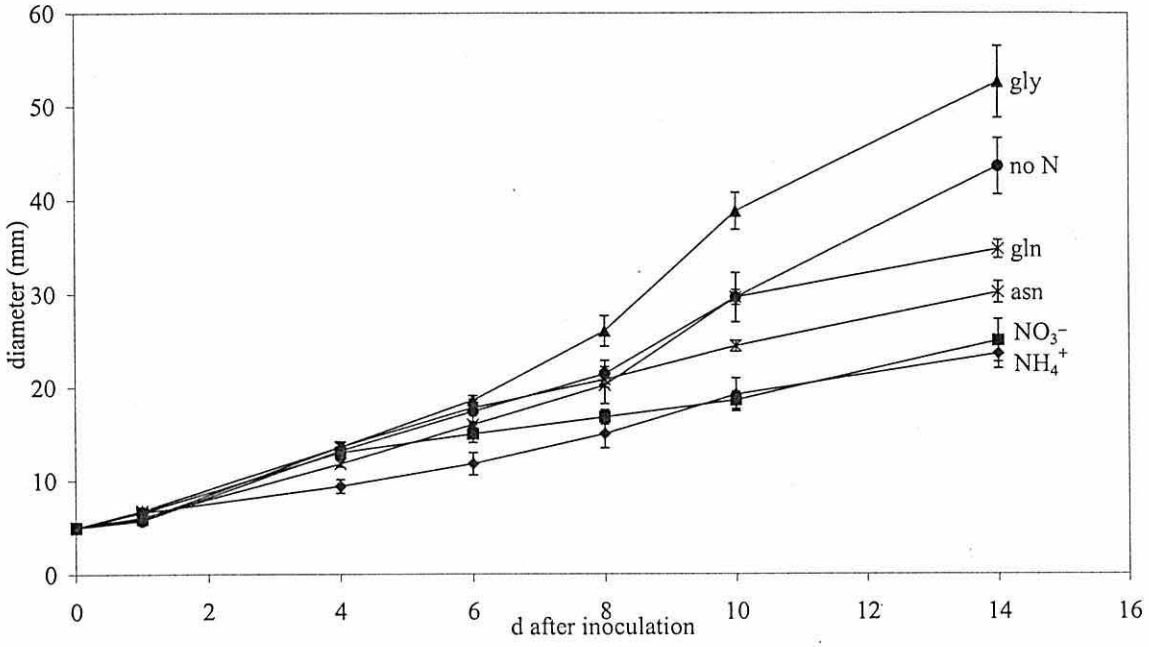

Figure 1 Mean ± SE diameter of *S. nodorum* colonies grown for 14 d on solid medium supplemented with glycine (gly), asparagine (asn), glutamine (gln), nitrate (NO_3^-), ammonium (NH_4^+) or no N. The total amount of N was 0.425 g dm^{-3} in each treatment except no N.

Pycnidial production

The first pycnidia became visible by 6 dai (**Fig. 2**). Pycnidial production was greatest on media containing asn, and then NO_3^-, but the difference in pycnidial numbers between these two treatments was only significant 6 and 8 dai ($P < 0.001$). Mean pycnidial coverage of colonies grown on asn reached 80 % by 14 dai and for colonies grown on NO_3^- the mean coverage was 70 %. Pycnidia were visible in two replicates of the gly treatment from 6 dai. The other three replicates remained non-reproductive. At 6 and 8 dai there was no significant difference in pycnidial production between colonies grown on NO_3^- and gly, but by 10 dai and at 14 dai colonies grown on NO_3^- had significantly higher pycnidial production than colonies grown on gly ($P < 0.01$). Colonies grown on media with no added N produced a few pycnidia in 4 out of 5 replicates, but they covered no more than 5 % of the colony area. No pycnidia were produced by cultures grown on media containing gln or NH_4^+. Spores exuded from the pycnidia in all replicates of the asn treatment, two replicates of the NO_3^- treatment, one replicate of the gly treatment, and in none of the other treatments.

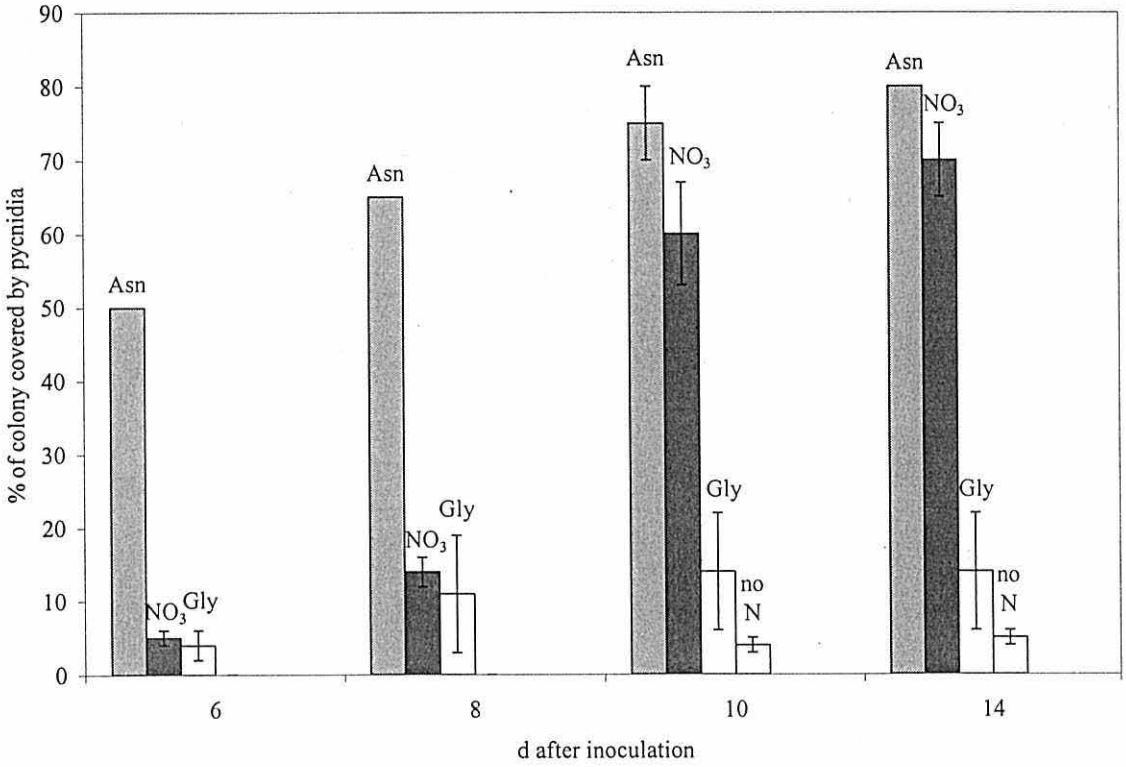

Figure 2 Area (%) (mean ± SE) of *S. nodorum* colonies covered by pycnidia. Colonies were grown for 14 d on solid medium supplemented with glycine (gly), asparagine (asn), glutamine (gln), nitrate (NO_3^-), ammonium (NH_4^+) or no N, to provide 0.425 g N dm^{-3} in each treatment except No N. Note that no pycnidia were produced by colonies grown on gln or NH_4.

2. Comparison of amino acids, singly and in mixtures, based on amino acid profiles of leaves as N sources for S. nodorum

Colony appearance

Colony morphology was generally round with dense, fluffy mycelium. By 10 dai colonies growing on asn and glu + asn had mycelium that looked more like aggregated strands, and appeared to be more branched than those of other treatments, but this was also common among the other treatments by 14 dai. By 10 dai some colonies appeared to have different sectors of growth. The colour of the colonies was initially white with a little yellow. By 7 dai most colonies were pink surrounded by yellow and at the end of the experiment all colonies were mostly pink surrounded by a thin band of yellow.

Colony growth

Growth, measured as increase in colony diameter (**Fig. 3**) was best favoured by ala, asn, glu and glu + asn. There were no significant differences in colony diameter between these treatments. Media that contained asp produced the smallest colonies, all of which were smaller than colonies grown on media that did not contain asp; at 7, 10 and 14 dai these differences were highly significant ($P < 0.01$). Of the four treatments producing the smallest colonies, at 7 , 10 and 14 dai, colonies grown on asp alone were significantly smaller than those grown on asp + glu ($P < 0.01$) and at 7 and 14 dai, colonies grown on asp alone were significantly smaller than those grown on glu + asp + asn ($P < 0.01$).

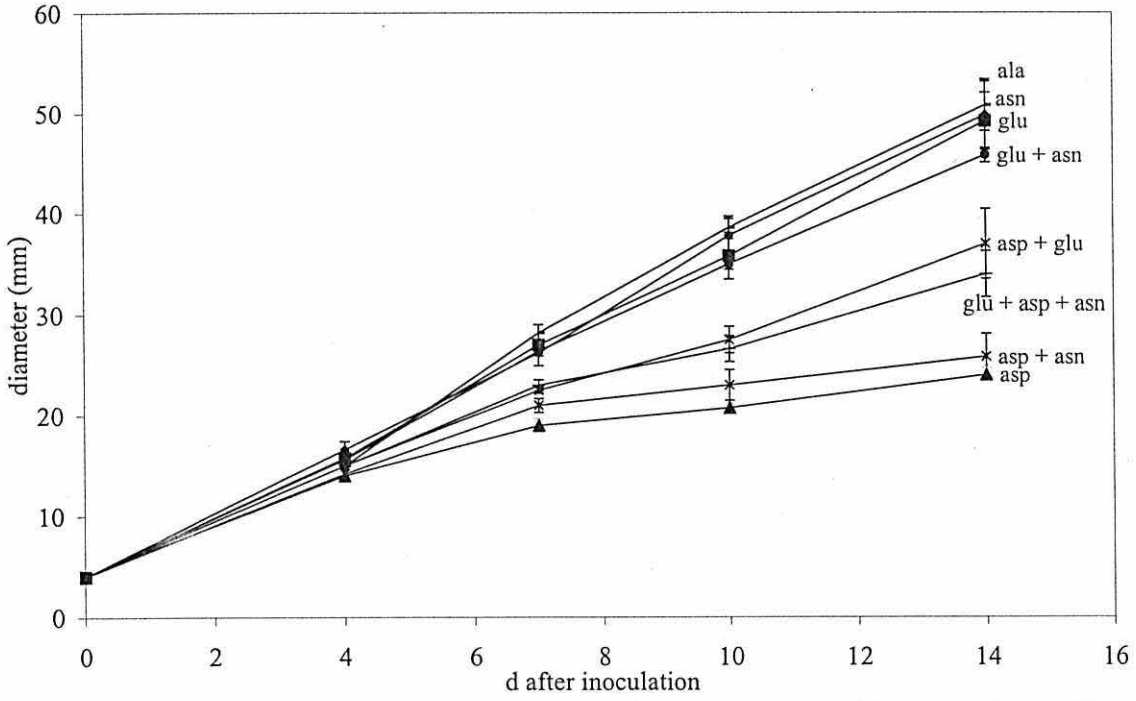

Figure 3 Mean ± SE diameter of *S. nodorum* colonies grown for 14 d on solid medium supplemented with alanine (ala), asparagine (asn), glutamate (glu), aspartate (asp), glu + asn, asp + glu, glu + asp + asn or asp + asn to provide 0.425 g N dm^{-3} in each treatment.

The increases in diameter of colonies from all treatments were best described using a linear equation (**Table 3.**). Colonies growing on media containing asp had the slowest growth rates. The goodness of fit (r^2) and significance of fit (P) of the linear equation are less good for treatments containing asp than for the other treatments. The four treatments not containing asp having the highest growth rates and these were quite similar; growth

rates of colonies grown on asp + glu and glu + asp + asn were lower than the above but similar to each other, and the remaining two treatments containing asp had the lowest growth rates which, again, were similar to each other.

Table 3. Goodness (r^2) and significance (P) of fit of, and radial growth from, a linear equation (y = a + b*x) fitted to change in mean diameter of *S. nodorum* colonies (mm) over time (0-14 d). Colonies were grown on solid medium where N was supplied as alanine (ala), asparagine (asn), glutamate (glu), aspartate (asp) or combinations of these amino acids. The total amount of N provided was 0.425 g dm^{-3} in all treatments except no N.

Treatment	r^2	P	fitted radial growth rate (mm d^{-1})
ala	0.99	0.0003	3.25 ± 2.0
asn	0.99	<0.0001	3.30 ± 2.0
glu	0.99	<0.0001	3.20 ± 2.0
glu + asn	0.99	<0.0001	3.05 ± 1.9
asp+ glu	0.99	0.0001	2.50 ± 1.6
glu + asp + asn	0.98	0.0006	2.40 ± 1.5
asp + asn	0.96	0.0039	1.90 ± 1.3
asp	0.95	0.0044	1.70 ± 1.2

Pycnidial production

Colonies grown on media containing all N sources except glu produced pycnidia (**Fig. 4.**). Pycnidial size varied between treatments. Pycnidial production was greatest in colonies grown on asn; these produced many pycnidia, and were generally larger than colonies in other treatments. Mean coverage of the colonies growing on asn by pycnidia was 83 % 10 dai and 79 % 14 dai. At both 10 and 14 dai, pycnidial production in colonies grown on asn was significantly higher ($P < 0.001$) than in all other treatments (with the exception of asp + asn). Pycnidial production by colonies grown on asp + asn was also high, with mean values of 65 % and 67 % pycnidial coverage at 10 and 14 dai respectively. This was significantly higher ($P < 0.01$) than the pycnidial production in all other treatments except asn. Combining glu with asn inhibited pycnidial production, as mean pycnidial coverage in this treatment was 49 % 10 dai and 41 % 14 dai; this was significantly lower than in colonies grown on asn alone ($P < 0.01$) and at 14 dai it was lower than in colonies grown on asp + asn ($P < 0.01$). Colonies grown on ala produced few pycnidia 7 dai, but by 14 dai pycnidial coverage was comparable to that in colonies grown on glu + asp + asn at

just under 30 %. Colonies grown on media containing glu as the only source of N produced few pycnidia, with a mean colony coverage of 1 % 14 dai. Colonies grown on media containing asp produced more pycnidia than colonies grown on media that did not contain asp. The stimulatory affect of asn on pycnidial production seemed to be inhibited by the presence of glu and asp. Spores exuded from pycnidia in all treatments.

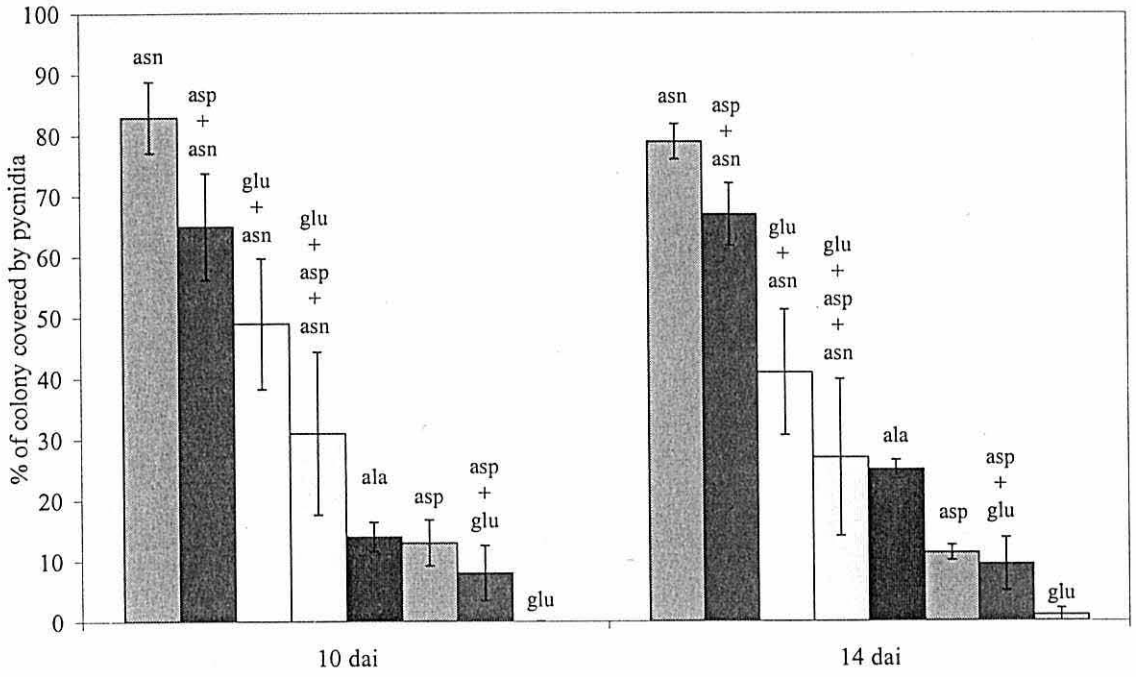

Figure 4 Pycnidial production (% of colony area covered by pycnidia: mean ± SE) by *S. nodorum* colonies grown for 14 d on solid medium supplemented with alanine (ala), asparagine (asn), glutamate (glu), aspartate (asp), glu + asn, asp + glu, glu + asp + asn or asp + asn to provide 0.425 g N dm^{-3} in each treatment.

Discussion

The *in vitro* development and reproduction of *S. nodorum* is sensitive to the form of N supplied. In both experiments there were significant differences in growth and reproduction between colonies grown on media containing different sources of N. It is important to note here that radial growth is a poor measure of colony development; the measurement of fungal biomass would have been better. Comparisons of radial growth rates between colonies grown on different N sources may not be significant, as the radial growth of a colony is a function of the width of the peripheral zone and the specific growth rate of the culture; anything which may affect the frequency of hyphal branching will influence the width of the peripheral zone and the relationship between radial extension and specific growth rate (Isaac, 1992). Radial growth is therefore used here as a guide to the development of the colonies. The assessment of pycnidial production is more useful in assessing the response of the fungus to the N sources, as reproductive success is highly important for a pathogen. Further measurement of pycnidial production e.g. assessment of numbers of pycnidia and sporulation would improve the robustness of the data.

It has been pointed out that the amount and type of C provided will affect the ability of fungi to use different N sources (Berredgem et al., 1998). For example, the use of certain amino acids by *Armillaria mellea* is more effective when ethanol is supplied as a source of C, compared with glucose (Weinhold and Garraway, 1966), and mycelial growth of *Trichoderma viride* on asparagine increased with increasing C supply (Aube and Gagnon, 1969).

How is radial growth of S. nodorum *colonies affected by N source and how can we relate this to growth of the fungus* in planta*?*

In both experiments the increase in colony diameter was linear, as expected. In the first experiment, the fit of the linear equation was highly significant in all treatments except gly, which also had the lowest r^2 value; in this treatment data were slightly better described by an exponential equation. Goodness (r^2) and significance (P) of fit of linear equations were all very high for the treatments asn, glu and glu + asn and slightly less

good for asp + glu and ala. The treatment with the lowest r^2 and P, and lowest predicted growth rate was asp, which also had the smallest mean colony diameter.

Radial growth of colonies at 14 dai was greatest on amino acids. Colonies grown on NO_3^- and NH_4^+ had poor radial growth relative to the other treatments. Growth rates of colonies on media containing NH_4^+ were slowest (although the increase in colony diameter was still linear) and the colonies did not look healthy. As the NH_4^+ was provided as $(NH_4)_2 SO_4$ it is likely that sulphuric acid was released into the medium as the NH_4^+ was assimilated by the fungus (Richards, 1951). When NH_4^+ is provided (in combination with an anion, e.g. sulphate) as an N source in growth media, acidification of the medium can occur; often only the NH_4^+ ion is taken up, which is balanced by extrusion of H^+ (Carlile and Watkinson, 1994), thus the sulphate provided as the anion in this experiment may have formed sulphuric acid. This may explain the unhealthy appearance and poor growth in this treatment

Radial growth rate was fast in N-deprived colonies, with the second largest mean colony diameter and growth rate by 14 dai. However, the colony was of very poor quality, with very pale, mostly submerged mycelium. The agar plugs which provided the initial inoculum on the Petri dishes may have contained N that was available to the fungus, as would the hyphae and pycnidia in the plug. The medium still contained C and micronutrients, so growth was possible, but the absence of N in this treatment had a striking effect on the morphogenesis of the fungus.

The growth of a fungal colony on a Petri dish is clearly an artificial situation, far removed from the real situation of growth into and inside a wheat leaf. After a spore of *S. nodorum* has successfully penetrated the epidermal cell of a wheat leaf it must grow, intercellularly at first, between the cells of the epidermis and into the mesophyll, before growing intracellularly and ramifying through the leaf. There are cell walls, membranes and organelles to contend with, in addition to the deposition of lignin in cell walls as a mechanism against the invasion of the fungal hyphae (Bird and Ride, 1981). The colonies with the slowest *in vitro* radial growth rates were those grown on NH_4^+, NO_3^- and

aspartate, with mean increases in colony diameter of 1.3, 1.5 and 1.7 mm d^{-1} respectively. If we convert these rates from mm d^{-1} to μm d^{-1} they become 1300 to 1700 μm d^{-1}, or, taking into account the standard errors, 300-2900 μm d^{-1}. This increase in colony diameter is due to the extension of all the hyphal tips at the edge of the colony. If we consider the growth of the fungus *in planta* and consider the dimensions of epidermal and mesophyll cells within a wheat leaf, an average epidermal cell could be 170 μm long by 38 μm wide (Miralles *et al.*, 1998), and an average mesophyll cell could be 150 μm long with a cross sectional area of 750 μm (Masle, 2000). *S. nodorum* spores penetrate the epidermal cells of wheat leaves within 10 h of germination (Bird and Ride, 1981) and initially grow intercellularly. Hyphae are not usually seen within the mesophyll cells until 6 to 9 dai (Karjalainen and Lounatmaa, 1986). If the fungus could grow *in planta* at the rates seen in this experiment *in vitro* then colonisation of the leaf would be very rapid indeed, but growth of *S. nodorum in planta* is inherently much slower than growth *in vitro*, and at the above growth rates the fungal colony would fill the width of a young wheat leaf within 3 - 10 d.

Which N-compounds promote pycnidial production?

To answer this question I will initially consider single N sources. See below for a further discussion relating to mixtures of amino acids.

Asparagine clearly promotes pycnidial production. In both experiments it was the N source on which colonies produced the most abundant pycnidia. Colonies with the second greatest pycnidial production were, surprisingly, those grown on NO_3^-, with pycnidial production being as high as in colonies grown on asparagine by 14 dai (experiment 1). Richards (1951) found that colonies grown on media containing NO_3^- produced fewer pycnidia than colonies grown on glycine or asparagine; however, the total numbers of pycnidia found were much greater in Richards' experiment, so comparisons are difficult. Mycelial growth of colonies grown on NO_3^- was poor, so perhaps vegetative growth of the colony was replaced by reproductive growth. Pycnidia were produced by colonies grown on glycine, but in low numbers and not in all replicates, and pycnidial production was significantly lower in this treatment than for asn and NO_3^-. Colonies grown on

glutamine (the uncharged derivative of glutamate) produced no pycnidia, and in the second experiment colonies grown on glutamate produced very few pycnidia. A few tiny pycnidia were produced by N-deprived colonies, which was surprising. As mentioned previously however, C and micronutrients were available in the media, and some N would possibly have been diverted from the mycelium and pycnidia contained in the initial plug of inoculum.

