Biochemical events associated with rust resistance in sunflower

By

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List of Abbreviations

AGIS	Agricultural geo-referenced information service
AOS	Active oxygen species
ASSV	Aborted substomatal vesicle
BABA	DL-β-amino-n-butyric acid
BSA	Bovine serum albumin
BTH	Benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester
CA	Cinnamic acid
cv	Cultivar
EDTA	Ethylenediaminetetraacetic acid
GlcNAc	N-acetylglucosamine
HI	Hypersensitivity index
HMC	Haustorium mother cell
hpi	Hours post inoculation
HPLC	High performance liquid chromatography
HR	Hypersensitive response
ICS	Isochorismate synthase
INA	2,6-dichloroisonicotinic acid
IWF	Intercellular wash fluid
kDa	kilodalton
LOX	Lipoxygenase
NDA	National Department of Agriculture
NPA	Nonpenetrating appressorium
NSA	Nonstomatal appressorium
PAL	Phenylalanine ammonia-lyase
PBST	Phospahate buffered saline containing Tween-20
PMSF	Phenylmethyl sulfonyl fluoride
PR	Pathogenesis-related
PVP	Polyvinylpyrrolidone
SA	Salicylic acid
SAGIS	South African Grain Information Service
SAR	Systemic acquired resistance
SD	Standard deviation

SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
SOD	Superoxide dismutase
TBS	Tris buffered saline
TBST	Tris buffered saline containing Tween-20
TMV	Tobacco mosaic virus
Tris	Tris(hydroxymethyl)aminomethane

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Introduction

Sunflower (*Helianthus annuus* L.) is a major oilseed-producing crop worldwide. It originated in the South-west United States-Mexico area where the seeds were used as food by native Indians (Heiser, 1976; Vranceaunu, 1974). Sunflower was introduced to Europe in the 16th century. It became popular as an ornamental and was first established as an oilseed crop in Eastern Europe (Weiss, 1983). Worldwide production of sunflower resulted primarily from the development of varieties that could be grown under different climatic conditions.

In South Africa, sunflower is largely cultivated in the summer rainfall regions of the country, that is, the North-West, Free State, Limpopo and the Mpumalanga Highveld (Burger, 2002). It is not only cultivated for oil-seed and oil-cake production, but also as animal feed, and to a lesser extent for the floristic industry. Sunflower seed is the most important oil-seed in South Africa with respect to gross value of production, followed by groundnuts and soyabeans (National Department of Agriculture, 2000). Sunflower seed production fluctuated between 170 035 and 839 500 tons between 1988/89 and 1990/00 (National Department of Agriculture, 1999). A large proportion of sunflower seed is destined for oil production with the remainder used directly for seed production and feed. In 1999 total producer sales, amounted to R1 109 000 and R 1 025 000 of this was from sales to local oil expressers. South Africa is nonetheless a predominant importer of sunflower seed even though exports do occasionally occur. Exports of sunflower seed and products are mainly concentrated in the Southern African Development Community (SADC). The European Union (EU 15) still plays an important role with respect to sunflower import trade with South Africa (National Department of Agriculture, 1999; 2000)

Sunflower rust caused by *Puccinia helianthi* Schw., poses a serious economic threat to sunflower producing areas worldwide, including Australia (Kong and Kochman, 1996), U.S.A (Gulya and Viranyi, 1994), Canada (Rashid, 1993) and South Africa (Los *et al.*, 1995). Yield losses as high as 50% due to reduction in the size of the capitulum

and seeds, as well as to the decline in oil content, have been reported in Canada and Argentina (Zimmer and Zimmerman, 1972; Siddiqui and Brown, 1977).

The initial symptoms of rust infection appear as small chlorotic leaf spots. These spots become cinnamon-brown as they begin to actively produce urediospores. Uredial pustules appear on both leaf surfaces, but more commonly on the lower surface, and may be surrounded by yellow haloes. The pustules may coalesce to occupy most of the leaf surface. The uredial stage is the most conspicuous and damaging stage of sunflower rust. In severe infestations pustules may occur on petioles, stalks, bracts and floral parts. Severely infected leaves die prematurely. The telial stage produces black-coloured pustules later in the growing season or when plants are under physiological stress (Sood and Sackston, 1970).

The rust pathogen is an obligate biotroph and enters the plant through appressoria that form over the stomata. Inside the leaf the infection peg develops into a vesicle from which infection hyphae develop. In contact with host cells, infection hyphae differentiate into haustorium mother cells (HMCs) that form haustoria inside host cells. These are the feeding structures used by the fungus to obtain nutrients from the plant cells (Staples, 2000).

The degree of rust damage is related to growing conditions and the seed variety used. Adjusting sowing times to avoid periods of high temperature and humidity will reduce disease incidence. Disease control is, however, possible to a certain degree with several applications of fungicides such as oxycarboxin, mancozeb or zineb. The use of resistant cultivars is nonetheless the best method of combating the disease. Although rust resistance genes have been identified and introduced into hybrids, the pathogenic variability of the fungus can lead to the occurrence of new virulent pathotypes that overcome host resistance (Gulya *et al.*, 1997). In some instances commercially viable hybrids fail to be released due to breakdown of resistance during the final stages of development, placing a huge burden on the development of new hybrids (Kong *et al.*, 1999). The use of fungicides to control rust diseases has proven uneconomical and there is an urgent need to develop disease resistant sunflower lines.

Disease resistance in plants can be either constitutive or induced. There is huge interest in the mechanisms of induced resistance responses of plants. This information may be of great value both in designing new agrochemicals that stimulate plant resistance responses, and in developing genetically engineered plants with enhanced disease resistance. The induced resistance mechanisms act both locally and systemically. Amongst the pathogen-induced defence responses, the hypersensitive response (HR) is the most efficient. This is characterised by host cell death around the infection point and serves to restrict further spread of the invading pathogen (Durner *et al.*, 1997). The HR occurs in plants in response to infection by pathogenic fungi, bacteria and viruses (Slusarenko, 2000). Associated with the HR is the induction of a diverse group of defence related genes, such as the pathogenesis-related (PR) proteins, the products of which are important in destroying the pathogen. Furthermore, a massive increase in the active oxygen species is induced. Over a period of time after the primary infection, the plant develops resistance to subsequent infection throughout, including the uninfected parts. This is termed systemic acquired resistance (SAR) and manifests itself as a long-lasting resistance to the same or even unrelated pathogens (Durner *et al.*, 1997).

A new pathotype, combining virulence for the R1 and R3 resistance genes in sunflower (Putt and Sackston, 1963; Goulter, 1990), was detected in 1998 near Potchefstroom, South Africa. Subsequent screening of entries in the South African cultivar trials showed that 79% of these were susceptible to the prevailing rust pathotypes. In view of the above-mentioned, the establishment of research programmes directed at improving genetic resistance in sunflower against rust and alternative methods of disease control, are considered essential. Genetic engineering provides opportunities to manipulate expression in plants of compounds with varying degrees of toxicity to pathogens. For instance, levels of endogenous phytoalexins could be increased or phytoalexin molecules could be modified to increase potency by introducing genes encoding appropriate enzymes into plants. Another alternative could be the introduction of genes into plants encoding proteins such as chitinases and glucanases that have a direct antifungal activity on pathogens (Jach *et al.*, 1995). Biotechnology approaches have in fact shifted the emphasis towards biochemical

and/or molecular marker-assisted breeding and the construction of vectors with highly regulated transgenes that confer resistance in various ways.

In this study, the main objective was to identify some of the biochemical components of the resistance response of sunflower against the fungal pathogen *P. helianthi*. This includes the possible involvement of the pathogenesis-related proteins, signalling molecules such as salicylic acid and the induction of the oxidative burst. The study also investigated and related disease development in both susceptible and resistant sunflower cultivars to the induced resistance-related events. In addition, the effect of chemicals, referred to as plant activators, on the resistance responses of sunflower was investigated. It is anticipated that the forthcoming results could contribute to a better understanding of the resistance response of sunflower to rust needed to eventually manipulate resistance or to design new and effective disease control strategies.

A review of resistance mechanisms in plants to diseases:

2.1 Sunflower rust

A number of serious diseases such as rust, downy mildew, grey headspot, *Sclerotinia* head rot and *Sclerotinia* stem rot, occur on sunflower (Agricultural Geo-referenced Information System, 2002). The most serious is sunflower rust, caused by *Puccinia helianthi* Schw. Rust occurs in all sunflower producing-areas of the United States of America and Canada as well as on wild sunflower. In the Northern Great Plains of America most oilseed, ornamental and confectionary hybrids have had well to excellent resistance to the prevailing rust races, but changes in rust populations in the late 1980s have resulted in greater rust severity and in substantial losses in seed yield and quality. In South Africa rust occurs in most sunflower production areas where susceptible cultivars are cultivated and environmental conditions conducive to rust proliferation exist (Burger, 2002).

Sunflower rust is a typical macrocyclic, autoecious rust where telial, pycnial, aecial and uredial stages are all produced on one host. The telial and uredial stages occur on older plants, while the pycnial and aecial stages are not easily recognised and usually occur on young seedlings early in the growing season (Zimmer and Hoes, 1978).

2.1.1 Life cycle

Rust fungi are obligatory biotrophic pathogens, and naturally grow and reproduce only on living host plants (Staples, 2001). This biotrophy, however, requires a high degree of cellular interaction between host and parasite. Rusts are generally foliar pathogens with a complex life cycle that involves two parasitic stages, dikaryotic and monokaryotic.

2.1.1.1 Dikaryotic stage

The urediospore germ tube of many rust fungi responds to topographical features of the leaf surface (Read *et al.*, 1992) allowing it to grow towards the stoma, and recognising stomatal presence by responding to the ridges around its lips (Terhune *et al.,* 1991). Adhesion to the leaf surface is required for this contact-sensing (Read *et al.,* 1992). Even though the perception and transduction of the signal is not fully elaborated, there is evidence for the involvement of stretch-activated calcium channels in the fungal plasma membrane (Corrêa *et al.,* 1996) and alteration to the fungal cytoskeleton (Read *et al.,* 1992). In response to stomatal lips, the fungus sequentially forms an appressorium over the stomatal opening, an infection peg that grows between the guard cells into the leaf tissue, a substomatal vesicle in the substomatal space, and an infection hypha that grows intercellularly between mesophyll cells. This morphological differentiation of the fungus is associated with changes in fungal wall composition (Littlefield and Heath, 1979) and the secretion of a variety of hydrolases (Mendgen *et al.,* 1996). In the absence of differentiation, the germ tube continues to grow on the leaf surface until its endogenous reserves are exhausted and it dies (Heath, 1997).

Inside the host, the fungus forms an intercellular mycelium from which intracellular haustoria are formed (Fig. 2.1). These are generally regarded as feeding structures and the last of a series of the development of infection structures. They develop from haustorium mother cells that adhere to the plant cell surface. In some rust fungal species, an unknown signal on the plant cell surface may be necessary for haustorium mother cell induction (Mendgen, 1982; Read *et al.,* 1992).

2.1.1.2 Monokaryotic stage

The basidiospore of the rust fungus usually penetrates directly into epidermal cells of the host. The only pre-penetration infection structure is the appressorium produced by the basidiospore germ tube. Even though very little is known about the signals required for appressorium formation, surface hardness is important (Freytag *et al.,* 1988).

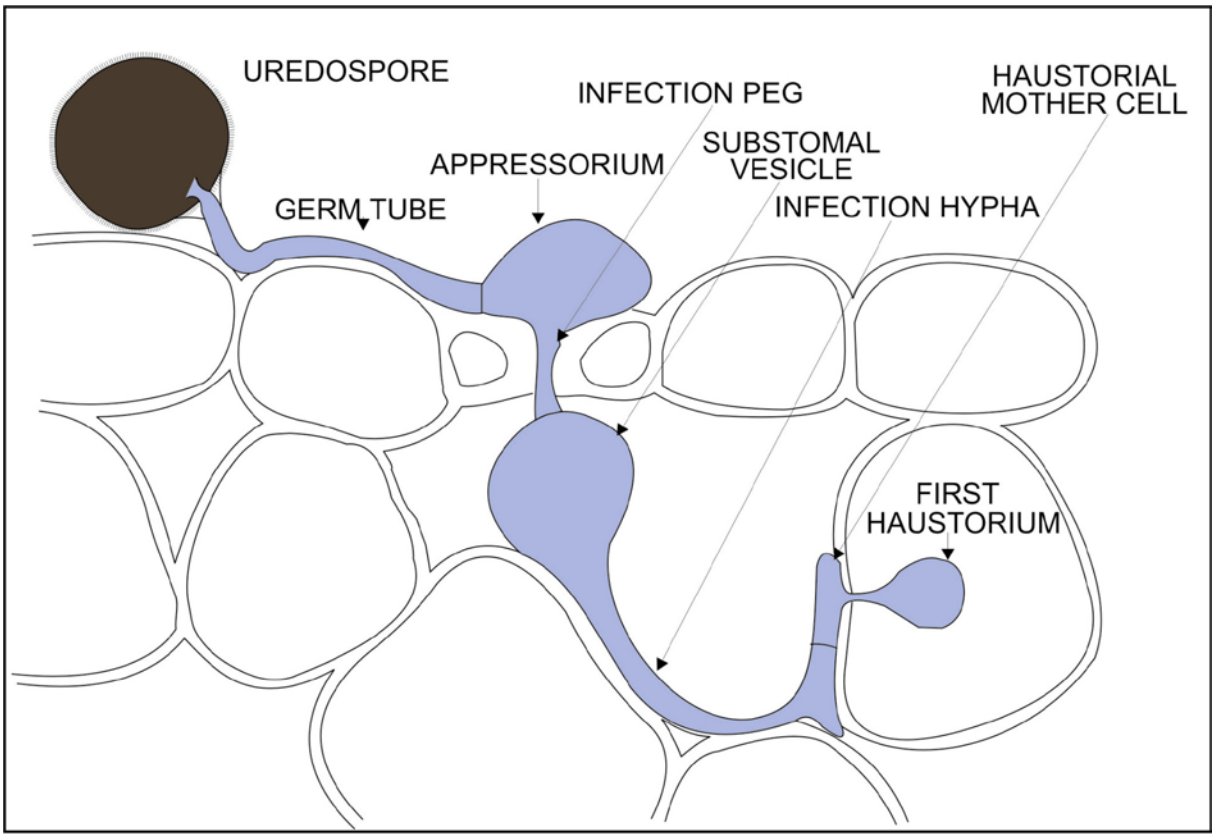


Fig. 2.1: Diagrammatic representation of a cross section showing the infection structures originating from a rust spore on the leaf surface. The structures include the germ tube and appressorium on the surface of the leaf, and the infection peg, substomatal vesicle, infection hypha, haustorium mother cell and haustorium, which develop inside the leaf (adopted from Mendgen *et al*., 1985).

2.1.2 Sunflower rust infection

Urediospores germinate within 4 h after plant inoculations in the greenhouse (Yang and Dowler, 1992). The germ tube is produced from one, rarely from two, of the equatorial germ pores (Sood and Sackston, 1970). Six to 8 h after inoculation germ tubes form irregularly shaped appressoria above the stomata. A small penetration peg grows from the lower surface (in rare cases from a lateral surface), passes through the stomatal opening into the substomatal cavity, and develops an H-shaped substomatal vesicle. The appressorium empties its contents into the substomatal vesicle. Twenty hours post inoculation, two or more infection hyphae emerge from the vesicle, which empties its contents into hyphae and collapses thereafter (Sood and Sackston, 1970). The manner in which germ tube penetrates in older leaves is similar to that in cotyledons. There are no differences in the urediospore germination, appressorium formation, or in the penetration process on susceptible and resistant lines (Sood and Sackston, 1970).

Haustoria form within 24 h of infection. In susceptible hosts they are elongate and numerous. They arise from the infection hyphae. In a resistant host they are spherical and few in numbers. The tip of intercellular hyphae in contact with a mesophyll cell distends and a septum is laid down, forming a haustorium mother cell (HMC). This gives rise to a fine tube that enters the host cell and enlarges to form a round or knob-shaped haustorium. Some haustoria in the susceptible host elongate and branch with age. Haustoria in resistant plants remain round and fewer in numbers than in a susceptible line (Sood and Sackston, 1970).

2.1.3 Host colonisation

Mycelium growth has been found to be rapid in susceptible plants, reaching the lower epidermis within 96 h after inoculation. Mature hyphae form cushions of sporogenous tissue under both the upper and lower epidermis 120 h after inoculation.

The cytoplasm in the young haustoria and hyphae become dense and stain red with fuchsin or safranin. Older hyphae are highly vacuolated and stain light red (Sood and Sackston, 1970). Urediospores are formed approximately 144 h after inoculation. They are initially slightly elongated and hyaline, but as they age they become oval, turn reddish brown, and eventually break through the host epidermis. In a resistant line, mycelial growth is slower and more restricted. Collapse of hyphae has been observed 96 h after inoculation and hyphae did not reach the lower epidermis (Sood and Sackston, 1970).

In a resistant line no apparent differences have been observed between invaded and control cells up to 96 hours post inoculation (hpi) (Sood and Sackston, 1970). At 120 hpi there was a general collapse of host cells underlying the incipient pustules under both the upper and the lower epidermis. There appeared to be fewer chloroplasts in infected than uninfected mesophyll cells. In the resistant line, a few collapsed host cells were observed in infection sites 60 hpi. The number of collapsed cells increased with time. In the susceptible line host cell collapse was not observed except under developing pustules. By 96 hpi nuclei of invaded cells were larger than those of healthy cells. The difference in size was greatest by 230 hpi and was much greater in susceptible than resistant lines. Chlorotic flecks were visible on resistant leaves 120 hpi. Sections of the affected tissues showed that chloroplasts had degenerated in the infected and neighbouring cells. Hyphae in contact with degenerated host cells were highly vacuolated and collapsed (Sood and Sackston, 1970).

2.1.4 Elicitors from rust fungi

The fungal components associated with development of infection structures are capable of acting as elicitors to induce host defence responses. Fungal wall components, especially chitin, act as non-specific elicitors (Bohland *et al.*, 1997), but exudates and wall fragments of chitin-containing infection hyphae of the cowpea rust fungus, *Uromyces vignae,* were found not to mimic the fungus in its ability to trigger

silica deposition in non-host bean leaves (Ryerson and Heath, 1992). Nevertheless, wall components of germ tubes of the wheat stem rust fungus, *Puccinia graminis* f. sp. *tritici*, and apoplastic fluids from rust-infected susceptible wheat leaves will trigger lignification in wheat plants irrespective of the presence or absence of the Sr5 gene for rust resistance (Sutherland *et* al.,1989; Beissmann *et al.*, 1992). This elicitor also stimulates lipoxygenase activity in wheat, apparently by a different signalling pathway than chitin oligosaccharides (Bohland *et al.*, 1997).

2.2 Plant resistance mechanisms

Plants can defend themselves in a very efficient manner against most plant pathogens. The defence can be based on constitutive resistance factors or pathogen-induced resistance reactions. Pre-formed and induced defence reactions can be structural or chemical in nature. The resistance of many plants to phytopathogens is due to pre-existing structural properties such as surface hydrophobocity or topography, as well as cell wall resilience to physical and chemical attack, which prohibit pathogen entry. If a pathogen somehow manages to overcome these barriers, then the plant invariably switches on its second line of defence, thus induction of active resistance reactions. These reactions may also involve structural aspects such as cell wall thickening and reinforcement (Bowles, 1990).

