Pathogen and Chemical Induction of Systemic Protection in Broad Bean and Barley, and Effects on Plant Growth and Development

A thesis submitted to the University of Glasgow for the degree of Doctor of Philosophy, in respect of research undertaken in the Department of Plant Biology at the Scottish Agricultural College, Auchincruive, Ayr.

by

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Abbreviations Used in the Text

ALA, aminolevulinic acid

AOPP, α-aminooxy-β-phenylpropionic acid

BA, benzoic acid

CAD, cinnamyl alcohol dehydrogenase

F_m, maximum fluorescence

F_o, minimal fluorescence

Fru 1,6bisPase, fructose-1,6-bisphosphatase

F_v, variance between F_o and F_m

HR, hypersensitive reaction

LAR, leaf area ratio

LAR, local acquired resistance

NAR, net assimilation rate

OH-PAS, [[(2-hydroxyphenyl)amino]sulphinyl]acetic acid, 1,1-din ethylethyl ester

PAL, phenylalanine ammonia-lyase

PGA, 3-phosphoglycerate

PGT, primary germ tube

Pi, inorganic phosphate

POX, peroxidase

PR, pathogenesis-related proteins

q_I, photoinhibition

q_N, non- photochemical quenching

q_o, F_o quenching

q_p, photochemical quenching

RGR, relative growth rate

ROS, reactive oxygen species

Rubisco, ribulose-1,5-bisphosphate carboxylase / oxygenase

RuBP, ribulose-1,5-bisphosphate

SA, salicylic acid

SAR, systemic acquired resistance

TAL, tyrosine ammonia-lyase

t-CA, trans-cinnamic acid

TMV, tobacco mosaic virus

triose-P, (triose phosphates) sum of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate

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Abstract

The efficacy of different fungal and chemical elicitors of systemic protection and their effects on plant growth and development were examined in barley (*Hordeum vulgare*) and broad bean (*Vicia faba*). Powdery mildew (*Blumeria graminis* syn. *Erysiphe graminis* f. sp. *hordei*) of barley and rust (*Uromyces viciae fabae*) of broad bean, two biotrophic pathogens, were used as fungal elicitors to induce systemic protection in barley and bean respectively, and the upper leaves of plants were subsequently challenged with the same pathogens.

Powdery mildew applied to the lower leaves of barley was found to induce systemic protection against powdery mildew in upper leaves. However, whole plant fresh and dry weight, and total leaf area was approximately 50% lower in barley 14 d after inoculation of the first leaves with mildew. The total number of leaves and tillers were also reduced by approximately 30%. Consequently, although there was a reduction in total infection in the mildew treated plants this was probably due to the reduction in growth.

Rust infection was reduced in upper leaves of broad bean plants following inoculation of the lowest leaf with rust. However, it was only in plants challenged 6 d following the inducer inoculation that the reduction in infection was significant. This was consistent with reports that maximal gene expression associated with SAR in tobacco was attained for most of the genes 6 d after inoculation. The strength of the protection response was lower than in previous reports of systemic protection in bean induced by rust. The degree of protection produced in upper leaves may be related to the number of lower

leaves inoculated with the inducing pathogen. Induction of resistance with rust had no significant effect on bean growth, except an increase in total leaf area in plants challenged 14 d after rust treatment.

Due to the limited effect of rust induced protection on growth of bean plants, it was decided to investigate further the effect of rust on plant growth. Whole plant fresh weight, dry weight and total leaf area increased 1 wk and decreased 3 wk after inoculating three leaves of the plants with rust. This may be consistent with a transient increase followed by a decrease in photosynthesis that is thought to occur in rust infected leaves. Changes in water uptake were in accordance with inhibition of stomatal opening in the early stages of infection and later, increased transpiration due to rupture of the leaf cuticle caused by sporulation.

Chlorophyll fluorescence analysis identified that rust infection resulted in F_o quenching and photoinhibition. In addition, rust infection was found to affect the photochemical efficiency of PSII reaction centres from 1 wk after rust inoculation. Non-photochemical quenching was higher in controls compared with leaves on rust infected plants, except in healthy leaves on rust infected plants 4 wk after inoculation. This is indicative of increased metabolic activity and increased demand for ATP in the infected tissue.

Phosphate, Salicylic acid (SA), and saccharin applied as a drench did have some detrimental effect on barley growth. In contrast, phosphate, SA and foliar applications of saccharin had no significant effect on growth of bean plants. Saccharin applied as a drench was more effective as an inducer of systemic protection compared with foliar

application of saccharin. However, associated with the increased efficacy of saccharin when applied as a drench, was a detrimental effect on plant growth.

In barley, chemical induced protection was found to be stronger in lower leaves, whereas in mildew induced protection, a significant response was only observed in leaves 3 and 4 and not in leaf 2. In broad bean, regardless of whether resistance was pathogen or chemically induced, the resistance response was stronger in the lower leaves, except with saccharin when the reverse was true. The differences observed may be related to signal strength.

Saccharin was found to be a very effective inducer of systemic protection, but the mode of action is unknown. Consequently, the effects of saccharin on phenylpropanoid metabolism were examined. Saccharin was found to have no significant effect on lignin or free phenolic compounds. Despite the lack of increased PAL activity, enhanced CAD and peroxidase activity were observed. It is possible that the increase in PAL activity is transient and had occurred before the period examined. The increase in CAD activity in saccharin treated plants occurred both prior to and after inoculation with powdery mildew, which suggests that saccharin is priming CAD activity prior to pathogen challenge.

Chapter 1

Introduction

1 Introduction

Despite the use of pesticides and disease resistant cultivars, crop losses due to pathogens are estimated to account for 10% of potential, world-wide crop production, with an estimated world cost of $6 billion annually in fungicides alone (Day, 2001). Disease also reduces crop quality and hence the value of the crop. The availability of existing crop protection methods continues to decline as pathogens overcome plant resistance or develop resistance to pesticides. For example, in 1988 previously resistant barley crops in Britain and northern Europe were devastated by mildew (Day, 2001). Plants bred for resistance also increases genetic uniformity and the risk of severe disease epidemics occurring, such as the southern corn leaf blight epidemic of 1970. It is necessary therefore to replace most resistant varieties with an alternative variety containing a different genetic base, within 3-5 years (Agrios, 1988). Additionally, it is not always possible to grow resistant varieties, as genetic resistance has not been identified in all crops (Ward, *et al.*, 1994).

Although fungicide resistance started to appear in the 1960s, it was not until the introduction of systemic fungicides that resistance became widespread. The reason for this is that systemic fungicides are very specific in their mode of action and consequently resistance is quicker to develop (Agrios, 1988). Hence, there is a need to develop improved methods of disease control. One possible solution is to utilise the plant's own natural defence system. However, it has been assumed that inducing plant defence responses has a cost in terms of reduced fitness, growth, yield or increased vulnerability to insect pests (Thaler *et al.*, 1999; Heil *et al.*, 2000). Therefore, in addition to effectively reducing plant disease, inducing plant defences must also compete economically with existing crop protection methods. Furthermore,

environmental implications must be fully considered, such as possible increased susceptibility to insect attack.

1.1 Plant Resistance to Disease

Defence responses may be local or systemic, some are expressed constitutively and others are produced in response to pathogen attack (Kessmann *et al.*, 1994; Peltonen, 1999; Mayda *et al.*, 2000). Most plant defence responses are non-specific and may be induced in response to various environmental factors and stress conditions, not just plant pathogens (Peltonen, 1999). In addition, some defence genes are controlled by plant hormones and consequently resistance may not develop until the plant reaches maturity (Lamb *et al.*, 1989). Resistance to powdery mildew increases with plant maturity. Consequently, disease severity is usually greatest on lower leaves (Large and Doling, 1962; Shaner, 1973). All plants are capable of resistance to disease (van Loon *et al.*, 1998; Peltonen, 1999) although the degree of resistance or susceptibility may be an indication of the host's ability to identify and rapidly respond to pathogen attack (de Wit and Van der Meer, 1986; Slusarenko and Longland, 1986; Collinge and Slusarenko, 1987). Additionally, defence responses may be delayed and then produced at a later stage as a means of limiting infection (Lamb *et al.*, 1989). As pathogens continue to evolve to ensure their survival, concomitant changes in the defence mechanisms utilised by plants are necessary. Consequently, although some defence mechanisms are common amongst plants, other responses are specific to particular host pathogen interactions (Keen, 1990).

1.2 Constitutive Defence Mechanisms

The plant cell wall provides a physical barrier to pathogen penetration. The cuticle which covers epidermal cells is hydrophobic and consequently prevents water accumulation and nutrient flow to the cell surface, which inhibits the growth of micro-organisms on the plant surface (Kolattukudy, 1980). Germination and penetration of fungal pathogens may also be chemically inhibited by cuticular waxes and cutin acids (Bell, 1981). Lignin also accumulates in cell walls during secondary thickening and as the plant matures. Esterification of lignin precursors produces a hemicellulose matrix, the 'lignin-carbohydrate complex'. Cellulose fibrils bind to this matrix to strengthen the plant secondary wall and help to prevent fungal penetration (Jefferies, 1990). Extensins, which are hydroxyproline-rich glycoproteins, may be oxidised and cross-linked to cell walls, again to strengthen and increase cell wall rigidity (Bradley *et al.*, 1992). Antimicrobial compounds may be used in constitutive defence and may also accumulate as part of an induced defence response (Bowles, 1990). These include phenolic compounds, phenolic glucosides, polyunsaturated compounds, glucose esters and hydroxamic acids (Schönbeck and Schlösser, 1976). These antimicrobial compounds are thought to be used primarily in resistance responses to insects and other herbivores, but may additionally provide some resistance against plant pathogens (Métraux and Raskin, 1993). Certainly, phenolics are lignin precursors (Friend, 1981; Lewis and Yamamoto, 1990) and may directly inhibit spore germination and mycelial growth (Peltonen, 1999). Furthermore, proanthocyanidins may inhibit the hydrolytic enzymes produced by fungal pathogens (Métraux and Raskin, 1993). Other instances of antimicrobial substances providing constitutive defence have included salicylic acid in rice (Silverman *et al.*, 1995), hydroxamic acid in wheat, maize and rye (Niemeyer,

1988), hordatines in barley seedlings (Friend, 1981) and various cysteine-rich antimicrobial proteins such as defensins (Broekaert *et al.*, 1995).

1.3 Systemic Acquired Resistance

In 1901, Ray and Beauverie (1901) identified a natural resistance phenomenon in plants. Beauverie amended the virulence of a strain of *Botrytis cinerea*, which was then used to inoculate Begonia plants. When subsequently challenged with a virulent strain of the pathogen, the begonias were found to have developed resistance. Chester (1933) produced a review in 1933 of a phenomenon he referred to as 'physiological acquired immunity'. However, it was not until the 1960s that more detailed analysis of this phenomenon was undertaken (Kessmann *et al.*, 1994). When, in 1961, Ross inoculated tobacco with tobacco mosaic virus (TMV), the plants exhibited a hypersensitive response that resulted in the induction of disease resistance in distal parts of the plant. This he called **S**ystemic **A**cquired **R**esistance (SAR). Resistance that was induced in the TMV inoculated leaf, he termed **L**ocal **A**cquired **R**esistance (LAR).

It is often assumed that the inducer pathogen has to be necrotrophic. However, this is clearly not true, for there are a few reported instances of biotrophic pathogens inducing a systemic resistance response. For example, Hwang and Heitefuss (1982) induced systemic protection in barley with powdery mildew and Murray and Walters (1992) induced systemic protection in broad bean with rust. Thordal-Christensen and Smedegaard-Peterson (1988) found that non-host pathogens were more effective in inducing local resistance in barley to *Erysiphe graminis* f. sp. *hordei,* than virulent or avirulent pathogens. In addition, although purely saprophytic fungi could induce a

defence response it was much weaker than either avirulent or non-pathogenic fungi (Gregersen and Smedegaard, 1989).

SAR produces protection against a broad spectrum of fungal, bacterial and viral pathogens, irrespective of the pathogen that induced SAR. However, it does not provide resistance to all diseases (Uknes *et al.*, 1996). In tobacco, SAR provides resistance against the fungal pathogens, *Phytophthora parasitica, Cercospora nicotianae* and *Peronospora tabacina.* There is also resistance to the bacterial pathogens, *Pseudomonas syringae* pv. *tabaci* and *Erwinia carotovora* and tobacco mosaic virus (TMV), and tobacco necrosis virus (TNV) (Vernooij *et al.*, 1995). However, SAR does not provide significant protection against *Botrytis cinerea* or *Alternaria alternata* in tobacco (Ryals *et al.*, 1996). Furthermore, SAR may be induced by fungal, bacterial or viral pathogens (Cruikshank and Mandryk, 1960; Kuc, 1982; Kessmann *et al.*, 1994).

1.4 Induced Systemic Resistance

Van Loon *et al.* (1998) has described a second form of systemic resistance, called induced systemic resistance (ISR). ISR differs from SAR, in that it is induced by certain nonpathogenic rhizobacteria that colonise plant roots. Although the pathogenesis related (PR) genes are not thought to be implicated in ISR, activation of the *npr1* gene (non-expresser of PR-1), also known as *nim1* (non-inducible immunity) in *Arabidopsis*, was found to inhibit both SAR and ISR (Pieterse *et al.*, 1998). ISR uses the jasmonate and ethylene signalling pathways, but is not dependent on salicylic acid (SA) (Pieterse *et al.*, 1998).

1.5 Initial Responses to Pathogen Attack

Pathogen attack results in the immediate release of elicitors, from either the pathogen or the plant or both, such as cryptogein, an elicitin produced by *Phytophthora cryptogea* (Lebrun-Garcia *et al.*, 1998; Chrispeels *et al.*, 1999). Elicitors can be polysaccharides, glycoproteins or products from the pathogen cell walls such as β- glucans, chitin or chitosan and released through the activity of plant enzymes, or produced and released by the pathogen in response to signals from the host (Lamb *et al.*, 1989; Dixon *et al.*, 1994). Conversely, elicitors may be released from the plant cell wall by the action of the pathogen's enzymes (Lamb *et al.*, 1989). Elicitors bind to plant receptors, either on the plasma membrane or in the cytosol, which subsequently leads to gene activation. Transcription factors move from the cytosol to the nucleus to activate genes involved in pathogen defence (Chrispeels *et al.*, 1999).

Within minutes of pathogen contact, plants react to the presence of a potential pathogen by plasma membrane depolarisation, changes in membrane permeability, production of oxygen radicals and activation of defence gene transcripts, (Dixon *et al.*, 1994). The first line of plant defence is localised accumulation of defensive compounds at the site of infection to prevent or inhibit growth of the pathogen and to allow time for plant secondary defence (Matern and Kneusel, 1988).

As a result of pathogen attack changes in ion fluxes occur as cell membrane integrity is affected. This permits an influx of Ca^{2+} ions stimulated by the elicitor, which act as a secondary signal in the activation of defence genes (Jabs *et al.*, 1997; Zimmermann *et al.*, 1997; Yano *et al.*, 1998). A change in cellular Ca^{2+} mediates the activation of callose synthase (β-1,3-glucan synthase), resulting in increased callose deposition at the

pathogen penetration site (Köhle *et al.*, 1985). In cereals papillae may be formed in response to fungal leaf pathogens, which inhibit penetration of fungal appressoria. This usually occurs prior to callose deposition (Aist, 1976; Carver *et al.*, 1994b). Papillae vary in composition and may contain callose, autofluorogenic substances (possibly phenolic compounds), hydrolytic enzymes, proteins and elements including calcium, silicon and manganese (Carver *et al.*, 1994b).

1.6 The Oxidative Burst and the Hypersensitive Reaction

Following the change in permeability of the plasma membrane and the resultant increase in ion fluxes across the membrane, plant cells release reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide anions (O_2^-), and hydroxyl free radicals ($OH^\cdot$). Reactive oxygen species are chemically active substances, and their accumulation is known as 'the oxidative burst' and is thought to be essential to the hypersensitive reaction (HR) (Mehdy, 1994). Necrotic lesions produced at the site of infection are known as the hypersensitive reaction (Slusarenko *et al.*, 1991). The HR inhibits pathogen growth by depriving the pathogen of access to plant nutrients. HR also inhibits growth of necrotrophic pathogens despite their ability to obtain nutrients from necrotic plant cells (Hammond-Kosack and Jones, 1996). It is possible that the necrotic cells accumulate phenolic compounds or other substances toxic to the pathogen and thereby limit the growth of necrotrophic pathogens (Nicholson and Hammerschmidt, 1992). H_2O_2 may also act as a diffusible signal to activate transcription of protectant genes in surrounding cells (Tenhaken *et al.*, 1995). In powdery mildew infection of barley an accumulation of H_2O_2 has been observed in both papillae and cells involved in the HR (Thordal-Christensen *et al.*, 1997).

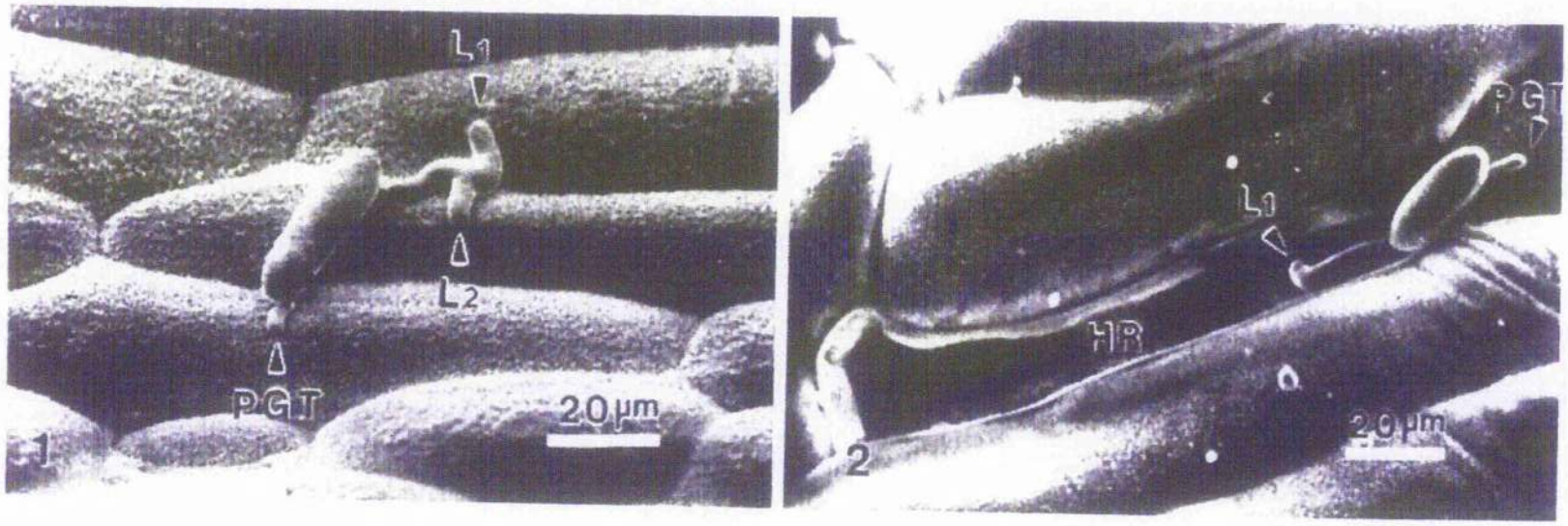


Figure 1 and 2 Scanning electron micrographs of the surfaces of barley leaves attacked by powdery mildew, 36 h after inoculation. Fig. 1 is the RISØ-R barley line containing the recessive *mlo5* allele conferring race non-specific penetration resistance without a hypersensitive cell death response. The fungal germling has produced a typical primary germ tube (PGT) and an appressorial germ tube. The appressorium produced an initial lobe (L1), which was unsuccessful in penetration, and later attempted penetration from a second appressorial lobe (L2) was also unsuccessful. Fig. 2 is the Alg-R barley line containing the dominant *Mla1* allele conferring race-specific, hypersensitive cell death resistance. The fungal germling produced a PGT and an appressorial germ tube. The appressorium produced an initial lobe (L1), which successfully penetrated the epidermal cell. This resulted in a hypersensitive response (HR), a loss of turgor pressure and the penetrated cell collapsed (from Zeyen *et al.*, 1995).

1.7 Secondary Defence Responses

Changes in gene expression result in synthesis of enzymes responsible for mediating the production of pathogenesis-related proteins (PR), proteinase inhibitors, hydrolytic enzymes, and phytoalexins. Structural polymers such as lignins, wall-bound phenolics and extensins may also be produced (Lamb *et al.,* 1989). Most of these products are derived from the plant phenylpropanoid pathway (Hahlbrock and Scheel, 1989; Nicholson and Hammerschmidt, 1992; Dixon and Paiva, 1995).

1.8 The Phenylpropanoid Pathway and the Defence Response

Phenylpropanoids are phenolic compounds found only in plants (Christensen, 1997). The phenylpropanoid pathway involves the synthesis of phenylpropanoids from phenylalanine through to substituted cinnamic acid CoA (coenzyme A) esters and peroxidase (Grisebach, 1981; Northcote, 1985; Christensen, 1997). From the CoA esters may be synthesised precursors of lignin, cell wall hydroxycinnamyl esters or soluble glucosides. The lignin precursors may subsequently be polymerised by way of the lignin biosynthetic pathway. Alternatively, the CoA esters may enter the flavonoid biosynthetic pathway to be synthesised into various flavonoid derivatives (Christensen, 1997). It has been suggested that salicylic acid (SA), which is involved in plant defence responses, may be derived from the phenylpropanoid pathway (Yalpani *et al.*, 1993). Alternatively, other biosynthetic pathways have also been proposed (Malamy and Klessig, 1992; Wildermuth *et al.*, 2001).

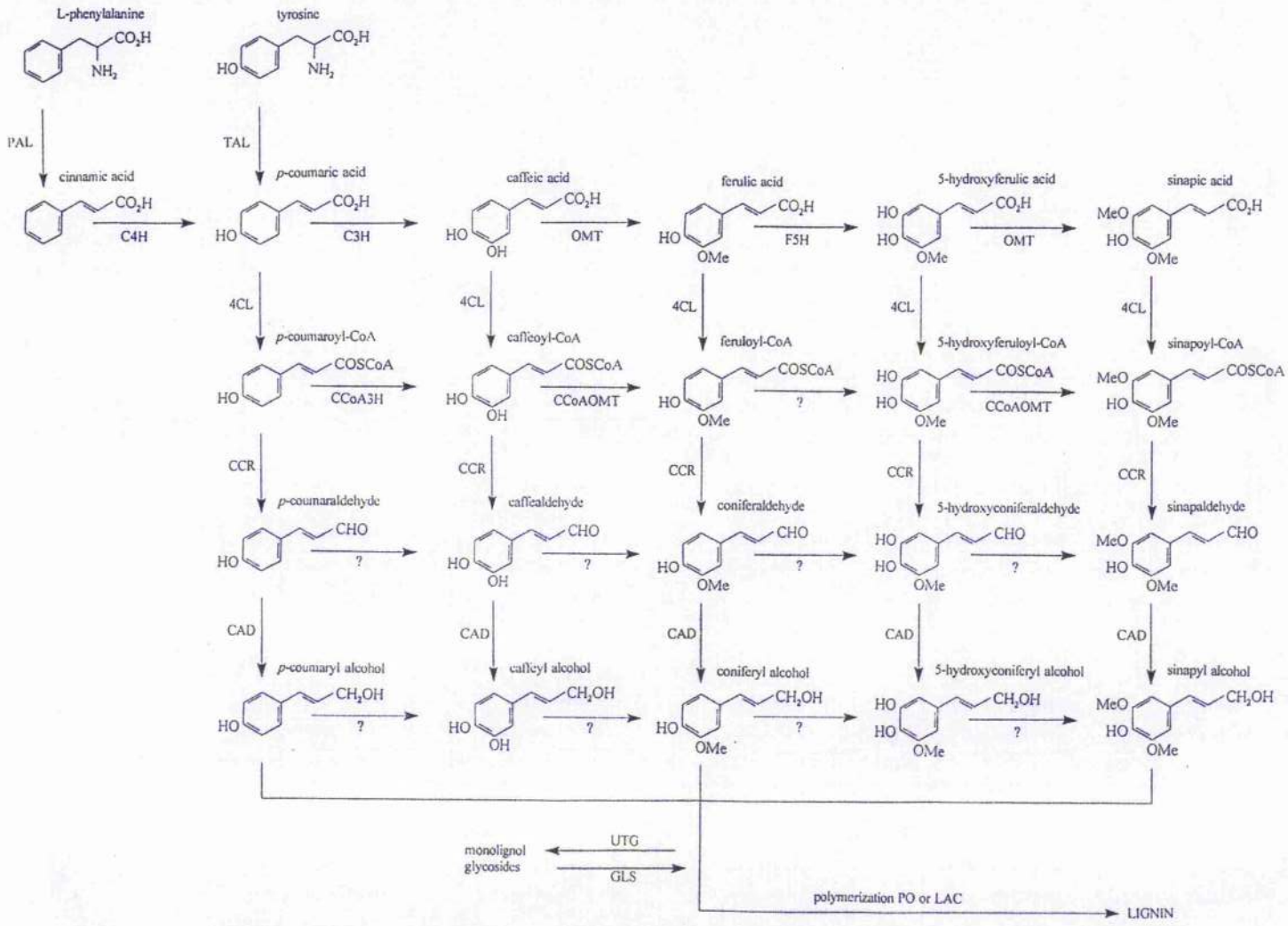


Figure 3. Phenylpropanoid pathway (from Barber and Mitchell, 1996)

1.8.1 Phenolics

Preformed phenolics are often released as an early defence response, but synthesis of substantial new phenolic compounds occurs later following up-regulation of phenylpropanoid metabolism (Peltonen, 1998; Graham and Graham, 1991a). Due to the potential cytotoxicity of preformed phenolics, they are stored in the central vacuole or in or on the cell wall until required. During the initial stages of a defence response preformed phenolics are released into the cytoplasm to be incorporated into the cell wall (Sedlářová and Lebeda, 2001). Consequently, there is a rapid increase in cell-wall phenolics following fungal infection. One of their functions is thought to be to modify cell wall polysaccharides to withstand the action of lytic enzymes produced by the pathogen (Matern, *et al.*, 1995). Phenolic acids or cinnamyl alcohols form ester or ether links to cell wall polysaccharides or hemicelluloses, or alternatively they may be polymerised into lignin (Lewis and Yamamoto, 1990; Cvikrova *et al.*, 1991; Lam *et al.*, 1992). Phenolics are also important in the HR, and the accumulation of lignin-like polyphenolics results in local cell necrosis inhibiting pathogen progress (Moerschbacher *et al.*, 1990; Nicholson, 1992; Levine *et al.*, 1994; Wei *et al.*, 1994; Zeyen *et al.*, 1995). Furthermore, the accumulation of phenolics together with toxins from the necrotic cells may also limit pathogen growth (Nicholson and Hammerschmidt, 1992).

1.8.2 Phenylalanine ammonia-lyase (PAL)

PAL is the catalyst that mediates the deamination of phenylalanine to cinnamic acid which is then hydroxylated to *p*-coumaric acid, and may be considered the first step in the phenylpropanoid pathway (Carver *et al.*, 1994b; Christensen, 1997). Alternatively, tyrosine ammonia-lyase (TAL), which has been detected in cereals, may control the

synthesis of tyrosine directly to *p*-coumaric acid (Camm and Towers, 1973; Maule and Ride, 1976; Guerra *et al.,* 1985; Kolfalvi and Nassuth, 1995). However, activity of TAL *in vitro* was found to be considerably lower than PAL and hence may only have a comparatively minor role in phenylpropanoid biosynthesis (Green *et al.,* 1975; Maule and Ride, 1976; Dickerson *et al.*, 1984; Rösler *et al.*, 1997).

The pattern of PAL induction may vary depending on whether the pathogen is necrotrophic or biotrophic. PAL activity is often linked with the hypersensitive response and cell necrosis, although facultative parasites induce a stronger PAL response than obligate parasites (Hadwiger *et al.,* 1970). In addition, genotype, tissue types and plant developmental stage all affect PAL activity (Camm and Towers, 1973).

In wheat inoculated with the non-host pathogenic fungus *Botrytis cinerea,* the increase in PAL activity was confined to the area surrounding lignified tissue and was rapid, occurring prior to the development of lignified HR cells (Maule and Ride, 1976). Similarly, the increase in PAL activity was rapid in barley, and occurred 4 h after inoculation with powdery mildew. Additionally, increases in both PAL and TAL were also observed in wheat within 4 h of powdery mildew inoculation (Green *et al.*, 1975; Shirishi *et al.*, 1989). PAL transcripts were also found to be specific to the area surrounding necrotic lesions in tobacco inoculated with TMV (tobacco mosaic virus) or induced with an elicitor (Pellegrini *et al.*, 1994). In addition, in potato leaves, mRNA for PAL was detected at the infection site within 1-2 h after inoculation with *Phytopthora infestans* or treatment with an elicitor. Furthermore, accumulation of the mRNA was faster and more narrowly confined to the infection site when penetration was unsuccessful than when penetration was successful (Cuypers *et al.*, 1988). Tobacco

plants transformed to suppress PAL activity had reduced lignin and were more susceptible to fungal infection than plants that had not been transformed (Bate *et al.*, 1994; Maher *et al.*, 1994). However, SAR could not be induced in plants in which PAL activity had been suppressed (Dixon andPaiva, 1995; Pallas *et al.*, 1996). It is, therefore, possible that PAL may be involved in SA synthesis in some plants (Mauch-Mani and Slusarenko, 1996).

Carver *et al.* (1994b) found that inhibition of PAL with α-aminooxy-β-phenylpropionic acid (AOPP) (PAL inhibitor) or cinnamyl alcohol dehydrogenase (CAD) with [[(2-hydroxyphenyl) amino] sulphinyl]acetic acid, 1,1-din ethylethyl ester (OH-PAS) (CAD inhibitor) in oat resulted in a considerable increase in successful penetration and haustoria formation of powdery mildew. This suggests that products produced in the phenylpropanoid pathway inhibit penetration of appressoria. The frequency and intensity of autofluorescence that occurs in leaf epidermal cells in response to either the primary germ-tube or appressoria was also reduced. This indicates that host autofluorogens may be phenolic compounds (Carver *et al.*, 1994b). However, adult leaves treated with AOPP remained more resistant than seedling leaves also treated with AOPP, suggesting that there is an additional factor or factors involved in adult plant resistance (Carver *et al.*, 1996).

PAL activity may be regulated by intermediates of the phenylpropanoid pathway through a feedback mechanism (Christensen, 1997). Elicitor treatment of alfalfa cell suspension cultures induced transcription of genes for enzymes of the phenylpropanoid pathway. When cinnamic acid was applied to the elicitor treated suspension cultures this resulted in inhibition of both PAL transcripts and PAL activity. However, treatment of the elicited cultures with the PAL inhibitor (AOPP) reduced cinnamic acid levels and resulted in an increase in PAL transcripts and PAL activity (Orr *et al.*, 1993).

1.8.3 Peroxidase

Peroxidase is thought to perform several functions in plant defence. The increase in peroxidase activity as a result of an incompatible plant pathogen interaction is often the result of incorporation into the cell wall of phenolic compounds (Fink *et al.*, 1991; Graham and Graham, 1991b; Reimers *et al.*, 1992; Milosevic and Slusarenko, 1996). Peroxidase also catalyses cross-linking of cell wall proteins and cross-links between other cell wall components (Iiyama *et al.*, 1994). It has also been suggested that peroxidase may be involved in lignin polymerisation (Monties, 1989; Christensen, 1997). Furthermore, peroxidase in thought to oxidise phenols into pathogen toxic derivatives. In addition to metabolising reactive oxygen species such as H_2O_2 (Gaspar *et al.*, 1985; Patykowski *et al.*, 1988; Baker *et al.*, 1995), peroxidase is also capable of oxidation resulting in the generation of H_2O_2 (Pedreño *et al.*, 1990; Vianello and Macri, 1991; Pichorner *et al.*, 1992; Jiang and Miles, 1993).

Various isoforms of peroxidase are known to exist. In barley inoculated with powdery mildew, a 16-fold increase in extracellular peroxidase activity was observed due to an increase in an anionic and a cationic peroxidase isoform (Kerby and Somerville, 1989). Transcripts for the cationic peroxidase were found to accumulate in barley within 4 h of inoculation with mildew and prior to fungal penetration (Thordal-Christensen *et al.*, 1992).

1.8.4 Phytoalexins

Phytoalexins accumulate in plants as a defence response to pathogen attack. Phytoalexins are low molecular weight compounds with antimicrobial activity. The

majority of phytoalexins that have been characterised are associated with dicotyledons, although some have been identified in several cereal species (Mayama *et al.*, 1981; Nicholson *et al.*, 1987; Kodama *et al.*, 1988, 1992; Bücker and Grambow, 1990; Hipskind *et al.*, 1990; Grayer and Harborne, 1994). It may be that phytoalexin accumulation occurs as a second line of defence if the hypersensitive response fails (Nicholson and Hammerschmidt 1992). Phytoalexins usually combine with other disease resistance mechanisms to produce a synergistic effect (Darvill and Albersheim, 1984). Furthermore, there are examples of pathogens producing enzymes that degrade phytoalexins produced by the host plant such as *Nectria haematococca* in pea, *Fusarium solani* in French bean, and *Botrytis cinerea* in grapevine (Yoder and Turgeon, 1985; VanEtten *et al.*, 1989; Turbek *et al.*, 1992; Jeandet *et al.*, 1993).

1.8.5 Cinnamyl alcohol dehydrogenase (CAD)

CAD is the catalyst for the reaction in which substituted hydroxycinnamyl aldehydes are reduced to hydroxycinnamyl alcohols such as coniferyl, *p*-coumaryl and sinapyl alcohols (Moerschbacher *et al.*, 1986; Christensen, 1997). These hydroxycinnamyl alcohols are subsequently polymerised to form lignin (Christensen, 1997). Several CAD isoforms have been identified in different species, which differ in substrate specificity thus affecting the structure of lignin produced (Hawkins and Boudet, 1994). Consequently, activity of CAD from gymnosperms is higher when coniferaldehyde is the substrate, whereas CAD from angiosperms tends to be equally active when either coniferaldehyde or sinapaldehyde are the substrates (Lüderitz and Grisebach, 1981; Kutsuki *et al.*, 1982; Walter, 1991).

1.8.6 Lignins

The basic lignin structure is comprised of the covalent inter-linkage of three alcohols, coniferyl alcohol, *p*-coumaryl alcohol and sinapyl alcohol, although there are structural differences between lignins from gymnosperms, angiosperms and grasses. Angiosperm lignins are derived from coniferyl and sinapyl alcohol, and gymnosperm lignins are based primarily on coniferyl alcohol. In contrast, grass lignins contain a ratio of: 40-50%, 40-50%, and 5-10% of coniferyl, sinapyl and *p*-coumaryl alcohol derivatives, respectively. Grass lignins also have a high content of coumaric and ferulic acid derivatives which are bound to different components of the cell wall with ester bonds (Boudet *et al.*, 1995; Christensen, 1997). These feruloyl and *p*-coumaroyl components of cell walls may be toxic to fungal pathogens and, in addition, may inhibit degradation caused by fungal enzymes (Ride, 1983; Nicholson and Hammerschmidt, 1992; Lyons *et al.*, 1994). Furthermore, a special defence lignin, a high proportion of which is derived from sinapyl alcohol, has been found to accumulate in wheat and maize in response to pathogen attack. It is possible that this defence lignin may have chemical and physical properties more suited to defence against pathogens (Ride, 1975; Lyons, *et al.*, 1994; Mitchell *et al.*, 1994).

Lignins provide strength and rigidity to cell walls and provide a chemical and physical barrier to pathogen ingress (Ride, 1983). In cereals, inhibition of enzymes involved in lignin biosynthesis resulted in weakened papillae and increased fungal penetration (Carver *et al.*, 1992; Zeyen *et al.*, 1995). Furthermore, when barley, wheat or oats were treated with inhibitors of lignin precursors the HR was suppressed, suggesting that lignin or lignin-like compounds are involved in HR (Moesbacher *et al.*, 1990; Carver *et al.*, 1992; Zeyen *et al.*, 1995). In barley and wheat inoculated with powdery mildew,

polyphenolics were found to accumulate in HR cells, but were not apparent in non-HR cells until after fungal penetration (Wei, *et al.*, 1994). The accumulation of polyphenolics similarly diverges between rice cultivars susceptible or resistant to *Xanthomonas oryzae*. Deposition of lignin was not apparent in the susceptible interaction until 96 h after inoculation, whereas lignin started to accumulate within 18-24 h of inoculation in the resistant plants (Reimers and Leach, 1991).

In cereals, the composition of polyphenolics that accumulate in response to pathogen attack may vary between species (Christensen, 1997). In some plant species, a lignin like polymer called suberin is found bound to cell walls. It is also thought to be involved in pathogen defence, but there have been limited studies of suberin (Christensen, 1997).

1.9 Barley and Powdery Mildew

Barley is an important cereal crop in temperate areas of the World. Powdery mildew is the most serious disease that affects barley and it is estimated that without fungicides losses to the disease would account for approximately 10% in cooler climates (Jørgensen *et al.*, 1988; Thordal-Christensen *et al.*, 1999).

Powdery mildew is from the genus *Erysiphe* which has been split into *Blumeria* and *Erysiphe* based on morphological characteristics (Golovin, 1958), although both names are still valid. According to host range the species is divided into *formae speciales* (f. sp.). Hence, f. sp. *hordei* cause disease on barley (*Hordeum* species) and f. sp. *tritici* on wheat (*Triticum* species) (Thordal-Christensen *et al.*, 1999).

Figure 4. Barley infected with powdery mildew

Powdery mildew is a biotrophic, obligate parasite and asexual spores (conidia) are carried in the air and deposited on the leaf surface of their potential host. From inoculation to development of conidia takes only 5-6 days at 20°C and infection occurs even in dry conditions, which are possibly two of the reasons that powdery mildew is so successful (Thordal-Christensen *et al.*, 1999).

Within 2-6 h after inoculation, powdery mildew conidia that germinate on the leaf surface produce a primary germ-tube (PGT). The PGT does not penetrate the epidermal cell, although it dissolves the epidermal cuticle and waxes (Carver *et al.*, 1994). The reason for production of the PGT is not fully understood, although it has been suggested that contact between the leaf surface and the PGT is a mechanism used by the fungus to

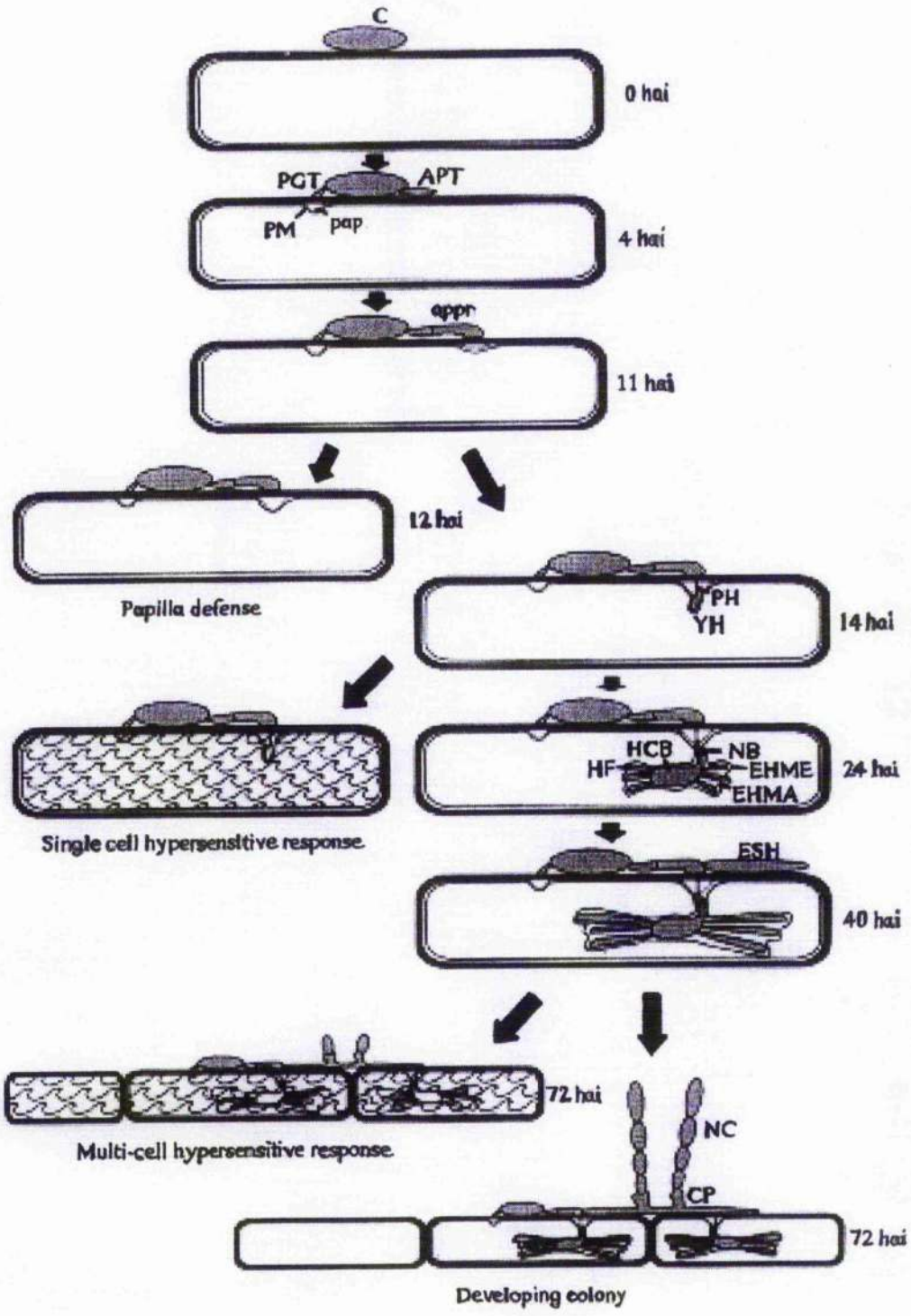


Figure 5. Development of powdery mildew on the barley leaf epidermis. C, conidium; PGT, primary germ tube; APT, appressorial germ tube; pap., papilla; PM, plasma membrane; appr., appressorium; PH, penetration hypha; YH, young haustorium; HCB, haustorial central body; HF, haustorial fingers; NB, neck band; EHME, extrahaustorial membrane (plant origin); EHMA, extrahaustorial matrix; ESH, elongating secondary hyphae; NC, new conidia; CP, conidiophore; hai, hours after inoculation (from Thordal-Christensen *et al.*, 1999).

recognise a potential host (Carver and Ingerson, 1987). The PGT may also take up cutin monomers from the epidermal leaf surface as a prerequisite for differentiation of the appressorial germ-tube (Francis *et al.*, 1996). It is also likely that PGT may take up nutrients (Kunoh *et al.*, 1978; Kunoh and Ishizaki, 1981) and water (Carver and Bushnell, 1983). However, the PGT is also known to activate plant defence responses and this is apparent in the production of papilla (Thordal-Christensen *et al.*, 1999).

Appressorial germ-tubes (appressoria) are specialised structures for infection and are produced 10-12 h after inoculation (Carver *et al.*, 1991). However, it is only appressorial germ tubes that develop infection pegs and attempt to penetrate the epidermal cell; this occurs at about 12-20 h following inoculation. If penetration is successful a fungal feeding structure called a haustorium starts to develop within the epidermal cell. The haustorium extracts nutrients from the host plant to supply hyphae and allow them to branch out and colonise the leaf surface (Carver *et al.*, 1991; Vanacker *et al.*, 2000).

The host, as a resistance mechanism to inhibit fungal penetration, forms papillae below the appressorium. Papillae are produced irrespective of whether the host pathogen interaction is compatible or incompatible, and in fact most cereals have some degree of penetration resistance (Zeyen *et al.*, 1995; Vanacker *et al.*, 2000). If the host-pathogen relationship is incompatible this often results in a HR and cell death, inhibiting further progress of the pathogen (Johnson *et al.*, 1979; Zeyen and Bushnell, 1979; Koga *et al.*, 1990; Hippe-Sanwald *et al.*, 1992). Autofluorescent compounds are associated with both penetration resistance and HR (Koga *et al.*, 1988, 1990). This autofluorescence is indicative of phenolic compounds and was observed in the host cell wall beneath the tip

of the primary germ-tube, at the potential penetration site and in the papillae beneath both of these points of fungal contact (Zeyen *et al.*, 1995). It has been suggested that these phenolic compounds have antifungal activity, increasing resistance in the leaf epidermal cells to fungal penetration (Mayama and Shishiyama, 1978; Aist and Israel, 1986). It is possible that cell wall strengthening also occurs due to esterification of phenolic compounds as an early response to fungal attack (Matern *et al.*, 1995). Furthermore, when epidermal cells die as a result of HR, the whole of the cell autofluoresces (Koga *et al.*, 1988; Carver and Zeyen, 1993). Consequently, this suggests that phenolic substances are implicated in hypersensitive cell death (Zeyen *et al.*, 1995).

1.10 Rust

Rust fungi cause some very important plant diseases and have been responsible for famine and even economic ruin of entire countries (Agrios, 1988). Rust hosts include cereals, vegetables, cotton, soybeans, coffee, and ornamentals (Agrios, 1988). Rusts are caused by Basidiomycetes of the order Uredinales. There are approximately 4000 species of rusts, most of which are very specialised and only attack specific plant genera or certain varieties. Rusts that attack different host genera, but otherwise are identical morphologically are considered special forms (*formae speciales*). These include *Puccinia graminis* f. sp. *tritici* that infect wheat (*Triticum* species) and *Puccinia graminis* f. sp. *hordei* that infect barley (*Hordeum* species). Each special form is then subdivided into pathogenic races according to the host varieties within the species (Agrios, 1988).

Figure 6. Rust infection on broad bean

Rusts are biotrophic obligate parasites. Most rusts produce five different fruiting structures and spores, also some rusts are heteroecious and complete their life-cycles on different hosts (Agrios, 1988). Microcyclic (short-cycled) rusts only produce teliospores and basidiospores. Macrocylic (long-cycled) rusts in addition to the teliospores and basidospores produce spermatia, and may also form aeciospores and uredospores. The fungus overwinters on plant debris as teliospores and following germination the basidium is produced. The basidium undergoes meiosis to produce basidiospores. The basidiospores are carried by the wind to susceptible plants. Following infection, the basidiospores produce mycelium on which is formed spermogonia containing receptive hyphae and haploid spermatia. The spermatia fertilise receptive hyphae and as a result the mycelium produced forms aecia containing dikaryotic aeciospores. Again, following infection, mycelium is formed only with uredia, the characteristic rust coloured pustules, containing dikaryotic uredospores. Subsequent infections produce more uredia and uredospores until host maturity

approaches when telia and teliospores are produced to complete the cycle (Agrios, 1988). In addition, the uredospores may also act as inoculum to spread infection to other plants.

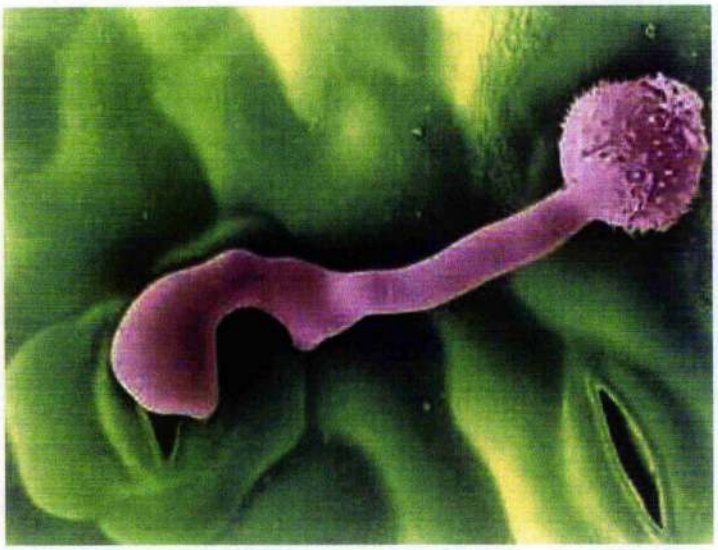


Figure 7. Bean rust fungus invading host leaf through the stomata (from Mendgen, 2002)

Uredeospores of the rust fungus must imbibe water to germinate (Staples and Macko, 1984). Webb and Nutter (1997) found that rust infection increased in alfalfa inoculated with rust as the length of time that the leaf surface remained wet was increased from 4 to 24 h. Basidiospore germ-tubes may directly penetrate the leaf surface stimulated by contact with the leaf surface (Wynn and Staples, 1981). However, appressoria produced by uredospores of the bean rust fungus penetrate the leaf through stomata. Therefore, following germination of the uredospore, the germ-tube grows across the leaf surface in response to leaf topography to locate the stomata (Littlefield and Heath, 1979; Wynn and Staples, 1981). This orientation of the germ-tube to locate stomata occurs in many

rusts such as the uredospore germ-tubes of the wheat rust fungus, which orientates itself by growing at right angles to veins in the leaf (Johnson, 1934). It is possible that the directional growth of the germ-tube is in response to a lattice of wax crystals that covers the cuticle of wheat leaves (Lewis and Day, 1972). Furthermore, the production of appressoria in some rust species may also be dependent on the topography of the leaf surface. Allen *et al.* (1991) found that optimal development of appressoria in nine rust species was initiated on ridges 0.4-0.8 μm high, whereas appressoria formation was inhibited on ridge heights above and below this range. After penetration through the leaf stomata the fungus colonises the mesophyll tissues both inter and intracellularly. The intercellular hyphae include haustorial mother cells and haustoria for the absorption of host nutrients (Silva *et al.*, 1999; Mendgen and Hahn, 2002).

HR is often associated with wheat resistance to rust (Tiburzy *et al.*, 1991). Similarly, Heath (1998) observed a HR in cowpea resistant to cowpea rust. However, her results suggested that in this particular interaction HR is not triggered by an oxidative burst. Sillero and Rubiales (2002) found that difference in resistance of nine faba bean lines was mainly due to inhibition of the formation of haustoria, reduced number of haustoria and smaller colonies. Furthermore, necrosis was also apparent in some host cells associated with hyphal infection. Similarly, acquired resistance in broad bean plants treated with salicylic acid (SA) or 2, 6-dichloroisonicotinic acid (INA) resulted in inhibition of infection hyphae and haustorial mother cells (Rauscher *et al.*, 1999).

1.11 Time-Course of Resistance

Local induced resistance, in the area surrounding the site of infection, usually develops within 2-3 days of primary inoculation (Agrios, 1988). The time-course for SAR in dicots and monocots is very similar (Kessmann *et al.*, 1994). Ward *et al.* (1991b) looked at gene expression in the primary inoculated leaves and uninfected systemic leaves of tobacco in which resistance was induced with TMV. In the primary TMV-inoculated leaves, maximum gene expression associated with SAR occurred 6 days after inoculation, although this varied to some degree depending on the gene. In the uninfected systemic tissue, high levels of gene expression of the genes associated with the onset of SAR also occurred 6 days after inoculation.

The length of time that protection is afforded once SAR has been induced varies. The chemical activator of SAR, BTH (benzo [1,2,3] thiadiazole-7-carbothioic acid-S-methyl ester), was found to provide protection against *Erysiphe graminis* (powdery mildew) in wheat for up to 10 weeks (Ruess *et al.*, 1996), whereas in cucumber inoculated with *Colletotrichum lagenarium*, protection against a broad spectrum of diseases lasted for 4-6 weeks. A further inoculation with *C. lagenarium*, 2-3 weeks after the initial inoculation, extended protection for the whole season (Kué and Richmond, 1977). It is not possible to elicit a SAR response once the cucumber plants are fruiting (Guedes *et al.*, 1980). However, plants inoculated at the two leaf stage, and given a booster inoculation 3-4 weeks later, maintained their resistance throughout the fruiting period (Kué and Richmond, 1977). Guedes *et al.* (1980) suggest that, either the chemical signal is not produced in fruiting plants, or the cells are unable to respond to the signal, which may be due to hormonal changes at the time of fruiting.

1.12 The Effect of Inoculation Position on the Induction of SAR

Tobacco leaves inoculated with *Thielaviopsis basicola* resulted in systemic resistance to TMV and tobacco necrosis virus. However, SAR could not be induced when the roots were inoculated (Hecht and Bateman, 1964). Guedes *et al.* (1980) found that infecting the second true leaf of cucumber with *Colletotrichum lagenarium* provided disease resistance in both the first and the third true leaf. However, the level of protection provided in the third leaf was higher than in the first leaf. When the stem was girdled above the inoculated leaf, this had no effect on the level of resistance in the leaf below the inoculated leaf. Inoculating leaf five provided protection in leaves 1-4 and 6-10, and the protection provided was highest in leaves 9 and 10. This increase in resistance towards the plant apex may be an indication of increasing sink strength at the growing point. Alternatively, this may be due to greater disease susceptibility in fully expanded leaves (Guedes *et al.*, 1980).

1.13 Pathogen Induced SAR

A number of examples of pathogen induced SAR have been characterised. Host plants have included cucumber, melon, bean, tobacco, *Arabidopsis*, red clover, soybean, potato, tomato, pearl millet and alfalfa. SAR has also been studied in monocots such as rice, barley and wheat. It is possible to induce SAR with pathogen strains that are not host specific. For example, bean inoculated with the cucumber pathogen *Colletotrichum lagenarium* became resistant to anthracnose (Kessmann *et al.*, 1994). Cucumbers inoculated with tobacco necrosis virus, *Pseudomonas lachrymans*, or *Colletotrichum lagenarium*, resulted in systemic resistance against at least 13 bacterial, fungal and viral diseases (Kessmann *et al.*, 1994).

1.14 The Effects of Wounding on the Induction of SAR

SAR is not thought to be a general stress response, as it is not induced by either wounding or phytohormones in dicots (Uknes *et al.*, 1993). In studies of the effects of girdling on signal transduction in SAR, the girdling did not induce a resistance response (Guedes *et al.*, 1980).

1.15 Systemic Protection Induced by Chemicals

Various chemicals and extracts have been considered for their potential to induce disease resistance. These include phosphate, unsaturated fatty acids, yeast extracts, polyacrylic acid, oxalate, 2-thiouracil etc. However, they do not necessarily fulfil the criteria for inducing SAR as proposed by Kessmann *et al.* (1994). Kessmann *et al.* (1994) determined that the chemical must induce resistance to the same range of pathogens and elicit the same biochemical markers (such as marker genes) as those produced biologically. Moreover, neither the chemical, nor its significant metabolites should have a direct antimicrobial effect on the pathogen (Ryals *et al.*, 1996).

1.15.1 Salicylic acid

Exogenous applications of SA are known to induce a defence response in plants. Moreover, neither SA nor its metabolites have significant antimicrobial properties. Consequently, SA fulfils the criteria established by Kessmann *et al.*, (1994) for a SAR activator. However, exogenous applications of SA are rapidly metabolised, and the resultant metabolites, mainly β-glucosides, lack phloem mobility (Enyedi and Raskin, 1993). Hence, exogenously applied SA may only provide limited protection to treated

tissue. A further problem exists with the practical use of SA for disease control; there is only a marginal difference between the concentration at which SA is efficacious in inducing SAR, and that at which it is extremely phytotoxic (Kessmann *et al.*, 1994).

1.15.2 INA (2,6-dichloroisonicotinic acid)

Known as INA, 2,6-dichloroisonicotinic acid, and its methylester, were developed at the laboratories of Ciba-Geigy (now Sygenta), as compounds to induce systemic resistance (Métraux *et al.*, 1991; Staub, Ahl-Goy and Kessmann, 1992; Kessmann *et al.*, 1993). INA provides resistance against the same spectrum of diseases as that provided by biological SAR (Staub *et al.*, 1992). Consequently, the same genes that are switched on by biological SAR in tobacco, cucumber, and *Arabidopsis,* are also switched on by INA (Métraux *et al.*, 1991; Ward *et al.*, 1991b; Uknes *et al.*, 1992). Additionally, INA and its metabolites do not appear to have significant antimicrobial activity (Métraux *et al.*, 1991). In tobacco, INA was found to stimulate the activity of β-1,3-glucanases, chitinase and 6-phosphogluconate-dehydrogenase (6-PGD). However, phenylalanine ammonia-lyase, acidic protease, peroxidases, and polyphenoloxidases, were not upregulated (Staub *et al.*, 1992). Kessmann *et al.* (1994) suggest that this is due to the fact that these enzymes are consistent with those expressed in local defence response, such as the hypersensitive response, and hence, that INA is stimulating SAR responses without affecting local necrotic responses.