Is there evidence that some N-compounds inhibit pycnidial production?

Again, I will initially consider the effects of single N compounds on pycnidial production, but see below for further discussion. The compounds that did not support pycnidial production at all were the inorganic N source NH_4^+, the amide glutamine (experiment 1) and the amino acid glutamate (experiment 2). Pycnidial production was poor on alanine and aspartate (experiment 2).

NH_4^+ is widely used as a source of N by fungi (Isaac, 1992; Carlile and Watkinson, 1994; Jennings and Lysek, 1996; Watkinson, 1999), and is often the preferred source (Isaac, 1992; Jennings and Lysek, 1996). Ammonium salts do not always support fruit body production however (Moore, 1998). That glutamine did not support pycnidial production was a surprising result, as this amide should be abundant in wheat leaves and therefore a good source of N for the fungus; plus glutamine is important for fungal development e.g. for the biosynthesis of purines and chitin (Farkas and Kiraly, 1961). It is unfortunate that glutamine was not used in the second experiment to provide more data, as this may have been an anomalous result. Glutamate has a carboxyl side chain and is therefore an acidic amino acid, which perhaps makes it unfavourable to the fungus. Like glutamate, aspartate is another acidic amino acid with a carboxyl side chain. The roles of glutamate and aspartate in inhibiting pycnidial production are discussed below, but it may be important that they are both acidic amino acids whilst the others used in this study are neutral.

Alanine is the second simplest amino acid. It has a ratio of 3 C: 1 N and a methyl side chain. Wheat leaves contain a moderate amount of alanine (Chapter 3). Alanine contributed to 5 and 17 % of the total amino acid profile in healthy leaves from plants

supplied NH_4^+ and NO_3^- respectively, and 16 % and 19 % of the total amino acid profiles in diseased leaves of leaves from plants supplied NH_4^+ and NO_3^- respectively. Although alanine did not support pycnidial growth as well as asparagine and mixtures containing asparagine, by 14 dai colonies grown on alanine had significantly more pycnidia than colonies grown on asp, asp + glu and glu, so I suggest that whilst alanine is a relatively poor source of N for pycnidial production, it certainly does not inhibit pycnidial production.

How does in vitro *pycnidial production respond to mixtures of amino acids and how can this be related to reproduction of the fungus* in planta*?*

Pycnidial production was most abundant in colonies grown on asn in both experiments. In experiment 2 the four treatments with the greatest pycnidial production are those containing asparagine. At 10 dai the combination of asn and asp resulted in a reduction in the mean colony area covered by pycnidia from 83 % on asn alone to 65 % on asp + asn. Pairing asn with glu yielded even less pycnidia (49 % coverage) and providing asn + asp + glu in combination resulted in a mean of 31 % of the colony area being covered by pycnidia. At 14 dai figures were similar. There seems to be compelling evidence for a stimulatory effect of asparagine on pycnidial production. Pycnidial production by colonies grown on asparagine alone was over 6 times greater than by colonies grown on aspartate, and 100 % greater than by colonies grown on glutamate. The relatively high pycnidial production by colonies grown on asp + asn and asn + glu must therefore be a product of the stimulatory effect of asparagine. The presence of two unfavourable N compounds alongside asparagine was sufficient to inhibit pycnidial production to almost three times lower than that on asn alone; however pycnidial production in this treatment was higher than the sum of the mean values of pycnidial production in colonies grown on aspartate alone and glutamate alone. In support, the combination of asp + glu without asn yielded poor pycnidial production with a mean of only 8 % of the colony covered by pycnidia 10 dai and 9 % 14 dai. I suggest that both aspartate and glutamate have an inhibitory effect on pycnidial production, with the latter having a greater effect, and this may be due to their low pH. Fungi have a number of different amino acid transport systems with differing characteristics and affinities depending on the type of amino acid

(i.e. whether acidic, basic or neutral) and there may be preferential uptake of a particular amino acid. A high concentration of the preferred amino acid will inhibit uptake of the others (Jennings and Lysek, 1999). Amino acids are taken up via permeases (Watkinson, 1999). Permeases that are pH sensitive have been found in the rust fungus *Uromyces fabae* (Struck *et al.*, 2004) and in *Saccharomyces cerevisiae* (Karagiannis *et al.*, 1999).

The results presented in this chapter are not entirely meaningful unless we relate them to the likely behaviour of the fungus *in planta* with respect to available nutrients. Wheat leaves contain the amino acids and amides used in this experiment, and the contents of these amino acids within leaves depends on the form of N that is supplied to the plant, and whether the plant is infected or healthy. We know from results presented in Chapter 3 that during the first 7 d after inoculation of a leaf its total protein content declines whilst the amino acid content of the leaf increases. Between 7 and 10 dai, amino acid contents decrease slightly and protein contents correspondingly show a slight increase. However, the same trend was seen in both healthy and diseased plants, so this cannot be attributed to the fungus. Increased sample numbers may have improved the data, as although differences were not significant, diseased plants appeared to have higher amino acid contents than healthy plants.

Between 7 and 10 dai the fungus should have invaded intracellularly and begun destruction of the mesophyll cells. Prior to intracellular ramification it is likely that the fungus will rely on the availability of free amino acids and sugars, but with the use of proteases it can break down cell membranes and absorb the released products of protein degradation (Bindschedler *et al.*, 2003). Asparagine is a good marker of protein degradation, particularly after C starvation or under stress conditions when it can accumulate in plant cells (Brouquisse *et al.*, 1992; Genix *et al.*, 1990).

In the apoplast of tomato leaves infected with *Cladosporium fulvum*, amino acid concentrations increased after 7 dai (Solomon and Oliver, 2001), and the source of these amino acids was thought to be increased protease activity, which increased after 4 dai in a compatible interaction. Most amino acids increased 4 to 5 fold in concentration between 7

and 14 dai, and during this time fungal biomass increased. It was suggested that the fungus was using apoplastic amino acids and amides such as glutamate, glutamine, aspartate and asparagine, and possibly γ-aminobutyric acid (GABA) which was present at high concentrations (Solomon and Oliver, 2001). GABA was found to be an efficient N source for the fungus in vitro, and it has been suggested that the fungus somehow manipulates the host plant's metabolism to increase apoplastic amino acids, particularly GABA in the case of *C. fulvum* in tomato (Solomon and Oliver, 2002; Solomon *et al.*, 2003*b*).

In the experiments described in chapters 2 and 3, plants supplied N as NH_4^+ were more susceptible to infection by *S. nodorum* than plants supplied N as NO_3^-. Plants supplied NH_4^+ had more amino acids (both when measured as total free amino acids, or as the total of amino acids in the profile), more soluble proteins, and also more soluble carbohydrates in their second leaves (the site of inoculation) than plants supplied NO_3^-. The amino acid profiles of the plants supplied NH_4^+ and NO_3^- were discussed in the introduction, but as a reminder, leaves from plants supplied NH_4^+ had 100 % (healthy) and 87 % (diseased) more asparagine than second leaves of plants supplied NO_3, and plants supplied NO_3^- had 85 % (diseased) and 78 % (healthy) more aspartate and 81 % (diseased) more glutamate than plants supplied NH_4^+. The results may be anomalous due to small sample numbers; however other researchers have found higher amino acid contents in plants supplied NH_3 than in plants supplied NO_3^- (Barneix and Causin 1996; Huber and Watson, 1984) and soybean [*Glycine max* (L.) Merr.] supplied NH_3 had 5 times more glutamine and asparagine, and 24 times less aspartate than plants supplied NO_3^- (Kpodar *et al.*, 1993, cited in Barneix and Causin, 1996).

Conclusions

In conclusion, I suggest that asparagine is the most favourable N source for pycnidial production by *S. nodorum in vitro*, and that glutamate and to a lesser extent aspartate inhibit pycnidial production. I also suggest that the increased susceptibility of wheat plants supplied N as NH_4^+ to *S. nodorum* is due, at least partly, to the high levels of free amino acids, particularly the high concentrations of asparagine within leaves.

Chapter 5 Alteration of leaf carbon status by shading changes resistance of wheat to *Stagonospora nodorum*

Introduction

During the lifetime of a wheat crop most of the leaves will experience shading due to either their position within the canopy, or cloud cover. This shading will have implications for the productivity of the crop, and also for quantitative disease resistance. The reduced light intensity itself may affect resistance, as may the changes in microclimate within the crop canopy (see below). The light intensity will be lower, and there will be differences in temperature and relative humidity (RH) low in the canopy. *S. nodorum* is a fungal pathogen that is favoured by humid conditions and an epidemic of the disease begins on the lower leaves of wheat plants and moves upwards through the crop, transmitted by rain splash (Eyal, 1999; Pietravalle *et al.*, 2001). Yield losses due to the disease are most severe when the flag leaf is infected, as it is this leaf (along with the sheath and ears) that provides the main proportion of assimilates used during grain filling. Therefore understanding factors that may increase quantitative resistance to the pathogen and reduce the upward spread through the canopy is important. Shading experiments also provide a convenient way of altering the C status of leaves for investigations into the effects of host plant C status on infection. Applying shading at different times during the infection process should provide insight into whether the timing of reduced leaf C status is important for the development of disease.

Effects of light intensity on disease resistance

The susceptibility of plants to pathogenic fungi will be in part due to the availability of C to the pathogen as well as the allocation of resources to defence mechanisms. Van der Plank (1984) reviewed the concept of high-sugar and low-sugar disease processes in plants. High sugar concentrations in leaves tend to delay senescence and inhibit the enzymatic degradation of plant cell walls by pathogens (Marschner, 1995). This provides partial resistance to low-sugar disease processes, which are nearly always due to necrotrophs (Van der Plank, 1984). Necrotrophs often infect very young or senescing tissues, which have limited resources available for defence. The high-

sugar-resistance theory would suggest that lowering the sugar content of a leaf by shading, particularly prior to inoculation, would increase its susceptibility to a necrotroph. Shading plants after inoculation will lower the sugar status of the infected leaf and perhaps inhibit infection by limiting resources available to the fungus. In contradiction to the theory however, shading a wheat crop prior to inoculation with *S. nodorum* resulted in delayed and reduced incidence of disease (Beed *et al.*, unpublished data). In contrast to low-sugar disease processes, high-sugar disease processes (high-sugar-susceptibility) are generally related to biotrophic pathogens. In order to keep their hosts alive, biotrophs (or obligate parasites) can only grow and reproduce within a living host and must therefore not deplete the host of all its nutrients. Thus plants with low leaf sugar contents are more likely to show resistance to biotrophs (Van der Plank, 1984).

Some shaded plants have low contents of C-based defence compounds e.g. tannins. Two tree species grown in shade had lower leaf dry matter, leaves were less tough, and leaves had significantly lower amounts of total phenolics than trees grown in full sunlight (Dudt and Shure, 1994)

Effect of light intensity on plant biomass, photosynthesis and leaf characteristics

Decreased irradiance decreases the rate of net photosynthesis (Lambers *et al.*, 1998; Lewis *et al.*, 2000; Marschner, 1995) which often leads to lower concentrations of non-structural carbohydrates in the leaves (Lambers *et al.*, 1998; Lewis *et al.*, 1999). Lower rates of photosynthesis will also lead to slower growth rates and less biomass production. If a plant is grown in low light it will respond by maximising capture of light for photosynthesis (Poorter and Van der Werf, 1998), by a greater allocation of biomass to leaf area and therefore higher shoot: root (S: R) ratios (Marschner, 1995; Poorter and Van der Werf, 1998). Leaves of plants grown in low light are often thinner, with smaller and fewer mesophyll cells (Lambers *et al.*, 1998) and high specific leaf areas (SLA; leaf area per unit leaf weight) (Gunn *et al.*, 1999; Poorter and Van der Werf, 1998). The chlorophyll content of leaves depends on light intensity and although irradiance alters the structure of leaves it does not affect chlorophyll content per unit leaf area (Evans, 1998). Plants grown in high compared with low light do

however have less chlorophyll per unit N, a higher chlorophyll a: b ratio, increased electron transport capacity per unit chlorophyll and slightly greater ratio of electron transport capacity to Rubisco activity (Evans and Poorter, 2001). Transpiration rates of shaded leaves will be slower than those of leaves grown in high light. At the top of a canopy the temperature will be higher than lower down in the canopy, which increases the partial pressure of water vapour inside the leaf, and greater difference in vapour pressure between the leaf and the air will cause transpiration to increase (Lambers *et al.*, 1998). Plants grown in full sun tend to have higher N contents per leaf area than plants grown in shade, partly due to the increased supply of N via the transpiration stream (Lambers *et al.*, 1998). There is a linear relationship between N content and photosynthetic capacity, and the N content of leaves is adjusted according to the photosynthetic photon flux density (PPFD) encountered during growth, in order to maximise radiation interception (Grindlay, 1997); thus plants grown in low light will have lower leaf N contents.

Fungal requirements for carbon

Carbohydrates in leaves ultimately arise from photosynthetically fixed C (Farrar and Lewis, 1987; Farrar, 1995). The supply of C to a phytopathogen may come directly from the products of photosynthesis; from translocation of C within the leaf or plant; or from the products of cell degradation by the fungus (Farrar, 1995). A pool of C comprising soluble sugars and insoluble storage carbohydrates is available in apoplastic and symplastic compartments (Ayres *et al.*, 1996). The favoured form of C for most fungi is glucose (Ayres *et al.*, 1996; Carlile and Watkinson, 1994; Hall *et al.*, 1992) whilst carbohydrate in plants is transported mainly as sucrose (Ehness *et al.*, 1997; Farrar, 2001). In wheat plants infected with stem rust, concentrations of sucrose in infected leaves decrease followed by an increase in glucose and fructose concentrations. Sucrose is converted to hexoses by invertase, the activity of which is commonly increased in infected leaves (Heisteruber *et al.*, 1994; Lucas, 1998; Sheard and Farrar, 1987; Whipps and Lewis, 1981).

Wheat leaves infected by *S. nodorum* retain assimilates (Scharen and Taylor, 1968), as in many diseases, particularly those caused by biotrophs (Doodson *et al.*, 1964; Hale and Whitbread, 1973; Hibberd *et al.*, 1996*b*). When leaves are fed $^{14}CO_2$, the

subsequent export of fixed C is often reduced in infected leaves when compared with healthy leaves (Lucas, 1998). Wafford and Whitbread (1976) found that in spring wheat infected with *S. nodorum* there was no significant difference in the total fixation of $^{14}CO_2$ between infected and healthy leaves. There was nearly a 10 % reduction in the proportion of assimilates exported by infected leaves compared with healthy leaves however (Wafford and Whitbread, 1976). In barley leaves infected with brown rust export of ^{14}C was dramatically reduced 8 dai (Owera *et al.*, 1983) and in barley leaves infected with powdery mildew the export of photosynthetically fixed ^{14}C was also reduced in infected compared with healthy leaves, but to a lesser extent (Hibberd *et al.*, 1996).

Hypotheses

Photosynthesis will be reduced by disease but will be unaffected by shading several days before measurement.

Shading will reduce the amount of soluble carbohydrates in leaves compared with the leaves of unshaded plants.

Shaded plants will have reduced plant biomass and will favour partitioning of resources to shoots compared with unshaded plants.

The reduced carbohydrate status of shaded plants will be reflected in lower C: N ratios.

Shaded plants will have higher specific leaf areas than unshaded plants, but the chlorophyll contents of unshaded and shaded leaves will not be significantly different.

The reduced carbohydrate status of shaded plants will increase susceptibility to *S. nodorum* if the shading is applied prior to inoculation, and will decrease susceptibility if the shading is applied after inoculation.

The partitioning of resources to phenolics will be greater in unshaded plants.

Methods

Plant growth

The data in this chapter are from three experiments. In experiment 1, plants were grown in compost. Due to limited space only one treatment (shading post-inoculation) had both inoculated and uninoculated plants. In experiments 2 and 3 plants were grown in hydroponics and in each treatment half of the plants were inoculated and half were uninoculated.

4-d old wheat seedlings were planted in 7 dm^3 troughs containing B&Q multipurpose compost (experiment 1) or in 1/2 strength Long Ashton nutrient solution (experiments 2 and 3). Nutrient solutions were changed every 3 d, and aerated at a rate of 1 dm^3 min^{-1}. Plants were grown in controlled environment cabinets (Sanyo Gallenkamp PG660) at a photosynthetic photon flux density (PPFD) of 450 μmol photons m^{-2} s^{-1} (unless being shaded), 70% relative humidity (RH), and ambient CO_2 concentration (350 ppm) provided by drawing air from above the roof of the building at a rate giving four air changes per hour. Plants were shaded using neutral-density filters (layers of muslin draped over a wire frame) as follows:

Treatment	*Conditions*
1.) Shading pre-inoculation	Plants were shaded to approximately 100 μmol m^{-2} s^{-1} for the 48 h immediately prior to inoculation
2.) Shading post-inoculation	Plants were shaded to approximately 100 μmol m^{-2} s^{-1} for 48h, commencing immediately after the 48 h period of high RH that followed inoculation
3.) No shading	Plants were not shaded

Production of inoculum and inoculation procedure

See previous chapters for detail, but briefly:

Cultures were maintained on Czapek-Dox agar supplemented with Campbell's V8 juice (CZD-V8). Spore suspensions were made from 14 d old cultures; the spore concentration was measured using a haemocytometer, and adjusted to 10^6 (experiments 1 and 2) or 5 x 10^5 (experiment 3) spores cm^{-3} if necessary. Plants were

inoculated with *S. nodorum* when the second seedling leaf was fully expanded, using a squirrel hair paintbrush dipped in the suspension. After inoculation, plants were returned to growth cabinets. A humid atmosphere (required for spore germination) was maintained for the 48 h period after inoculation by enclosing troughs in Perspex boxes, which had been sprayed inside with distilled water.

Disease assessment

Leaves were assessed for lesion numbers and necrosis (percentage of leaf covered by necrotic lesions). The day after inoculation on which the first lesions appeared was noted and lesion numbers on each infected leaf were counted at each harvest. Necrosis was assessed visually.

Analysis of plant material

Plants were harvested on several occasions after inoculation (depending on the experiment and the analysis required, see Table 1), with the first harvest being 0 or 2 days after inoculation (dai), and the final harvest being 10, 12 or 14 dai. At each harvest three to six diseased and healthy plants per treatment were selected at random. Each plant was separated into roots (not experiment 1), shoots, and blade of leaf 2. Leaf 2 of each plant was assessed for disease (number of lesions and percentage area of leaf covered by lesions and associated chlorosis), chlorophyll content (using a chlorophyll meter Spad-502, Minolta) and leaf area (using a Delta T scanner). Blades of second leaves were dried, frozen or freeze-dried prior to analysis. The remaining shoot material and roots of each plant were then dried in an oven at 60 °C to constant weight. Unless otherwise stated, second leaves from 3 replicate plants per treatment were used for each chemical analysis.

Table 1 Treatments and analyses for each of the experiments. For each analysis the number of days after inoculation (dai) that the plant material was harvested or non-destructive assessments were carried out is given.

Analysis	Experiment 1	Experiment 2	Experiment 3
Treatments used	As for expts 2 and 3, but plants in treatments 1 and 3 were all diseased.	1. Shading to ~ 100 μmol m^2 s^{-1} for 48 h pre-inoculation (diseased + healthy) 2. Shading for 48 h post-inoculation (after the 48 h period of high RH) (diseased + healthy) 3. No shading (diseased + healthy)	
Disease assessment	7, 10, 12 dai	7, 9, 14 dai	7, 10 dai
Specific leaf area	5, 12 dai		2, 7, 10 dai
Soluble carbohydrates	0, 2, 7, 12 dai	2, 6 dai	
Total Carbon and Nitrogen (CHN)	0, 2, 7, 12 dai	2, 6 dai	
Chlorophyll content (using SPAD meter)		2, 7, 14 dai	2, 7, 10 dai
Photosynthesis		8, 9, 10 dai	
Total soluble polyphenols			2, 7 dai
Efflux of $^{14}CO_2$		8, 9, 10 dai	
Dry weights	5, 12 dai (no root weights)	2, 6, 14 dai	2, 7, 10 dai
Shoot: root ratios and allometry		2, 6, 14 dai	2, 7, 10 dai

Extraction and analysis of soluble carbohydrates

Soluble carbohydrates were extracted from the middle 3 cm of the dried leaf blade, which was cut into three roughly equal portions and weighed. Soluble carbohydrates were extracted from the leaf portions in ethanol and water and measured as in Chapter 3.

CHN analysis

Leaves were chopped to pass through a 1-mm sieve. Each sample was then re-weighed and sealed in a foil cup and analysed for C, hydrogen (H) and N content using a LECO CHN 2000 combustion analyser, calibrated using EDTA.

Photosynthesis

Measurements began approximately 3 h and ended approximately 7 h into the photoperiod. The rates of photosynthesis, leaf surface conductance, internal CO_2 concentration and transpiration were determined for the second fully expanded leaf by means of an infra red gas analyser with narrow leaf cuvette (CIRAS, PP Systems, Hitchin, Herts., UK). It was intended that three healthy and three diseased plants would be measured for each treatment, but due to constraints on equipment and time diseased plants were measured on two days (three plants per treatment each day) and healthy plants were only measured on one day (three plants per treatment). See Chapter 2 for full method.

Measurement of translocation by ^{14}C efflux

Due to equipment constraints ^{14}C efflux could only be measured on two plants at a time. $^{14}CO_2$ was fed to three healthy leaves and three diseased leaves per treatment for 20 min. At 8, 9 and 10 d after inoculation, one infected and one healthy plant from treatment 2 were taken and the second leaves were fed $^{14}CO_2$ to measure efflux. One week later (8, 9 and 10 dai) plants from treatment 3 (planted and inoculated one week later than the plants from the main experiment, so they were at the same age and stage of disease progression) were also taken for measurement of ^{14}C efflux. See chapter 2 for full method.

Autoradiography

See Chapter 2 for full method. Ilford FP4 was exposed for 5 d and autoradiographs were developed after using Ilford Ilfotec LC29 developing fluid.

Determination of soluble polyphenols (Folin-Ciocalteau method) (Experiment 3 only)

Oven-dried leaves weighing approximately 20 mg were chopped into pieces to pass a 1-mm sieve, placed in tubes and weighed. See chapter 3 for details of extraction and analysis.