The induced resistance mechanisms act both locally and systemically. More often local resistance is manifested as a hypersensitive response (HR). This is characterised by the development of cell suicide-associated lesions around the point of pathogen entry and serves to restrict further spread of the invading pathogen (Durner *et al.*, 1997). The HR occurs in plants in response to infection by pathogenic fungi, bacteria and viruses (Slusarenko, 2000). Associated with the HR is the induction of a diverse group of defence related genes, the products of which are important in destroying the pathogen. Furthermore, a massive increase in the active oxygen species is induced. Over a period of time after the primary infection, the plant develops resistance to subsequent infection throughout, including the uninfected parts. This is termed systemic acquired resistance (SAR) and manifests

itself as a long-lasting resistance to the same or even unrelated pathogens (Durner *et al.*, 1997). Systemic signals are involved in the induction of SAR.

In this chapter the induced resistance reactions are grouped into the upstream and downstream defence related responses. The upstream events include the eliciting and signalling events, and the downstream events encompass activation of defence genes and physiological responses of the defence gene products.

2.2.1 Constitutive defence responses

Many fungal pathogens gain entrance into their hosts by direct penetration of the host cuticle, which forms the first barrier to be overcome by many plant pathogenic fungi. The role of the cuticle as a barrier to fungal invasion has been supported by a direct correlation between disease susceptibility and cuticle thickness. In the *Solanaceae*, cuticle thickness of the New Mexican-type pepper (*Capsicum annuum*) increased from immature green fruit (12 µm) to mature red fruit (24 µm). Biles *et al.* (1993) have shown that the susceptibility of unwounded fruit to infection by *Phytopththora capsici* decreased with increased ripening. In *Poaceae*, the cuticle of the *Sorghum bicolor* bloomless mutant bm-22 is approximately 60% thinner and one-fifth the weight of the wild type parent P954035 cuticle. This reduction in cuticle size is linked to an increase in disease susceptibility to *Exserohilum turcicum* (Jenks *et al.*, 1994). Antagonistic reports, however, are abundant. For instance, no significant correlations between cuticle thickness and resistance have been observed in pathogenesis of the powdery mildew pathogen (*Erysiphe cichoracearum*) in *Phlox* (Jarosz *et al.*, 1982).

2.2.2 Induced defence responses

2.2.2.1 Upstream defence responses

2.2.2.1.1 Elicitors

Elicitors are defined as molecules that can induce physiological or biochemical responses associated with the expression of resistance (Kogel *et al.*, 1988). Many elicitors originate from plant pathogens. Fungal cell wall elicitors include β-linked glucans (Anderson, 1978; Sharp *et al.*, 1984), chitosan (Friestensky *et al.*, 1985; De

Wit *et al.*, 1985; Mayama *et al.*, 1986) and the unsaturated lipids, arachidonic and eicosopentanoic acids. A number of metabolites of pathogenic origin also act as elicitors and these include polysaccharides (Hadwiger and Beckman, 1980; Sharp *et al.*, 1984), galactose and mannose-rich glycoproteins (Darvil and Albersheim, 1984; Dixon, 1986; Hamdan and Dixon, 1987), fatty acids (Bostock *et al.*, 1981) and hydrolytic enzymes (Collmer and Keen, 1986). Elicitor activity has also been correlated with pectic fragments that arise from the degradation of the plant cell wall by the invading pathogens (Collmer and Keen, 1986).

There are two types of elicitors, the general (non-specific) elicitors which do not differ in their effects on different cultivars within a plant species, and may overall be involved in general resistance, and specific elicitors which are unique to a pathogenic race or strain and function only in plant cultivars carrying a matching disease resistance gene. They account for gene-for-gene interactions and specific resistance (Boller, 1995; Hahn, 1996).

2.2.2.1.2 Gene-for-gene interactions

In plant-pathogen interactions the highly specialised form of recognition between the elicitor and the host is governed by the gene-for-gene interactions. If the plant and the pathogen carry complementary genes specifying disease resistance (*R* genes) and avirulence (*Avr* genes) respectively, then the plant recognises the pathogen (Crute and Pink, 1996). The perception of the elicitors by the high-affinity binding receptors on the host plasma membrane then initiates an intracellular signal cascade which eventually results in the co-ordinate transcription of a large number of defence related genes and rejection of the pathogen (Lamb *et al.*, 1989; Dixon *et al.*, 1994). This interaction is referred to as incompatible and the plant is resistant to the disease. If either of the complementary pairs of genes is absent or carried in a recessive form, there is neither recognition nor induction of the resistance response and the pathogen is able to colonise the host. Such is a compatible interaction and is equivalent to disease susceptibility (Lawton, 1997).

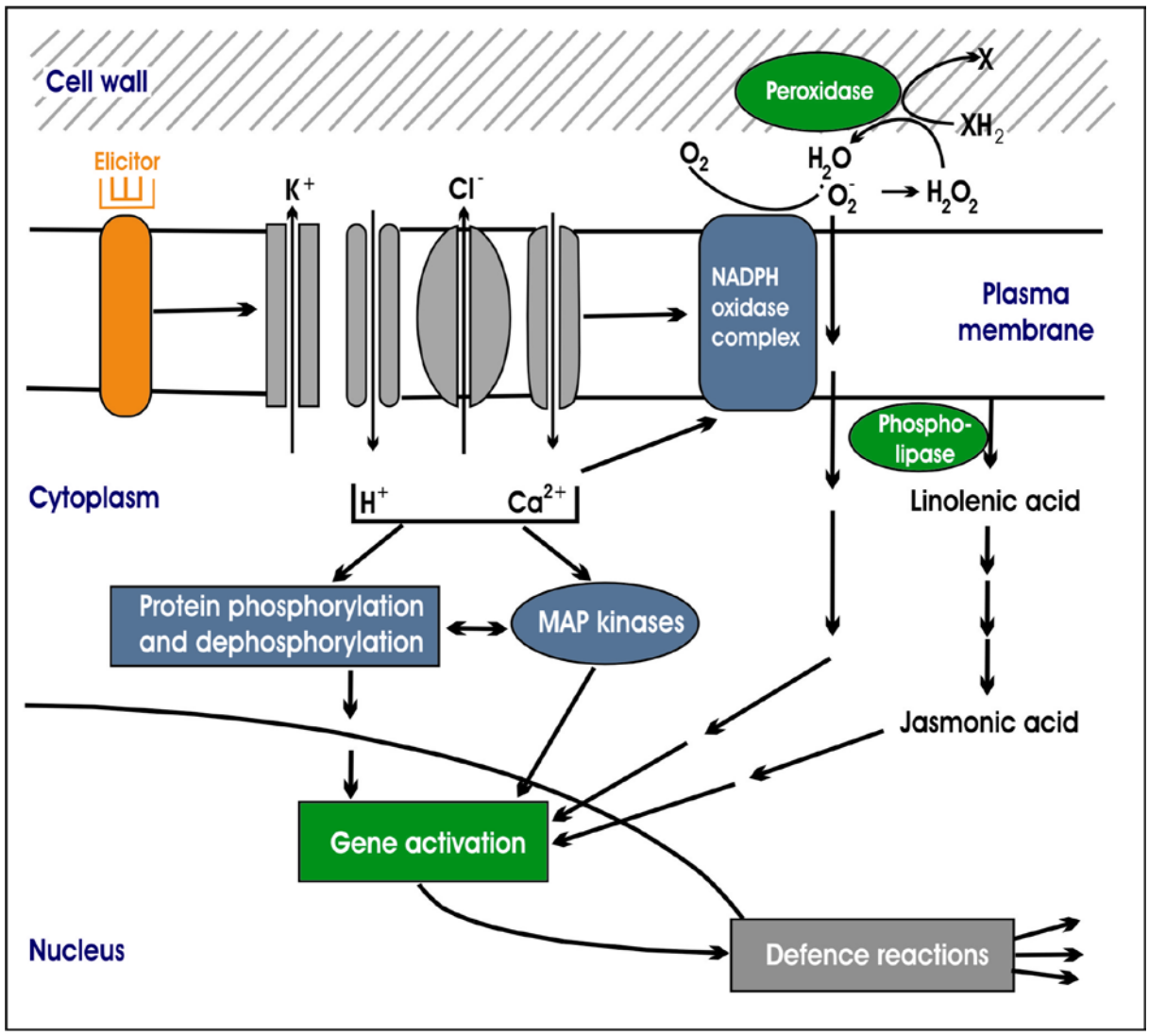


Fig. 2.2: Schematic representation of the generation of AOS and their involvement in signalling downstream defence responses (Somssich and Hahlbrock, 1998).

2.2.2.1.3 Signalling events

The signalling events in plant-pathogen interactions begin with the elicitor binding to the receptors in the plasma membrane. This initiates a signal transduction cascade that involves the production of active oxygen species such as O_2^- and H_2O_2. The activation of certain kinases and lipases also signal the activation of genes whose products are involved in defence reactions (Fig. 2.2).

2.2.2.1.4 Oxidative burst

Several rapid processes characteristic of the HR appear to involve primarily activation of pre-existing components rather than changes in gene expression. One of these rapid processes is the release of active oxygen species (AOS) termed the oxidative burst. This response to elicitors or pathogens has been observed in diverse monocotyledonous and dicotyledonous species including rice, tobacco, soybean and spruce (Mehdy, 1994).

The AOS are toxic intermediates that result from successive one-electron steps in the reduction of molecular O_2. The predominant species detected in plant-pathogen interactions are superoxide anions (O_2^-), hydrogen peroxides (H_2O_2) and hydroxyl radicals ($OH^\bullet$) (Bolwell and Wojtaszek, 1997; Bolwell, 1999).

Active oxygen species are routinely generated at low levels by plant cells due to electron transport in chloroplasts, mitochondria and enzymes in other cell compartments involved in reduction-oxidation processes. The first reaction during the pathogen induced oxidative burst is believed to be the reduction of molecular O_2 to form superoxide anion (O_2^-). In aqueous solutions O_2^- undergoes spontaneous dismutation in an overall reaction written as:

$$2\ O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$$

This reaction occurs at a higher rate at acidic pH such as found in the cell wall where the O_2^- half-life is less than 1s (Sutherland, 1991). The reaction can also be catalysed by superoxide dismutase (SOD) enzymes that originate from the cytosol, chloroplasts and mitochondria (Scandalios, 1993).

Superoxide anion can act as a reducing agent for transition metals such as Fe^{3+} and Cu^{2+}. These metals may be reduced even if they are complexed with proteins or low molecular weight chelators. One important consequence of metal reduction is that it can lead to the H_2O_2-dependent formation of hydroxyl radicals ($OH^{\bullet}$). For instance;

$$O_2^- + Fe^{3+} \longrightarrow O_2 + Fe^{2+} \quad \text{(Reduction)}$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + OH^{\bullet} \quad \text{(Formation of } OH^{\bullet}\text{)}$$

The above equation is known as the Fenton reaction. Due to its extreme reactivity and its formation in cells producing O_2^- and H_2O_2, $OH^{\bullet}$ is believed to be a major AOS responsible for modifications of macromolecules and cellular damage. The $OH^{\bullet}$ initiates radical chain reactions including lipid peroxidation, enzyme inactivation and degradation of nucleic acids. By comparison, O_2^- and H_2O_2 are weaker oxidising agents, but O_2^- has been shown to react with proteins containing Fe-S_4 clusters or heme groups, and H_2O_2 can attack thiol groups of proteins or glutathione (Thompson *et al.*, 1987).

2.2.2.1.5 Oxidative species

2.2.2.1.5.1 Hydrogen peroxide (H_2O_2)

Hydrogen peroxide is a stable, partially reduced form of oxygen produced in cells by both the dismutation of superoxide anion radical and by several enzymatic routes (Halliwell and Gutteridge, 1989). It is removed from the cell by catalases and various peroxidases such as glutathione peroxidase (Halliwell and Gutteridge, 1989).

Little is known about the mechanisms responsible for generating O_2 radicals during resistance responses. One possibility is that a specific membrane-located NAD(P)H oxidase catalyses the single electron reduction of molecular O_2 to O_2^- (Doke, 1985) in a manner roughly similar to the oxidase found in neutrophils. Peroxidases have also come under scrutiny as possible sources of oxygen radicals in plants (Halliwell, 1978). Hydrogen peroxide is an important substrate for peroxidases in the oxidation of coniferyl alcohol to initiate the lignification chain reaction. In one pathway, NAD(P)H is oxidised in the presence of peroxidase and Mn^{2+} to reduce molecular O_2 to O_2^- (Halliwell, 1978). Hydrogen peroxide is further reduced by peroxidases to

water at the expense of electrons from coniferyl alcohol. The alcohol radical then initiates the lignification chain reaction (Gross *et al.*, 1977). In fact, H_2O_2 biosynthesis has been localised in plant tissues to sites of active lignin deposition (Olson and Varner, 1993).

A major obstacle to infection resides in the physical barriers to pathogen penetration and H_2O_2 released during the oxidative burst can significantly strengthen these barriers. Thus H_2O_2 has been found to cross-link soluble proteins of about 33 and 100 kDa into the plant cell wall (Bradley *et al.*, 1992). The source of the H_2O_2 is likely the oxidative burst, since complete insolubilisation of the two hydroxyproline-rich glycoproteins can be stimulated with fungal elicitors. Following the oxidative cross-linking, digestion of the plant cell wall is significantly retarded even to the extent that protoplasts are difficult to prepare from elicitor-treated cells (Brisson *et al.*, 1994). Since the cross-linking is only observed in incompatible plant-microbe interactions, it seems reasonable to assume that it constitutes part of a successful pathogen defence response.

Furthermore, addition of H_2O_2 has been shown to stimulate transcription of genes encoding proteins that protect against oxidant stress (Levine *et al.*, 1994). These authors have shown that H_2O_2 can induce transcription of glutathione-S-tranferase and glutathione peroxidase in cultured soybean cells. Since both enzymes can ameliorate H_2O_2 toxicity, their expression may help a host plant escape its own biocidal activity.

Another function of pathogen-induced H_2O_2 may be its participation in the HR. This is a defence strategy where plant cells in the immediate vicinity of an infection undergo programmed cell death in order to eliminate the most immediate source of energy and nutrients for the invading microbe (Greenberg *et al.*, 1994). In support of this, Levine *et al.* (1994) found that levels of H_2O_2 sufficient to trigger soybean cell death are generated by elicited cells and that the consequent programmed cell death can effectively be inhibited by catalase.

Hydrogen peroxide has also been implicated in the production of phytoalexins. In cell suspension cultures of soybean (*Glycine max*) responding to a preparation from *Verticillium dahliae* which elicited glyceolin, Apostol *et al.* (1989) noted a rapid destruction of fluorescent probes used to monitor the progress of elicitation. This destruction could be attributed to the action of cell wall peroxidases catalysing the oxidation of the probes. The oxidation reactions were dependent on a flux of exogenous H_2O_2, produced by the plant cells within minutes of exposure to the elicitor. Exogenous H_2O_2, in the absence of the elicitor, was also effective at eliciting glyceollin production in soybean cells. Addition of catalase to the cultures prior to elicitor addition partially inhibited glyceollin production. However some cultures of the same soybean cells displayed no capacity to synthesise phytoalexins in response to H_2O_2 (Low and Merida, 1996).

Furthermore, since the function of the oxidative burst in human neutrophils is clearly microbicidial (Rossi, 1986), as evidenced by the enhanced susceptibility to infectious disease in individuals with a compromised oxidative response (Baggiolini and Wymann, 1990), it is assumed that at least part of the role of H_2O_2/O_2^- synthesis in plants is to directly damage the attacking pathogen. Studies indicate that H_2O_2 produced by elicited plants *in vitro* is sufficient to significantly retard microbial growth (Peng and Kuc, 1992). Also in cultures of the potential biocontrol agent *Talaromyces flavis* which produces large quantities of glucose oxidase, an enzyme which can oxidise glucose to produce H_2O_2 in the surrounding medium, inhibition of the growth of competitors such as *Verticilium dahliae* have been observed (Kim *et al.*, 1988).

2.2.2.1.6 Enzymes generating AOS

2.2.2.1.6.1 NADPH oxidase (EC 1.6.3.1)

Two candidates exist for the generation of reactive oxygen species on plasma membranes during stress. One line of evidence is in favour of the complex that is responsible for ferric ion reduction during its uptake. The flavoprotein involved is NADH-dependent and active at pH 4.5-5.0. It oxidises NADH, and this process is linked to the univalent oxidoreduction of iron ions by a reduced flavoprotein, which is

then converted to a radical form. The latter, reacting with O_2, generates superoxide anions, which dismutate to H_2O_2 (Vianello and Macri, 1991).

The second more favoured candidate is the homologue of the mammalian NADPH oxidase catalysing the reaction;

$$NADPH + 2O_2 \longrightarrow 2O_2^- + 2H^+$$

The respiratory burst is attributed to the activation of NADPH oxidase that transfers electrons from NADPH on the inside of the membrane to molecular O_2 on the outside of the membrane leading to the generation of O_2^- (Segal and Abo, 1993).

There are some similarities between the oxidative burst in the neutrophil and plant systems, and Keller *et al.* (1998) proposed that plants have NADPH oxidase very similar to the neutrophil oxidase in animals, but with novel regulatory mechanisms. In both systems, O_2 and NADPH are consumed, while O_2^- and H_2O_2 are generated (Doke and Chai, 1985; Apostol *et al.*, 1989; Dwyer *et al.*, 1996). Further, the kinetics of the reactions in two cell types is very similar. The generation of AOS in both systems can be detected within 3 min of elicitor or microbial treatment (Low and Heinstein, 1986; Rossi, 1986; Apostol *et al.*, 1987), and both plant and animal cells generate H_2O_2 at a rate of approx. 10^{-14} mol cell^{-1} min^{-1} (Legendre *et al.*, 1993).

The strongest evidence for similarity between the oxidative burst in plants and animals comes from immunological studies. Dwyer *et al.* (1996) have observed that antibodies raised against human NADH-oxidase proteins (p47-phox and p67-phox) recognise proteins of the same molecular mass in various plant species. Also using an alkaline phosphatase reporter system, Tenhaken *et al.* (1995) have shown that an antibody raised against human p22-phox cross-reacts with a protein of corresponding molecular weight in soybean. Since diphenyliodonium and α-naphthol, selective inhibitors of the human neutrophil oxidase, block the elicitor-induced oxidative burst in plants (Levine *et al.*, 1994; Dwyer *et al.*, 1996), there is reason to say that the two systems employ homologous polypeptides.

Doke (1985) described the production of reactive oxygen species in the interaction of potato with avirulent races of *Phytophthora infestans*. In potato tuber tissue NADPH oxidase activity could be induced by wounding, infection with an incompatible race of *P*. *infestans* and treatment with an elicitor consisting of hyphal wall components of the fungus. No increases in NADPH oxidase activity were observed if the tissue was treated with a compatible race of the pathogen. Infection induced activity was strongly inhibited by superoxide dismutase (SOD), a scavenger of superoxide anions (Doke, 1985; Doke and Miura, 1995). In cell cultures of tomato treated with apoplastic fluid containing AVR5 elicitor preparation, an activation of the plasma membrane NADPH oxidase (Xing *et al.*, 1997) was observed. Further, an ectopic expression of AK1-6H, an *Arabidopsis thaliana* calmodulin-like domain protein kinase in tomato significantly expressed NADPH oxidase (Xing *et al.*, 2001).

2.2.2.1.7 Antioxidant enzymes

Several enzymatic and non-enzymatic systems can be utilized by plants to remove the AOS. Enzymes such as SOD, catalases, peroxidases and enzymes of the ascorbate-glutathione cycle are all involved in the removal of AOS (del Rio *et al.*, 2002). Here we describe the involvement of SOD and peroxidase in the removal of AOS.

2.2.2.1.7.1 Superoxide dismutase (SOD, EC 1.15.1.1)

Superoxide dismutase (SOD) was first isolated from bovine blood as a green copper protein (Mann and Keilin, 1938) of which the biological function was believed to be copper storage. Over the years, the enzyme has been variably referred to as erythrocuprein, indophenol oxidase and tetrazolium oxidase. McCord and Fridovich (1969) discovered the catalytic function of the enzyme. The enzyme is ubiquitous among O_2-consuming organisms, aerotolerant anaerobes and some obligate anaerobes (Fridovich, 1986).