1.15.3 Bion [BTII] (CGA 245704)

Benzo [1,2,3] thiadiazole-7-carbothioic acid-S-methyl ester [BTH] was developed at Ciba Geigy as the product Bion®50 WG in Germany and Unix Bion® 63 WG in Switzerland. BTH protects cereals, rice, tobacco, banana, and some vegetable crops, against fungal and bacterial pathogens (Ruess *et al.*, 1995). It has no direct antimicrobial affect and induces the same gene markers as biological SAR (Métraux *et al.*, 1991; Ward *et al.*, 1991b; Uknes *et al.*, 1992). BTH fails to induce SAR in plants with a dysfunctional SAR signalling pathway. This suggests that BTH uses the same signalling pathway as biological SAR (Lawton *et al.*, 1996).

Both BTH and INA are thought to act downstream of SA, in the SAR signalling pathway and, therefore, work independently of SA. Consequently, SA is not increased in plants treated with BTH or INA. Additionally, BTH and INA induce SAR even when applied to *NahG* transformed plants (transformed to express the *nahG* gene which codes for salicylate hydroxylase, the enzyme that converts SA to catechol) (Vernooij *et al.*, 1995; Friedrich *et al.*, 1996). SA, BTH and INA are unable to induce SAR gene expression in *nim1* (non-indicible immunity) mutant *Arabidopsis*. It is therefore possible that all three use the same signalling pathway to induce SAR (Delaney *et al.*, 1995; Lawton *et al.*, 1996). It has been suggested that all three compounds may bind to the same receptor, given the structural similarities between the three (Görlach *et al.*, 1996).

1.15.4 Phosphate

Phosphate has been found to induce systemic resistance to *Colletotrichum lagenarium* in cucumber, and to rust in bean (Gottstein and Kuć, 1989; Walters and Murray, 1992). EDTA, a calcium chelator, similarly induced systemic resistance to rust in bean. However, the subsequent addition of calcium to phosphate or EDTA treated plants inhibited the induction of systemic resistance (Walters and Murray, 1992). Consequently, it may be that phosphates sequester calcium in lower leaves, affecting membrane integrity and effecting the synthesis or release of hydrolytic enzymes. The enzymes may in turn cause the release of pectic fragments from cell walls, which may lead to the production of a signal, or in fact be the signal for instigating defence responses (Gottstein and Kuć, 1989). Doubrava *et al.* (1988) have suggested a similar function for oxalates in the induction of systemic resistance. However, Kessmann *et al.* (1994) suggest that phosphate may not fulfil the criteria of a SAR activator.

1.15.5 Saccharin

Cultured parsley cells have been used as a system to identify substances with the potential to activate SAR. Treatment of the cells with known SAR activators such as INA and BTH sensitise the cells so as to substantially increase their response to an elicitor (Kauss *et al.*, 1992, 1993; Kauss and Jeblick, 1995; Katz *et al.*, 1998). Siegrist *et al.* (1998) looked at the effects of different chemicals on the accumulation of coumarin, in parsley cells, compared with the levels produced by the SAR activator BTH. Results indicated the artificial sweetener saccharin as a possible SAR activator. Cucumber, tobacco and French bean plants challenged 8 d after applying a saccharin drench exhibited reductions of 79.1% in *Colletotrichum lagenarium*, 63.7% in tobacco

mosaic virus, and 73% reduction in *Uromyces appendiculatus*. Saccharin was also found to be effective in protecting against infection by *Uromyces appendiculatus*. The degree of protection in cucumber and bean was comparable with that produced by BTH, but less effective than BTH in tobacco.

1.16 Physiological Basis of SAR

The SAR response utilises a distinct signalling pathway and necrotic lesions are often produced prior to activation of this pathway (Ryals *et al.*, 1996). When *Arabidopsis* was inoculated with *Peronospora parasitica* following treatment with the SAR activator 2,6-dichloroisonicotinic acid (INA), the result was single cell necrosis at the site of attempted infection (Uknes *et al.*, 1992). The necrotic cells surrounded and prevented the growth of any hyphae that managed to penetrate the leaf. This is comparable to the hypersensitive response that results from the interaction of incompatible host and pathogen genotypes (Kessmann *et al.*, 1994). The success rate of hyphae penetrating the plant tissue was increased when lower concentrations of INA were used. Pathogen progress was still halted, but at a later stage of infection. This manner of inhibition is similar in other biological and chemically induced SAR interactions (Kovats *et al.*, 1991; Madamanchi and Kuć, 1991).

Fungal penetration and formation of primary and secondary haustoria of *Erysiphe graminis* f. sp. *tritici* was also inhibited when the SAR activator BTH was applied to wheat. However, spore germination and appressorium formation were unaffected (Görlach *et al.*, 1996). In tobacco, spore germination and appressorium formation by *Peronospora hyoscyami* f. sp. *tabacina* was unaffected by BTH. Here, epidermal

penetration and vesicle formation was reduced by approximately 50% compared with untreated plants (Ruess *et al.*, 1996). Similarly, SAR had no effect on the subsequent germination or appressorium formation of *Colletotrichum lagenarium* in cucumber. However, due to the formation of papilla-type material beneath the appressoria, penetration in systemically induced leaves was greatly reduced (Madamanchi and Kuć, 1991). This papilla type material contains lignin and silicon (Stein *et al.*, 1993). Lignification has been found to be rapid in SAR induced plants and is accompanied by an increase in peroxidase activity (Dean and Kuc, 1988; Smith and Hammerschmidt, 1988).

1.17 Biochemical Basis of SAR

The effect of SAR on phytoalexin and lipoxygenase accumulation in plants varies. When tobacco was inoculated with TMV this resulted in an increase in lipoxygenase in uninoculated tissue (Staub *et al.*, 1992). However, when SAR was induced by *Pseudomonas syringae* in rice, no new proteins were detected (Smith and Metraux, 1991). Contrary to this, Hofmann and Babuin (1993) found that lipoxygenase was induced in both inoculated and uninoculated tissue, in interactions between tobacco and *P. syringae* pv. syringae. In rice, inoculated with *Pyricularia oryzae*, there was found to be an accumulation of hydroxylated fatty acids followed by phytoalexin accumulation (monilactone class). A lipoxygenase inhibitor prevented the accumulation of both the fatty acids and the phytoalexins. This may suggest that the fatty acids are a precursor of the phytoalexins as well being antimicrobial in their own right (Wen Xin *et al.*, 1991). It has been suggested that the same resistance mechanism occurs with SAR against rice blast disease (Namai *et al.*, 1993). Certainly, Seguchi *et al.* (1992) also found that SAR

induced in rice following treatment with either the SAR activator N-cyanomethyl-2-chloroisonicotinamide (an isonicotinic acid derivative), or rice blast, resulted in increased activation of lipoxygenase(s), general lipid metabolism and peroxidase(s) activity. Also, phytoalexins have been found to accumulate as a localised response (at the inoculation site) in SAR induced green bean (Elliston *et al.*, 1977). However, there was no change in the levels of phytoalexins in SAR induced tobacco (Stolle *et al.*, 1988).

It is likely, therefore, that chemical changes in the plant in response to SAR, are dependent on the specific plant pathogen interaction, and consequently the defence mechanisms employed. For example, superoxide dismutase and peroxidase are induced systemically in potato inoculated with *Phytophthora infestans*. Consequently, generation of O_2 may be important to SAR defence responses in this species (Chai and Doke, 1987). When SAR was induced in tobacco with *Peronospora tabacina*, there was an accumulation of glucose, fructose and derivatives of β-ionone in the systemic tissue (Salt *et al.*, 1986; Wyatt and Kuć, 1992). β-ionone, when injected into tobacco stems, provided protection against blue mould (Salt *et al.*, 1986).

1.18 Molecular Basis of Disease Resistance

An accumulation of a specific set of proteins was identified in virus infected tobacco (Gianinazzi *et al.*, 1970; Van Loon, and Van Kammen, 1970). These were called pathogenesis-related proteins (PR proteins) and accumulation of the acidic-extracellular form of the PR proteins was found to coincide with the onset of resistance (Van Loon and Antoniw, 1982).

1.18.1 Gene expression associated with defence responses in *Arabidopsis*

It was not until 2000 that gene expression involved in defence was characterised in *Arabidopsis thaliana* using microarray analysis. Schenk *et al.* (2000) identified changes in the degree of expression of 705 mRNAs following inoculation with *Alternaria brassicicola* (an incompatible pathogen), treatment with SA, methyl jasmonate (MJ), or ethylene. It was found that 126 genes were induced by more than one of the treatments. This suggests a high degree of interaction between the different defence signalling pathways. The two treatments that had the most similarity in gene expression were salicylic acid and methyl jasmonate. This is perhaps surprising, as the salicylate and jasmonate pathways have been thought to be to some degree antagonistic. However, the antagonism may be gene specific, since eight genes that were significantly induced by SA, were significantly repressed by MJ (Schenk *et al.*, 2000). Genes already associated with plant defence accounted for approximately 10% of the changes in gene expression. A further 35% of the changes were attributable to genes responsible for cell maintenance and development, and more than 7% for cell wall synthesis and modification. In excess of 25% of the genes may be implicated in signal recognition and transduction. There were found to be significant changes in the genes involved in the oxidative burst, hypersensitive response, programmed cell death and the production of antioxidant enzymes (Schenk *et al.*, 2000).

Gene family	Product function	References
PR-1 (PR-1a, PR-1b, and PR1-c)	Acidic, extracelular; function unknown; most abundant PR protein in tobacco; >90% identical to PR-1b and PR-1c	Payne *et al.*, (1988b)
PR-2 (PR-2a, PR-2b and PR-2c)	Acidic, extracelular β-1,3-glucanase; >90% identical to PR-N and PR-O	Kauffmann *et al.*, (1987); Ward *et al.*, (1991a)
PR-3 (PR-3a, and PR-3b)	Acidic, extracelular chitinase; also known as PR-Q; >90% identical to PR-P	Legrand *et al.*, (1987) Payne *et al.*, (1990a)
PR-4 (PR-4a and PR-4b)	Acidic, extracelular; unknown function; homologous to C-terminal domain of Win1 and Win2 of potato	Friedrich *et al.*, (1991)
PR-5 (PR-5a and PR-5b)	Acidic, extracellular; homologous to thaumatin and bifunctional amylase / proteinase inhibitor of maize; also known as PR-R or PR-S	Richardson *et al.*, (1987); Payne *et al.*, (1988a)
PR-1 basic	Basic isoform of acidic PR-1	Payne *et al.*, (1989)
Basic class III chitinase	Homologous to cucumber chitinase (Métraux *et al.*, 1989); structurally unrelated to PR-3	Lawton *et al.*, (1994)
Acidic class III chitinase	Extracellular; approximately 60% identical to basic isoform	Lawton *et al.*, (1994)
PR-Q'	Acidic extracelular β-1,3-glucanase; approximately 55% identical to PR-2 group	Payne *et al.*, (1990b)

Table 1. SAR Genes in Tobacco (from Ward *et al.*, 1991b)

1.19 Molecular Basis of SAR

Not all defence related genes are expressed during SAR (Ryals *et al.*, 1996). Ward *et al.* (1991b) used cDNA clones to make probes from 13 gene families that were potentially induced at the onset of SAR. SAR was induced in tobacco plants by inoculation with TMV. In the primary TMV inoculated leaves, all the genes were detected, except acidic peroxidase. However, in the secondary uninfected leaves there

was a progressive accumulation of mRNA from nine of the gene families. The accumulation of the mRNA was concomitant with the expression of SAR. Both the basic forms of β-1,3-glucanase and chitinase mRNA failed to accumulate, and the acidic peroxidase mRNA was not induced in the systemically protected tissue. The mRNA for SAR 8.2 was present in the uninfected controls. Therefore, although SAR 8.2 was induced earlier than the other genes in the systemic tissue of infected plants, it was induced to a lesser extent (Ward *et al.*, 1991b).

In tobacco, exogenous applications of SA or INA (both activators of SAR) induced the same nine gene families. Again, there was a high accumulation of mRNA for these genes accompanying the development of resistance. The lowest expression of these genes, induced with SA, occurred at the end of the photoperiod (Ward *et al.*, 1991b). Certainly, it has been found that light levels affect PR protein accumulation (Asselin *et al.*, 1985). TMV, SA and INA all induced the same nine gene families, but the levels of accumulation varied. Further studies found a positive correlation between the degree of mRNA accumulation and the level of resistance achieved. These nine gene families have come to be known as the SAR genes, several of which code for antimicrobial products (Table 1-Ward *et al.*, 1991b).

Studies made of the different SAR genes appear to substantiate the claim that they code for disease resistance products. For instance, tobacco and brassica seedlings transformed with a chitinase gene from bean, provided resistance to damping off caused by *Rhizoctonia* sp. (Broglie *et al.*, 1991). Similarly, tobacco transformed with acidic and basic chitinases from tobacco or cucumber increased resistance to *Rhizoctonia* (Uknes *et al.*, 1996). Tobacco over expressing SAR 8.2 had increased resistance to *P.*

parasitica and expression of tobacco PR-1a increased resistance to *Peronospora tabacina* and *Phytophthora parasitica* (Alexander *et al.*, 1993a,b). Antimicrobial activity *in-vitro* has also been identified for PR proteins (Schlumbaum *et al.*, 1986; Mauch *et al.*, 1988; Roberts and Selitrennikoff, 1988; Richardson, 1991; Sandoz, 1991; Wooloshuk *et al.*, 1991; Ponstein *et al.*, 1994).

Resistance that occurs with SAR may be the result of individual or a combined anti-microbial activity of SAR genes. It has been suggested that combinations of SAR genes may have a synergistic rather than an additive effect (Zhu *et al.*, 1994; Uknes *et al.*, 1996). Tobacco transformed with PR-1 (basic), Class III chitinase (basic) and PR-5 (acidic) provided resistance against *P. syringae,* whereas PR-Q', PR-3 and PR-5 (acidic) gave protection against *C. nicotiana* (Uknes *et al.*, 1996). Uknes *et al.* (1992) identified the proteins found in the intercellular wash fluid from SAR induced by INA in *Arabidopsis*. Four new proteins were identified and sequenced and were found to be similar to tobacco PR-1, PR-5, PR-2, and the fourth was thought to be a breakdown product of PR-2.

In the same way that some defence mechanisms are specific to particular host pathogen interactions, the SAR genes that are induced and the degree of expression may be similarly species specific. In tobacco and *Arabidopsis*, there is greater expression of the PR-1 gene, whereas a class III chitinase is more highly expressed in cucumber (Métraux *et al.*, 1988; Ward *et al.*, 1991b; Uknes *et al.*, 1992). Alexander *et al.* (1993) suggest that PR-1 may be particularly effective against oomycetes, whereas tobacco transformed to over-express PR-1 and PR-5, did not have increased resistance to TMV (Cutt *et al.*, 1989; Linthorst *et al.*, 1989). This specificity between SAR genes and the host-

pathogen interaction was also apparent when the over-expression of tobacco osmotin partially inhibited growth of *P. infestans* in potato, but not in tobacco (Liu *et al.*, 1994).

1.20 The Signalling Events that Occur with SAR

When SAR is induced, it has been suggested that a signalling molecule moves through the phloem from the site of infection to distal parts of the plant, to induce SAR. (Kessmann *et al.*, 1994). This was supported by the fact that plants failed to develop SAR when the petiole of the inoculated leaf had been previously girdled. Similarly, resistance failed to develop in leaves that were girdled prior to plant infection (Guedes *et al.*, 1980; Dean and Kuć, 1986). Moreover, SAR developed in a healthy scion grafted onto a pathogen-inoculated rootstock (Jens and Kuć, 1979).

1.20.1 The long distance signalling molecule in SAR

There has been considerable speculation as to the identity of the signalling molecule involved in SAR. Salicylic acid (SA) was considered a prime candidate and, indeed, was found to accumulate in tobacco inoculated with TMV, and was also found in cucumber phloem at the onset of SAR induced by TNV or *Colletotrichum lagenarium* (Malamy *et al.*, 1990; Metraux *et al.*, 1990; Leon *et al.*, 1993). When SA was applied to tobacco, this resulted in resistance to TMV and the accumulation of the PR-proteins (White, 1979). Further studies found that exogenous applications of SA induced the same PR-proteins as were induced by biological SAR (Ward *et al.*, 1991b; Uknes *et al.*, 1992, 1993;). A correlation was identified between the amount of SA and PR-protein induction (Yalpani *et al.*, 1991; Klessig and Malamy, 1993).

To clarify the necessity for SA in SAR, tobacco plants were transformed to express the *nahG* gene, which codes for salicylate hydroxylase, the enzyme that converts SA to catechol. When subsequently inoculated with a pathogen there was no accumulation of SA, no induction of SAR genes in systemic tissue and systemic resistance did not occur (Gaffney *et al.*, 1993). This led to the supposition that SA may be the endogenous signal that initiates the resistance response (Kessmann *et al.*, 1994).

It was necessary to ascertain whether SA was the translocated signalling molecule. Certainly, significant levels of SA were found in the phloem sap of tobacco and cucumber plants following pathogen infection (Métraux *et al.*, 1990; Yalpani *et al.*, 1991). Up to 70% in tobacco, and 50% in cucumber, of the increase in SA in uninfected tissue, following pathogen inoculation, was found to originate from the infected leaves (Shulaev *et al.*, 1995; Mölders *et al.*, 1996). In contrast, the findings of other studies suggest that SA is not the translocated signal. For example, in cucumber inoculated with *P. syringae,* the induced leaf was removed shortly after inoculation and prior to significant accumulation of SA. This had no effect on SA accumulation or the expression of an acidic peroxidase gene in distal tissue. The acidic peroxidase gene is considered to be the SAR marker for this species (Rasmussen *et al.*, 1991).

Further evidence against SA being the long-distance signal was obtained in grafting experiments. Rootstocks transformed to express salicylate hydroxylase failed to inhibit SAR in the grafted scion. However, when the scion was transformed to express salicylate hydroxylase, SAR could not be induced in the scion, irrespective of whether the rootstock was transformed or not. From this, it has been concluded that SA is not the primary long-distance signal. Moreover, the production of this signal is not

dependent on SA accumulation. However, SA is a prerequisite for the transduction of the signal into gene expression, necessary for the SAR response (Vernooij *et al.*, 1994). The identity of the long-distance signal is still to be discovered.

1.21 Salicylic Acid Synthesis

Although SA is not the translocated signalling molecule involved in SAR, SA still has an important role in both disease resistance and SAR. The biosynthesis of SA may perhaps be considered to start when phenylalanine is metabolised by phenyalanine ammonia-lyase (PAL) to produce *trans*-cinnamic acid (*t*-CA) (Ryals *et al.*, 1996). Although the mechanism by which benzoic acid (BA) is synthesised from *t*-CA is unclear, Ryals *et al.* (1996) suggest two possibilities (Figure 8). The first is that BA is a result of β oxidation of *t*-CA. Certainly, in cell free extracts of *Quercas pedunculata*, acetyl-CoA and ATP act upon *t*-CA to produce SA (Alibert and Ranjeva, 1971). The second possibility is a nonoxidative method. In suspension cultures of *Daucus carota* and *Lithospermum erythrorhizon*, *p*-hydroxybenzaldehyde is synthesised from *p*-coumarate (similar to *t*-CA, only with the addition of a 4-hydroxyl group) from which *p*-hydroxybenzoic acid is produced. This does not require acetyl-CoA (Yazaki *et al.*, 1991; Schnitzler *et al.*, 1992). As a result of pathogen infection, the enzyme 2-hydroxylase that converts BA to SA has been found to increase several-fold (Léon *et al.*, 1993).

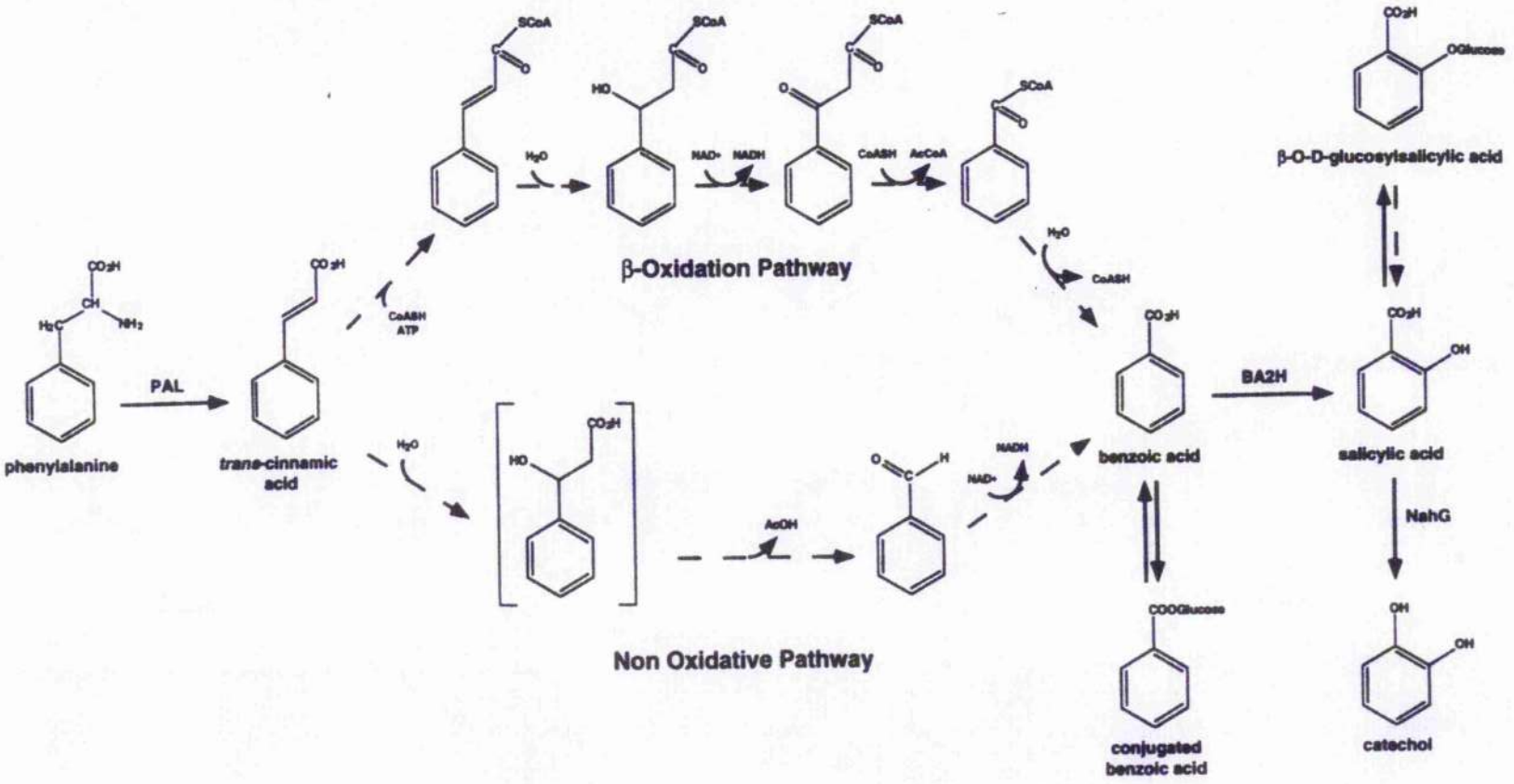


Figure 8. Biosynthetic pathways of SA proposed by Ryals *et al.* (1996)

Solid arrows indicate established biochemical reactions, whereas broken arrows indicate possible steps not yet described (see Yazaki *et al.*, 1991)

Both SA and BA may be conjugated to glucose, which is potentially relevant to the regulation of SA concentration. In tobacco following pathogen infection, there is a transient decrease in the store of conjugated BA resulting in an increase in free BA and therefore SA (Yalpani *et al.*, 1993a). Alternatively, SA may be stored as β-*O*-D-glucosylsalicylic acid (SAG), to be converted back to free SA as required. SAG itself has no affect on disease resistance (León *et al.*, 1993a).

1.22 Role of SA in the Induction of SAR

It has been suggested that hydrogen peroxide (H_2O_2) may be involved as a second messenger for SA to induce SAR. Certainly, a SA binding protein, identified in tobacco, is a catalase. Therefore, it was suggested that SA could, by binding to the catalase, prevent the breakdown of H_2O_2 and hence induce SAR. Furthermore, H_2O_2 has been found to induce expression of the SAR gene PR-1 (Chen *et al.*, 1993, 1995). However, in tobacco inoculated with TMV, there was no concomitant increase in H_2O_2 to coincide with the induction of SAR. Also, concentrations of 1M H_2O_2 required to induce PR-1 mRNA in tobacco, was also sufficient to cause severe damage to the plant. Concentrations of 1 M H_2O_2 did not significantly induce expression of PR-1 in *nahG* transformed plants (transformed to express the *nahG* gene which codes for salicylate hydroxylase, the enzyme that converts SA to catechol). This suggests that SA is required for H_2O_2 induction of PR-1 (Neuenschwander *et al.*, 1995b). It is possible that, rather than acting as a second messenger of SA in the induction of SAR, H_2O_2 may moderate SA production. In both tobacco and *Arabidopsis*, high concentrations of H_2O_2 were found to induce SA synthesis (León *et al.*, 1995; Neuenschwander *et al.*, 1995a).

It is possible that benzoic acid-2-hydroxylase (BA2-H) which catalyses the synthesis of SA is induced by H_2O_2 (Hammond-Kossack and Jones, 1996).

The inhibition of catalase by SA may have limited relevance to SAR. Instead, it may have a primary role in localised pathogen defence responses. *In vitro* activity of enzymes containing heme-iron, such as catalase and ascorbate peroxidase, is inhibited by high concentrations of SA (Chen *et al.*, 1993; Durner and Klessig, 1995). This is in accordance with the suggestion that SA is an iron chelator (Rueffer *et al.*, 1995). The concentration of SA required to bind either catalase or ascorbate peroxidase is similar to that which occurs adjacent to lesions produced in pathogen infected leaves. However, concentrations of SA found in uninoculated leaves of SAR induced plants, are between 10-100 times lower than that required for enzyme inhibition (Enyedi *et al.*, 1992; Neuenschwander, *et al.*, 1995b).

At the site of infection, high concentrations of SA may inhibit catalase activity, extending the life of H_2O_2. This would intensify the oxidative burst, which may instigate localised defences such as the hypersensitive response including programmed cell death, activation of defence genes and SA synthesis in adjacent cells (Mehdy, 1994; Hammond-Kossack and Jones, 1996; Levine *et al.*, 1996). This increase in SA could then result in an escalation of localised defence responses. Perhaps, not surprisingly, in parsley cell cultures and cucumber cotyledons, priming with SA followed by treatment with an elicitor resulted in a higher concentration of H_2O_2 and increased resistance in cucumber to *Colletotrichum lagenarium*. However, the increase in H_2O_2 was not the result of reduced degradation, but increased H_2O_2 synthesis. Moreover, priming with SA was also found to be dependent on protein synthesis. (Kauss and Jeblick, 1995;

Fauth *et al.*, 1996). Consequently, it may be that SA has more than one mode of action in pathogen defence responses. In uninfected tissue, SA may bind to a receptor which results in the moderation of SAR gene expression (Ryals *et al.*, 1996).

1.23 Gene for Gene Resistance and SAR

Physiological responses which occur with SAR include localised necrosis at the site of infection, increased deposition of papilla-like material, and increased auto-fluorescence. These responses are similar to those that occur when resistance is conferred by a single dominant resistance gene (Dean and Kuc, 1988; Uknes *et al.*, 1992; Stein *et al.*, 1993; Dietrich *et al.*, 1994).

To further examine the role of salicylic acid (SA) and SAR in general disease resistance, *Arabidopsis* plants were transformed to express the *nahG* gene. The transformed plants became susceptible to avirulent bacteria, or races of *Peronospora parasitica* to which the untransformed plant would normally be resistant. However, when the transformed plants were treated with INA, a chemical known to activate SAR, SAR gene expression and normal disease resistance was restored. Moreover, *nahG* transformed plants displayed more severe disease symptoms than non-transformed plants when inoculated with virulent fungal and bacterial pathogens (Delaney *et al.*, 1994). Further evidence as to the requirement of SA was obtained when *Arabidopsis* plants were treated with 2-aminoindan-2-phosphonic acid (AIP). AIP inhibits phenyalanine ammonia-lyase (PAL) activity and, therefore, prevents phenylpropanoid metabolism and potentially, SA synthesis. It was then possible to infect the treated plants with a previously incompatible isolate of *P. parasitica* and reverse the effect with

exogenous applications of SA (Mauch-Mani and Slusarenko, 1996). Activation of SAR in a susceptible host-pathogen interaction may make the plant resistant to that pathogen. However, inhibition of the SAR pathway can make a normally incompatible plant pathogen interaction susceptible (Delaney *et al.*, 1994; Mauch-Mani and Slusarenko, 1996).

These results suggest that SAR may be necessary for some dominant resistance genes to induce resistance, and SA, or SAR gene expression may be important to general disease resistance in plants (Uknes *et al.*, 1996). Uknes *et al.* (1996) speculate that virulent pathogens may have the ability to inhibit the SAR response, possibly by affecting SA or the systemic signal. It is possible that some dominant resistance genes may, by utilising SA, induce a rapid SAR gene response. Alternatively, the resistance gene may be important in recognition, which subsequently allows SAR to prime the signalling pathway and culminates in resistance.

1.24 Costs of Induced Defence Responses

Plant defences consist of those that are constitutively expressed and others that are induced in response to pathogen attack (Christensen, 1997; Stieher *et al.*, 1997). Heil *et al.* (2000) ask why induced resistance is not constitutive and suggest that fitness costs to the plant may be too great (Bazzaz *et al.*, 1987; Simms and Rausher, 1987; Marquis, 1991; Heil *et al.*, 2000). Heil uses the term fitness costs as the ability to reproduce, which may be estimated indirectly by assessing plant growth (Coley, 1986; Skogsmyr and Fagerström, 1992; Sagers and Coley, 1995; Steinberg, 1995). The cost to the plant

of induced resistance is thought to be the result of competition for limited resources (Herms and Mattson, 1992).

Smedegaard-Peterson and Tolstrup (1985) found that highly resistant barley plants subjected to continuous inoculation with powdery mildew resulted in significant reductions in yield. In resistant barley plants increased respiration has generally been found to be higher and produced earlier following inoculation with powdery mildew, compared to susceptible plants (Millerd and Scott, 1956; Paulech, 1967; Smedegaard-Peterson, 1980; Smedegaard-Peterson, and Stølen, 1981) although there are exceptions (Scott and Smillie, 1966). Smedegaard-Peterson and Stølen, (1981) propose that the increase in respiration and resultant reduction in both grain yield and quality is necessary to provide the energy required for defence responses.

However, although there is a paucity of work on the effects of induced resistance on plant growth, available data appears contradictory. Reglinski *et al.* (1994) found that yeast derived elicitors produced up to 95% reduction in powdery mildew infection in barley. Although this was not as high as that achieved by fungicides, in some instances yields were comparable with that obtained by the use of fungicides (Reglinski *et al.*, 1994). Dehne *et al.* (1984), Steiner *et al.* (1988), Oerke *et al.* (1989) and Kehlenbeck *et al.* (1994) all observed an increase in yield associated with induced resistance in barley. It has been suggested that this may be the result of increased efficiency in incorporating assimilates into kernels (Steiner and Schönbeck, 1995). However, contrary to this Heil *et al.* (2000) found that the SAR inducer BION had a detrimental effect on wheat growth and yield. Similarly, *Arabidopsis* mutants lacking the gene *MPK4* exhibit constitutive SAR as SA levels remain constantly elevated, but were also found to be

severely stunted (Petersen *et al.,* 2000; Day, 2001). Furthermore, the mode of induction may also affect plant growth. When SAR was induced by injecting a spore suspension of the pathogen *Peronospora tabacina* through the cambium into the stems of tobacco plants, this resulted in severe stunting and premature senescence (Cohen and Kuć, 1981). SAR was still induced when the spore suspension was injected outwith the cambium. However, there was also an increase in plant weight and number of leaves (Tuzun and Kuć, 1985; Tuzun *et al.*, 1986).

1.24.1 Potential antagonism between two defence signalling pathways

Concern has been expressed recently regarding the possible antagonism that may exist between the two signalling pathways involved in plant resistance to pathogens and insect herbivores. Resistance to pathogens involves the salicylate pathway and insect resistance the octadecanoid pathway, which produces jasmonic acid (Felton *et al.*, 1999; Thaler *et al.*, 1999; Hatcher and Paul, 2000). Thaler *et al.* (1999) found that it was possible to separately induce resistance to *Pseudomonas syringae* (bacterial speck pathogen) and *Spodoptera exigua* (noctuid caterpillar) in tomato. However, when the inducers were applied simultaneously both resistance responses were compromised. It is possible that the conflict between the two resistance pathways may in fact be the result of resource limitation or cross-talk preventing both pathways from optimum induction simultaneously (Heil *et al.*, 2000).

1.25 Photosynthesis in Plants Infected by Biotrophic Fungal Pathogens

Plants infected by biotrophic fungi may initially exhibit a transient increase in the rate of net photosynthesis. However, most plant pathogen interactions result in a progressive decline in both the rate of net photosynthesis and chlorophyll content (Ahmad *et al.*, 1983; Scholes and Farrar, 1986; Tang *et al.*, 1996). So and Thrower (1976) found that the rate of decline in photosynthesis is not substantial in *Vigna sesquipedalis* infected with rust (*Uromyces appendiculatus*), until sporulation occurs, when the rate of photosynthesis declines dramatically. The initial increase in net photosynthesis observed in some interactions has been attributed to a reduction in photorespiration, or increased stomatal opening (Ayres, 1977). However, Berghaus and Reisener (1985) found the transient increase to be cultivar specific, and could find no correlation with stomatal resistance.

Photosynthesis is inhibited, and chlorophyll predominantly lost within rust pustules of *Arabidopsis thaliana* (Tang *et al.*, 1996), bluebell leaves (Scholes and Farrar, 1985), and leeks (Roberts and Walters, 1988). In contrast, brown rust pustules in barley retain chlorophyll in preference to surrounding unaffected areas, and photosynthesis decreases in the areas between pustules (Scholes and Farrar, 1986).

1.25.1 Photosynthesis in green-islands

In the latter stages of a biotrophic infection, when leaves are senescing, green-islands become apparent (Coghlan and Walters, 1990), and chlorophyll is retained in the infection sites. In rust infected leaves, the rate of photosynthesis within infection sites may be comparable, or higher, than in leaves of healthy plants (Scholes, 1985). However, in powdery mildew infected barley leaves, photosynthesis within green

islands is reduced. Although total chlorophyll within infection sites was unchanged, it is possible that there was an increase in light-harvesting chlorophyll. The chlorophyll *a* : *b* ratio was affected, and chlorophyll *b* increased by 70%. It is interesting to note that the barley leaves had been kept under low intensity light (Coghlan and Walters, 1992). A similar change in the chlorophyll ratio was also found by Hewitt, (1976) in mildewed oak leaves. Additionally, in barley leaves infected with powdery mildew, the quantum yield within green-islands was also reduced by 47%, which is symptomatic of damage to the electron transport chain (Coghlan and Walters, 1992).

1.25.2 Rate of photosynthesis in the healthy tissue of an infected plant

The rate of photosynthesis in a healthy plant increases to compensate for leaf loss (Khan and Sagar, 1969). It is, therefore, possible that healthy leaves may compensate for photosynthetic reduction in infected tissue. Photosynthesis in the healthy leaves of a rust infected bean plant, is higher than in a healthy plant (Livne, 1964). Similar results occur in mildewed barley (Williams and Ayres, 1981; Walters and Ayres, 1983), and rusted leek (Roberts and Walters, 1986). Net photosynthesis in healthy leaves on an infected plant increases due to stimulation of gross photosynthesis and a reduction in photorespiration (Williams and Ayres, 1981). Increased photosynthesis may be stimulated by an increased demand for photosynthates by the pathogen (Williams and Ayres, 1981). However, no examples of net imports of sugars into infected leaves of a monocot have been found (Farrar and Lewis, 1987). When $^{14}CO_2$ was fed to the upper unaffected leaves of broad bean infected with rust, it was translocated to rust infected leaves, young developing leaves, and roots (Murray and Walters, 1992). In addition to that translocated, more of the assimilate remained in the healthy leaves on the infected

plants than in the control. This is of particular relevance because resistance to rust infection in the upper uninfected leaves of the barley together with the young developing leaves increased. However, when the rate of photosynthesis in the upper leaves was reduced to that of the control, resistance also reduced. Consequently, it is possible that the increase in photosynthesis is necessary to fund increased resistance (Murray and Walters, 1992).

In mildew resistant barley, net photosynthesis was unaffected for up to 6 days after infection, but reduced immediately in a susceptible cultivar (Ayres, 1979). However, oat, with minor gene resistance to powdery mildew, exhibited no significant difference in the rate of photosynthesis or respiration between resistant and susceptible genotypes, when infected with the pathogen (Haigh *et al.*, 1991).

1.25.3 Respiration

Biotrophic pathogens increase respiration (Scholes, 1992; Scholes and Rolfe, 1996), mostly within infected areas of the leaf (Scholes and Farrar, 1986). The increase in respiration provides energy and carbon skeletons necessary for biosynthesis (Farrar, 1985). Removal of surface mycelium of powdery mildew from barley has shown the host to be responsible for much of the increase in respiration (Farrar and Rayns, 1987). In powdery mildew resistant barley, respiration increased sharply within 24h following inoculation, peaked after 24 or 48h, and then dropped to normal levels. In comparison, respiration in susceptible cultivars did not start to increase until 3 days after infection (Smedegaard-Petersen, 1980). However, the effect of infection on photorespiration is less certain. In oak (Hewitt and Ayres, 1975), and sugar beet leaves (Gordon and

Duniway, 1982b), infected with powdery mildew, photorespiration decreased. However, in barley infected by brown rust (Owera *et al.*, 1981), and pea infected with powdery mildew (Ayres, 1976), photorespiration increased.

1.25.4 Diffusion of CO_2 into the leaf

Stomatal opening affects CO_2 flux into the leaf, and therefore photosynthesis. Powdery mildew causes a progressive reduction in stomatal opening. Stomatal aperture in mildewed sugar beet leaves declined by 50% (Gordon and Duniway, 1982a). Peas infected with powdery mildew produce the phytoalexin pisatin, which may affect membrane permeability, or electron transport, limiting energy production in chloroplasts. This inhibits the uptake of solutes by guard cells, and hence stomatal opening (Ayres, 1980). There are changes in the chloroplast RNA of barley infected with powdery mildew within 24 h of infection (Simpson *et al.*, 1979), whereas the changes in stomatal behaviour do not occur until about 72 h after infection (Ayres, 1979). Consequently, stomatal resistance is not thought to be an important factor in the reduction of photosynthesis (Gordon and Duniway, 1982a).

1.25.5 Inhibition of photosynthetic electron transfer

Noncyclic photophosphorylation was reduced 20-30% by rust, and 50% by powdery mildew in sugar beet. In contrast, cyclic photophosphorylation was unaffected by powdery mildew, and reduced by 10% or less by rust infection (Montalbini and Buchanan, 1974; Magyarosy *et al.*, 1976). Photosynthesis progressively declines in rust pustules of bluebell leaves. However, fluorescence emitted from chlorophyll *a*

molecules in photosystem II initially increases, then falls below that in the control tissue, once sporulation begins. The increase may result from a reduction of the primary electron acceptor, inactivating the reaction centre, which may be triggered by a compound produced by pathogen or host in response to infection. Infection may also disorientate some of the chlorophyll *a* molecules in the thylakoid membranes. The reduction in fluorescence may be caused by chlorophyll loss (Scholes and Farrar, 1985).

At low light levels, the amount of light reaching the reaction centre chlorophyll will determine the rate of electron transport. If light-harvesting chlorophyll is lost, this reduces the basic rate of photosynthesis. When light is saturating, photosynthesis is limited by the amount of reaction centre chlorophyll. In rust infected bluebell leaves, the rate of photosynthesis is reduced irrespective of light levels, and the ratio of chlorophyll *a* / *b* is reduced, although the concentration of carotenoid is relatively unaffected. Although chlorophyll *a* / *b* and carotenoids may be lost from the light harvesting complex, it is thought likely that chlorophyll *a* is also lost from the antenna matrices of photosystem II, resulting in a decline in non-cyclic photophosphorylation (Scholes and Farrar, 1985). Alternatively, a reduction in cytochromes (chloroplast electron carriers), in infected tissue, may be responsible for inhibiting noncyclic electron transport and photophosphorylation (Goodman *et al.*, 1986).

1.25.6 Altered solutes within cells

The reduction in chlorophyll in rusted leaves corresponds with a reduction in the size of chloroplasts (Whitney *et al.*, 1962). This may be indicative of a change in the level of solutes, and therefore the osmotic status within diseased cells. If the loss of chlorophyll

is due to a loss of structural integrity of membranes, this suggests that other components of the electron transport chain may be affected (Scholes and Farrar, 1985).

1.25.7 Proposed sequence of events involved in the inhibition of photosynthesis in cereal leaves infected with powdery mildew.

Although, various mechanisms have been proposed for the decrease in photosynthesis following pathogen infection, it has been unclear which are primary, and which secondary events. Studies by Scholes (1992), Scholes *et al.* (1994) and Wright *et al.* (1995) have suggested a possible sequence of events and the mechanisms involved in the inhibition of photosynthesis in barley and wheat infected with powdery mildew.

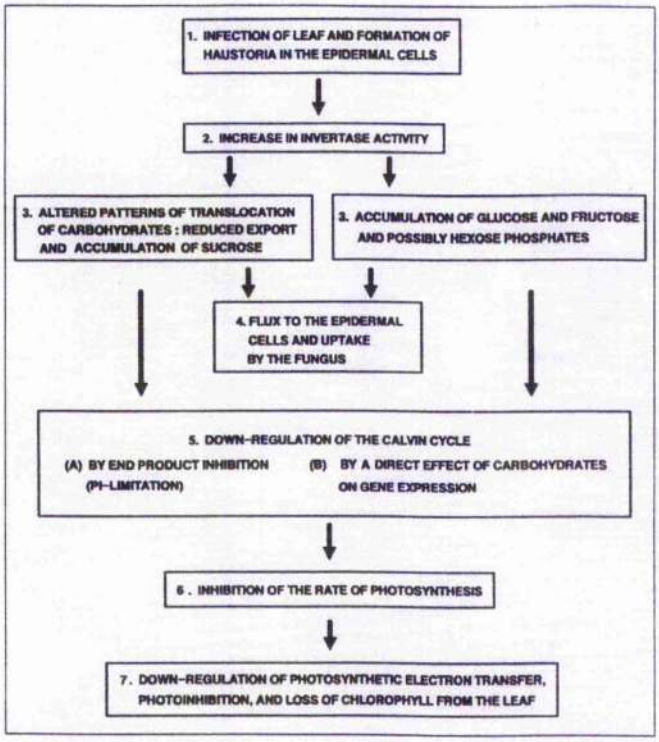


Figure 9. Proposed sequence of events involved in the inhibition of photosynthesis in cereal leaves infected with powdery mildew (Scholes, 1992).

1.25.8 Invertase and the accumulation of carbohydrate

Although photosynthesis is reduced in cereal leaves infected with powdery mildew, soluble carbohydrates accumulate (Scholes, 1992). This may be due to an increase in the activity of invertase, an enzyme involved in carbohydrate metabolism. Invertase activity has also been found to increase in plants infected with rust and downy mildew (Long *et al.*, 1975; Greenland and Lewis, 1981, 1983). Carbohydrates accumulated in tobacco plants transformed to express yeast invertase, resulting in down-regulation of Calvin cycle enzymes, and up-regulation of the enzymes involved in glycolysis. Consequently, the rate of photosynthesis decreased and respiration increased (Stitt *et al.*, 1990).

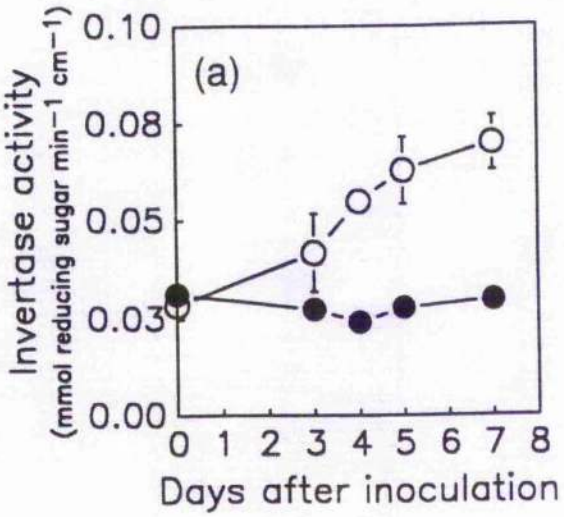


Figure 10. Effect of powdery mildew infection on the activity of invertase in barley leaves. Control (●) and infected (○) leaves. Results are the mean ±s.e. of four measurements (Scholes *et al.*, 1994).

Both the host and pathogen are capable of producing invertases. In cereals infected with mildew, there was no correlation between invertase activity and number of haustoria within epidermal cells. In addition, when mycelium, together with the

epidermis was removed from barley leaves, there was no significant reduction in invertase activity in mesophyll cells. This suggests that at least some of the increase in invertase is of host origin (Scholes *et al.,* 1994). Biotrophic pathogens are known to produce, or induce host production of auxin (Pegg, 1981), which can control invertase activity. Therefore, it is possible that auxin may be involved as a signal between pathogen and host (Weil and Rausch, 1990).

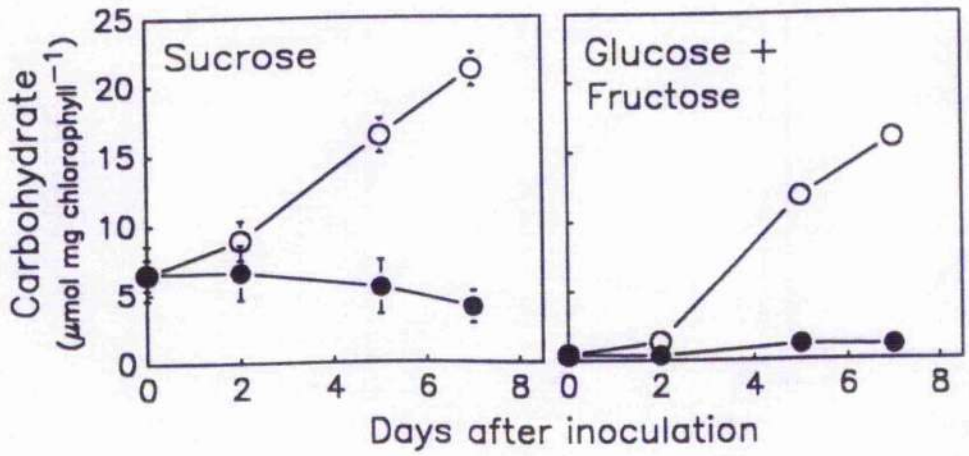


Figure 11. Effect of powdery mildew on amount of carbohydrate in barley leaves during infection. Control (●) and infected (○) leaves. Results are the mean ±s.e. of four measurements (Scholes *et al.*, 1994).

The increase in invertase may inhibit the export of sucrose from infected leaves. Additionally, it increases the hydrolysis of sucrose to glucose and fructose, the preferred forms for fungal uptake (Scholes *et al.*, 1994). In wheat and barley infected with powdery mildew, the sucrose concentration increases as the disease progresses. However, sucrose concentration often decreases in plants infected with rust or downy mildew (Brem *et al.*, 1986). An increased concentration of carbohydrate in the cytosol

may limit inorganic phosphate (P_i) resulting in end-product inhibition. Additionally, a high concentration may also restrict photosynthetic gene expression reducing the activity of the Calvin cycle enzymes, and consequently the rate of photosynthesis (Scholes *et al.*, 1994; Tang *et al.*, 1996).

1.25.9 Pi limitation and end-product inhibition

Triose phosphate, produced during photosynthesis, is used in the synthesis of sucrose in the cytosol. In the production of sucrose, inorganic phosphate (P_i) is released, and returns to the chloroplast in exchange for more triose phosphate (Stitt *et al.*, 1987). An accumulation of sucrose would result in an increase in cytosolic hexose-P by holding onto P_i (Wright *et al.*, 1995). Fru6P and Glc6P increased in barley leaves as infection with powdery mildew progressed (Scholes *et al.*, 1994). In addition biotrophic pathogens also take up P_i, and therefore may add to depletion (Whipps and Lewis, 1981). If export of P_i to the chloroplast is reduced, this limits the production of ATP, necessary for the reduction of PGA to triose phosphate. If the concentration of P_i in the chloroplast is very low this may eventually inhibit the Calvin cycle, and hence photosynthesis.

However, P_i limitation is unlikely to be the primary cause of the reduction in photosynthesis (Scholes, 1992). This would explain why supplying P_i to rust infected barley had limited effect, suggesting that P_i deficiency had not been the limiting factor on photosynthesis (Scholes and Farrar, 1986). Additionally, in wheat and barley infected with powdery mildew, the concentration of P_i in the leaves was unaffected or increased slightly at sporulation (Walters and Ayres, 1981; Zulu *et al.*, 1991; Scholes *et*

al., 1994; Wright *et al.*, 1995). In contrast, the P_i concentration was found to double in barley and wheat leaves infected with rust (Bennet and Scott, 1971; Ahmad *et al.*, 1983).

1.25.10 Restriction of photosynthetic gene expression and down regulation of the Calvin cycle

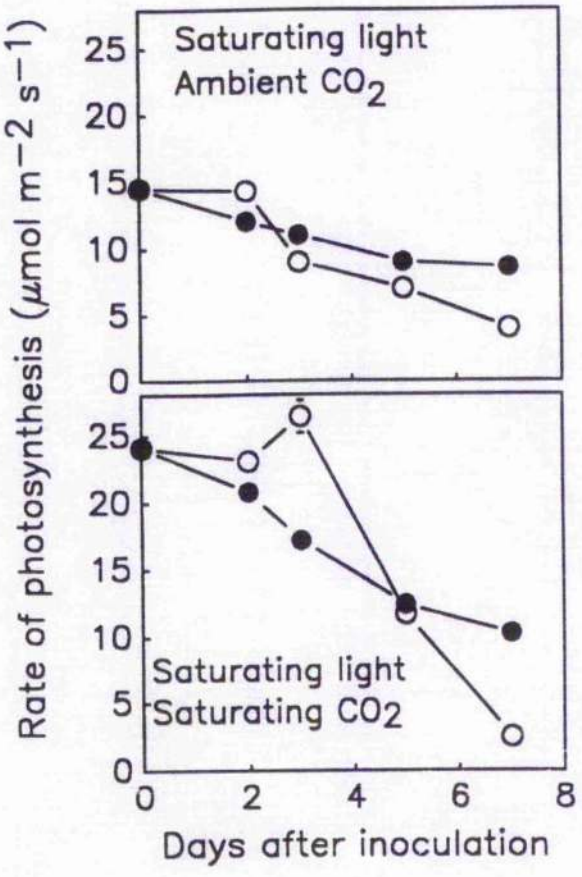


Figure 12. Effect of powdery mildew on the rate of photosynthesis in barley leaves at saturating light (1000 µmol m^{-2} s^{-1}) and either ambient or saturating CO_2 throughout infection. Control (●) and infected (○) leaves. Results are the mean ±s.e. of four measurements (Scholes *et al.*, 1994).

At ambient CO_2 levels and light saturation, the rate of carboxylation is the limiting factor on photosynthesis. Inhibition of photosynthesis is greater in an infected plant, when light is saturated and CO_2 at ambient level, indicating that carboxylation is limiting (Scholes *et al.*, 1994).

Carbohydrate accumulation often coincides with a decrease in Rubisco and other enzymes involved in the Calvin cycle (Besford, 1990). Sheen (1990) suggests that high concentrations of sucrose and glucose may suppress transcription by photosynthetic gene promoters in maize (including those for small subunits of Rubisco). When 50 mM glucose was added to a cell suspension of *Chenopodium rubrum* the transcript levels of ribulose bisphosphate carboxylase small subunit (*rbcS*), chlorophyll *a/b* binding protein (*cab*), and the δ subunit of thylakoid ATPase (*atp-δ*) genes were found to be significantly reduced within 5 h (Krapp *et al.*, 1993). Inoculation of melon leaves with *Colletotrichum lagenarium* reduced the mRNA encoding for the large and small subunits of which Rubisco is comprised (Roby *et al.*, 1988). A similar reduction in host mRNA was also found in potato infected with *Phytophora infestans* (Kombrink, and Hahlbrock, 1990), and powdery mildew infected barley (Higgins *et al.*, 1985).

The activity of enzymes in the reductive pentose phosphate pathway, 3-Phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase (NAD^+ and $NADP^+$), were reduced in barley infected with powdery mildew. This limits the regeneration of Rubisco (Walters and Ayres 1984). Gordon and Duniway (1982a) found a reduction in Rubisco protein in sugar beet leaves infected with mildew, but not in enzyme activity. Rubisco activity and protein was reduced in *Arabidopsis thaliana* inoculated with *Albugo candida* (Tang *et al.*, 1996). A similar reduction was also

observed in wheat and barley infected with powdery mildew (Gordon and Duniway, 1982a; Scholes *et al.*, 1994), rust infected leek (Roberts and Walters, 1988), and melon infected with *Colletotrichum lagenarium* (Roby *et al.*, 1988).

The down regulation of the Calvin cycle in mildewed wheat leaves, produced an initial 66% reduction in the activity of Rubisco (maximum activity was reduced by 70%), and consequently increased the ratio of RuBP/PGA. Hence, there was a reduction in the amount of PGA and the activity of NADP-GAPDH. There was also an increase in the ratio of triose-P/RuBP, and a 57% reduction in the activity of stromal Fru 1,6bisPase. This resulted in a reduction in the regeneration of the CO_2 acceptor molecule RuBP. The reduction in PGA produced would decrease the demand for assimilatory power to reduce PGA to triose-P. Reduced demand for assimilatory power is likely to down-regulate the electron transfer chain (Wright *et al.*, 1995). Wright *et al.* (1995) suggest that the loss of chlorophyll from infected leaves may be a result of the decreased demand for assimilatory power causing increased energisation of the thylakoids. In the early stages following infection of barley leaves with powdery mildew, there was no change in light response to photosynthesis (Scholes *et al.*, 1994), and chlorophyll fluorescence failed to indicate any effect on PS II (Scholes *et al.*, 1990). This suggests that down regulation of the electron transfer chain may be an effect of reduced activity in the Calvin cycle, rather than a direct result of the pathogen (Scholes, 1992).

In cereal leaves infected with powdery mildew it would appear that an increase in invertase activity causes the accumulation of carbohydrates, which mediates down regulation of the Calvin cycle, electron transport chain, and hence photosynthesis. It is not possible to assume that all interactions between host and biotrophic fungi will

adhere to the same sequence of events, and many conflicting results suggest they may not. As carbohydrate accumulation has been found to be significant in the proposed sequence of events, differences in the uptake of carbohydrate may be relevant to the sequence of events and mechanisms involved in different host pathogen interactions. Powdery mildew is an ectoparasite, therefore carbohydrate uptake through haustoria occurs within epidermal cells. Endoparasites, such as downy mildew and rusts, form haustoria in epidermal, mesophyll, and bundle sheath cells and, therefore, have access to a more direct carbohydrate supply, which may modify the events involved (Scholes, 1992). In addition, variation may occur to the proposed sequence for powdery mildew in cereals, due to differences between fungal pathogens, environmental or host factors.

1.26 Potential Applications for Systemic Induced Protection

SAR is potentially a very stable means of crop protection, as it requires to be induced as well as utilising the plant's multiple defence responses (Uknes *et al.*, 1996). Used as a preventative measure, it provides long-lasting protection against a range of diseases without recourse to pesticides (Kessmann *et al.*, 1994). It is already possible to transform plants to be constantly primed to respond rapidly to pathogen attack. Alternatively, there is the potential to develop suitable chemicals for application to the crop to induce SAR (Kuć, 1982; Métraux *et al.*, 1991; Ryals *et al.*, 1991).

However, induction of plant defence responses will only be considered a viable alternative to traditional crop protection methods if it can be shown that there is not a detrimental effect on crop quality or yield. Furthermore, environmental issues have to be addressed such as potential antagonism between defence pathways. Increased

protection from pathogens may increase susceptibility to insect pests and impact on plant fitness.

1.27 Aim of Study

It is more than a century since the possibility of inducing a plant's natural defence responses was realised. It has been generally assumed that induction of plant resistance results in costs to the plant in terms of fitness, growth and yield, as resources are reallocated to fund defence responses. However, there is a paucity of work undertaken in this area and what exists appears contradictory. This study was, therefore, designed to consider the efficacy and effects on plant growth and development of different fungal and chemical elicitors of systemic protection. It was decided to use two host-pathogen systems that had been used previously in studies of induced resistance, broad bean/rust and barley/powdery mildew.