Data analysis

Data were compared using one-way ANOVA with post-hoc tests of least significant difference (SPSS 9.0, SPSS Inc, Chicago, USA. Differences were considered statistically significant at $P < 0.05$. Data for efflux of ^{14}C were fitted to double exponential decay curves using Sigmaplot (Sigmaplot 8.0, SPSS Inc, Chicago, USA). From curves of net counts per second (cps), data were used to plot curves of the percentage of the total assimilation which was retained in the leaf with time.

Results

The results are divided into three sections:

1. What shading does to leaf 2

The first section describes the effects of shading on leaf 2 and compares characteristics of healthy shaded and unshaded leaves. Differences between healthy and diseased leaves are also presented. Results presented in this section include dry weights of leaf 2, specific leaf area (SLA), photosynthesis, chlorophyll, soluble carbohydrates, soluble polyphenols and total C and N.

2. What shading does to disease on leaf 2

The second section describes the effects of shading on the development of disease symptoms.

3. What shading and disease do to export from leaf 2 and whole plant biomass, growth rates, shoot: root partitioning and allometry

The third section describes the effects of both shading and disease on the whole plant.

1. What shading does to leaf 2

Dry weight

Experiment 1

Dry weights of leaf 2 (**Fig. 1**) were up to 33 % heavier in unshaded plants from the other treatments 5 dai ($P < 0.05$).

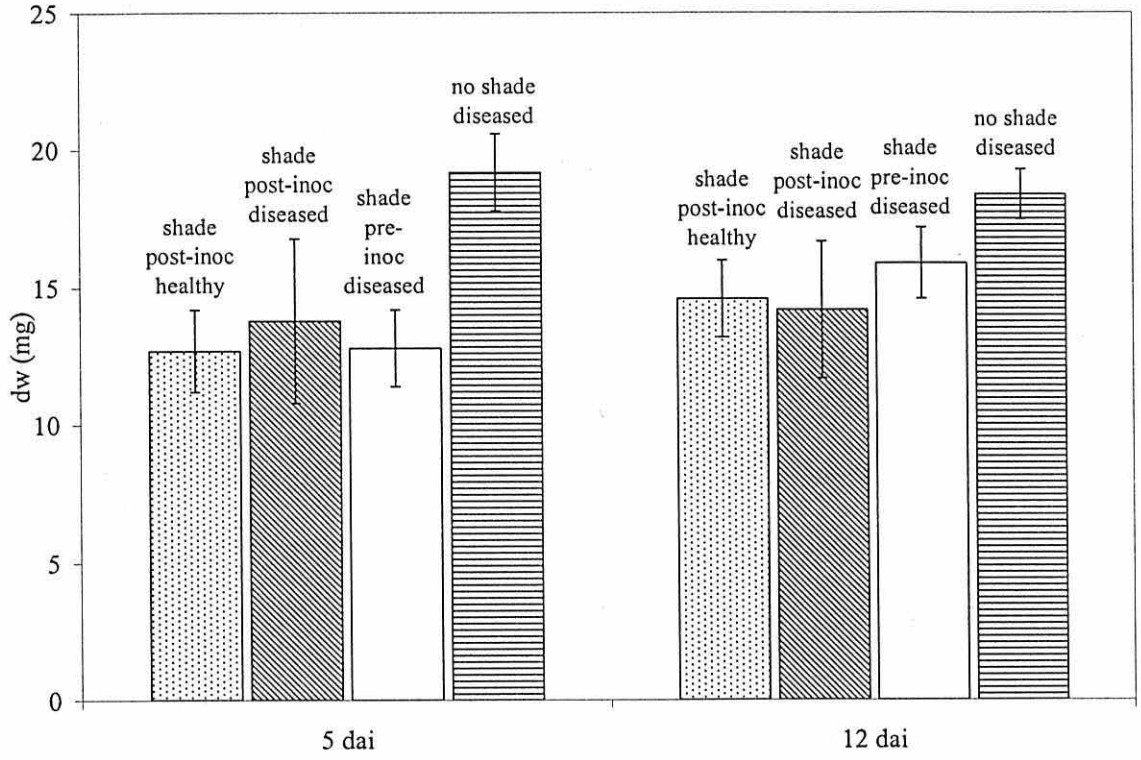

Figure 1 Dry weights (mean ± SE) of second leaves of wheat plants that were either shaded for 48 h immediately before inoculation with *S. nodorum*; shaded for 48 h after inoculation (commencing after the 48 h period of high relative humidity required following inoculation), or not shaded.

Experiment 2

The mean dry weights of leaf 2 were not significantly affected by shading or disease, with the exception that at 6 dai, second leaves of healthy plants shaded post-inoculation had significantly lower (up to 24 %) dry weights ($P < 0.05$), than those of other treatments. In all other treatments, leaf weight was highest 6 dai, but in healthy plants shaded post-inoculation, the mean dry weight of leaf 2 was lowest 6 dai (data not shown).

Experiment 3

There were no significant differences in dry weights of leaf 2 between any of the treatments (data not shown).

Specific leaf area (SLA)

In experiment 1 there was no significant difference in SLA between treatments. In experiment 3, SLA **(Fig. 2)** was not significantly affected by shading or disease, with the exception that at 2 dai unshaded healthy plants had significantly lower SLA than unshaded diseased plants. Healthy plants shaded pre-inoculation had higher SLA than healthy plants from the other two treatments ($P < 0.05$).

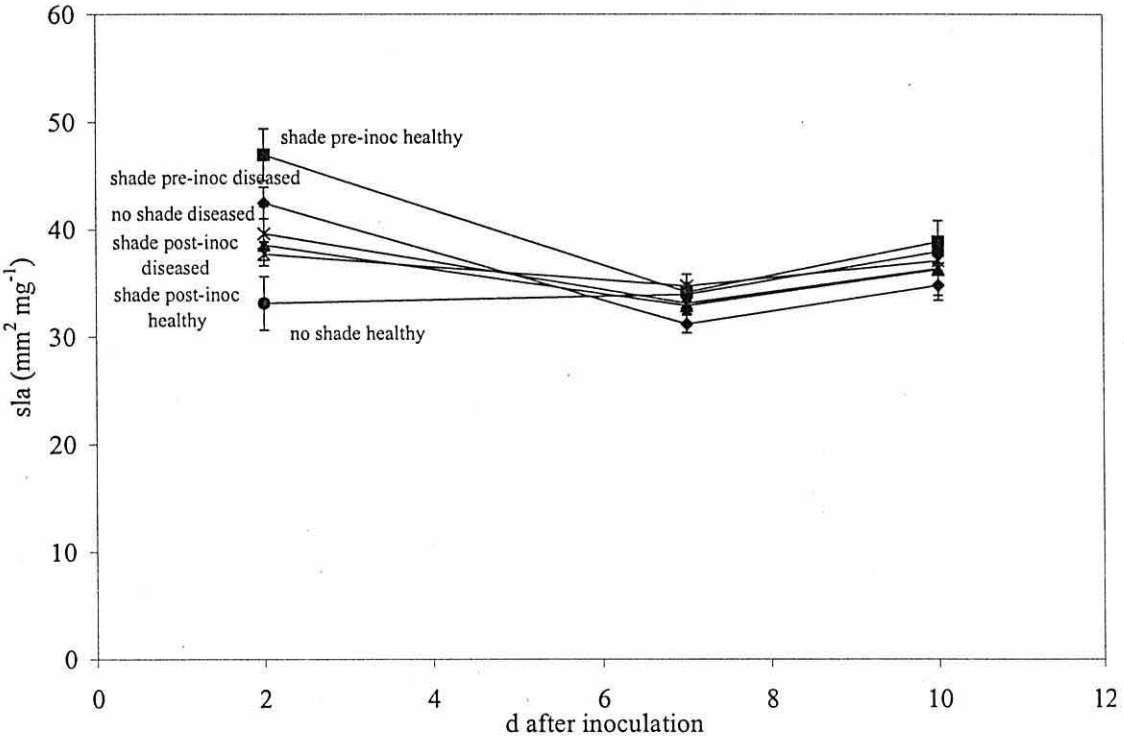

Figure 2 Specific leaf area (mean ± SE) of second leaves of wheat plants that were either shaded for 48 h immediately before inoculation with *S. nodorum*; shaded for 48 h after inoculation (commencing after the 48 h period of high relative humidity required following inoculation), or not shaded.

Photosynthesis:

For all photosynthesis data, comparisons between healthy and diseased plants have been made with caution, as the photosynthesis measurements of healthy and diseased plants were made on different days.

Net photosynthesis

Net photosynthesis was depressed slightly by the combination of shading and disease **(Fig. 3)**.

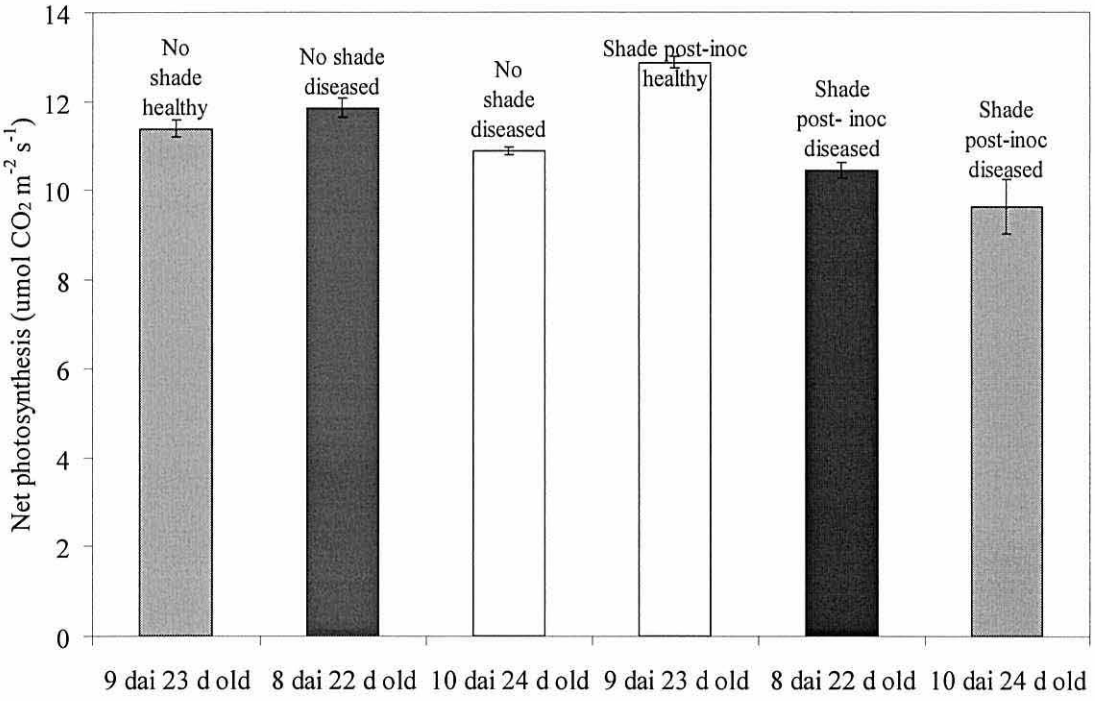

Figure 3 Net CO_2 assimilation rates (mean ± SE) of leaves from plants that were either shaded to ~ 100 µmol m^{-2} s^{-1} for 48 h after inoculation with *S. nodorum* (commencing after the 48 h period of high relative humidity required following inoculation), or not shaded. Plants were inoculated at 14 d old, on the day after full expansion of leaf 2

Effect of shading

There was no significant difference between healthy unshaded and healthy shaded plants. Photosynthetic rate was significantly higher in diseased unshaded plants than in diseased shaded plants, both 8 and 10 dai ($P < 0.01$).

Effect of disease

In shaded plants, photosynthetic rate was higher in healthy than diseased plants by approximately 23 % ($P < 0.001$). There was no significant difference in photosynthetic rate between healthy and diseased unshaded plants.

Sub-stomatal CO_2 concentration

Effect of shading

There was no significant difference in sub-stomatal CO_2 concentration between healthy shaded and healthy unshaded plants (**Fig. 4**); CO_2 concentration was just over 200 ppm in both treatments. Sub-stomatal CO_2 concentration was 10 % higher in diseased shaded ($P < 0.001$), than diseased unshaded plants, but this had reversed by 10 dai when diseased unshaded had significantly higher ($P < 0.001$) sub-stomatal CO_2 concentrations than diseased shaded plants.

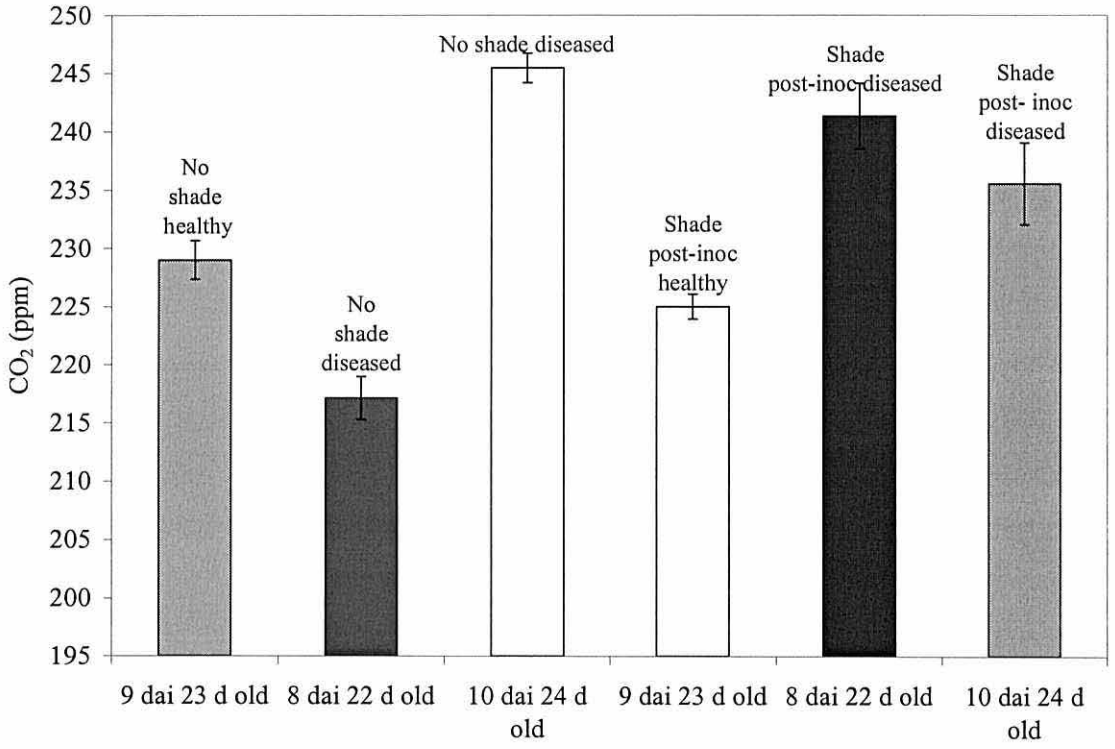

Figure 4 Sub-stomatal CO_2 concentrations (mean ± SE) of leaves from plants that were either shaded to ~ 100 μmol m^{-2} s^{-1} for 48 h after inoculation with *S. nodorum* (commencing after the 48 h period of high relative humidity required following inoculation), or not shaded.

Effect of disease

Sub-stomatal CO_2 concentrations in healthy shaded plants were 10 to 16 ppm lower than in diseased shaded plants ($P < 0.001$). However in unshaded plants, sub-stomatal CO_2 concentrations were slightly higher in healthy plants (9 dai) than in diseased plants at 8 dai, but 20 ppm lower in healthy plants than in diseased plants at 10 dai ($P < 0.001$).

Transpiration rate

Shading had a noticeable effect on the transpiration rates of healthy, but not of diseased, plants (**Fig. 5**).

Effect of shading

Mean transpiration rates were 17 % faster in healthy shaded plants (1.8 mmol H_2O m^{-2} s^{-1}) than in healthy unshaded plants ($P < 0.001$) . Transpiration rates of diseased plants were approximately 1.5 mmol H_2O m^{-2} s^{-1}. There was no significant difference in transpiration rate between diseased shaded and diseased unshaded plants.

Effect of disease

Healthy shaded plants had significantly higher transpiration rates than diseased shaded plants ($P < 0.001$). There was no significant difference between diseased and healthy unshaded plants.

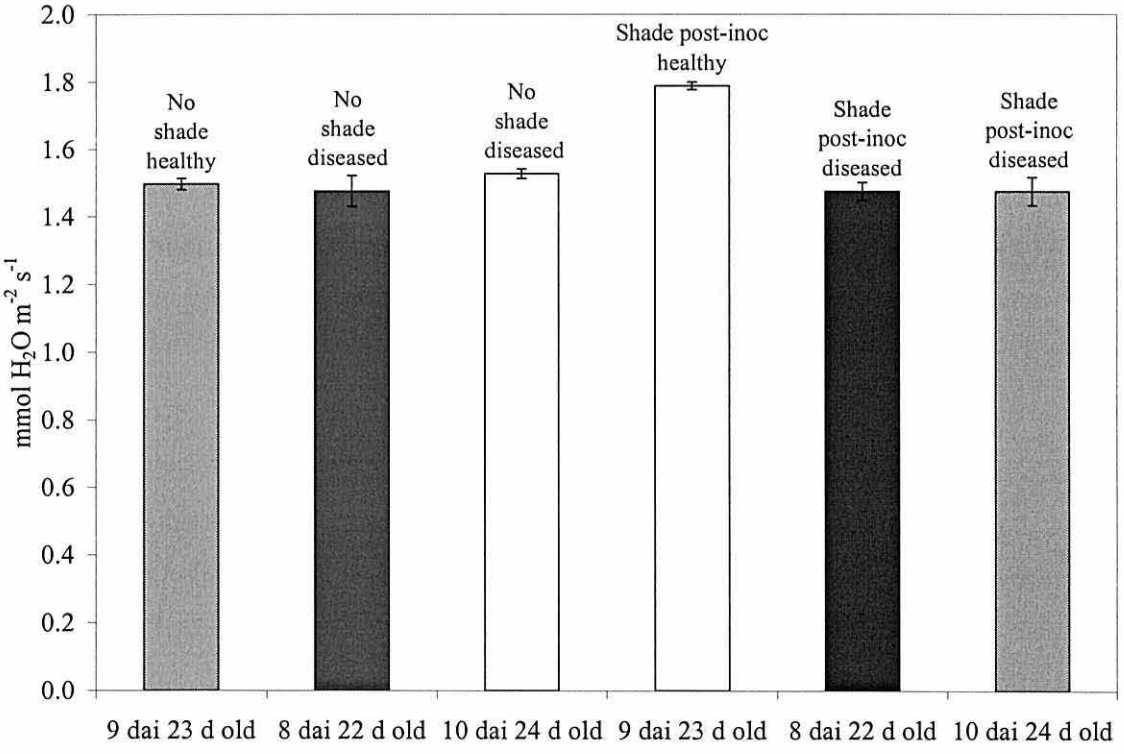

Figure 5 Transpiration rates (mean ± SE) of leaves from plants that were either shaded to ~ 100 µmol m^{-2} s^{-1} for 48 h after inoculation with *S. nodorum* (commencing after the 48 h period of high relative humidity required following inoculation), or not shaded.

Leaf surface conductance

Shading and disease (Fig. 6) affected leaf surface conductance.

Effect of shading

Healthy unshaded plants had a mean leaf surface conductance of 227 mmol H_2O m^{-2} s^{-1} cv healthy shaded plants at 253 mmol H_2O m^{-2} s^{-1}, but the difference is not significant. At 8 dai conductance was higher in diseased shaded plants, with a mean value 12 % higher than that of diseased unshaded plants ($P < 0.005$). This was reversed at 10 dai when diseased unshaded plants had a much higher mean leaf surface conductance of 262 mmol H_2O m^{-2} $s^{-1}$and the mean leaf surface conductance of diseased shaded plants had dropped to only 195 mmol H_2O m^{-2} s^{-1} ($P < 0.001$).

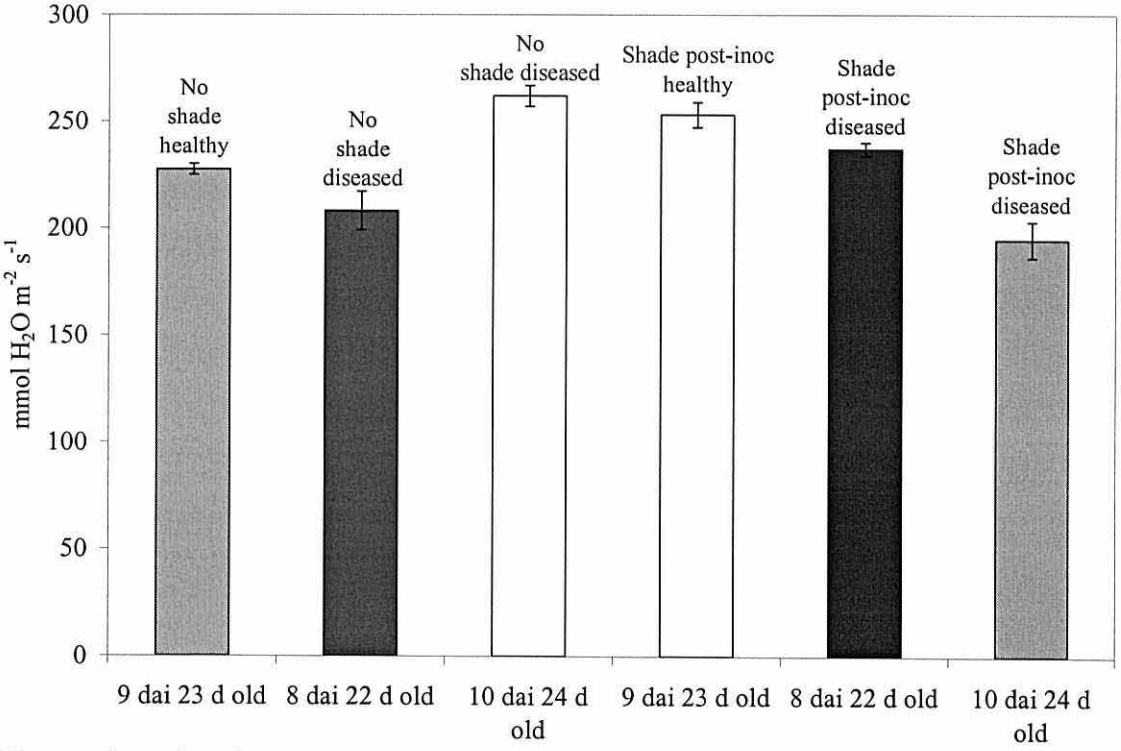

Figure 6 Leaf surface conductance (mean ± SE) of leaves from plants that were either shaded to ~ 100 µmol m^{-2} s^{-1} for 48 h after inoculation with *S. nodorum* (commencing after the 48 h period of high relative humidity required following inoculation), or not shaded.