Superoxide dismutases are a group of metalloenzymes that protect cells from superoxide radicals by catalysing the dismutation of the superoxide radicals to

molecular O_2 and H_2O_2. All SODs, irrespective of source are multimeric metalloproteins that are very efficient at scavenging the superoxide radical. The enzyme reacts with superoxide radicals at almost diffusion-limited rates to produce H_2O_2. To accomplish this reaction, the mechanism employs an alternating reduction/oxidation of the respective metal associated with the enzyme (McCord and Fridovich, 1969);

$$2O_2^{\bullet -} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

In the presence of metal ions superoxide radicals and H_2O_2 can react in a Haber-Weiss reaction to form hydroxyl radicals ($OH^{\bullet}$), which can react indiscriminately with all macromolecules leading to lipid degradation, denaturation of proteins and mutation of DNA (Bowler *et al.*, 1992).

$$H_2O_2 + O_2^{\bullet -} \xrightarrow{Fe^{2+},\ Fe^{3+}} OH^- + O_2 + OH^{\bullet}$$

The types of SODs present are based on the metal ion present in their active sites. There are SODs that contain copper and zinc (Cu/ZnSOD), manganese (MnSOD) or iron (FeSOD).

In higher plants, there are multiple enzymic forms of SOD. The existence of SOD isozymes in plants and their genetic basis was first demonstrated in maize (Baum and Scandallios, 1979; 1982). Cu/ZnSOD is located mainly in the cytosol and chloroplasts of plants, whereas the other family contains either MnSOD in the mitochondria or FeSOD in the chloroplasts (Fridovich, 1986). There are also reports of peroxisomal and extracellular SODs (Streller and Wingsle, 1994; Bueno *et al.*, 1995).

Both chloroplasts and mitochondria can produce AOS either under normal growth conditions or during exposure to various stress conditions. Photosystem I electron-transport chain contains a number of auto-oxidisable enzymes that reduce O_2 to superoxide anion (Badger, 1985; Asada and Takahashi, 1987; Asada, 1994) and

evidence also shows that superoxide anion and H_2O_2 can also be produced by photosystem II under high light intensities (Landgraf *et al.*, 1995). During mitochondrial respiration, reactive oxygen species are also generated via the reactions of the electron transport chain (Rich and Bronner, 1978; Bowler *et al.*, 1991). Pathogen invasion as well as exposure to photoinhibitory light and ozone, increases superoxide levels in plants (Yruela *et al.*, 1996; Lamb and Dixon, 1997; Runeckles and Vaartnou, 1997).

Superoxide dismutase is a major antioxidant catalysing the conversion of O_2^- to H_2O_2, hence control of O_2^- and limiting of potential damage. Superoxide dismutase is also believed to function with NADPH oxidase to generate an H_2O_2 signal during the pathogen-induced oxidative burst (Desikan *et al.*, 1996; Lamb and Dixon, 1997). These AOS then work in conjunction with nitric oxide to induce hypersensitive response cell death in response to avirulent pathogens (Delledonne *et al.*, 1998; Durner *et al.*, 1998).

Pathogen infection induces increases in the levels of activity of various SODs and peroxidases in plants. Buonaurio *et al.* (1987) observed significant increases in activity of cyanide-sensitive Cu/Zn SOD during the HR of bean (*Phaseolus vulgaris*) to bean rust (*Uromyces appendiculatus*, formerly *U. phaseoli*), which were observed only during pustule eruption in compatible interactions. In tobacco leaves infected with tobacco mosaic virus (TMV), hypersensitively reacting tissues showed higher activities of both Cu/ZnSOD and MnSOD than in the susceptible leaves. In both investigations the relative enhancement of SOD activity in hypersensitive tissue was greatly exceeded by an increase in peroxidase activity, suggesting that despite the rise in SOD levels, a net increase in the level of cellular oxidants, due to increased peroxidase activity was likely. Increased MnSOD expression has also been reported for cultured tobacco cells during conditions of stress caused by both pathogenic and non-pathogenic factors (Bowler *et al.*, 1988).

2.2.2.1.7.2 Peroxidases (EC 1.11.1.7)

The family of peroxidase proteins (donor: H_2O_2 oxidoreductases; EC 1.11.1.7) has a broad range of functions in lignification, wound responses, pathogen attack and growth regulator activities (Bolwell, 1999). Extracellular peroxidase activity in plant tissues has been associated with several biochemical reactions including lignin and suberin synthesis (Espelie *et al.*, 1986; Lagrimini, 1991), formation of bridges between cell wall matrix components by oxidative coupling of pectins or hemicelluloses through the formation of diferuloyl cross-links, and by oxidative coupling of tyrosine residues of extensin monomers (Fry, 1986; Everdeen *et al.*, 1988), and NADH-dependent formation of H_2O_2 (Pedreno *et al.*, 1989). These biochemical reactions have some physiological roles such as defence against pathogen attack (Mohan and Kolattukudy, 1990), wound healing (Espelie *et al.*, 1986), stiffening of load-bearing tissues (Zieslin and Ben-Zaken, 1991; McDougall, 1992), and control of extension growth (Fry, 1986; Morrow and Jones, 1986; Kim *et al.*, 1989).

2.2.2.1.8 Signals from fatty acid metabolism

2.2.2.1.8.1 Lipoxygenase (LOX, EC 1.13.11.12) pathway

Lipoxygenases (linoleate: oxygen oxidoreductase, EC 1.13.11.12) are omnipresent in eukaryotes. They are non-heme Fe-containing dioxygenases that catalyse the regio- and stereo-selective dioxygenation of 1,4-pentadiene *cis*-polyunsaturated fatty acids (Siedow, 1991). The potential substrates for LOX activity are linoleic and α-linolenic acids, which are main constituents of plant storage and membrane lipids (Gardner, 1991). The arachidonic acid found in lipids of some plant pathogenic fungi might also serve as substrates (Choi *et al.*, 1994). Lipoxygenase oxidises polyunsaturated fatty acids into their hydroperoxides. Sequential reactions following LOX activity are involved in separate pathways and lead to formation of different products including a wide range of keto- and hydroxy-compounds (Hamberg, 1993).

Lipoxygenase activity promotes lipid peroxidation, a deterioration of membranes, which is also activated during the HR (Keppler and Norvaky, 1986; Croft *et al.*,

1990). Compounds formed through LOX activity may also serve in the resistance mechanisms of plants in several ways; direct inhibition of pathogen growth has been reported (Ohta *et al.,* 1991), as well as induction of phytoalexin synthesis (Li *et al.*, 1991). Products of LOX activity can go through further metabolism into synthesis of signalling compounds such as jasmonic acid (Farmer and Ryan, 1992) or cell wall components such as cutin (Blee and Schuber, 1993). Even though the involvement of jasmonates in plant pathology is still equivocal, induction of LOX isoforms (Fournier *et al.*, 1993; Meier *et al.*, 1993; Peng *et al.*, 1994) and increased LOX activity (Croft *et al.*, 1990; Ohta *et al.*, 1991; Avdiushko *et al.*, 1993) following pathogen infection have been observed.

A major biocidal compound formed on the LOX pathway is (*E*)-2-hexenal. It is a C6 α-β unsaturated aldehyde, which is very reactive with pronounced effects on biological systems (Schauenstein *et al.*, 1977). Major *et al.* (1960) isolated (*E*)-2-hexenal from *Ginkgo biloba* and showed that it can inhibit growth of the fungal pathogen *Monilinia fructicola*. (*E*)-2-hexenal has also been shown to be antiprotozoal (Von Schildknecht and Rauch, 1961).

Phaseolus vulgaris inoculated with *Pseudomonas syringae* pv *phaseolicola* showed an increase in LOX activity. This was only apparent in the incompatible interaction (Croft *et al.*, 1990). In tomato seedlings inoculated with an incompatible pathogen, *Pseudomonas syringae* pv *syringae,* the pathogen-induced increases in LOX activity followed increases in LOX mRNA transcripts. The hypersensitive cell collapse was observed when LOX activity had reached its maximum levels (Koch *et al.*, 1992). These reactions indicated the involvement of LOX in the induction of the defence responses. The products of LOX also interact with other signals or metabolites in the production of AOS, signal substances or in contributing to membrane damage in the HR (Feussner *et al.*, 1997).

2.2.2.2 Systemic signalling molecules

2.2.2.2.1 Salicylic acid (SA)

Salicylic acid (SA) is known as one of the signalling molecules in plant defence responses, mainly involved in the establishment of the systemic acquired resistance. It also interferes with fatty acid metabolism by inducing LOX activity, thus producing products which may function in the inhibition of pathogen growth, induction of phytoalexin and cell wall component synthesis, and internal signalling (Feussner *et al.*, 1997). Salicylic acid may also interact with active oxygen species in the amplification of signalling reactions.

2.2.2.2.2 Origins of Salicylic acid

Plants are one of the world's richest sources of natural medicines. Historically, the American Indians and ancient Greeks independently discovered that the leaves and bark of the willow (*Salix*) tree cured aches and fevers. The use of the willow tree bark to relieve pain is believed to date as far back as the 4th century B.C., when Hippocrates purportedly prescribed it for women during child birth (Rainsford, 1984).

The active principle of the willow remained a mystery until the 19th century when Johann Buchner isolated salicilin, the glucoside of salicyl alcohol, which was the major salicylate in willow bark. The name salicylic acid (SA) from the Latin word *Salix* for the willow tree, was given to this active ingredient by Raffaele Piria in 1838. The first commercial production of synthetic SA began in Germany in 1874. Aspirin, a trade name for acetylsalicylic acid, which is not a natural product, was introduced by Bayer company in 1898 and the compound rapidly became one of the world's best-selling drugs. In spite of the fact that the mode of medicinal action of salicylates is a subject of continual debate, they are used to treat human diseases ranging from common cold to heart diseases. In aqueous solutions aspirin undergoes spontaneous hydrolysis to SA.

2.2.2.2.3 Salicylic acid in plants

Salicylic acid (SA) belongs to a diverse group of plant phenolics. These are defined as substances that possess an aromatic ring bearing a hydroxyl group or its derivative.

Many plant phenolics play an essential role in the regulation of plant growth, development and interaction with other organisms (Harborne, 1980). For instance, phenolics are essential for the biosynthesis of lignin, an important structural component in plant cell walls. In addition, phenolics, especially phytoalexins (Hahlbrock and Scheel, 1989) have been associated with the chemical defences of plants against microbes, insects and herbivores (Metraux and Raskin, 1993). Experimental evidence increasingly suggests that penolics function as signals in plant-microbe interactions.

2.2.2.2.4 Salicylic acid (SA) biosynthesis

The potential to manipulate the levels of SA in plants depends on the understanding of its biosynthetic pathway. Salicylic acid is synthesised via the general phenylpropanoid pathway, which involves the conversion of phenylalanine to coumaryl-CoA. This reaction is initiated by the enzyme phenylalanine ammonia lyase (PAL, EC 4.3.1.5) which catalyses the deamination of L-phenylalanine to produce (*E*)-cinnamic acid that feeds into different branches of the phenylpropanoid pathway (Hahlbrock and Scheel, 1989). Phenylalanine ammonia-lyase activity provides precursors for synthesis of structural components such as lignin (Hahlbrock and Scheel, 1989) and other phenolics that accumulate in response to infection such as SA (Malamy *et al.*, 1990; Yalpani *et al.*, 1993).

The SA biosynthetic pathway has been more thoroughly studied in tobacco. In the phenylpropanoid pathway, *trans*-cinnamic acid is converted to benzoic acid via β-oxidation, which is subsequently converted to SA through the catalysis of benzoic acid-2-hydroxylase (Yalpani *et al.*, 1993), which functions as a cytochrome P_{450} monoxygenase (León *et al.*, 1993). Benzoic acid-treated plants induced the

accumulation of PR-1 proteins and increased resistance to tobacco mosaic virus (TMV). *Ortho*-coumaric acid did not induce any of these responses, or any increase in SA levels and therefore was ruled out as a precursor of SA (Yalpani *et al.*, 1993). Labelling experiments using [^{13}C] phenylalanine in TMV inoculated plants showed that benzaldehyde in the phenylpropanoid pathway is not an intermediate of SA biosynthesis (Léon *et al.*, 1995). Furthermore, high levels of H_2O_2 induced the accumulation of free benzoic acid and SA in tobacco leaves (Léon *et al.*, 1995). This accumulation was preceded by the rapid activation of benzoic acid-2-hydroxylase in the H_2O_2-infiltrated tissues. This enzyme catalyses the conversion of benzoic acid to SA (Léon *et al.*, 1995).

In potato (*Solanum tuberosum* L.) leaves treated with arachidonic acid, there was a rapid local increase in free SA and conjugated SA (Coquoz *et al.*, 1998). Radiolabeling studies with untreated leaves showed that SA was synthesised from phenylalanine and that both cinnamic acid and benzoic acid were intermediates in the biosynthesis of SA (Coquoz *et al.*, 1998).
In cucumber (*Cucumis sativus* L.) radiolabeling studies showed that SA is synthesised from phenylalanine. An inhibitor of PAL activity, 2-aminoindan-2-phosphonic acid completely inhibited the incorporation of [^{14}C] phenylalanine into [^{14}C] SA, even though plants treated with the inhibitor still produced [^{14}C] SA from benzoic acid (Métraux *et al.*, 1995).

Rice (*Oryza sativa* L.) seedlings on the other hand have high constitutive levels of SA. Rice is a monocot, but the biosynthetic pathway of SA is still the same as that of tobacco. However in rice leaves large amounts of cinnamic acid (CA) are directed to the production of lignin precursors such as *ortho*-coumaric acid and ferulic acid (Silverman *et al.*, 1995).

Recently it has been shown that the biosynthetic pathway from phenylalanine via cinnamic acid and benzoic acid is not the exclusive route in pathogen-induced SA biosynthesis. Some bacteria such as *Pseudomonas aeruginosa* synthesise SA

through isochorismate synthase (ICS) and pyruvate lyase. Wildermuth *et al.* (2001) have mapped the SA-induction-deficient *Sid 2* mutation to a gene (*ICS 1*) encoding isochorismate synthase in *Arabidopsis thaliana*. *ICS 1* is induced both locally and systemically during infection. Salicylic acid accumulation observed in the *Sid 2* mutants after infection has been found to be much lower (5-10% of the wild-type levels) and resistance to fungal or bacterial pathogens was also reduced (Nawrath and Metraux, 1999). This provides some evidence that SA produced by the ICS is required for local and systemic acquired resistance. The possibility that higher plants also produce pathogen-induced SA from ICS, a biosynthetic pathway typical for bacteria, has been highlighted by the annotation of expressed sequence tags for ICS in soybean and wild tomato (Wildermuth *et al.*, 2001).

2.2.2.2.5 Salicylic acid (SA) translocation and mode of action

Salicylic acid is a likely endogenous signal in the development of systemic acquired resistance (SAR). Salicylic acid levels increased systemically following inoculation of a single leaf with TMV (Malamy *et al.*, 1990). To determine the extent to which systemic increases in SA result from SA export from the inoculated leaf, SA produced in TMV-inoculated or healthy leaves was labelled with $^{18}O_2$. Largest increase in SA level occurred in the leaf located directly above the inoculated leaf. The fact that the highest SA increase occurred in the strongest sink leaf to the inoculated leaf further supports the hypothesis that the SAR-inducing signal is translocated in the phloem. Salicylic acid accumulation in the upper leaves could be prevented by removing the inoculated leaf before it accumulated substantial amounts of SA, this lead to the loss of PR-1 gene expression and TMV resistance (Shulaev *et al.*, 1995). However, in cucumber inoculated with *Pseudomonas syringae* pv *syringae* systemic increases in SA accumulation were observed even when the inoculated leaf was cut off before SA could be detected in its phloem (Rasmussen *et al.*, 1991).

In cucumber, labelling studies with [^{14}C] benzoic acid injected into cotyledons showed that SA increases in the upper uninfected leaf result from both synthesis in

the leaf and transport from the infected cotyledon (Mölders *et al.*, 1996). This transport occurs before the establishment of SAR. While these results show that SA is transported, they also indicate that only part of SA found in non-inoculated leaves is derived from the inoculated leaf.

Contrary to this, some evidence exists that favours SA not being the mobile signal. Grafting experiments in tobacco between *nahG* and wild type plants together with leaf excision experiments in cucumber supported the notion that SA is not the mobile signal exported from the infection site to systemic parts of the plant (Rasmussen *et al.*, 1991; Vernooij *et al.*, 1994).

In an effort to elucidate mechanisms through which SA induces defence responses, several SA effector proteins were identified. The first to be found was a cytosolic tobacco catalase (Chen *et al.*, 1993a) referred to as SA-binding protein (SABP). Salicylic acid and all its functional analogues inhibit catalase activity of degrading H_2O_2 and it was speculated that inhibition of H_2O_2 degradation leads to elevated levels of H_2O_2 observed during the HR. Assuming that systemic induction of PR proteins and disease resistance relies on salicylate's action on plant catalases, then it logically follows that the consequent rise in H_2O_2 levels must be critical to induction of genes involved in systemic resistance. Kauss and Jeblick's (1995) studies support this assumption by documenting that salicylate can indeed promote elevated accumulation of H_2O_2 in elicited suspension cultured parsley cells. Despite this, Bi *et al.* (1995) and Neuenschwander *et al.* (1995) have shown that a SA-sensitive catalase is unlikely to play a role in SAR. However, since much higher levels of SA accumulate around infection sites, inhibition of catalase at such sites may yield significant amounts of H_2O_2, possibly promoting HR-associated cell death (Levine *et al.*, 1994).

One consequence of SA interaction with catalase is the production of SA radicals, formed as SA donates an electron to enzyme intermediates of the peroxidative cycle (Anderson *et al.*, 1998). Free SA radicals (Savenkova *et al.*, 1994) primarily target

membrane lipids resulting in the initiation of lipid peroxidation (Halliwell and Gutteridge, 1990).

The second SA-binding protein 2 (SABP 2) (Du and Klessig, 1997) was also discovered in tobacco cytoplasm. It has a relatively high affinity for SA and may play a role in the transport or metabolism of SA. On the other hand, SABP 2 might be involved in other SA-mediated reactions instead of/or in addition to defence signalling. Recently, Slaymaker *et al.* (2002) have identified yet another SA-binding protein, SABP 3 in the stroma of tobacco chloroplasts. Purification and partial sequencing of this protein shows that it is a chloroplast carbonic anhydrase with some antioxidant activity.

Although SA has received much attention as a signalling molecule in the resistance mechanisms in plants, it is not the exclusive determining metabolite for resistance or susceptibility. Plants defective in SA-dependent defence responses such as *nah*G and *npr* 1 are more susceptible to a range of biotrophic pathogens, but they are not susceptible to all pathogens. For instance, they are not highly susceptible to the necrotrophic fungal pathogen *Botrytis cinerea* (Thomas *et al.*, 1998) showing that SA is not required for resistance against this pathogen. Genetic studies in *Arabidopsis thaliana* have revealed that resistance responses that operate independent of SA accumulation are mediated by jasmonic acid and ethylene (Dong, 1998). In fact SA has been noted to antagonise jasmonic acid synthesis and/or its action (Peña-Cortés *et al.*, 1993; Doares *et al.*, 1995), and SA and methyl jasmonate, a derivative of jasmonic acid, mutually antagonise each other's ability to induce gene expression in tobacco (Niki *et al.*, 1998).