Chapter 2

Pathogen Induced Systemic Protection:

Effects on Plant Growth and Development

2 Pathogen Induced Systemic Protection: Effects on Plant Growth and Development

2.1 Introduction

It is often assumed that disease resistance exerts a cost to the plant due to diversion of energy resources required to maintain defence mechanisms. Contrary to this assumption, some work with induced resistance in barley detected increased yields (Dehne *et al.*, 1984; Steiner *et al.*, 1988; Oerke *et al.*, 1989; Kehlenbeck *et al.*, 1994). However, studies of the cost of pathogen induced systemic protection in terms of plant growth and yields are limited. One of the few examples concerns systemic induced resistance in tobacco. It was observed that field grown tobacco plants, naturally induced for systemic resistance to blue mold, were often severely stunted (Cruickshank and Mandryk, 1960; Cohen and Kuć, 1981). Tuzun and Kuć (1985) subsequently found that amending the manner of inoculation dramatically increased growth of unchallenged plants compared with controls.

Other studies have compared the effects of variations in plant disease resistance on plant growth and yield. For example, a negative correlation was found to exist between growth rate and degree of resistance to fungal pathogens in a study of 15 radish cultivars (Hoffland *et al.*, 1996). Similarly, Smedegaard-Petersen and Stølen (1981) found that following inoculation, levels of respiration and biosynthesis simultaneously increased in barley highly resistant to mildew. It was suggested that the increase in respiration was necessary to fund energy-requiring defence responses and that this

impacted on the plant's energy resources and resulted in poorer grain quality and lower yields.

2.1.1 Objective

In view of the paucity of information on the effects of induced resistance on growth of plants, it was decided that these experiments should examine the effects of pathogen induced systemic protection on the growth and development of barley and broad bean.

2.2 Materials and Methods

2.2.1 Growing conditions

Plants were grown in a ventilated glasshouse at a temperature of 15°C, venting at 22°C. Daylight was supplemented with 400W sodium lamps to produce a 16h photoperiod.

In order to afford protection against aphids, a pesticide drench of 0.2g litre^{-1} of Intercept 70WG (imidacloprid 70% w/w) (Scotts UK. Professional, Ipswich, UK.), a systemic pesticide, was applied to all experimental plants as soon as they were actively growing. In addition blue sticky traps 'Blue Trappits' (Koppert UK. Ltd., Wadhurst, East Sussex, UK.) were suspended above the plants, for some degree of protection against thrips.

2.2.2 Powdery mildew induced systemic protection of barley

2.2.2.1 Plant material and experimental design

Barley seeds (*Hordeum vulgare* L. cv. Golden Promise) were sown in 10 cm pots, 1 plant per pot, in Levington M3 compost (Scotts UK. Professional, Ipswich, UK.). To minimise environmental effects on results a random experimental design was created in Minitab 12.

× 2	Treatments, mildew inoculation of first leaves and controls (i.e. not inoculated)
× 4	Pathogen challenge at 6, 8, 10, and 14 d after treatment
× 15	Replicates
120	Total plants required

2.2.2.2 Maintenance of pathogens

Powdery mildew (*Erysiphe graminis* f. sp. *hordei*) was maintained on barley (cv. Golden Promise) sown in seed trays. The seedlings were inoculated with powdery mildew, by dusting with the mildew conidia, at the first leaf unfolded growth stage 11 (Zadocks *et al.*, 1974). The infected plants were grown under the same conditions as described in section 2.2.1 above.

2.2.2.3 Inoculation with powdery mildew

Barley plants at the first leaf unfolded growth stage 11 (Zadocks *et al.*, 1974) were placed in an infection chamber and the first leaves dusted with powdery mildew conidia.

Upper leaves on the barley plants were covered in clingfilm to avoid infection. Controls were similarly covered in clingfilm but were not inoculated. The clingfilm remained in place for 24 h.

Barley plants, both treated and controls, were challenged with powdery mildew at 6, 8, 10 and 14 d after mildew inoculation of first leaves.

2.2.2.4 Harvest

Plants were destructively harvested and assessed for infection and growth 10 d after pathogen challenge. Infection intensity and leaf area were determined using a scanner and the Winfolia leaf analysis software (Regent, Canada). For plants challenged 6 and 8 d after mildew inoculation, assessment of infection was only undertaken on the leaves known to be present and fully expanded at the time of challenge inoculation. In hindsight, it seemed reasonable to assess the whole plant for infection. Therefore, information on infection on all leaves on plants is only available for plants challenged 10 and 14 d after mildew inoculation.

2.2.3 Rust induced systemic protection of broad bean

2.2.3.1 Plant material and experimental design

Broad bean seeds (*Vicia faba* cv. Aquadulce) were sown in 2 l pots, 1 plant per pot, in Levington M3 compost. To minimise environmental effects on results a random experimental design was created in Minitab 12.

× **2** Treatments, rust inoculation of first leaves and controls (i.e. not inoculated)

× **5** Pathogen challenge at 1, 2, 6, 10, and 14 d after treatment

× **11** replicates

110 Total plants required

2.2.3.2 Maintenance of pathogen

Rust (*Uromyces viciae fabae*) was maintained on bean plants (cv. Aquadulce and Bunyard Exhibition) and, in addition, rust spores were collected and stored at –80°C. Bean plants were inoculated with rust spores at approximately the 5-leaf stage. A spore suspension in distilled water was applied to the leaves using a soft camel-hair brush. Inoculated plants were then covered with a polythene bag for 24 h to maintain the high humidity required for spore germination. The infected plants were grown under the same conditions as described in section 2.2.1 above.

2.2.3.3 Inoculation with rust

The lowest leaves of the bean plants were inoculated as described above. The spore suspension contained 0.3 g of rust spores in 100 ml of 0.01% Tween 20. A solution of 0.1% Tween 20 was also applied to the lowest leaves of control plants. The treated

leaves, both on inoculated and control plants, were then enclosed in polythene bags for 24 h.

Bean plants, both treated and controls, were challenged with rust at 1, 2, 6, 10 and 14 d after rust inoculation of first leaves. The number of leaves challenged depended on the number of leaves present at challenge.

challenge 1 d	leaves 2 and 3 inoculated with rust
challenge 2 d	leaves 2 and 3 inoculated with rust
challenge 6 d	leaves 2 - 4 inoculated with rust
challenge 10 d	leaves 2 - 5 inoculated with rust
challenge 14 d	leaves 2 - 6 inoculated with rust

2.2.3.4 Harvest

Plants were destructively harvested and assessed for infection and growth 14 d after pathogen challenge. Infection intensity and leaf area were determined using a scanner and the Winfolia leaf analysis software (Regent, Canada). Infection intensity was assessed on leaves challenged with rust. One of the growth parameters was the total number of "leaflets". As bean leaves may comprise of between 1-6 or more leaflets, this seemed a more reasonable measurement of growth compared with total number of leaves.

2.2.3.5 Growth analysis

Growth analysis was undertaken based on the FW/DW ratio, relative growth rate (RGR), net assimilation rate (NAR) and the leaf area ratio (LAR).

Note the following abbreviations:

dry weight (DW), fresh weight (FW), leaf area (La) and t (time)

1. FW / DW ratio

As fresh weight varies according to water content, this ratio is an indication of plant turgor.

2. Relative growth rate (RGR)

The RGR measures the increase in weight in terms of original weight, over time, and therefore measures growth efficiency. RGR is the product of LAR and NAR, therefore changes in LAR and NAR affect RGR (Leopold and Kriedemann, 1985).

$$\mathbf{RGR} = \frac{\ln([DW]_2 - \ln[DW]_1}{t_2 - t_1}$$

3. Net assimilation rate (NAR)

NAR represents the result of photosynthetic gain over respiratory loss and is an indication of plant efficiency at producing dry matter, i.e. the rate of increase in dry weight per unit of leaf area produced.

$$\mathbf{NAR} = \frac{[DW]_2 - [DW]_1}{[La]_2 - [La]_1} \times \frac{\ln [La]_2 - \ln [La]_1}{t_2 - t_1}$$

4. Leaf area ratio (LAR)

LAR is the rate of increase in leaf area in relation to the increase in the plants dry weight. It is an indication of the proportion of the plant receptive to light for photosynthesis.

$$\mathbf{LAR} = \frac{[La]_2 - [La]_1}{\ln [La]_2 - \ln [La]_1} \times \frac{\ln [DW]_2 - \ln [DW]_1}{[DW]_2 - [DW]_1}$$

2.2.4 Statistical analyses

An analysis of variance (ANOVA) was applied to data, using the Genstat 5 statistical program (Lawes Agricultural Trust). The differences between the treatment and control means were tested for significance using the Student's *t*-test. Data are presented as means ± standard error (SEM).

2.3 Results

2.3.1 Powdery mildew induced systemic protection of barley

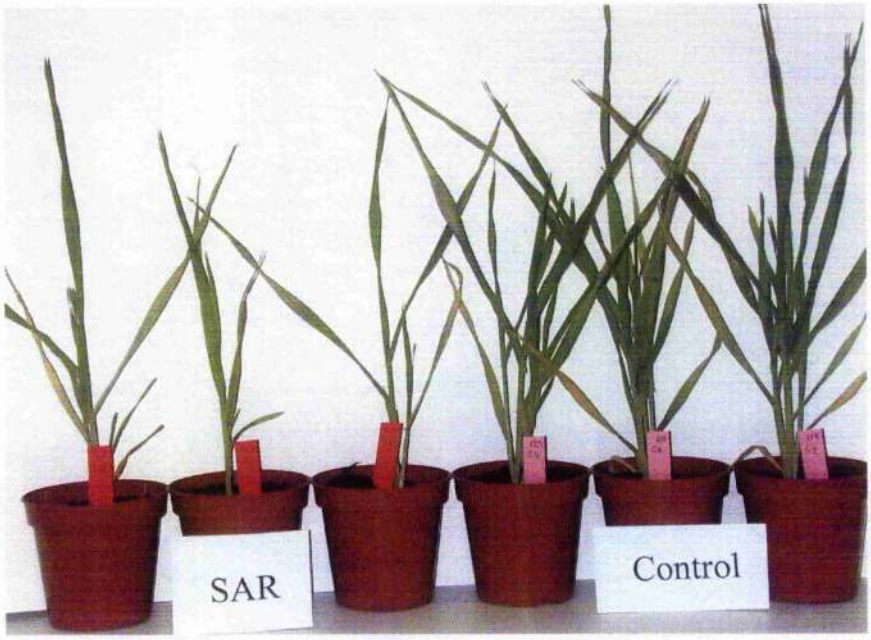


Figure 13. Powdery mildew induced systemic protection of barley plants. Induced plants are on the left and controls are on the right of picture.

Powdery mildew induced plants were visibly smaller than controls (Figure 13). All aspects of plant growth examined were significantly reduced in the mildew treated plants. Whole plant fresh weight, whole plant dry weight and total leaf area were approximately 50% of control values, and numbers of leaves and tillers were 30% lower in plants challenged 14 d after treatment compared with controls (Figure 16a-e).

A strong positive correlation was observed between the total leaf area infected with mildew and total leaf area. It was possible to establish that if total leaf area remained constant the total leaf area infected was higher in treated plants compared to controls

(Figure 17a). As expected there was less of a correlation between the % of the total leaf area infected and total leaf area, especially in the mildew treated plants (Figure 17b).

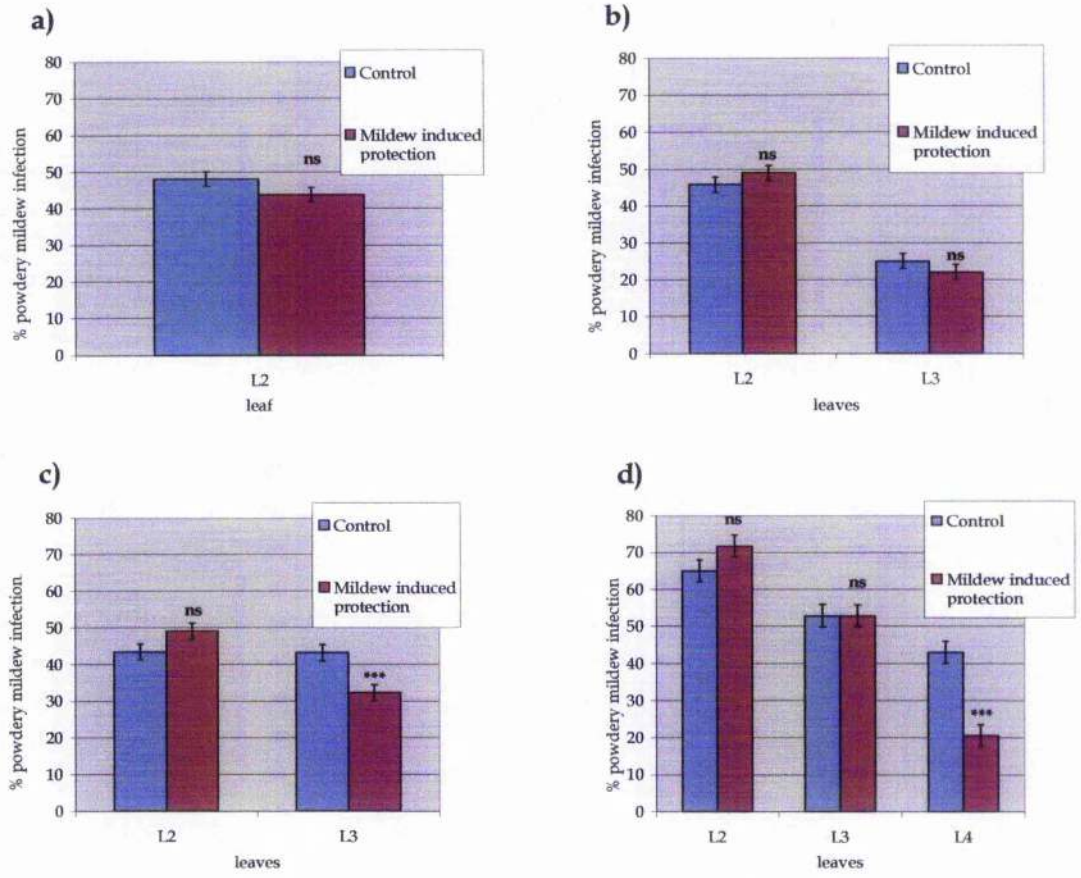


Figure 14. (a-d) Effect of powdery mildew induced systemic protection of barley on % infection on leaves challenged with powdery mildew 6 d (a); 8 d (b); 10 d (c); and 14 d (d) after induction of systemic protection. Plants were assessed for infection 10 d after pathogen challenge. Leaf numbers refer to order in which leaves were produced on the plant. Systemic protection induced on L1 (first leaf); subsequent leaves assessed for infection L2 (second leaf); L3 (third leaf) and L4 (fourth leaf) etc. Values are the mean ± SEM of 15 replicates. Significant differences from controls were determined using the Student's *t*-test and are shown as $P < 0.001$ ***, $P < 0.01$ **. $P < 0.05$ * and ns not significant.

Inoculation with powdery mildew to induce a resistance response had no significant effect on the % of leaf area infected on leaves of plants challenged with the pathogen 6 and 8 d after treatment (Figure 14a-b). Significant reductions in % infection were

detected in leaves 3 and 4 of plants challenged with mildew 10 and 14 d after induction of resistance, respectively (Figure 14c-d).

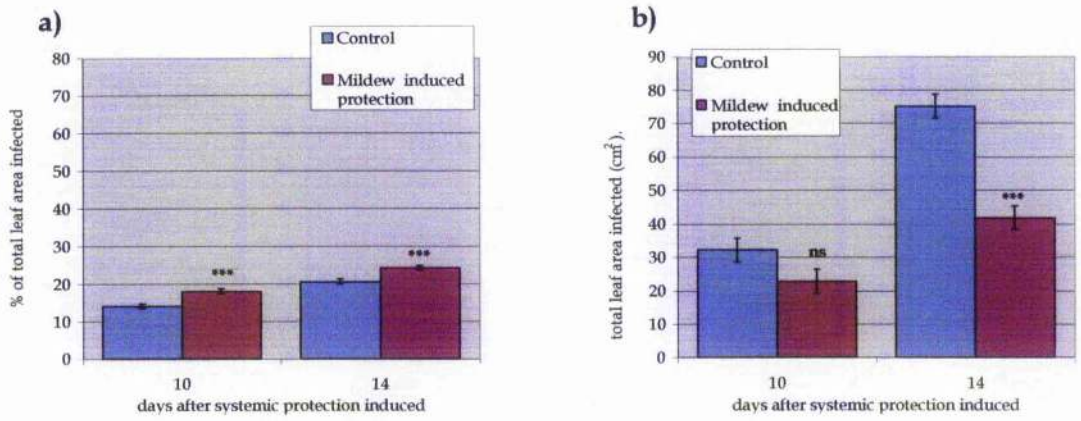


Figure 15. (a-b) Effect of powdery mildew induced systemic protection of barley on the % of the total leaf area infected (a); and on the total leaf area infected with mildew (cm^2) (b) (excluding leaf 1). Plants were assessed for infection 10 d after pathogen challenge. Values are the mean ± SEM of 15 replicates. Significant differences from controls were determined using the Student's *t*-test and are shown as $P < 0.001$ ***, $P < 0.01$ **. $P < 0.05$ * and ns not significant.

The effect of mildew treatment on mildew levels on the whole plant (excluding L1) were only examined in plants challenged with mildew 10 and 14 d after treatment. Significant reductions in the total leaf area covered with mildew were observed in plants challenged 14 d after treatment (Figure 15b). However, there was a significant increase in the % of the total leaf area infected 10 and 14 d following treatment (Figure 15a).

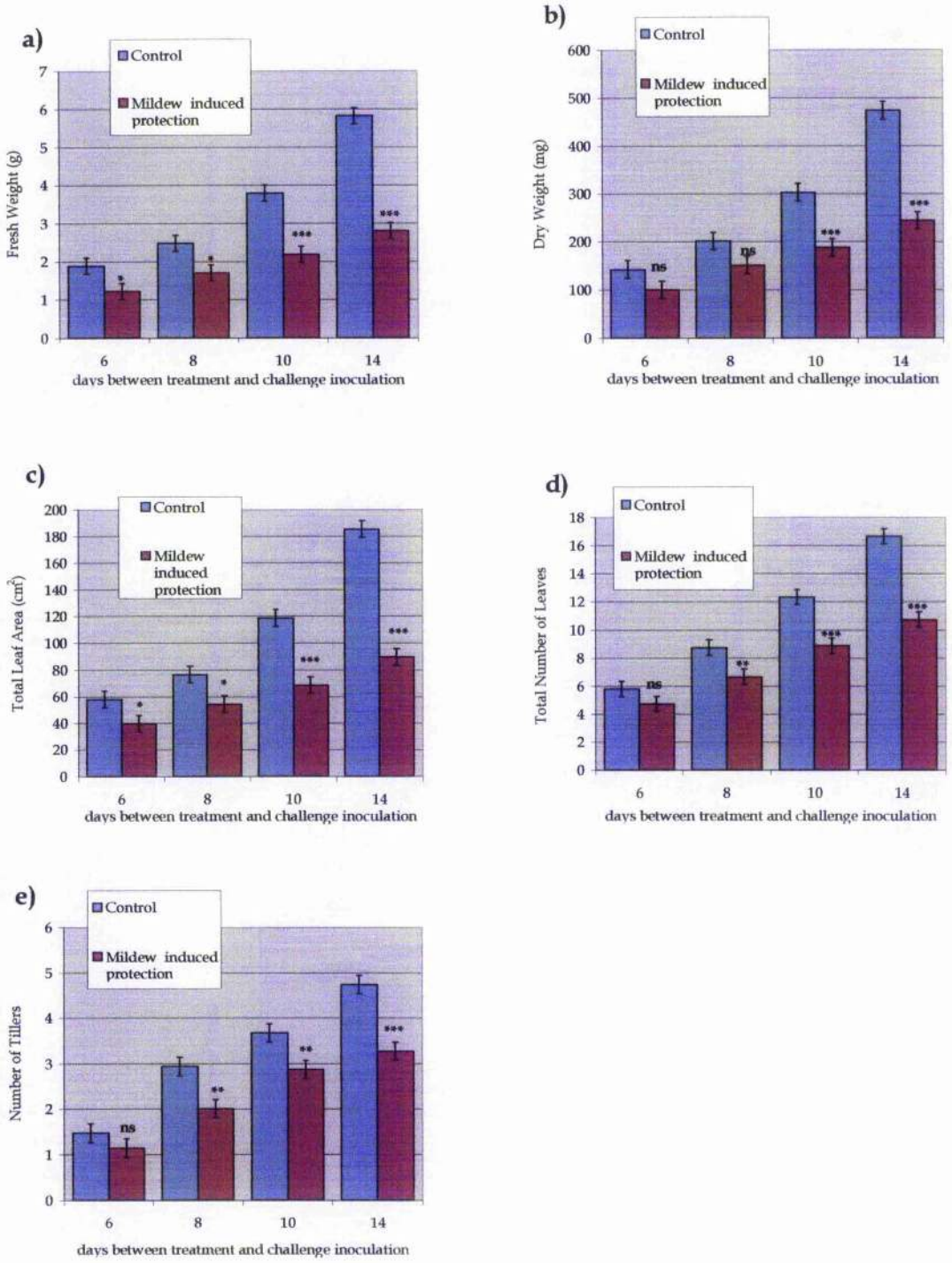
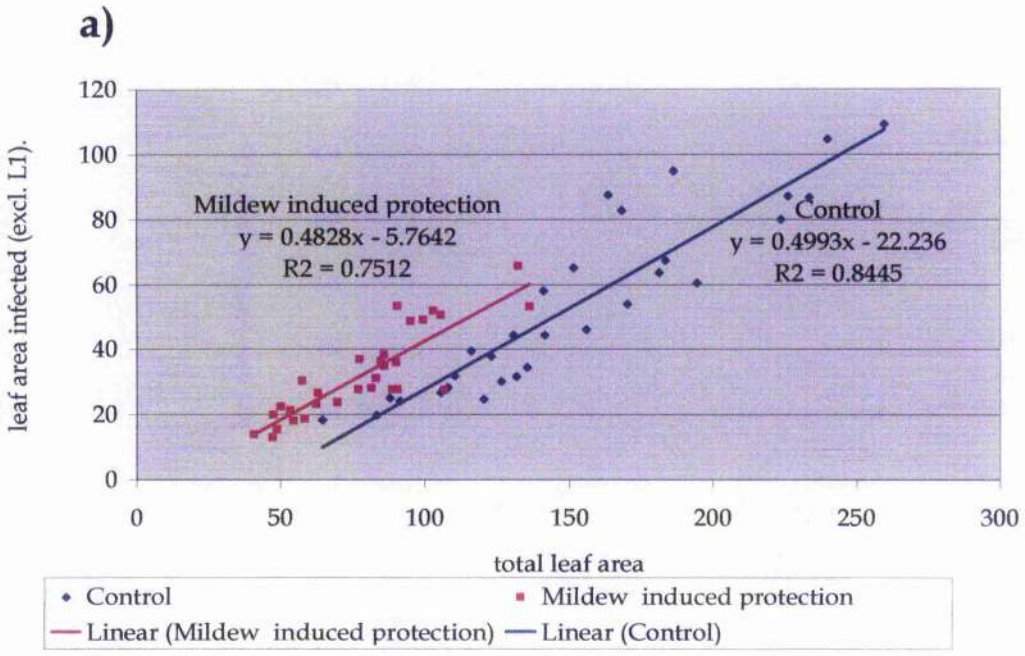


Figure 16. (a-e) Effects of powdery mildew induced systemic protection in barley on whole plant fresh weight (a), whole plant dry weight (b), total leaf area (c), total numer of leaves (d) and number of tillers (e). Plants were assessed for growth 10 d after pathogen challenge. Values are the mean ± SEM of 15 replicates. Significant differences from controls were determined using the Student's *t*-test and are shown as $P < 0.001$ ***, $P < 0.01$ **. $P < 0.05$ * and ns not significant.



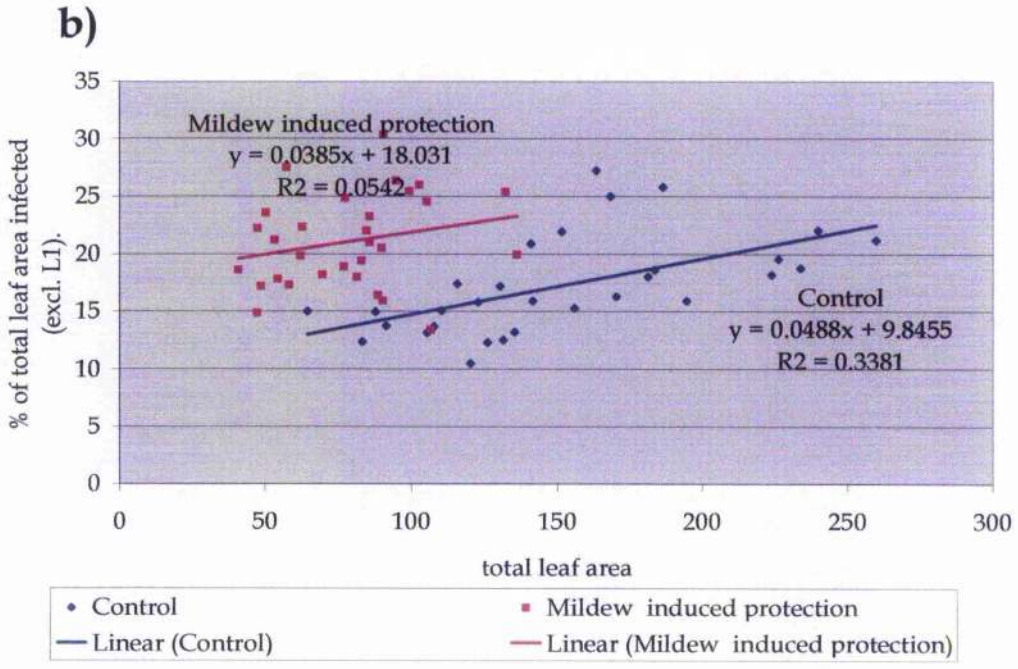


Figure 17. (a-b) The relationship between infection severity and total leaf area of barley plants in which systemic protection had been induced with powdery mildew. Total leaf area infected (a), % of total leaf area infected (b) (excluding leaf 1), in plants challenged 10 and 14 d after induction of systemic protection.

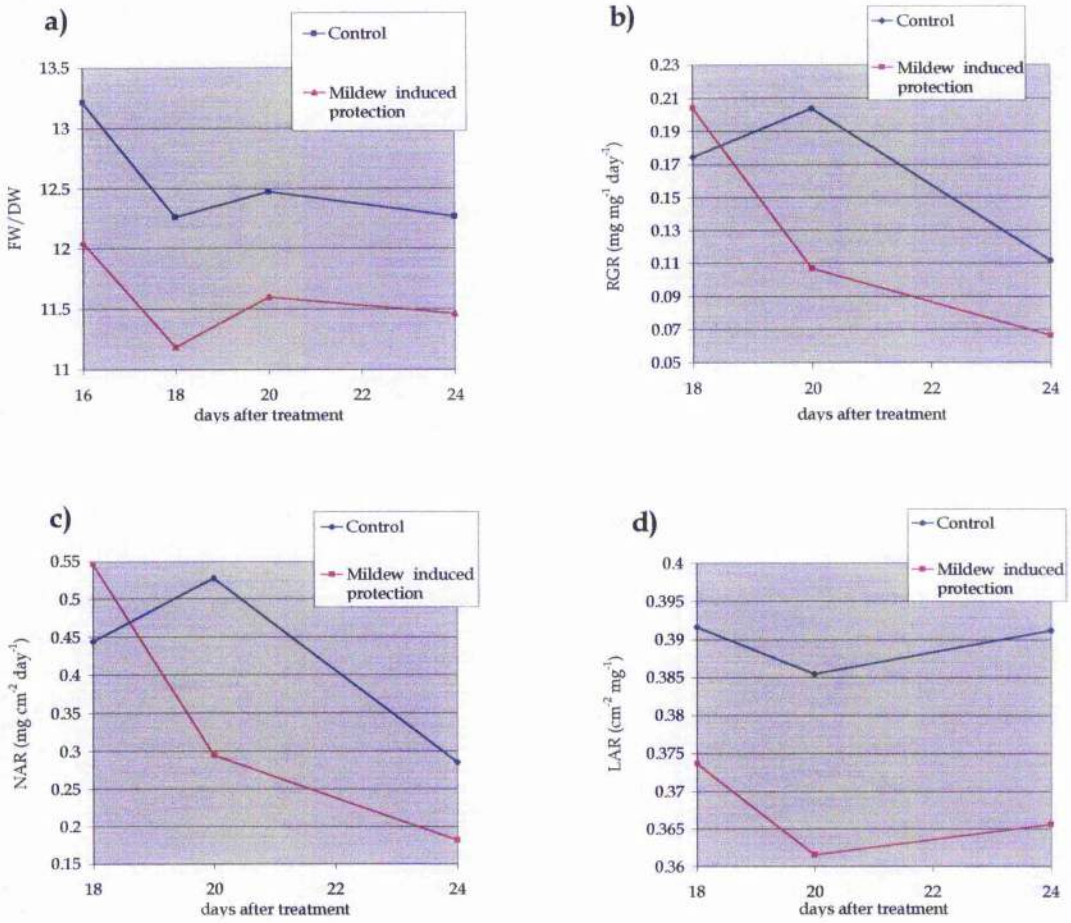


Figure 18. (a-d) Growth analysis of barley inoculated with powdery mildew to induce systemic protection. Effect of induced protection on fresh wt. / dry wt. ratio (a); relative growth rate (b); net assimilation rate (c); and leaf area ratio (d). Plants were harvested 10 d after pathogen challenge and assessed for growth parameters. Therefore, plants harvested at 16 d, 18 d, 20 d, and 24 d were respectively challenged 6 d, 8 d, 10 d and 14 d after treatment.

The fresh weight/dry weight (FW/DW) ratio was higher in controls compared with treated plants, but declined in all plants over the experimental period (Figure 18a). Both the relative growth rate (RGR) and the net assimilation rate (NAR) generally declined over the 6 d. Apart from the initially higher RGR and NAR in the plants at 18 d, RGR and NAR declined more rapidly in the mildew induced plants compared to controls (Figure 18b-c). The leaf area ratio (LAR) was also lower in the treated plants compared with controls (Figure 18d).

2.3.2 Rust induced systemic protection of broad bean.

Infection intensity was assessed as the percentage of leaf area infected, based upon the leaves available for inoculation at time of challenge. Significant reductions in levels of infection were observed in plants pathogen challenged 6 d after rust induction of systemic protection (Figure 19).

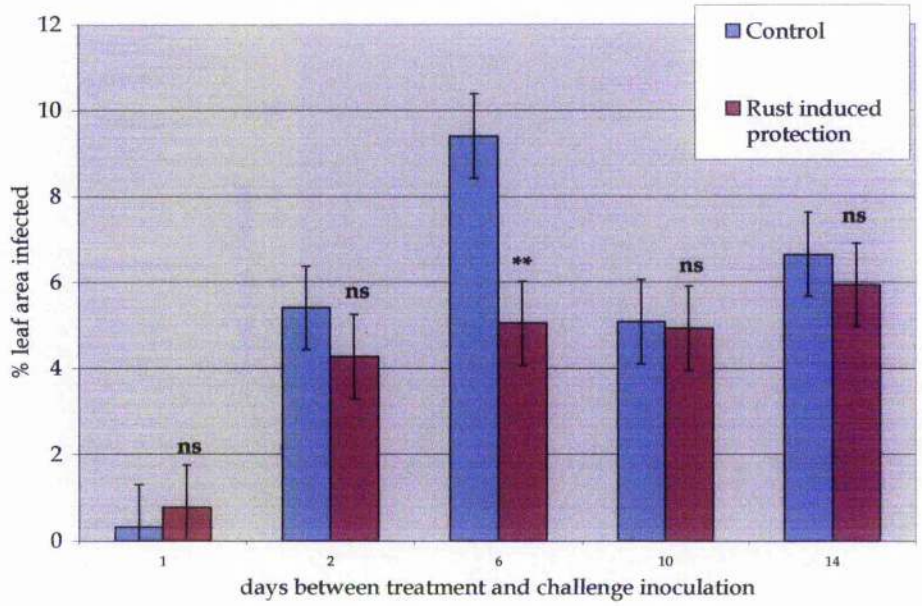


Figure 19. Effect of rust induced systemic protection on % rust infection in broad bean. Plants were assessed for infection 14 d after pathogen challenge. Values are the mean ± SEM of 11 replicates. Significant differences from controls were determined using the Student's *t*-test and are shown as $P < 0.001$ ***, $P < 0.01$ **. $P < 0.05$ * and ns not significant.

Leaf position was a significant factor in disease severity. Rust infection on leaves 2 and 4 (L2 and L4) was significantly lower than in non induced controls (Figure 20a). Infection was significantly reduced in plants challenged 6 d after treatment in all leaves challenged with the pathogen (L2, L3 and L4) (Figure 20b-d).

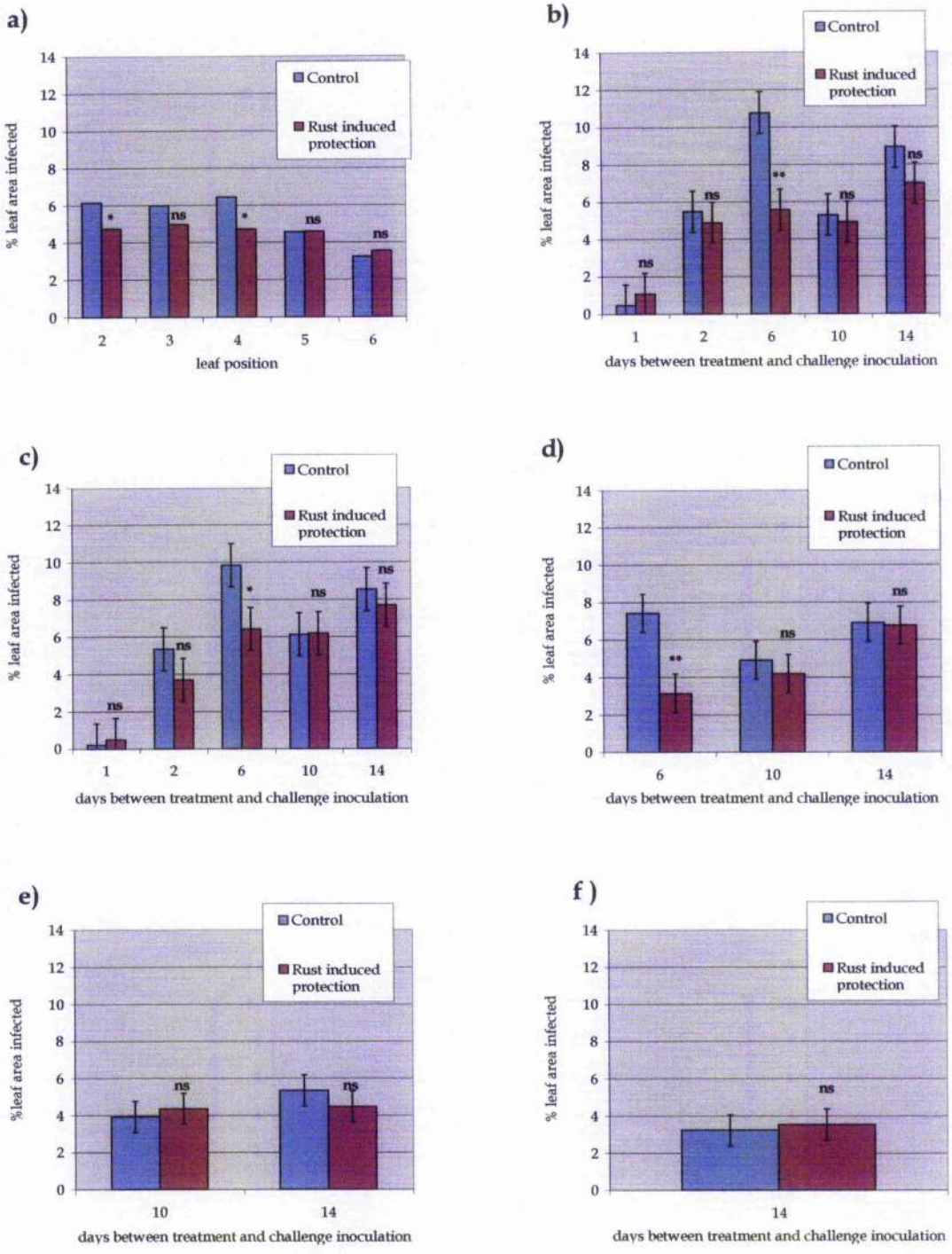


Figure 20. (a-f) Effect of leaf position on % leaf area infected with rust in rust induced systemic protection of broad bean. Plants were assessed for infection 14 d after pathogen challenge. Leaf position refers to the order of leaves from the base of the main stem, leaf 1 being the lowest leaf. % leaf area infected on leaves 2-6 for all 5 challenge periods (a), infection on leaf 2 (b), leaf 3 (c), leaf 4 (d), leaf 5 (e), and leaf 6 (f). Values are the mean ± SEM of 11 replicates. Significant differences from controls were determined using the Student's *t*-test and are shown as $P < 0.001$ ***, $P < 0.01$ **. $P < 0.05$ * and ns not significant.

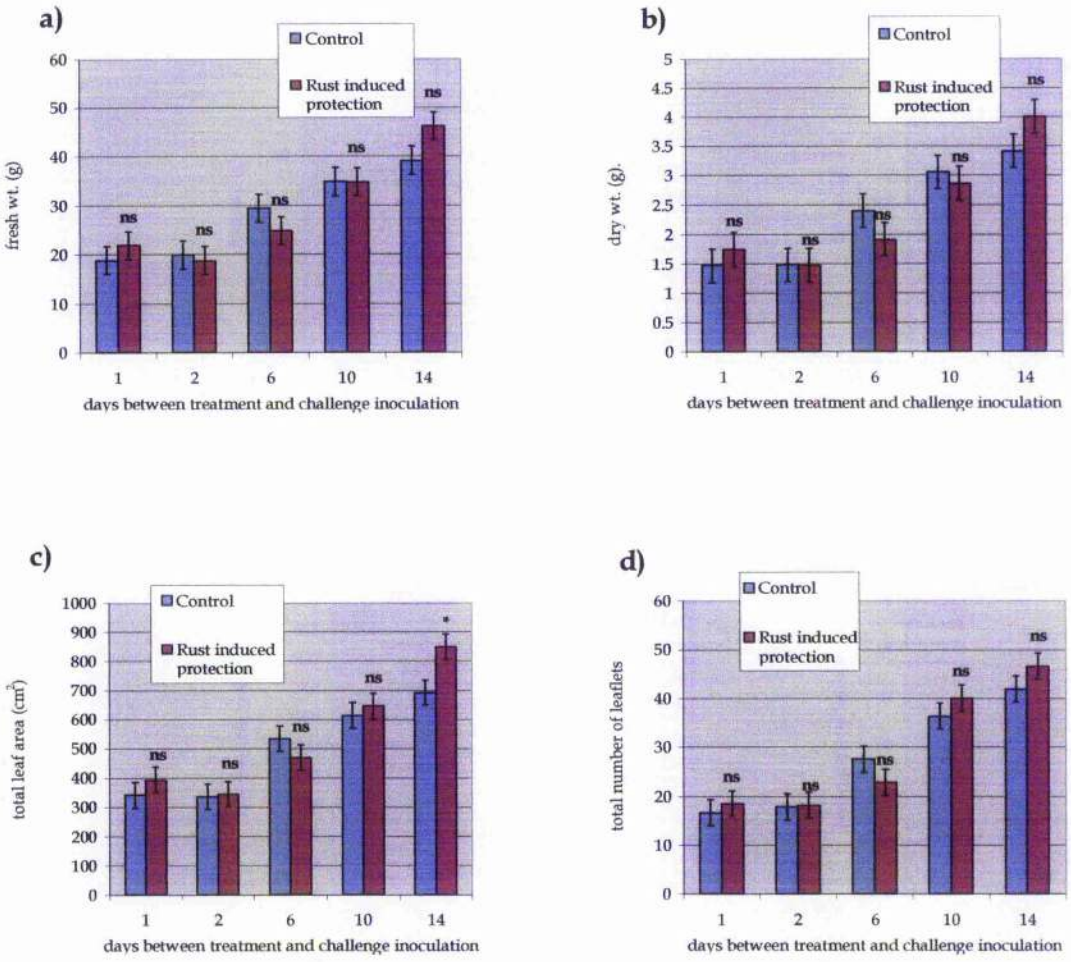


Figure 21. (a-d) Effect of rust induced systemic protection in broad bean on whole plant fresh weight (a), whole plant dry weight (b), total leaf area (c), and total number of leaflets (d). Plants were assessed for growth 14 d after pathogen challenge. Values are the mean ± SEM of 11 replicates. Significant differences from controls were determined using the Student's *t*-test and are shown as $P < 0.001$ ***, $P < 0.01$ **. $P < 0.05$ * and ns not significant.

Rust induction of systemic protection had no significant effect on growth. The only exception to this was a significant increase in total leaf area in plants challenged 14 d after protection was induced (Figure 21a-d).

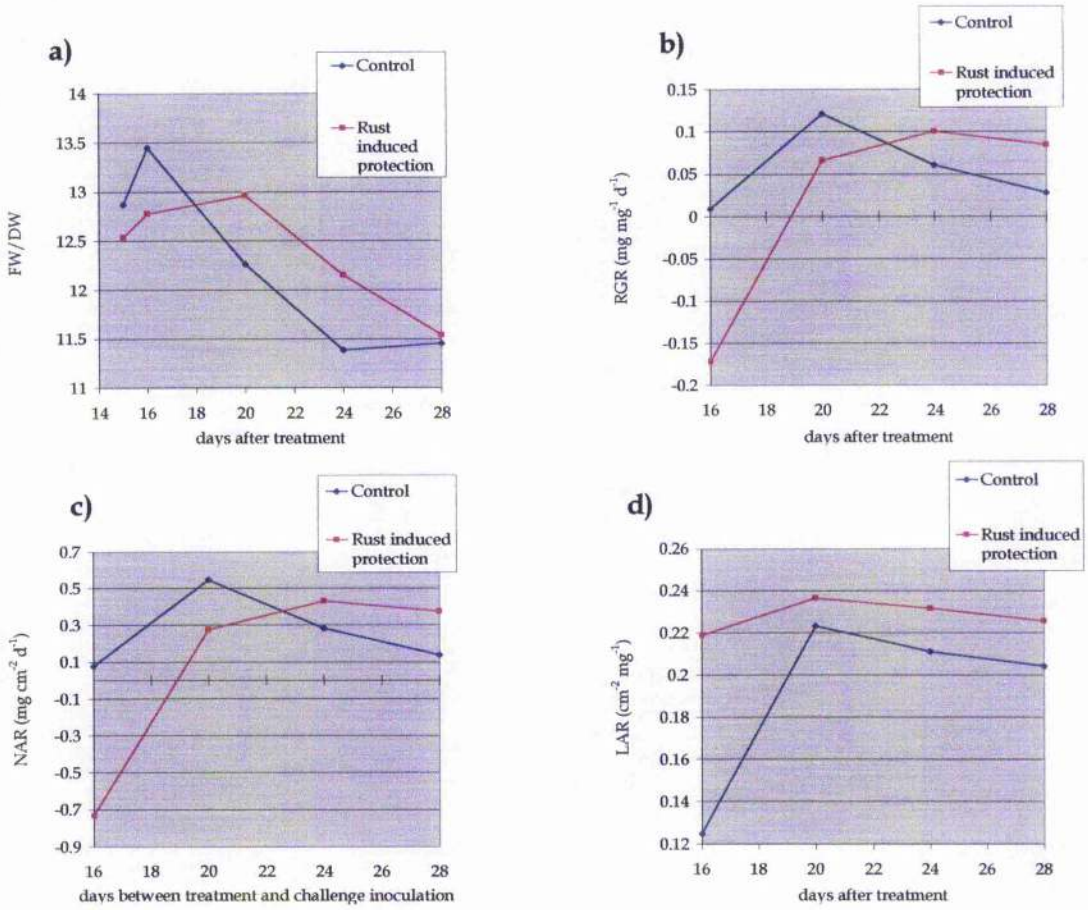


Figure 22. (a-d) Growth analysis of broad bean plants exhibiting rust induced systemic protection. Effect of induced protection on fresh wt. / dry wt. ratio (a); relative growth rate (b); net assimilation rate (c); and leaf area ratio (d). Plants were harvested 14 d after pathogen challenge and assessed for growth parameters. Therefore, plants harvested at 15 d, 16 d, 20 d, 24 d, and 28 d were respectively challenged 1 d, 2 d, 6 d, 10 d and 14 d after treatment.

The FW/DW ratio declined in both treated and control plants during the 14 d period and although it was lower than non induced controls at 15 and 16 d after treatment, it was greater than controls as time progressed (Figure 22a). There was an overall increase in both RGR and NAR over the time examined. RGR and NAR were both initially lower in the rust treated plants, but the positions had reversed in plants 24 and 28 d after treatment (Figure 22b-c). LAR remained higher in the protected plants compared with controls irrespective of time after treatment (Figure 22d).

2.4 Discussion and Conclusions

2.4.1 Powdery mildew induced systemic protection of barley

When systemic resistance in barley to powdery mildew was induced with application of salicylates and phenolic acids to the first leaves, this resulted in reduced mildew infection on second leaves (Walters *et al.*, 1993). This is contrary to the effects found in this work where resistance was induced by prior inoculation with powdery mildew. There was no significant effect on powdery mildew infection on second leaves. However, there was a significant reduction in disease on third leaves on plants challenged 10 d and fourth leaves on plants challenged 14 d after inoculation of first leaves. This, therefore, is only the second report of the induction of systemic protection in barley by prior inoculation with a biotrophic pathogen, and confirms the previous work of Hwang and Heitefuss (1982).

Inoculation of the first leaves of barley seedlings with powdery mildew resulted in a significant reduction in the total leaf area infected with mildew on upper leaves of plants challenged 14 d after inoculation of first leaves. However, the percentage of total leaf area infected was significantly higher in treated plants challenged 10 and 14 d after inoculation of first leaves, compared with controls. This apparent anomaly can be explained by the reduction in plant growth caused by treatment with mildew. All aspects of plant growth examined increased in both treated and control plants as the experiment progressed. However, the relative growth rate (RGR) was lower in the mildew-induced plants compared with controls. Fresh and dry weight and total leaf area were approximately 50% lower in plants challenged 14 d after inoculation of first

leaves. In addition, the total number of leaves and tillers on treated plants was approximately 30% lower. Consequently, the reduction in growth accounted for the reduction in total infection levels in the mildew treated plants. Results showed a strong positive correlation between total leaf area covered with mildew and total leaf area. Indications are that if total leaf area remained constant, total leaf area covered with mildew would be higher in the mildew treated plants.

This reduction in growth in response to mildew induced systemic protection is consistent with the concept of funding defence responses proposed by Smedegaard-Petersen and Stølen (1981), although in the present work little increased resistance was observed in the treated barley. These findings are certainly at variance with the work of Dehne *et al.* 1984; Steiner *et al.* 1988; Oerke *et al.* 1989; Kehlenbeck *et al.* 1994, since they observed an increase in yield associated with induced resistance in barley, although the present work was not taken through to yield and was conducted in the glasshouse and not in the field.

The effect of mildew inoculation on the growth factors examined was echoed in the growth analysis. The mildew treated plants in general exhibited reduced FW/DW ratios, RGR, NAR and LAR, compared with controls. NAR is an indication of the plant's efficiency at producing dry matter, i.e. the rate of increase in dry weight per unit of leaf area produced. The reduction in NAR in mildew treated plants was possibly due to the reduction in photosynthesis and increase in respiration that is known to occur in powdery mildew infected leaves (Bushnell and Allen, 1962; Walters and Ayres, 1984; Farrar and Rayns, 1987; Scholes *et al.*, 1994). These changes are accompanied by an altered pattern of translocation, with most of the newly formed assimilate remaining in

the infected leaf (Farrar, 1992). This results in decreased amounts of assimilate available for transport to roots and to young leaves and as a result, will have an effect on growth of both roots and shoots (Walters, 1985). But what of changes occurring in uninfected leaves on these plants? There have been reports of transient increases in rates of net photosynthesis in uninfected leaves of barley plants where the first leaves were mildewed (e.g. Walters and Ayres, 1983), but what contributions such changes might make to offsetting the changes in mildewed leaves is not known. Also of interest here is the fact that in leaves of barley expressing resistance to powdery mildew, large, transient increases in respiration have been reported (Smedegaard-Petersen and Stolen, 1981). This increased respiration is thought to be involved in funding the energy requirements of plant defence responses. Rates of photosynthesis and respiration were not measured in the present work, but in addition to measurement on individual leaves, both infected and induced, what is required in the future are measurements of photosynthesis and respiration for whole plants.

LAR is the rate of increase in leaf area in relation to the increase in the plant dry weight. LAR is much lower in the mildew induced plants, which suggests that treatment has a detrimental effect on leaf expansion and is confirmed in the lower leaf area in the treated plants. The smaller leaf area reduces photosynthetic capacity, which in turn affects NAR.

2.4.2 Rust induced systemic protection of bean

A decrease in infection was detected in bean plants pathogen challenged 6 d after inoculation of the lowest leaf with rust. This is consistent with the findings of Ward *et*

al. (1991) on the up-regulation of SAR gene expression. In tobacco inoculated with tobacco mosaic virus (TMV), maximal gene expression associated with the SAR response was attained for most of the genes 6 d after inoculation (Ward *et al.*, 1991). However, in tobacco in which systemic protection had been induced with *Peronospora tabacina*, full protection against blue mould did not become fully developed until 2-3 wk after induction (Tuzun *et al.*, 1986).

Murray and Walters (1992) found significantly reduced infection in upper leaves of bean plants challenged with rust 1 d, 3 d, 6 d, and 9 d after inoculation of the lowest 2 leaves with rust, although rust was not significantly reduced in plants challenged 12 d after inoculation. In the above experiment the plants were challenged 2 d, 6 d, 10 d, and 14 d after inoculation of the lowest leaf with rust. However, although rust infection was reduced in the upper leaves it was only in plants that were challenged 6 d following the inducer inoculation that the reduction in infection was significant. This may perhaps be explained by the work of Kováts *et al.* (1991) on the optimisation of induction of systemic resistance in tomato to *Phytophthora infestans*. They found a relationship between the number of pre-inoculated leaves and the strength of the resistance response. Infection in control leaves averaged 85% compared to 73% in plants induced with 1 leaf and 40% in plants with 3 leaves pre-inoculated.

Induction of resistance with rust had no significant effect on all aspects of bean growth examined. The exception to this was a significant increase in total leaf area in plants challenged 14 d after rust treatment. For all 4 parameters of plant growth, values were higher in plants challenged 14 d after rust treatment although, apart from total leaf area, the results were not statistically significant. However, as all 4 growth parameters show

very similar trends, it is perhaps possible that in plants challenged 14 d after rust treatment there is a small increase in growth. Additionally, the RGR is higher in plants 24 and 28 d (challenged 10 and 14 d) after rust treatment compared with controls. This is contrary to the effects of resistance to *Streptomyces ipomoeae* on the growth of infected sweet potato. Both shoot and fibrous root dry weight decreased in a *S. ipomoeae* resistant variety compared to a susceptible variety. In healthy plants the dry weight of storage roots was similar in both varieties. However, storage roots from the infected resistant variety were smaller (Ristaino, 1993). Similarly, growth reductions were found in resistant alfalfa clones infected with *Verticillium albo-atrum* compared to pathogen susceptible clones (Pennypacker *et al.*, 1988, 1990).

NAR increased in rust treated bean plants, whereas in the controls there was an initial increase after which NAR started to decline. The increase in NAR in the rust treated plants is consistent with an increase in photosynthesis. This is supported by the findings of Murray and Walters (1992) on rust induced resistance in broad bean. They found both increased resistance to rust infection and increased rates of photosynthesis in upper leaves after inoculation of lower leaves with rust. LAR was also higher in the rust treated plants, which would support the increase in photosynthesis. Murray and Walters (1992) also found that rates of net photosynthesis were significantly reduced in rust infected leaves. In the present work, it would appear that reductions in net photosynthesis in rusted leaves were offset by possible increases in net photosynthesis in upper, uninfected leaves.

2.4.3 Conclusions

It is interesting that in the two plant/pathogen systems chosen for study, the effects on growth are totally different. Inoculation of the first leaves of barley with mildew resulted in a reduction in all aspects of growth, whereas with rust induced resistance in broad bean there was either no effect or a small increase in growth. It is possible that effects on growth are specific to the particular interaction of plant and elicitor of the resistance response. However, this is confounded by the work of Tuzun and Kué (1985) on systemic protection of tobacco against blue mould, by injection of plants with a spore suspension of *Peronospora tabacina*. They found that the location of the injection had a major impact on plant growth and development. Unchallenged plants injected outwith the xylem produced 4 –6 more leaves, developed 15% greater leaf area, and were 40% taller than controls. In addition, there was a 30% increase in fresh weight and 40% increase in dry weight compared with controls. Whereas, when injections were made into the xylem, this resulted in severe stunting of the tobacco plants (Tuzun and Kué, 1985). As the number of studies undertaken on resistance and the effects on plant growth are limited and often appear conflicting, it is not possible at present to draw any conclusions as to the underlying causes of the variance observed. To date, little work has been carried out on the effects of inducing systemic resistance on growth and yield of plants. However, in some interesting recent work, Heil *et al.* (2000) found that the treatment of wheat plants with the SAR inducer Bion (benzothiadiazole) led to reduced growth and yield. The authors speculate that such losses could be the result of metabolic competition between different processes involved in plant growth and the synthesis of compounds for plant defence.

Chapter 3

The Effect of Rust Induced Systemic Protection on Plant Growth, Chlorophyll Fluorescence and Water Uptake

3 The Effect of Rust Induced Protection on Plant Growth, Chlorophyll Fluorescence and Water Uptake.

3.1 Introduction

Biotrophic pathogens such as rusts and mildews utilise host nutrients, affect translocation of plant assimilates and it is, therefore, assumed that this results in reduced plant growth (Ayres, 1976; Bushnell and Gay, 1978; Gay and Manners, 1981; Farrar and Lewis, 1987; Scholes, 1992). A further factor that may affect growth of an infected plant is the proportion of leaves on the plant that are uninfected relative to those that are infected. For although photosynthesis may be reduced in infected leaves, photosynthesis has been found to be higher in uninfected leaves of a rust infected bean plant than in the leaves of a healthy plant (Livne, 1964; Murray and Walters, 1992).

The effect of the pathogen on water uptake in the plant is another factor to be considered in relation to plant growth, since leaf expansion is dependent to some degree on water uptake. Powdery mildew infection of barley was found to reduce the diameter of xylem vessels in barley roots by 25% (Walters, 1981). However, although resistance to axial flow increased, overall resistance in the whole root system was reduced by infection due to increased root permeability and an increase in the osmotic force driving exudation (Walters and Ayres, 1982). Another important aspect to consider is the effect of the pathogen on stomatal closure and transpiration. Water uptake within the plant is dependent on evaporation of water from the leaf surface, as this creates a water potential gradient necessary for water to be drawn up through the xylem from the roots (Walters, 1985). Duniway and Durbin (1971) found that rust and powdery mildew infection

resulted in inhibition of stomatal opening in the light. Although stomatal apertures eventually remained smaller in rust infected leaves, this became less important after fungal sporulation ruptured the cuticle resulting in increased transpiration (Johnson and Miller, 1934, 1940; Murphy, 1935; Duniway and Durbin, 1971). Hence, after sporulation water uptake may no longer be indicative of leaf turgor and, therefore, a gauge of leaf expansion.

3.1.1 Objective

Studies in this report suggest that rust induced systemic protection results in either no effect or a small increase in growth of broad bean. Consequently, it was decided to investigate further the effect of rust induced protection on plant growth, water uptake and chlorophyll fluorescence. Analysis of chlorophyll fluorescence is a means of examining the effect of rust on the photosynthetic system.

3.2 Materials and Methods

3.2.1 Growing conditions

Plants were grown in a ventilated glasshouse as previously described (section 2.2.1).

3.2.2 Maintenance of pathogens

Rust was maintained on broad bean as previously described (section 2.2.3.2).

3.2.3 Plant material and experimental design

Broad bean seeds (*Vicia faba* cv. Bunyard Exhibition) were sown in 13 cm pots, 1 plant per pot, in Levington M3 compost. To minimise environmental effects on results a random experimental design was created in Minitab 12.