Effect of disease

Differences between healthy and diseased plants were only significant when healthy plants were compared with diseased plants 10 dai (24 d old); healthy shaded plants had significantly higher leaf surface conductance than diseased shaded plants, and

healthy unshaded plants had significantly lower leaf surface conductance than diseased unshaded plants.

Water use efficiency (WUE)

Neither shading nor disease significantly affected WUE (**Fig. 7**), although WUE was slightly lower and therefore more efficient (fewer moles of water used per mol of CO_2 assimilated) in unshaded plants.

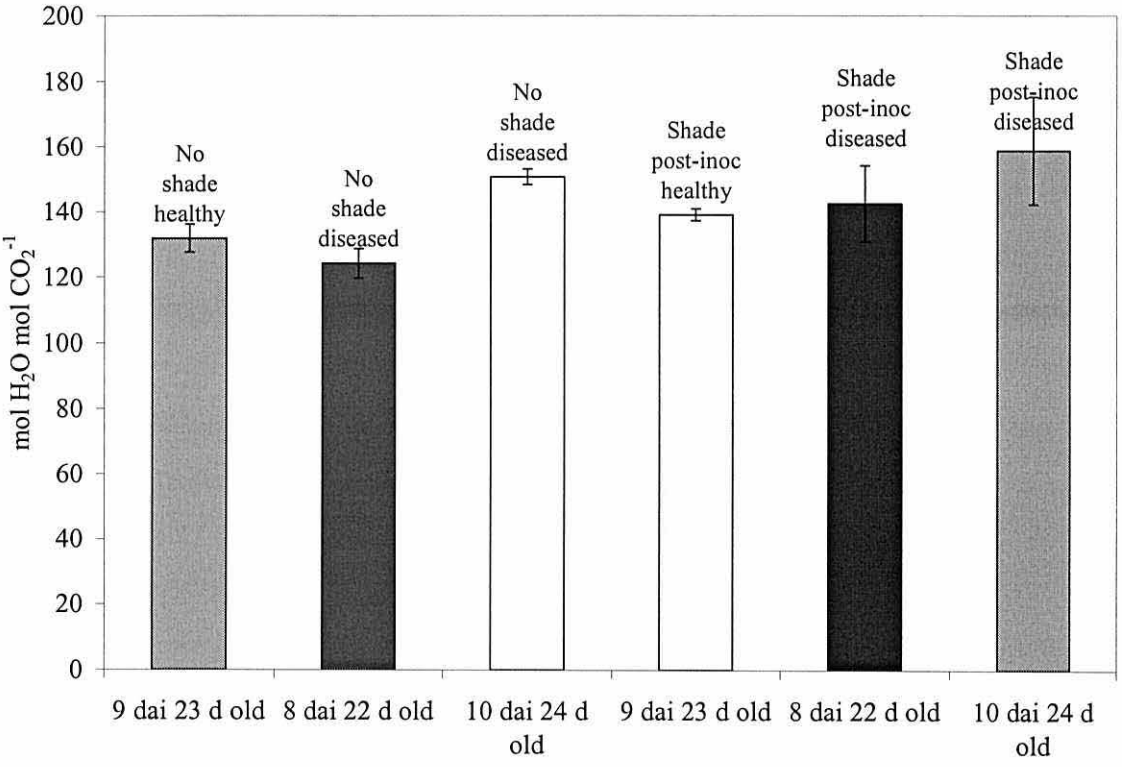

Figure 7 WUE (mean ± SE) of leaves from plants that were either shaded to ~ 100 µmol m^{-2} s^{-1} for 48 h after inoculation with *S. nodorum* (commencing after the 48 h period of high relative humidity required following inoculation), or not shaded.

Chlorophyll contents of leaf 2

Experiment 2

Both shading and disease reduced the chlorophyll contents of leaf 2 to some extent (Table 2).

Effect of shading

Shading only significantly reduced chlorophyll 6 dai, when second leaves of plants shaded pre-inoculation had significantly less chlorophyll than unshaded p lants ($P <$ 0.01 in diseased plants and < 0.05 in healthy plants).

Table 2 Chlorophyll contents (SPAD meter readings; mean ± SE) of second leaves of wheat plants that were either shaded for 48 h immediately before inoculation with *S. nodorum*; shaded for 48 h after inoculation (commencing after the 48 h period of high relative humidity required following inoculation), or not shaded. N.B. to convert SPAD readings to mg chlorophyll m^{-2} multiply value by 6.2. n = 6

	2 dai		6 dai		14 dai	
	healthy	diseased	healthy	diseased	healthy	diseased
shading pre-inoculation	45 ± 1	47 ± 2	48 ± 1	46 ± 1	45 ± 1	39 ± 2
shading post-inoculation	52 ± 1	51 ± 2	50 ± 1	49 ± 1	48 ± 1	32 ± 8
no shading	48 ± 3	51 ± 1	51 ± 1	51 ± 2	46 ± 2	38 ± 4

Effect of disease

At 14 dai second leaves of diseased plants that were shaded post-inoculation had a mean SPAD meter reading of 32. This figure was significantly lower than the mean of 48 for healthy plants shaded post-inoculation ($P < 0.001$). Diseased plants shaded post-inoculation also had significantly less chlorophyll than healthy unshaded plants and healthy plants shaded pre-inoculation ($P < 0.01$)

Experiment 3

Both shading and disease had the following effects on chlorophyll contents of leaves (Table 3).

Effect of shading

Shading reduced chlorophyll contents of leaves at 2 dai, when leaves from plants shaded pre-inoculation had significantly lower chlorophyll contents than all other plants. At 10 dai second leaves of diseased plants shaded post inoculation had significantly more chlorophyll than those of diseased unshaded plants ($P < 0.05$). This

is more likely to be as a result of the lower levels of disease in plants shaded post-inoculation in this experiment, rather than as a result of shading.

Table 3 Chlorophyll contents (SPAD meter readings; mean ± SE) of second leaves of wheat plants that were either shaded for 48 hours immediately before inoculation with *S. nodorum*; shaded for 48 h after inoculation (commencing after the 48 h period of high relative humidity required following inoculation), or not shaded. N.B. to convert SPAD readings to mg chlorophyll m^{-2} multiply value by 6.2. n = 6

	2 dai		7 dai		10 dai	
	healthy	diseased	healthy	diseased	healthy	diseased
shading pre-inoculation	44 ± 1.0	42 ± 1.7	48 ± 0.4	46 ± 0.6	49 ± 0.7	48 ± 0.5
shading post-inoculation	49 ± 0.7	49 ± 1.1	50 ± 0.8	47 ± 2.7	50 ± 0.8	50 ± 0.6
no shading	48 ± 0.8	47 ± 1.5	50 ± 1.8	48 ± 1.4	51 ± 0.5	48 ± 0.8

Effect of disease

The only case in which there was a significant reduction of chlorophyll by disease within a treatment was in unshaded plants, where at 10 dai second leaves of diseased plants had significantly less chlorophyll than second leaves of healthy plants ($P < 0.005$)

Soluble carbohydrates

Experiment 1

Second leaves of unshaded plants maintained the same soluble carbohydrate content throughout the experiment, whilst shading for 48 h resulted in a transient fall in soluble carbohydrate contents; the presence of disease caused an accumulation of soluble carbohydrates in an infected leaf as compared to a healthy leaf. Soluble carbohydrate contents of diseased plants shaded before inoculation were initially significantly lower than those of diseased unshaded plants (**Fig. 8**). However, by 2 dai there was no significant difference in soluble carbohydrate contents of second leaves between diseased plants shaded pre-inoculation and diseased unshaded plants. There were no healthy plants available for direct comparison in these two treatments. At 2 and 5 dai plants shaded post-inoculation had significantly lower soluble carbohydrate

contents in their second leaves than plants from the other treatments. At 2 dai there was 50 % less soluble carbohydrate in diseased and healthy leaves of plants shaded post inoculation than in leaves from the other treatments. Between 2 and 5 dai (during the period of shading for plants shaded post-inoculation) soluble carbohydrate contents of diseased leaves from plants shaded post inoculation increased, whereas in healthy leaves less carbohydrate was present at 5 dai than at 2 dai. By 12 dai both diseased and healthy leaves of plants shaded post-inoculation had the same soluble carbohydrate contents, but these were still lower than the carbohydrate contents of leaves from the other treatments by approximately 1 μg mm^{-2}.

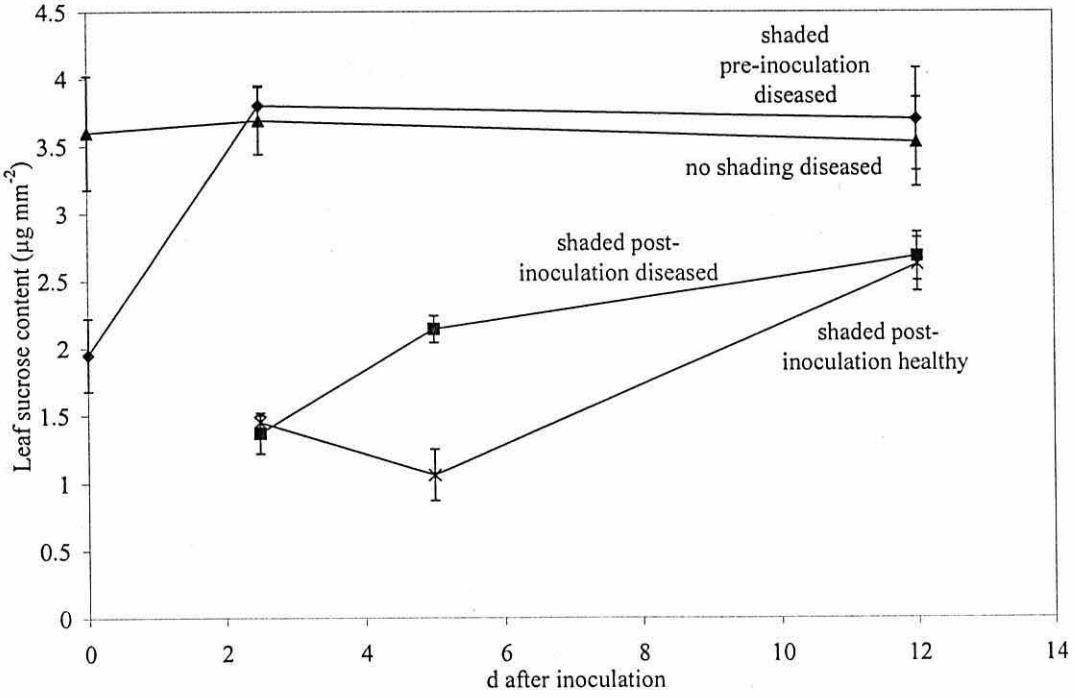

Figure 8 Soluble carbohydrate contents (mean ± SE) of second leaves of wheat plants that were either shaded for 48 h immediately before inoculation with *S. nodorum*; shaded for 48 h after inoculation (commencing after the 48 h period of high relative humidity required following inoculation, marked by black up arrow), or not shaded. Down arrows indicate removal of shades. n = 3

Experiment 2

Effects of shading

At 2 dai soluble carbohydrate contents were around 0.06 g g^{-1} in plants from both shading treatments (**Fig. 9**). Healthy unshaded plants had significantly higher soluble carbohydrate contents (mean value 0.10 g g^{-1}) than all shaded plants 2 dai ($P < 0.05$),

but by 6 dai diseased plants that were shaded pre-inoculation had the highest soluble carbohydrate contents (a mean value of 0.2 g g^{-1}), which was significantly higher than both healthy and diseased plants from the other two treatments ($P < 0.05$). Soluble carbohydrate contents increased over time much more markedly in leaves from shaded plants than in leaves from unshaded plants. At 6 dai, soluble carbohydrate contents of second leaves from plants shaded pre-inoculation were 62 % (healthy plants) and 67 % (diseased plants) higher than they had been at 2 dai; second leaves from plants shaded post-inoculation had 56 % (healthy plants) and 50 % (diseased plants) more soluble carbohydrates than they had had at 2 dai; but in unshaded plants, the soluble carbohydrate contents of second leaves were only 27 % (healthy plants) and 17 % (diseased plants) higher than they had been at 2 dai.

Effect of disease

There was no significant difference in soluble carbohydrate contents between diseased and healthy plants in any of the treatments.

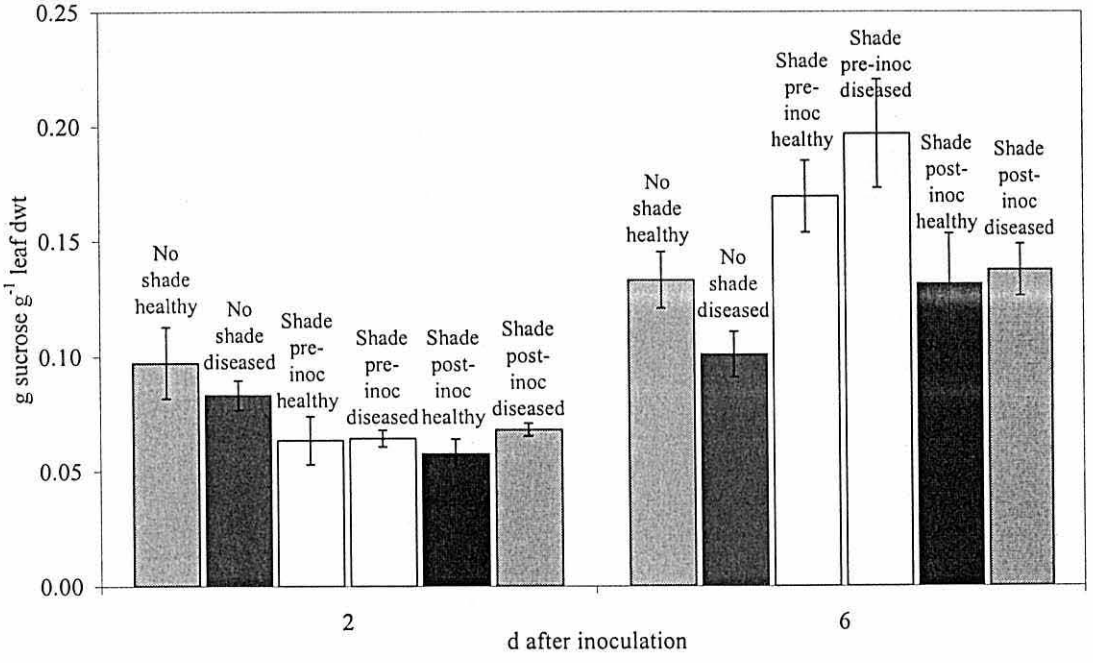

Figure 9 Soluble carbohydrate contents (mean ± SE) of second leaves of wheat plants that were either shaded for 48 h immediately before inoculation with *S. nodorum*; shaded for 48 h after inoculation (commencing after the 48 h period of high relative humidity required following inoculation), or not shaded. n = 3

Soluble polyphenols

Soluble polyphenol contents of the wheat leaves in this experiment accounted for a very small proportion of the leaf dry weight (**Fig. 10**). Shading tended to increase polyphenol contents.

Effect of shading

There were no significant differences between the shading treatments 2 dai. At 7 dai healthy plants shaded during the pre-inoculation period had significantly higher soluble polyphenol contents than plants shaded post-inoculation ($P < 0.05$). In diseased plants there were no significant differences between the shading treatments.

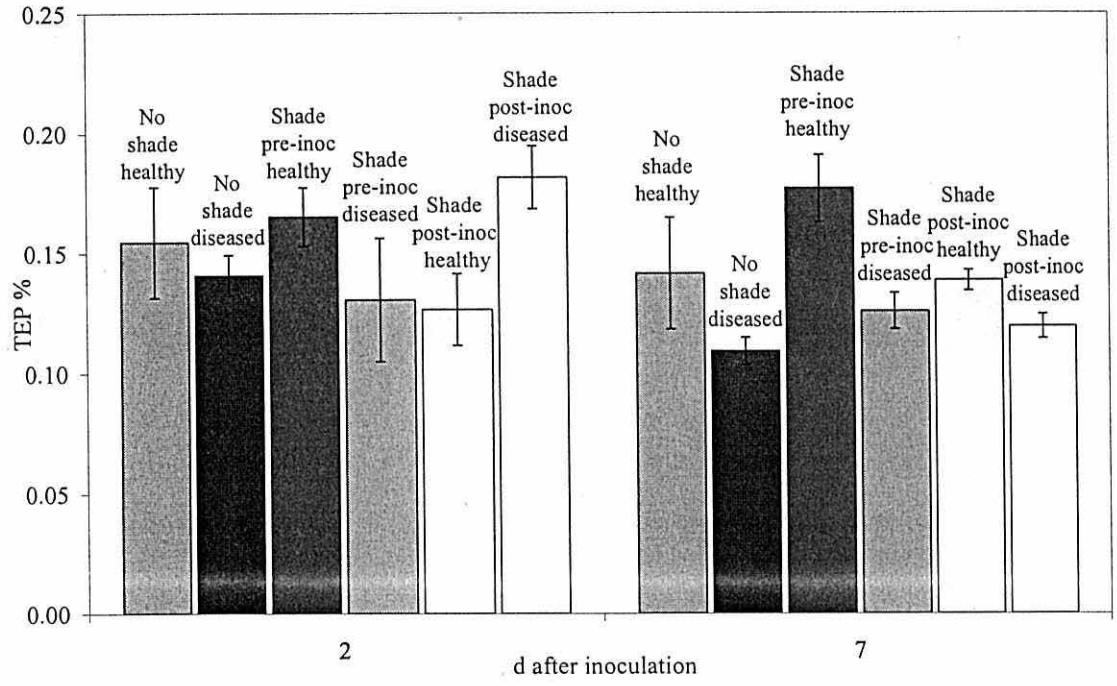

Figure 10 Soluble polyphenol contents [Total exchangeable polyphenols (TEP) as a percentage of leaf dry wt; mean ± SE] of second leaves of wheat. Plants were shaded for 48 h immediately before inoculation with *S. nodorum*; shaded for 48 h after inoculation or not shaded. Plants were inoculated at 14 d old and leaves were harvested 2 and 7 days after inoculation (dai) for analysis of polyphenols. Samples were read against a standard curve of tannic acid. n = 3.

Effect of disease

At 2 dai, diseased plants that were shaded post-inoculation (which were most resistant to *S. nodorum* in this experiment) had significantly higher polyphenol contents than healthy plants shaded during the post-inoculation period ($P < 0.05$). At 7 dai diseased

plants shaded pre-inoculation had significantly lower polyphenol contents than healthy plants shaded during the pre-inoculation period ($P < 0.01$). Unshaded diseased plants and diseased plants that were shaded post-inoculation showed a reduction in the polyphenol contents of their second leaves between 2 and 7 dai.

Total Carbon and Nitrogen contents of leaf 2

Experiment 1

Carbon

Shading had no effect on C content, with leaves from all treatments being approximately 38 % C, falling to approximately 35 % by 12 dai.

Nitrogen

Shading (Fig 1 1) altered the N content of leaves. On the day of inoculation leaves from shaded plants had significantly higher N contents (7 %) than leaves from unshaded plants, in which N contributed to 6 % of leaf dry weight. After this harvest leaves from plants shaded before inoculation and from unshaded plants had the lowest N contents. The N contents of leaves that were shaded post-inoculation were significantly higher than those of unshaded plants and those shaded pre-inoculation ($P < 0.05$), but there was no difference in N content between healthy and diseased plants shaded post-inoculation. By 14 dai N contents of leaves had fallen to approximately 5.3 % in plants shaded post-inoculation, 4.7 % in unshaded plants and 4 % in plants shaded pre-inoculation. Lesion numbers were plotted against the N contents of leaves and fitting a linear equation to the data revealed a negative relationship between disease and N content (over a limited range of N contents) (**Fig 12**).

C: N ratios

C: N ratios followed the same trend as N in terms of significant differences, but this time plants shaded post inoculation had the lowest C: N ratios and unshaded plants and plants shaded pre-inoculation had the highest C: N ratios (**Fig. 11**). C: N ratios rose slightly with time. Lesion numbers were plotted against C: N ratios and fitting a linear equation to the data revealed a positive relationship (**Fig. 12**).