2.2.2.3 Downstream defence related events

2.2.2.3.1 Pathogenesis-related (PR) proteins

During the HR a number of proteins referred to as "pathogenesis-related" are also induced. These proteins were first observed in tobacco plants reacting hypersensitively to tobacco mosaic virus (TMV) in the early 1970s (Gianinazzi *et al.*,

1970; Van Loon and Van Kammen, 1970). This biological system has been used to characterise the majority of PR proteins.

The PR proteins are produced during infection of plants by viruses, viroids, bacteria or fungi (Van Loon, 1985; Carr and Klessig, 1989; Bol *et al.*, 1990; Bowles, 1990; Linthorst, 1991; White and Antoniv, 1991). Apart from pathogens, PR proteins can be induced by gall mites in *Solanum dacara* (Bronner *et al.*, 1991), root cyst nematodes in leaves of potato plants (Rahimi *et al.*, 1996) and Russian wheat aphid in wheat plants (Van der Westhuizen *et al.*, 1998a,b). In addition, treatment with chemicals such as polyacrylic acid (Dumas *et al.*, 1985), amino acid derivatives (Asselin *et al.*, 1985), heavy metal salts (Nasser *et al.*, 1990), aspirin and salicylic acid (Bol *et al.*, 1990; Uknes *et al.*, 1992) induced the expression of PR proteins. Furthermore, high osmotic or salt stress conditions (King *et al.*, 1986) as well as ethylene (Boller *et al.*, 1983) are potent inducers of PR protein accumulation. The production of PR proteins is developmentally regulated in healthy plants during physiological processes such as senescence of leaves (Côté *et al.*, 1991) and flowering (Fraser, 1981; Leung, 1992). PR proteins display characteristic physico-chemical properties, which aid in their detection and isolation. They are stable at low pH and less susceptible to denaturation; relatively resistant to the action of proteolytic enzymes (both endogenous and exogenous); monomers of low molecular mass (8-50 kDa); and localised in compartments such as vacuoles and cell walls (Stintzi *et al.*, 1993).

The PR proteins were first described in tobacco plants reacting hypersensitively to tobacco mosaic virus and have so far been classified as PR 1 to PR 14-types according to amino acid sequences, serological relationship, and/or enzymatic or biological activity (Van Loon *et al.*, 1987; Bol *et al.*, 1990; Van Loon *et al.*, 1994; Van Loon and Van Strien, 1999). A number of members of the PR families have been shown to exert antimicrobial activities (e.g. PR proteins 1 to 5 and PR-11 to 14), while for other PR families no intrinsic antimicrobial activity has been observed, suggesting those may fulfil a different function. For instance, proteinase inhibitors of

the PR-6 family are believed to be involved mainly in defence against insect herbivores (Johnson *et al.*, 1989; Duan *et al.*, 1996; Van Loon and Van Strien, 1999).

2.2.2.3.2 β-1,3-glucanases (EC 3.2.1.39) and chitinases (EC 3.2.1.14)

The PR-2 family consists of proteins with β-1,3-glucanase activity and the PR-3, PR-4, PR-8 and PR-11 families consist of proteins with chitinase activity. The members of the families are hydrolytic in nature and are able to catalyse β-D-glucosidic linkages in β-1,3-D-glucans and β-1,4-(2-acetamido-2-deoxy)-D-glucosidic linkages in chitin (Boller, 1988; Collinge *et al.*, 1993). Both enzymes are constitutively expressed in different organs and tissues of higher plants, and are regulated by normal developmental processes, ethylene and other plant hormones (Côté *et al.*, 1991). In addition to being constitutively expressed, these enzymes are typically induced to higher levels in plants after pathogen attack, insect infestation and exposure to various biotic and abiotic elicitors (Boller *et al.*, 1983; Boller, 1988; Vogelsang and Barz, 1990; Côté *et al.*, 1991; Van der Westhuizen *et al.*, 1998a,b).

A general characteristic of plant chitinases and β-1,3-glucanases is their complicated isoenzymatic composition with isoforms differing in physical properties, enzyme activity, antigenicity, cellular compartmentation, tissue localisation and antifungal activity, encoded by a small multigene family (Linthorst, 1991). The physiological implication is that plants may respond in a tissue-specific or stimulus-specific manner (Margis-Pinheiro *et al.*, 1993). At the protein level the enzymes are classified into two main groups, the acidic and basic groups. The acidic group includes the extracellular enzymes while the basic isoforms occur in the intracellular matrix. Specific roles in defence against pathogens have been proposed for these two groups (Mauch and Staehelin, 1989).

The products released from hydrolysis of fungal cell walls are oligosaccharides, some of which have been shown to be perceived by the plant cell as signals i.e. elicitors and to induce active defence responses in the absence of any infection. The

biologically active oligochitins are thought to be released during plant-pathogen interactions by the action of chitinases and chitosanases (Kendra *et al.*, 1989; Hahn *et al.*, 1993). This might lead to an amplification of the defence responses, some of the induced proteins being again chitinases (Parker *et al.*, 1988).

The growth in culture of some fungi is inhibited by the presence of the β-1,3-glucanase and/or chitinases (Mauch *et al.*, 1988; Schlumbaum *et al.*, 1986; Roberts and Selitrennikoff, 1988), and the two enzymes working together have a greater inhibitory effect (Leah *et al.*, 1991; Sela-Buurlage *et al.*, 1993). In transgenic plants, over-expression of one of these enzymes enhanced resistance against certain fungal pathogens (Broglie *et al.*, 1991), and constitutive co-expression of the two enzymes provided enhanced resistance against certain pathogens (Zhu *et al.*, 1994; Jach *et al.*, 1995).

The 35S-chitinase gene was introduced into *Nicotiana tabacum*. Assays of protein extracts indicated an elevated chitinase activity in the 35S-chitinase transformed plants. Homozygous progeny of transgenic tobacco plants harbouring the 35S-chitinase gene showed enhanced resistance to *Rhizoctonia solani* infection which appeared to be correlated with the level of bean chitinase expression (Broglie *et al.*, 1986).

Complimentary DNAs encoding three proteins from barley, a class II chitinase, class II β-1,3-glucanase and a type-I ribosome-inactivating protein were expressed in tobacco plants under the control of CaMV 35S-promoter. Fungal infection assays revealed that expression of the individual genes, resulted in each case, in an increased protection against the soil borne fungal pathogen *Rhizoctonia solani*. Transgenic tobacco lines co-expressing genes coding for ribosome-inactivating protein and chitinase as well as glucanase and chitinase revealed significantly enhanced protection against fungal attack when compared with the protection levels obtained with corresponding isogenic lines expressing a single barley transgene to a similar level (Jach *et al.*, 1995).

Immunocytochemical studies have shown that chitinase and β-1,3-glucanase accumulate in the form of electron-dense aggregates within vacuoles. In ethylene-stressed bean leaves, β-1,3-glucanases and the basic isoforms of chitinase are localised primarily in the vacuole (Boller and Vogeli, 1984; Mauch and Staehelin, 1989). In the case of tobacco, the basic isoforms of β-1,3-glucanase induced by salicylate treatment or *Pseudomonas syringae* infection of leaves were also found in the vacuole (Van den Bulcke *et al.,* 1989). In tomato and eggplant infected by vascular wilt fungi the enzyme accumulated predominantly in host and fungal cell walls (Benhamou *et al.*, 1989). Immunocytochemistry has also demonstrated that chitinase accumulates in infected tomato plants and is often associated with altered fungal wall structure, indicating an active *in vivo* role in defence reactions (Benhamou *et al.*, 1990).

2.2.2.4 Plant activators

In addition to the existing measures of disease control, some knowledge on the biochemical events associated with defence responses has led to the establishment of a novel method of disease control. This employs the use of chemicals referred to as 'plant activators'. Chemicals that are considered as 'plant activators' have been proposed to exhibit the following characteristics:

- Induction of resistance against the same spectrum of pathogens as in the biological model
- Lack of antimicrobial activity in the compound or its significant metabolites
- Induction of the same expression of biochemical markers as in the biological model (Kessmann *et al.*, 1994)
- Inactivity of the compound in plant mutants with non-functional SAR-signalling pathway
- Presence of a lag time between application and protection.

Different chemicals have been labelled as activators of plant defence responses. Among them is benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester (BTH), SA,

aspirin, 2,6-dichloroisonicotinic acid (INA), DL-β-amino-n-butyric acid (BABA), paraquat, chitosan and silver nitrate. In barley, SA induced the accumulation of two of the six pathogen-induced proteins and one SA-specific protein. The induction of proteins by powdery mildew inoculation was comparable with SA treatment (Támás and Huttová, 1996). 2,6-Dichloroisonicotinic acid induced an almost similar set of gene expression as *Pseudomonas syringe* pv *syringae* infection of rice. 2,6-Dichloroisonicotinic acid treatment also induced lipoxygenase activity as well as high levels of unconjugated jasmonic acid (Schweizer *et al.*, 1997). Even though SA and INA were able to induce some defence responses, they failed to be commercialised due to intolerance by some plant species.

The 'plant activator', which has so far been commercialized as an agrochemical, is BTH. It is supplied in a formulation commercially known as Bion® (50 WG), developed and patented by Syngenta AG, Switzerland.

The spectrum of BTH-induced activity in tobacco involves the induction of resistance against *Cercospora nicotiniae* (frog eye leaf spot), *Erwinia carotovora, Phytophthora parasitica* (black shank), *Pseudomonas syringae* pv *tabaci* (bacterial wild fire) and TMV, and *Peronospora tabacina* (blue mould). Treatment with BTH as well as other biological activators, however, did not lead to resistance in tobacco plants against *Alternaria alternata* or *Botrytis cinerea* (Friedrich *et al.*, 1996).

Molecular and genetic studies carried out so far indicate that BTH acts as a functional analogue of the endogenous defence signalling molecule, SA, either binding to the same target protein as SA (Du and Klessig, 1997) or acting at some other site further down the signalling pathway (Friedrich *et al.*, 1996). Treatment of plants with SA or aspirin leads to the accumulation of the same set of PR proteins as achieved after treatment with BTH. However, effective amounts of aspirin are much higher than those of the plant activator, BTH, which is taken up by the plant more effectively than SA.

Induction of the plant defence responses by the plant activator is thought to occur through activation of the signal transduction pathway leading to SAR. Benzothiadiazole has been shown to be effective in protecting a wide range of plants including tobacco, tomato, cucumber, wheat and rice against a diverse spectrum of diseases (Friedrich *et al.*, 1996).

2.3 Conclusion

Even though mechanisms of defence responses have thoroughly been elucidated for many plant-pathogen interactions, little is known about the biochemical basis of resistance in sunflower-rust interaction. Plants employ a variety of defence related responses to defend themselves against disease-causing pathogens as well as other pests. These physiological responses involve a number of biochemical pathways, which interact with each other, amplifying certain responses and down-regulating or up-regulating other responses for the survival of the plant. All these reactions collectively induce the resistant state. A comprehensive study of the induced resistance mechanisms during incompatible plant-pathogen interactions should contribute towards a more effective and environmentally friendly approach towards disease management.

Sunflower rust development

3.1 Introduction

Plant-pathogen interactions are generally described as compatible and the host susceptible if the fungus produces characteristic symptoms and reproduces, or incompatible and the host resistant if it can react to the fungus by restricting symptom development and fungal reproduction (Lawton, 1997). Many fungal pathogens undergo a series of developmental and metabolic changes to which plants in turn have evolved a set of different counter attack responses. The most crucial steps in the successful establishment of pathogenicity, however, include attachment to the host surface, germination on the host and formation of infection structures, penetration of the host, and colonisation of the host tissue (Schafer, 1994). Biotrophic pathogens retrieve nutrients from living cells and have developed specialised feeding structures, or haustoria (Agrios, 1997). These pathogens aim to evade recognition by the host.

In this chapter the development of infection structures on two sunflower lines susceptible (S37-388) and resistant (PhRR3) to rust, *Puccinia helianthi* Schw. pathotype UVPhe 2 and host response were investigated. This would enable a comparison of the disease progression and the induction of biochemical defence related events in rust-susceptible and resistant sunflower plants.

3.2 Materials and methods

3.2.1 Biological material

Seeds of sunflower (*Helianthus annuus* L.) susceptible (cv S37-388) and resistant (cv PhRR3) to rust (*Puccinia helianthi*) (Kong *et al.*, 1999) were germinated and the seedlings transplanted into 4.2 L pots (three seedlings/pot). The seedlings were cultivated in a sterilised soil:peat (3:1) mixture in the glasshouse where the temperature was maintained at 25 ± 3°C. The plants were subjected to a photoperiod of 14 h light and 10 h darkness. Daylight was supplemented with 120 µmol m^{-2} s^{-1} photosynthetically active radiation provided by cool whitefluorescent tubes.The plants were watered daily and fertilised three times per week with 100 ml per pot of a

2 g L^{-1} hydroponics nutrient solution containing N, P and K (6.5:2.7:13) and micronutrients.

Sunflower rust (*Puccinia helianthi*), pathotype UVPhe 2, that is virulent to the line S37-388 and avirulent to PhRR3 (Los *et al.*, 1995) was used to inoculate the plants.

3.2.2 Rust inoculation

A source of fresh spores of pathotype UVPhe 2 of *Puccinia helianthi* was prepared by inoculating susceptible plants as follows: seeds of the susceptible cultivar S37-388 were densely planted in a soil:peat (3:1) mixture in small pots. When the seedlings were 10 d old, 50 ml of maleic hydrazide solution (0.3 g L^{-1}) was added to the soil to retard plant development and stimulate sporulation. At the beginning of the 2nd-leaf growth stage (15 d) the leaves were inoculated with a rust spore suspension on both sides of the leaf.

Spores were suspended in light mineral oil and the highly concentrated spore suspension was sprayed on both surfaces of the leaves using a pressurized atomiser. The sprayed plants were let to dry at room temperature (45 min) before being transferred to a dew simulation chamber (>96% relative humidity) where they were kept in darkness at 21°C for 16 h. From the dew chamber the plants were let to dry at room temperature (2 h), then transferred to the glasshouse. The spores were harvested after 14 d and used for inoculation in the subsequent experiments.

At the 3rd-leaf growth (3rd leaf pair) stage (30 d) the plants were inoculated with a sunflower rust spore suspension (8.9 x 10^5 spores ml^{-1}). Freshly harvested spores were suspended in sterile distilled water containing 0.05% (v/v) Tween 20. Control plants were sprayed with sterile distilled water containing 0.05% (v/v) Tween 20 only. The plants were let to dry at room temperature (2 h) and subsequently incubated in a dew simulation chamber (>96% relative humidity, 21ºC) for 16 h in the dark. After drying (2 h) at room temperature, plants were returned to the glasshouse.

3.2.3 Fluorescence microscopy

One leaf from three different plants (30 days) of each cultivar was sampled 6, 12, 18, 24, 48, 72, 96 and 144 h after infection. The leaves were cut into 1 cm^2 pieces and cleared and fixed in ethanol: dichloromethane (3:1 v/v) + 0.15% trichloroacetic acid for 24 h. The specimens were washed twice in 50% (v/v) ethanol for 15 min, twice for another 15 min each in 0.05 M sodium hydroxide and then rinsed three times in distilled water. Specimens were submerged in 0.1% (w/v) Uvitex 2B (Syngenta, Basel, Switzerland) (Niks and Dekens, 1991) prepared in 0.1 M Tris-HCl buffer, pH 5.8. Specimens were then rinsed four times in distilled water and washed for 30 min in 25% (v/v) aqueous glycerol and stored in 50% (v/v) glycerol containing trace amounts of lactophenol.

Observations on 10 infection sites on each of five randomly selected leaf pieces were carried out at 400 x, 200 x or 100 x magnification with a Nikon (Nikon Corp., Tokyo, Japan) Optiphot epifluorescence microscope. The filter combinations UV-1A (excitation filter 330-380 nm and barrier 420 nm) for fungal structures and B-2A (excitation filter 450-490 nm and barrier filter 520 nm) for autofluorescence measurements were used. Fungal structures fluoresced a bright light-blue colour. Haustorium mother cells fluoresced extremely brightly, while the haustoria in the cells were not visible. Using the B-2A filter, host cells fluorescing bright yellow colour were considered necrotic whereas unaffected healthy cells did not fluoresce (Rohringer *et al.*, 1977).

Two dimensions of fungal colonies and the necrotic leaf area (if present) were measured at 100 x magnification with a calibrated eyepiece micrometer. Since the colonies and the necrotic leaf area were not always a perfect circle, areas were estimated using the formula: $\pi \times l \times \omega / 4$, where, l = length and ω = width.

A hypersensitivity index (HI) (Kloppers and Pretorius, 1995) was calculated using the formula:

HI = necrotic leaf area (mm^2) /colony size (mm^2)

3.2.4 Scanning electron microscopy (SEM)

One leaf from three different plants (30 days) of each cultivar was sampled after 6, 12, 18, 24, 48, 72, 96 and 144 h of infection. The leaves were cut into 1 cm^2 pieces and fixed in 3% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.0, for 24 h, washed three times in the buffer, then dehydrated in a graded ethanol series. The specimens were critical-point dried with carbon dioxide as the transition fluid. Each dried specimen was mounted on a metal stub previously coated with a double-sided cellotape. A thin strip of the sticky tape was gently appressed to the dried leaf surface and pulled away, removing some portions of the epidermis. The tape was also mounted on stubs. Specimens for studying spores, germ tubes and appressoria *in situ* were mounted without the removal of the epidermis. All stubs were gold-coated in a Polaron Sputter Coater. Specimens were examined using Jeol (JSM-T200) scanning electron microscope operating at 10-15kV.

3.2.5 Disease rating

Five leaves were randomly selected from five different plants that were either infected or not infected with rust. After 15 days of infection the area of the leaf covered by pustules and chlorosis was estimated using WinDIAS Colour Image Analysis Software Programme (Delta-T Devices, Cambridge, England).

3.2.6 Statistical analysis

Data were analysed for variance using NCSS statistical software (Kaysville, Utah). Standard deviations were used to compare the means. The entire experiment was done twice.

3.3 Results

3.3.1 Disease symptoms

Rust-inoculated susceptible and resistant sunflower lines together with their controls were photographed 15 days post inoculation (dpi). The symptoms began as small chlorotic spots, which developed into reddish brown uredial pustules. The pustules were associated with dead leaf tissue at severe infection sites. Symptoms were visible on both the abaxial and adaxial leaf surfaces (Fig. 3.1) even though pustules developed relatively faster on the lower surface of the leaf (Fig. 3.1D). In the resistant

line rust infection induced only necrotic lesions both on the lower and upper surfaces of the leaf (Fig. 3.2B and C).