× **3**	Treatments, rust inoculation of first leaves, rust inoculation of first 3 leaves and controls (i.e. not inoculated)
× **5**	Harvests prior to and 1, 2, 3, and 4 wk after rust inoculation.
×20	Replicates
300	Total plants required

3.2.4 Rust inoculation

A spore suspension containing 0.3 g of rust spores to 100 ml distilled water was applied to the first leaves or the first 3 leaves of bean plants, using a soft camel-hair brush. Distilled water was applied to the lowest 3 leaves of control plants and the second and third leaves of treated plants, which had only the first leaf inoculated with rust. The treated leaves, both on inoculated and control plants, were then enclosed in polythene bags for 24 h.

3.2.5 Water uptake

Water uptake was measured weekly during the course of the experiment. Plants due to be harvested in the final week of the experiment were used to assess water uptake. The surface of the compost was covered with pea gravel to minimise evaporation. The

compost in the pots was saturated with water and then allowed to fully drain. A polythene bag was then placed over the base of the pot and secured with an elastic band, to minimise drainage losses before weighing. After 24 h, the pots were reweighed to calculate transpiration losses and therefore water uptake.

3.2.6 Chlorophyll fluorescence

Chlorophyll fluorescence was measured weekly throughout the experiment (Plant Efficiency Analyser, Hansatech Instruments Ltd., Norfolk, UK). Plants due to be harvested in the final week of the experiment were used to monitor changes in chlorophyll fluorescence. Fluorescence was measured on leaves 3 and 4 of plants with 3 leaves rust inoculated and controls (not inoculated). Measurements were made on dark-adapted leaves, based upon thirty saturating light pulses (650nm) at 20 s intervals and 3 % background light.

Calculation of fluorescence parameters

Note the following abbreviations:

F_o minimal fluorescence

F_m maximum fluorescence

F_v variance between F_o and F_m

t time

Photosystem II (PSII) efficiency

This indicates the photochemical efficiency of photosystem II (PSII) open reaction centres by measuring the proportion of absorbed energy used in photochemistry (Jones, 1992).

$F_v/F_m = (F_m(t) - F_o)/F_m(t)$

Photochemical quenching (q_p)

This is a measurement of the redox state of the primary electron acceptor. When the primary electron acceptor is fully reduced, all reaction centres are closed and maximum fluorescence is therefore emitted, and q_p is equal to zero. Fluorescence emitted is minimal and q_p equal to one, when the primary electron acceptor is oxidised and all PSII reaction centres open to excitation energy (Jones, 1992).

$q_p = (F_m(t) - F(t))/(F_m(t) - F_o)$

Non-photochemical quenching (q_N)

When photosynthesis is inhibited, non-photochemical quenching employs various mechanisms to dissipate the excess energy absorbed by chloroplasts, and minimise damage to the photosynthetic system (Jones, 1992).

$q_N = (F_m \text{ (dark adapted)} - F_m(t))/F_m \text{ (dark adapted)} - F_o)$

F_o quenching (q_o)

$q_o = (F_o - F'_o)/F_o = 1 - (F'_o/ F_o)$

if F_o quenching occurs

$q_p = (F_m(t) - F(t))/ (F_m(t) - F'_o)$

$q_N = (F_m \text{ (dark adapted)} - (F_m(t) \times F_o/ F'_o))/ F_m \text{ (dark adapted)} - F_o)$

Calculations for fluorescence parameters from Jones (1992).

3.2.7 Harvest

Plants were destructively harvested and assessed for growth prior to inoculation and 1, 2, 3 and 4 wk after rust inoculation. Leaf area was determined using a scanner and the Winfolia leaf analysis software (Regent, Canada).

3.2.8 Growth analysis

Growth analysis was undertaken as previously described (section 2.2.3.5).

3.2.9 Statistical analyses

An analysis of variance (ANOVA) was applied to data, using the Genstat 5 statistical program (Lawes Agricultural Trust). The differences between the treatment and control means were tested for significance using the Student's *t*-test. Data are presented as means ± standard error (SEM).

3.3 Results

3.3.1 Effects on growth and water uptake

The fresh weight, dry weight and total leaf area were significantly higher in plants with 3 leaves infected compared with controls, 1 wk after rust inoculation (Figure 23a, 24a, 25a). There was also a significant decrease in dry weight 3 wk after inoculation, in plants with 3 leaves infected with rust (Figure 24c). In contrast, rust had no significant effect on the number of leaflets, irrespective of the time after inoculation (Figure 26a-d). The only significant effect on water uptake was a decrease that occurred in plants with only 1 leaf infected with rust 1 wk after inoculation (Figure 27a).

Senescence became apparent in plants at week 4. Due to the number of shrivelled and dropped leaves, replication was reduced in the final harvest from 20 to 10 replicates for each of the rust treatments, and to only 3 replicates for the controls.

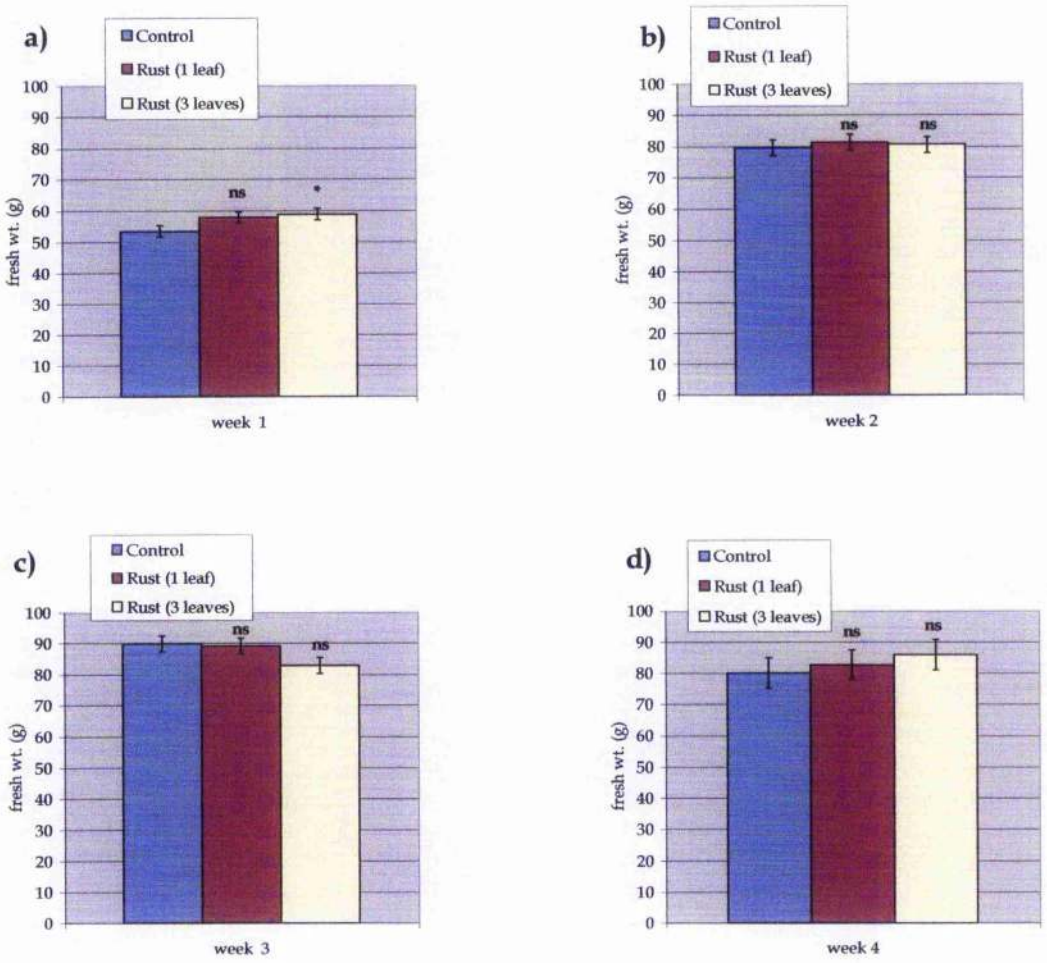


Figure 23. (a-d) Effect of rust on fresh weight of broad bean, 1 wk (a), 2 wk (b), 3 wk (c) and 4 wk (d) after rust inoculation. Bean plants were inoculated with rust on 0, 1 or 3 lowest leaves. Values are the mean ± SEM of 20 replicates, except during week 4 when they were reduced to10 replicates of rust inoculated plants and 3 replicates of controls. Significant differences from controls were determined using the Student's *t*-test and are shown as $P < 0.001$ ***, $P < 0.01$ **. $P < 0.05$ * and ns not significant.

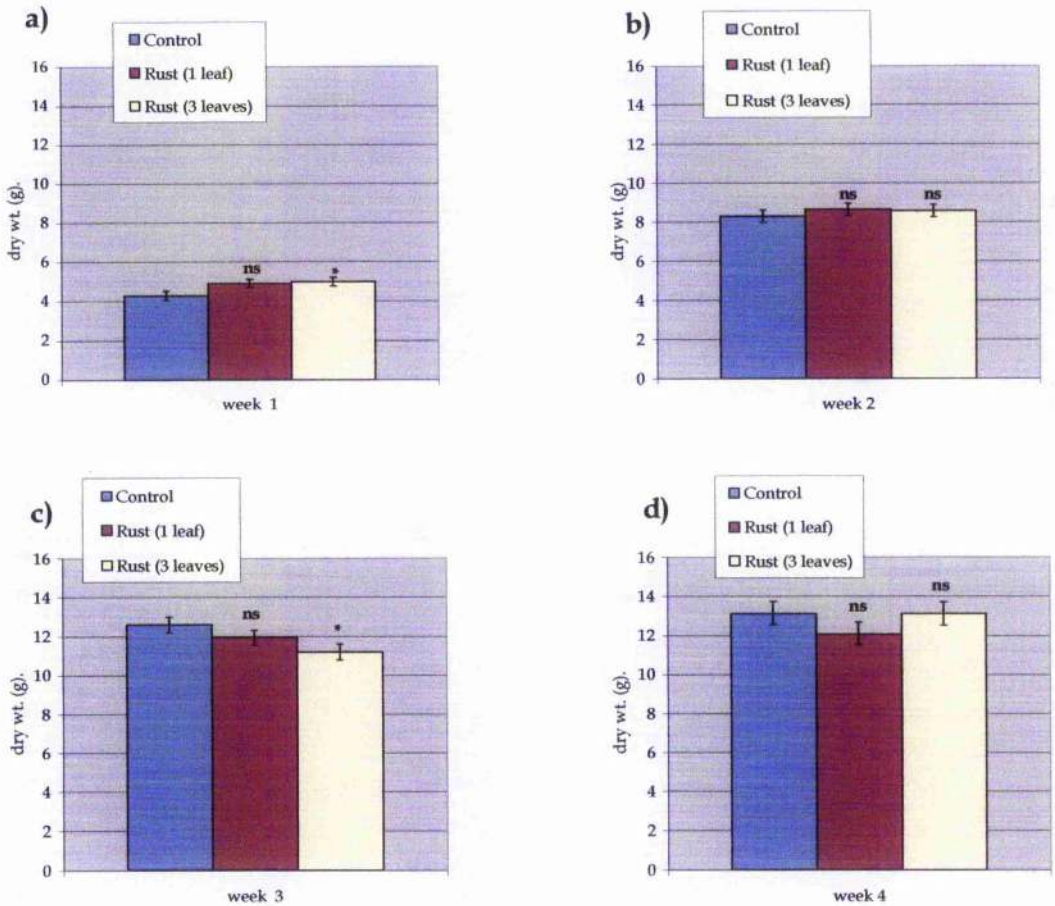


Figure 24. (a-d) Effect of rust on dry weight of broad bean, 1 wk (a), 2 wk (b), 3 wk (c) and 4 wk (d) after rust inoculation. Bean plants were inoculated with rust on 0, 1 or 3 lowest leaves. Values are the mean ± SEM of 20 replicates, except during week 4 when they were reduced to10 replicates of rust inoculated plants and 3 replicates of controls. Significant differences from controls were determined using the Student's *t*-test and are shown as $P < 0.001$ ***, $P < 0.01$ **. $P < 0.05$ * and ns not significant.

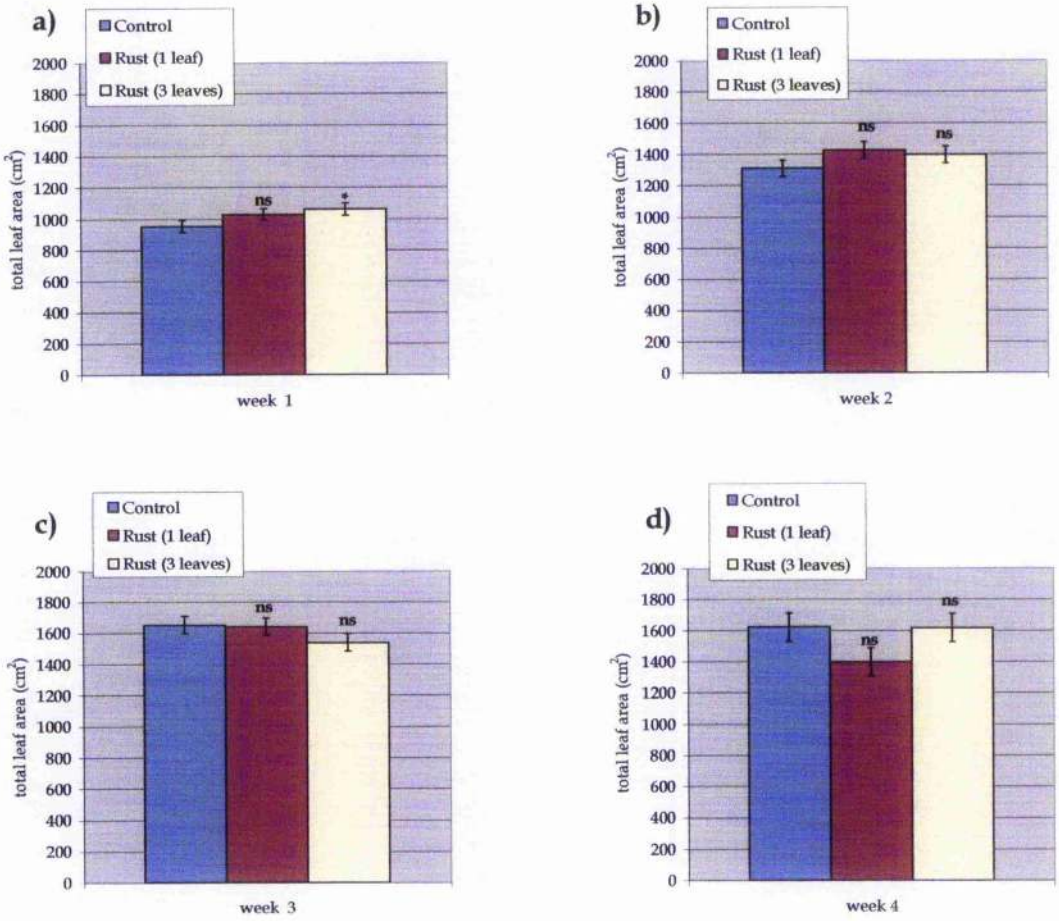


Figure 25. (a-d) Effect of rust on total leaf area of broad bean, 1 wk (a), 2 wk (b), 3 wk (c) and 4 wk (d) after rust inoculation. Bean plants were inoculated with rust on 0, 1 or 3 lowest leaves. Values are the mean ± SEM of 20 replicates, except during week 4 when they were reduced to10 replicates of rust inoculated plants and 3 replicates of controls. Significant differences from controls were determined using the Student's *t*-test and are shown as $P < 0.001$ ***, $P < 0.01$ **. $P < 0.05$ * and ns not significant.

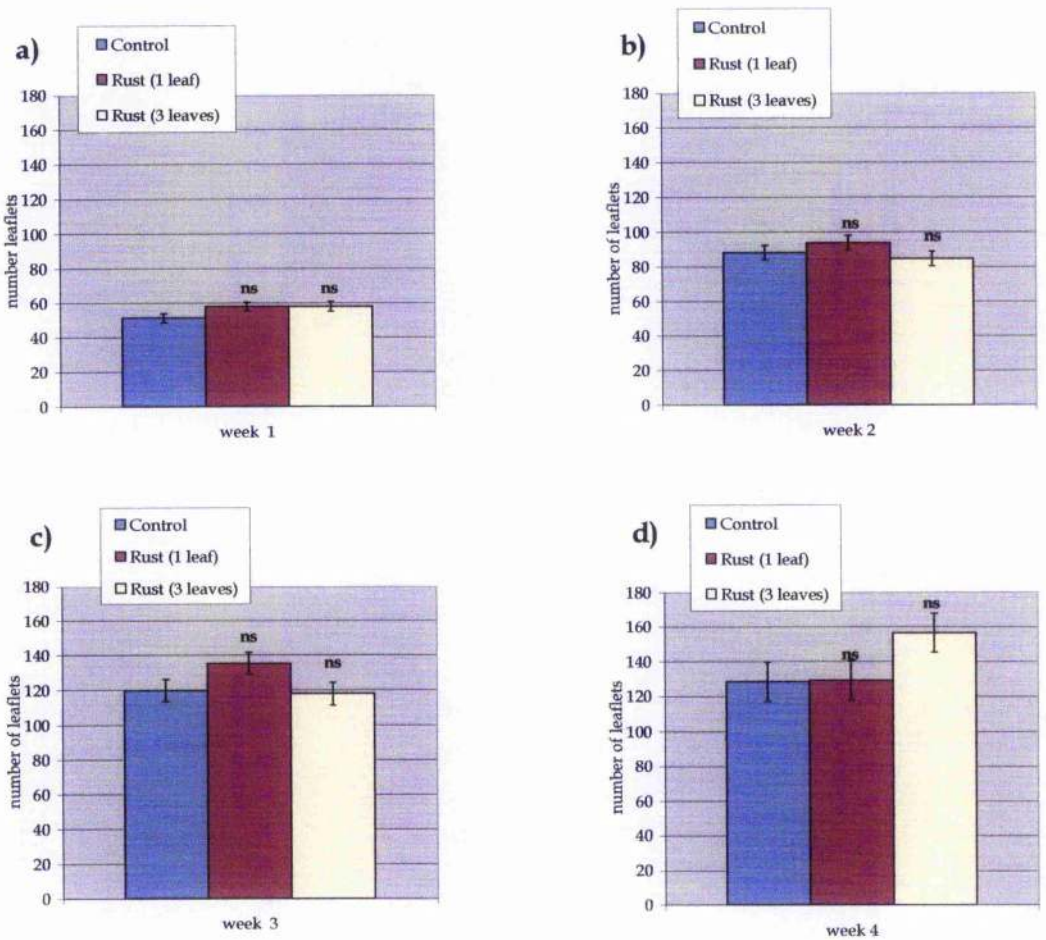


Figure 26. (a-d) Effect of rust on number of broad bean leaflets, 1 wk (a), 2 wk (b), 3 wk (c) and 4 wk (d) after rust inoculation. Bean plants were inoculated with rust on 0, 1 or 3 lowest leaves. Values are the mean ± SEM of 20 replicates, except during week 4 when they were reduced to10 replicates of rust inoculated plants and 3 replicates of controls. Significant differences from controls were determined using the Student's *t*-test and are shown as $P < 0.001$ ***, $P < 0.01$ **. $P < 0.05$ * and ns not significant.

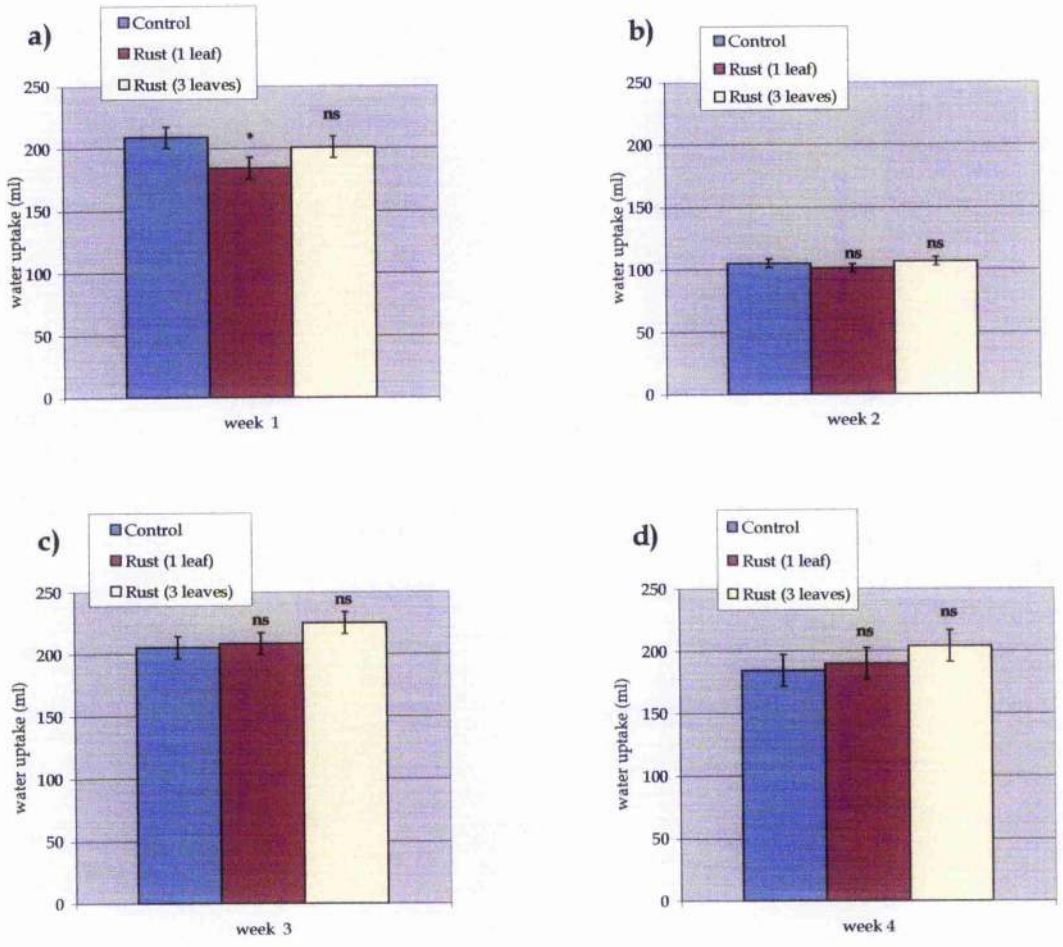


Figure 27. (a-d) Effect of rust on water uptake of broad bean, 1 wk (a), 2 wk (b), 3 wk (c) and 4 wk (d) after rust inoculation. Bean plants were inoculated with rust on 0, 1 or 3 lowest leaves. Values are the mean ± SEM of 20 replicates. Significant differences from controls were determined using the Student's *t*-test and are shown as $P < 0.001$ ***, $P < 0.01$ **. $P < 0.05$ * and ns not significant.

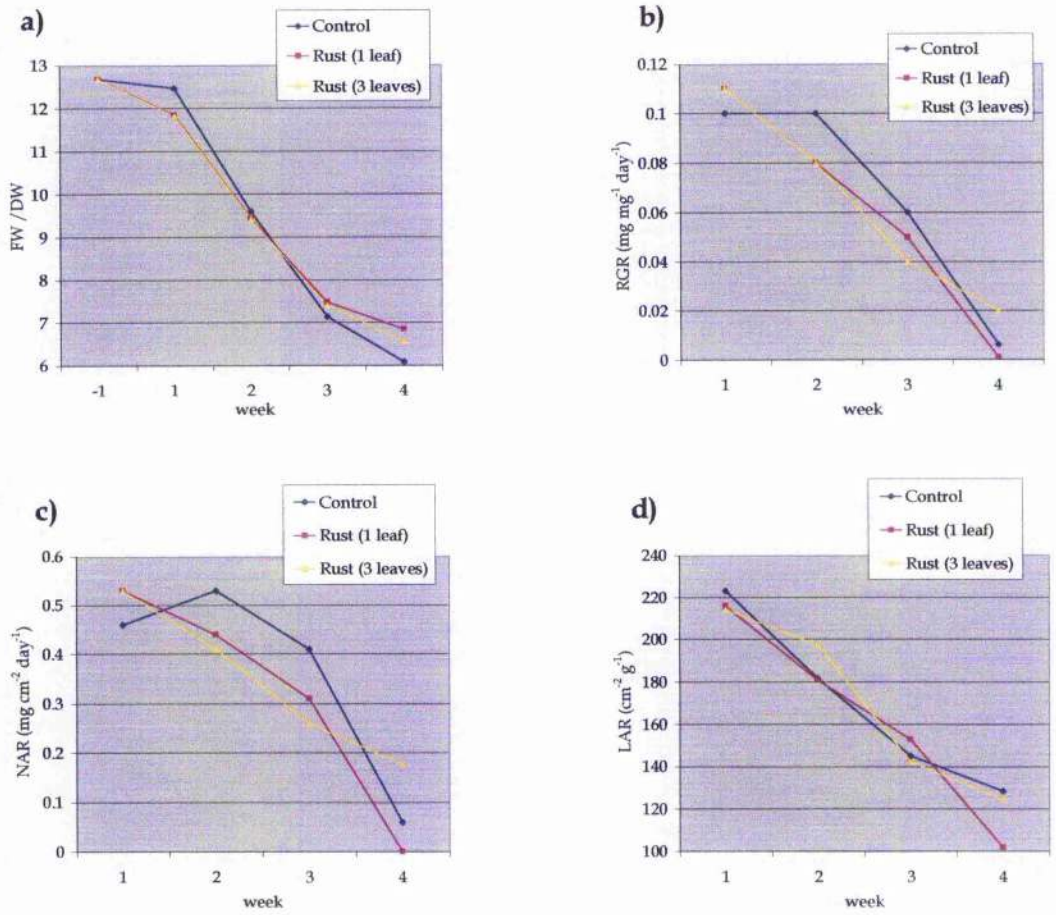


Figure 28. (a-d) Growth analysis of rust infected broad bean plants. Bean plants were inoculated with rust on 0, 1 or 3 lowest leaves. Plants were harvested prior to and 1 w, 2 w, 3 w and 4 w after pathogen inoculation and assessed for growth parameters. Effect of induced protection on fresh wt. / dry wt. ratio (a); relative growth rate (b); net assimilation rate (c); and leaf area ratio (d).

During the course of the experiment there was an overall decrease in all aspects of growth analysis considered, irrespective of treatment. There was very little difference observed in growth analysis variables between the different treatments. However, NAR and RGR were slightly higher in controls compared to plants infected with rust, but only at weeks 2 and 3. In addition, 4 wk after rust inoculation RGR, NAR and LAR were all lower in plants with one rust infected leaf compared to plants with three rust infected leaves (Figure 28a-d).

3.3.2 Effect of rust on chlorophyll fluorescence

The F_v (difference between F_o and F_m) declined in the rust infected leaf (leaf 3) from 2 wk after inoculation (Figure 31b). At week 4, F_v was largest in the healthy leaf on the rust infected plant (leaf 4) (Figure 33a-d).

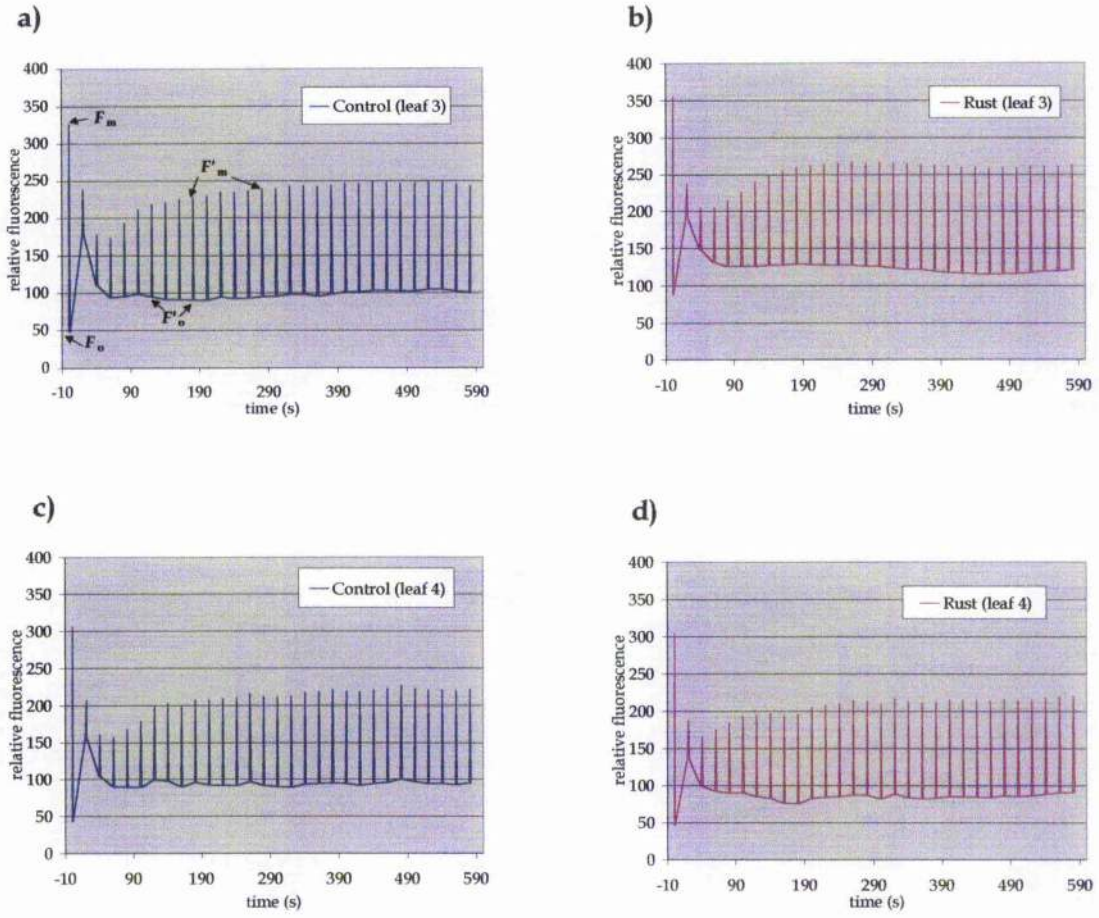


Figure 29 (a-d) Effect of rust infection on chlorophyll fluorescence in broad bean plants 3 d after rust inoculation. Controls were not inoculated with rust and treated plants were inoculated with rust on the lowest three leaves. Measurements were made on control leaf 3 (a) rust infected plant leaf 3 (b) control leaf 4 (c) and healthy leaf 4 on rust infected plants (d).

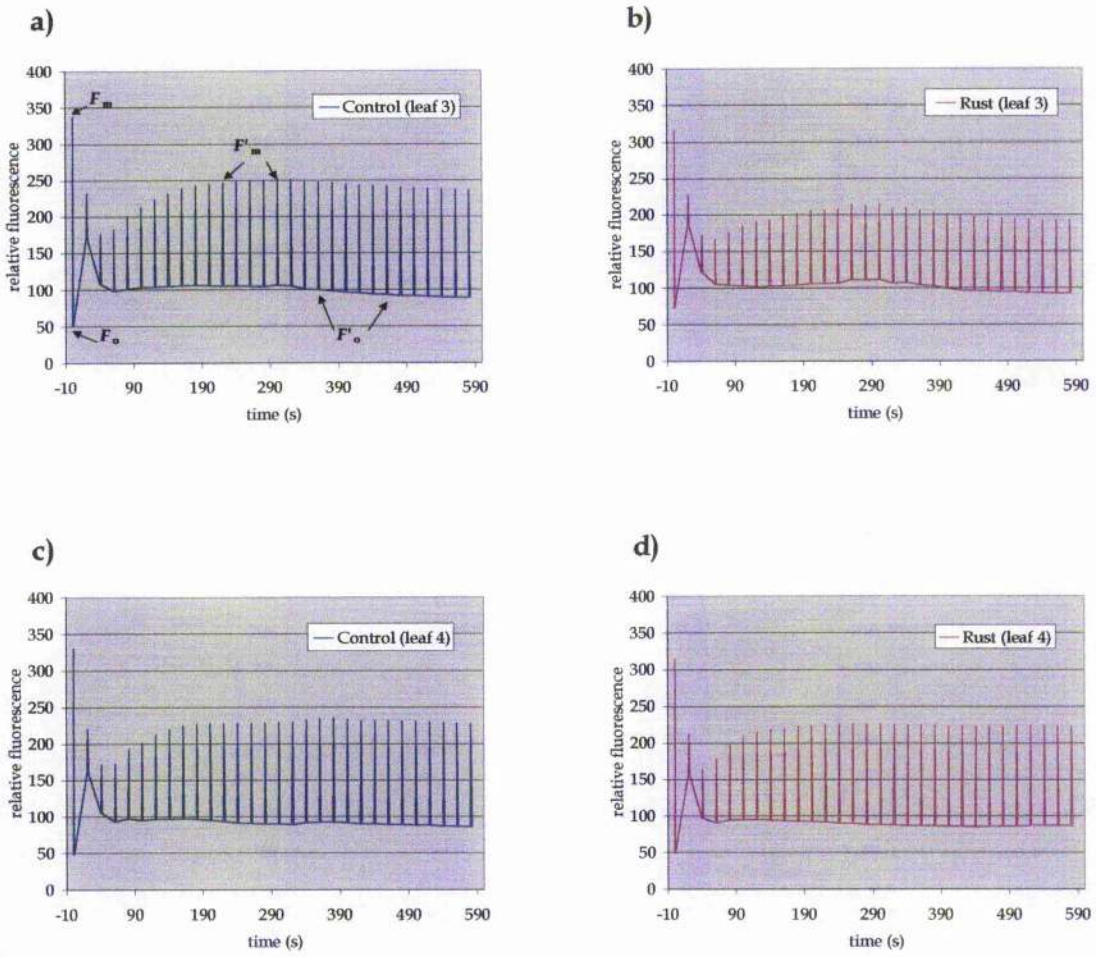


Figure 30. (a-d) Effect of rust infection on chlorophyll fluorescence in broad bean plants 1 wk after rust inoculation. Controls were not inoculated with rust and treated plants were inoculated with rust on the lowest three leaves. Measurements were made on control leaf 3 (a) rust infected plant leaf 3 (b) control leaf 4 (c) and healthy leaf 4 on rust infected plants (d).

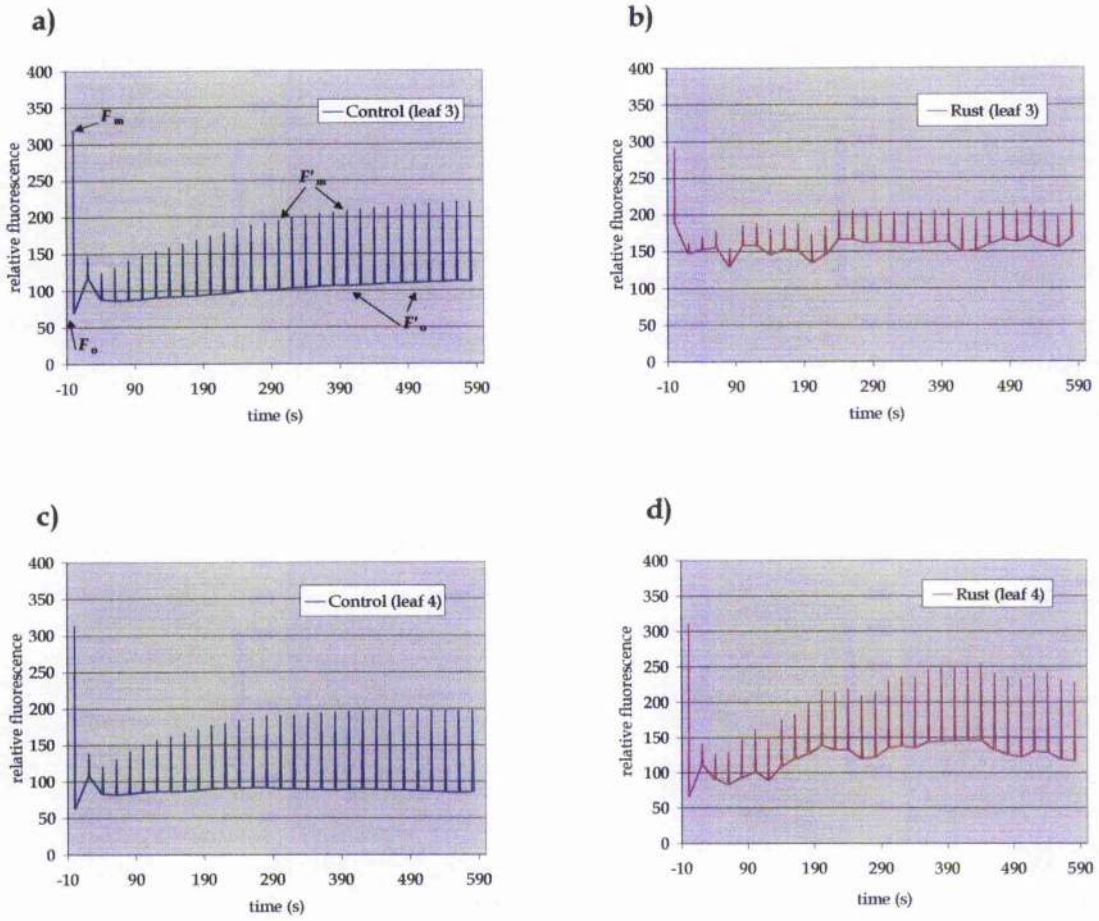


Figure 31. (a-d) Effect of rust infection on chlorophyll fluorescence in broad bean plants 2 wk after rust inoculation. Controls were not inoculated with rust and treated plants were inoculated with rust on the lowest three leaves. Measurements were made on control leaf 3 (a) rust infected plant leaf 3 (b) control leaf 4 (c) and healthy leaf 4 on rust infected plants (d).

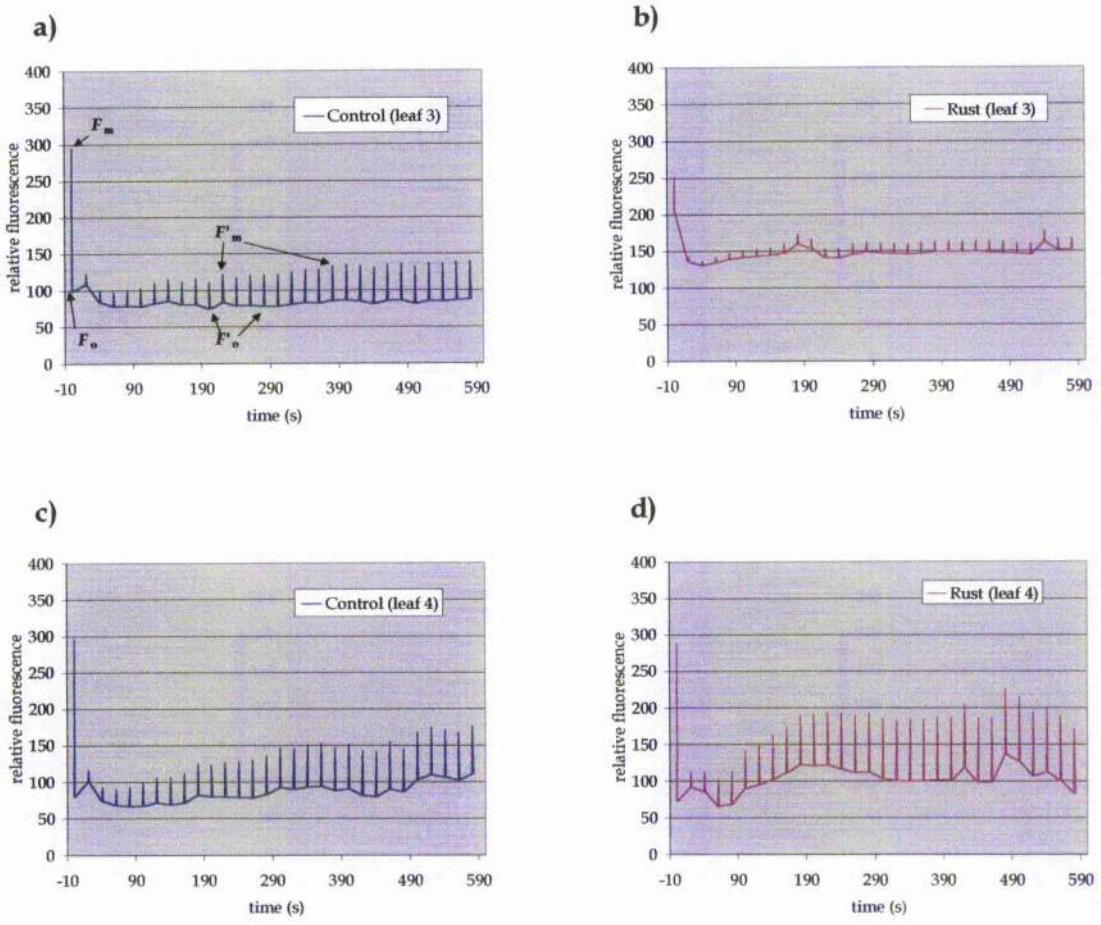


Figure 32. (a-d) Effect of rust infection on chlorophyll fluorescence in broad bean plants 3 wk after rust inoculation. Controls were not inoculated with rust and treated plants were inoculated with rust on the lowest three leaves. Measurements were made on control leaf 3 (a) rust infected plant leaf 3 (b) control leaf 4 (c) and healthy leaf 4 on rust infected plants (d).

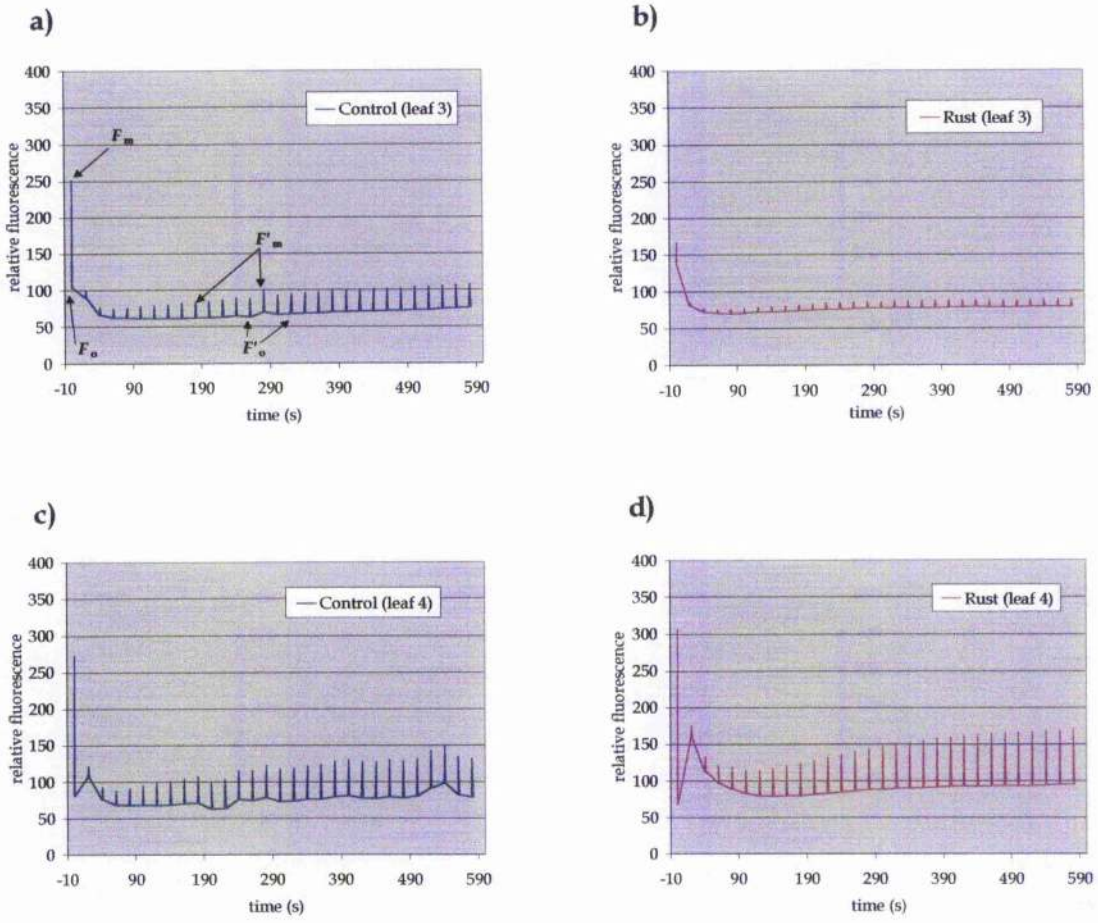


Figure 33(a-d) Effect of rust infection on chlorophyll fluorescence in broad bean plants 4 wk after rust inoculation. Controls were not inoculated with rust and treated plants were inoculated with rust on the lowest three leaves. Measurements were made on control leaf 3 (a) rust infected plant leaf 3 (b) control leaf 4 (c) and healthy leaf 4 on rust infected plants (d).

The F_v/F_m ratio for dark-adapted plants is a measure of photochemical efficiency. There were significant reductions in F_v/F_m in leaf 3 (infected leaf) of the rust infected plants from 1 w after rust inoculation. The ratio for dark-adapted healthy plants is normally 0.83 (Björkman and Demmig, 1987) and, therefore, the ratio was lower than expected from 2 wk after infection, irrespective of treatment or leaf position (Figure 34a-e).

F_o quenching (q_o) was observed throughout the experimental period. After 2w q_o was higher in infected leaves, compared with leaf 3 on the controls. However, the increase was only statistically significant 2 wk after inoculation (Figure 35a-e). Photoinhibition lowers F_m in a dark-adapted leaf below the level found for F_m in a dark-adapted leaf on a healthy plant. Photoinhibition was observed in infected leaves 1 wk and 4 wk after rust inoculation (Figure 36a-e).

Rust had no significant effect on photochemical quenching (q_p) (Figure 37a-e). However, non-photochemical quenching (q_N) was significantly increased in rust infected leaves, compared with the control leaf 3, 1 wk after inoculation. A reduction in q_N was observed 2 w after treatment in healthy leaves on infected plants (leaf 4) and in week 4 in rust infected leaves (leaf 3), compared with controls (Figure 38a-e).

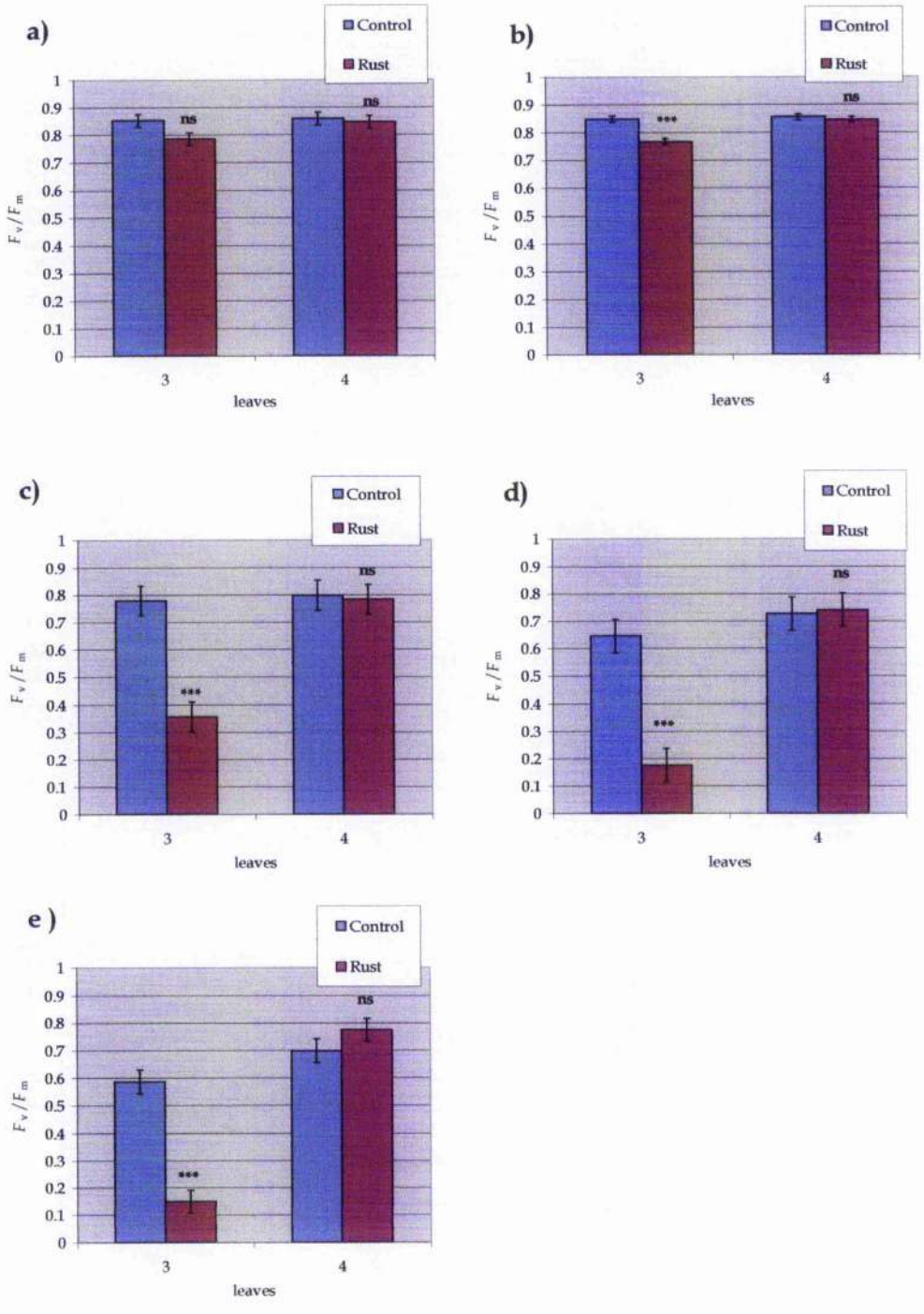


Figure 34 (a-e). Effect of rust infection on F_V/F_M ratio in broad bean plants 3 d (a), 1 wk (b), 2 wk (c), 3 wk (d) and 4 wk (e) after rust inoculation. Controls were not inoculated with rust and treated plants were inoculated with rust on the lowest 3 leaves. Measurements were made on control leaf 3, rust infected plant leaf 3, control leaf 4 and healthy leaf 4 on rust infected plants. Values are the mean ± SEM of 8 replicates, except week 4 when 6 replicates were used. Significant differences from controls were determined using the Student's *t*-test and are shown as $P < 0.001$ ***, $P < 0.01$ **. $P < 0.05$ * and ns not significant.

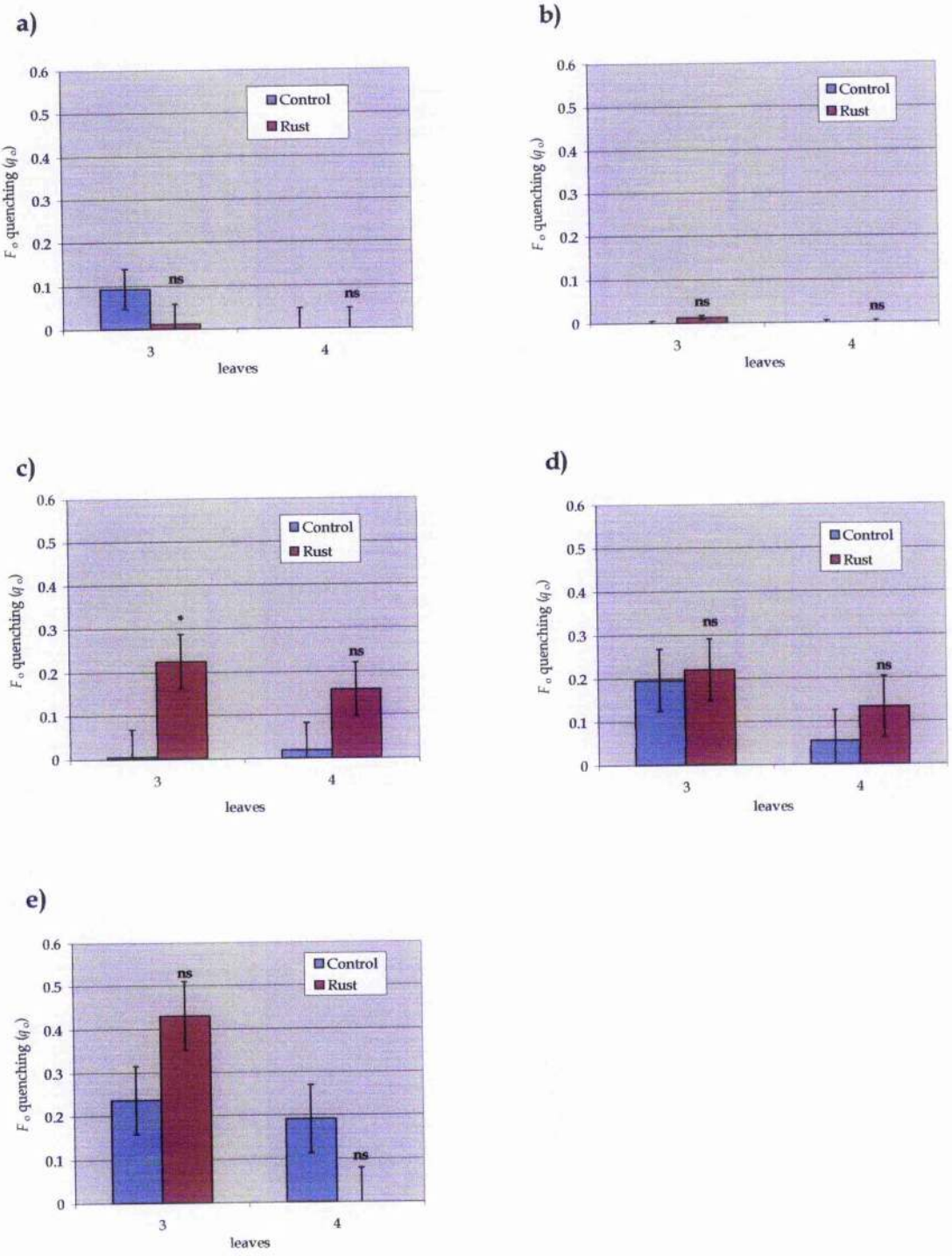


Figure 35 (a-e). Effect of rust infection on F_o quenching (q_o) in broad bean plants 3 d (a), 1 wk (b), 2 wk (c), 3 wk (d) and 4 wk (e) after rust inoculation. Controls were not inoculated with rust and treated plants were inoculated with rust on the lowest 3 leaves. Measurements were made on control leaf 3, rust infected plant leaf 3, control leaf 4 and healthy leaf 4 on rust infected plants. Values are the mean ± SEM of 8 replicates, except week 4 when 6 replicates were used. Significant differences from controls were determined using the Student's *t*-test and are shown as $P < 0.001$ ***, $P < 0.01$ **. $P < 0.05$ * and ns not significant.