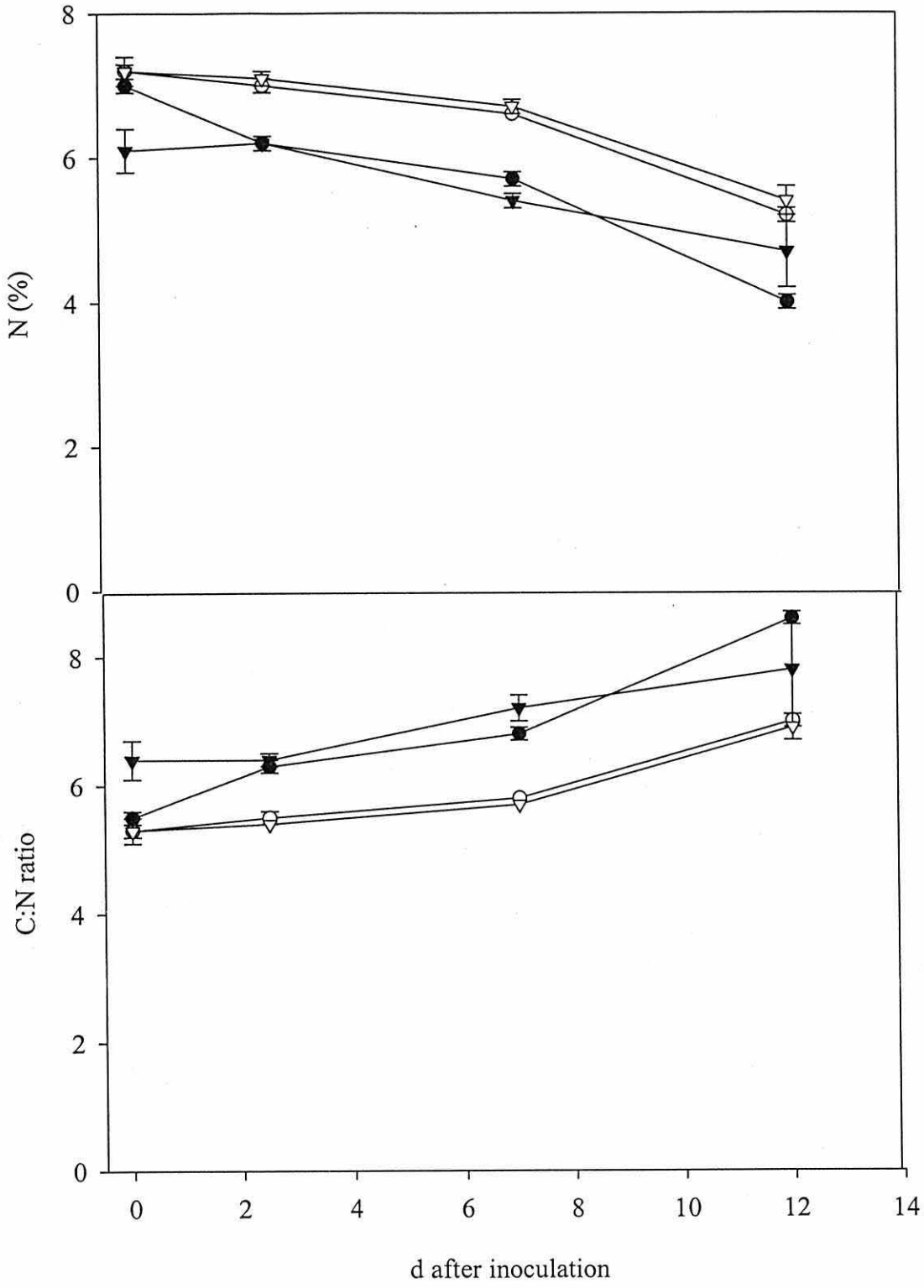

Figure 11 N content (% of leaf dry weight; mean ± SE) and C:N ratios (mean ± SE) of leaves from plants that were either shaded for 48 h immediately before inoculation with *S. nodorum* (•); shaded for 48 h after inoculation (commencing after the 48 h period of high relative humidity required following inoculation) (healthy ↗, diseased ○), or not shaded (▼). n = 3

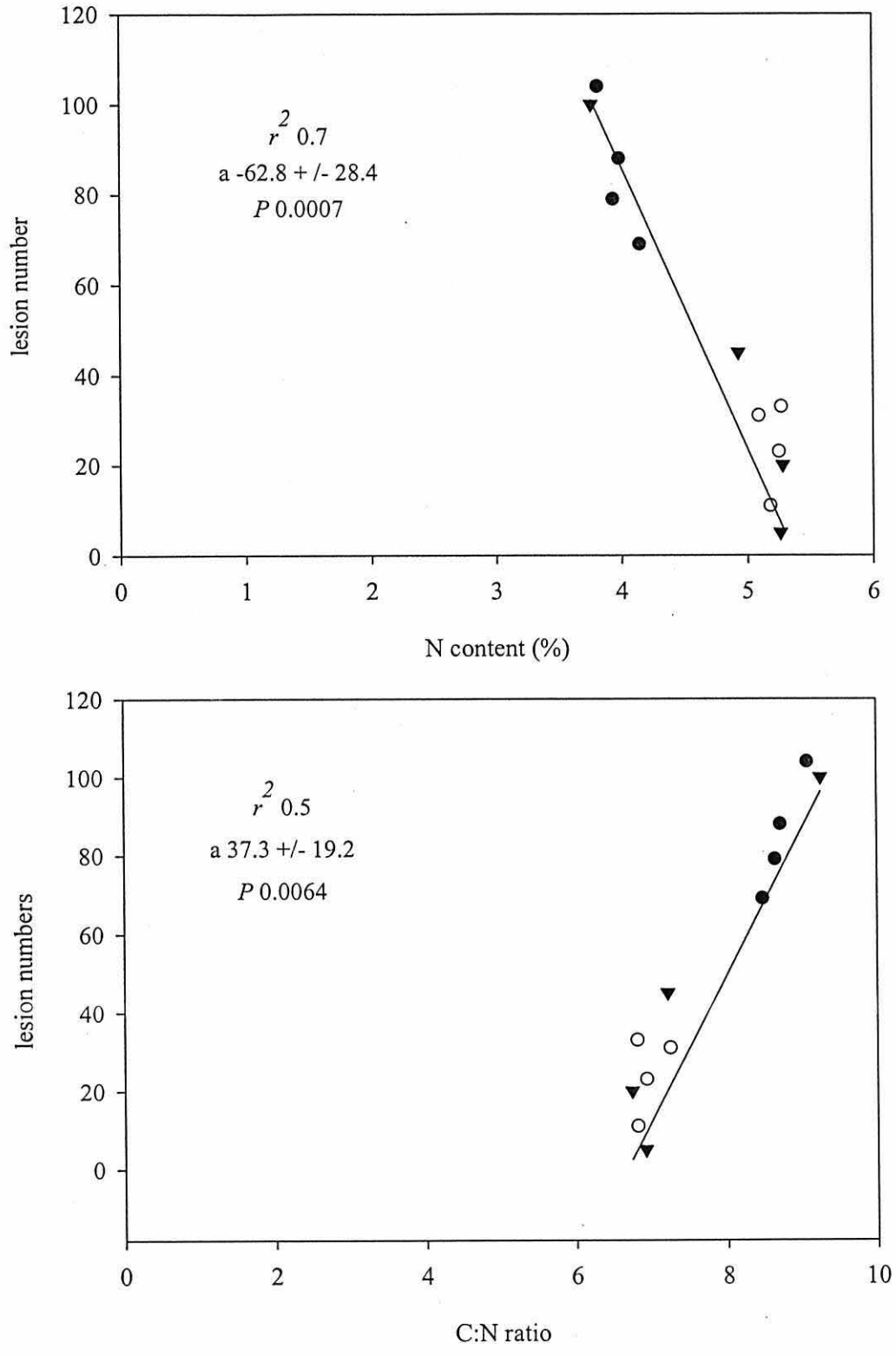

Figure 12 Linear regressions of lesion numbers against leaf N content (top) and C: N ratios (bottom) at 12 dai of leaves from plants that were either shaded for 48 h immediately before inoculation with *S. nodorum* (•); shaded for 48 h after inoculation (commencing after the 48 h period of high relative humidity required following inoculation) (○), or not shaded (▼). Plants were inoculated at 14 d old.

Experiment 2

Carbon

Leaves in this experiment were approximately 39 % C. Shading and disease had very little effect on leaf C contents. At 2 dai healthy unshaded plants had significantly lower C contents than healthy plants from both shading treatments ($P < 0.05$) and at 6 dai diseased unshaded plants had higher C contents than diseased plants from both shading treatments ($P < 0.05$).

Effect of disease

The only significant difference in C contents between diseased and healthy plants was in plants shaded post-inoculation, where healthy plants had significantly higher C contents than diseased plants (data not shown).

Nitrogen

Disease had no effect on N contents of leaves. Shading did have an effect on the N content of leaves, but only at 2 dai (**Table 4**). Plants shaded pre-inoculation had mean N contents of 6.9 % of leaf dry weight (healthy) and 6.8 % (diseased) which were both significantly higher than those of plants shaded post-inoculation and unshaded plants, which had mean N contents of 6.5 % and 6.4 % respectively ($P < 0.05$). At 6 dai there were no significant differences in N content, but N contents were lower than they had been at 2 dai, at approximately 5 % of leaf dry weight. There was no correlation between lesion numbers and N content of leaf 2.

C: N ratios

C: N ratios were affected by shading but not by disease (**Table 5**). At 2 dai plants shaded pre-inoculation had mean C: N ratios of 5.8 (diseased) and 5.7 (healthy), which were significantly lower ($P < 0.05$) than the C: N ratios of plants shaded post-inoculation (diseased and healthy) and unshaded plants (diseased only), which had C: N ratios of approximately 6. There was no correlation between lesion numbers and C: N ratios of leaf 2.

Table 4 Nitrogen contents (% dry weight; mean ± SE) of second leaves of wheat from plants that were either shaded for 48 h immediately before inoculation with *S. nodorum*; shaded for 48 h after inoculation (commencing after the 48 h period of high relative humidity required following inoculation), or not shaded. n = 3

	2 dai		6 dai	
	diseased	healthy	diseased	healthy
Shading pre-inoculation	6.8 ± 0.10	6.9 ± 0.09	4.9 ± 0.23	5.2 ± 0.32
Shading post-inoculation	6.5 ± 0.11	6.5 ± 0.04	5.3 ± 0.17	4.9 ± 0.05
No shading	6.4 ± 0.01	6.4 ± 0.03	5.0 ±0.16	5.5 ± 0.14

Table 5 C: N ratios (mean ± SE) of second leaves of wheat from plants that were either shaded for 48 h immediately before inoculation with *S. nodorum*; shaded for 48 h after inoculation (commencing after the 48 h period of high relative humidity required following inoculation), or not shaded. n = 3.

	2 dai		6 dai	
	diseased	healthy	diseased	healthy
Shading pre-inoculation	5.8 ± 0.06	5.7 ± 0.08	7.9 ± 0.45	7.7 ± 0.44
Shading post-inoculation	6.1 ± 0.15	6.1 ± 0.08	7.2 ± 0.21	7.9 ± 0.16
No shading	6.1 ± 0.08	5.9 ± 0.03	7.9 ± 0.29	7.2 ± 0.22

What shading does to leaf 2 – summary of results.

The time course of each experiment was slightly different and samples were taken or measurements made between

Time course of experiment	*Expt 1:* 0 - 12 dai *Expt 2:* 2 - 14 dai *Expt 3:* 2 - 10 dai
Analysis	**Results**
Dry weight (all expts)	*Expt 1:* Shading reduced leaf 2 weight. *Expt 2:* Leaf 2 wt reduced 6 dai in healthy plants shaded post-inoc. *Expt 3*: No significant difference (NSD) between treatments
Specific leaf area Expts 1 and 3	*Expt 1:* NSD between treatments. *Expt 3*: Shading pre-inoc increased SLA 2 dai.
Net photosynthesis Expt 2	Reduced in diseased shaded plants
Sub stomatal [CO_2] (Ci) Expt 2	NSD between healthy plants. Disease increased Ci. Diseased shaded plants had higher Ci than in diseased unshaded plants 8 dai, but lower 10 dai.

Transpiration ***Expt 2***	Shading increased transpiration in healthy plants. Combination of shading and disease reduced transpiration.
Leaf surface conductance ***Expt 2***	NSD between healthy plants. Shading increased leaf surface conductance in diseased plants 8 dai, but decreased it 10 dai. Disease reduced surface conductance in shaded plants and increased it in unshaded plants.
Water use efficiency ***Expt 2***	NSD between treatments.
Chlorophyll content (using SPAD meter) ***Expts 2 and 3***	*Expt 2:* Shading pre-inoc reduced chlorophyll early in expt. Disease reduced chlorophyll in most infected plants *Expt 3:* As above
Soluble carbohydrates ***Expts 1 and 2*** ***Soluble carbohydrates*** ***Expts 1 and 2***	*Expt 1:* Shading caused transient drop in carbohydrates. Diseased plants shaded post-inoc retained carbohydrates while carbohydrates in healthy plants temporarily dropped. *Expt 2:* Shading caused initial reduction in carbohydrates. 6 dai plants shaded pre-inoc had highest carbohydrate contents. No significant accumulation of carbohydrates in diseased leaves.
Total soluble polyphenols ***Expt 3***	Shading pre-inoc reduced polyphenols in diseased plants (compared to healthy plants shaded pre-inoc). Shading pre-inoc increased polyphenols healthy plants (compared to healthy plants shaded post-inoc). Polyphenols in diseased unshaded plants and diseased plants shaded post-inoc decreased between 2 and 7 dai.
Total Carbon ***Expts 1 and 2***	*Expt 1*: NSD between treatments *Expt 2*: Shading increased C contents of healthy leaves 2 dai and decreased C contents of diseased leaves 6 dai. Shading post-inoc and disease in combination reduced C contents in comparison to healthy leaves shaded post-inoc.
Total Nitrogen ***Expts 1 and 2***	*Expt 1*: Shading pre-inoc caused a transient increase in N content compared to unshaded plants. By 6 dai plants shaded post-inoc had highest N contents. N contents decreased over time in all treatments. There was a negative correlation between lesion numbers and N content. *Expt 2*: Shading pre-inoc caused a transient increase in N content. NSD between treatments by 6 dai. Disease did not affect N or correlate with N.

C: N ratios ***Expts 1 and 2***	*Expt 1*: There was a positive correlation between lesion numbers and C: N ratios. *Expt 2*: Differences only significant 2 dai when plats shaded pre-inoc had the lowest C: N ratios. Disease did correlate with C: N ratios.

2. What shading does to disease on leaf 2

Summary of the effects of shading on disease

The first lesions were visible 6 dai. Disease results were variable between different experiments (**Fig. 13**), at least in part because of the different duration after inoculation that measurements ceased. Although results were variable between the three experiments, overall, shading pre-inoculation increases susceptibility of wheat seedlings to *S. nodorum*, and shading post-inoculation reduces susceptibility. In two out of the three experiments, disease symptoms were greater on plants shaded pre-inoculation than on plants shaded post-inoculation, and in the same two experiments plants shaded post-inoculation had fewer disease symptoms than control plants.

Experiment 1

Plants with the most disease symptoms were those shaded pre-inoculation, with nearly 40 % of the leaf being covered by necrotic lesions at the end of the experiment. The least disease symptoms were found on plants that were shaded post-inoculation and unshaded plants had intermediate disease symptoms. At 10 dai the area of leaf covered by necrotic lesions in unshaded plants was significantly higher than in plants shaded post-inoculation ($P < 0.05$). Disease symptoms were greatest in plants shaded before inoculation, which by 10 dai were significantly higher than in plants shaded post-inoculation ($P < 0.01$), and by 12 dai were significantly higher than unshaded plants as well as plants shaded post-inoculation ($P < 0.01$).

Experiment 2

Differences in disease symptoms between treatments were not significant and data were variable. Disease severity was similar to experiment 1.

Experiment 3

Disease symptoms were slight in this experiment when compared to the other two, although symptoms on unshaded plants were at similar levels in all three experiments at 10 dai. The most infected leaves (on unshaded plants) showing only just over 10 % necrosis at the end of the experiment. Plants shaded before inoculation had more disease symptoms 10 dai than plants shaded after inoculation (as in experiment 1) although this was not statistically significant. Unshaded plants had slightly more disease symptoms (though not significantly) than plants shaded before inoculation and significantly greater lesion numbers ($P < 0.005$) and necrosis ($P < 0.05$) than plants shaded after inoculation 10 dai.

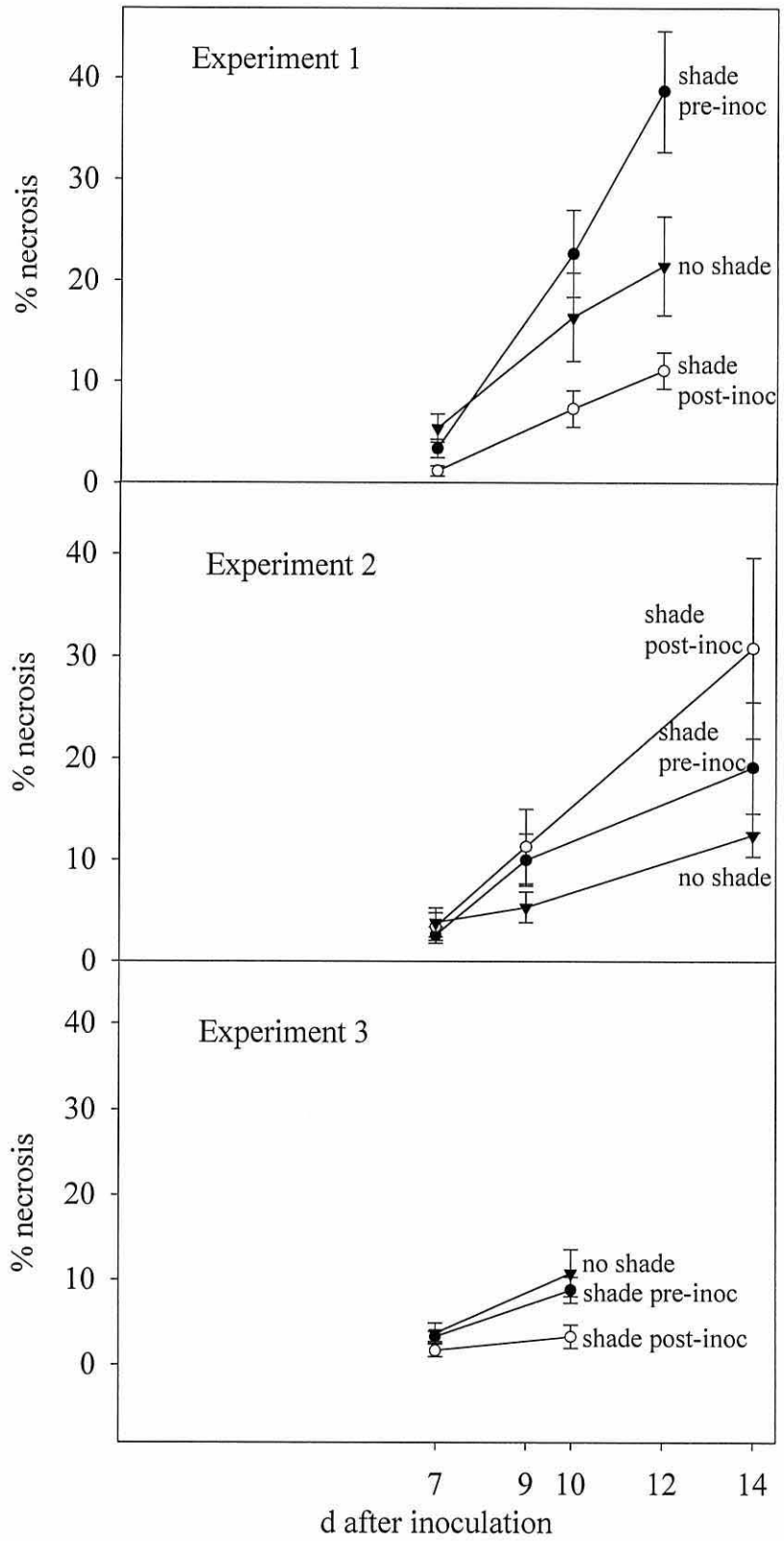

Figure 13 Necrotic area (mean ± SE) of second leaves of wheat inoculated with *S. nodorum*. Leaves were inoculated when plants were 14 d old. Plants were either shaded to ~ 98 μmol m^{-2} s^{-1} for 48 h immediately before inoculation (●); for 48 h after inoculation (commencing after the 48 h period of high relative humidity required following inoculation) (○), or not shaded (▼). n = 6-12

3. What shading and disease do to export from leaf 2, whole plant biomass, growth rates, shoot: root partitioning and allometry

Efflux of ^{14}C

Substantial efflux of ^{14}C from leaf 2 occurred over a 20 h period following feeding $^{14}CO_2$ for 20 min to leaf 2 (**Figure 14**). The loss of ^{14}C followed a double exponential decay curve which had 4 parameters (**Table 6**): A (representing ^{14}C partitioned into a readily transported pool of C), B (the exponential coefficient of A), C (^{14}C partitioned into a temporary storage pool) and D (the exponential coefficient of C). Table 1 shows the mean values of the parameters obtained from the curves that showed ^{14}C efflux as a percentage of the maximum taken up.

Table 6 Mean ± SE values of the four parameters of double exponential decay curves representing loss of $^{14}CO_2$ from fed leaves. Leaves were from plants that were either shaded to ~ 100 μmol m^{-2} s^{-1} for 48 h after inoculation with *S. nodorum* (commencing after the 48 h period of high relative humidity required following inoculation, or not shaded. n = 3

	A	B	C	D
No shading healthy	43.6 ± 4.8	1.8 ± 0.20	56.4 ± 4.8	0.03
No shading diseased	48.4 ± 8.3	1.0 ± 0.10	51.6 ± 8.3	0.02
Shading post-inoculation healthy	50.1 ± 6.3	1.2 ± 0.12	49.9 ± 6.3	0.03
Shading post-inoculation diseased	36.9 ± 4.2	1.1 ± 0.03	63.2 ± 4.2	0.03

There were no significant differences in parameters A, C and D between the treatments, or between healthy and diseased plants within the treatments. Parameter B (the exponential coefficient for the first phase) was significantly higher in healthy unshaded plants, than in all other treatments. Disease alone reduced B in unshaded plants, but did not add to the reduction in B brought about by shading post-inoculation. There was no other significant difference in ^{14}C efflux between diseased and healthy plants or between shaded and unshaded plants.

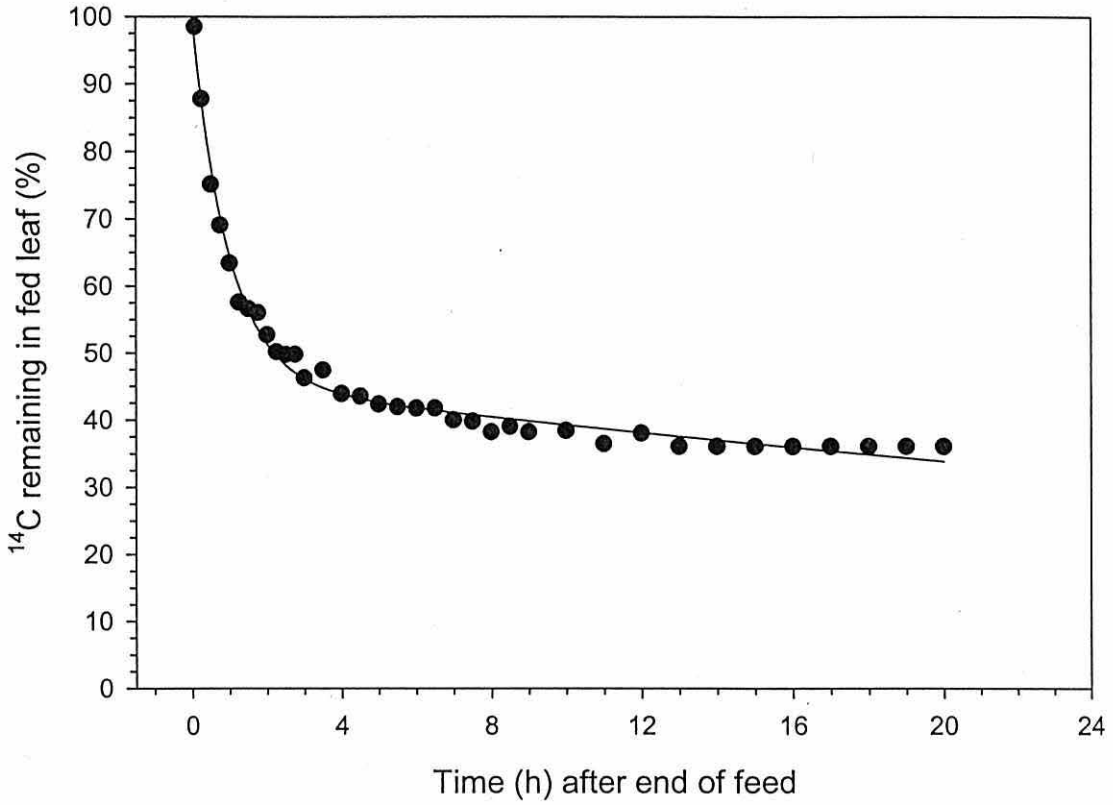

Figure 14 Example of a double exponential decay curve showing loss of ^{14}C from the second seedling leaf of a wheat plant that was shaded to ~ 100 μmol m^{-2} s^{-1} for 48 h after inoculation with *S. nodorum* (shading began after the 48 h period of high relative humidity required following inoculation). The leaf was fed $^{14}CO_2$ for approximately 20 min, 8 d after inoculation with *S. nodorum*.