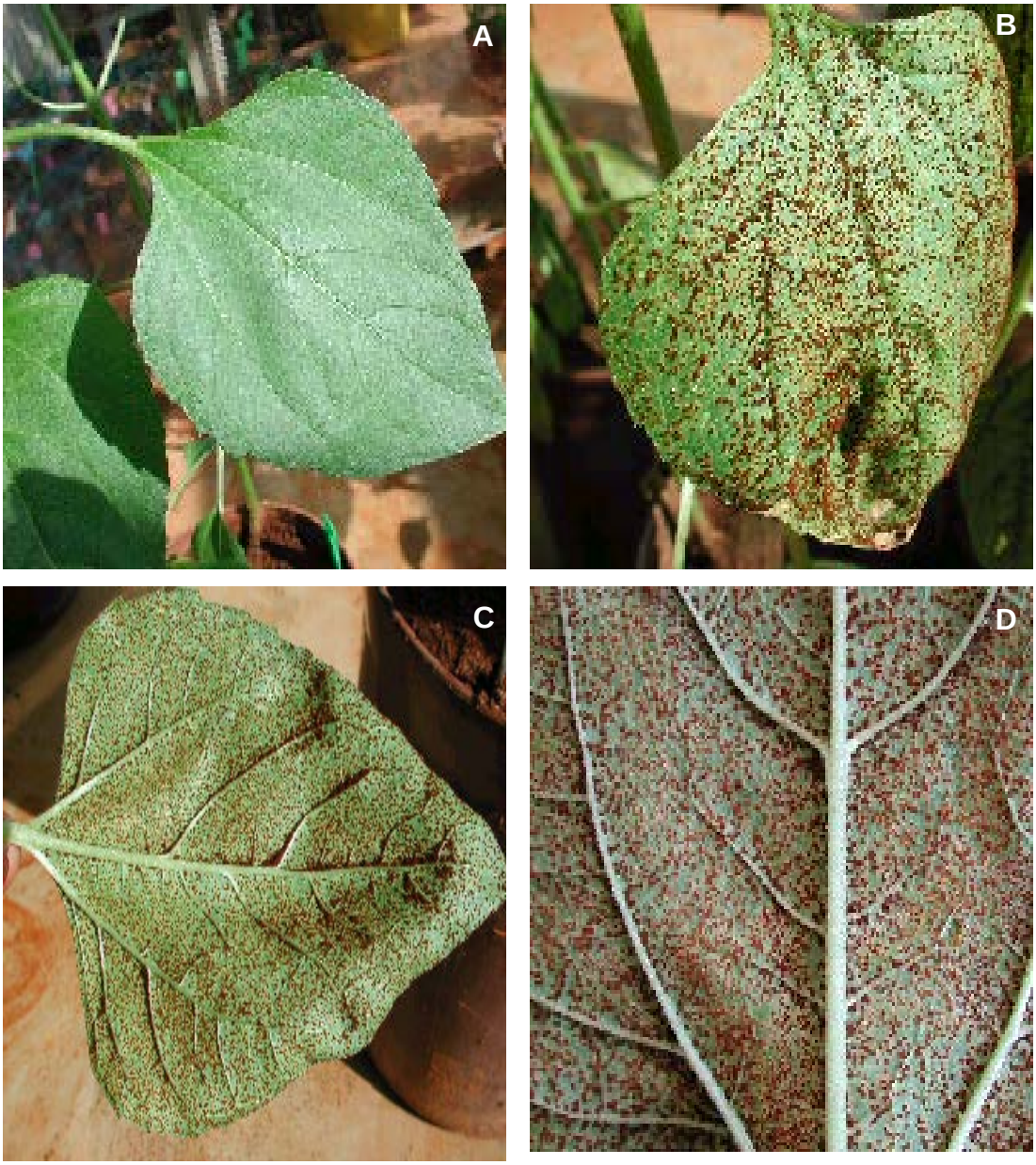


Fig.3.1: Rust (*Puccinia helianthi* Schw., pathotype UVPhe 2) infection symptoms on the susceptible sunflower line (S37-388). A: uninfected control; B: rust pustules on the upper surface of the leaf; C: rust pustules on the lower surface of the leaf; D: rust pustules on the lower surface of the leaf at close proximity.

Fig. 3.2: Rust (*Puccinia helianthi* Schw., pathotype UVPhe 2) infection symptoms on the resistant sunflower line (PhRR3). A: uninfected control; B: necrotic lesions on the upper surface of the leaf; C: necrotic lesions on the lower surface of the leaf at close proximity.

Fig 3.3: Rust (*P. helianthi*, pathotype UVPhe 2) infection structures and host (*Helianthus annuus* cvs S37-388 and PhRR3) response. The scale bar represents 10 µm:

a: Germinating rust (*Puccinia helianthi* Schw., pathotype UVPhe 2) spores on the lower leaf surface of a susceptible sunflower (S37-388) cultivar, 6 hpi.

b: A germinating rust (*P. helianthi*, pathotype UVPhe 2) spore has differentiated into a germ tube (GT) and an appressorium (arrow) on the lower leaf surface of a susceptible sunflower (S37-388) cultivar, 6 hpi.

c: Inside the stomata (S), an H-shaped stomatal vesicle (arrow), 12 hpi.

d: Aborted substomatal vesicle (arrow), in the susceptible (S37-388) sunflower line, 6 hpi.

e: The interior of a leaf of susceptible sunflower (S37-388) cultivar showing infection hyphae (arrows) developing from the substomatal vesicle, 18 hpi.

f: The inner part of a leaf of susceptible sunflower (S37-388) cultivar showing the development of two brightly fluorescing haustorium mother cells (arrows) at the tips of infection hyphae and in contact with host mesophyll cells, 24 hpi.

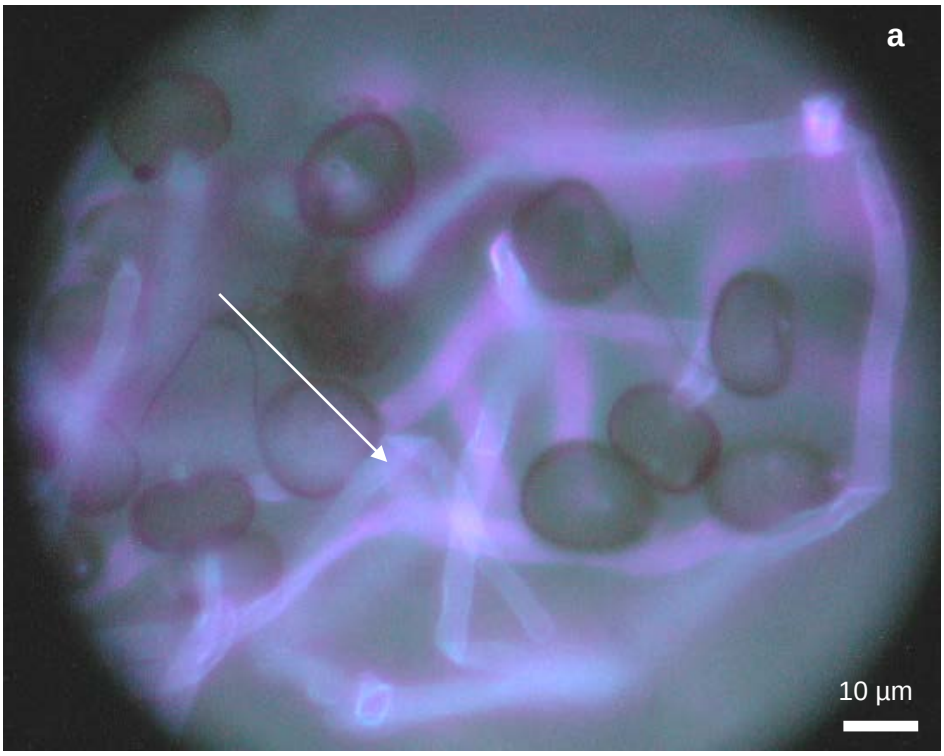
g: Three haustorium mother cells (arrows) in the interior of the leaf of a susceptible sunflower (S37-388) cultivar, 36 hpi.

h: Initiation of a rust (*P. helianthi*, pathotype UVPhe 2) colony inside a leaf of a susceptible sunflower cultivar (S37-388) formed by at least six haustorium mother cells, 48 hpi.

i and j: Larger colonies of rust (*P. helianthi*, pathotype UVPhe 2) within the inner part of a leaf of susceptible sunflower (S37-388) cultivar, 72 hpi.

k: Host cell necrosis of the resistant sunflower (PhRR3) cultivar fluorescing a bright yellow colour, 72 hpi.