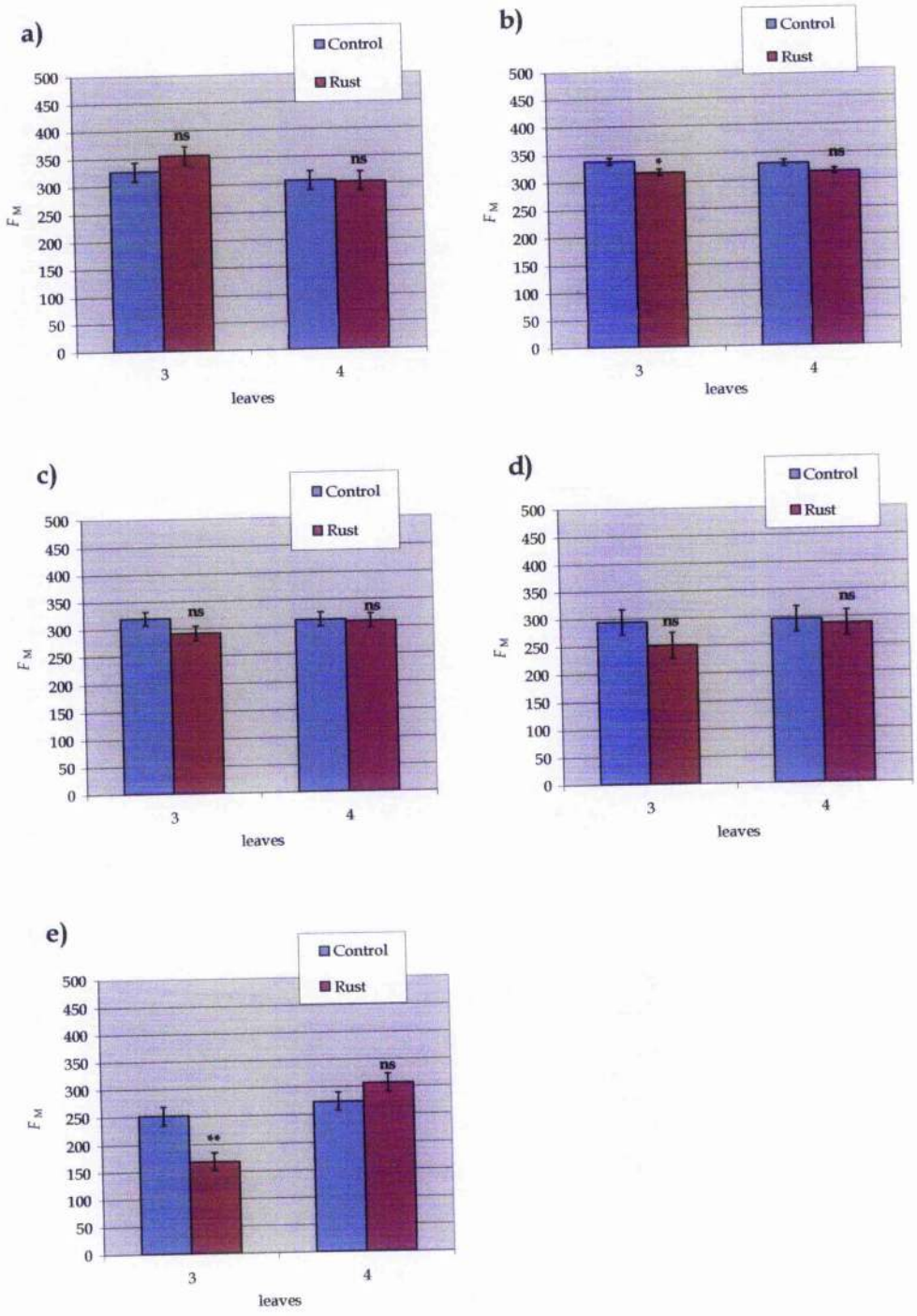


Figure 36 (a-e). Effect of rust infection on maximal fluorescence (F_M) in broad bean plants 3 d (a), 1 wk (b), 2 wk (c), 3 wk (d) and 4 wk (e) after rust inoculation. Controls were not inoculated with rust and treated plants were inoculated with rust on the lowest 3 leaves. Measurements were made on control leaf 3, rust infected plant leaf 3, control leaf 4 and healthy leaf 4 on rust infected plants. Values are the mean ± SEM of 8 replicates, except week 4 when 6 replicates were used. Significant differences from controls were determined using the Student's *t*-test and are shown as $P < 0.001$ ***, $P < 0.01$ **. $P < 0.05$ * and ns not significant.

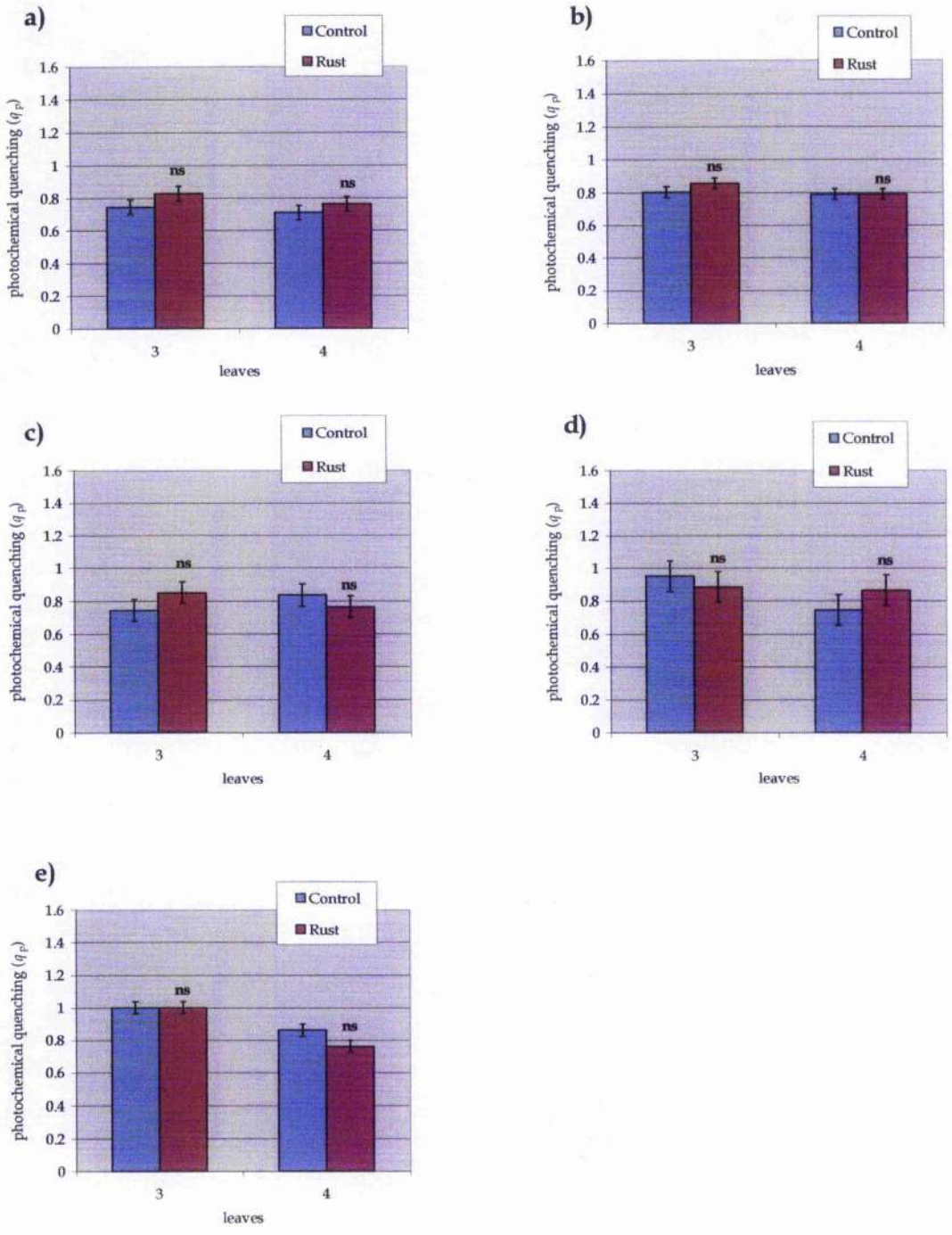


Figure 37 (a-e). Effect of rust infection on photochemical quenching (q_P) in broad bean plants 3 d (a), 1 wk (b), 2 wk (c), 3 wk (d) and 4 wk (e) after rust inoculation. Controls were not inoculated with rust and treated plants were inoculated with rust on the lowest 3 leaves. Measurements were made on control leaf 3, rust infected plant leaf 3, control leaf 4 and healthy leaf 4 on rust infected plants. Values are the mean ± SEM of 8 replicates, except week 4 when 6 replicates were used. Significant differences from controls were determined using the Student's *t*-test and are shown as $P < 0.001$ ***, $P < 0.01$ **. $P < 0.05$ * and ns not significant.

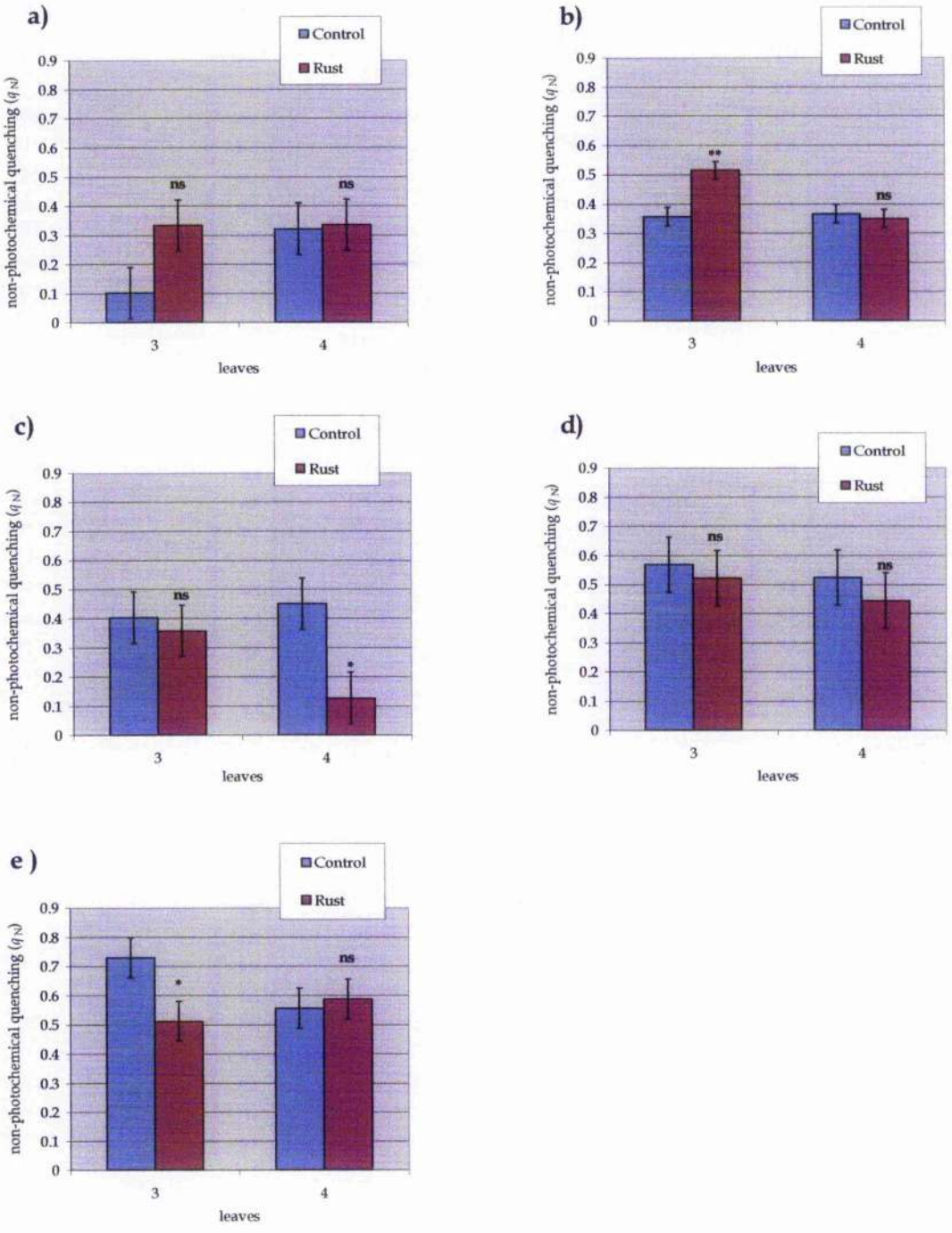


Figure 38 (a-e). Effect of rust infection on non-photochemical quenching (q_N) in broad bean plants 3 d (a), 1 wk (b), 2 wk (c), 3 wk (d) and 4 wk (e) after rust inoculation. Controls were not inoculated with rust and treated plants were inoculated with rust on the lowest 3 leaves. Measurements were made on control leaf 3, rust infected plant leaf 3, control leaf 4 and healthy leaf 4 on rust infected plants. Values are the mean ± SEM of 8 replicates, except week 4 when 6 replicates were used. Significant differences from controls were determined using the Student's *t*-test and are shown as $P < 0.001$ ***, $P < 0.01$ **. $P < 0.05$ * and ns not significant.

3.4 Discussion and Conclusions

There was an increase in fresh weight, dry weight and total leaf area, in plants with 3 leaves infected with rust, 1 wk after inoculation. There was also a decrease in these 3 growth parameters in plants with 3 leaves rust infected, 3 wk after inoculation, athough only the decrease in dry weight was statistically significant. Both the initial increase in growth followed by a decrease is consistent with the findings of Scholes (1992), who found a transient increase in photosynthesis followed by a subsequent decrease in rust infected leaves. However, there was also no significant effect on growth in weeks 2 or 4, and no significant effect on the number of leaflets throughout the experimental period.

Water uptake increased in rust infected plants from 3 wk after rust inoculation, although the only significant effect was a reduction in water uptake 1 wk after inoculation, in plants with 1 rust infected leaf. Certainly, the increase in water uptake 3 wk after rust inoculation is consistent with increased transpiration due to rupture of the cuticle caused by fungal sporulation (Johnson and Miller, 1934, 1940; Murphy, 1935; Duniway and Durbin, 1971). During the early stages of rust infection, inhibition of stomatal opening may account for the reduction in water uptake observed (Duniway and Durbin, 1971). In contrast, Bassanezi *et al.* (2002) found no changes in either the transpiration rate or stomatal conductance during rust infection of French bean. However, in infection by the angular leaf spot and anthracnose pathogens of French bean, both transpiration and stomatal conductance were found to decrease during lesion development (Bassanezi *et al.*, 2002). Although water uptake is directly related to plant fresh weight and total leaf area, no parallels between the pattern of water uptake and these two aspects of plant growth were observed in the present work.

There was a general decline in all derived growth analysis variables over the experimental period. Photosynthesis decreases as plants age but respiration does not, and so NAR declines with time. This is due to the increasing number of older leaves that are senescing, with lower rates of photosynthesis and increasing respiration. Thorne (1960) found that NAR declined with time in barley, potato and sugar beet, regardless of environmental conditions.

Only very limited effects on growth analysis were observed as a result of rust infection. Surprisingly, 4 wk after rust inoculation, RGR and NAR were highest in plants with 3 leaves infected with rust and lowest in plants with only 1 leaf infected. This suggests that at week 4, net photosynthesis was higher in plants with 3 leaves infected than either the controls or those plants with only 1 leaf infected. Although, a transient increase in photosynthesis may occur in rust infected leaves, this is followed by a subsequent reduction in the rate of photosynthesis (Scholes, 1992). Bassanezi *et al.* (2002) also found that rust, angular leaf spot and anthracnose all reduced net photosynthesis and increased dark respiration in French bean leaves after the appearance of visible symptoms of the diseases. They also found that differences in net photosynthesis and dark respiration in infected leaves compared with healthy leaves increased as the diseases progressed. However, to compensate, photosynthesis has been found to increase in the uninfected leaves on an infected plant (Livne, 1964; Murray and Walters, 1992). It was, therefore, surprising that 4 wk after inoculation, NAR was higher in the rust infected plants with the lower proportion of healthy leaves, i.e. those with 3 leaves infected. It is possible that the level of increase in photosynthesis in the upper healthy leaves of infected plants may be affected by the severity of infection in the lower leaves.

Another confounding factor was the increased senescence apparent in control plants at week 4. Plants for this experiment were grown in 13 cm pots and it is possible that nutrient availability became a limiting factor just prior to the final harvest. Consequently, for all subsequent bean experiments the plants were grown in 2 l pots. However, due to senescence and the number of shrivelled and dropped leaves, replication was reduced in the final harvest from 20 to 10 replicates for each of the infected treatments, and to only 3 replicates for controls. It is surprising that controls were more susceptible to senescence as there was no major increase in growth of the controls, compared with infected plants, to justify a higher nutrient usage and, therefore, more rapid depletion of the available nutrients. However, NAR and RGR were higher in controls at weeks 2 and 3 compared to the infected plants. Conversely, there was no major reduction in growth of the infected plants to suggest that they had been more conservative in nutrient uptake. In addition, it is assumed that demand for nutrients by the pathogen would have been more likely to hasten senescence in infected plants.

The F_v/F_m ratio determines the proportion of absorbed energy that is used in photochemistry. Therefore, it measures the photochemical efficiency of open PSII reaction centres (Jones, 1992). Non-photochemical quenching will reduce this ratio. However, non-photochemical quenching that takes in excess of 1 h or so to relax in a dark adapted leaf is usually designated as photoinhibition (Jones, 1992). The F_v/F_m ratio was significantly reduced in the dark-adapted rust infected leaf from 1 wk after inoculation, indicating that rust infection was affecting photochemical efficiency of PSII reaction centres. This is similar to the findings of Nogues *et al.* (2002) that, during the early stages of infection of tomato with *Fusarium oxysporum*, there was no significant damage to PSII, but that the efficiency of PSII photochemistry decreased as the disease

progressed. Bauer *et al.* (2000) also found that the efficiency of PSII was impaired in Norway spruce seedlings as symptoms of the rust, *Chrysomyxa rhododendri*, became apparent.

The optimal F_v/F_m ratio for dark-adapted healthy plants is usually approximately 0.83 (Björkman and Demmig, 1987). However, the F_v/F_m ratio was slightly below the optimal level from week 2, and reduced further in week 3 and 4, irrespective of treatment. It is possible that this was an early indication of the onset of senescence.

In plants that are stressed it is possible for F_o (minimal fluorescence) to be quenched. This occurs when the value for F_o subsequently falls below that produced immediately after illumination of the dark-adapted leaf. F_o quenching became particularly apparent 2 wk after treatment in the rust infected leaves. From 1 wk after inoculation quenching was higher in the rust infected leaf 3 compared to the control leaf 3, although the difference was only statistically significant in week 2. From week 3, F_o quenching was also more evident in control leaves and was possibly due to nutrient stress resulting in senescence. Similarly, Bassanezi *et al.* (2002) found that in bean leaves with angular leaf spot, minimal fluorescence was reduced.

Photoinhibition (q_I) is a form of quenching that results from damage to the photosynthetic system. Consequently, the damage sustained is generally considered to either permanent or only slowly reversible (Jones, 1992). Reductions in F_m (maximum fluorescence) in a dark adapted leaf, below that found in a healthy plant are the result of photoinhibition. Obviously, if the controls were also subject to stress, such as limitation in nutrients toward the end of the experiment, it is likely they were also affected by q_I.

Certainly, a progressive decline in F_m was observed from 1 wk after treatment, in the controls (Figure 36). In addition, F_m was lower in the rust infected leaf compared with leaf 3 on the control plants from 1 wk after inoculation, although the decrease was only significant at week 1 and 4. Similarly, Bassanezi *et al.* (2002) found that maximal fluorescence and optimum quantum yield of PSII were reduced in bean leaves infected with rust, angular leaf spot or anthracnose.

Photochemical quenching (q_p) is an indication of the redox state of the primary electron acceptor. If the electron acceptor is fully reduced, the photosystem II (PS II) reaction centres are closed, the maximal amount of incident energy is, therefore, emitted as fluorescence and quenching is low. In contrast, if the primary electron acceptor is oxidised, the PSII reaction centres are open to accept excitation energy and, consequently, a minimal amount of excitation energy is emitted as fluorescence and quenching is high (Jones, 1992). Photochemical quenching varies from zero, when the primary electron acceptor is fully reduced to 1 when it is oxidised. Photochemical quenching was high throughout the experimental period and was not affected by rust infection. In French bean leaves infected with rust, angular leaf spot and anthracnose, assessment of chlorophyll fluorescence by Bassanezi *et al.* (2002) suggested that in healthy areas of infected leaves there was no effect on electron transport capacity and generation of ATP and NADPH. However, in areas of leaf that were visibly diseased fluorescence was reduced. In leaves with angular leaf spot, minimal fluorescence was reduced and all three diseases reduced maximal fluorescence and the optimal quantum yield of PSII (Bassanezi *et al.*, 2002).

In high light conditions, when plants are stressed photosynthesis is inhibited. Non-photochemical quenching (q_N), or down regulation, dissipates excess energy absorbed by the chloroplasts that cannot be used for photosynthesis. Without quenching this excess energy would result in increased photoinhibitory and photobleaching damage (Jones, 1992). This may involve energy-dependent quenching which relies upon the pH gradient across the thylakoid membrane or the degree of energisation of the thylakoids. An increase in the pH gradient is thought to result in the dissipation of radiant energy as heat, although the mechanism is not fully understood. Alternatively, some of the excitation energy may be transferred from PSII to PSI. The third main method of non-photochemical quenching is thought to involve carotenoids within the chloroplast. Production of zeaxanthin, which quenches excitation in the antenna chlorophyll, is increased as a result of excess light (Jones, 1992).

Omari *et al.* (2001) found that infection of oak with the fungal pathogens *Cryphonectria parasitica* or *Phomopsis* resulted in higher levels of q_N. In the present work, non-photochemical quenching was significantly increased in the rust infected leaf 1 wk after rust inoculation. From 2 wk after treatment, q_N was found to be higher in control leaves compared with leaves on the rust infected plants, except in healthy leaves on the rust infected plant at week 4. However, the increases in q_N in control leaves were only statistically significant in leaf 4 at 2wk and in leaf 3 at 4 wk. In addition, there was a progressive increase in q_N in control leaves during the course of the experiment. Similarly, Scholes and Rolfe (1996) also found, in rust infected oats, that q_N was much lower in infected leaves compared to healthy leaves 5 d after inoculation. However, 8 d after infection, although q_N remained low in the parts of the leaf infected with the

pathogen, in uninfected areas of the infected leaf q_N was higher than that found in healthy leaves.

Scholes and Rolfe (1996) also found PSII photochemical efficiency was reduced in rust infected oat leaves. They suggested that this might result from interference with the trans-thylakoid pH gradient, such as increased demand for ATP or the production of an uncoupler by the pathogen. However, it is unlikely that biotrophic fungal pathogens produce the necessary metabolites to uncouple electron transport from the production of ATP (Scholes and Rolfe, 1996). Scholes and Rolfe (1996) also identified a transient increase in non-photochemical quenching in uninfected regions of an infected leaf, which also suggested the absence of an uncoupling agent. Furthermore, in isolated chloroplasts from mildewed sugarbeet leaves and rust infected broad bean leaves, the ratio of ATP formed to reduced NADP, indicated a reduction in the rate of non-cyclic electron transport without affecting photophosphorylation (Montalbini and Buchanan, 1974; Magyarosy *et al.*, 1976; Scholes and Rolfe, 1996). However, infection by biotrophic pathogens usually stimulates host cell respiration and host metabolic activity (Daly, 1976; Mitchell, 1979; Scholes and Farrar, 1986; Roberts and Walters, 1988). Increases in respiration may provide the energy and carbon skeletons required for enhanced biosynthetic activity in infected leaves and, therefore, result in an increased demand for ATP (Scholes and Rolfe, 1996). Scholes and Rolfe (1996) found that changes in non-photochemical quenching showed that infection altered the trans-thylakoid ΔpH, which they suggest indicates high metabolic activity and an increased demand for ATP. However, contrary to this, Scholes and Rolfe (1996) found in uninfected regions of the infected leaf non-photochemical quenching was enhanced.

3.4.1 Conclusions

Fresh weight, dry weight and total leaf area were increased in week 1 and reduced in week 3 after inoculation, in plants with 3 leaves infected with rust. This may be consistent with a transient increase followed by a decrease in photosynthesis that is thought to occur in rust infected leaves (Scholes, 1992). There was no apparent link between water uptake and plant growth. Changes in water uptake appeared to be consistent with inhibition of stomatal opening in the early stages of infection and later, increased transpiration due to rupture of the cuticle by fungal sporulation. Rust infection did result in F_o quenching and photoinhibition as apparent in the reduced values for F_m. Non-photochemical quenching was higher in controls compared with leaves on rust infected plants, except in healthy leaves on the rust infected plant at week 4. This is indicative of increased metabolic activity in the infected tissue, consistent with the findings of Scholes and Rolfe (1996). In addition, rust infection was found to affect the photochemical efficiency of PSII reaction centres from 1 wk after rust inoculation. Scholes and Rolfe (1996) also found that 5 d after inoculation PSII photochemical efficiency was slightly reduced in infected areas of rust infected oat leaves, but that values were comparable with healthy leaves in uninfected areas of the infected leaves. However, by 8 d after inoculation PSII efficiency was reduced throughout the infected leaf, although the reduction was greatest in visibly infected areas of the leaf. Scholes and Rolfe (1996) also observed variation in q_N within the leaf dependent on whether disease symptoms were visible or not and also the length of time that had elapsed since inoculation. Therefore, the relationship between pathogen infection and plant growth may be more complex than a simple relationship between the proportion of leaves within a plant that are infected and those that are not.

Chapter 4

Chemical Induced Systemic Protection

4 Chemical Induced Systemic Protection

4.1 Introduction

If the potential that exists in plants to defend themselves is to be utilised it will be necessary to establish the associated costs in terms of plant fitness, growth, yield, effects on the nutritional value of the crop and even possible resultant toxicity. One such study involving *Arabidopsis* transformed to constitutively express systemic resistance resulted in stunted growth (Bowling *et al.*, 1994). Heil *et al.* (2000) have also studied the effects of the SAR activator BION on the growth and fitness of spring wheat. They found that induction of systemic resistance on uninfected plants resulted in reduced growth and /or seed production. Contrary to this are the findings of Mitchell and Walters (1995) on the effects of methyl jasmonate on barley yield. Methyl jasmonate applied to barley in a field experiment was found to reduce mildew levels, and although plant height was reduced, grain yield was significantly increased.

A number of chemicals have been identified that induce systemic protection including salicylic acid (SA), 2,6-dichloroisonicotinic acid (INA), β-aminobutyric acid, BION (a benzothiadiazole, Sygenta, Germany), phosphate, and unsaturated fatty acids. The four chemicals chosen for this study are phosphate, aminolevulinic acid (ALA), salicylic acid (SA) and saccharin. Phosphate has been shown to induce resistance to *Colletotrichum lagenarium* in cucumber (Gottstein and Kuć, 1989) and rust in broad bean (Walters and Murray, 1992). SA is known to be involved in the endogenous signalling response that occurs with SAR. Exogenous applications of SA have been found to induce systemic resistance in tobacco, cucumber and barley (Metraux *et al.*,

1990; Rasmussen *et al.*, 1991; Walters *et al.*, 1993). Siegrist *et al.* (1998) were the first to identify saccharin as an inducer of systemic resistance in cucumber, tobacco and French bean. To date no further examples of the use of saccharin as an inducer of systemic resistance are known to exist. The fourth chemical chosen, ALA has no previous history as an inducer of systemic protection. It was decided to test ALA to check if production of a necrotic response was sufficient to induce a pathogen defence response, as it has been suggested that at sufficiently high concentrations ALA may cause necrosis.

4.1.1 Objectives

These experiments examine the efficacy of different chemicals in inducing systemic protection and their subsequent effect on plant growth and development. In addition, a comparison is made between the effectiveness of two methods of chemical application and any resulting differences in plant growth.

4.2 Materials and Methods

4.2.1 Growing conditions

Plants were grown in a ventilated glasshouse as previously described (section. 2.2.1)

4.2.2 Maintenance of pathogens

Powdery mildew was maintained on barley and rust on broad bean as previously described (sections. 2.2.2.2 and 2.2.3.2).

4.2.3 Chemical induction of systemic protection in barley (phosphate, ALA, salicylic acid and saccharin)

Barley seeds (*Hordeum vulgare* L. cv. Golden Promise) were sown in 10 cm pots, 1 plant per pot, in Levington M3 compost. To minimise environmental effects on results a random experimental design was created in Minitab 12.

An experiment on saccharin induced systemic protection had already been undertaken. However, the opportunity was taken in this experiment to include the effects of pathogen challenge 1, 2 and 3 d after treatment with saccharin. These particular challenge times had not been considered in the previous saccharin experiment.

× **4** Treatments, phosphate, ALA, SA, controls (i.e. water)

× **6** Pathogen challenge at 1, 2, 3, 6, 10, and 14 d after treatment

× **11** replicates

264 plants required

× 1 Treatment, saccharin

× 3 Pathogen challenge at 1, 2, and 3 d after treatment

× 11 replicates

33 plants required

297 Total plants required

4.2.3.1 Treatment and challenge inoculation

Solutions of 10 mM potassium phosphate, 3 mM ALA, 15 mM SA, 3 mM saccharin or distilled water (for controls) containing 0.01% Tween 20 were applied to the first leaves of barley seedlings, using a soft camel hair brush. Following treatment, plants were watered from the base into a saucer. Barley plants, both treated and controls, were challenge inoculated with powdery mildew at 1, 2, 3, 6, 10 and 14 d after treatment of first leaves. The exception to this was that saccharin treated plants were only challenged 1, 2 and 3 d after application of saccharin, as additional challenge times were considered in a previous experiment. The barley plants were inoculated with mildew, by placing them in an infection chamber and dusting with powdery mildew conidia.

4.2.3.2 Harvest

Plants were destructively harvested and assessed for infection and growth 10 d after pathogen challenge. Infection intensity and leaf area were determined using a scanner and the Winfolia leaf analysis software (Regent, Canada).

4.2.4 Saccharin applied to leaves and as a drench to induce systemic protection in barley

This experiment compared two different methods of chemical application. Barley seeds (*Hordeum vulgare* L. cv. Golden Promise) were sown in 10 cm pots, 1 plant per pot, in Levington M3 compost. To minimise environmental effects on results a random experimental design was created in Minitab 12.

× **3**	Treatments, saccharin (leaf application), saccharin (applied as soil drench), and controls (i.e. water)
× **4**	Pathogen challenge at 6, 8, 10, and 14 d after treatment
× **11**	replicates
132	Total plants required

4.2.4.1 Treatment and challenge inoculation

Saccharin was applied as a soil drench, or to the first leaves of the barley seedlings using a soft camel hair brush. For the soil drench, each plant received either 30 ml of a 3 mM saccharin solution or 30 ml distilled water. For foliar application, the first leaf of each plant was treated with either 3 mM saccharin or distilled water. Following treatment, plants were watered from the base into a saucer. Barley plants, both treated and controls, were challenge inoculated with powdery mildew at 6, 8, 10 and 14 d after treatment of first leaves. The barley plants were inoculated with mildew, by placing them in an infection chamber and dusting with powdery mildew conidia.

4.2.4.2 Harvest

Plants were destructively harvested and assessed for infection and growth 10 d after pathogen challenge. Infection intensity and leaf area were determined using a scanner and the Winfolia leaf analysis software (Regent, Canada).

4.2.5 Phosphate induction of systemic protection in broad bean

Broad bean seeds (*Vicia faba* cv. Bunyard Exhibition) were sown in 2 l pots, 1 plant per pot, in Levington M3 compost. To minimise environmental effects on results a random experimental design was created in Minitab 12.

× **2**	Treatments, phosphate, and controls (i.e. water)
× **5**	Pathogen challenge at 1, 6, 10, 14 and 21 d after treatment
× **12**	replicates
120	Total plants required

4.2.5.1 Treatment and challenge inoculation

A solution of 10 mM potassium phosphate, or distilled water (for controls) was applied to the first leaves of broad bean plants, using a soft camel hair brush. Following treatment, plants were watered from the base into a saucer. Plants were challenge inoculated with rust at 1, 6, 10, 14 and 21 d after treatment of first leaves. The spore suspension contained 0.3 g of rust spores in 100 ml distilled water. The spore suspension was applied to the leaves using a soft camel hair brush. Upper leaves were then covered with a polythene bag for 24 h to maintain the high humidity required for

spore germination. The number of leaves challenged depended on the number of leaves present at challenge.

challenge 1 d	leaves 2-4 inoculated with rust
challenge 6 d	leaves 2-5 inoculated with rust
challenge 10 d	leaves 2-6 inoculated with rust
challenge 14 d	leaves 2-7 inoculated with rust
challenge 21 d	leaves 2-10 inoculated with rust

4.2.5.2 Harvest

Plants were destructively harvested and assessed for infection 14 d after pathogen challenge. Infection intensity and leaf area were determined using a scanner and the Winfolia leaf analysis software (Regent, Canada). Infection intensity was assessed on leaves challenged with rust. Assessment of growth was not made on plants challenged 1, 6, 10 and 14 d after treatment with phosphate. In hindsight, it seemed reasonable to measure whole plant growth. Therefore, information on plant growth is only available for plants challenged 21 d after application of phosphate.

4.2.6 Salicylic acid induction of systemic protection in broad bean

Broad bean seeds (*Vicia faba* cv. Aquadulce) were sown in 2 l pots, 1 plant per pot, in Levington M3 compost. To minimise environmental effects on results a random experimental design was created in Minitab 12.

× **2** Treatments, salicylic acid, and controls (i.e. water)

× **5** Pathogen challenge at 1, 2, 6, 10, and 14 d after treatment

× **11** replicates

110 Total plants required

4.2.6.1 Treatment and challenge inoculation

A solution of 15 mM salicylic acid, or distilled water (for controls) were applied to the first leaves of broad bean plants, using a soft camel hair brush. Following treatment, plants were watered from the base into a saucer. Plants were challenge inoculated with rust at 1, 2, 6, 10 and 14 d after treatment of first leaves. The spore suspension contained 0.3 g of rust spores in 100 ml distilled water. The spore suspension was applied to the leaves using a soft camel hair brush. Upper leaves were then covered with a polythene bag for 24 h to maintain the high humidity required for spore germination. The number of leaves challenged depended on the number of leaves present at challenge.

challenge 1 d	leaves 2 and 3 inoculated with rust
challenge 2 d	leaves 2 and 3 inoculated with rust
challenge 6 d	leaves 2-4 inoculated with rust
challenge 10 d	leaves 2-5 inoculated with rust
challenge 14 d	leaves 2-6 inoculated with rust

4.2.6.2 Harvest

Plants were destructively harvested and assessed for infection 14 d after pathogen challenge. Infection intensity and leaf area were determined using a scanner and the Winfolia leaf analysis software (Regent, Canada). Infection intensity was assessed on leaves challenged with rust.

4.2.7 Saccharin applied to leaves and as a drench to induce systemic protection in broad bean

Broad bean seeds (*Vicia faba* cv. Aquadulce) were sown in 2 l pots, 1 plant per pot, in Levington M3 compost. To minimise environmental effects on results a random experimental design was created in Minitab 12.

× **3**	Treatments, saccharin (leaf application), saccharin (applied as soil drench), and controls (i.e. water)
× **4**	Pathogen challenge at 1, 6, 10, and 14 d after treatment
× **10**	replicates
120	Total plants required

4.2.7.1 Treatment and challenge inoculation

Saccharin was applied as a soil drench, or to the first leaves of the bean plants using a soft camel hair brush. For the soil drench, each plant received either 200 ml of a 3 mM saccharin solution or 200 ml distilled water. For foliar application, the first leaf of each plant was treated with either 3 mM saccharin or distilled water. Following treatment,

plants were watered from the base into a saucer. Plants were challenge inoculated with rust at 1, 6, 10 and 14 d after treatment of first leaves. The spore suspension contained 0.3 g rust spores in 100 ml distilled water. The spore suspension was applied to the leaves using a soft camel hair brush. Upper leaves were then covered with a polythene bag for 24 h to maintain the high humidity required for spore germination. The number of leaves challenged depended on the number of leaves present at challenge.

challenge 1 d	leaves 2 and 3 inoculated with rust
challenge 6 d	leaves 2-5 inoculated with rust
challenge 10 d	leaves 2-6 inoculated with rust
challenge 14 d	leaves 2-7 inoculated with rust

4.2.7.2 Harvest

Plants were destructively harvested and assessed for infection 14 d after pathogen challenge. Infection intensity and leaf area were determined using a scanner and the Winfolia leaf analysis software (Regent, Canada). Infection intensity was assessed on leaves challenged with rust.

4.2.8 Statistical analyses

An analysis of variance (ANOVA) was applied to data, using the Genstat 5 statistical program (Lawes Agricultural Trust). The differences between the treatment and control means were tested for significance using the Student's *t*-test. Data are presented as means ± standard error (SEM).

4.3 Results

4.3.1 Chemical induced systemic protection of barley

Treatment with phosphate, salicylic acid (SA) and saccharin had a significant effect on the total % of leaf area infected with powdery mildew (Figure 39a-d). ALA had no significant effect on the % of leaf area infected with mildew (Figure 39b).

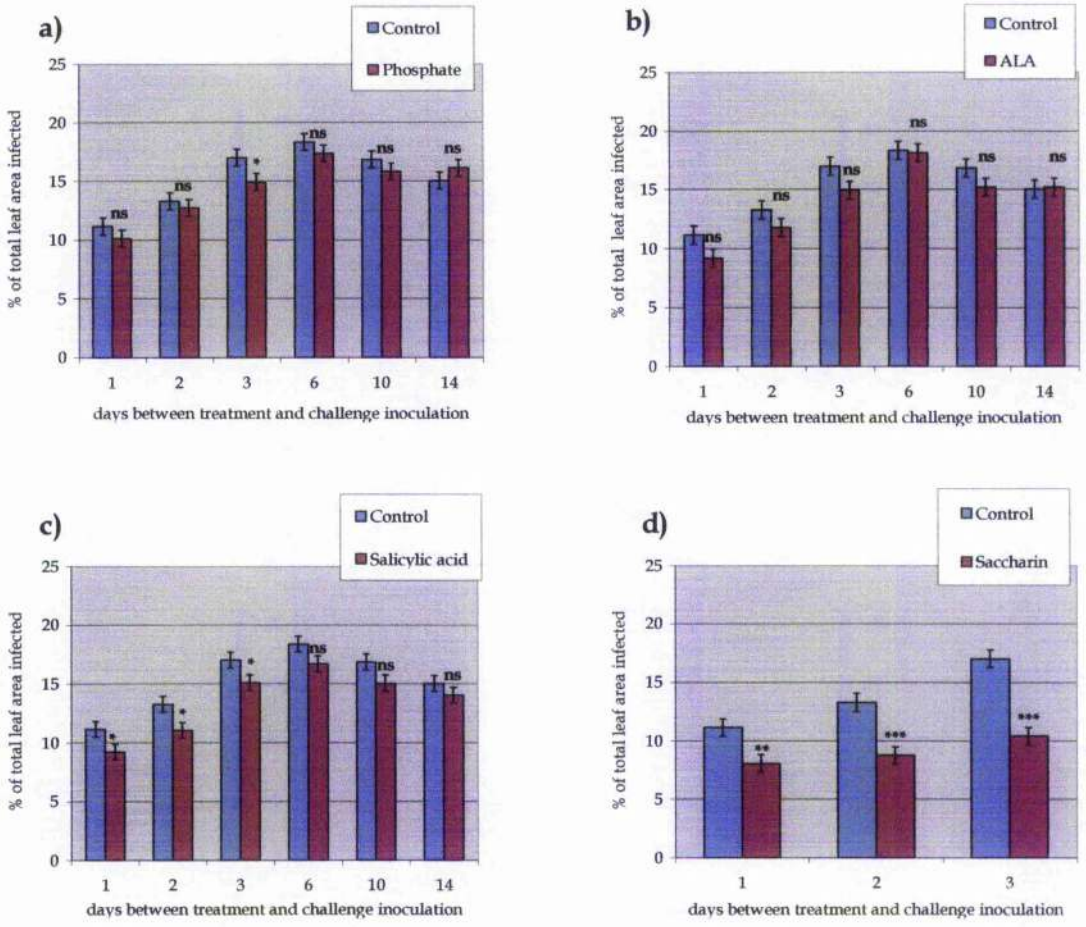


Figure 39. (a-d) Effect of phosphate (a), ALA (b), salicylic acid (c) and saccharin (d) induced systemic protection on % total barley leaf area infected with mildew. Plants were assessed for infection 10 d after pathogen challenge. Values are the mean ± SEM of 11 replicates. Significant differences from controls were determined using the Student's *t*-test and are shown as $P < 0.001$ ***, $P < 0.01$ **. $P < 0.05$ * and ns not significant.

Significant reductions in % infection were observed in SA treated plants challenged with mildew 1 d following treatment. Although % infection remained lower in SA treated plants throughout the experiment, the reduction was no longer significant if plants were challenged 6 d or more after treatment (Figure 39c). Treatment with phosphate reduced % infection in plants challenged 3 d after treatment (Figure 39a). Saccharin produced significant reductions in % infection regardless of the period between treatment and challenge (Figure 39d). Although plants were not challenged with mildew more than 3 d after treatment with saccharin in this experiment, in a subsequent experiment the % leaf area infected was also significantly reduced in plants challenged 6, 8, 10 and 14 d after treatment with saccharin (Figure 50 a).

Similarly, phosphate, SA and saccharin also had a significant effect on total leaf area infected, whereas ALA did not (Figure 40a-d). However, the effect was only significant in phosphate treated plants challenged 14 d after treatment and in SA treated plants challenged 10 d after treatment (Figure 40a, c). Saccharin significantly reduced the total leaf area infected with mildew, but the reduction was no longer significant in plants challenged 1 d after treatment (Figure 40d).

In phosphate treated plants, challenged 14 d after treatment, the % of leaf area infected was higher, but the total leaf area infected lower compared with controls. However, plant total leaf area was also significantly reduced in the phosphate treated plants challenged 14 d after treatment (Figure 44a).

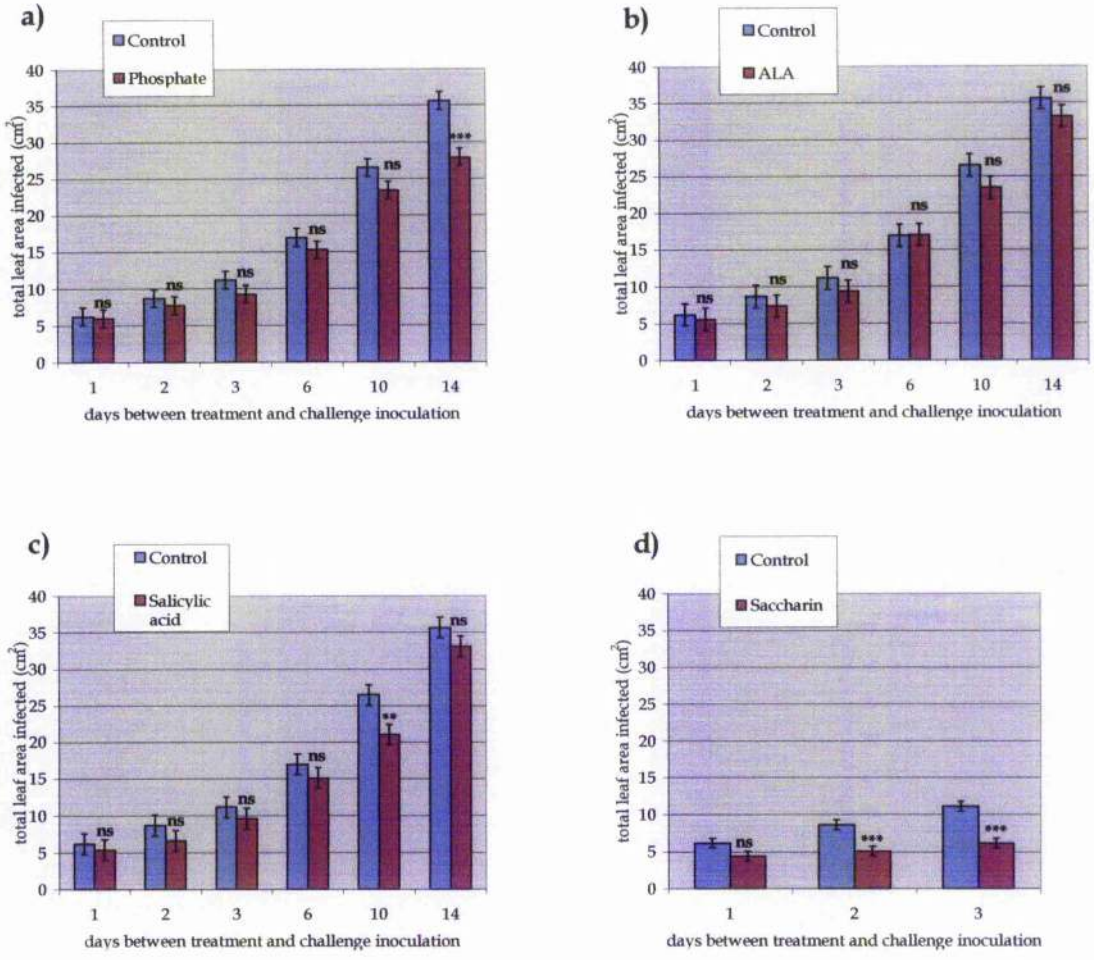


Figure 40. (a-d) Effect of phosphate (a), ALA (b), salicylic acid (c) and saccharin (d) induced systemic protection on total barley leaf area infected with mildew. Plants were assessed for infection 10 d after pathogen challenge. Values are the mean ± SEM of 11 replicates. Significant differences from controls were determined using the Student's *t*-test and are shown as $P < 0.001$ ***, $P < 0.01$ **. $P < 0.05$ * and ns not significant.

The % leaf area infected was not significantly reduced in plants challenged 10 d after treatment with SA, but there was a significant reduction in total leaf area infected. This similarly coincided with a reduction in total leaf area in plants 10 d after treatment with SA, although the reduction was not significant (Figure 44c).

Chemical treatments were applied to leaf 1 and the whole plant subsequently challenged with powdery mildew. All four chemical treatments significantly reduced infection in leaf 1 (the leaf to which the chemicals were applied) and, in addition, SA and saccharin also reduced infection in leaf 2 (Figure 41). There was a significant reduction in infection in leaf 1 in plants challenged more than 6 d after treatment with ALA (Figure 41d-f). Phosphate significantly reduced infection in leaf 1 compared with controls in plants challenged 3 d and 6 d after treatment (Figure 41c, d). However, infection on leaf 1 was significantly increased in plants challenged 14 d after phosphate treatment (Figure 41f), while there was no significant effect of phosphate on infection in leaves 2, 3 or 4 irrespective of the time between treatment and challenge (Figure 41a-f).

Infection was reduced in leaf 1 of plants challenged 2 d or more after treatment with SA. In addition, there was reduced infection in leaf 2 of plants challenged with mildew 14 d after treatment with SA, compared with controls (Figure 41b-f). Saccharin significantly reduced infection in leaf 1 in plants challenged 1, 2 and 3 d after treatment. Infection was also significantly reduced in leaf 2 in plants challenged 2 d after treatment (Figure 41a-c). In the subsequent experiment, saccharin applied to the first leaf was found to reduce mildew infection significantly in leaves 1 and 2 in plants challenged 6, 8, 10 and 14 d after treatment. However, there was no significant effect on mildew in leaves 3 and 4 irrespective of the time between treatment and challenge (Figure 41d-f, 51a-f).

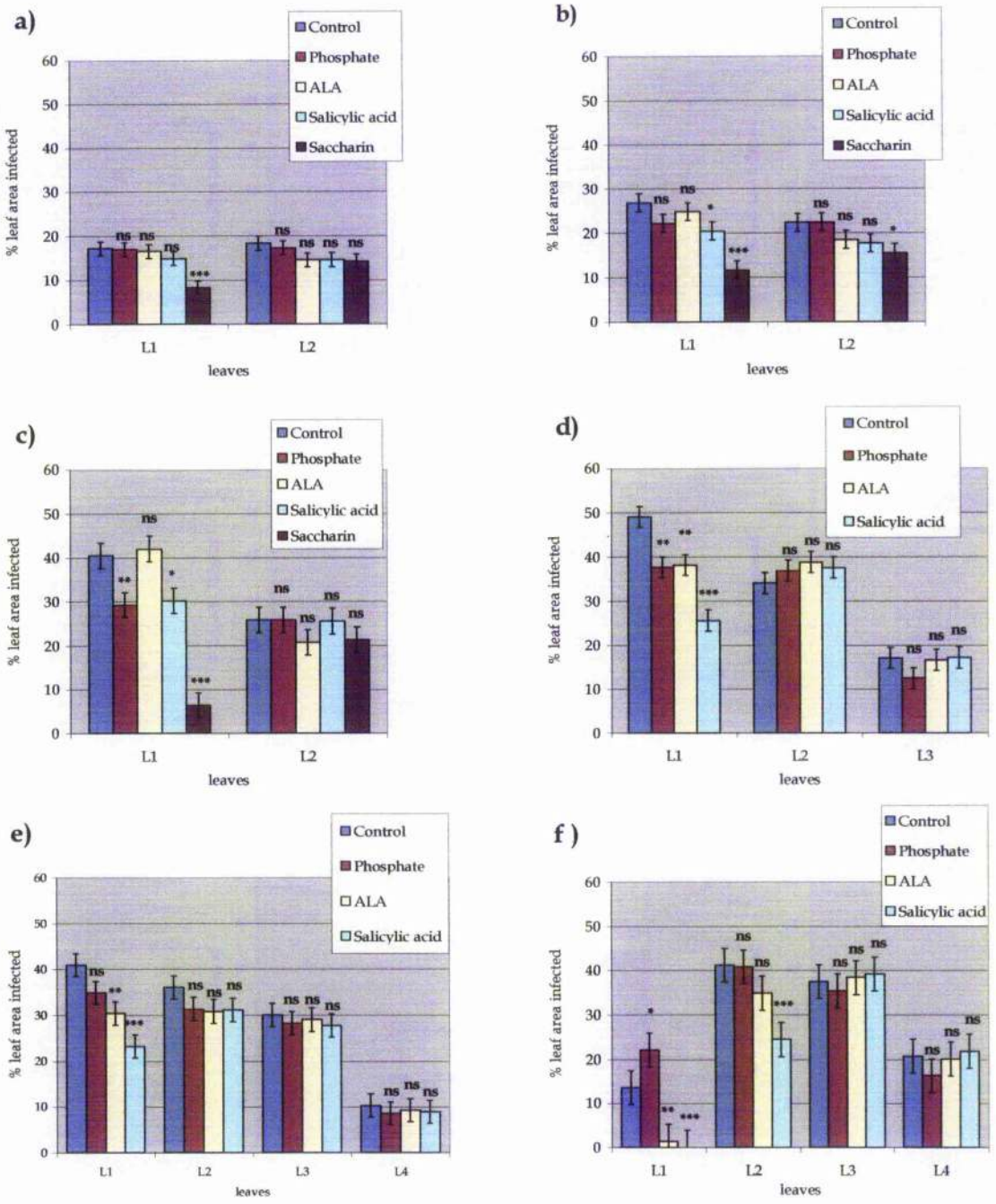


Figure 41. (a-f) Effect of chemical induced systemic protection of barley on % infection on leaves challenged with powdery mildew 1 d (a); 2 d (b); 3d (c), 6d (d), 10 d (e); and 14 d (f) after induction of systemic protection. Plants were assessed for infection 10 d after pathogen challenge. Leaf numbers refer to order in which leaves were produced on the plant. Systemic protection induced on L1 (first leaf); all leaves including the first leaf challenged with powdery mildew, L2 (second leaf); L3 (third leaf) and L4 (fourth leaf). Values are the mean ± SEM of 11 replicates. Significant differences from controls were determined using the Student's *t*-test and are shown as $P < 0.001$ ***, $P < 0.01$ **. $P < 0.05$ * and ns not significant.

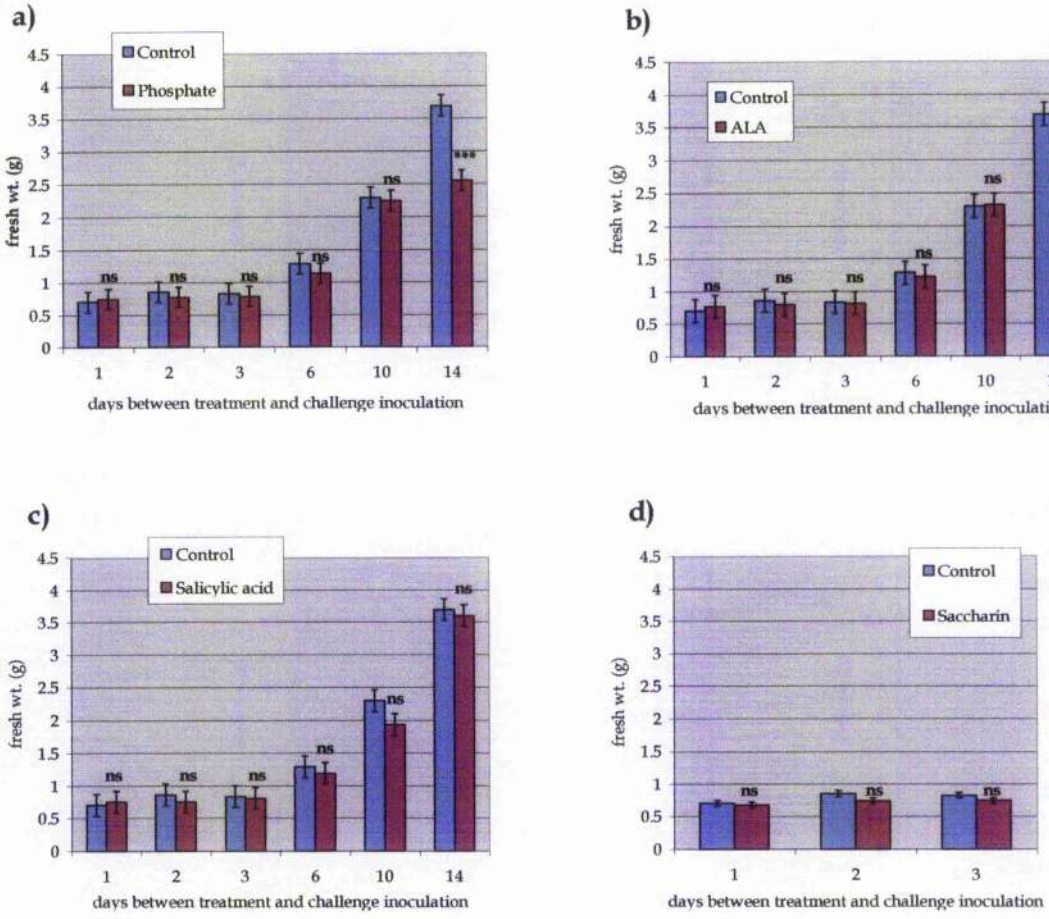


Figure 42. (a-d) Effect of phosphate (a), ALA (b), salicylic acid (c) and saccharin (d) induced systemic protection in barley on whole plant fresh weight. Plants were assessed for growth 10 d after pathogen challenge. Values are the mean ± SEM of 11 replicates. Significant differences from controls were determined using the Student's *t*-test and are shown as $P < 0.001$ ***, $P < 0.01$ **. $P < 0.05$ * and ns not significant.

Phosphate, ALA, SA and saccharin generally had no significant effect on whole plant fresh weight, whole plant dry weight or total leaf area, compared with controls. The exception to this was a reduction in fresh weight, dry weight and total leaf area in plants challenged with mildew 14 d after treatment with phosphate (Figure 42a, 43a, 44a). The numbers of leaves and tillers were similarly affected. However, in addition to the significant reduction in leaves and tillers in plants challenged 14 d after treatment with

phosphate, there was also a reduction in leaves and tillers in plants challenged 10 d after treatment with SA (Figure 45c, 46c).

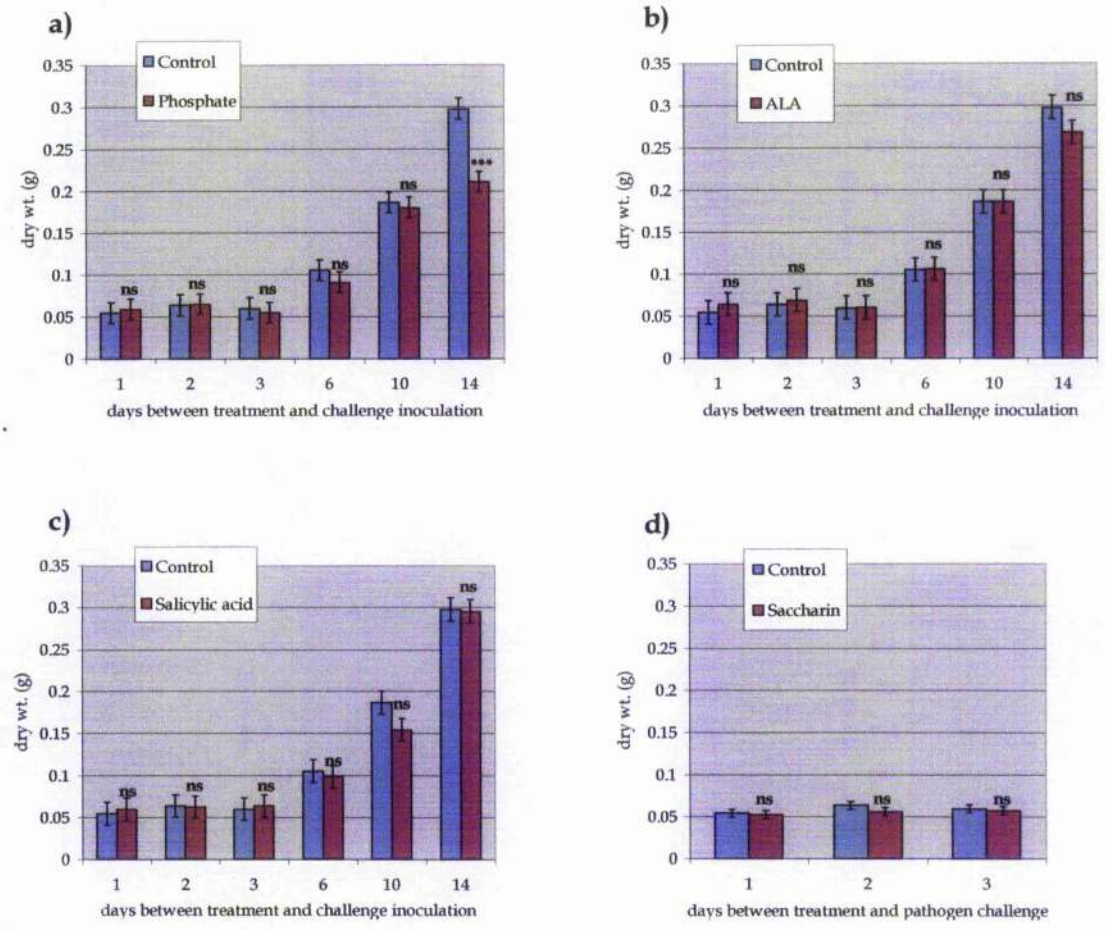


Figure 43. (a-d) Effect of phosphate (a), ALA (b), salicylic acid (c) and saccharin (d) induced systemic protection in barley on whole plant dry weight. Plants were assessed for growth 10 d after pathogen challenge. Values are the mean ± SEM of 11 replicates. Significant differences from controls were determined using the Student's *t*-test and are shown as $P < 0.001$ ***, $P < 0.01$ **. $P < 0.05$ * and ns not significant.