Autoradiography of leaves that had been fed $^{14}CO_2$ showed accumulation of fixed ^{14}C around lesion sites 20 h after supplying the ^{14}C (data not shown).

Whole plant dry weights

Experiment 1

No roots were harvested in this experiment so data are only available for dry weights of shoots and leaf 2 (see below).

Experiment 2

Shading plants had a significant effect on whole plant dry weights, and disease affected dry weights to some extent (**Fig. 15**).

Effect of shading

At 6 dai all plants that received shading had significantly lower dry weights than unshaded plants. By 14 dai unshaded healthy plants had the highest dry weights and with a mean value of 1.45 g, they were significantly higher ($P < 0.05$) than the dry weights of all other plants, with the exception of healthy plants shaded post-inoculation.

Effect of disease

Disease significantly reduced dry weights in two treatments. At 6 and 14 dai diseased unshaded plants had much lower dry weights (6 dai $P < 0.05$; 14 dai $P < 0.01$) than healthy unshaded plants, with a mean value at 14 dai 56 % lower than their healthy counterparts. At 14 dai diseased plants shaded pre-inoculation had a mean dry weight which was 26 % lower ($P < 0.05$) than healthy plants shaded pre-inoculation.

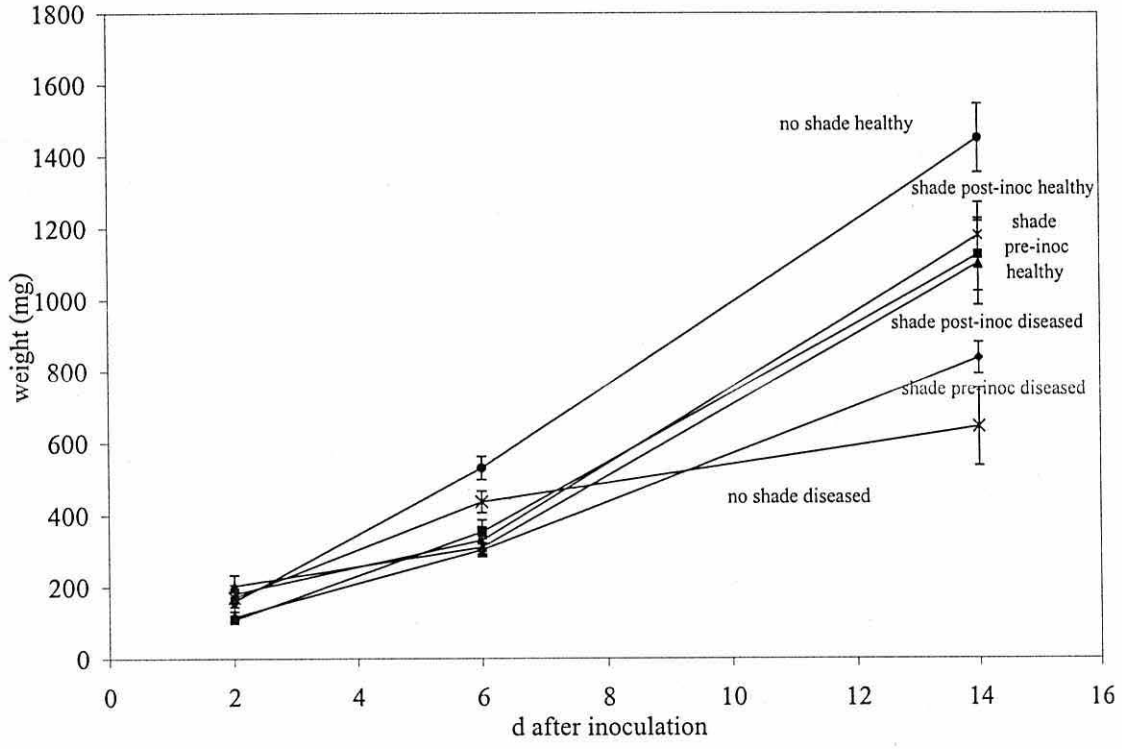

Figure 15 Dry weights (mean ± SE) of whole wheat plants that were either shaded for 48 h immediately before inoculation with *S. nodorum*; shaded for 48 h after inoculation (commencing after the 48 h period of high relative humidity required following inoculation), or not shaded

Experiment 3

Effect of shading

The effects of shading on dry weight were much smaller in this experiment. At 2 dai plants shaded pre-inoculation (diseased and healthy) had significantly lower dry weights than all plants from the other two treatments ($P < 0.01$ diseased plants and < 0.05 healthy plants); at 7 dai there was no significant difference between any of the treatments, and at 10 dai healthy unshaded plants had significantly higher dry weights than all other plants ($P < 0.001$) (data not shown).

Effect of disease

Disease did not reduce dry weights with the exception that unshaded diseased plants had significantly lower (by almost 30 %) dry weights than healthy unshaded plants ($P < 0.01$).

Relative growth rates (RGR)

RGR of whole plants in experiment 3 was higher between 2 and 7 dai than between 7 and 10 dai (**Fig. 16**).

Effects of shading

Plants with the highest RGR between 2 and 7 dai were those shaded pre-inoculation and healthy unshaded plants. Between 7 and 10 dai unshaded plants had the highest RGR, followed by plants that were shaded post-inoculation, then plants shaded pre-inoculation.

Effects of disease

Healthy plants had faster growth rates than diseased plants between 2 and 7 dai and this was most marked in unshaded plants, which had the most disease symptoms in this experiment (although between 2 and 7 dai symptom severity was very similar for unshaded plants and plants shaded pre-inoculation). Between 7 and 10 dai diseased plants had slightly higher growth rates than healthy plants.

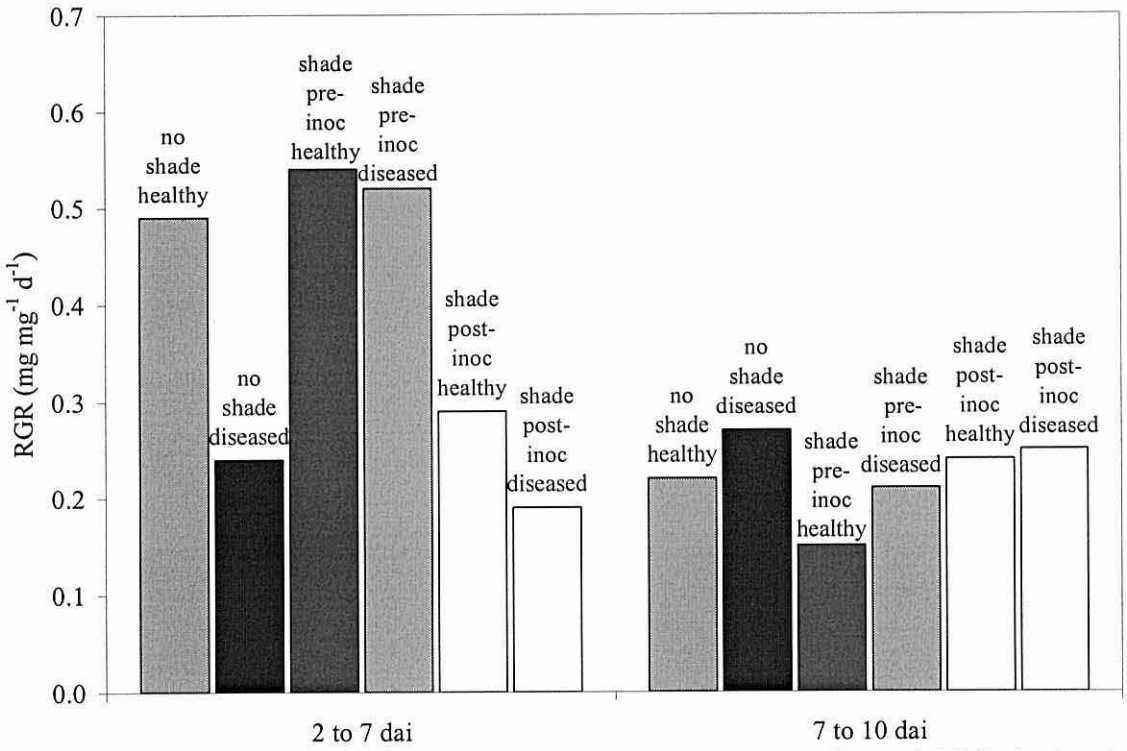

Figure 16 Relative growth rates (RGR) over the period 2 - 7 and 7 - 10dai (calculated from mean dry weights) of whole wheat plants that were either shaded for 48 h immediately before inoculation with *S. nodorum*; shaded for 48 h after inoculation (commencing after the 48 h period of high relative humidity required following inoculation), or not shaded.

Shoot dry weights

Experiment 1

At 12 dai shoot weights of unshaded plants were significantly higher than the shoot weights of plants shaded pre-inoculation and post-inoculation (diseased plants); there were no significant differences in shoot weights between unshaded plants and healthy plants shaded post-inoculation (data not shown).

Shoot dry weights followed the same trend in experiments 2 and 3; just data from experiment 2 is presented here. Both shading and disease significantly affected shoot dry weight (**Fig. 17**).

Effect of shading

Healthy, unshaded plants had the highest shoot dry weight of all the treatments, with a mean weight of just over 1 g by 14 dai. At 6 and 14 dai shoot dry weight of healthy unshaded plants was significantly higher than that of plants from the two shading

treatments ($P < 0.05$). There was no significant difference in shoot dry weights between healthy plants from the two shading treatments. At 6 dai diseased unshaded plants had significantly higher shoot dry weights than diseased shaded plants ($P < 0.05$), but by 14 dai unshaded diseased plants had the lowest shoot dry weights. There was no significant difference in shoot dry weights between diseased plants from the two shading treatments.

Effect of disease

At 6 dai unshaded healthy plants had significantly higher shoot dry weights than unshaded diseased plants ($P < 0.01$), but there was no significant difference between diseased and healthy plants of the two shading treatments. At 14 dai diseased plants shaded pre-inoculation had significantly lower shoot dry weights than healthy plants shaded pre-inoculation ($P < 0.05$); diseased unshaded plants had significantly lower shoot dry weights than healthy unshaded plants ($P < 0.05$), but there was no significant difference between diseased and healthy plants shaded post-inoculation.

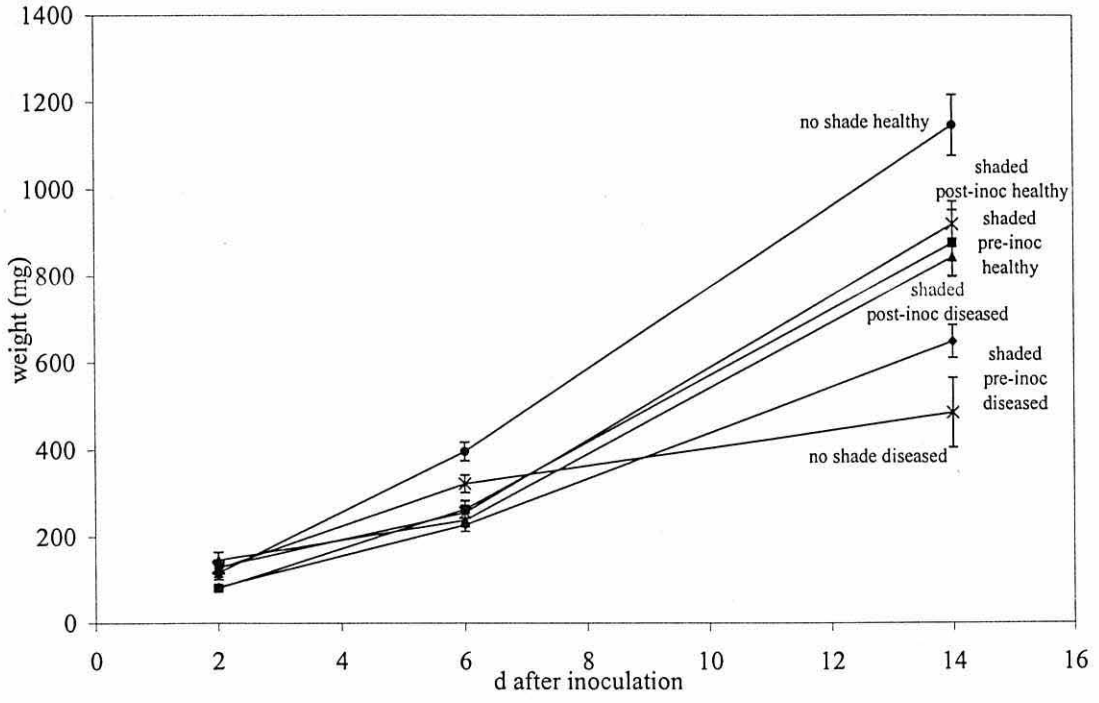

Figure 17 Dry weights of shoots of wheat plants that were either shaded for 48 hours immediately before inoculation with *S. nodorum*; shaded for 48 h after inoculation (commencing after the 48 h period of high relative humidity required following inoculation), or not shaded.

Leaf and tiller numbers

Shading did not have a great impact on leaf and tiller numbers. Tiller numbers (**Table 7**) were only significantly different at 10 dai, when healthy unshaded plants had more tillers than plants that were shaded pre-inoculation (both diseased and healthy) and healthy plants that were shaded post-inoculation. There were no differences between diseased and healthy plants within the treatments.

Table 7 Number of tillers (mean ± SE) on wheat plants that were either shaded for 48 h immediately before inoculation with *S. nodorum*; shaded for 48 h after inoculation (commencing after the 48 h period of high relative humidity required following inoculation), or not shaded. Plants labelled healthy were not inoculated. N = 6.

	2 dai		7 dai		10 dai	
	diseased	healthy	diseased	healthy	diseased	healthy
Shading pre- inoculation	1.0 ± 0	1.0 ± 0	3.0 ± 0	3.0 ± 0	4.0 ± 0.3	4.0 ± 0.3
Shading post-inoculation	1.0 ± 0	1.0 ± 0	2.7 ± 0.3	2.7 ± 0.3	4.3 ± 0.5	3.8 ± 0.2
No shading	1.0 ± 0	1.0 ± 0	3.0 ± 0	3.0 ± 0	4.5 ± 0.4	5.0 ± 0.3

At 2 dai plants shaded pre-inoculation (diseased and healthy) had significantly fewer leaves (**Table 8**) than plants from all other treatments, with the exception of diseased plants that were shaded post-inoculation. Healthy unshaded plants had significantly more leaves than plants from all other treatments, including diseased unshaded plants; this was the only case where there was a difference between diseased and healthy plants however. By 7 dai the only significant difference was between healthy unshaded plants and diseased plants that were shaded post-inoculation, where the healthy unshaded plants had the most leaves.

Table 8 Number of leaves (mean ± SE) on wheat plants that were either shaded for 48 h immediately before inoculation with *S. nodorum*; shaded for 48 h after inoculation (commencing after the 48 h period of high relative humidity required following inoculation), or not shaded. Plants labelled healthy were not inoculated. n = 6.

	2 dai		7 dai		10 dai	
	diseased	healthy	diseased	healthy	diseased	healthy
Shading pre- inoculation	2.0 ± 0	2.0 ± 0	3.3 ± 0.3	3.3 ± 0.3	4.2 ± 0.2	4.0 ± 0
Shading post-inoculation	2.3 ± 0.2	2.5 ± 0.2	3.0 ± 0	3.3 ± 0.3	3.8 ± 0.2	4.0 ± 0
No shading	2.5 ± 0.2	3 ± 0	3.7 ± 0.3	4.0 ± 0.0	4.0 ± 0.3	4.2 ± 0.2

Shoot: root (S:R) ratios and allometry

Neither shading nor disease significantly affected S: R ratio in experiment 2 or 3, and S: R did not vary much over time. Plotting root weights and shoot dry weights allometrically (**Fig. 18**) shows that neither shading nor disease significantly affected the partitioning of biomass to shoots or roots. A linear equation was fitted to the data with the following parameters:

	r	P	Y_0	a
Experiment 2	0.98	<0.0001	0.84 ± 3.7	1.06 ± 0.8
Experiment 3	0.99	<0.0001	0.91 ± 2.9	1.02 ± 0.7

The parameters of the linear equation were very similar for the two experiments. Although roots and shoots were heavier at the end of experiment 2 (due to a later final harvest than in experiment 3) there is no difference in S:R allometry between the two sets of plants.

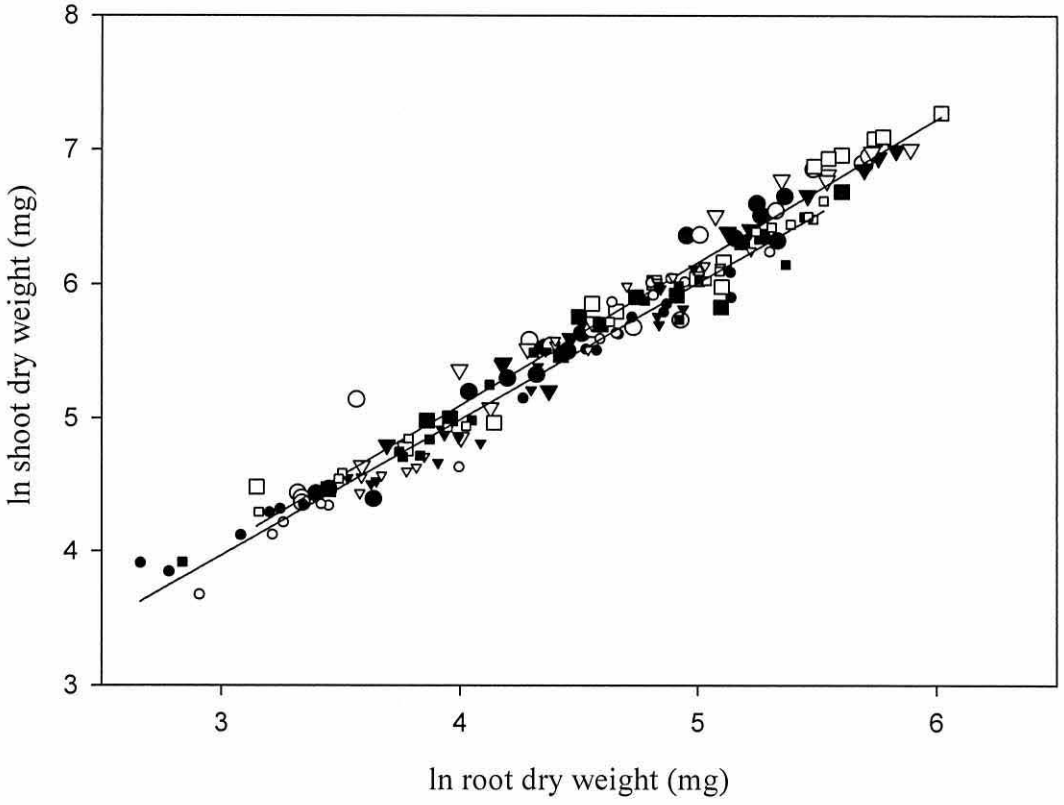

Figure 18 The allometric relationship between shoot and root dry weight for wheat plants. Plants were either shaded for 48 hours immediately before inoculation with *S. nodorum* (diseased ●, healthy ○); shaded for 48 h after inoculation (commencing after the 48 h period of high relative humidity required following inoculation) (diseased ▼, healthy ▽), or not shaded (diseased ■, healthy □). Large symbols are data from experiment 2 and small symbols are data from experiment 3 and a regression line for each experiment is shown.

What shading and disease do to export from leaf 2, whole plant biomass, growth rates, shoot: root partitioning and allometry: summary of results

Efflux of ^{14}C

The only significant treatment effect was that the exponential coefficient of the parameter representing ^{14}C partitioned into a readily transportable pool of C was reduced by shading and disease.

Dry weights

Shading (before and after inoculation) reduced the dry weights of whole plants. Disease reduced dry weights in unshaded plants in both experiments and in plants shaded pre-inoculation in experiment 2. Shoot weights were reduced by shading. Disease only reduced dry weights of shoots in unshaded plants.

RGR

Overall, RGR was reduced by shading, however RGR was initially increased by early shading. Disease initially decreased RGR, then caused a slight increase in RGR between 7 and 10 dai.

Leaf and tiller production

Shading (before and after inoculation) reduced tiller numbers by 10 dai. Shading pre-inoculation caused a temporary reduction in leaf numbers.

Shoot: root partitioning and allometry

No significant treatment effects

Discussion

Shading influenced disease incidence and plant physiology, but results were variable.

What shading does to leaf 2

The first section of this chapter described the effects of shading on leaf 2 and compared dry weights of leaf 2, specific leaf area (SLA), photosynthesis, chlorophyll, soluble carbohydrates, soluble polyphenols and total C and N contents of leaf 2. Differences between healthy and diseased leaves were also presented.

The data partially support the hypothesis that photosynthesis will be reduced by disease but will be unaffected by shading several days before measurement. Photosynthesis was reduced only in diseased shaded plants. Sub-stomatal CO_2 concentrations were initially (at 8 dai) higher in diseased shaded than diseased unshaded plants, but 2 d later sub-stomatal CO_2 concentrations were highest in diseased unshaded plants. Transpiration rate was increased by shading in healthy plants. Leaf surface conductance was affected in diseased shaded leaves, where it was initially higher in diseased shaded plants at 8 dai, then at 10 dai leaf surface conductance was higher in diseased unshaded plants.

Plants grown in shade will have lower rates of photosynthesis than plants grown in full sun (Grindlay, 1997). However, here the plants are not long term shade-adapted plants, but plants that have been subjected to short term shading. Photosynthesis was measured a minimum of 4 d after the shades were removed, which should have been adequate time for the net photosynthetic rate to stabilise. Plants that had been shaded only had depressed rates of photosynthesis when they were infected, which suggests that infection only reduced photosynthesis. There was no difference in net photosynthesis between diseased and healthy unshaded plants, which seems to suggest the combination of disease and shading caused photosynthesis to be reduced. However, at 8, 9 and 10 dai when photosynthesis was measured, approximately 5 % of the area of second leaves of unshaded plants was necrotic, but approximately 10 % of the area of second leaves of shaded plants was necrotic, so disease alone reduced photosynthesis in shaded plants. Other parameters measured alongside photosynthesis

were also affected by disease: diseased shaded plants had variable sub-stomatal CO_2 concentrations, lower transpiration rates and lower leaf surface conductances than healthy shaded plants, so partial closure of the stomata may have accounted for the reduction in photosynthesis. Diseased unshaded plants had variable sub-stomatal CO_2 concentrations and higher leaf surface conductance than healthy unshaded plants, but there was no difference in transpiration rates between healthy and diseased unshaded plants.