l: Host cell necrosis of the resistant sunflower (PhRR3) cultivar fluorescing a reddish orange colour, 72 hpi.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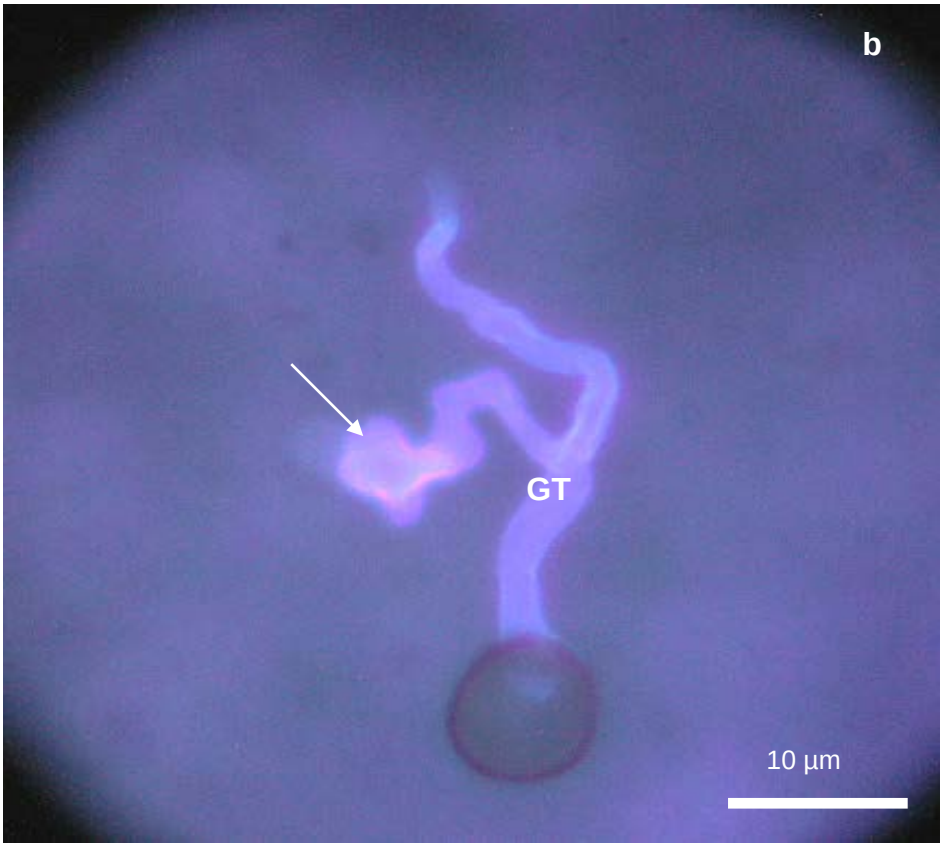
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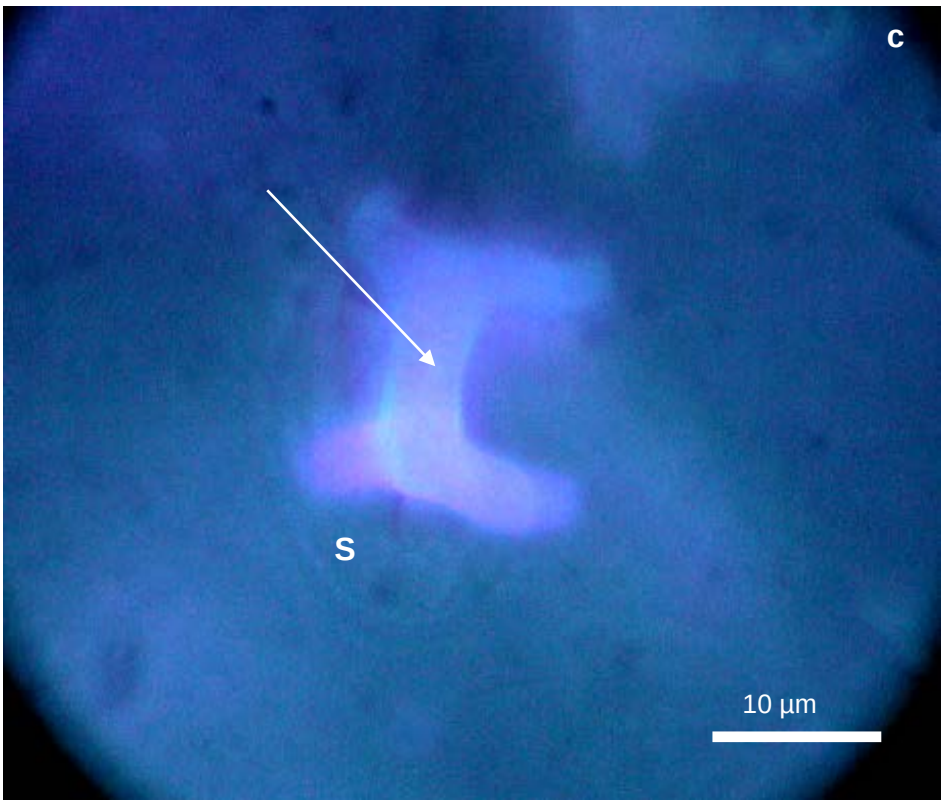
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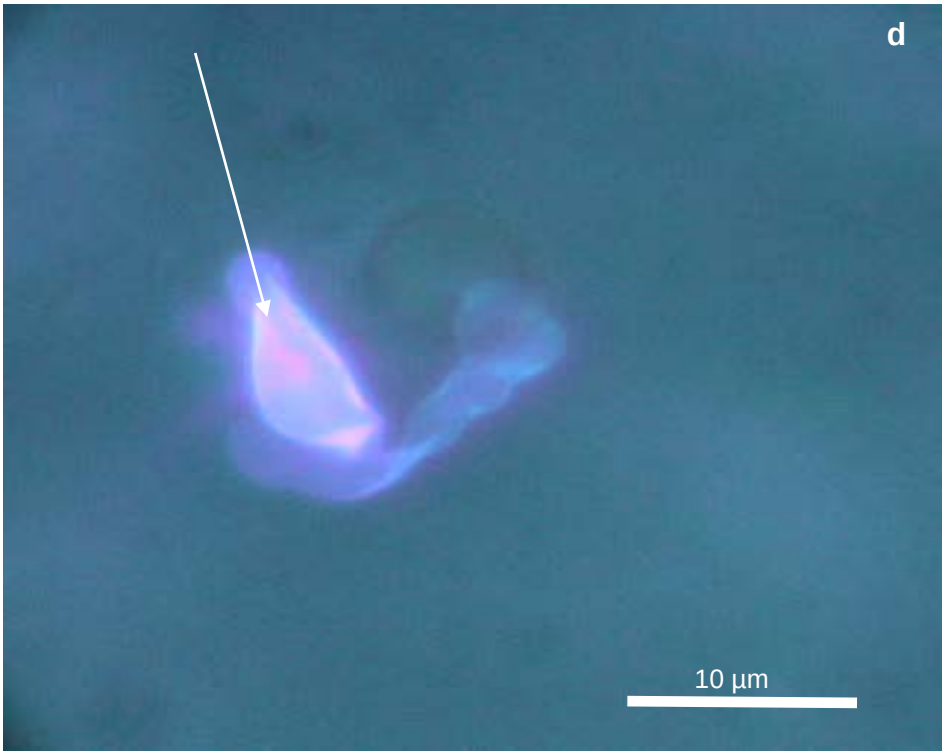
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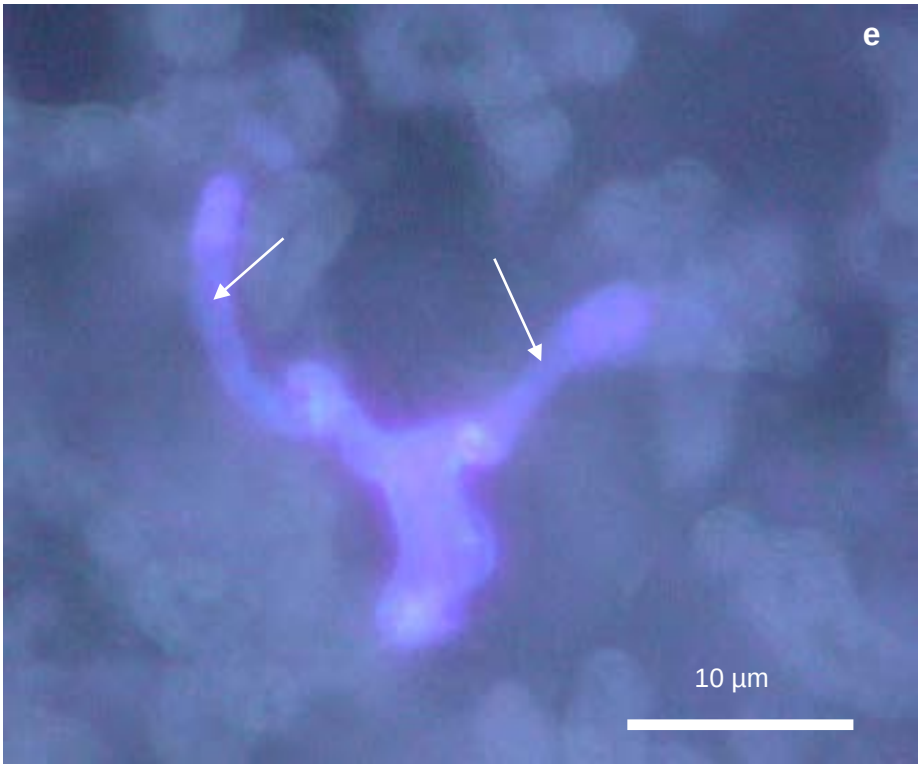
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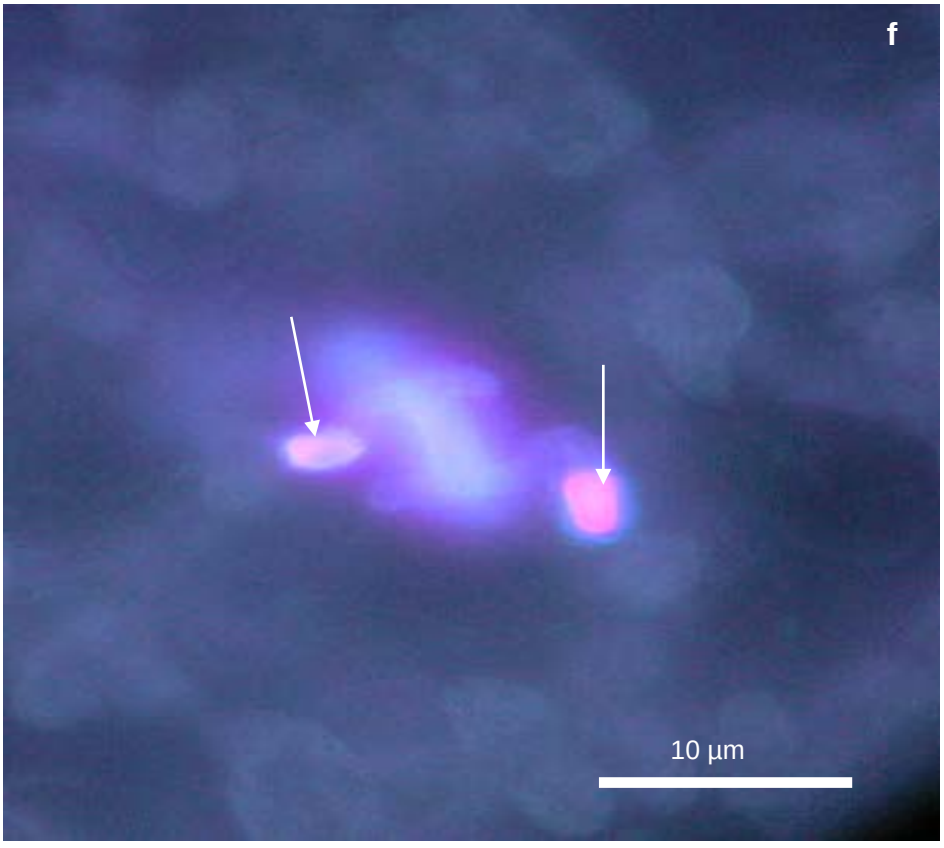
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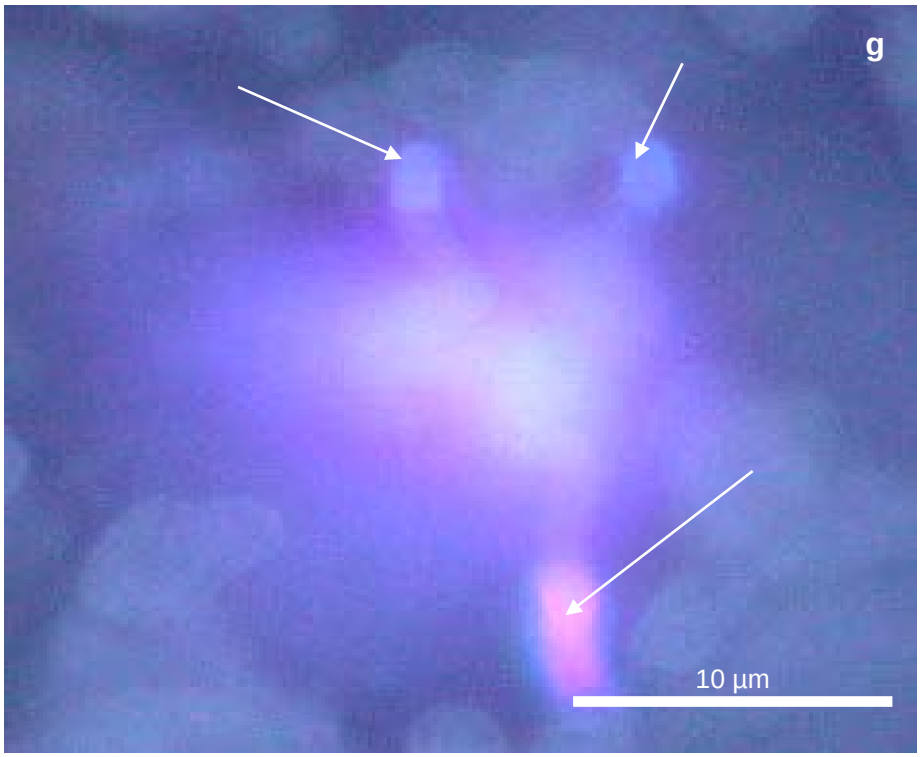
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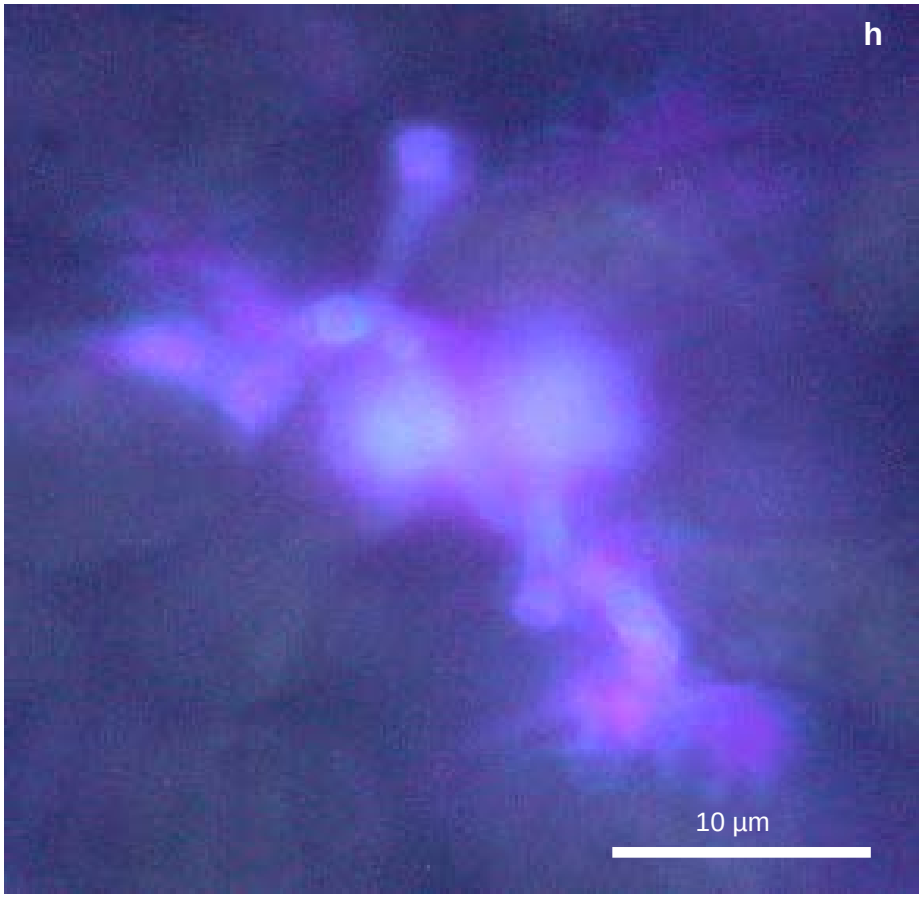
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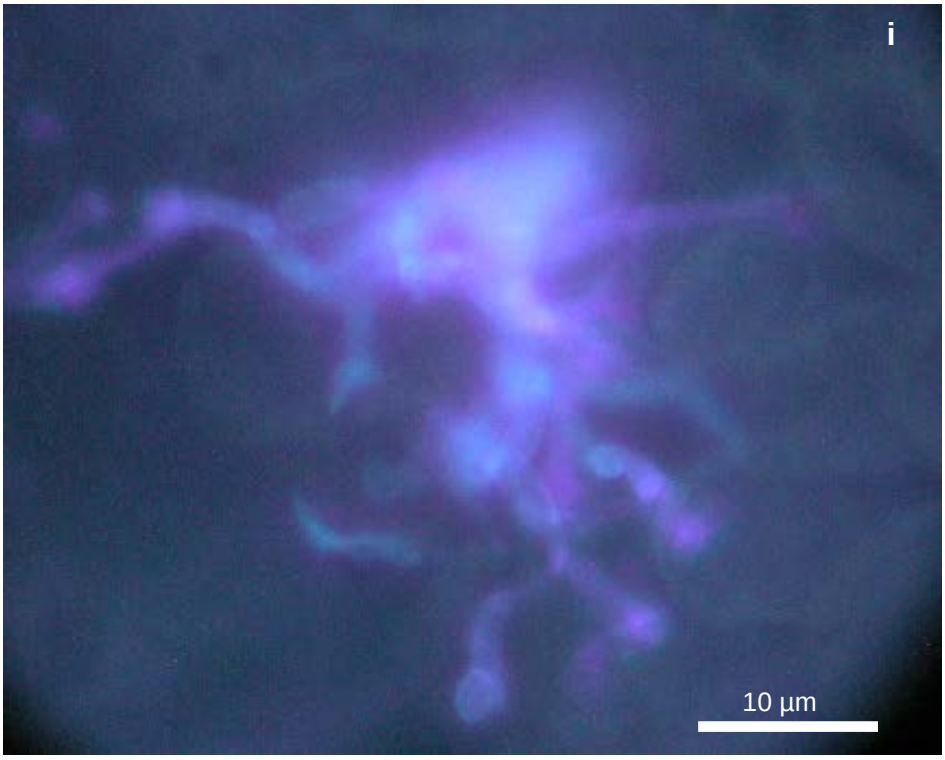
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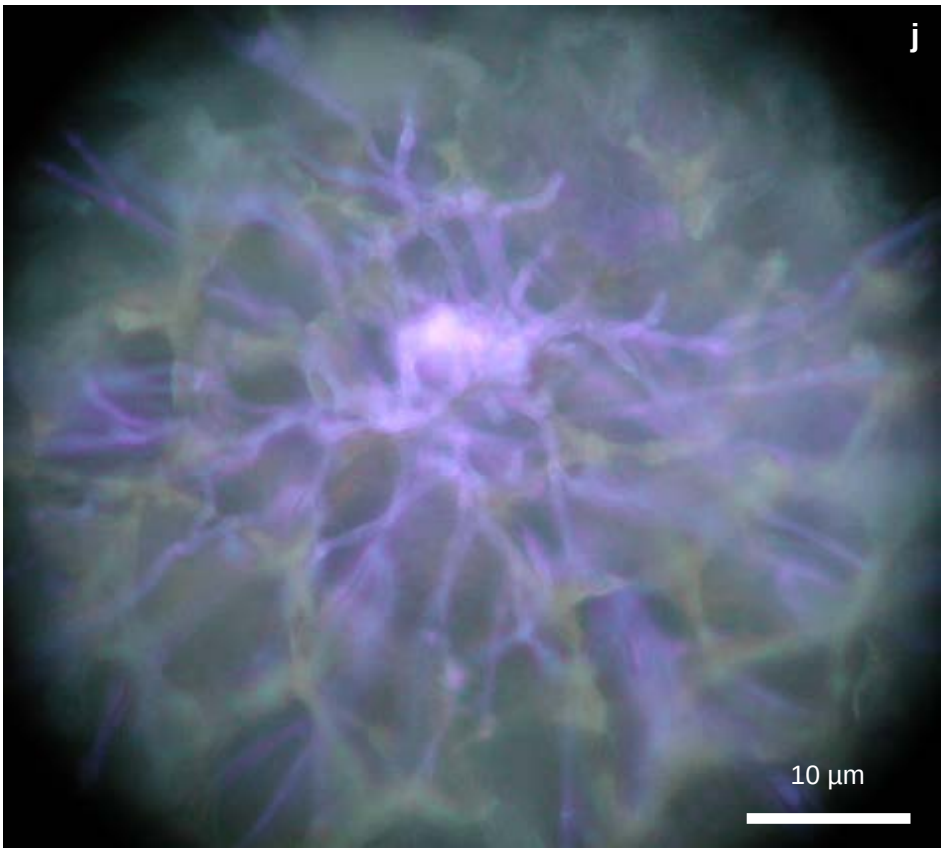
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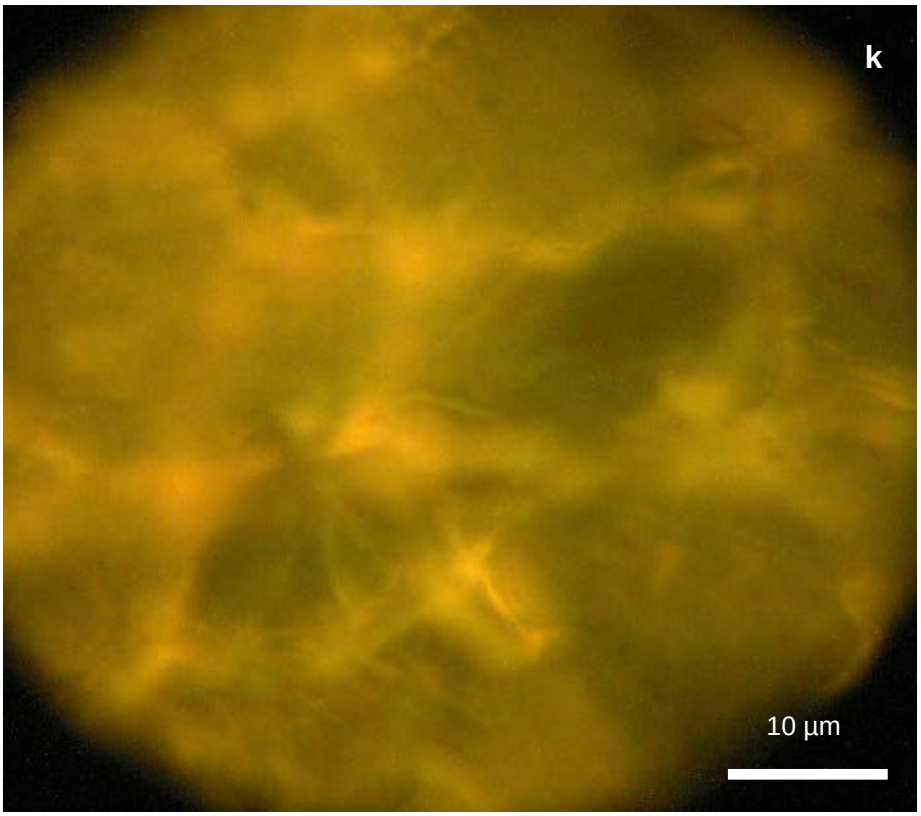
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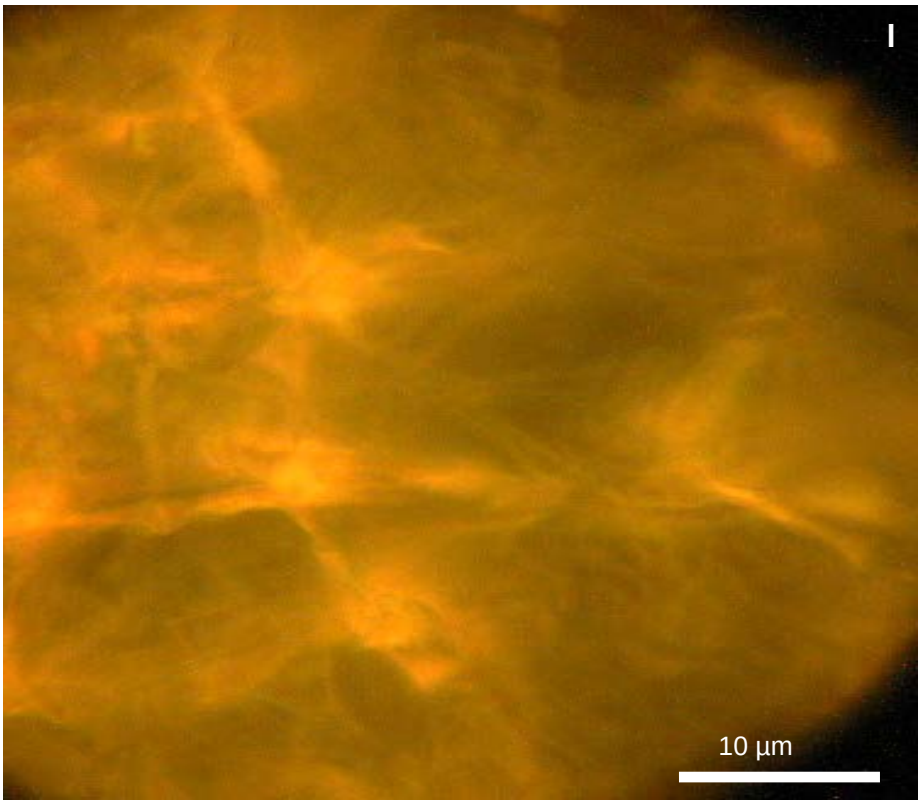
l
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3.3.2 Description of infection structures

The development of rust (*P. helianthi*, pathotype UVPhe 2) infection structures in the susceptible (S37-388) and resistant (PhRR3) sunflower cultivars was similar, and representative images are from the interaction of the rust (*P. helianthi*, pathotype UVPhe 2) with the susceptible host (S37-388).

During rust infection, a germ tube emerged through the germ pore (Fig. 3.3a) and extended along the surface of the leaf until it came across a stoma where it formed an irregularly shaped appressorium (Fig. 3.3b). In some instances the germ tube failed to recognise the immediate stomata and extended to the next one, whereas in other cases the appressorium formed without the presence of a stoma forming a nonstomatal aprressorium. All these events were referred to as prestomatal behaviour of rust spores. The appressorium formed on both susceptible (S37-388) and resistant (PhRR3) sunflower lines had irregular shapes, including a knob and a club shape.

Only stomatal appressoria developed further infection structures. A small penetration peg developed from the lower surface of the appressorium forming an H-shaped substomatal vesicle inside the leaf (Fig. 3.3c). The structures formed after after the substomatal vesicles were also observed under scanning electron microscopy (SEM). The results shown were viewed under fluorescence microscopy where fungal structures fluoresced bright blue on a dark background.In some instances, the substomatal vesicle collapsed and could not develop further, forming the aborted substomatal vesicle (Fig. 3.3d). This beviour was referred to as abortive penetration.

Infection hyphae developed from the substomatal vesicle (Fig. 3.3e) and at 24 hpi, the primary infection hyphae had already formed at the four sides of the substomatal vesicle. The number of infection hyphae was not uniform under all the infection sites, some sites had a high density of primary infection hyphae while others had low density.

As infection progressed (36 hpi) the number of infection hyphae per substomatal

vesicle increased and at the tip of the infection hyphae, and in contact with a host cell, a haustorium mother cell developed (Fig. 3.3 f, g). The haustorium mother cells fluoresced extremely brightly.

At 72 hpi infection hyphae had branched and increased in length to ramify the intercellular spaces. At this stage more than six haustorium mother cells had formed per substomatal vesicle and developed rust colonies (Fig 3.3 h, I, j). The dimensions of the colonies differed, increasing in size with disease progression. In the susceptible cultivar larger colonies than in the resistant plants were observed. In the resistant (PhRR3) plants rust colonies formed were much smaller and surrounded by necrosis (Fig 3.3 k,l), which was not present in the susceptible (S37-388) line. At 144 hpi, pockets of urediospores had formed on the surface of the leaves in the susceptible (S37-388) plants. This was not observed in the resistant (PhRR3) line. The urediospores had not formed at all the infection sites and therefore were not quantified.

3.3.3 Distribution and frequency of infection structures

The infection structures observed under fluorescence microscopy showed that the average number of germinating spores did not significantly differ in the susceptible and resistant sunflower lines (Fig.3.4a). The period of infection (hpi) however significantly ($P<0.05$) influenced the germination of spores in both susceptible and resistant sunflower lines. The number of germinating spores increased from 6 hpi to 48 hpi (Fig. 3.4b). In the second experiment the same pattern of results was obtained even though the values were slightly higher than in the first experiment.

The average number of stomatal apprressoria (Fig. 3.5a,A) significantly ($P<0.05$) differed between susceptible and resistant plants. The values were higher in the susceptible than resistant plants. The duration of infection (hpi) significantly ($P<0.05$) influenced the development of appressorium in the two sunflower lines. The number of stomatal appressoria was higher in the susceptible than resistant line from 6 hpi to 48 hpi (Fig. 3.5b).

The mean numbers of nonstomatal appressoria (NSA) did not differ significantly between the susceptible and resistant lines (Fig. 3.6a).

The time period after infection (hpi) significantly ($P<0.05$) influenced the development of nonstomatal appressoria in both susceptible and resistant lines. The number declined in both lines from 6 hpi to 48 hpi with a steeper decline in the susceptible line (Fig. 3.6b). Host genotype (susceptible or resistant) did not significantly influence the formation of nonpenetrating appressoria (Fig. 3.7a). The time period after inoculation (hpi) nonetheless significantly ($P<0.05$) influenced the development of nonpenetrating appressoria in both susceptible and resistant lines. The values increased between 48 hpi to 72 hpi (Fig. 3.7b).

Host genotype significantly ($P<0.05$) influenced the formation of aborted substomatal vesicles (ASSVs) in these two sunflower lines. Higher numbers of ASSVs were observed in the resistant than susceptible line (Fig. 3.8a and b). The number of ASSVs significantly ($P<0.05$) increased as the period of infection lengthened. After 48 h and 72 h of inoculation more ASSVs were observed in the resistant than susceptible line (Fig. 3.8b). The results in the first and second experiments followed the same pattern, but values were higher in the latter.

A significantly ($P<0.05$) higher number of haustorium mother cells (HMCs), which were not surrounded by host necrotic cells, were formed in the susceptible than resistant plants. As the duration of infection proceeded from 48 h to 72 h, significantly ($P< 0.05$) higher numbers of HMCs not associated with necrosis, were formed in the susceptible than resistant plants (Figs. 3.9a and b).