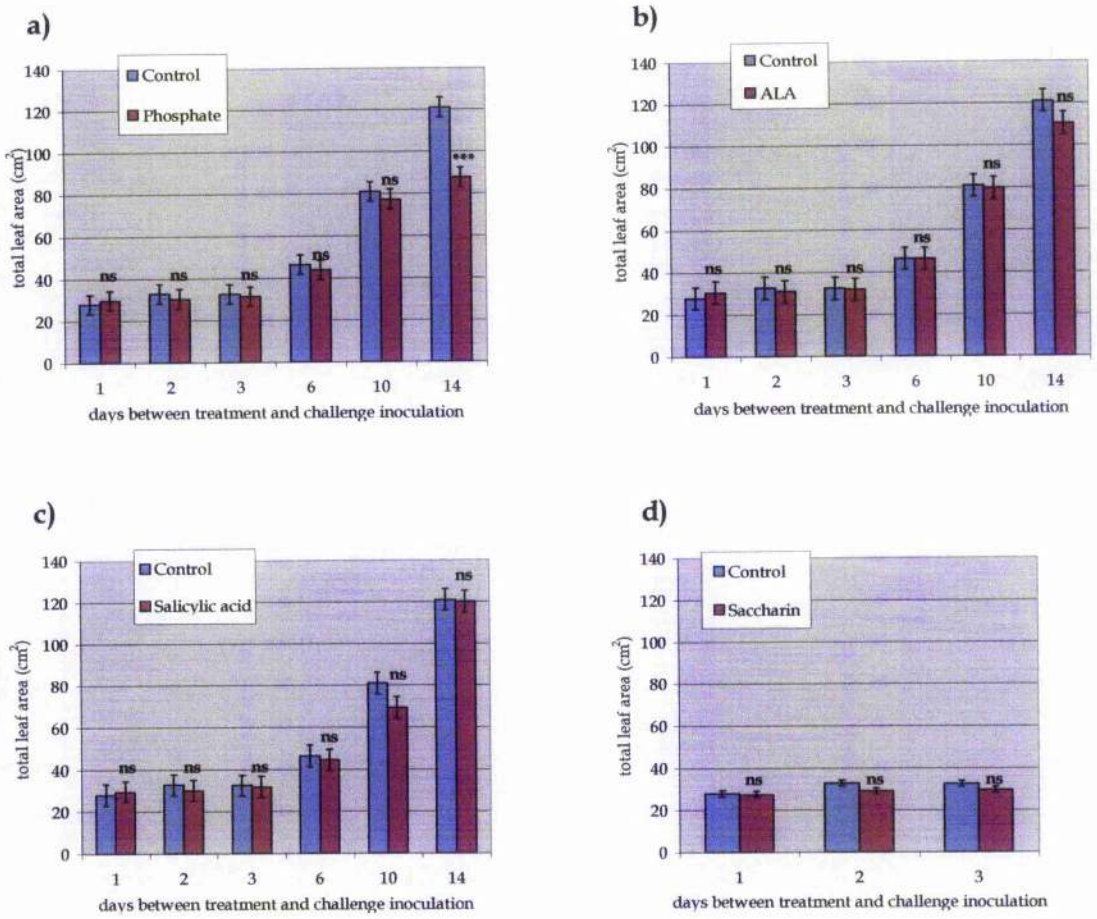


Figure 44. (a-d) Effect of phosphate (a), ALA (b), salicylic acid (c) and saccharin (d) induced systemic protection in barley on total leaf area. Plants were assessed for growth 10 d after pathogen challenge. Values are the mean ± SEM of 11 replicates. Significant differences from controls were determined using the Student's *t*-test and are shown as $P < 0.001$ ***, $P < 0.01$ **. $P < 0.05$ * and ns not significant.

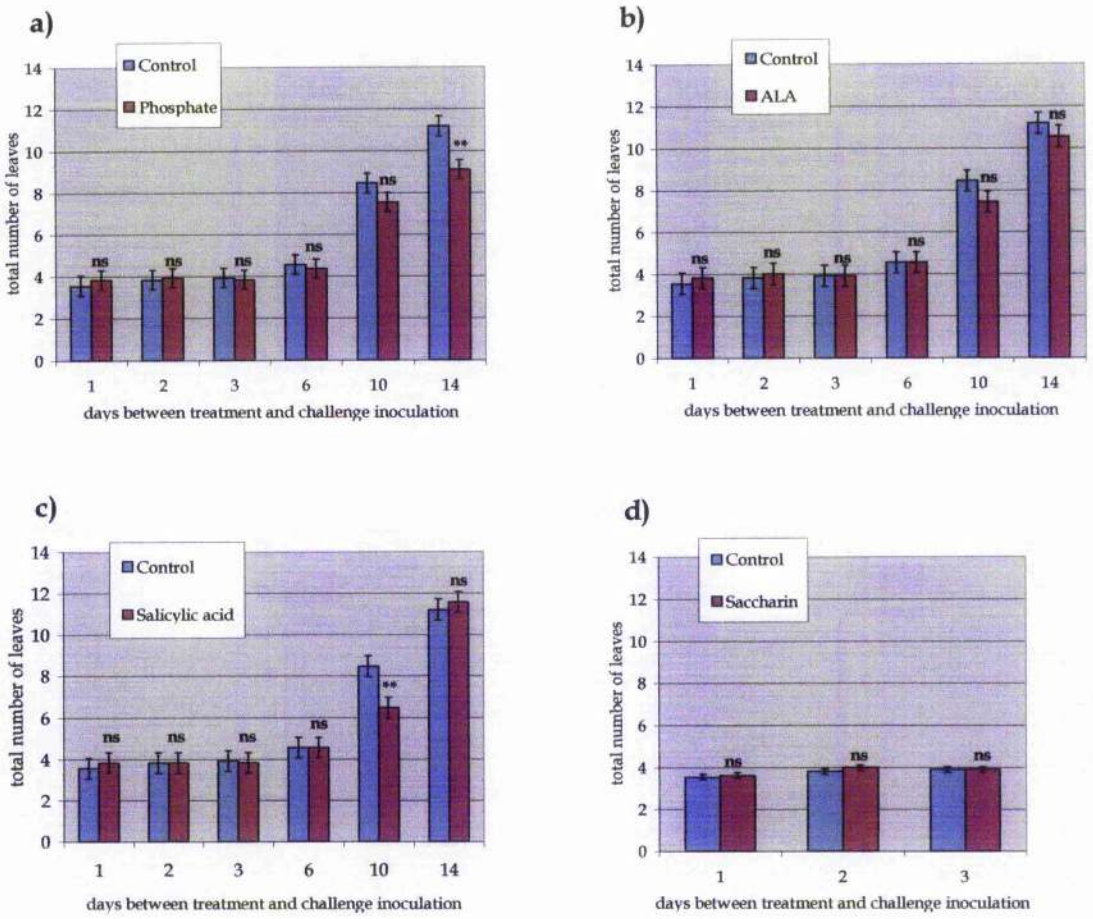


Figure 45. (a-d) Effect of phosphate (a), ALA (b), salicylic acid (c) and saccharin (d) induced systemic protection in barley on the number of leaves produced per plant. Plants were assessed for growth 10 d after pathogen challenge. Values are the mean ± SEM of 11 replicates. Significant differences from controls were determined using the Student's *t*-test and are shown as $P < 0.001$ ***, $P < 0.01$ **. $P < 0.05$ * and ns not significant.

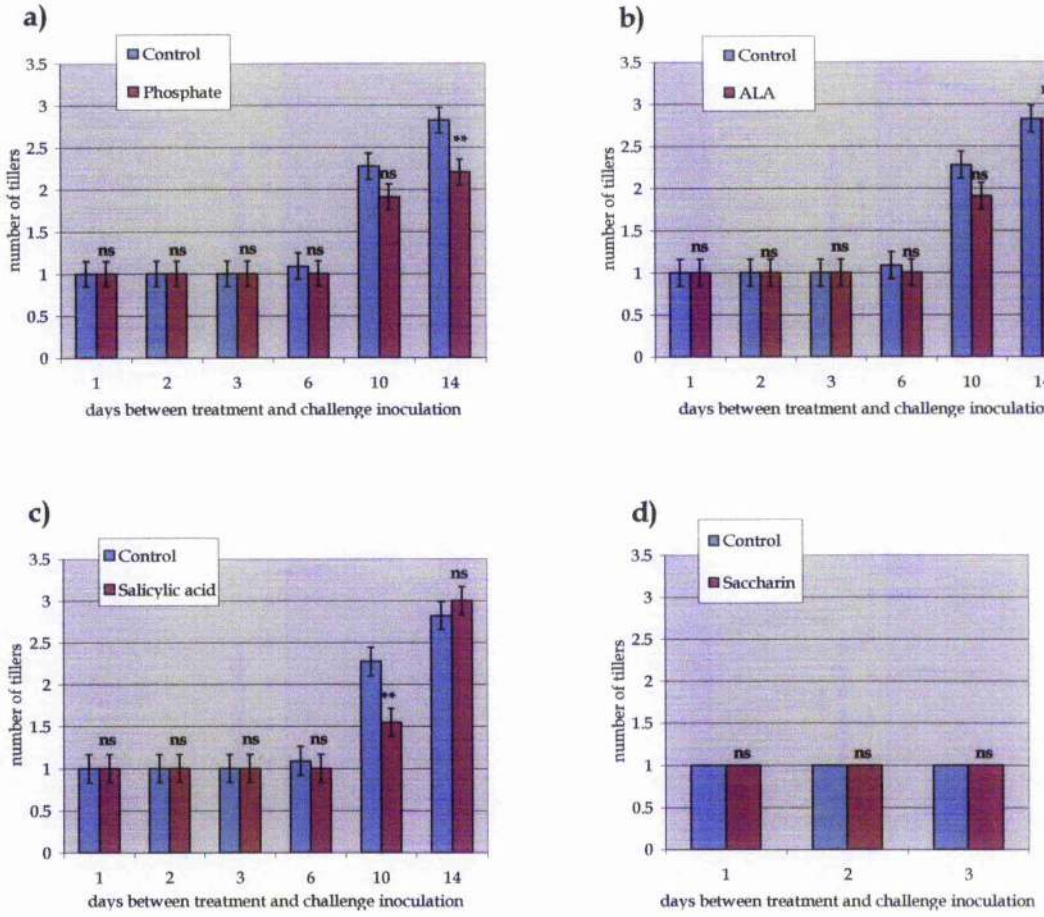


Figure 46. (a-d) Effect of phosphate (a), ALA (b), salicylic acid (c) and saccharin (d) induced systemic protection in barley on the number of tillers produced per plant. Plants were assessed for growth 10 d after pathogen challenge. Values are the mean ± SEM of 11 replicates. Significant differences from controls were determined using the Student's *t*-test and are shown as $P < 0.001$ ***, $P < 0.01$ **. $P < 0.05$ * and ns not significant.

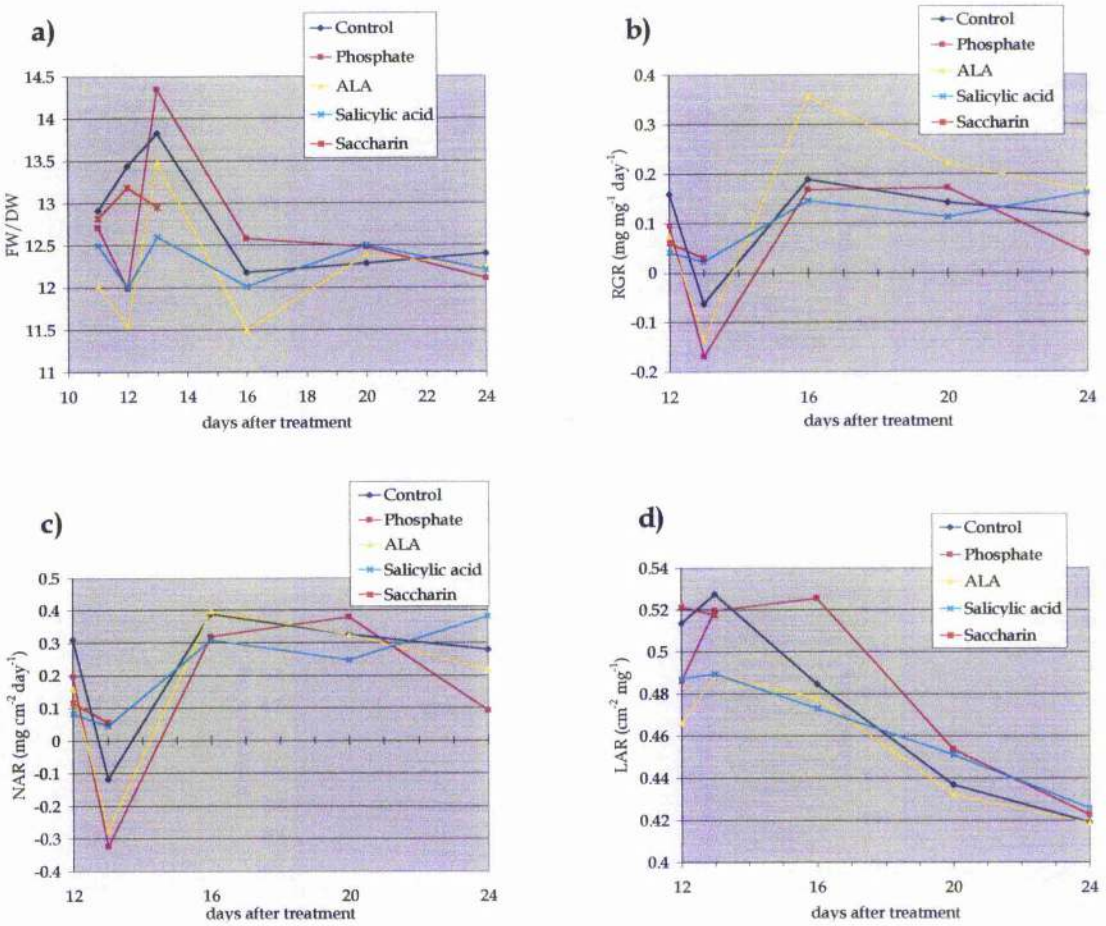


Figure 47. (a-d) Growth analysis of barley plants exhibiting chemical induced systemic protection. Plants were harvested 10 d after pathogen challenge and assessed for growth parameters. Therefore, plants harvested at 11 d, 12 d, 13 d, 16 d, 20 d and 24 d were respectively challenged 1 d, 2 d, 3 d, 6 d, 10 d and 14 d after treatment. Effect of induced protection on fresh wt. / dry wt. ratio (a); relative growth rate (b); net assimilation rate (c); and leaf area ratio (d).

In plants harvested after 11 d the FW/DW ratio was lowest in those treated with ALA. The FW/DW ratio peaked at 13 d, irrespective of treatment (Figure 47a). This coincided with a reduction in both RGR and NAR in plants harvested 13 d after all treatments (Figure 47b, c). After 20 d, the FW/DW ratio was very similar for all plants, regardless of treatment (Figure 47a). There was an overall decrease in LAR for all treatments over the time examined (Figure 47d).

4.3.2 Saccharin applied to leaves and as a soil drench to induce systemic protection of barley.

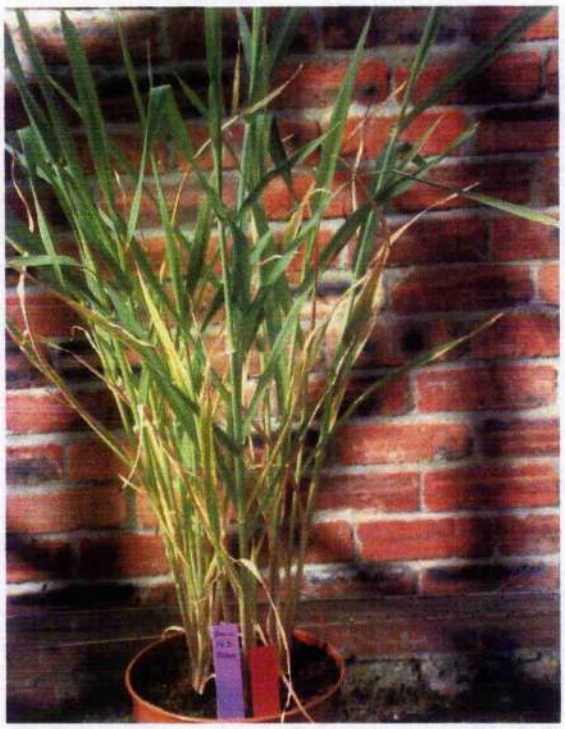


Figure 48. Saccharin induced systemic protection of barley.

Figure 49. Control barley plant infected with powdery mildew.

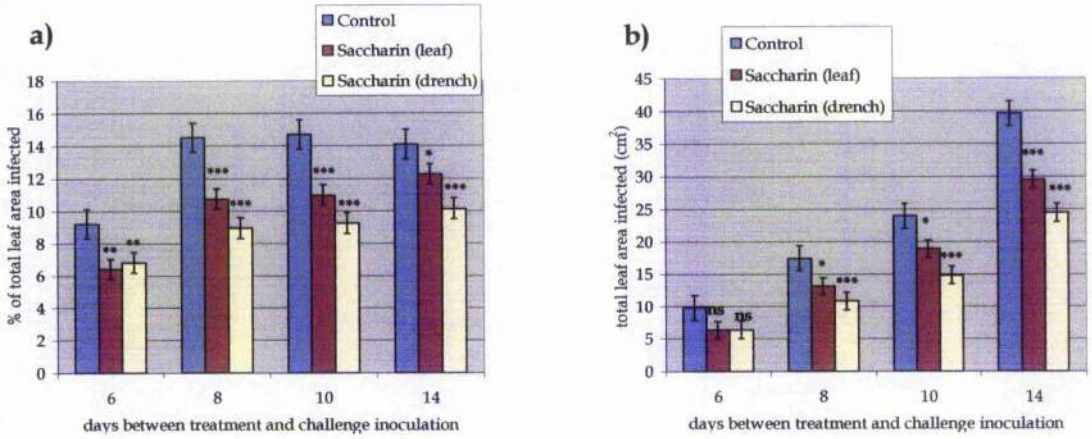


Figure 50. (a, b) Effect of saccharin induced systemic protection on % total leaf area infected with mildew(a), and total leaf area infected with mildew (b). Plants were assessed for infection 10 d after pathogen challenge. Values are the mean ± SEM of 11 replicates. Significant differences from controls were determined using the Student's *t*-test and are shown as $P < 0.001$ ***, $P < 0.01$ **. $P < 0.05$ * and ns not significant.

Saccharin significantly reduced the % of total leaf area infected with powdery mildew irrespective of whether the treatment was applied to the plant's first leaf or used as a soil drench (Figure 50a). The total leaf area infected was also significantly reduced with both methods of treatment. The exception was in plants challenged 6 d after application of saccharin, since although there was still a reduction in infection, the effect was not significant regardless of the mode of treatment (Figure 50b). There was no significant effect of saccharin treatment on total leaf area to explain the difference observed between the % of total leaf area and the total leaf area infected (Figure 52c). Mildew infection was generally lower in the plants treated with the saccharin drench compared to those where saccharin was applied to the leaves (Figure 50a, b).

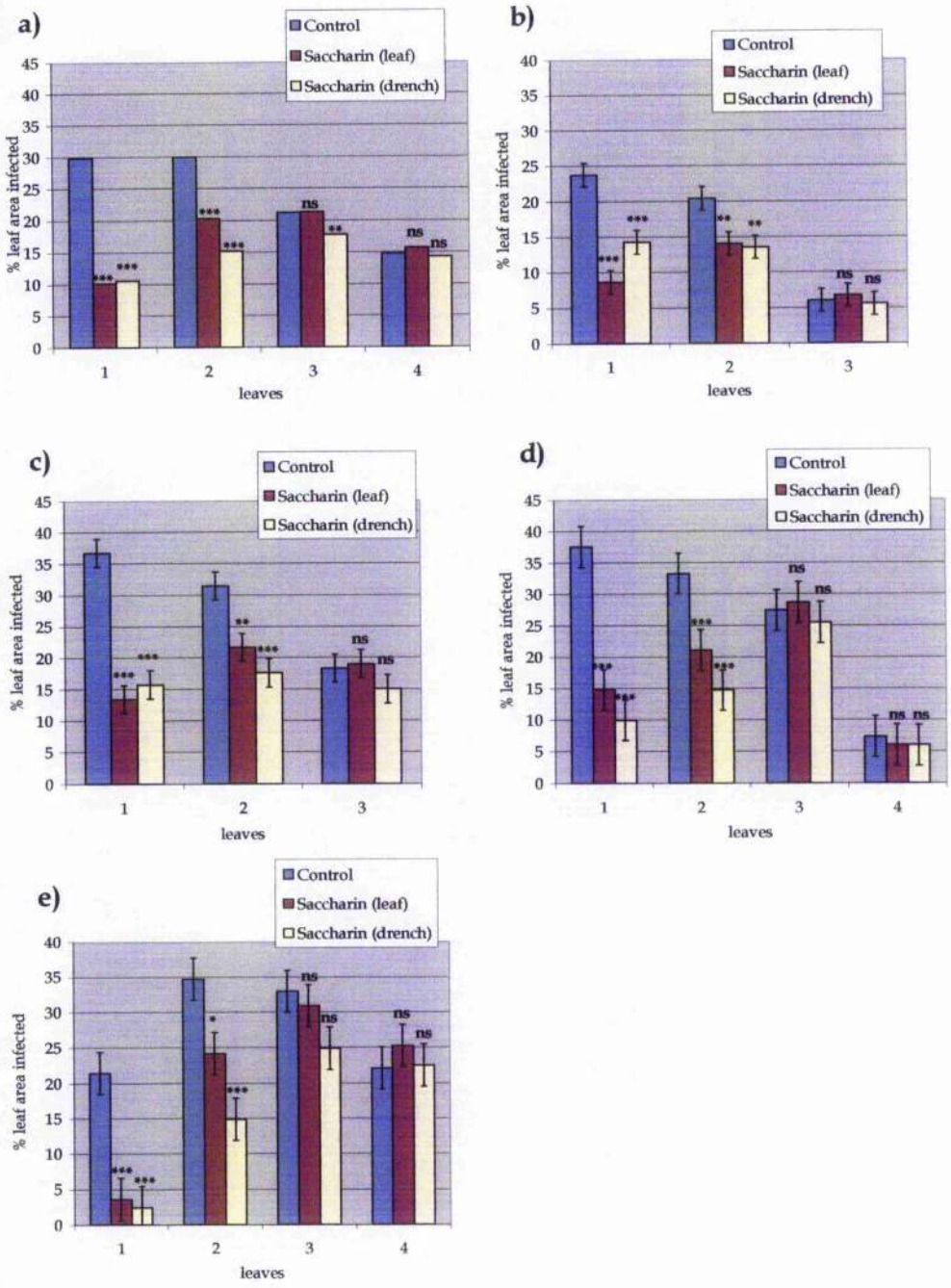


Figure 51. (a-e) Effect of saccharin and leaf position on % leaf area infected on leaves 1-4 for all challenge periods (a) and on leaves challenged with powdery mildew 6 d (b); 8 d (c); 10 d (d), and 14d (e) after induction of systemic protection. Plants were assessed for infection 10 d after pathogen challenge. Saccharin was applied either to leaf 1 or as a soil drench. The whole plant was challenged with mildew and at 6 or 8 d, plants had a minimum of 3 leaves and at 10 or 14 d a minimum of 4 leaves. Leaf numbers refer to order of leaves from the base of the first tiller produced, leaf 1 being the lowest leaf. Values are the mean ± SEM of 11 replicates. Significant differences from controls were determined using the Student's *t*-test and are shown as $P < 0.001$ ***, $P < 0.01$ **. $P < 0.05$ * and ns not significant.

Saccharin applied either to leaves or as a drench significantly reduced the level of infection in leaves 1 and 2, irrespective of the time between challenge and treatment. Although, the reduction in infection in leaf 3 following application of the saccharin drench was not significant for any one of the 4 challenge periods (Figure 51b-e), overall the reduction in infection in leaf 3 was significant (Figure 51a). Saccharin had no significant effect on mildew in leaf 4 (Figure 51d, e).

Saccharin induction of systemic protection had no significant effect on growth regardless of how saccharin was applied to the plants (Figure 52). The only exception to this was a reduction in total leaf area in plants challenged 14 d after treatment with the saccharin drench (Figure 52c).

The FW/DW ratio was higher in controls compared with either of the two saccharin treatments in plants 16 d after treatment (Figure 53a). However by the end of the experimental period the FW/DW ratio was highest in plants that had received foliar saccharin treatment and lowest when a saccharin drench was used (Figure 53a). Similarly, LAR was highest in controls at the start of the experiment and declined in all treatments during the course of the experimental period (Figure 53d). At the final harvest, LAR was lowest in the plants to which a drench was applied, whereas LAR was the same for both controls and foliar treated plants (Figure 53d). However, after 18 d RGR and NAR were lowest in controls but this position was reversed by the end of the period examined, when RGR and NAR was highest in controls. There was a peak in both the RGR and the NGR at 20 d, irrespective of treatment (Figure 53b, c).

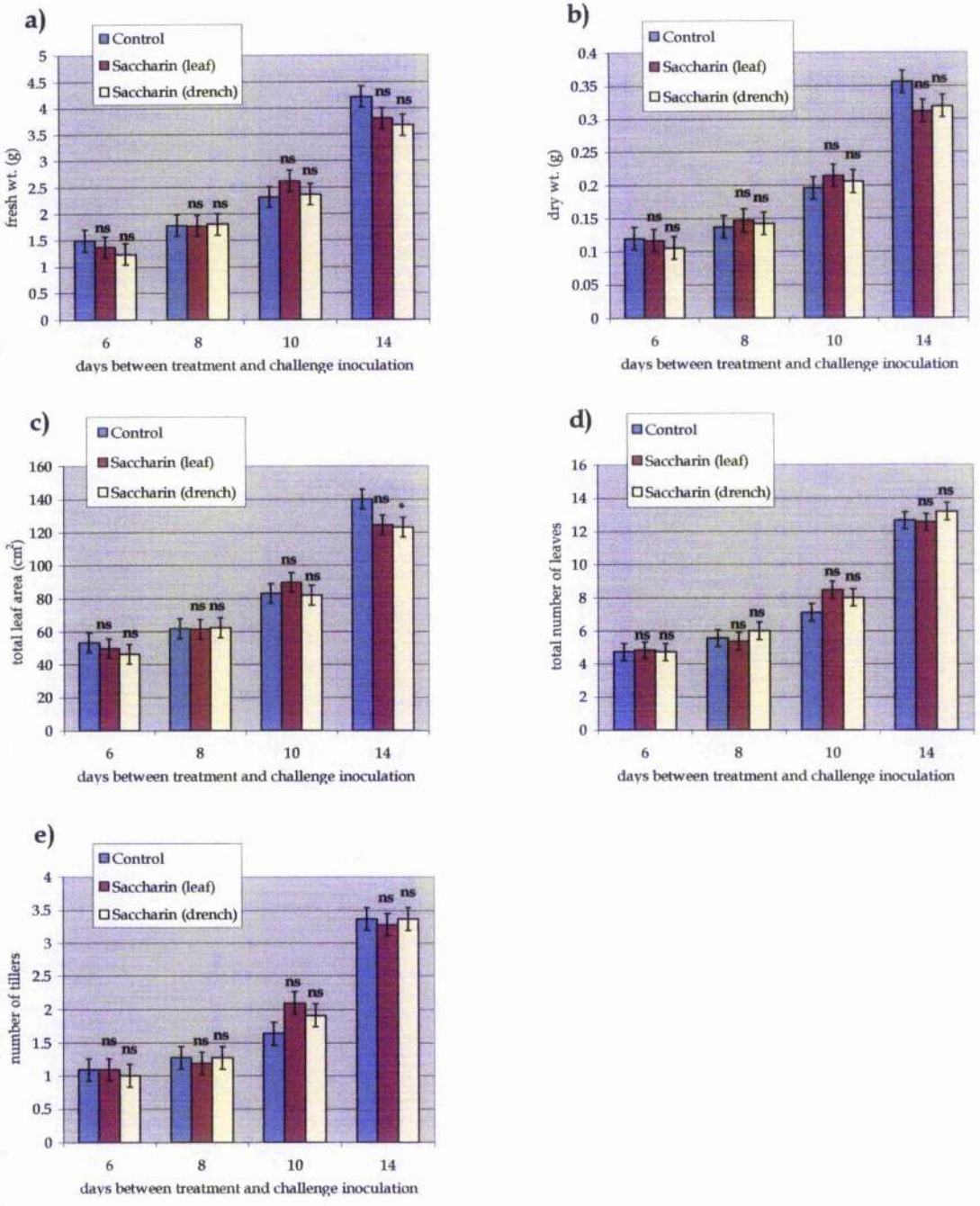


Figure 52. (a-e) Effect of saccharin induced systemic protection in barley on fresh weight (a), dry weight (b), total leaf area (c), total number of leaves (d) and number of tillers (e). Plants were assessed for growth 10 d after pathogen challenge. Values are the mean ± SEM of 11 replicates. Significant differences from controls were determined using the Student's *t*-test and are shown as $P < 0.001$ ***, $P < 0.01$ **. $P < 0.05$ * and ns not significant.

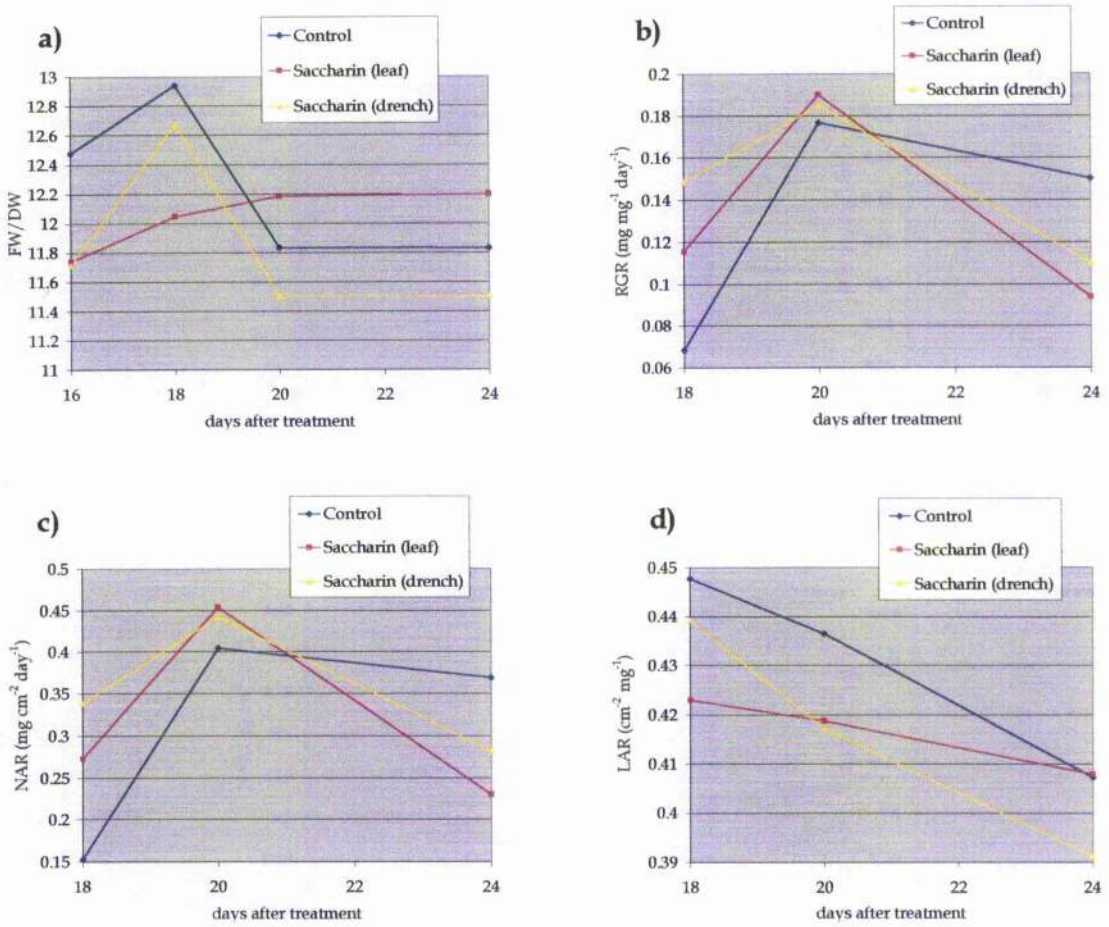


Figure 53. (a-d) Growth analysis of barley plants exhibiting saccharin induced systemic protection. Plants were harvested 10 d after pathogen challenge and assessed for growth parameters. Therefore, plants harvested at 16 d, 18 d, 20 d and 24 d were respectively challenged 6 d, 8 d, 10 d and 14 d after treatment. Effect of induced protection on fresh wt. / dry wt. ratio (a); relative growth rate (b); net assimilation rate (c); and leaf area ratio (d).

4.3.3 Chemical induced systemic protection of broad bean

Infection intensity was assessed as the percentage of leaf area infected, based upon the leaves available for inoculation at time of challenge.

4.3.4 Phosphate

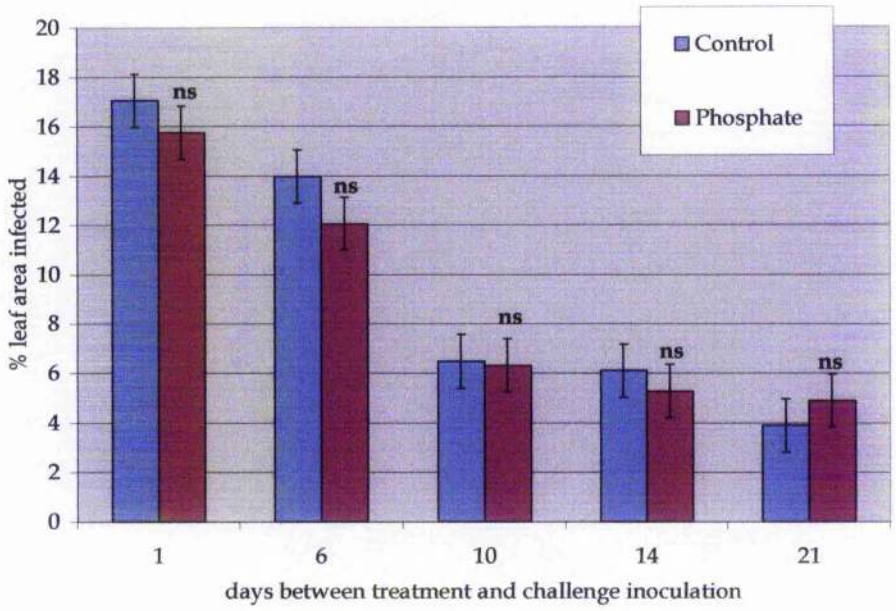


Figure 54. Effect of phosphate application to first leaves on % rust infection in upper leaves of broad bean. Based upon the total number of leaves challenge inoculated with rust. Plants were assessed for infection 14 d after pathogen challenge. Values are the mean ± SEM of 12 replicates. Significant differences from controls were determined using the Student's *t*-test and are shown as $P < 0.001$ ***, $P < 0.01$ **. $P < 0.05$ * and ns not significant.

Phosphate had no significant effect on the overall level of infection in rust challenged leaves (Figure 54). Similarly, leaf position was not a significant factor in overall disease severity (Figure 55a). However, in leaf 4 there was a significant reduction in infection in plants challenged with rust 6 d after treatment (Figure 55c).

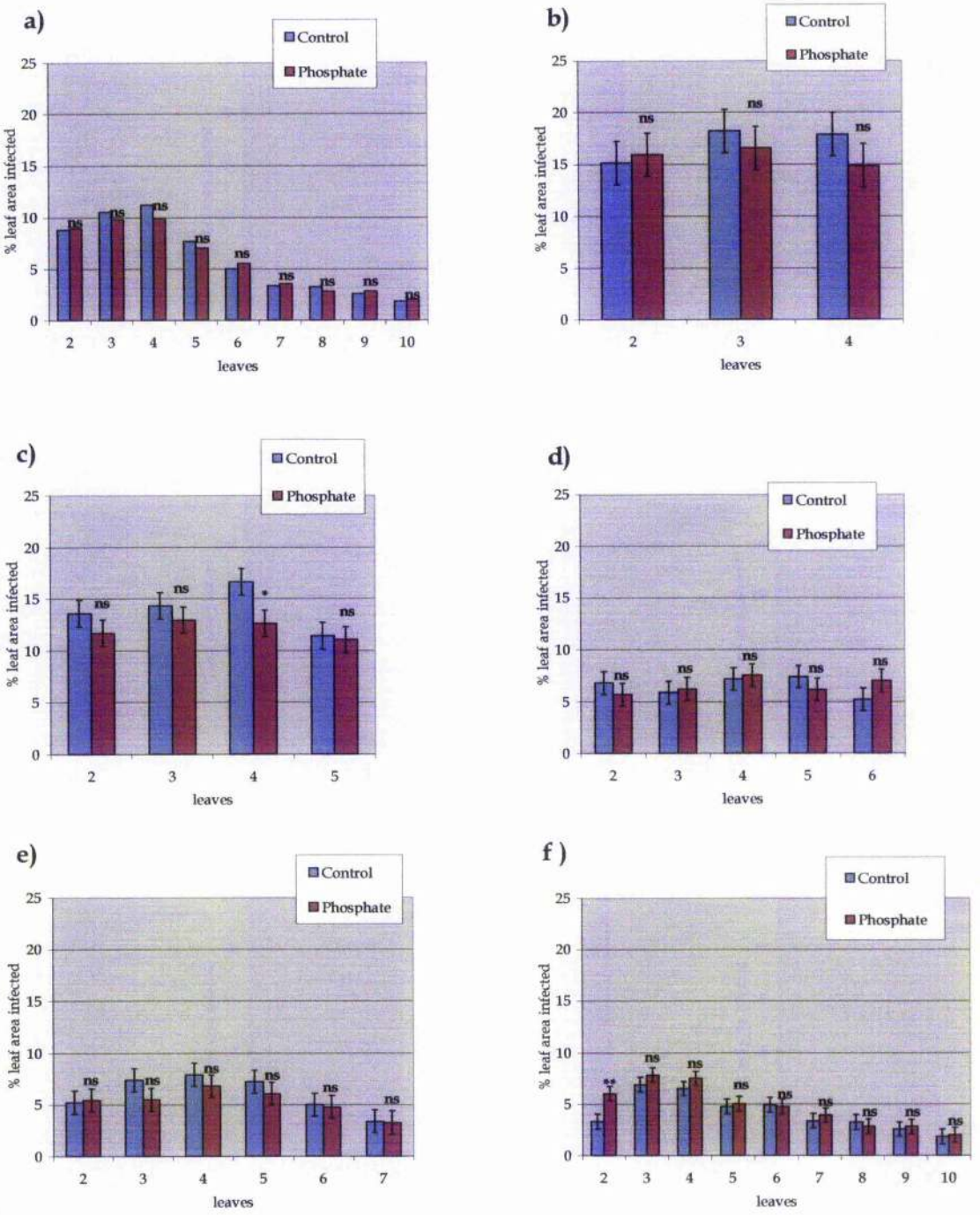


Figure 55. (a-f) Effect of phosphate and leaf position on % leaf area infected on leaves 1-10 for all challenge periods (a) and on leaves challenged with rust 1 d (b); 6 d (c); 10 d (d), 14d (e) and 21 d (f) after phosphate treatment. Plants were assessed for infection 14 d after pathogen challenge. Leaf numbers refer to the order in which leaves were produced on the main stem of the plant, leaf 1 being the lowest leaf. Phosphate applied to leaf 1 and upper leaves challenged with rust. Values are the mean ± SEM of 12 replicates. Significant differences from controls were determined using the Student's *t*-test and are shown as $P < 0.001$ ***, $P < 0.01$ **. $P < 0.05$ * and ns not significant.

However, there was also a significant increase in infection in leaf 2 in plants challenged with rust 21 d after the application of phosphate (Figure 55f).

There was no significant effect on growth in plants challenged 21 d after treatment with phosphate (Figure 56a-d). Growth was not measured for earlier challenge times.

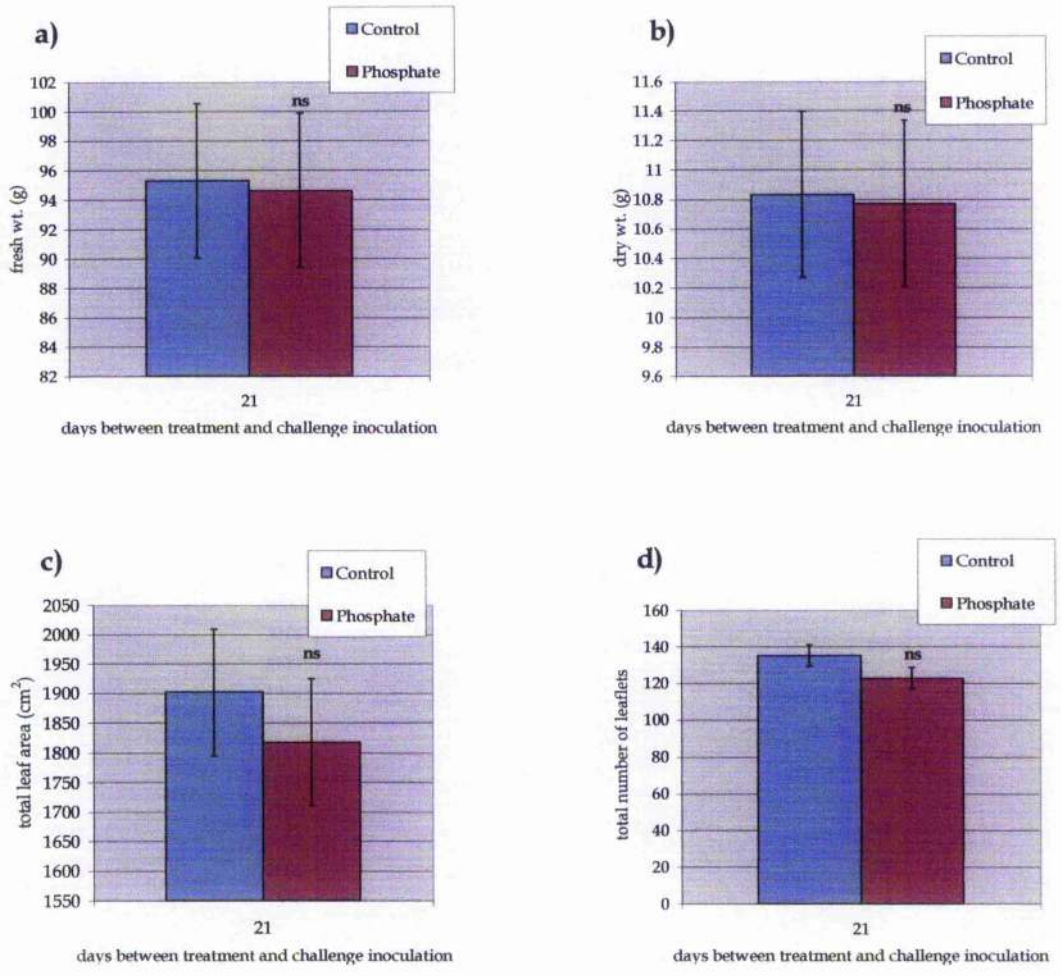


Figure 56. (a-d) Effect of phosphate application to first leaves of broad bean on whole plant fresh weight (a), dry weight (b), total leaf area (c), and total number of leaves (d). Plants were assessed for growth 14 d after pathogen challenge. Values are the mean ± SEM of 12 replicates. Significant differences from controls were determined using the Student's *t*-test and are shown as $P < 0.001$ ***, $P < 0.01$ **. $P < 0.05$ * and ns not significant.

4.3.5 Salicylic acid

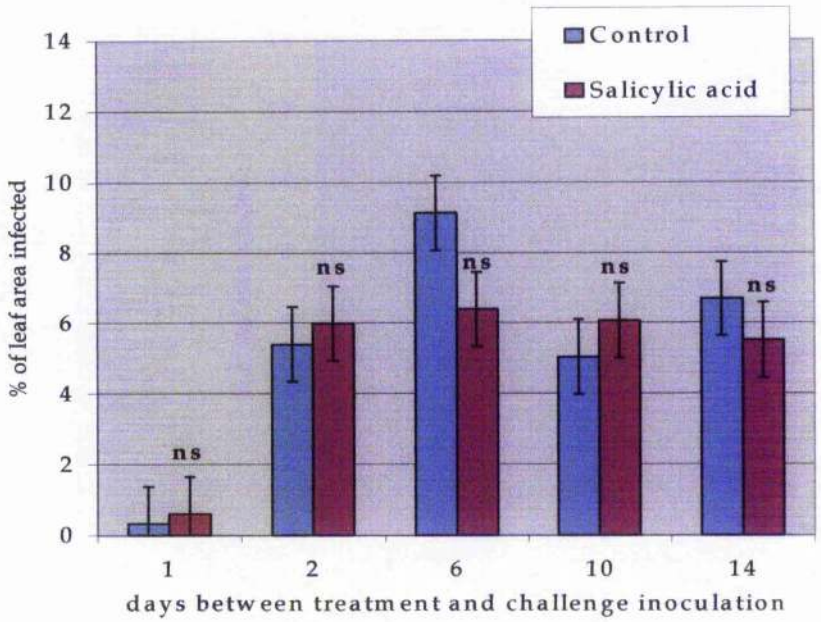


Figure 57. Effect of salicylic acid application to first leaves on % rust infection in upper leaves of broad bean. Based upon the total number of leaves challenge inoculated with rust. Plants were assessed for infection 14 d after pathogen challenge. Values are the mean ± SEM of 11 replicates. Significant differences from controls were determined using the Student's *t*-test and are shown as $P < 0.001$ ***, $P < 0.01$ **. $P < 0.05$ * and ns not significant.

SA applied to first leaves had no significant effect on the overall level of infection in challenged leaves (Figure 57). Leaf position was a significant factor in disease severity and rust infection was significantly reduced in leaves 2 and 4 in plants challenged 6 d after treatment with SA (Figure 58b, d). However, when the effect of SA on rust infection was considered on the basis of each challenge period, SA had no significant effect on rust except in leaf 2 in plants challenged 14 d after treatment (Figure 59e).

Necrotic patches were observed on some of the leaves to which a foliar application of SA had been applied.

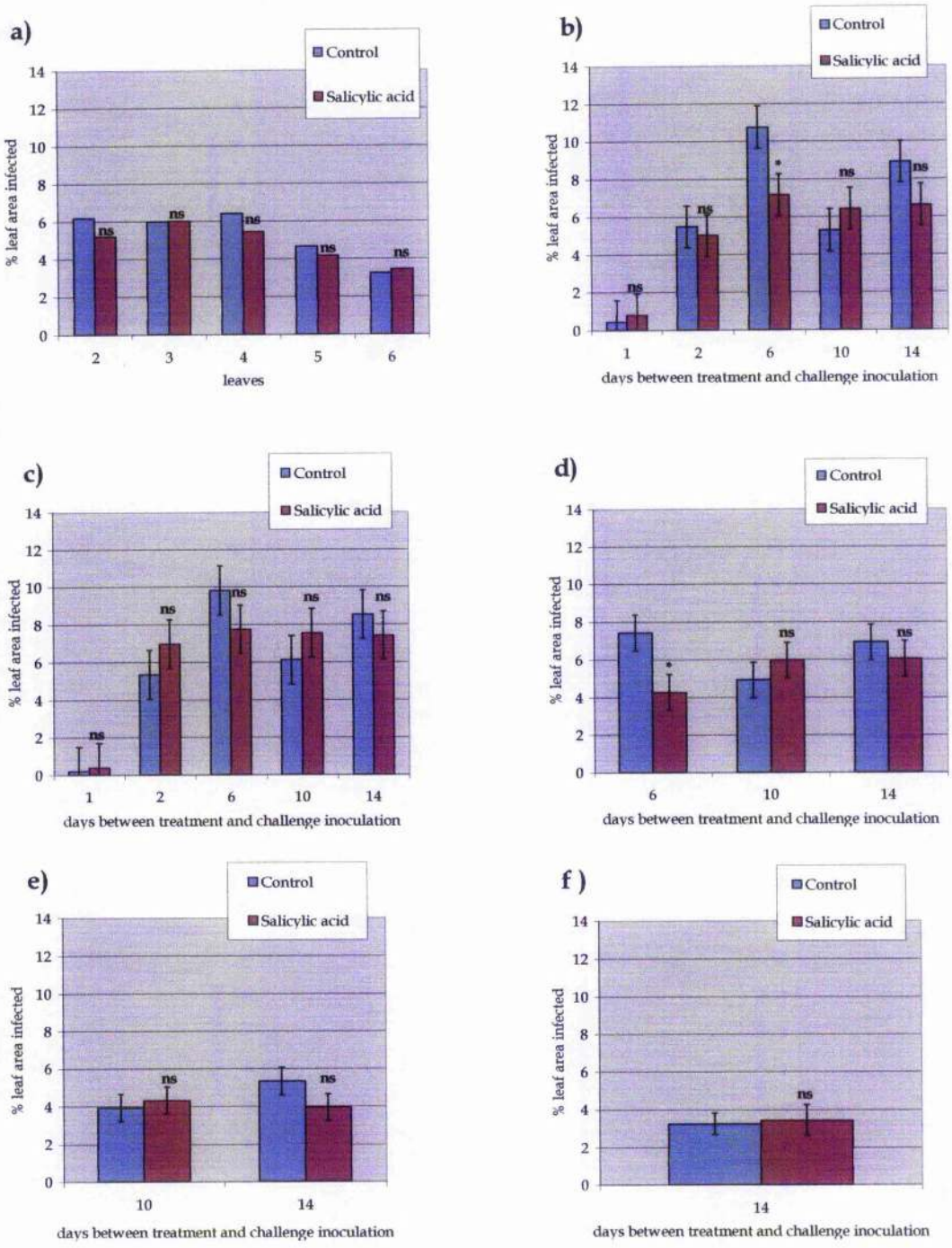


Figure 58. (a-f) Effect of leaf position on % leaf area infected with rust in salicylic acid induced systemic protection of broad bean. Plants were assessed for infection 14 d after pathogen challenge. Leaf position refers to the order of leaves from the base of the main stem, leaf 1 being the lowest leaf. % leaf area infected on leaves 2-6 for all 5 challenge periods (a), infection on leaf 2 (b), leaf 3 (c), leaf 4 (d), leaf 5 (e), and leaf 6 (f). Values are the mean ± SEM of 11 replicates. Significant differences from controls were determined using the Student's *t*-test and are shown as $P < 0.001$ ***, $P < 0.01$ **. $P < 0.05$ * and ns not significant.

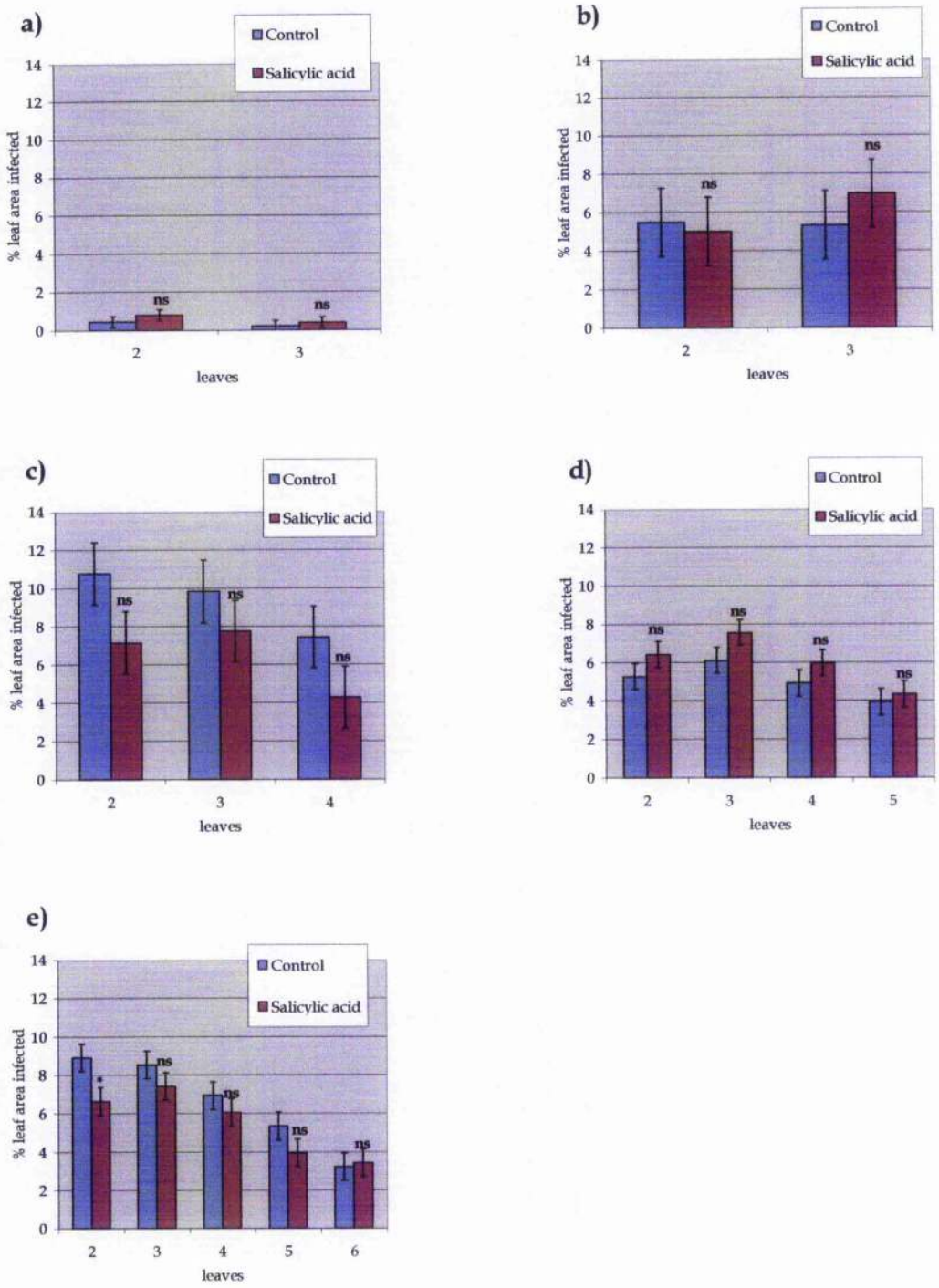


Figure 59. (a-e) Effect of salicylic acid application to first leaves of bean on % infection on leaves challenged with rust 1 d (a); 2 d (b); 6 d (c), 10 d (d) and 14 d (e) after induction of systemic protection. Plants were assessed for infection 14 d after pathogen challenge. Leaf numbers refer to order in which leaves were produced on the main stem of the plant. Values are the mean ± SEM of 11 replicates. Significant differences from controls were determined using the Student's *t*-test and are shown as $P < 0.001$ ***, $P < 0.01$ **. $P < 0.05$ * and ns not significant.