The reduction of photosynthesis by biotrophs is well known (Ahmad *et al.*, 1983; Hibberd *et al.*, 1996*d*; Owera *et al.*, 1981; Scholes and Farrar, 1985), but there has been less work on reduction of photosynthesis by necrotrophs. Pathogens reduce photosynthesis by reducing the leaf area available to intercept radiation (Ayres *et al.*, 1996) due to the presence of pustules (biotrophs) or spots / blotches (necrotrophs), and by physical disruption of the photosynthetic machinery e.g. degradation of chloroplasts (Ahmad *et al.*, 1983; Karjalainan and Lounatmaa, 1986) or chlorophyll (Scholes and Farrar, 1985; Tang *et al.*, 1996). Net photosynthesis of thale cress (*Arabidopsis thaliana* L.) infected with white blister rust (*Albugo candida*) was reduced and both chlorophyll and Rubisco were lost from infected regions of leaves (Tang *et al.*, 1996).

Some pathogens can affect the radiation use efficiency (RUE – the fraction of solar energy converted to chemical energy or g of biomass / MJ of intercepted radiation) of tissues that are not visibly colonised by the pathogen (Garry *et al.*, 1998; Meyer *et al.*, 2001), in the case of necrotrophs by means of mobile toxins (Ayres *et al.*, 1996). Reduction of photosynthesis in wheat plants infected by *S. nodorum* has been reported (Scharen and Taylor, 1968; Scharen and Krupinsky, 1969; Krupinsky *et al.*, 1973), but the pathogen appears not to affect respiration (Scharen and Taylor, 1968). In addition to the effect of chlorosis in infected leaves, fungal toxins such as ochracin cause stomatal closure and consequently reduce net photosynthesis (Jørgensen and Smedegaard-Petersen, 1999). The direct inhibiting effect was suggested to be in the Calvin cycle (Bethenod *et al.*, cited by Jørgensen and Smedegaard-Petersen, 1999).

These data support the hypothesis that shading will reduce the amount of soluble carbohydrates in leaves compared with the leaves of unshaded plants. In both experiments where soluble carbohydrates were measured there was a transient fall in soluble carbohydrate contents of second leaves during the shading period, but when the shades were removed soluble carbohydrate contents began to recover to that of unshaded plants. Soluble carbohydrate contents of second leaves of plants shaded post inoculation were low when measured the day after the shading commenced, then subsequently increased between 2 and 12 dai (experiment 1) but remained lower than those of second leaves of unshaded plants and plants shaded post-inoculation however. The soluble carbohydrate contents of second leaves of plants shaded pre-inoculation increased to higher than the soluble carbohydrate contents of second leaves of unshaded plants at 6 dai (experiment 2). The reduced carbohydrate content of shaded leaves is probably accounted for by reduced photosynthetic rates during the shading period as light intensity was reduced to approximately 1/5 of ambient in the growth cabinets.

Accumulation of carbohydrates by diseased leaves was seen in experiment 1, where during the period of shading there was a decrease in soluble carbohydrates in second leaves of healthy plants shaded post-inoculation and an increase in soluble carbohydrates in second leaves of diseased plants shaded post-inoculation. The same phenomenon was not found in experiment 2 however. In biotrophic infections infected leaves can accumulate carbohydrate (Abood and Losel, 2003; Tang *et al.*, 1996; Wright *et al.*, 1995; Zulu, Farrar and Whitbread, 1991), but in barley infected with powdery mildew (Hibberd *et al.*, 1996*b*) and brown rust (*Puccinia hordei*) (Tetlow and Farrar, 1993) there was a reduction in total soluble carbohydrate concentrations in infected leaves. In *Arabidopsis thaliana*, leaves infected with white blister rust (*Albugo candida*) accumulated soluble carbohydrates and starch, and invertase activity was elevated in infected leaves (Tang *et al.*, 1996). Necrotrophic fungi have a different mode of nutrition to biotrophs. Unlike biotrophs they do not need to keep their hosts alive, and can break down cell walls and absorb the released nutrient, so alteration of the host's metabolism to engineer accumulation of carbohydrates is not as necessary. This is reflected in the shortage of literature available on the effects of infection by necrotrophic fungi on the plant leaf carbohydrate metabolism.

The carbohydrate profiles of leaves can be altered by infection, in particular infected leaves often accumulate fructans (Hibberd *et al.*, 1996*b*) and have reduced concentrations of sucrose (Heisterüber *et al.*, 1994; Tetlow and Farrar, 1993). Fungi also tend to accumulate polyols, particularly mannitol (Abood and Losel, 2003; Clark *et al.*, 2003), and as many plants cannot metabolise these it is a way of sequestering C away from plant use (Clark *et al.*, 2003). The carbohydrate profiles of leaves were not measured here, but analysing the different types of carbohydrate within healthy and infected leaves would provide further insight into alteration of leaf carbohydrate status by shading and infection.

The hypothesis that reduced carbohydrate status of shaded plants will be reflected in lower C: N ratios is tentatively accepted. Shaded plants did have the lowest C: N ratios in both of the experiments where total C and N were measured; however in one experiment C: N ratios were only reduced in plants shaded pre-inoculation and in another C: N ratios were only reduced in plants shaded post-inoculation. Unshaded plants had consistently high C: N ratios however, so in general it can be concluded that shading reduced C: N ratios. Shading and disease had very little effect on the total C contents of leaves. In experiment 2 healthy unshaded plants initially had the lowest C contents (but also the highest soluble carbohydrate contents) and at 6 dai diseased unshaded plants had the highest C contents (and the lowest soluble carbohydrate contents) but there was no other effect attributable to shading. Disease caused a reduction in C content in plants shaded post-inoculation, and soluble carbohydrate contents of diseased plants shaded post-inoculation were slightly (though not significantly) higher than those of healthy leaves. The positive relationship between lesion numbers and C: N ratios seen in experiment 1 suggests the importance of high C availability for the growth of the pathogen *in planta*. This is supported in experiment 1 by the low C: N ratios and low soluble carbohydrate contents of leaves from plants shaded post-inoculation which, in two out of three experiments, had the lowest disease severity. The data from experiment 2 neither corroborate, nor support this evidence. It is important to note that whilst the suggested correlation between lesion numbers and C: N ratio may be important, it is difficult to be confident about a relationship when inconsistencies exist and at the time when measurements are made

the pathogen is undoubtedly influencing host metabolism. The apparent discrepancy between soluble carbohydrate contents and total C contents, and variability between experiments could be explained by investigating the ratio of soluble to structural and storage carbohydrate in leaves.

Shading caused a transient increase in leaf N contents which persisted beyond the shading period in plants shaded post-inoculation in experiment 1. As with C data however, results were different in experiment 2, although the increase in N contents of leaves immediately after shading pre-inoculation was seen in both experiments. In experiment 1 there was a negative relationship between lesion numbers and N content of second leaves, which is supported by the low N contents and low disease severity of plants shaded post-inoculation. There was no relationship between lesion numbers and N content in experiment 2. Disease symptoms were highly variable and there were no significant differences in disease severity between treatments in experiment 2, which may partially explain why no relationship was found here. Given the increase in disease severity on plants supplied high N (Chapter 2) it is unlikely that there is a negative relationship between disease and N content.

The data generally do not support the hypothesis that leaves of shaded plants will have higher specific leaf areas than unshaded plants, as in one experiment there were no significant differences in SLA between shaded and unshaded plants, but in another, shading plants prior to inoculation caused an increase in SLA at 2 dai, but only in healthy plants. After 2 dai there was no significant difference between shaded and unshaded plants. The SLA of shaded plants is often increased, with leaves being thinner and having smaller and fewer mesophyll cells (Lambers *et al.*, 1998), however, as previously mentioned, here the plants were only subjected to short-term shading and are therefore not likely to have all the characteristics of shade-adapted plants, and here, the leaves measured were already fully expanded when shading was applied, so changes were unlikely. Gunn *et al.*, (1999) found that shading of whole barley plants increased the SLA of some leaves that were already expanded at the time of shading, but had no effect on the oldest leaf which was the same leaf (leaf 2) on which SLA was measured here (Gunn et al., 1999).

The hypothesis that the chlorophyll contents of unshaded and shaded leaves will not be significantly different is contradicted by the data. Both shading treatments affected chlorophyll contents of second leaves. In both of the experiments where chlorophyll contents were measured, shading pre-inoculation caused a transient drop in chlorophyll contents (at 6 dai in one experiment and 2 dai in another) relative to unshaded plants and plants shaded post-inoculation. At 10 dai (experiment 3), second leaves of diseased plants shaded post-inoculation had higher amounts of chlorophyll than second leaves of diseased unshaded plants. This may be attributable to the lower disease symptoms (and therefore less chlorosis) in plants shaded post-inoculation in this experiment, rather than an effect of shading however. In low light plants maximise their light capture and light use efficiency by altering their chloroplast protein and pigment composition (Paul and Foyer, 2001).

The data do not fully support the hypothesis that partitioning of resources to phenolics will be greater in unshaded plants, but there is a suggestion that shading decreases polyphenols in second leaves. Polyphenol contents were similar in all treatments, and were very low (< 0.2 % of leaf dry wt). At 7 dai healthy plants shaded pre-inoculation had higher polyphenol contents than plants shaded post-inoculation. At this point plants shaded pre-inoculation had been in full light for 7 d but plants shaded post-inoculation had only been in full light for 3 d. Polyphenols were increased at 2 dai in diseased plants shaded post inoculation (which at that time had not been shaded), where infection was least in the experiment in which polyphenols were measured, and decreased at 7 dai in diseased plants shaded pre-inoculation. Plotting soluble polyphenol contents of leaves against soluble carbohydrate contents of leaves revealed a weak positive, but not significant, relationship between soluble polyphenols and soluble carbohydrates in second leaves of plants shaded pre-inoculation and unshaded plants (not shown). There was no relationship between soluble polyphenols and disease symptoms. A weak negative (but again, not significant) relationship was found between polyphenol contents and leaf N contents. Although the relationships between polyphenols and carbohydrates (positive) and polyphenols and N (negative) are not significant, the trends suggest that perhaps there is a greater investment of resources to C based defence compounds when C supply is plentiful, or N supply is limiting. This is supported by the negative (but again, not statistically significant) relationship

between soluble polyphenol contents and N contents found in second leaves of plants supplied 2 mmol dm^{-3} NO_3^- and NH_4^+ (Chapter 3).

The content of phenolic compounds in shaded leaves of water lily (*Nymphaea alba* L. and *Nuphar lutea* L.) was investigated in relation to their susceptibility to the pathogens *Colletotrichum nymphaeae* and *Pythium* sp. respectively (Vergeer and van der Velde, 1997). Shaded leaves had significantly lower contents of phenolics than unshaded leaves, and higher incidence of disease. A significant negative correlation was found between the polyphenol contents of leaves and their N contents. There was also a negative relationship between polyphenols and infection (Vergeer and van der Velde, 1997). The influence of irradiance on the production of phenolics in leaves is likely to be associated with the rate of net photosynthesis, where reduction of photosynthesis by shading reduces photosynthetically fixed C in leaves and thus there is less C available for allocation to C-based defence compounds (Vergeer and van der Velde, 1997). In addition, the principal source of secondary metabolites involved in defence is the enzyme phenylalanine ammonia-lyase (PAL), the first enzyme in the phenylpropanoid pathway, which is stimulated by light (Kervinen et al., 1998) and depressed by high N (Matsuyama and Dimond, 1973).

What shading does to disease on leaf 2

The effects of shading on the development of disease symptoms were examined. Disease symptoms were variable between experiments but in general the data support the hypothesis that the reduced carbohydrate status of shaded plants will increase susceptibility to *S. n odorum* if the shading is applied prior to inoculation, and will decrease susceptibility if the shading is applied after inoculation. Ignoring experiment 2, where there were no significant differences in disease severity between treatments, there was consistently more infection on plants shaded pre-inoculation and on unshaded plants, than on plants shaded post-inoculation. Where plants were shaded post-inoculation the shades were in place 2 – 4 dai, during which time it is likely that the fungus was growing intercellularly, and therefore reliant on apoplastic sugars. In this treatment leaf sugars were reduced during the shading period which may have limited available resources for the fungus. Indeed there was a positive correlation between disease symptoms and C: N ratio suggesting that the leaf C status is

important for growth of the pathogen within the leaf. This has been discussed above but to reiterate, there were inconsistencies in the effects of shading on disease and physiology between experiments so this correlation must be viewed with caution as it could be due to pathogen-induced changes, rather than being a reason for susceptibility. Disease symptoms were similar between unshaded plants and plants shaded pre-inoculation. In the latter case, although leaf sugar status was reduced at the time of infection (penetration), sugar concentrations were increasing from then onwards and may have been at sufficient concentrations to provide adequate C for the fungus during the intercellular growth stage. Light has little effect on symptom expression of *S. nodorum*, but reduction in light to 40 % of ambient slightly increased susceptibility (Rosielle, 1969 cited by Shipton *et al.*, 1971).

Other researchers have found shading increased susceptibility of plants to disease. Squash (*Cucurbita pepo* L.) was more susceptible to powdery mildew infection when plants were shaded (Leibovich et al., 1996). Water lily [*Nymphoides peltata* (Gmel.) O. Kuntze] was more susceptible to infection by *Septoria villarsiae* during a cloudy summer (Vergeer and van der Velde, 1997). The effects of wounding and shading the shoots of kiwi fruit (*Actinidia deliciosa*) on susceptibility to infection by grey mould (*Botrytis cinerea*) were investigated (Greaves *et al.*, 2001). Plants shaded to 33 % of ambient photosynthetically active radiation (PAR) had 45 % greater incidence of infection by *B. cinerea* on wounded shoots inoculated immediately after wounding than unshaded plants. Shading also reduced the content of phenolics in leaves (Greaves *et al.*, 2001).

What shading and disease do to export from leaf 2 and whole plant biomass, growth rates, shoot: root partitioning and allometry

The third section described the effects of both shading and disease on the whole plant. Efflux of ^{14}C from second leaves was unaffected by shading or disease. The only significant effect of disease and shading was on the exponential coefficient of the parameter of the double exponential curve that represented ^{14}C partitioned into a readily transportable pool of C. This was reduced by disease in unshaded plants, and in both healthy and diseased plants the value was significantly lower than the value for healthy unshaded plants. Whilst there was no significant effect of disease on efflux of

^{14}C, in plants shaded post-inoculation, the mean size of the transport pool (parameter A) was smaller in diseased plants than healthy plants. Correspondingly, the mean size of the vacuolar storage pool (parameter C) was larger in diseased plants than healthy plants. Although this was not significant, it does suggest a reduction of export of ^{14}C in diseased shaded plants. This is partially supported by the data from experiment 1 which showed accumulation of soluble carbohydrates in diseased plants during the shading period and there is further evidence for reduced export from diseased leaves in the reduction of plant biomass in diseased plants compared to healthy plants. As *S. nodorum* uses toxins and enzymes to break down cells and chloroplasts (see above) the presence of the fungus in a source leaf will reduce both photosynthesis and export leading to a reduction of plant biomass.

The data support the hypothesis that the reduced carbohydrate status of shaded plants will reduce plant biomass, but do not support the hypothesis that shading will favour partitioning of resources to shoots compared with unshaded plants. Shading reduced whole plant dry weights and shoot dry weights, however disease reduced the dry weights of unshaded plants to lower than those of shaded plants. Whole plant dry weights were reduced dramatically in diseased unshaded plants in two experiments. Diseased plants shaded pre-inoculation also had reduced dry weights in experiment 2. Shoot dry weights were also reduced in diseased plants shaded pre-inoculation and diseased unshaded plants. It is odd that the difference between diseased and healthy plants was so dramatic in unshaded plants, particularly as in experiment 2 they had the least disease symptoms, although differences in disease symptoms between treatments were not significant in this experiment.

Relative growth rates (RGR) were reduced by shading, although shading caused a transient increase in RGR, as between 2 and 7 dai plants shaded pre-inoculation had the highest RGR. RGR was reduced in diseased plants early in the infection process (2 to 7 dai), but there was a slight increase in the RGRs of diseased plants relative to healthy plants between 7 and 10 dai.

Shading for just 48 h reduced tiller numbers at 10 dai but did not significantly affect leaf numbers except at 2 dai when unshaded plants had the most leaves. Winter wheat

seedlings were grown under three different irradiances for one month and the leaf and tiller development was monitored. Seedlings grown under the lowest irradiance (140 μ mol m^{-2} s^{-1}) produced fewer tillers than plants grown at 500 μmol m^{-2} s^{-1} (Rickman *et al.*, 1985). S: R ratios and allometry were not affected by shading or disease, so it is likely that although a brief period of shading was sufficient to reduce overall biomass, it was not long enough to affect partitioning. Shading tends to reduce root growth as a result of reduced sugar supply and preferential partitioning of C to the shoot (Pollock and Farrar, 1996)

Conclusions

Alteration of leaf C status by shading has a significant but inconsistent effect on the physiology and biochemistry of second leaves of wheat, and the susceptibility of wheat seedlings to *S. nodorum*. It could be suggested that lowering the C status of leaves prior to inoculation creates a favourable environment for the penetration of and initial colonisation of leaves by *S. nodorum*, or perhaps the low sugar status of leaves shaded during the period when *S. nodorum* is growing intercellularly in the apoplast inhibits growth of the pathogen. This could be due to a reduction in soluble carbohydrates, or perhaps due to the transient increase in the N contents of leaves seen in shaded plants, which was negatively correlated with disease in one experiment. Both the N and C contents of leaves affect the production of different defence compounds within leaves, but the evidence here suggests that perhaps the effect of shading on susceptibility is a nutritional, rather than defence-based response.

Chapter 6 Final Discussion

Aim of thesis

The primary aim of the thesis is to identify mechanisms by which the nutritional status or environmental conditions of wheat influences horizontal resistance to *S. nodorum,* and to answer the question: how do changes to the crop's environment affect the nutrients available to fungal pathogens? The broader aim of the thesis is to find ways in which to maximise the expression of horizontal resistance, or to allow plant breeders to select for traits that improve horizontal resistance, which may result in a reduction in fungicide requirements. This was achieved by carrying out experiments under controlled conditions that manipulated the C supply (by altering light intensity) or N supply to wheat seedlings, and examining the subsequent effect on infection by the pathogen. An attempt was made to identify the physiological or biochemical reasons for reduction of disease. The original hypothesis stated that a necrotroph is predominantly limited by C supply (or high sugar inhibits disease). During the study this hypothesis was revised to state that resistance of wheat to a necrotroph (*Stagonospora nodorum*) is influenced by both N and C supply. I suggested that the C and N status of leaves affect both the nutrients available to the fungus and the production of secondary metabolites, which in turn affect resistance to *S. nodorum*.

There were questions raised by the aims and hypotheses of the thesis, which were:

Does N decrease resistance by affecting the resources available to the fungus, or by affecting the resistance mechanisms of the plant?

What form of N does the fungus use preferentially (NO_3, NH_4, amides, amino acids)?

What morphological, chemical or physiological differences are there between leaves or plants fed NO_3^- and plants fed NH_4^+ that might increase or decrease resistance?

What morphological, chemical or physiological differences are there between plants fed 'low' and 'high' nitrogen N that might influence resistance?

Is reduced C status important in reducing pathogen growth?

How do C and N interact within plants to affect resistance?

Brief summary of experimental results

Three types of experiment were performed. In order to effectively control the nutrient supply, hydroponics was used in all experiments investigating the role of N in resistance. To investigate the effects of altered C supply on resistance, shading was used and plants were grown in hydroponics in all but one experiment, where they were grown in compost. The third type of experiment was to investigate the effect of N supply on growth and reproduction of the pathogen *in vitro*, where the pathogen was cultured on solid medium containing different N sources, in conditions of controlled temperature and lighting.

Chapter 2 describes experiments to investigate the effects of form and concentration of N on susceptibility of wheat seedlings to *S.nodorum*. Plants were supplied NO_3^- or NH_4^+ at 3 different concentrations. Infection was greatest when N (NO_3^- or NH_4^+) was supplied at 2 and 8 mmol dm^{-3} as compared with 0.04 mmol dm^{-3} N. There was no significant difference in disease symptoms between plants supplied 2 mmol dm^{-3} N and plants supplied 8 mmol dm^{-3} N. The form of N is extremely important in determining susceptibility; infection was greatest on plants grown on NH_4^+.

Several biochemical and physiological parameters were measured (Chapters 2 and 3) to try and address the questions outlined above. Results varied slightly between experiments. In general, the effects of N concentration agreed with the hypotheses. Increasing N supply increased net photosynthesis, chlorophyll, plant dry weights, total N and C contents and amino acid contents, and reduced soluble carbohydrates, C: N ratios, and soluble polyphenols.

N form affects the chemical composition of leaves (Chapter 3). The total C and total N contents of leaves were higher in plants supplied NO_3^- than NH_4^+, but soluble carbohydrates, amino acids and proteins were highest in plants supplied NH_4^+, where infection was greatest. The positive linear relationship between disease symptoms and soluble carbohydrate contents of leaves perhaps suggests this is an important source of C for the pathogen, but may simply be a reflection of the sugars accumulated in diseased leaves. As mentioned in Chapter 1, *S. nodorum* can synthesise proteases (Bindschedler *et al.*, 2003), and proteins are known to be used as N sources by fungi (Marzluf, 1997;

Solomon *et al.*, 2003*b*). Supplying NH_4^+ to plants altered the amino acid profiles such that second leaves contained more asparagine and glutamine than second leaves of plants supplied NO_3^-. Plants supplied NO_3^- contained more aspartate, and in diseased plants more glutamate than plants supplied NH_4^+. It may be that the availability of a high concentration of free amino acids and proteins, plus the availability of amides that the fungus possibly uses preferentially, in the leaves of plants supplied NH_4^+ will provide the fungus with a more nutritionally favourable environment in comparison to plants supplied NO_3^-. Defence compounds (soluble polyphenols) were also measured and were present in greater quantities in second leaves of diseased plants supplied NO_3^- than in second leaves of healthy plants supplied NO_3^-, and in second leaves of healthy and diseased plants supplied NH_4^+.

The most significant finding of these experiments was that the form of N supplied to wheat plants significantly affects their leaf chemistry, more so than the concentration of N supplied. In conclusion, I suggest that the greater availability of soluble carbohydrates, soluble N and amides in leaves of wheat plants supplied NH_4^+ compared with leaves from plants supplied NO_3^- provides a more favourable environment for *S. nodorum*, and this possibly in addition to lower contents of defence compounds renders these plants more susceptible to infection. Plants supplied very low amounts of N (0.04 dm^{-3} mmol) are poor hosts for the fungus, probably due to a combination of low nutrient availability and high concentrations of phenolic defence compounds. The nutritional status of wheat plants is an important factor in modifying its susceptibility to *S. nodorum*, and it is perhaps as important as the presence of defence compounds, in determining the success of the pathogen *in planta.*

Chapter 5 describes experiments designed to investigate the impact of reduced C supply before and after inoculation, to ascertain whether any resistance mechanisms were acting during the initial penetration of the leaf by the fungus and its early growth, or during the colonisation period. Shading was applied immediately before inoculation or 2 d after inoculation. Many of the physiological and biochemical parameters described above were also measured here, with the exception of total amino acids, amino acid profiles and total soluble proteins.