The genotype of the host significantly ($P<0.05$) motivated the development of necrosis around HMCs. More HMCs with necrosis were formed in the resistant line whereas in the susceptible line no necrosis formed (Fig. 3.10a). This development was also dependent on the duration of infection. At 72 hpi, more ($P<0.05$) HMCs associated with necrosis developed in the resistant plants while no necrotic cells were observed around HMCs in the susceptible plants (Fig. 3.10b)

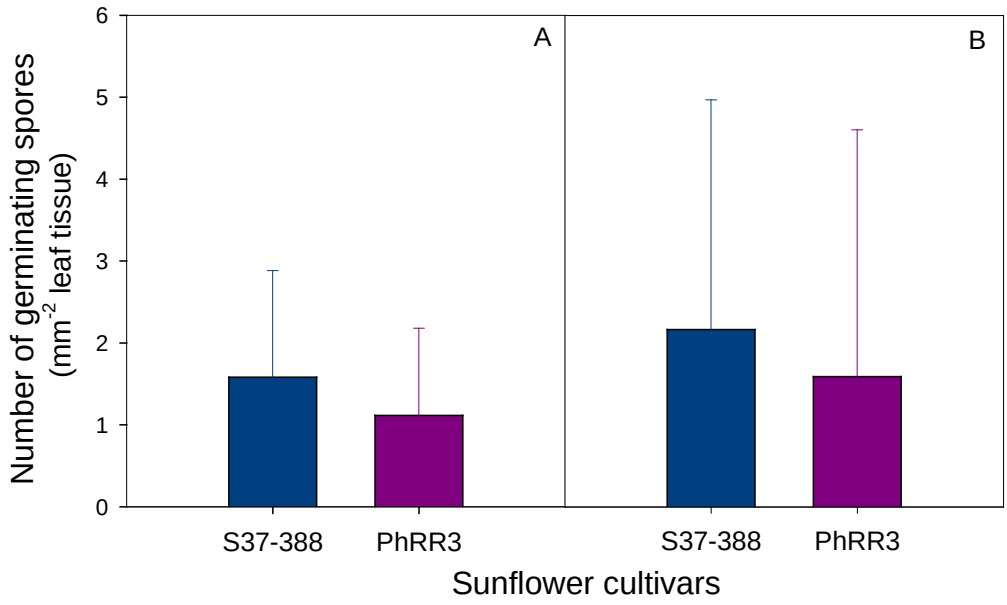

Fig. 3.4a: The mean number of spores (*Puccinia helianthi* Schw., pathotype UVPhe 2) germinating in the susceptible (S37-388) and resistant (PhRR3) sunflower lines during an infection period beginning at 6 h and extending to 48 h after infection. A and B represent two independent experiments. The values are means ± SD (n=50).

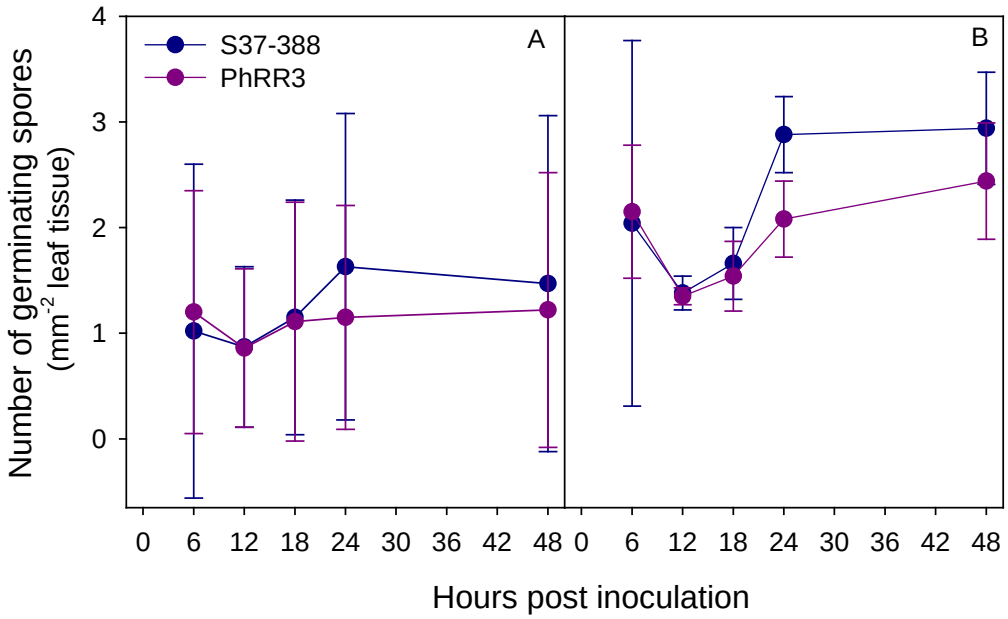

Fig. 3.4b: The number of spores (*Puccinia helianthi* Schw., pathotype UVPhe 2) germinating on the susceptible (S37-388) and resistant (PhRR3) sunflower lines. A and B represent two independent experiments. The values are means ± SD (n=50).

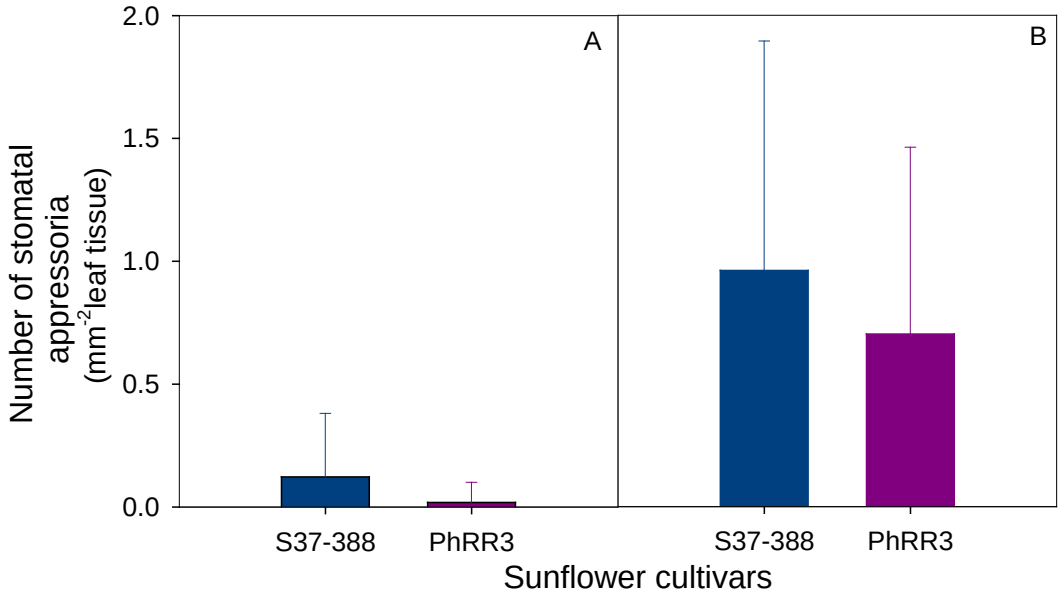

Fig. 3.5a: The mean number of stomatal appressoria of rust (*Puccinia helianthi* Schw., pathotype UVPhe 2) in the susceptible (S37-388) and resistant (PhRR3) sunflower lines at an infection period extending from 6 to 48 hpi. A and B represent two independent experiments**.** The values are means ± SD (n=50).

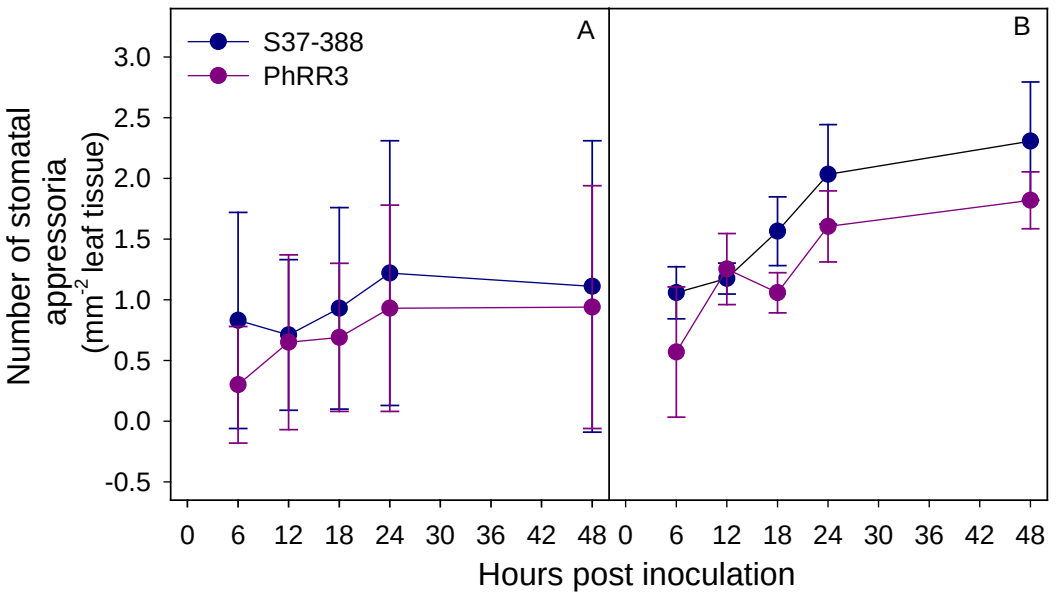

Fig. 3.5b: The number of stomatal appressoria of rust (*Puccinia helianthi* Schw., pathotype UVPhe 2) in the susceptible (S37-388) and resistant (PhRR3) sunflower lines. A and B represent two independent experiments. The values are means ± SD (n=50).

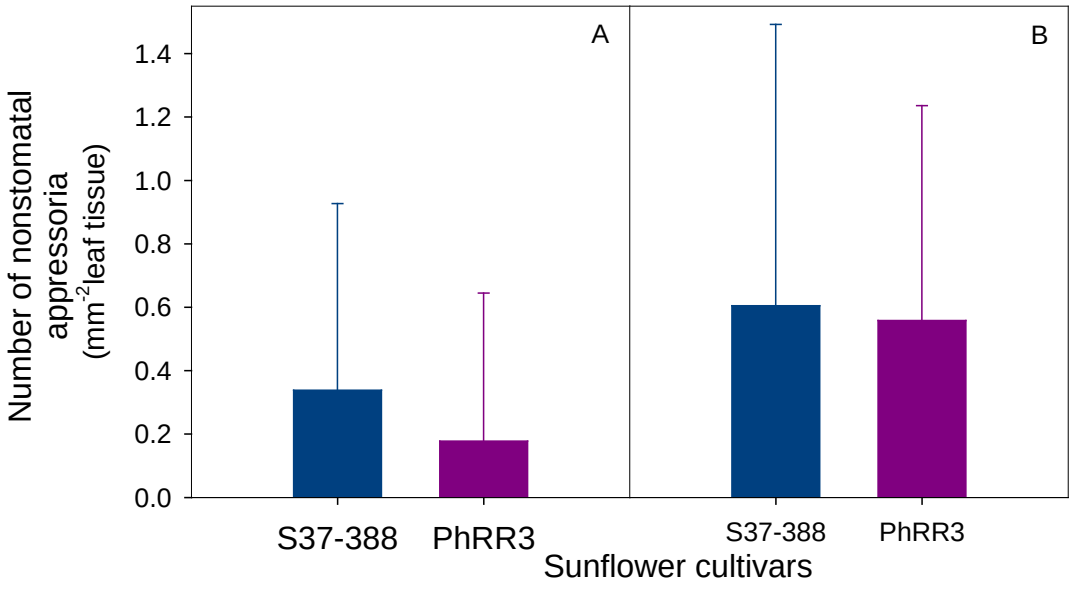

Fig. 3.6a: The mean number of nonstomatal appressoria of rust (*Puccinia helianthi* Schw., pathotype UVPhe 2) in the susceptible (S37-388) and resistant (PhRR3) sunflower lines at an infection period of 6 to 48 hpi. A and B represent two independent experiments. The values are means ± SD (n=50).

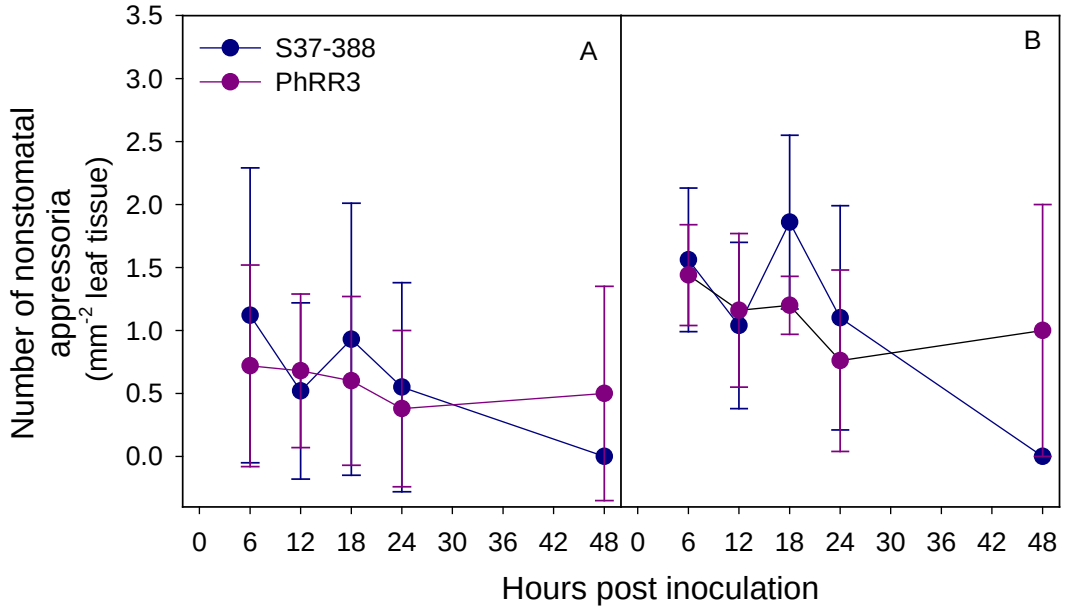

Fig. 3.6b: The number of nonstomatal aprressoria of rust (*Puccinia helianthi* Schw., pathotype UVPhe 2) in the susceptible (S37-388) and resistant (PhRR3) sunflower lines. A and B represent two independent experiments. The values are means ± SD (n=50).

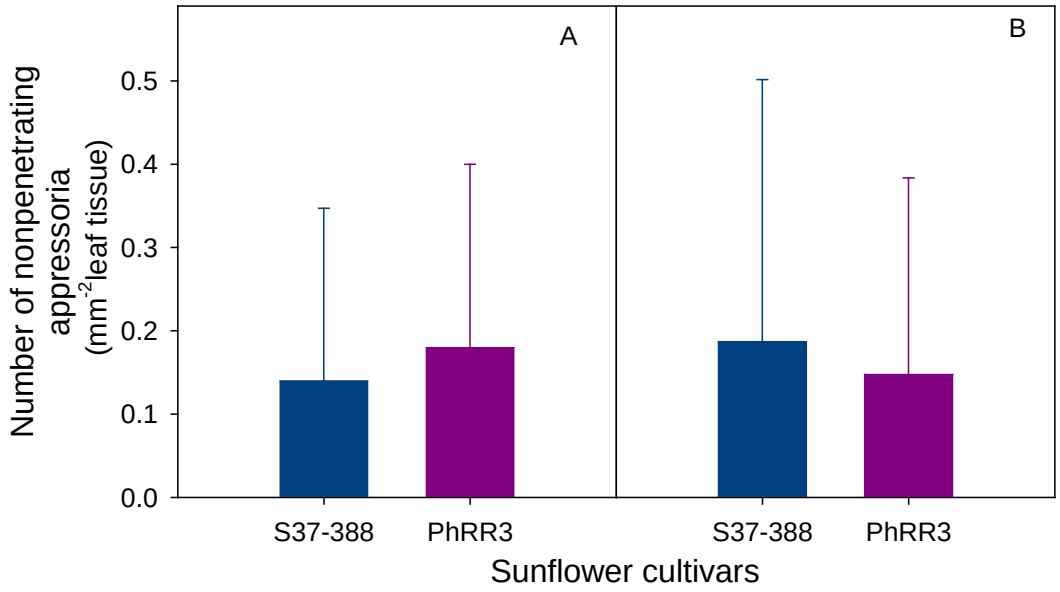

Fig. 3.7a: Mean number of nonpenetrating appressoria of rust (*Puccinia helianthi* Schw., pathotype UVPhe 2) in susceptible (S37-388) and resistant (PhRR3) sunflower lines at an infection period from 6 to 48 hpi. A and B represent two independent experiments. The values are means ± SD (n=50).

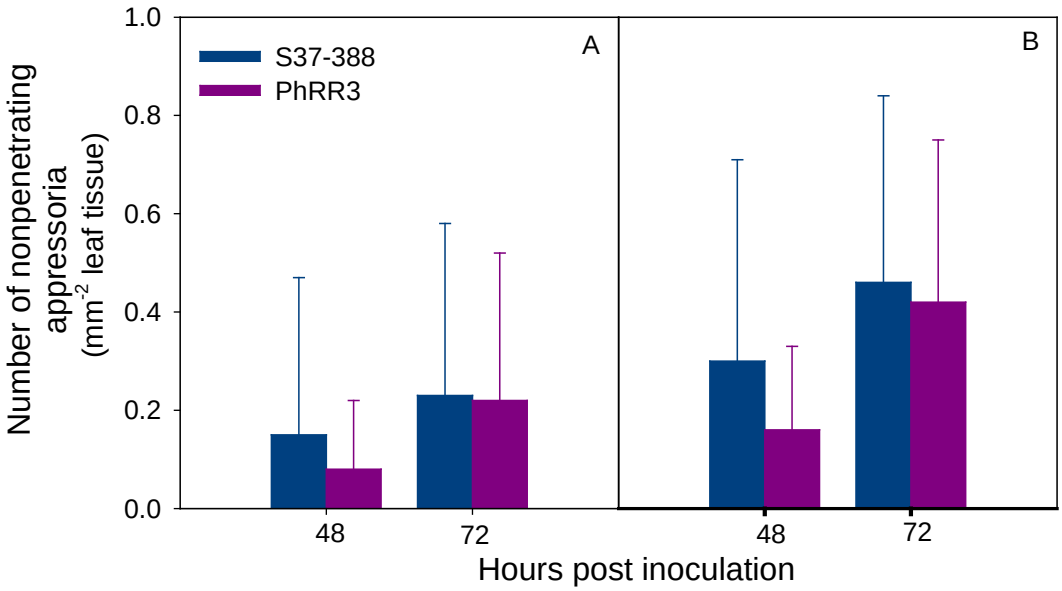

Fig. 3.7b: The number of nonpenetrating appressoria of rust (*Puccinia helianthi* Schw, pathotype UVPhe 2) in the susceptible (S37-388) and resistant (PhRR3) sunflower lines at 48 and 72 hpi. A and B represent two independent experiments. The values are means ± SD (n=50).