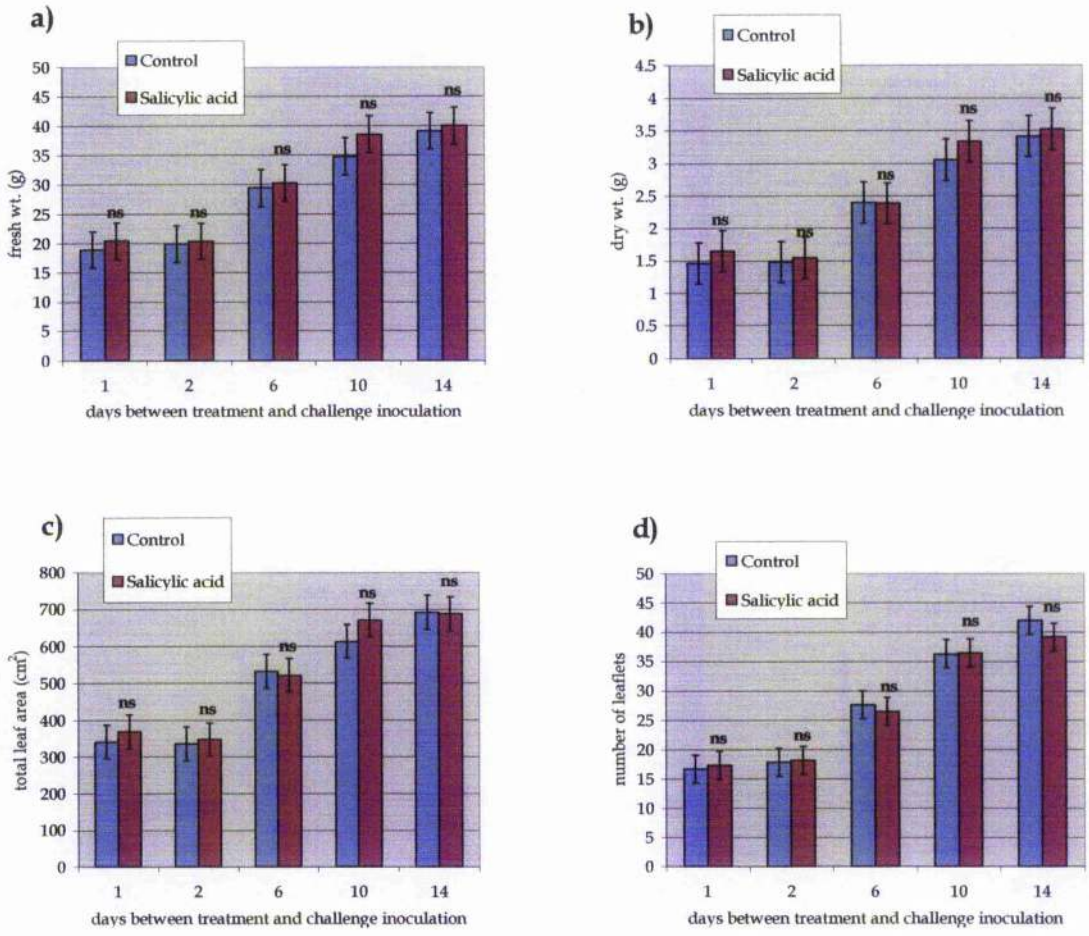


Figure 60. (a-d) Effect of salicylic acid application to first leaves of broad bean on whole plant fresh weight (a), dry weight (b), total leaf area (c), and total number of leaflets (d). Plants were assessed for growth 14 d after pathogen challenge. Values are the mean ± SEM of 11 replicates. Significant differences from controls were determined using the Student's *t*-test and are shown as $P < 0.001$ ***, $P < 0.01$ **. $P < 0.05$ * and ns not significant.

Growth was not significantly affected by SA application to first leaves of beans (Figure 60a-d). Growth analysis showed similar results for both controls and SA treated plants, although all four growth analysis parameters were initially lower in the SA treated plants. There was an overall decrease in both the FW/DW ratio and LAR over the time

examined (Figure 61a, d). After 20 d, RGR and NAR peaked, irrespective of treatment (Figure 61b, c).

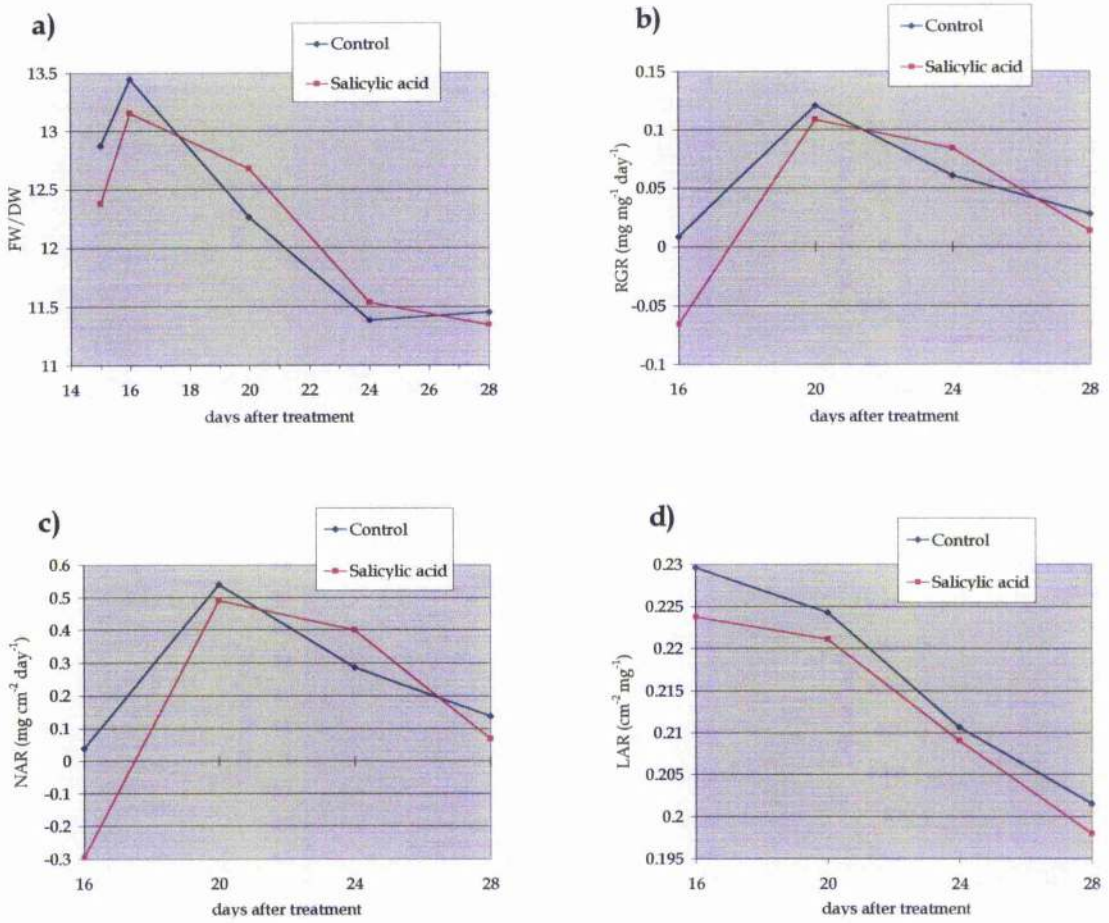


Figure 61. (a-d) Growth analysis of broad bean plants following treatment of first leaves with salicylic acid. Plants were harvested 14 d after pathogen challenge and assessed for growth parameters. Therefore, plants harvested at 15 d, 16 d, 20 d, 24 d and 28 d were respectively challenged 1 d, 2 d, 6 d, 10 d and 14 d after treatment. Effect of induced protection on fresh wt. / dry wt. ratio (a); relative growth rate (b); net assimilation rate (c); and leaf area ratio (d).

4.3.6 Saccharin applied to leaves and as a soil drench to induce systemic protection of broad bean.

Figure 62. Bean plant appeared unaffected by the saccharin drench.

Figure 63. Wilting caused by the saccharin drench

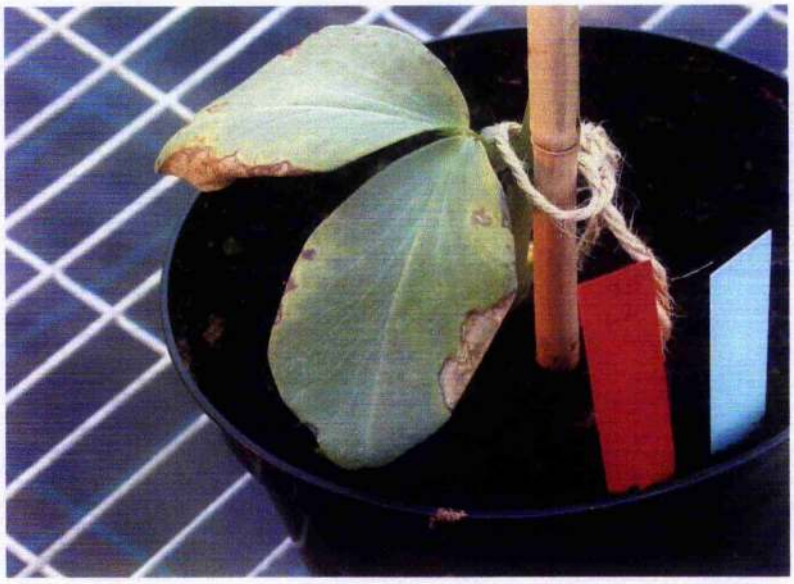


Figure 64. Necrosis caused by saccharin.

The saccharin drench resulted in severe wilting in some of the bean plants, from which they did not recover (Figure 63). Other bean plants appeared unaffected (Figure 62). Some necrosis was also observed in plants treated with saccharin, irrespective of the method of application (Figure 64).

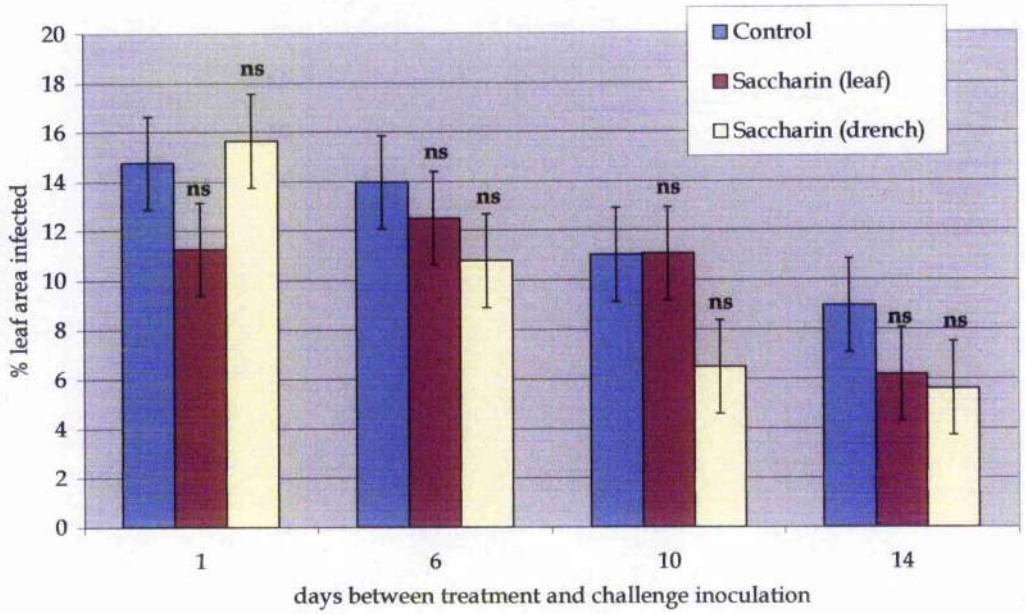


Figure 65. Effect of saccharin induced systemic protection on % rust infection in upper leaves of broad bean. Based upon the total number of leaves challenge inoculated with rust. Plants were assessed for infection 14 d after pathogen challenge. Values are the mean ± SEM of 11 replicates. Significant differences from controls were determined using the Student's *t*-test and are shown as $P < 0.001$ ***, $P < 0.01$ **. $P < 0.05$ * and ns not significant.

Disease severity was reduced by the saccharin drench in plants challenged 6 d or more after the drench was applied. Similarly, when saccharin was applied to leaves there was a decrease in rust except in plants challenged 10 d after treatment. However, none of the differences in rust infection were significant (Figure 65).

However, leaf position and mode of saccharin application were both significant factors in the severity of rust infection (Figure 66). When saccharin was applied as a drench, rust infection was significantly reduced in all leaves challenged with rust compared with controls. Although rust infection in all rust challenged leaves was also reduced by application of saccharin to the leaves, the reduction was only significant in leaf 5 (Figure 66).

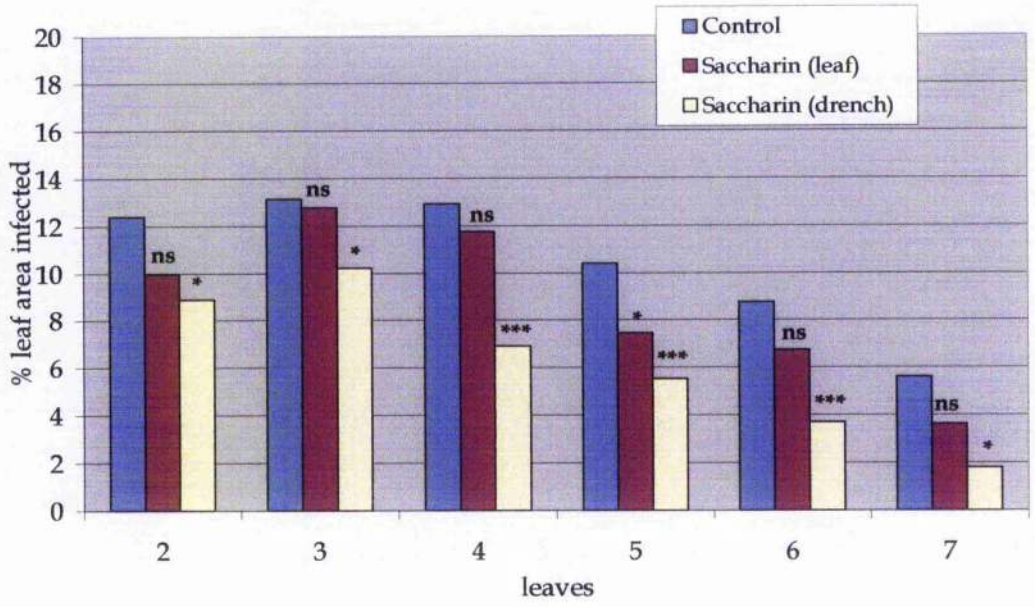


Figure 66. Effect of leaf position on % leaf area infected with rust in saccharin induced systemic protection of broad bean. Leaf position refers to the order of leaves from the base of the main stem, leaf 1 being the lowest leaf. % leaf area infected on leaves 2-10 for all 5 challenge periods. Plants were assessed for infection 14 d after pathogen challenge. Significant differences from controls were determined using the Student's *t*-test and are shown as $P < 0.001$ ***, $P < 0.01$ **. $P < 0.05$ * and ns not significant.

Saccharin did not significantly reduce rust infection until 6 d or more had elapsed between application of the saccharin and pathogen challenge (Figure 67a-d). Then, in the plants challenged 6 d after treatment, the reduction in infection was only observed in leaf 2 (Figure 67b). In plants challenged 10 d after treatment, rust infection was only significantly reduced by the saccharin drench, except in leaf 2 where the reduction was not significant (Figure 67c). Saccharin reduced disease severity in all leaves challenged 14 d after treatment. However, infection was only significantly reduced by the drench in leaves 4, 5, and 6 and leaf application in leaves 4 and 5 (Figure 67d).

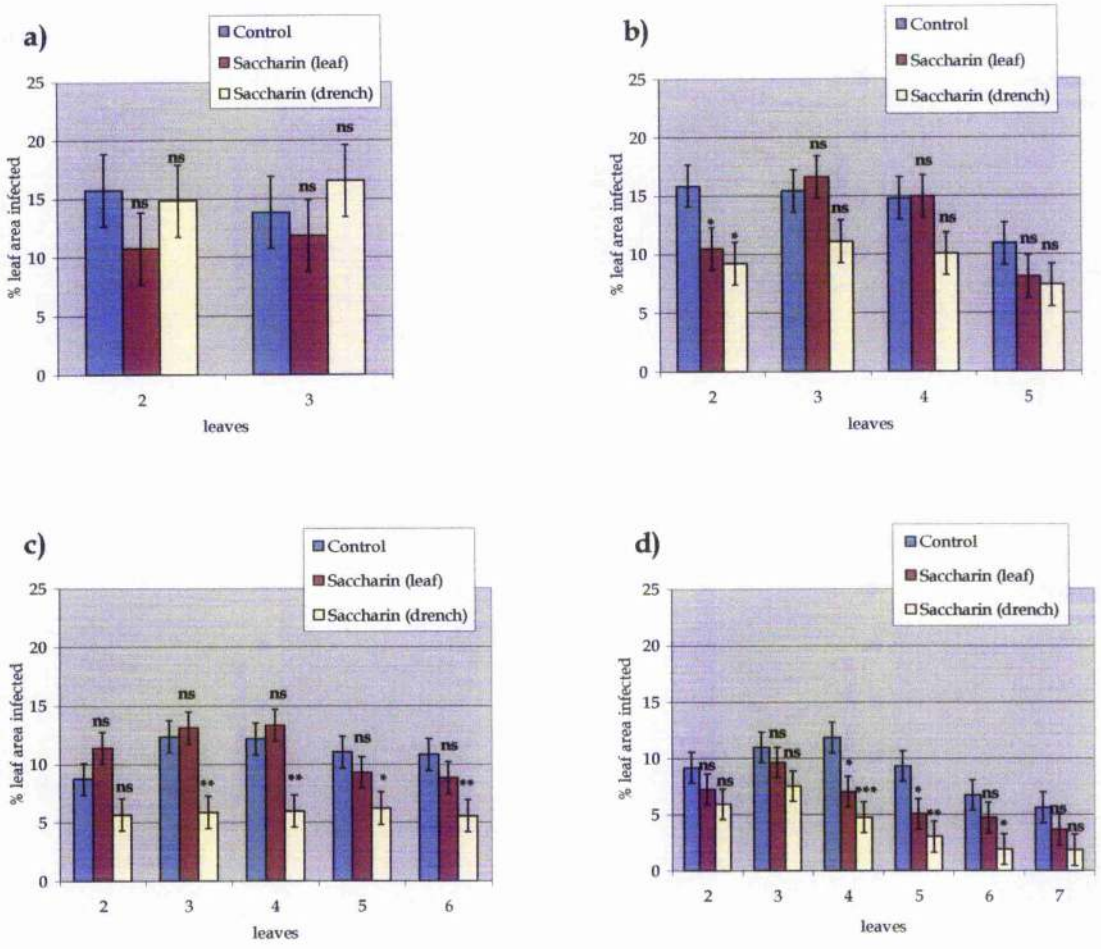


Figure 67. (a-d) Effect of saccharin induced systemic protection of bean on % infection on upper leaves challenged with rust 1 d (a); 6 d (b); 10 d (c), and 14 d (d) after induction of systemic protection. Plants were assessed for infection 14 d after pathogen challenge. Leaf positions refer to order in which leaves were produced on the main stem of the plant. Saccharin applied to L1 (first leaf) or as a soil drench. Values are the mean ± SEM of 10 replicates. Significant differences from controls were determined using the Student's *t*-test and are shown as $P < 0.001$ ***, $P < 0.01$ **. $P < 0.05$ * and ns not significant.

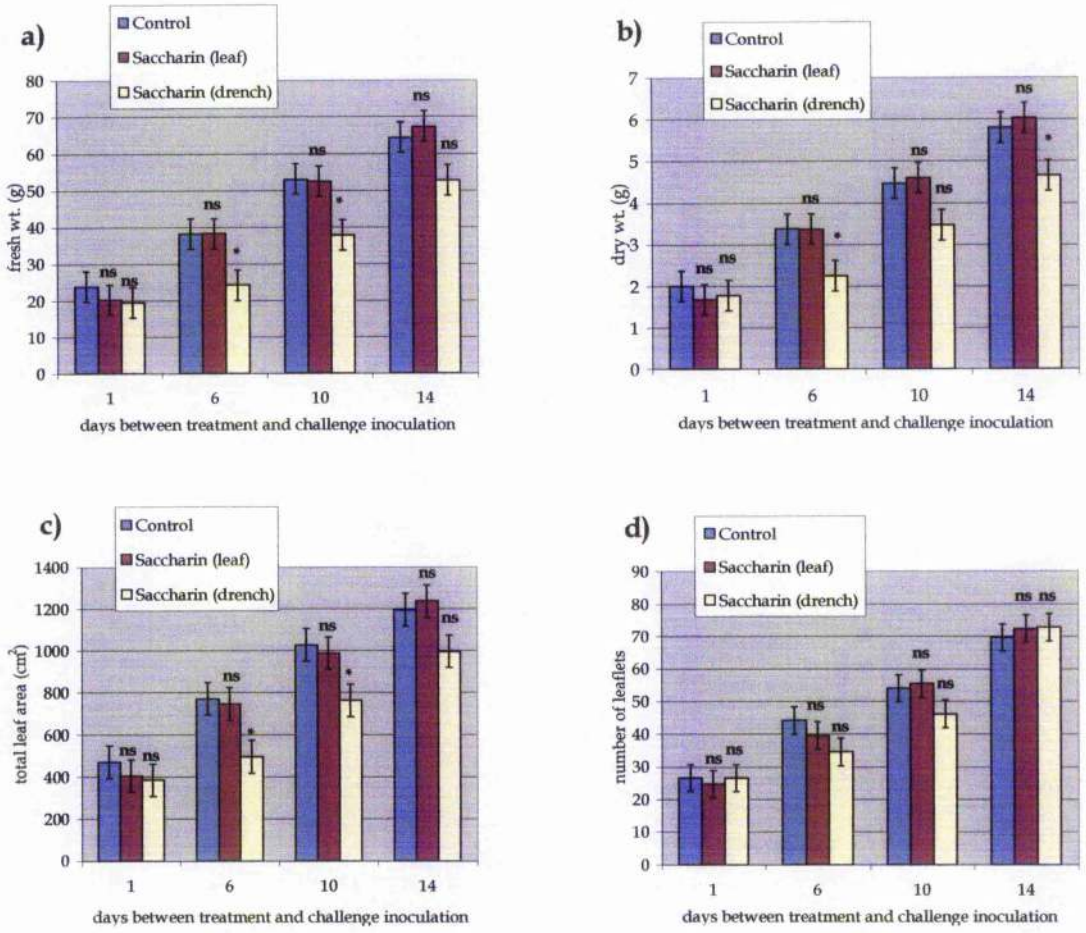


Figure 68. (a-d) Effect of saccharin induced systemic protection in broad bean on whole plant fresh weight (a), dry weight (b), total leaf area (c), and total number of leaflets (d). Plants were assessed for growth 14 d after pathogen challenge. Values are the mean ± SEM of 10 replicates. Significant differences from controls were determined using the Student's *t*-test and are shown as $P < 0.001$ ***, $P < 0.01$ **. $P < 0.05$ * and ns not significant.

Saccharin induced systemic protection affected plant growth depending on the method of application. Systemic protection when induced by the leaf application of saccharin had no significant effect on plant growth (Figure 68). Both fresh weight and total leaf area were significantly reduced in plants challenged with rust 6 and 10 d after application of the drench (Figure 68a, c). Dry weight was also significantly reduced in plants challenged 6 and 14 d after treatment with the drench (Figure 68b). However,

saccharin had no significant effect on the total number of leaflets produced (Figure 68d).

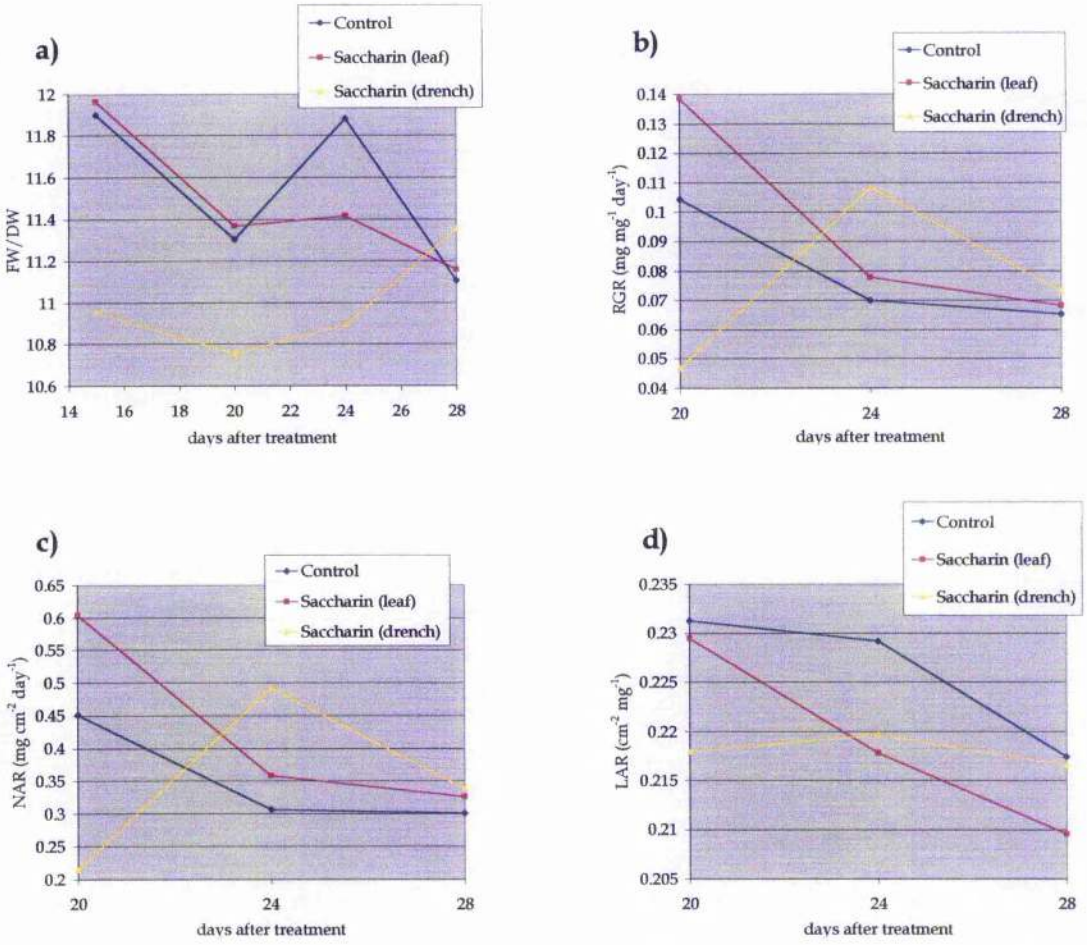


Figure 69. (a-d) Growth analysis of broad bean plants exhibiting saccharin induced systemic protection. Plants were harvested 14 d after pathogen challenge and assessed for growth parameters. Therefore, plants harvested at 15 d, 16 d, 20 d, 24 d and 28 d were respectively challenged 1 d, 2 d, 6 d, 10 d and 14 d after treatment. Effect of induced protection on fresh wt. / dry wt. ratio (a); relative growth rate (b); net assimilation rate (c); and leaf area ratio (d).

Initially, all aspects of growth analysis were lower in plants treated with the saccharin drench (Figure 69). However, by the final harvest, FW/DW ratio, RGR and NAR were all slightly higher in plants treated with the saccharin drench compared to controls or those that had saccharin applied to their leaves. There was a general decline in the

RGR, NAR and LAR, over the time examined, in both controls and plants that had received foliar applications of saccharin (Figure 69b-d).

4.4 Discussion and Conclusions

4.4.1 SA

Experiments have shown that exogenous applications of salicylate were sequestered or metabolised, such that very little of that applied was found in untreated leaves (Métraux *et al.*, 1990). Certainly, in the present work, a defence response occurred in leaf 1 of barley from 2 d after treatment, but a significant response was not apparent in leaf 2 until 14d after treatment with SA (Figure 41b-f). This is similar to the findings of Walters *et al.* (1993) who showed that mildew infection on the second leaves of barley seedlings were reduced after treatment of the first leaves with SA. They found that the greatest reduction in mildew occurred when plants were challenged with mildew within 1-2 d of treating the first leaf with SA. In the experiment reported here, a significant reduction in mildew was not apparent in leaf 2 until 14 d had elapsed between the time of treatment and challenge (Figure 41).

SA significantly reduced the percentage of the total leaf area infected with mildew when plants were pathogen challenged within 3 d of treatment (Figure 39c). What was perhaps surprising was that the resistance response that occurred in leaf 1 appeared to increase concomitant with increasing time between SA application and pathogen challenge (Figure 41). Hence, the reduction in mildew in leaf 1, in plants challenged 1

d after application of SA, was not even statistically significant. However, in plants challenged 14 d after treatment with SA the reduction in mildew, in leaf 1, was highly significant.

Both the total leaf area infected and the percentage of the total leaf area infected remained lower in SA treated plants throughout the experiment, although differences in statistical significance were apparent. Thus, the total leaf area infected with mildew was significantly reduced in plants challenged 10 d after treatment with SA (Figure 40c). The reason may be the concomitant reduction in leaf area in the plants challenged 10 d after the SA was applied, although the reduction in leaf area was not considered statistically significant (Figure 44c). However, there were no significant increases in leaf area to account for the fact that total leaf area infected in plants challenged within 3 d of treatment was not significant (Figure 40c), but when considered as a percentage of the total leaf area they were significant (Figure 39c).

SA was less effective in inducing a defence response in broad bean. SA did not significantly reduce the overall level of rust infection in challenged leaves. However, infection was significantly reduced in leaf 2 and 4 in plants challenged 6 d after application of SA (Figure 58b, d). Similarly, a significant reduction in rust occurred in plants challenged 6 d after treatment with phosphate (Figure 55c). A decrease in rust was also detected in the bean plants challenged 6 d after inoculation of the lowest leaf with rust (Section. 2.4.2). As previously mentioned, Ward *et al.* (1991) found that maximum expression of genes associated with the SAR response in tobacco occurred 6 d after induction of the defence response.

4.4.2 Phosphate

Phosphate significantly reduced the % of the total leaf area infected with mildew only in plants challenged with the fungus 3 d after treatment (Figure 39a). Mildew induction of systemic protection resulted in a reduction in total leaf area (section 2.3.1). However, there was no significant effect on total leaf area in the phosphate induced plants, except in plants challenged 14 d after treatment (Figure 44a).

Rust infection in broad bean was lower in the plants treated with phosphate except in plants challenged 21 d after treatment, but the reduction was not statistically significant (Figure 54). However, a significant reduction in rust did occur in leaf 4 of plants challenged with rust 6 d after application of phosphate (Figure 55c). These results are similar though less pronounced than those of Walters and Murray (1992). They found that treatment of the lower two leaves of broad bean plants with phosphate significantly reduced rust infection in upper leaves. Furthermore, the increased protection was still found to be effective when the plants were pathogen challenged 12 d after phosphate application. It is possible that the differences observed between the two experiments was due to the number of lower leaves treated with the phosphate. In the experiment undertaken by Walters and Murray, the two lower leaves were treated with phosphate instead of the leaf 1 in the experiment reported here. Indeed, Kováts *et al.* (1991) found that increasing the number of lower leaves of tomato induced with *Phytophthora infestans* increased the resistance response, and it is possible that the same relationship may hold true for chemically induced systemic protection.

4.4.3 ALA

Although mildew infection was generally lower in ALA treated plants, the results were not significant (Figures 39Figure 39b and 40Figure 40b). However, infection was significantly reduced in leaf 1 in plants challenged with mildew 6 d or more after application of ALA (Figure 41d-f). This is thought to be the first report of the induction of a defence response by the application of ALA. As the significant response only occurred in leaf 1, it is not possible to know if ALA is capable of inducing a systemic defence response. Since no previous reports are known to exist using ALA for protection against pathogens, the choice of concentration used, 3 mM, was completely arbitrary. Application of ALA at this concentration did not result in necrosis. It is, therefore, possible that a stronger concentration, treatment of more than one leaf, or application of ALA as a drench may increase efficacy or induce a systemic response.

4.4.4 Saccharin

Saccharin was a highly effective inducer of systemic protection in barley and the reduction in mildew infection was highly significant (Figures 39 andFigure 39 40d). Similarly, Reignault *et al.* (2001) found that powdery mildew infection of wheat was reduced by the application of trehalose (α-D-glucopyranosly-[1-1]-α-D-glucopyranoside). Trehalose is a non-reducing disaccharide found in various organisms, including fungi. Applying saccharin as a soil drench was more effective than leaf application, except in leaf 1 when plants were challenged within 8 d of applying the saccharin (Figure 51b, e). This is perhaps unsurprising as saccharin was applied directly to leaf 1, for the foliar application.

Saccharin produced significant reductions in % infection at all 3 challenge times tested (Figure 39d). In the second barley experiment, it was additionally found that the percentage leaf area infected was also significantly reduced in plants challenged 6, 8, 10 and 14 d after leaf application of saccharin (Figure 50a). These findings are contrary to the effect of mildew induced systemic protection in barley, which resulted in an overall increase in the percentage of leaf area infected (Section 2.3.1).

Saccharin also induced systemic protection in broad bean. Again, the saccharin drench was more effective in reducing rust infection than leaf application of saccharin. The exception to this was in plants challenged 1 d after application of the drench (Figures 65 and 67). This is believed to be the first report of saccharin induced systemic protection of both broad bean against rust and barley against powdery mildew.

4.4.5 Effect of leaf position

In control leaves of both bean and barley there was a progressive increase in pathogen resistance with increasing juvenility. This is consistent with the findings of Peltonen and Karjalainen (1995) that young barley leaves were more resistant to the necrotrophic pathogen *B. sorokiniana* and therefore exhibited higher levels of PAL activity than older leaves. Similarly, control leaves of cucumber, challenged with anthracnose exhibited increased resistance in the higher, younger leaves (Guedes *et al.*, 1980).

Leaf position was a significant factor in disease severity in systemic induced protection in both bean and barley. Mildew infection was significantly reduced in leaf 1 for all four treatments. This is perhaps not surprising as the chemicals were applied to the lowest leaf. Although reduced infection in leaf 1 is important, it is obviously not a

systemic response. In addition to the response in leaf 1, infection was also reduced in leaf 2 in both SA and saccharin treated plants. However, there was no significant effect on mildew infection observed in leaves 3 and 4. Even saccharin applied as a drench, which significantly reduced mildew infection in the whole plant, did not significantly reduce mildew in leaves 3 and 4. This suggests that the induced defence response is stronger in the lower leaves. This is contrary to the effect observed with mildew induced systemic protection, as a significant response was only observed in leaf 3 and 4 and not in leaf 2 (section 2.3.1). The effect with mildew induced protection is consistent with the effect observed in tobacco protected against blue mould by the application of *P. hyoscyami* to the soil around the stem base. This provided greater protection to upper leaves compared to lower leaves (Cohen and Kuć, 1981).

In bean plants rust challenged 14 d after treatment with SA, protection was significantly increased in leaf 2 but not in leaves 3 to 6. In fact, resistance was lower in leaf 6 of the treated plants compared to the controls. Similarly, in rust induced plants, protection was significantly increased in leaves 2 and 4 but not in leaves 5 and 6. Resistance was also higher in leaves 3, 4, and 5 of phosphate treated plants and lower in 6, 7, 9, and 10, compared to controls. Although these results were not statistically significant, combined with the previous results this suggests that in broad bean, regardless of whether resistance is produced by a pathogen or chemically induced by phosphate or SA, the resistance response is stronger in the lower leaves. This is contrary to the results of Murray and Walters (1992), who found that when systemic protection was induced in broad bean with rust, the young developing leaves were more resistant to rust than the lower fully developed leaves. It is possible that the differences observed may

be related to the strength of the signal. If the signal is strong it may travel further in the plant, whereas a weaker signal maybe limited to the lower leaves.

However, similar to the findings of Murray and Walters (1992), were the effects of saccharin on broad bean. The saccharin drench significantly increased protection in all leaves examined, although protection was higher in leaves 4-7 compared to leaves 2 and 3. When saccharin was applied to the first leaf, resistance was higher in leaves 5-7 compared to leaves 2-4. Additionally, Guedes *et al.* (1980) also found that systemic resistance to anthracnose in cucumber induced by inoculating the fifth leaf with *Colletotrichum lagenarium*, was produced in leaves 6 to 10, but the response was strongest in leaves 9 and 10. It has been suggested that this may be the result of increased pathogen susceptibility in fully expanded leaves compared to young developing leaves. The alternative suggestion is that the increased resistance in the upper leaves is due to a sink effect at the growing point (Guedes *et al.*, 1980).

4.4.6 Effect of chemically induced systemic protection on growth

Phosphate, ALA, SA and saccharin had limited effect on the growth of barley. Nevertheless, there was a significant reduction in all aspects of growth examined in plants challenged 14 d after treatment with phosphate. Also, in plants challenged 14 d after application of the saccharin drench, there was a significant reduction in total leaf area, although there was not a similar reduction in leaf area when saccharin was applied to leaves. In addition, there was also a significant reduction in the total number of leaves and tillers in plants challenged 10 d after treatment with SA.

Neither phosphate nor SA had any significant effect on the growth of broad bean, although the effects of phosphate on growth were only examined in plants 21 d after treatment. However, the effect of saccharin on plant growth was dependent on the method of application. Saccharin when applied to the leaves had no significant effect on plant growth. Although the saccharin drench produced no significant effect on the total number of leaflets, all other aspects of growth considered were significantly reduced compared with controls.

There was a general decline in NAR in barley plants 13 d after treatment (Figure 47c). The decline was greater in the ALA and phosphate treated plants, whereas the decrease was minimal in the SA and saccharin treated plants. This suggests a net reduction in photosynthesis and was accompanied by a similar reduction in RGR. This is possibly indicative of the reduced photosynthesis and increased respiration known to occur in mildew infected barley (Bushnell and Allen, 1962; Walters and Ayres, 1984; Farrar and Rayns, 1987; Scholes *et al.*, 1992), since despite the various treatments, all plants were mildew infected. Smedegaard-Petersen and Stølen (1981) also identified enhanced respiration in mildew resistant barley to fund defence responses, and in plants challenged 3 d (harvested at 13 d) following treatment with phosphate, salicylic acid and saccharin, significant protection against mildew was observed (Figure 39). However, the decrease in NAR was of short duration and 16 d after treatment NAR had been restored to a little above values at the start of the experiment. Both NAR and RGR were lower 24 d after treatment with phosphate and this was evident in the overall decrease in growth in these plants. Similarly, NAR and RGR were slightly lower 20 d after treatment with SA, and this is reflected in the decrease in numbers of leaves and tillers observed in plants challenged 10 d (harvested at 20 d) after treatment with SA.

In bean plants 16 d following treatment with salicylic acid, NAR was reduced compared to controls (Figure 61c). This difference was not apparent in plants 20 d after treatment and, indeed, had reversed after 24 d. Since salicylic acid treatment had little effect on rust infection it is impossible to attribute these changes in NAR and, indeed, in RGR and LAR compared to controls, to SA or to rust infection. In the work of Murray and Walters (1992) on rust induced resistance in broad bean, resistance in upper leaves coincided with increased photosynthesis, following inoculation of lower leaves with rust. They also found that net photosynthesis was reduced in rust infected leaves. In plants treated with the saccharin drench, NAR and RGR were initially lower at the beginning of the experiment, compared with the other treatments, but there was a subsequent increase in NAR and RGR in plants 24 d (challenged 10 d) after treatment with the drench. It is tempting to speculate that this increase in NAR may be the result of the reduction in rust infection following the saccharin drench, although the reduction observed was not significantly different from the control (Figure 65).

In barley, there was a general decline in LAR over the time examined. As LAR is an expression of leaf area in terms of dry weight, this is probably an indication of an increase in water stress and a reduction in leaf expansion due to pathogen infection. Water stress is a symptom associated with powdery mildew infection (Jenkyn and Bainbridge, 1978). However, LAR was lower in the plants treated with the saccharin drench compared to the control (Figure 53d) and this was apparent in the reduced total leaf area in plants challenged 14 d after treatment with the saccharin drench (Figure 52c). In accordance with this, the FW/DW ratio was lower in the drench treated plants compared with controls throughout the experiment (Figure 53a). There was also a decline in LAR in the bean plants during the period of the experiment, irrespective of

treatment (Figures 61d and 69d). This is perhaps also due to increased water stress as a result of pathogen infection, since it is well documented that rust infection greatly reduces cuticular resistance to water loss and leads to increased transpiration (Ayres, 1981). The exception to this was in the plants treated with the saccharin drench. Here, although, LAR was initially lower in the plants treated with the drench, there was very little change during the experimental period. This is in agreement with the decrease observed in both fresh weight and total leaf area in bean plants treated with the saccharin drench and the lower FW/DW ratio. What is less clear is the reason for the increased water stress in both barley and bean plants treated with the drench. It is unlikely that the increase in water stress is due entirely to pathogen infection, as the saccharin drench was the most effective of the chemicals tested in reducing pathogen infection. Consequently, it is possible that saccharin, applied as a drench, also inhibits water uptake possibly by damaging plant roots or the plant's vascular system.

4.4.7 Wilting

A few of the smaller bean plants became badly wilted within 24 h of saccharin application and died. This wilting symptom in bean plants adversely affected by saccharin perhaps indicates that the saccharin was damaging vascular tissue or roots. The effects on LAR mentioned above, which suggested that plants treated with the saccharin drench became water stressed, similarly support this. However, not all small plants were severely wilted, and indeed some small plants appeared unaffected. This suggests that plant size may be only one of the factors involved. There is perhaps a similarity between plant response to saccharin and that observed in tobacco when *Peronospora tabacina* was used to induce resistance to blue mould. Tuzun *et al.* (1986)

found that if either the plants were very small or the injection of the spore suspension too deep, infection of vascular tissue resulted and necrosis spread through vascular tissues resulting in stunting. However, as with the bean experiment, not all small plants were adversely affected, which suggests that in tobacco, plant size was not an exclusive factor.

4.4.8 Mode of chemical application

Ward *et al.* (1991) found that injecting tobacco leaves with the SAR inducer INA (methyl-2,6-dichloroisonicotinic acid), was more effective than spraying INA onto the leaves. Similarly, the effectiveness of saccharin depended on the mode of application. Saccharin applied as a drench was found to have greater efficacy in inducing systemic protection in both barley and broad bean, compared with leaf application of saccharin. However, saccharin applied as a drench was found to have a detrimental effect on plant growth. The reduction in growth is possibly associated with increased water stress, perhaps through damage caused to plant roots or the vascular system by the saccharin.

There are parallels with the work of Tuzun and Kuć (1985) on systemic protection of tobacco induced with *Peronospora tabacina,* against blue mould. They found that whether resistance, was induced by stem injection, application of a spore suspension to soil at the base of the stem or occurred naturally in the field, unless high levels of nitrogen fertiliser were applied, the plants were often severely stunted. In contrast, injection outwith the xylem resulted in a major increase in plant growth (Tuzun and Kuć, 1985). The present work has shown that the method of saccharin application alters the effectiveness of the defence response in barley and broad bean. However, further

work is required to determine the mechanisms responsible for the effects of saccharin on growth of some of the plants.

4.4.9 Necrosis

There is known to be only a comparatively small margin between the concentration rates at which SA is effective in inducing systemic resistance and that at which it is phytotoxic (Kessmann *et al.*, 1994). In the present work, within 24h of applying 15 mM of SA to barley plants the treated leaves were severely shrivelled from which they did not recover. The remaining plant leaves appeared unaffected. The effect was less noticeable with the other treatments. Similarly, necrosis was apparent on the treated leaves of bean plants within 24 h of SA application.

Within 24 h of applying 3 mM saccharin, the surface of some of the treated bean leaves appeared stretched and there was also a slight discolouration on some of the leaves. As the experiment progressed, some plants treated with saccharin developed necrosis, particularly on lower leaves. There are similarities with the work of Cohen *et al.* (1991) when they tested five fatty acids as inducers of systemic resistance in potato against *Phytophthora infestans*. The fatty acids were found to be phytotoxic to potato leaves. The most phytotoxic, arachidonic acid (AA) and eicosapentaenoic acid (EPA) were also found to be the most effective in inducing systemic resistance. The least phytotoxic failed to induce systemic resistance.

4.4.10 Conclusions

The effect of chemical induction of systemic protection on plant growth and development is not straightforward. Phosphate, SA, and saccharin applied as a drench to barley, did have some detrimental effect on plant growth. In contrast, phosphate, SA and leaf applied saccharin had no significant effect on bean growth. Perhaps what is more pertinent to both the effect on growth and the effectiveness of the defence response is the mode of chemical application. Saccharin applied as a drench was more effective as an inducer of systemic protection compared to leaf application of saccharin. However, when saccharin was applied to leaves there was no adverse effect on plant growth. Associated with the increased efficacy of saccharin applied as a drench was a detrimental effect on plant growth. There are parallels with the work of Tuzun and Kuć (1985). They found that injection of tobacco plants with a spore suspension of *Peronospora tabacina* outwith the xylem still induced protection against blue mould, but in addition there were dramatic increases in plant growth, compared with other modes of inducing resistance. In the present work, it is quite possible that the ability of the chemicals to induce systemic protection was related to their ability to damage the plant. There was not time to explore this further in the current work and consequently, further work is required to address this aspect of systemic induced protection.

Chapter 5

The Effect of Saccharin on the Phenylpropanoid Pathway in Barley

5 The Effect of Saccharin on the Phenylpropanoid Pathway in Barley

5.1 Introduction

5.1.1 Phenylpropanoids

Phenylpropanoids are phenolic compounds found only in plants. They include flavonoids, suberins, stilbenes, coumarins and polymeric lignins. Coumarins and stilbenes are thought to be active in plant defence responses. Lignins provide strength and rigidity in plant vascular tissues and are also implicated in defence against pathogens (Christensen, 1997).

5.1.2 Phenylalanine ammonia-lyase (PAL)

Phenylalanine ammonia-lyase (PAL) is the catalyst involved in the deamination of phenylalanine to cinnamic acid (Carver *et al.*, 1994). This may be considered the first step in the phenylpropanoid pathway leading to the production of substituted cinnamic acid CoA esters and peroxidase, and culminates in lignin synthesis from cinnamyl alcohols (Grisebach, 1981; Northcote, 1985). After conversion of L-phenylalanine into cinnamic acid, the cinnamic acid is converted into *p*-coumaric acid. Alternatively, in some plants, such as wheat, tyrosine ammonia-lyase (TAL) may act directly upon L-tyrosine to produce *p*-coumaric acid (Maule and Ride, 1976; Guerra *et al.*, 1985; Kofalvi and Nassuth, 1995;).

An increase in PAL activity may occur as a direct response to penetration by the pathogen (Hahlbrock and Scheel, 1989; Shiraishi *et al.*, 1995). In dicotyledons inhibition of PAL gene expression has resulted in reduced lignin and increased susceptibility to fungal disease (Bate *et al.*, 1994; Maher *et al.*, 1994). Additionally, resistance of barley to powdery mildew has been linked with an increase in PAL activity (Shiraishi *et al.*, 1995).

5.1.3 Cinnamyl alcohol dehydrogenase (CAD)

Cinnamyl alcohol dehydrogenase (CAD) catalyses the reaction to form the final lignin precursor (Moerschbacher *et al.*, 1986; Mitchell *et al.*, 1994). Substituted hydroxycinnamyl aldehydes are reduced to coniferyl, *p*-coumaryl and sinapyl alcohols (Moerschbacher *et al.*, 1986). In grasses these monolignols are then polymerised, mainly by isozymes of peroxidase (POX) in cell walls to form lignin (Sauter and Kende, 1992).

5.1.4 Peroxidases (POX)

Peroxidases have several different functions within plant defence responses. They are instrumental in the synthesis of cell wall components such as suberin, cross-linked extensin, and as previously mentioned lignin, all of which increase resistance to fungal penetration (Grisebach 1981; Gasper *et al.*, 1982; Lamport, 1986). In addition to their role in lignification and strengthening of cell walls, peroxidases also oxidise phenols into pathogen toxic derivatives and metabolise active oxygen species, particularly H_2O_2 (Baker *et al.*, 1985; Gaspar *et al.*, 1985; Patykowski *et al.*, 1988). However,

peroxidases are also involved in various oxidising reactions resulting in the generation of H_2O_2 (Pedreño *et al.*, 1990; Vianello and Macri, 1991; Pichorner *et al.*, 1992; Jiang and Miles, 1993). This is significant, as elevated levels of H_2O_2 are often associated with plant defence responses against pathogen attack (Lamb and Dixon, 1997).

5.1.5 Phenolic compounds

An initial response in many plant pathogen interactions is the release of pre-formed phenolics. However, it is not until later, after stimulation of the phenylpropanoid pathway that there are substantial increases in the synthesis of phenolics (Graham and Graham, 1991; Peltonen, 1998). Due to the toxicity of free phenolic compounds in the cytoplasm, these compounds are sequestered in the vacuole or the cell wall. At the cell wall, phenolic acids or cinnamyl alcohols may be polymerised into lignin or linked together with esters or ethers to cell wall polysaccharides or hemicelluloses (Lewis, and Yamamoto, 1990; Cvikrova *et al.*, 1991; Lam *et al.*, 1992). During the initial stages of the plant defence reaction, phenolics stored in the central vacuole are released into the cytoplasm to be subsequently incorporated into cell wall defences (Sedlářová and Lebeda, 2001). Phenolics are also instrumental in the hypersensitive response (HR). In cells penetrated by the pathogen there is an accumulation of lignin-like polyphenolics, resulting in single cell necrosis in an attempt to limit pathogen progress and development (Moerschbacher *et al.*, 1990; Nicholson, 1992; Levine *et al.*, 1994; Wei *et al.*, 1994; Zeyen *et al.*, 1995). In addition the release of toxic substances from dead cells together with accumulated phenolic compounds may inhibit pathogen growth (Nicholson and Hammerschmidt, 1992).

5.1.6 Lignins

Cereal lignins are more complex than those found in non-grass plants. They are derived from coniferyl, sinapyl and *p*-coumaryl alcohols purported to be in the ratio of 40-50%, 40-50%, and 5-10% respectively (Boudet *et al.*, 1995; Christensen, 1997). They also contain high concentrations of coumaric and ferulic derivatives from cell walls (Boudet *et al.*, 1995; Christensen, 1997). Esterified cell wall bound *p*-coumaroyl and feruloyl derivatives, may chemically inhibit enzymatic degradation of plant cell walls by fungal pathogens as well as being toxic to the pathogen (Ride, 1983; Nicholson and Hammerschmidt, 1992; Lyons *et al.*, 1994).

5.1.7 Objective

The way in which saccharin elicits a systemic defence response in barley has not previously been examined. In this study, it was hoped to elucidate the extent to which saccharin affects some of the constituents of the phenylpropanoid pathway, and in so doing gain an insight into the mode of action involved in the induction of systemic protection by saccharin.

5.2 Materials and Methods

5.2.1 Plant material

Barley plants (*Hordeum vulgare* L. cv. Golden Promise) were grown four plants to a 13 cm pot in Levington M3 compost. Plants were grown in a ventilated glasshouse as previously described (section 2.2.1).

5.2.2 Experimental design

× 2 Treatments, saccharin or water drench

× 5 Pathogen challenge at, 1, 3, 6, 10, 14 d after treatment

× 4 Samples taken prior to and 18, 24 and 48 hr after pathogen challenge

× 3 Replicates

× 16 Plants in one replicate

1920 Total plants required

It was considered important to sample across as wide a timescale as possible, as it was unknown when responses in phenylpropanoid metabolism might occur. In addition, a total of 16 plants comprised one replicate to provide sufficient plant material for enzyme assays. Consequently, only three replicates were possible to permit the number of sampling times to be maximised. To minimise environmental effects on results, a random experimental design was created in Minitab 12.

5.2.3 Treatment

Saccharin was applied to plants as a soil drench at the two leaves unfolded growth stage 12 (Zadocks *et al.*, 1974). Each plant received either 30 ml (120 ml per pot) of a 3 mM saccharin solution or water for the controls. Following treatment, plants were watered from the base into a saucer.

5.2.4 Pathogen challenge

Plants were inoculated with mildew at 1, 3, 6, 10 and 14 days after treatment with either a saccharin or water drench. The barley plants were inoculated with mildew, by placing in an infection chamber and dusting with powdery mildew conidia.

5.2.5 Sampling

Leaf samples were taken prior to inoculation and at 18, 24 and 48 h after inoculation. Sample material for enzyme extraction comprised of tissue (approx. 1 g) from between 7-8 leaves. For free phenolics and lignin extraction a further sample was taken from 3-4 leaves (approx. 0.5 g). In all cases, second leaves on barley plants were harvested. Leaves excised in the glasshouse were placed immediately in liquid nitrogen, before storage at –80°C.

5.2.6 Enzyme extraction

The enzyme extract was used to assay the activity of PAL, POX and CAD. The extraction protocol was based upon methods described by Cahill and McComb (1992), and Stadnik and Buchenauer (2000). Leaf material was ground in liquid nitrogen in a pre-cooled mortar and pestle. The sample was then transferred to a centrifuge tube and 5 ml of ice-cold extraction buffer (100 mM Tris-HCl buffer pH 7.5; 1 mM EDTA; 1.5% insoluble polyvinylpolypyrrolidone and 10 mM β-mercaptoethanol) was added. The samples were then centrifuged at 25 000*g* at 4°C for 20 min. The resulting supernatant was then stored at –40°C until required, since samples stored at –20°C precipitated out

and denatured. However, the PAL assay was undertaken immediately following enzyme extraction.

5.2.7 Phenylalanine ammonia-lyase (PAL) assay

The PAL assay was based on the method used by Stadnik and Buchenauer (2000). Into a test tube was placed 1 ml of freshly prepared enzyme extract together with 4 ml of 100 mM Tris-HCl buffer (pH 8.8) containing 34 µmol ofL-phenylalanine (Sigma Chemical Co.). To the reaction mixture was added 100 µl of Tris-HCl buffer with L-phenylalanine containing [^{14}C] L-phenylalanine (3.7×10^3 Bq; 17.4 GBq mmol^{-1}; Amersham Pharmacia Biotech UK Ltd.). The samples were incubated at 40°C for 2 h, when the reaction was stopped by the addition of 30 µl of 10% sulphuric acid. Cinnamic acid formed was extracted by the addition of 1ml of ethyl acetate. The samples were mixed, and after a short settling period the upper organic phase was removed. The upper phase was removed by pipette, allowed to resettle and the upper phase removed again. To the organic phase was added 10 ml of hionic fluor scintillation cocktail (Hewlett Packard, Netherlands), and enzyme activity measured in terms of ^{14}C cinnamic acid present in the organic phase using a liquid scintilation analyser (Canberra Packard Tri-carb 1900 TR).

5.2.8 Peroxidase (POX) assay

Peroxidase activity was measured based on a protocol described by Stadnik and Buchenauer (2000). To start the reaction 4-10 µl of enzyme extract was added to 1.5 ml of 100 mM sodium phosphate buffer (pH 6) containing 0.25% (v/v) guaiacol (2-

methoxyphenol) and 100 mM of H_2O_2. Changes in the rate of absorbance were measured at 1 s intervals over 60 to 180 s at 470 nm using a spectrophotometer (Hewlett Packard). Due to varying sample strength it was necessary to amend the concentration of the enzyme extract and reaction time measured. This ensured that the rate of absorbance was measured during the exponential (linear) phase of the reaction. Peroxidase activity expressed as nkat mg^{-1} protein was calculated using the molar extinction coefficient of 2.66×10^4 l mol^{-1} per cm for tetraguaiacol (Southerton and Deverall, 1990).

5.2.9 Cinnamyl alcohol dehydrogenase (CAD) assay

CAD was assayed according to the method of Mitchell, Hall and Barber (1994), based on the oxidation of coniferyl alcohol to coniferaldehyde. Controls were prepared for each sample containing 200 µl of 0.5 mM coniferyl alcohol (Sigma Aldrich), 200 µl of 0.5 M Tris HCl (pH 9.3), and 400 µl of 4.98 µM zinc nitrate (a CAD cofactor). Samples contained of 200 µl coniferyl alcohol, 200 µl of 0.5 M Tris HCl (pH 9.3), 200 µl of 0.5 M $NADP^+$ (sodium salt), and 200 µl of 4.98 µM zinc nitrate. Samples and controls were equilibrated at 30°C. One sample and one control reaction was prepared for each sample by adding 200 µl enzyme extract containing 10% ethylene glycol. Changes in absorbance at 400 nm were measured in a spectrophotometer (Hewlett Packard) at 20 s intervals for 30 min. After deducting the rate of absorbance observed in the control, CAD activity was expressed as pkat mg^{-1} protein, using the molar extinction coefficient for coniferaldehyde of 21×10^3 M^{-1} cm^{-1} (Wyrambik and Grisebach, 1975).

5.2.10 Protein assay

Protein concentrations were determined using the colorimetric assay developed by Bradford (1976), using a kit produced by Biorad. This assay utilises the principle that an acidic solution of Coomassie Brilliant Blue G-250 when bound to protein has a maximum absorbance at 595 nm.