Short-term shading of plants was sufficient to cause a reduction in overall dry matter production and temporarily reduced RGR, but its effect on soluble carbohydrates, chlorophyll, total C and N and soluble polyphenols did not persist much after removal of the shading. Photosynthesis was reduced only in plants which were diseased and shaded. This was likely to be more due to the disease than to shading as there was no reduction of photosynthesis in healthy shaded plants, and disease severity was twice as high on shaded plants as on unshaded plants at the time photosynthesis was measured, so perhaps disease alone reduced photosynthesis in shaded plants.

Polyphenol contents were similar in all treatments, and were very low (< 0.2 % of leaf dry weight). There was no overwhelming effect of shading or disease, but overall, shading decreased polyphenol contents of second leaves. There was no relationship between soluble polyphenols and disease symptoms.

Shading had a significant but variable effect on infection but disease symptoms were similar between unshaded plants and plants shaded pre-inoculation and there was consistently more infection on plants shaded pre-inoculation and on unshaded plants, than on plants shaded post-inoculation. In the latter, it is likely that growth of the fungus during shading period was inhibited by the reduced availability of soluble carbohydrates as it is likely that at this time the fungus was growing intercellularly and therefore reliant on apoplastic sugars. The positive correlation between disease symptoms and C: N ratio suggests that high leaf C content is important for growth of the pathogen within the leaf.

In conclusion, variable results make it difficult to fully interpret the effects of C status on susceptibility, but I suggest that high C status increases growth of the fungus and is important in determining the susceptibility of wheat to *S. nodorum* principally due to the nutritional requirements of the fungus rather than its influence on defence mechanisms.

Chapter 4 describes two experiments in which the response of *S. nodorum* to different N sources *in vitro* was investigated. The aim of this experiment was to investigate the N sources that promote growth and reproduction of *S. nodorum*, without the complication of plant tissues and metabolites. Growth *in vitro* is an artificial situation for the fungus, but

the aim was to use N sources found in the leaves of plants supplied N as NO_3^- or NH_4^+ to try to further investigate the nutritional requirements of the fungus. The pathogen consistently produced more pycnidia when supplied N as asparagine. Pycnidial production was also high when asparagine was supplied in combination with aspartate, a precursor to asparagine. This finding was particularly significant in the light of the amino acid profiles of leaves (Chapter 3) as plants supplied N as NH_4^+, where infection was greatest, had significantly more asparagine in their second leaves than plants supplied NO_3^-. There is evidence that pycnidial production was inhibited by the acidic amino acids glutamate and, to a lesser extent, aspartate.

In conclusion I suggested that asparagine is the most favourable N source for pycnidial production *in vitro* by *S. nodorum* and that acidic amino acids inhibit pycnidial production. I also suggest that the increased susceptibility of wheat plants supplied N as NH_4^+ to *S. nodorum* is due, at least partly, to the high levels of free amino acids and particularly the high concentrations of the amide asparagine within leaves.

Does N decrease resistance by affecting the resources available to the fungus, or by affecting plant resistance mechanisms?

Both plant nutrition and defence were affected by N. The only defence compounds measured here were total soluble polyphenols, which are thought to be synthesised as a rapid response to infection (Nicholson and Hammerschmidt, 1992). Their mode of action is to cause lignification of cell walls, thus obstructing the path of the fungus through the leaf, but does not stop growth entirely. Resistance of wheat to certain pathogens is correlated with phenolics (Kiraly and Farkas, 1962; Moerschbacher *et al.*, 1989; Rengel *et al.*, 1994). Here, the only difference in polyphenol contents of healthy and diseased plants was at 12 dai in plants supplied 2 mmol dm^{-3} NO_3^-, where in diseased plants polyphenol contents were 36 % higher than in healthy plants. At the same time, diseased leaves from plants supplied 2 mmol dm^{-3} NO_3^- had 54 % higher soluble polyphenol contents than diseased leaves from plants supplied 2 mmol dm^{-3} NH_4^+. As plants supplied NO_3^- were less susceptible to infection than plants supplied NH_4^+ this implies that polyphenols may have played a role in defence. The polyphenol concentrations were very

low, accounting for less than 0.5 % of leaf dry weight, so perhaps there were localised differences but that were initially diluted by the large volume of leaf (Hoffland *et al.*, 1999). These results imply that plants supplied NO_3^- are better defended than plants supplied NH_4^+, as whilst the polyphenol contents of healthy plants were similar, when the leaves encountered disease, leaves from plants supplied NO_3^- had a larger defence response than those from plants supplied NH_4^+, and this may have resulted in leaves from plants supplied NO_3^- having fewer disease symptoms. Plants supplied 0.04 mmol dm^{-3} N had significantly higher polyphenol contents than plants supplied 2 mmol dm^{-3} N, but there were no differences between N treatments or diseased and healthy plants at the lower concentration.

Polyphenols are positively correlated with resistance of wheat to certain pathogens (Sander and Heitefuss, 1998, Mckeehen *et al.*, 1999, Moerschbacher *et al.*, 1989; Kofalvi and Nassuth 1995). The analysis of specific phenolic compounds such as caffeic, ferulic and coumaric acids may provide more insight into the defence response of wheat plants to *S. nodorum*, and in particular, studying the time course of defensive reactions. Analysis of bulk soluble polyphenols at a time relatively late after application of spores to the leaf is of limited use as phenolics may be translocated to localised regions around penetration sites, and therefore their concentration may be diluted when the whole leaf is analysed. More sophisticated techniques to analyse specific compounds from small regions of tissue at a time scale more closely correlated to that of the initial infection process would provide a more accurate picture of the defence response. The defence response of four wheat cultivars inoculated with Karnal bunt (*Neovossia indica*) was investigated by measuring total polyphenols followed by qualitative analysis using thin-layer chromatography (TLC) at 0, 2, 6 and 10 dai (Gogoi *et al.*, 2001). Tyrosine and caffeic acids were present both in resistant and susceptible varieties of wheat, hydroquinone was only present in resistant cultivars and DL-phenylalanine was present only in the durum wheat. Total phenols varied between cultivar: in a resistant cultivar phenol contents were elevated from 2 dai to 6 dai, then decreased, in one susceptible cultivar phenol total phenols were highest at 0 dai then subsequently decreased, but in another there was no significant difference between 0 and 6 dai, but phenols declined between 6 and 10 dai

(although in this cultivar it seems that in this cultivar total phenol contents were higher at 6 dai than those of the resistant cultivar). Peroxidase (an enzyme that oxidises phenols to quinones) activity was also measured; it was maximal peak 2 dai in resistant cultivars and 6 dai in susceptible cultivars. It was concluded that biochemical defence mechanisms operate in both resistant and susceptible varieties, but are initiated immediately after inoculation in resistant varieties and the response is much slower in susceptible varieties (Gogoi *et al.*, 2001).

The other physiological and biochemical differences in leaves attributable to N supply that affect the nutrients available to the fungus are described in Chapters 2 and 3, and discussed further below.

What form of N does the fungus use preferentially (NO_3, NH_4, amides, amino acids?).

Nitrogen metabolism of fungi is described in Chapters 1 and 4. Comparisons between the nutritional profiles of leaves and the results of experiments investigating N supply to the fungus *in vitro* are encouraging. When these results are compared with the literature the results are supported further, and we can begin to understand how the growth of the fungus within the plant is affected by N supply. Despite the differences in severity, *S. nodorum* successfully infected leaves from plants supplied both NO_3^- and NH_4^+, so it must be able to use the forms of N available in both of these plants. However, there were significant differences in disease severity between plants supplied NO_3^- and NH_4^+, and as there were significant differences in the nutritional profiles of the plants, it is likely that nutrition played an important role in determining the growth of the pathogen. I suggest that amino acids (and possibly proteins) provide a major source of N for the fungus, and specifically there is evidence that asparagine is the most favourable amino acid used by *S. nodorum*.

What morphological, chemical or physiological differences are there between leaves or plants fed NO_3^- and plants fed NH_4^+ that might increase or decrease resistance?

This question has been partially answered above. Second leaves of plants fed NO_3^- were lighter, had higher rates of photosynthesis, higher total N contents and higher soluble

polyphenol contents (see above) than second leaves of plants supplied NH_4^+. Second leaves of plants supplied NH_4^+ had higher chlorophyll contents (initially), higher soluble carbohydrates, C: N ratios and total C (initially), soluble proteins, total amino acids and amides than second leaves of plants supplied NO_3^-. It is likely that a combination of more favourable N compounds, more soluble carbohydrates and lower soluble polyphenol contents in plants supplied NH_4^+ rendered them more susceptible to infection by *S. nodorum*. The high concentration of asparagine in leaves of plants supplied NH_4^+ cannot be ignored. As discussed in Chapter 3, asparagine accumulation in plants supplied NH_4^+ has been found by other workers, but of particular interest here is that plants supplied NH_4^+ were more susceptible to infection by *S. nodorum*, and when the pathogen was grown *in vitro*, pycnidial production was stimulated when asparagine was supplied as the sole N source (Chapter 4). Asparagine is a precursor for synthesis of other amino acids, is used for storage and transport of N and is also involved in remobilisation of N during leaf senescence (Snoeijers *et al.*, 2000). Asparagine is a good marker of proteolysis, particularly after C starvation when it can accumulate in plant cells (Brouquisse *et al.*, 1992; Genix *et al.*, 1990). Here, asparagine was found in high concentrations in both healthy and diseased leaves of plants supplied NH_4^+, but accumulation of asparagine as a result of pathogenesis was seen in leaves of tomato infected with *Pseudomonas syringae* pv. *tomato* (Snoeijers *et al.*, 2000). Asparagine concentrations were certainly higher in diseased leaves of plants supplied NO_3^- than healthy leaves of plants supplied NO_3^-, so perhaps in this case asparagine accumulated as a result of infection.

What morphological, chemical or physiological differences are there between plants supplied 'low' and 'high' N that might influence resistance?

N compounds (total N, amino acids and proteins) were increased in plants supplied high N, and C compounds (soluble carbohydrates, and soluble polyphenols) were decreased by high N supply. Results from this study show that infection by *S. nodorum* is greatest when organic N compounds are high and polyphenols are low, so plants supplied with low N are more resistant than plants supplied higher N. For plants supplied 2 and 8 mmol dm^{-3}, there was a positive correlation between severity of disease and leaf soluble carbohydrate content at 14 dai, but there was also significantly more soluble carbohydrate

in the leaves of diseased plants compared to healthy plants, so it is likely that the high sugar content is a consequence of disease rather than a cause of it. Here, disease symptoms could only be correlated with physiological parameters once infection was advanced enough to produce visible symptoms, by which time the presence of the pathogen within the leaf may have altered the amount and composition of metabolites that were being measured. Analysis of sugars and other compounds that may influence resistance earlier in the infection process would be useful as a way of addressing this problem.

Is reduced C status important in reducing pathogen growth?

Disease intensity was variable in shading experiments but overall the data suggest that shading a plant pre-inoculation increases infection and shading post-inoculation reduces infection. During germination, the main energy source for the fungus comes from stored lipids, so it is unlikely that the C status of a leaf prior to penetration is important for fungal nutrition (Solomon *et al.*, 2003*b*). Results were variable between experiments, so a statement about the effects of C supply on pathogen growth cannot be made with confidence. Photosynthesis was not measured during the shading period, but as light intensity was reduced to 1/5 of ambient, photosynthesis and thus sucrose synthesis will undoubtedly have been reduced. When a pathogen has penetrated a leaf sugars become the main energy source for the fungus (Solomon *et al.*, 2003*b*). Fungal mycelium present in the apoplast may have experienced a shortage of soluble carbohydrates during shading, and assessment of fungal biomass in leaves (by means of analysing chitin: ergosterol ratios, or using PCR) would shed light on whether fungal growth was reduced during shading. An assessment of pycnidial production may also have revealed retarded or delayed reproduction by the fungus in leaves shaded post-inoculation.

Shading tended to decrease leaf soluble polyphenol contents, and there was a weak positive correlation between soluble carbohydrates and soluble polyphenols, which suggests that the higher sugar status of unshaded leaves (or leaves that had recovered from shading) increases partitioning of resources to defence. This is supported by other workers who found decreased phenolics and increased infection in shaded leaves (Greaves *et al.*, 2001; Vergeer and van der Velde, 1997).

How do C and N interact within plants to affect resistance?

In one shading experiment there was a negative relationship between lesion numbers and leaf N content. Correspondingly, there was a positive relationship between lesion numbers and C: N ratios. In Chapter 3 there was no significant relationship between lesion numbers and N or lesion numbers and C: N ratio but there was a significant positive relationship between lesion numbers and soluble carbohydrates. It is unlikely that there is a negative relationship between disease and leaf N content, as here and in the literature a positive increase in disease severity with increasing N supply has been shown. The negative relationship found here was within a narrow range of N contents, and suggests that something other than N content *per se* may be important.

Under conditions of high light, both net photosynthesis and the concentration of leaf carbohydrates will be high. Glutamine synthetase (GS) and glutamate synthase (GOGAT) are stimulated; asparagine synthetase (AS) is inhibited and N assimilation into glutamine and glutamate is favoured. These compounds are rich in C and participate in the synthesis of new plant materials. Under conditions of low light or energy limited conditions there is inhibition of GS and GOGAT, stimulation of AS and increased assimilation of N into asparagine (Lam *et al.,* 1996), which is relatively N rich, stable and suitable for storage and transport (Sieciechowicz *et al.,* 1988). This implies that in shaded plants, asparagine would be more abundant than in unshaded plants, so whilst carbohydrate contents of shaded leaves would be low, the N compounds available may be favourable to *S. nodorum*. Perhaps this is partly why plants shaded prior to inoculation were generally more susceptible to *S. nodorum*, although the interactions between C and N in relation to disease resistance are complex.

Here, I have shown that in plants supplied high N, photosynthesis, total N, and soluble N compounds are increased and soluble carbohydrates and polyphenols are decreased. The form of N has also influenced plant physiology in a way that altered photosynthesis and its products, and the partitioning of N into different amino acids and proteins. N supply has affected the severity of infection by *S. nodorum*, and this can be attributed to changes in the nutritional status of the host plant as well as defence compounds. Light intensity also alters plant physiology and disease resistance but the mechanisms are less clear cut.

The research in a wider context

Limitations of visual disease assessments and other methods that could be used

Assessment of disease involves measurement of disease incidence, disease severity and yield loss (Agrios, 1997). The incidence of disease refers to the proportion of plants or leaves in a crop population showing symptoms. Disease severity is measured by the area of plant tissue affected by the disease, and may involve a distinction between 'symptoms' i.e. necrosis and chlorosis, and 'pathogen', i.e. actively sporulating region. Severity may be assessed at several stages of the growing season, and this data can be used to plot a disease progress curve. The area under the disease progress curve (AUDPC) can be used as a measure of yield loss (Agrios, 1997). Visual assessment of disease severity can be made with reference to one of the published disease severity scales. For example, Ma and Singh (1996) used the modified Cobb scale to assess the severity of yellow rust on wheat. There are also scales for infection types of yellow rust which give ratings of 0-9 according to infection type (Qayoum and Line, 1985). The use of visual disease assessments has been criticised (Parker, Shaw and Royle, 1995) but nevertheless visual assessments provide a rapid and simple way to assess disease, and continue to be used in conjunction with more sophisticated diagnostic methods.

There are several stages during the infection of a host plant by a fungal pathogen, including germination, penetration, colonisation and reproduction. Identifying the stage of infection at which environmental resistance is expressed may be important in understanding the mechanisms of such resistance. Visual assessments of disease severity are clearly useful, but give no indication of how much mycelium or inoculum is present in the tissues. Spore production can be monitored, but it is still not an accurate measurement of fungal abundance. Another complication is that nutritional deficiencies or natural senescence may easily be confused with symptoms caused by pathogens such as *Septoria* (Hollomon *et al.,* 1999) and leaves of field-grown crop plants will be infected by more than one pathogen at any one time (Dimmock and Gooding, 2002). Accurate detection of infection is important for the timing of fungicide applications, as once *S. nodorum* has begun to produce pycnidia, no fungicide will affect that generation of the

disease (Hollomon *et al.,* 1999). The three topmost leaves of a wheat plant are most important for providing assimilates to be used in grain filling, so detection of disease before it reaches these leaves is necessary (Fraaije *et al.,* 1999). The increasing sophistication of molecular techniques available to detect and quantify plant pathogens is such that pathogens may now be detected in symptomless leaves (Fraaije *et al.,* 2001). PCR methods for detection and quantification of pathogens including *S. nodorum* exist, and whilst they still require improvement their usefulness as a tool for resistance screening and disease forecasting must not be overlooked (Fraaije *et al.,* 2001). QTLs for resistance have also been identified which could be used as a tool for breeders to enhance partial resistance against *S. nodorum* (Schnurbusch *et al.*, 2003).

Usefulness of controlled environment studies and lack of comparability with real crops

The research presented in this thesis has focussed on assessing the effects of altered N and C supply to wheat on its resistance to *S. nodorum*. All experiments have been carried out on seedlings in controlled environments. This situation is far removed from that of a wheat plant growing as part of a crop in the field, so this research cannot be, and was not intended to be, extrapolated to a field situation. The intention was to study possible mechanisms of resistance without the complication of variable environmental and edaphic factors. My research will be discussed with reference to field studies that may corroborate my research, but with the knowledge that a simple comparison between field and controlled environments cannot be made.

The influence of N supply on the resistance of field grown wheat to foliar pathogens has been studied, and whilst only one published study has assessed the effect of N supply on *S. nodorum* (Leitch and Jenkins, 1995), several have investigated the impact of N supply on the now more common *Septoria tritici* (Simon *et al.,* 2003; Leitch and Jenkins, 1995; Tompkins *et al.,* 1993). The study by Leitch and Jenkins (1995), aimed to assess the effects of timing and rate of N fertiliser application on the development of septoria diseases on winter wheat (cv. Norman). N fertiliser was supplied at a rate of up to 300 kg ha^{-1}. Both *S. nodorum* and *S. tritici* were found on the wheat plants, with *S. tritici* being dominant. *S. nodorum* was found on the ears during grain filling. Timing of N application

did not significantly affect disease severity. Infection was increased with increasing N supply and in April and July, but not May, there was a positive correlation between disease severity and leaf N concentration. Yield loss in terms of individual grain weight was due to severity of disease on the flag leaf in July, which is likely to have caused a reduction in photosynthesis (Leitch and Jenkins, 1995).The study by Simón *et al.* (2003) aimed to assess the impact of N supply on the AUDPC for *S. tritici*, and the interactions between environmental conditions and the effects of N. Wheat was supplied no N, or N as urea at 100 kg ha^{-1} or 100 kg ha^{-1} plus a further 50 kg ha^{-1} in a second dose. Visual assessments of disease were made and the AUDPC was calculated. Rainfall, relative humidity (RH) and temperature were recorded during the experiment. Disease severity was increased by N fertilisation, but only in the year when environmental conditions were conducive to disease development, specifically, high rainfall after inoculation (Simón *et al.*, 2003).

Severity of powdery mildew infection (*Erysiphe graminis* f. sp. *hordei*) on spring barley (*Hordeum vulgare* L.) increased with increasing N supply (Jensen and Munk, 1997). The colony density, sporulation capacity and spore production all increased with increasing N, although the magnitude of the response varied between cultivars. Sporulation capacity was positively correlated with leaf N content even when colony density was unaffected, suggesting N nutrition is important for sporulation and that infection and sporulation may be affected by different resistance mechanisms (Jensen and Munk, 1997).

Altering N supply to a crop not only affects its nutritional status but also affects resistance by altering the microclimate of the crop. Increasing N supply increases leaf area which makes the canopy more dense and humid, which creates favourable conditions for germination of fungal spores. The closer proximity of leaves to one another increases the probability of spores being transmitted from leaf to leaf by splashy rainfall (Lovell *et al.*, 1997).

Potential use of the research

Environmental resistance does not provide full protection against a pathogen, but it is a more durable form of resistance than vertical resistance. With increasing levels of fungicide resistance by pathogens, and the burden of high agrochemical inputs on the environment, integrated pest management is becoming more important. Environmental resistance mechanisms could be exploited by breeders when attempting to breed varieties of wheat with durable resistance traits. The decreased resistance of wheat to *S. nodorum* when N was supplied as NH_4^+ may seem of little significance in light of the relatively low availability of NH_4^+ in agricultural soils. However, with increasing NH_4^+ deposition in the biosphere (Britto and Kronzucker, 2002) and the growing trend for organically produced food which tends to be grown with N amendments that provide relatively high concentrations of NH_4^+, such as animal manures (Sørensen, 2001), the role of NH_4^+ nutrition in crops may become more important. The relationship between asparagine and susceptibility of wheat to *S. nodorum* should be explored further.

Conclusions

The nutritional status of wheat is important in modifying its resistance to *S. nodorum*, and it is perhaps as important as the presence of defence compounds, in determining the success of the pathogen *in planta.*

Carbon supply has inconsistent effects on resistance of wheat to *S. nodorum*, and whilst carbon availability is important for fungal growth it is not limiting.

Supplying moderate and high concentrations of N, and supplying NH_4^+ increases susceptibility of wheat to *S. nodorum*.

Amino acids and perhaps proteins favour disease development, and asparagine may play an important role in the reproduction of the pathogen. Acidic amino acids may inhibit reproduction of the pathogen.

The mechanisms of environmental resistance of wheat are both N and C-based with emphasis on the availability of soluble N compounds and perhaps C-based defence compounds. The form of N supplied further complicates the relationship but points to the availability of amides as a putative resistance mechanism.

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Appendix

Poster and oral presentations

Posters

8th International congress of plant pathology (ICPP 2003), Christchurch, New Zealand.
(Poster presented on my behalf by Professor John Farrar)

'Nitrogen reduces resistance of wheat to *Stagonospora nodorum'*

British Society for Plant Pathology annual meeting 2000 (Imperial College, London, Wye campus).

'Nitrogen affects resistance of wheat (*Triticum aestivum* L.) seedlings to *Septoria nodorum* Berk'.

Oral presentations

British Ecological Society winter meeting 2001 (University of Warwick)

'Environmental resistance of plants to fungal pathogens: nitrogen and *Stagonospora nodorum* on wheat'.

Plant Science Wales 2002 (University of Wales, Bangor).

'Environmental resistance of plants to fungal pathogens: nitrogen and *Stagonospora nodorum* on wheat'.