To produce a standard curve, 5 ml of diluted dye reagent was added to 100 µl of four protein standards ranging from 200 to 800 µg ml^{-1} bovine serum albumin. After 5 min, the standards were measured on the spectrophotometer at 595 nm and a standard curve produced. Protein concentrations could then be determined by comparing the absorbance of the enzyme extract, with added dye reagent, with that produced by the standard curve at a particular protein concentration. A dilution factor of 5 was used to bring the enzyme extract samples within the protein concentration range of the calibration curve.

5.2.11 Assay of free phenolic compounds

Free phenolic compounds were extracted and assayed based on a modification of the protocol provided by Stadnik and Buchenauer (2000). Phenolics were extracted by the addition of 4 ml of 50% methanol to the frozen leaf samples. The samples were then incubated at 80°C for 1.5 h. The samples were then centrifuged for 5 min at 3000 *g*. The supernatant was removed from the leaf tissue and its volume measured. The remaining leaf tissue was dried for a minimum of 48 h at 60°C for quantification of lignin. The supernatant was then used to determine phenolic concentration. The reaction mixture consisted of 250 µl of each phenolic extract, 750 µl of distilled H_2O,

100 µl of 20% Na_2CO_3, and 50 µl of Folin Ciocalteau reagent. After 20 min at room temperature, absorbance of the samples was measured at 725 nm. A calibration curve was produced from *p*-coumaric acid, with standards of 10 to 40 µg ml^{-1}. The reaction mixture for the standards comprised of 1 ml of each standard, 100 µl of 20% Na_2CO_3, and 50 µl of Folin Ciocalteau reagent. This allowed the free phenolic compounds to be quantified allowing for a dilution factor of four.

5.2.12 Lignin assay

The lignification of leaf tissue was determined using the method of Stadnik and Buchenauer (2000). Dried leaf samples from the phenolics assay were weighed and then ground to a fine powder in a mortar and pestle using a small amount of silver sand. The dried, powdered leaf samples were then placed into a centrifuge tube together with 5 ml of 2 M HCl containing 10% (v/v) thioglycolic acid (mercaptoacetic acid). The leaf samples were then incubated for 4 h at 100°C, after which they were then cooled on ice and centrifuged at 27000 *g* for 5 min. The supernatant was removed and 2 ml of distilled H_2O added to wash the pellet by centrifuging for a further 5 min at 27000 *g*. After removing the water wash, the pellet was resuspended in 5 ml of 0.5 M NaOH overnight at 4°C to extract lignothioglycolic acid from the pellet. The following day, the samples were centrifuged for 5 min at 27000 *g*, 200 µl of concentrated HCl added to each sample, and then samples were incubated on ice for 4 h. After centrifuging at 27000 *g* for 10 min, the supernatant was discarded and the pellet resuspended in 1 ml 0.5 M NaOH, containing 10% ethanol. Lignin content was determined by measuring absorbance at 280 nm against a lignin calibration curve. Lignin standard (alkali, 2-hydroxypropyl ether, Aldrich) was dissolved in ethanol and then diluted with NaOH to

produce standards between 10 and 150 μg ml^{-1} lignin. The samples had to be diluted by a factor of 25 to be read on the calibration curve.

5.2.13 Statistical analyses

An analysis of variance (ANOVA) was applied to data, using the Genstat 5 statistical program (Lawes Agricultural Trust). The differences between the treatment and control means were tested for significance using the Student's *t*-test. Data are presented as means ± standard error (SEM).

5.3 Results

5.3.1 PAL

PAL activity was not significantly affected by the application of saccharin, except at 14 d after treatment when PAL activity was lower in the saccharin treated plants than untreated plants (Figure 70a).

Pathogen challenge did not significantly alter PAL activity (Figure 70b-f).

PAL activity was considerably higher in samples from both saccharin treated and control plants 14 d after treatment. This may reflect the reduction in soluble protein content observed in both treated and control plants 14 d after treatment (Figures 71 and 72).

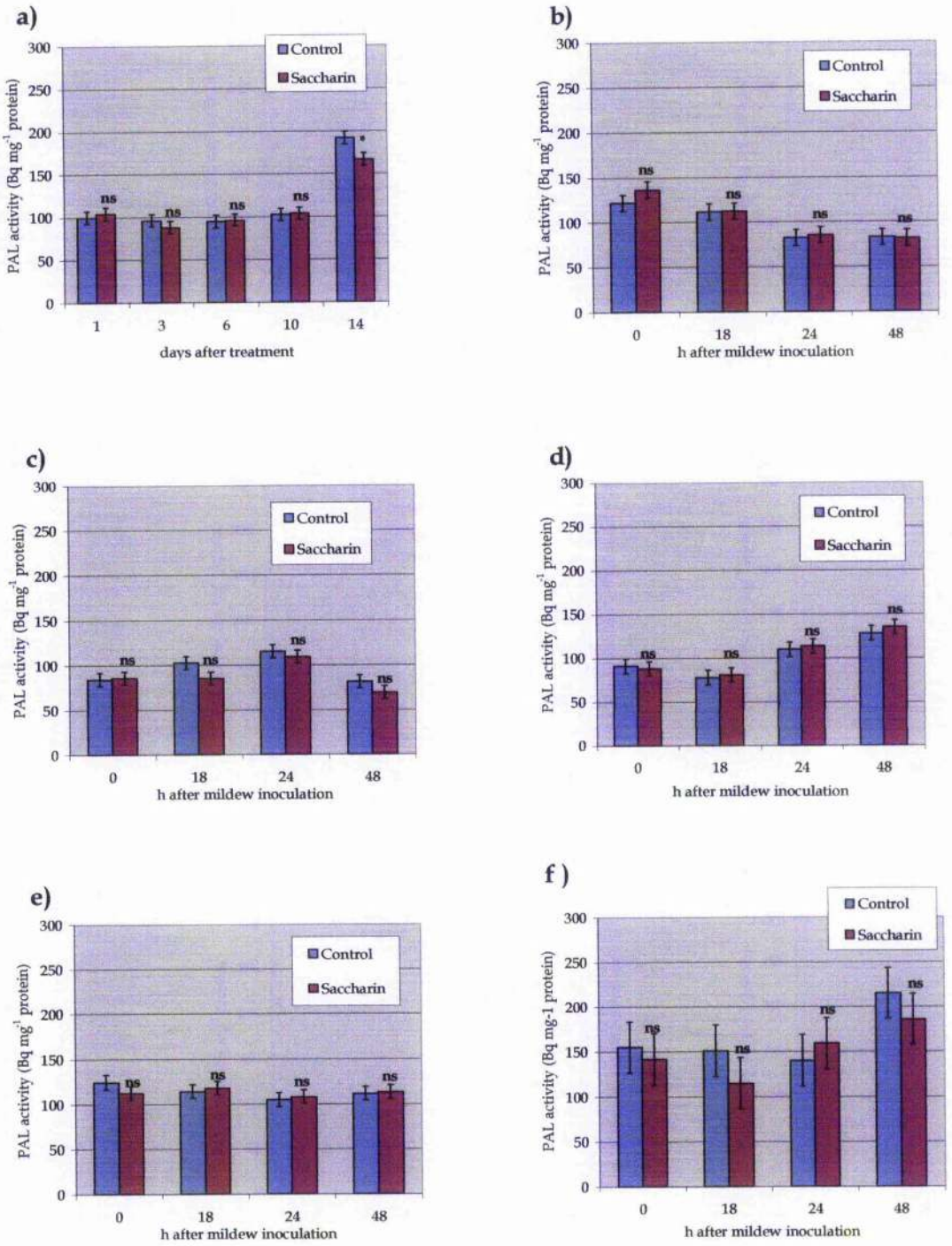


Figure 70(a) PAL activity in barley treated with 3 mM saccharin drench. Values are the mean ± SEM of 12 replicates. (b-f) Effects of powdery mildew inoculation on PAL activity in barley treated with 3mM saccharin drench. Plants were inoculated with powdery mildew 1 d (b), 3 d (c), 6 d (d), 10 d (e) and 14 d (f) after treatment with saccharin. Values are the mean ± SEM of 3 replicates. Significant differences from controls were determined using the Student's *t*-test and are shown as $P < 0.001$ ***, $P < 0.01$ **. $P < 0.05$ * and ns not significant.

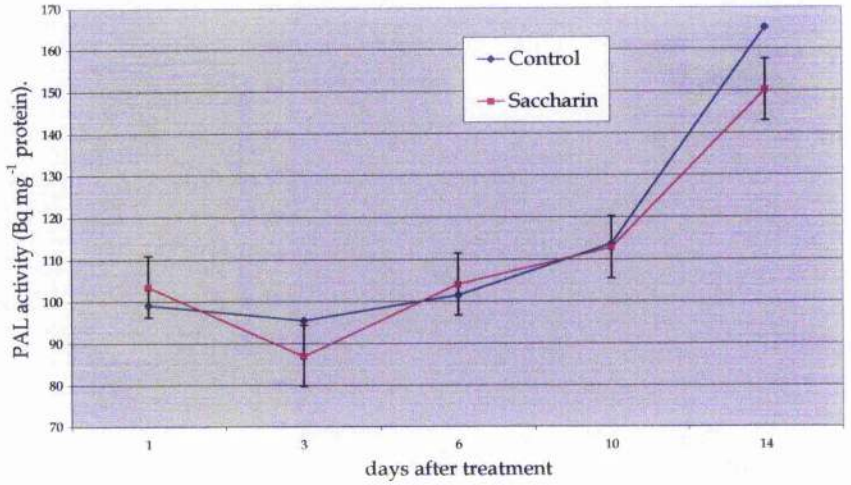


Figure 71. PAL activity in barley treated with 3mM saccharin drench. Values are the mean ± SEM of 12 replicates. (These are the same data that appear in Figure 70a).

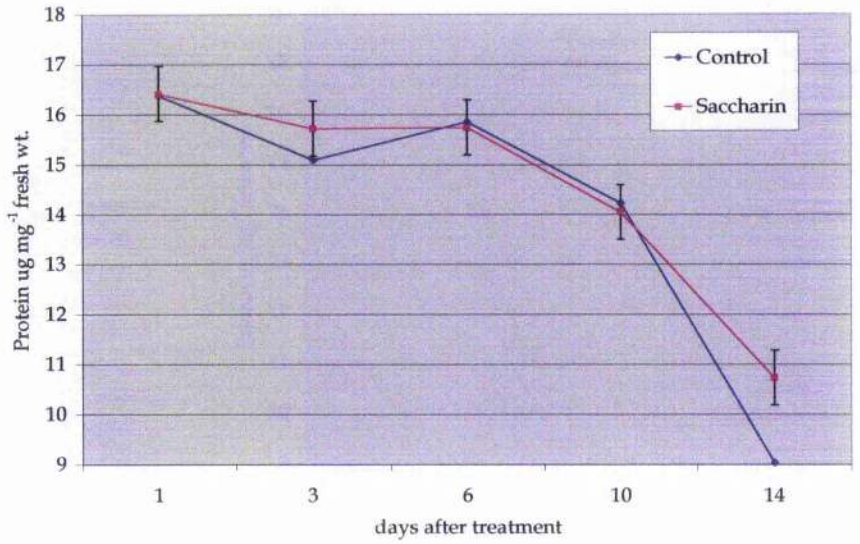


Figure 72. Protein concentration in barley samples used for the PAL assay. Values are the mean ± SEM of 12 replicates.

5.3.2 POX

Significant increases in POX activity were observed in saccharin treated plants 6 d following treatment (Figure 73d). However, this effect had disappeared by 10 d after treatment and by 14 d following saccharin treatment POX activity was significantly reduced compared to controls (Figure 73e, f).

Inoculation with powdery mildew produced no significant change in POX activity during the first 24 h, irrespective of the period between saccharin treatment and mildew inoculation (Figures73Figure 73b-d, f). The only exception to this was a significant increase in POX at 18 h in plants inoculated with mildew 10 d following saccharin treatment (Figure 73e). Significant increases in POX activity were detected at 48 h in plants inoculated with powdery mildew 1, 3, 6 d following treatment with saccharin (Figures 73Figure 73b-d). The effect was not observed when the interval between saccharin treatment and mildew inoculation had increased to 10 d, and by 14 d between saccharin treatment and mildew inoculation, POX activity 48h after inoculation had decreased significantly (Figure 73e, f).

POX activity was considerably higher in samples from both saccharin treated and control plants 14 d after treatment. This may reflect the reduction in soluble protein content observed in both treated and control plants 14 d after treatment (Figures 74 and 75).

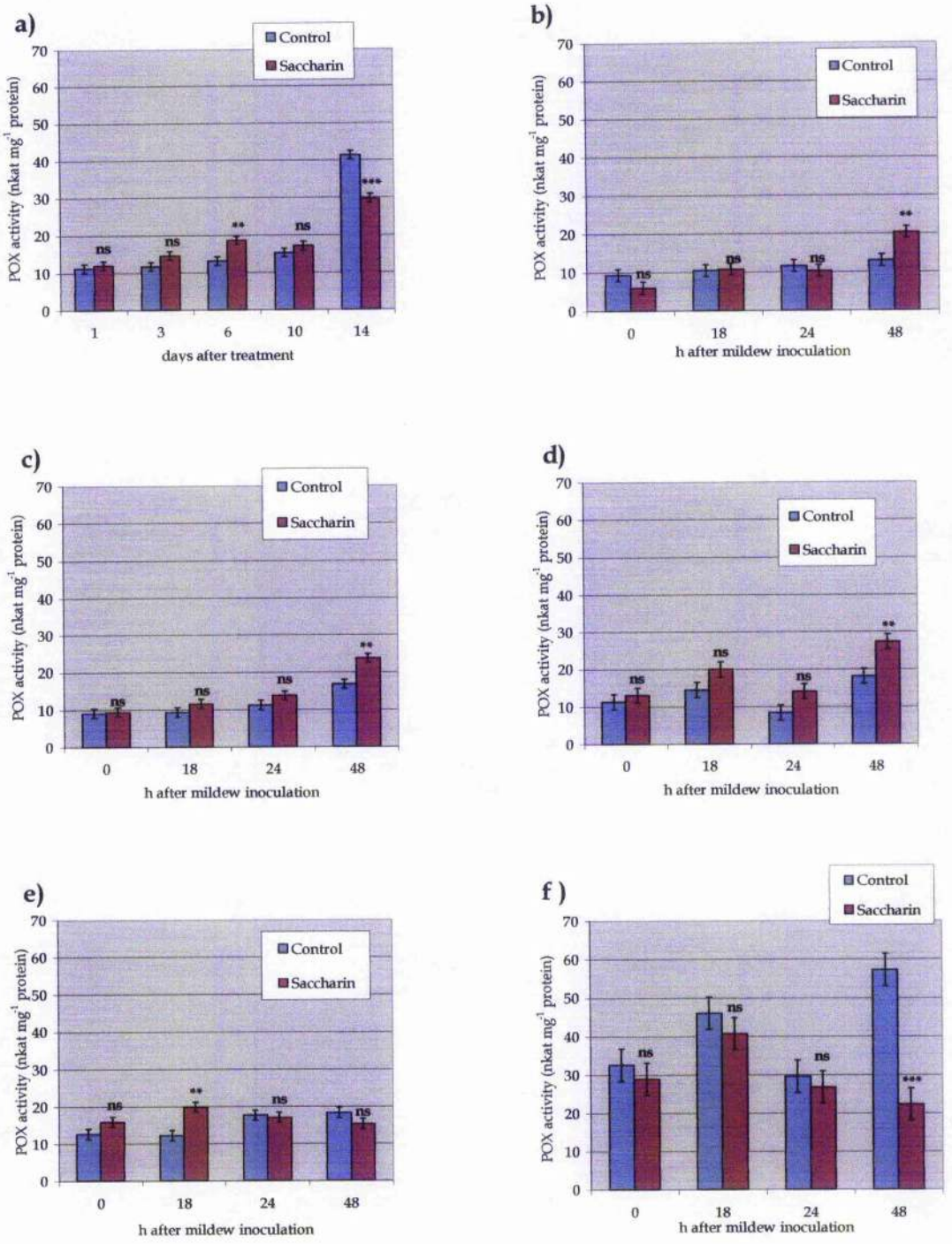


Figure 73(a) POX activity in barley treated with 3 mM saccharin drench. Values are the mean ± SEM of 12 replicates. (b-f) Effects of powdery mildew inoculation on POX activity in barley treated with 3mM saccharin drench. Plants were inoculated with powdery mildew 1 d (b), 3 d (c), 6 d (d), 10 d (e) and 14 d (f) after treatment with saccharin. Values are the mean ± SEM of 3 replicates. Significant differences from controls were determined using the Student's *t*-test and are shown as $P < 0.001$ ***, $P < 0.01$ **. $P < 0.05$ * and ns not significant.

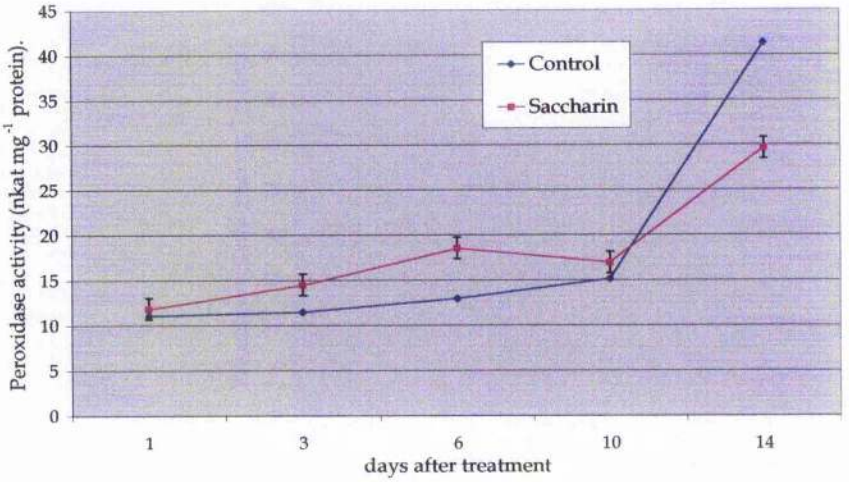


Figure 74. POX activity in barley treated with 3mM saccharin drench. Values are the mean ± SEM of 12 replicates. (These are the same data that appear in Figure 73a)

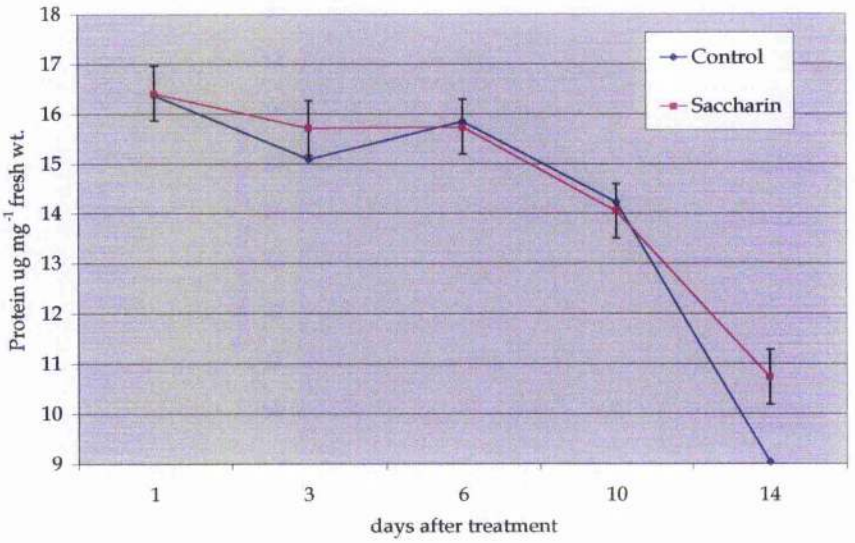


Figure 75. Protein concentration in barley samples used for the POX assay. Values are the mean ± SEM of 12 replicates.

5.3.3 CAD

Significant increases in CAD activity were detected in saccharin treated plants compared with controls from 3 d after treatment. CAD activity increased approximately 2-fold in saccharin treated plants compared with controls 14 d after treatment (Figure 76a).

Inoculation with powdery mildew produced no significant increase in CAD activity during the first 18 h, irrespective of the period between saccharin treatment and mildew inoculation (Figure 76Figure 76b-d, f). The only exception to this was a significant increase in CAD 18 h after inoculation with mildew 10 d following saccharin treatment (Figure 76c).

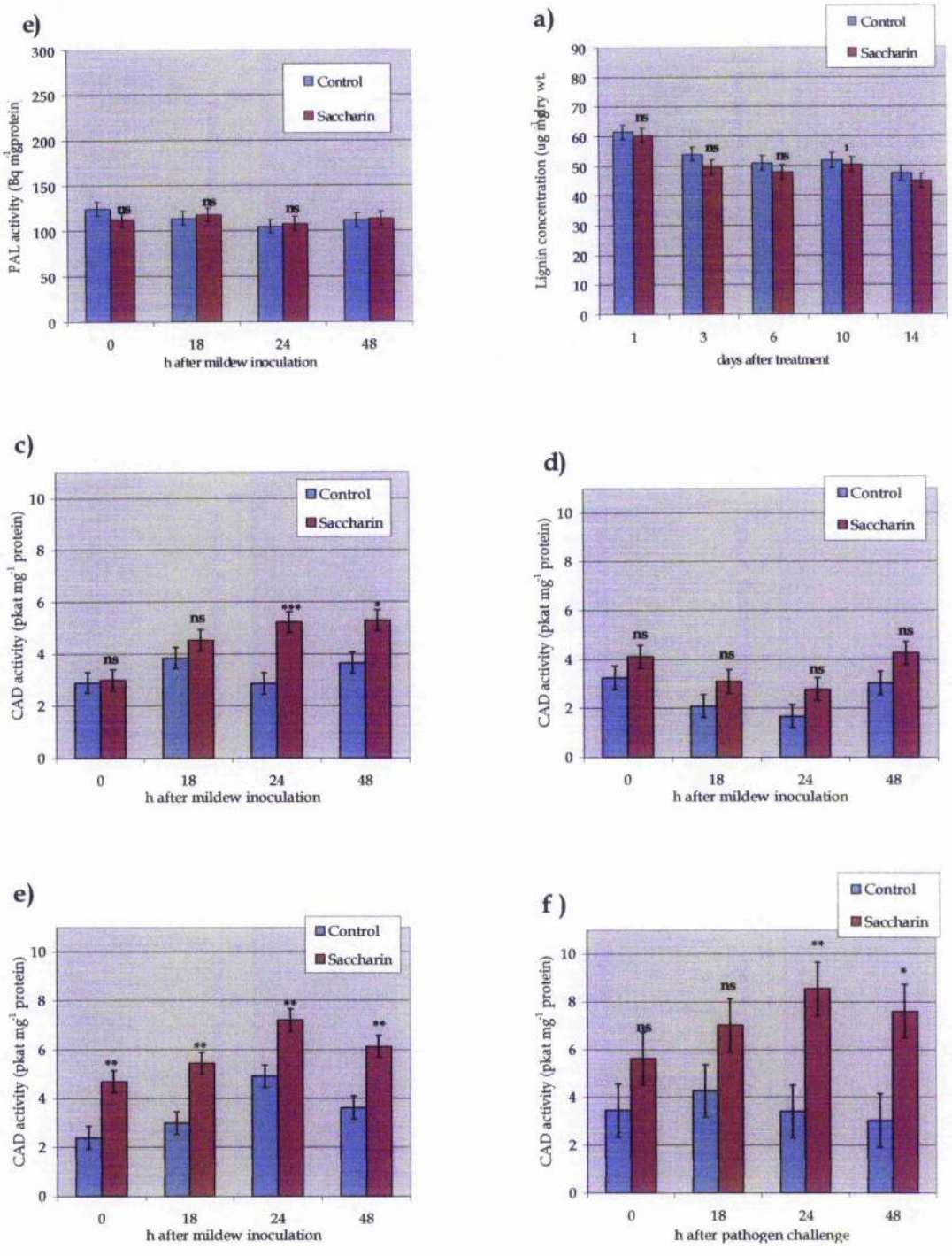


Figure 76 (a) CAD activity in barley treated with 3 mM saccharin drench. Values are the mean ± SEM of 12 replicates. (b-f) Effects of powdery mildew inoculation on CAD activity in barley treated with 3mM saccharin drench. Plants were inoculated with powdery mildew 1 d (b), 3 d (c), 6 d (d), 10 d (e) and 14 d (f) after treatment with saccharin. Values are the mean ± SEM of 3 replicates. Significant differences from controls were determined using the Student's *t*-test and are shown as $P < 0.001$ ***, $P < 0.01$ **. $P < 0.05$ * and ns not significant.

5.3.4 Free phenolic compounds

Levels of free phenolics were not significantly affected by the application of saccharin. In saccharin treated plants and controls the level of free phenolics increased during the first 3 d following treatment, irrespective of the time after mildew inoculation. However, the level of free phenolics had declined by 14 d after treatment and were lower in both treated and control plants compared with levels 1 d after treatment (Figure 77a-f).

Pathogen challenge did not significantly increase the level of free phenolics. The only exception to this was a significant increase in free phenolics at 48 h in plants challenged with mildew 14 d following saccharin treatment (Figure 77f).

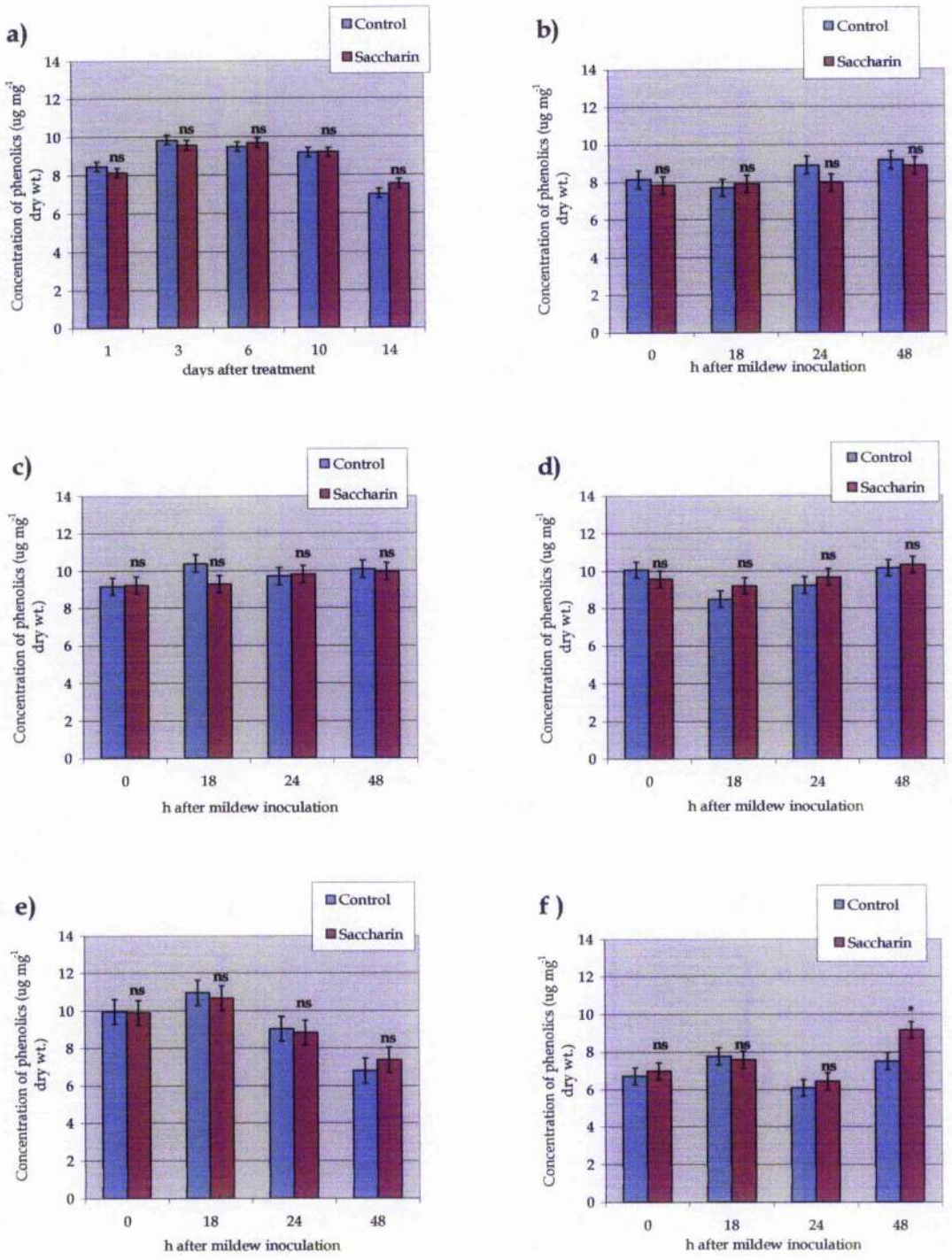


Figure 77 (a) Free phenolics in barley treated with 3 mM saccharin. Values are the mean ± SEM of 12 replicates. (b-f) Effects of powdery mildew inoculation on free phenolics concentration in barley treated with 3mM saccharin. Plants were inoculated with powdery mildew 1 d (b), 3 d (c), 6 d (d), 10 d (e) and 14 d (f) after treatment with saccharin. Values are the mean ± SEM of 3 replicates. Significant differences from controls were determined using the Student's *t*-test and are shown as $P < 0.001$ ***, $P < 0.01$ **. $P < 0.05$ * and ns not significant.

5.3.5 Lignin

Saccharin treatment had no significant effect on lignification. Lignification declined in treated and control plants during the course of the experiment. Lignification was highest 1 d after treatment, but by 14 d after treatment lignification was reduced by approximately 25% compared to plants at 1 d (Figure 78a).

Inoculation with powdery mildew had no significant effect on lignification, irrespective of the period between saccharin treatment and mildew inoculation (Figure 78b-f).

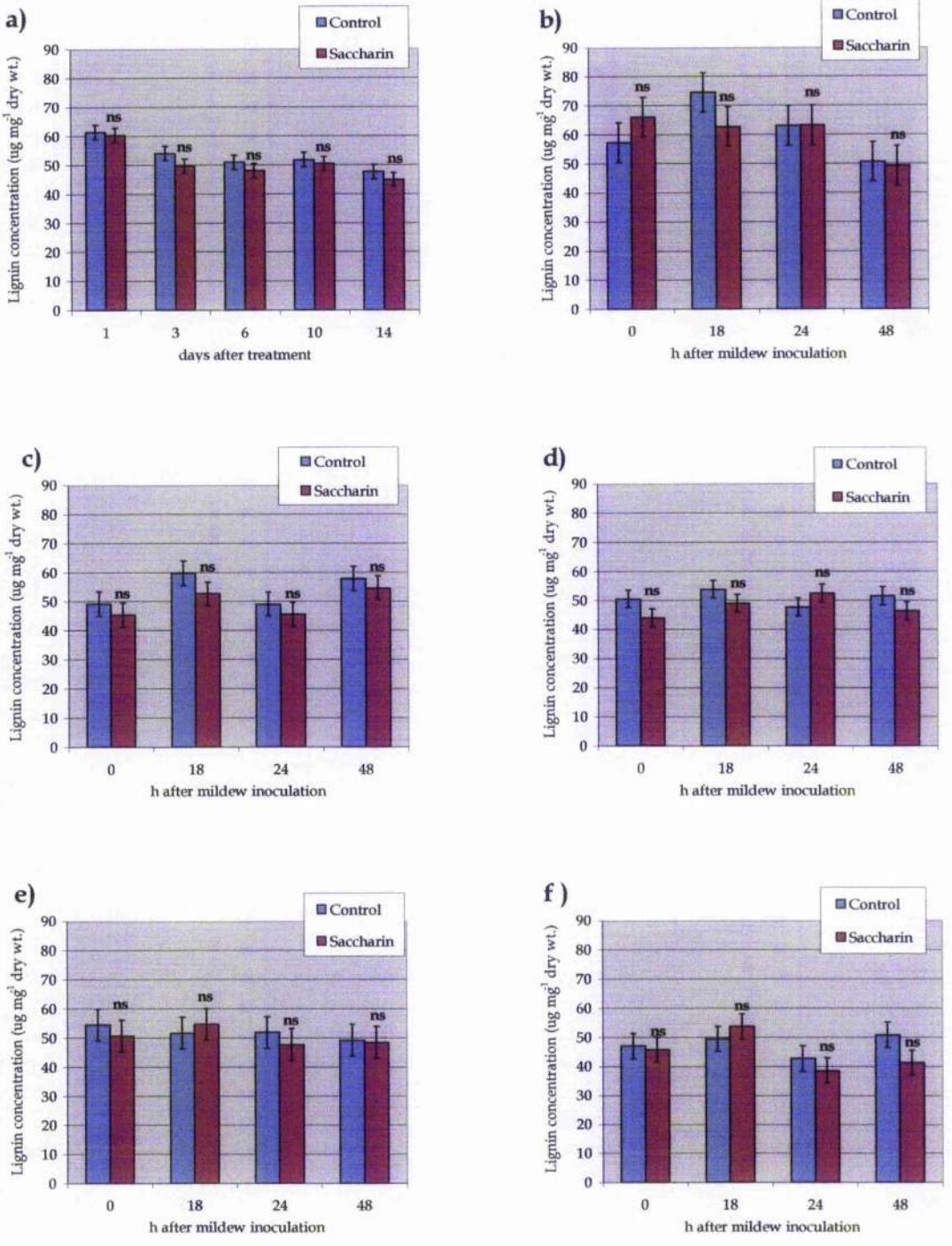


Figure 78 (a) Lignification of barley treated with 3 mM saccharin. Values are the mean ± SEM of 12 replicates. (b-f) Effects of powdery mildew inoculation on lignification in barley treated with 3mM saccharin. Plants were inoculated with powdery mildew 1 d (b), 3 d (c), 6 d (d), 10 d (e) and 14 d (f) after treatment with saccharin. Values are the mean ± SEM of 3 replicates. Significant differences from controls were determined using the Student's *t*-test and are shown as $P < 0.001$ ***, $P < 0.01$ **. $P < 0.05$ * and ns not significant.

5.4 Discussion and Conclusions

5.4.1 PAL

PAL catalyses the first step in the phenylpropanoid pathway. Where PAL activity has been increased, this often results in other enzymes further down the phenylpropanoid pathway also exhibiting an increase in activity (Maule and Ride, 1983; Moerschbacher *et al.*, 1988; Flott *et al.*, 1989). However, stimulation of PAL activity does not necessarily mean that all enzymes in the pathway will be similarly upregulated. For example, Stadnik and Buchenauer (2000) found increased PAL activity, whereas CAD activity was unaffected in powdery mildew infected wheat. Similarly, Zhang *et al.* (1997) found increased PAL activity but not CAD in oats and barley infected with powdery mildew.

In some plant defence responses, PAL activity does not appear to be enhanced but this does not exclude increased activity of other enzymes and products of phenylpropanoid metabolism. For example, Kofalvi and Nassuth (1995) found that PAL and TAL activity was unaffected in wheat infected with wheat streak mosaic virus (WSMV) although CAD activity was increased. Similarly, in the present work, there appeared to be no affect on PAL activity in barley treated with saccharin, even after the subsequent challenge with powdery mildew. Despite the lack of increased PAL activity, enhanced levels of CAD and peroxidase were still detected. Kofalvi and Nassuth (1995) suggest that activities of enzymes in the phenylpropanoid pathway are not necessarily correlated, and if background levels of cinnamic acid were sufficiently high, this would negate up-regulation of PAL activity.

However, the nature of PAL activity may vary widely according to plant development, genotype, type of tissue and environmental factors such as light levels (Camm and Towers, 1973; Podstolski and Brown, 1974; Liang *et al.*, 1989). Additionally, activity of PAL is thought to be stronger and appear earlier in resistant interactions than in susceptible interactions (Latunde-Dada *et al.*, 1987; Bhattacharyya and Ward, 1988; Ralton *et al.*, 1988).

Peltonen (1998) compared the induction pattern of PAL in barley and wheat with highly aggressive (HA) and weakly aggressive (WA) isolates of *B. sorokiniana.* The highly aggressive isolates produced a strong early induction of PAL activity 16-24 h after inoculation. Weakly aggressive isolates produced little or no activity in PAL at that time (Peltonen, 1998). In cereals, PAL activity is thought to increase as appressoria mature and autofluorecent responses become apparent and decline as penetration occurs (Peltonen and Karjalainen, 1995; Shiraishi *et al.*, 1995; Zang *et al.*, 1997; Stadnik and Buchenauer, 1999). A second peak of PAL activity could be clearly identified in the more resistant barley and wheat cultivars 40 h after pathogen inoculation. This response was irrespective of the aggressiveness of the isolate. The production of this second peak in PAL activity has previously been observed in more highly resistant plant varieties (Green *et al.*, 1975; Peltonen and Karjalainen, 1995). The second peak seems to coincide with the development of lesions (Lawton and Lamb, 1987; Boyd *et al.*, 1994; Peltonen, 1998). It is possible that the appearance of the second peak corresponds to a further attempt by the plant to limit progress of the pathogen (Peltonen, 1998).

Orr *et al.* (1993) suggest that increased activity of PAL is very transient. This is consistent with the work of Peltonen *et al.* (1997) where various elicitors were used to induce PAL in barley cell suspension cultures. They found a rapid but transient induction of PAL. Treatment with yeast extract and its purified glucan induced maximum PAL activity 4 h after treatment, but enzyme activity then decreased to control levels within 24 h. Similarly, in cell cultures treated with purified chitin or *B. sorokiniana* mycelium, PAL activity was detected after 2 h, reached maximum activity at 8 h and returned to control levels within 24 h.

It seems possible that the reason no increase in PAL activity was observed in barley treated with saccharin was due to the transient nature of the PAL response. It may be that the differences identified by researchers as to which combination of phenylpropanoid products are enhanced in a particular plant defence response, may be an indication of different rates of progress within the phenylpropanoid pathway. Since CAD activity was stimulated in treated barley it is possible that a transient increase in PAL activity had already occurred, its products metabolised, and levels of PAL returned to normal.

5.4.2 POX

Although saccharin had no affect on PAL activity differences were observed with POX activity. Moerschbacher *et al.* (1988) and Southerton and Deverall (1990), both found that increases in PAL activity preceeded increases in POX during rust infection of wheat, suggesting perhaps that if POX activity was stimulated in barley, an increase in PAL activity may have already taken place.

Significant increases in POX activity in saccharin treated barley plants, compared to controls, were only apparent 48 h after pathogen challenge, in plants that were inoculated with mildew 1, 3 and 6 d after treatment with the saccharin. On these occasions treatment with saccharin resulted in an approximately 33% increase in peroxidase activity compared with controls. Similarly, enhanced POX activity has been detected in SAR induced in *Nicotiana tabacum* against tobacco mosaic virus (TMV) (Buonaurio and Montalbini, 1995).

There was a considerable increase in POX activity in both control and treated plants 14 d after treatment. However, there was also a concomitant reduction in soluble protein concentrations at 14 d after treatment, especially in the controls. It is possible that the higher level of protein in treated plants compared to controls at 14 d is an indication of increased synthesis of defence proteins. However, the effect of reduced protein levels in samples was to elevate POX specific activity, as POX was expressed in terms of protein concentration. Although POX activity may have increased relative to protein concentration at 14 d, it was not possible to assess POX activity in the leaf as a whole. In future studies it would be useful to additionally restrict samples to a known number of whole leaves. This would allow a comparison to be made between levels of activity in the whole leaf as well as expressing activity in terms of protein.

It is interesting to note that a decrease in protein was also observed in the grain of mildew infected barley and wheat, and leaf rust infected wheat grain (Caldwell *et al.*, 1934; Johnson *et al.*, 1979; Smedegaard-Petersen and Stølen, 1981). Additionally, protein was also reduced in the grain of highly resistant barley plants inoculated with mildew (Smedegaard-Petersen and Stølen, 1981).

5.4.3 CAD

The CAD assay measured enzyme activity in terms of the oxidation of coniferyl alcohol to coniferaldehyde. CAD activity was significantly higher in saccharin treated plants compared with controls from 3 d after treatment. The increase in CAD activity was approximately 2-fold at 14 d after treatment. Similarly, Kofalvi and Nassuth (1995) found a 2-fold increase in levels of coniferaldehyde in wheat streak mosaic virus (WSMV) infected wheat, from 8 d after inoculation. Interestingly, CAD activity in wheat leaves in which an elicitor was used to induce a defence response produced no increase in either *p*-coumaraldehyde, or coniferaldehyde, but a 10-fold increase in sinapaldehyde (Mitchell *et al.*, 1994). In addition, it would be interesting to measure CAD activity in barley on the substrates *p*-coumaryl and sinapyl alcohols to see if an isoform of CAD exists in barley with a specific preference for one particular substrate.

However, in contrast to the results with saccharin and barley described in this work, treatment of wheat with the SAR inducer BTH and the pathogen *Blumeria graminis* had no significant effect on CAD activity. Leaf segments treated *in-vitro* with the CAD inhibitor 2-hydroxyphenyl amino sulphinyl acetic acid (OH-PAS), or OH-PAS and BTH, resulted in reduced CAD activity (Stadnik and Buchenauer, 2000). Similarly, Zhang *et al.* (1997) found no accumulation of gene transcripts for CAD in either powdery mildew infected oat or barley.

CAD activity remained high throughout the barley / saccharin experiment, in both treated and control plants infected with powdery mildew. In work done by Kofalvi and Nassuth (1995), CAD activity was high in the healthy and WSMV infected plants 2 d

after inoculation, and remained high in infected plants but declined in the healthy plants 8 d after inoculation (Kofalvi and Nassuth, 1995).

5.4.4 Free phenolic compounds

Levels of free phenolic compounds peaked in plants 3 days after treatment, but by day 14 had declined to levels below those present one day after treatment. However, there was no discernible effect on concentrations of free phenolic compounds either due to application of the saccharin drench or as a result of pathogen challenge. The only possible exception to this was a significant increase in phenolic compounds in saccharin treated plants 14 d after saccharin treatment, and then only at 48 h after pathogen challenge. It is possible that this is an indication that increases in free phenolic compounds, as a result of treatment with saccharin, do not become apparent until later than the timescale covered by this experiment. However it is equally likely, especially given the level of significance involved, that this is merely an aberrant result and not significant at all. It is therefore possible that changes in free phenolics were entirely due to the developmental stage of the barley and not treatment or pathogen challenge.

Certainly, early on in a plant defence reaction phenolic compounds accumulate at the fungal penetration site, but these are in the walls of epidermal cells and would consequently not be identified by our assay. Phenolics subsequently accumulate in cells when a hypersensitive response (HR) occurs. In barley infected with powdery mildew, cells expressing a HR accumulated cell wall bound polyphenolics (Keon and Hargreaves 1984; Hargreaves and Keon, 1986). Again, as these phenolics are bound to cell walls, they would not be identified by this assay.

Stadnik and Buchenauer (2000) also found that levels of free phenolics and lignin in wheat treated with BTH were not significantly different from inoculated controls. However, Kofalvi and Nassuth (1995) detected elevated concentrations of methanol extracted free phenolics in WSMV infected wheat. This coincided with increased CAD activity. This is not surprising as methanol extracted free phenolics include the cinnamyl alcohols which are synthesised by CAD and precursors of lignin (Kofalvi and Nassuth, 1995).

5.4.5 Lignin

Following fungal pathogen attack lignin deposition is generally regarded as a relatively slow process (Sherwood and Vance, 1982; Ride and Barber, 1987), although, a HR caused by stem rust in wheat resulted in cell lignification in less than 2-3 h after fungal penetration (Reisener *et al.*, 1986; Moerschbacher *et al.*, 1990). However, the compositions of polyphenolic compounds that accumulate in cereals in response to pathogen attack vary according to species, and for example, barley and oat cell walls did not accumulate lignin in response to infection (Keon and Hargreaves 1984; Hargreaves and Keon, 1986).

Lignification was found to be highest in barley one day after saccharin treatment. Lignification subsequently declined and was lowest 14 d after treatment, with a reduction of approximately 25% compared to day one. However, mildew inoculation had no effect on lignification. Kofalvi and Nassuth (1995) also found that increased lignification was not detected during WSMV infection of wheat. Similarly, there was

no significant difference in lignin levels in wheat treated with BTH (Stadnik and Buchenauer, 2000).

Peroxidases involved in lignin biosynthesis are likely to be restricted to cell walls and would therefore not be present in the enzyme extract (Bruce and West, 1989; Salzwedel and Dazzo, 1993; Sato *et al.*, 1993). This is consistent with the elevated levels of POX activity seen in saccharin treated barley, without leading to a concomitant increase in lignin.

5.4.6 Conclusions

The nature of the protection afforded by SAR varies in different plant pathogen interactions. Some researchers have found that induction of SAR resulted in reduced fungal penetration (Richmond *et al.*,1979; Hammerschmidt and Kuc, 1982; Cloud and Deverall, 1987; Latunde-Dada and Lucas, 2001), whereas, increased resistance in other SAR responses occurred after penetration and was related to necrosis and the accumulation of phytoalexins (Elliston *et al.*, 1976; Elliston *et al.*, 1977; Malamy and Klessig, 1992; Urbanek *et al.*, 1996). Therefore, it is possible that systemic protection primes the plant's endogenous resistance mechanisms to respond more rapidly (Siegrist *et al.*, 1997; Sticher *et al.*, 1997; Katz *et al.*, 1998; Latunde-Dada and Lucas, 2001). This is consistent with the effects of saccharin on aspects of phenylpropanoid metabolism in barley, found in this work. So, CAD activity generally increased in barley controls following powdery mildew inoculation. However, higher CAD activity occurred in saccharin treated plants, compared with controls, both prior to and after

inoculation with powdery mildew, which suggests that saccharin is priming CAD activity prior to pathogen challenge.

Final Discussion and Conclusions

Final Discussion and Conclusions

Many reports of the induction of systemic resistance by plant pathogens seem to assume that it is a prerequisite for the pathogen to be necrotrophic. Indeed, there are very few reported examples of biotrophic pathogens inducing a defence response. However, in this work it has been shown that both mildew and rust, two biotrophic pathogens, are capable of inducing systemic protection in the upper leaves of barley and broad bean, respectively. Results from the current work might suggest that biotrophic pathogens are less effective in inducing systemic protection. Thus, both mildew and rust were found to be less effective in inducing a defence response in the present work, compared with the previous studies namely, Hwang and Heitefuss (1982) on powdery mildew and barley and Murray and Walters (1992) on rust and broad bean. Consequently, this discrepancy may have less to do with the type of pathogen involved and more to do with the strength of the inducer, as affected by the severity of the inducing infection or the number of pre-inoculated leaves. Certainly, Kováts *et al.* (1991) found the existence of a relationship between the number of pre-inoculated leaves and the strength of the defence response. In both of the previous studies, systemic protection was induced by inoculation of the two lowest leaves, compared with only one leaf in the present work.

Leaf position was also a significant factor in the strength of defence response produced. The defence response was stronger in lower leaves of barley induced with chemicals, while a significant response was only observed in leaf 3 and 4 and not in leaf 2 when protection was induced with mildew. The resistance response was also found to be stronger in the lower leaves of broad bean irrespective of whether the response was induced chemically or with rust, although the reverse was true when saccharin was used

as the inducer. Furthermore, Murray and Walters (1992) found that the young upper leaves were more resistant than lower leaves following induction of systemic resistance in broad bean with rust. This suggests that the differences observed were not dependent on the particular plant or inducer, and it may be that signal strength is more pertinent to the distance travelled by the defence signal within the plant.

Siegrist, Mühlenbeck and Buchenauer (1998) using cultured parsley cells, identified saccharin as a potential inducer of SAR. They then proceeded to confirm that saccharin induced SAR in cucumber to *Colletotrichum lagenarium*, French bean to *Uromyces appendiculatus* and tobacco to tobacco mosaic virus (TMV). No further reports on the use of saccharin as an inducer of systemic resistance appear to have been made. This is therefore believed to be the first report of saccharin induced systemic protection in both broad bean and barley. However, the mode of action is unknown, and so it was decided to investigate the effects of saccharin on the phenylpropanoid pathway. The concentration of free phenolic compounds and lignin were not significantly affected by saccharin. There was also no increase in PAL activity, although it is possible that a transient increase in PAL activity had already occurred. Nevertheless, an increase in CAD and peroxidase activity was observed in response to saccharin. The increase in CAD activity, in saccharin treated plants, occurred both prior to and after inoculation with powdery mildew, which suggests that saccharin is priming CAD activity prior to pathogen challenge.

Unlike saccharin, phosphate and SA, ALA is not known as an inducer of pathogen defence. In plants treated with ALA, mildew infection was generally lower compared with controls, although the results were not significant. Furthermore, in plants

challenged more than 6 d after treatment with ALA, mildew infection in leaf 1 was significantly reduced. Therefore, ALA does induce a defence response, although the response observed was not systemic. As the choice of concentration, 3mM was completely arbitrary, the effects of a stronger concentration of ALA, application to more than one leaf, or ALA applied as a drench are unknown. Certainly, ALA applied at 3 mM did not produce necrosis.

It has been shown that necrosis is not essential to the induction of systemic protection. Nonetheless, it is often apparent that a relationship does exist between necrosis and the induction of systemic protection. Indeed, necrosis was observed with the application of both SA and saccharin, and it could be argued that these chemicals were in fact the most effective in inducing a defence response. Similarly, Cohen *et al.* (1991) found that although fatty acids induced systemic resistance in potato leaves against *Phytophthora infestans*, they were also found to be phytotoxic, and the most phytotoxic were also the most effective at inducing a defence response.

Tuzun and Kuć (1985) reported that systemic protection against blue mould induced in tobacco by stem inoculation with *P. tabacina,* external to the xylem, resulted in necrosis of the outer phloem and cortical tissue. However, when the inducer was injected into the xylem, necrosis was observed to extend outwith the xylem. Tuzun *et al.* (1986) also found that injecting some small tobacco plants with *P. tabacina,* or if the injection was too deep, resulted in necrosis spreading to the vascular tissues, the consequence of which was stunting. It is, therefore, possible that the wilting observed in some small bean plants following application of the saccharin drench, may have been the result of root damage. Equally, saccharin may have been causing necrosis in vascular tissue. In

small plants, damage to the vascular system may severely inhibit water uptake resulting in plant death. Although foliar application of saccharin induced systemic protection and produced necrotic areas on some of the leaves, it did not cause wilting.

The effect of systemic induced protection on growth of barley was inconsistent and varied not only in respect of inducer, but also the mode of application. Powdery mildew induced systemic protection resulted in reduced plant growth, whereas phosphate, ALA, SA and saccharin had a limited effect on barley growth. Although the effects on growth were not specific to barley or bean, the effect on growth of barley inoculated with mildew was in direct contrast to the effect of rust on the growth of broad bean. For rust had no significant effect on the growth of broad bean, apart from an increase in total leaf area in plants challenged 14 d after induction. Phosphate, SA and foliar application of saccharin similarly had no effect on bean plant growth, although the effects of phosphate on growth were only examined in plants 21 d after treatment. However, the saccharin drench significantly reduced all aspects of plant growth considered, except the total number of leaflets.

A further more in-depth study was undertaken on the effects of rust induction on bean plant growth, although these plants were not subsequently challenged with the pathogen. Plant growth was not significantly affected by the inoculation of one leaf with rust, whereas, when three leaves were inoculated this resulted in an increase in total plant fresh weight, total plant dry weight and total leaf area one week after inoculation and a reduction in dry weight at week 3. However, by the end of the experiment at week 4, there was no significant difference in growth parameters between the rust induced plants and controls.

Water uptake was reduced 1 w after inoculation and increased from week 3, in the rust infected plants. This was consistent with reduced stomatal opening in the early stages of infection, followed by increased transpiration due to rupture of the cuticle caused by fungal sporulation (Johnson and Miller, 1934, 1940; Murphy, 1935; Duniway and Durbin, 1971). In this further experiment on bean, senescence became apparent, particularly in the controls, towards the end of the experimental period. This became noticeable due to the number of shrivelled and dropped leaves, and was thought to be the result of the limited nutrient supply in the 13-cm pots. Consequently, it is surprising that there was no detrimental effect on growth in the rust induced bean plants, compared with controls, particularly at the end of the experimental period when nutrient supply would be at its lowest. This is contrary to the findings of Heil *et al.* (2000) who found that although BION reduced the growth of wheat, the effects on growth were more severe if the plants were short of nitrogen. Similarly, Mandryk (1961) observed that stunting associated with induced resistance of tobacco to blue mold could be partially alleviated by the application of nitrogen fertiliser.

A progressive decline in the rate of photosynthesis is often observed following infection of leaves with biotrophic pathogens (Whipps and Lewis, 1981; Farrar and Lewis, 1987; Scholes, 1992). In bean leaves infected with rust, F_0 quenching and photoinhibition were apparent. In addition, the photochemical efficiency of PSII reaction centres was reduced in the rust infected leaves from 1 w after rust inoculation. Non-photochemical quenching was also significantly lower at week 4 in the rust infected leaves. It was therefore apparent that the rust was causing severe damage to the photosynthetic apparatus. Nonetheless, there was no indication that photosynthesis was enhanced in the uninfected leaf on the infected plant as might have been suggested if photochemical

quenching had been increased. However, Scholes and Rolfe (1996) using chlorophyll fluorescence imaging of the whole leaf surface were able to identify that changes in photosynthesis were far more complex that had previously been realised. Changes in fluorescence occurred not just over time and depending on whether the leaf was infected or healthy, but there were also differences within an infected leaf. They found that photosynthesis was lowest in areas of the leaf invaded by the fungal mycelium, and at sporulation non-photochemical quenching was enhanced in uninfected regions of the leaf compared with healthy leaves.

Conclusions

In conclusion, the present work found saccharin to be particularly effective at inducing a defence response in both barley and broad bean against powdery mildew and rust respectively. Furthermore, it was apparent that the method of application was not only pertinent to the efficacy of the induced response but was also an important factor in determining the subsequent effect on plant growth. Saccharin when applied as a drench was found to be more effective in inducing the defence response compared to foliar applications. Although foliar applications of saccharin had limited effect on the growth of broad bean, treatment with the saccharin drench was found to be detrimental to plant growth. The saccharin drench was also found to induce severe wilting resulting in the death of some of the smaller bean plants, whereas other bean plants of a similar size were unaffected as were barley plants treated with the saccharin drench. Further work is therefore required to ascertain the cause of these severe wilting symptoms and whether the saccharin drench is perhaps causing damage to the plant's roots or to the vascular system.

Saccharin was found to prime CAD activity in barley prior to pathogen challenge. It would also be valuable to determine if *p*-coumaryl and sinapyl alcohols are used as substrates in addition to coniferyl alcohol and so identify isoforms of CAD that might exist in barley with a preference for a particular substrate. Further investigation is also necessary to consider if a different time-course to that examined in the present work is involved in the up-regulation of PAL by saccharin. Additional data on the level of SA in the plants in relation to any up-regulation of PAL could further produce a keener insight into whether SA is synthesised from phenylalanine or chorismate as proposed by Wildermuth *et al.* (2001).

Although necrosis is not a prerequisite to the induction of a defence response, necrosis often develops as was found following application of saccharin or SA in the present work. It has been suggested that at sufficiently high concentration ALA will cause necrosis. As only a limited study was possible on the effects of ALA on inducing a defence response, further work is required to consider a range of concentrations and examine whether a correlation exists between the level of necrosis and the induction of the resistance response. The nature of the defence response, in terms of whether it is local or systemic when induced by a higher concentration of ALA, is still to be determined, as is the effect of ALA on other plant species in addition to barley.

There was a striking contrast between the detrimental effect of powdery mildew on the growth of barley and the limited effect of rust on the growth of broad bean. It was apparent from the measurement of chlorophyll fluorescence that the photochemical efficiency of PSII was compromised in the rust infected leaves. However, there was no apparent increase in the photochemical efficiency of PSII or in photochemical

quenching in the uninfected leaves on the infected plant to compensate. Future work requires either both spatial and temporal analysis of the whole leaf with chlorophyll fluorescence to allow this to be related to pathogen progress within an infected leaf. Alternatively, measurements of both photosynthesis and respiration are required in individual leaves together with measurements of photosynthesis and respiration in the whole plant.

It is only by a radical rethink of existing crop protection methods that we can hope to sustain an increasing world population. For example, China has to provide for 22% of the world's population from 7% of the world's arable land and China's population is continuing to increase at a rate of 16 million a year (Leeming, 1994). The current use of pesticides, resistant cultivars and agricultural practices as the three main methods of crop protection have their limitations as pathogens overcome plant resistance or develop resistance to pesticides (Ward *et al.*, 1994). Thus, it is important to establish that any new method of crop protection is economically viable and that yield and efficacy compares favourably with existing crop protection methods. It is also important to ensure that ecological implications are fully explored, that fitness is not compromised, or that the crop is not protected from one pathogen only to be devastated by an insect pest. Furthermore, although not within the scope of the current work, that the integrity of the final food product is not compromised, such that nutritional value is maintained and induced compounds are not toxic to the consumer.

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