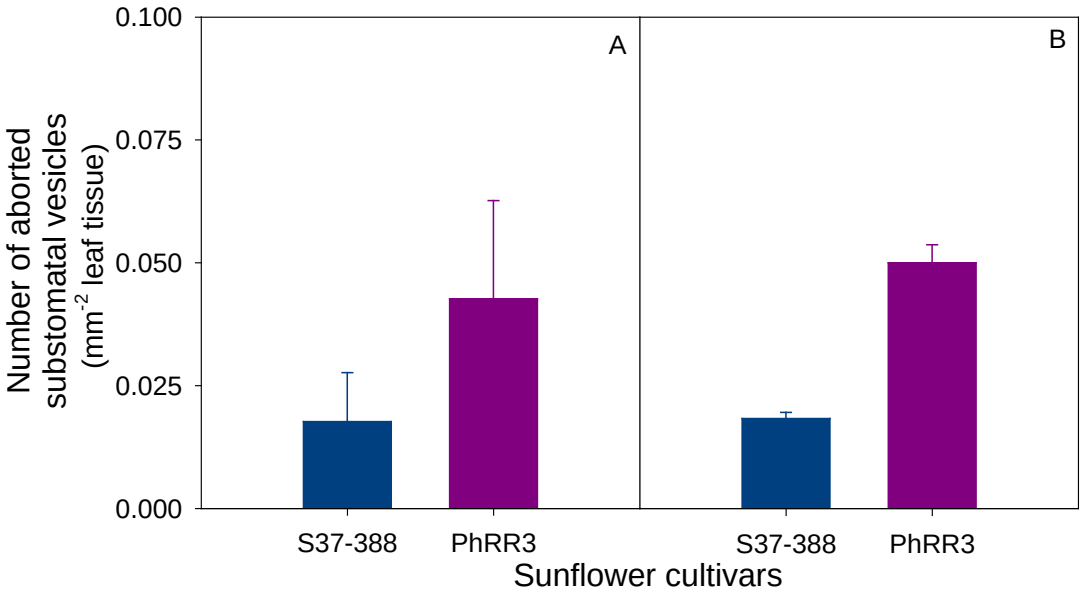

Fig. 3.8a: The mean number of aborted substomatal vesicles of rust (*Puccinia helianthi* Schw., pathotype UVPhe 2) in the susceptible (S37-388) and resistant (PhRR3) sunflower lines at an infection period of 48 and 72 hpi. A and B represent two independent experiments. The values are means ± SD (n=50).

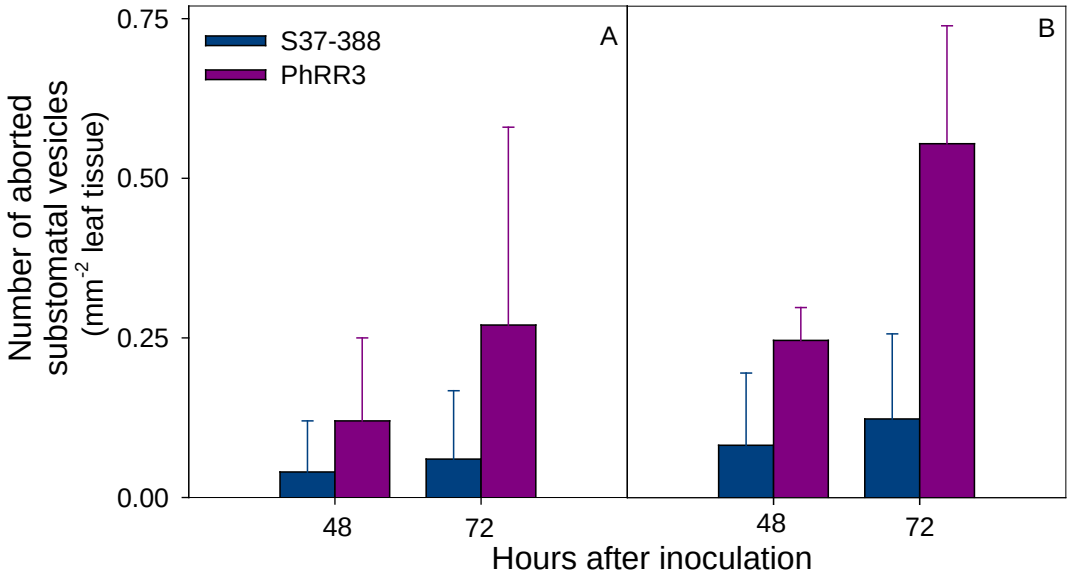

Fig. 3.8b: The number of aborted substomatal vesicles of rust (*Puccinia helianthi* Schw., pathotype UVPhe 2) in susceptible (S37-388) and resistant (PhRR3) sunflower plants at 48 and 72 hpi. A and B represent two independent experiments. The values are means ± SD (n=50).

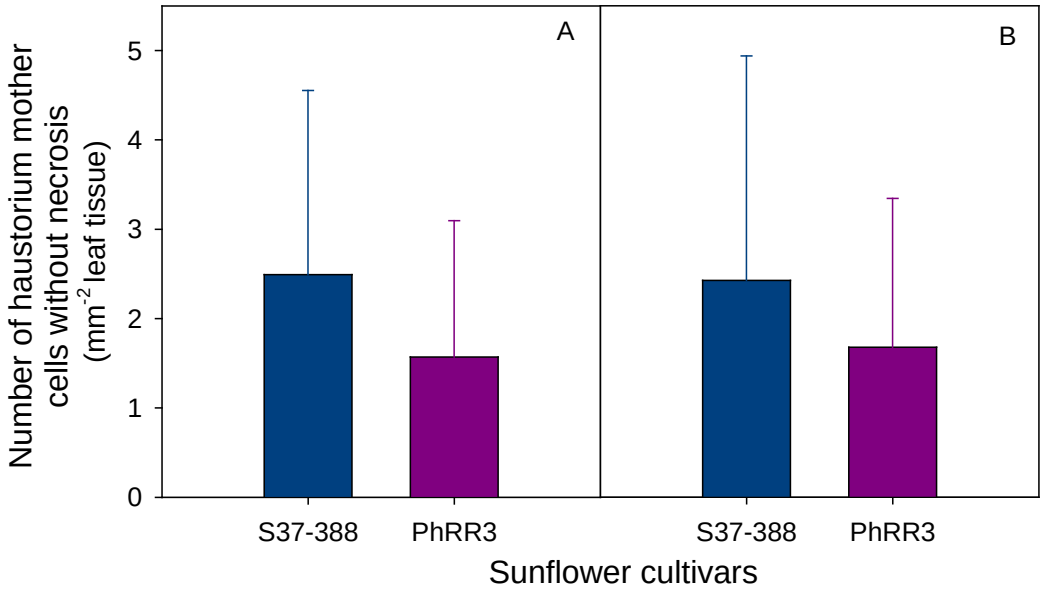

Fig. 3.9a: The number of haustorium mother cells of rust (*Puccinia helianthi* Schw., pathotype UVPhe 2) not associated with necrosis in susceptible (S37-388) and resistant (PhRR3) sunflower plants at an infection period comprising 48 and 72 hpi. A and B represent two independent experiments. The values are means ± SD (n=50).

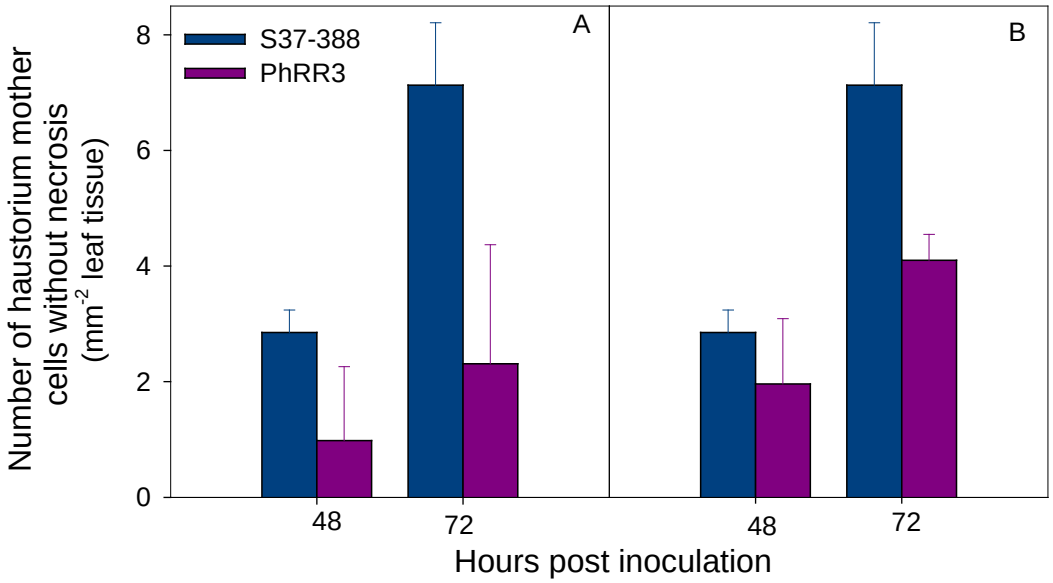

Fig. 3.9b: The number of haustorium mother cells of rust (*Puccinia helianthi* Schw., pathotype UVPhe 2) not associated with necrosis in susceptible (S37-388) and resistant (PhRR3) sunflower plants at 48 and 72 hpi. A and B represent two independent experiments. The values are means ± SD (n=50).

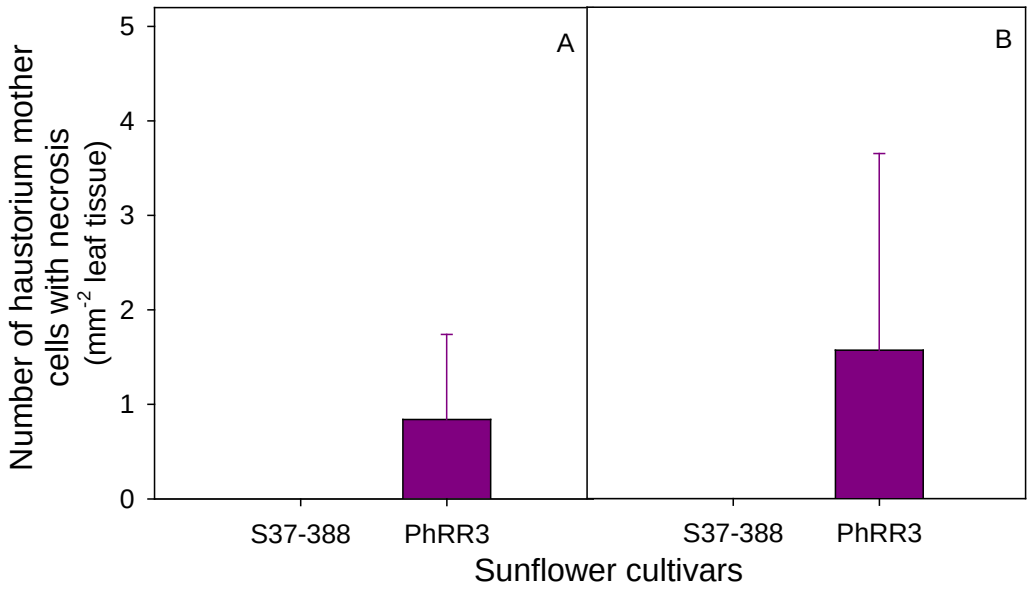

Fig. 3.10a: The number of haustorium mother cells of rust (*Puccinia helianthi* Schw., pathotype UVPhe 2) associated with necrosis in susceptible (S37-388) and resistant (PhRR3) sunflower plants at an infection period including 48 and 72 hpi. A and B represent two independent experiments. The values are means ± SD (n=50).

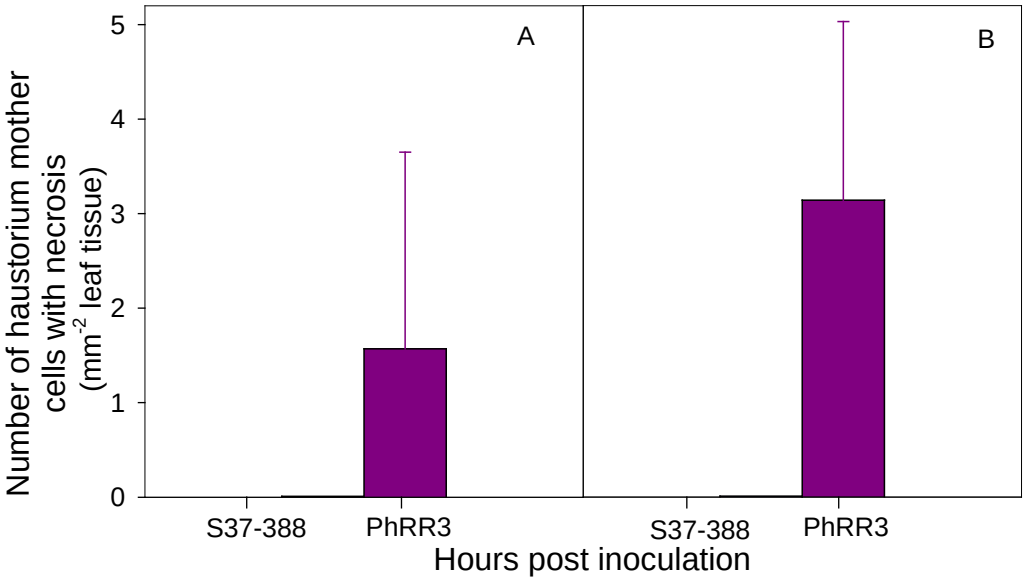

Fig. 3.10b: The number of haustorium mother cells of rust (*Puccinia helianthi* Schw., pathotype UVPhe 2) associated with necrosis in susceptible (S37-388) and resistant (PhRR3) sunflower plants at 72 hpi. A and B represent two independent experiments. The values are means ± SD (n=50).

The sizes of rust colonies differed significantly ($P<0.05$) between susceptible and resistant plants. Larger colonies were observed in the susceptible than resistant line at 72 and 96 hpi (Fig. 3.11). Duration of infection (hpi) also significantly ($P<0.05$) directed formation and size of colonies as well as the necrotic area in both the susceptible and resistant lines. The different host genotypes had a significant ($P<0.05$) impact on the colony sizes and necrotic areas formed during infection. In the susceptible line the colony sizes were much larger at 72 hpi and 96 hpi than in the resistant line (Fig. 3.11). In the resistant line at infection periods of 72 h and 96 h the necrotic areas were much bigger than in the susceptible line where no necrotic areas were observed (Fig. 3.11). In the second experiment (Fig. 3.11ii.) the pattern of colony development and colony sizes as well as the necrotic area were similar, but the values were slightly higher.

The hypersensitivity index (HI) in the resistant plants increased from less than one at 72 hpi and increased to 1.2 at 96 hpi (Table 3.1). In the susceptible plants the index was not calculated due to the absence of necrosis.

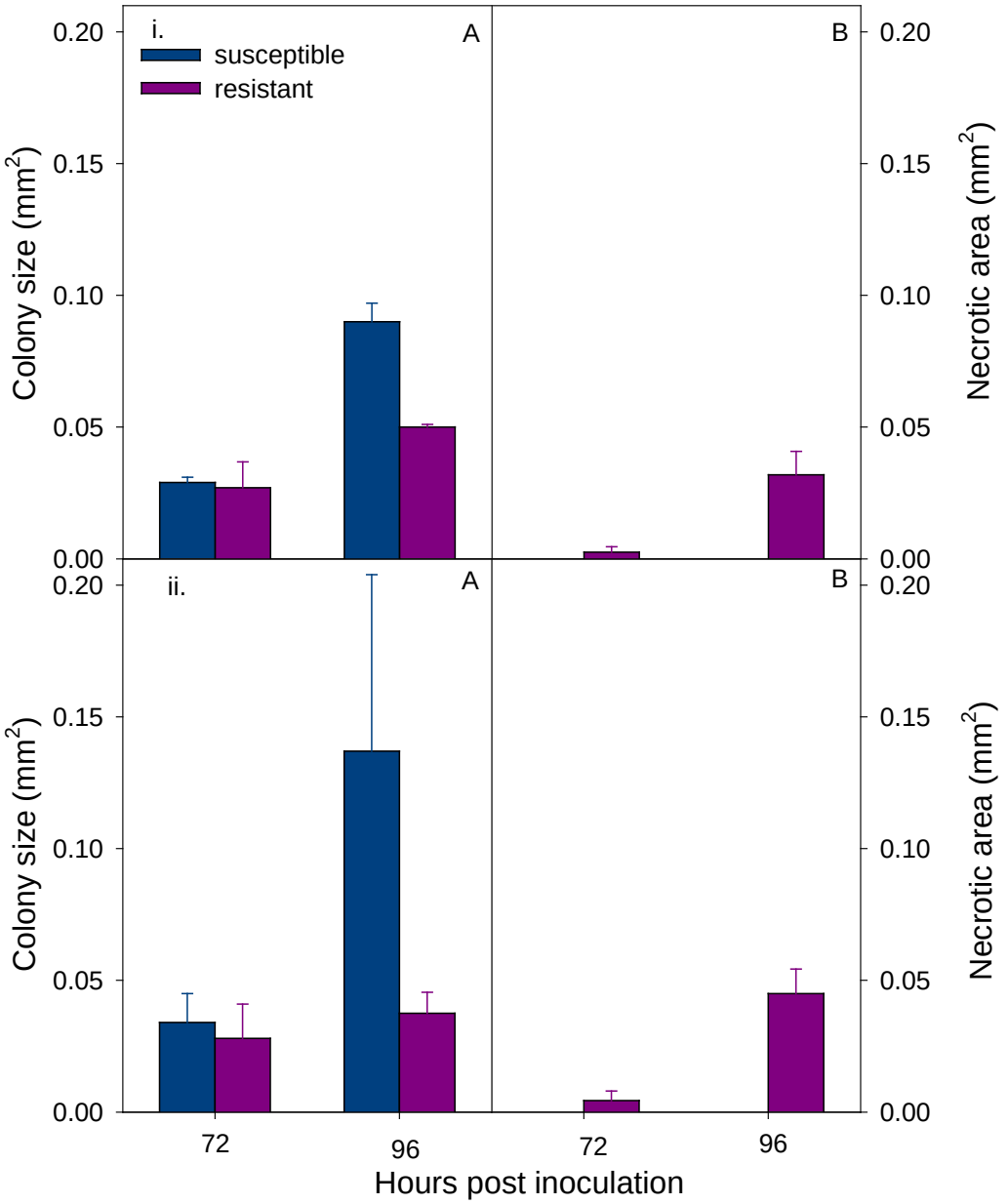

Fig. 3.11: Colony size (A) of rust (*Puccinia helianthi* Schw., pathotype UVPhe 2) and necrotic leaf area (B) in susceptible (S37-388) and resistant (PhRR3) sunflower plants. i. and ii. represent two independent experiments. The values are means ± SD (n=50).

Table 3.1: Hypersensitivity index in the resistant (PhRR3) sunflower line at two different time periods after inoculation with rust (*Puccinia helianthi* Schw., pathotype UVPhe 2). The values are means ± SD (n=50).

Hours after inoculation	72	96
Hypersensitivity index	0.2 ± 0.06	1.200 ± 0.201

3. 4 Discussion

The infection process of sunflower rust can be described under the following headings: the prestomatal behaviour, abortive penetration, early abortion and colony development and host response. Spore germination, stomatal appressoria and nonstomatal appressoria are all grouped into prestomatal behaviour. All the three stages were significantly dependent on time, i.e., hpi in both the resistant and susceptible sunflower lines. The germination of spores did not differ significantly in the two sunflower lines indicating that this process may not be triggered by any specific signal controlled by the host genotype. Sood and Sackston (1970) also found no differences in average germination of rust spores on the leaves of susceptible and resistant sunflower plants. In coffee plants infected with coffee orange rust (*Hemileia vastatrix* Beck and Br.), the germination of urediospores and appressoria formation did not differ between the susceptible and resistant cultivars (Silva *et al.*, 2002).

The ability of the germ tube to locate a stoma in many rust fungi is governed by the surface topography of the leaf (Wynn and Staples, 1981). Tight attachment to the plant cuticle would allow the germ tube to sense host topographical or chemical signals and/or stimulate tube growth and induce appressorium differentiation. In sunflower-rust interaction, the ability to locate a stoma may also be governed by some signal in the host genotype since the average numbers of stomatal appressoria were significantly different between the susceptible and resistant sunflower lines. The development of nonstomatal appressoria was generally the same in the susceptible and resistant lines and this may be an unimportant structure in the establishment of rust resistance in sunflower.

The germ tube differentiates into an appressorium, from which further infection structures inside the leaf develop. A higher density of stomatal appressoria in the susceptible line poses agreater chance for further differentiation inside the leaf. The higher number of aborted substomatal vesicles in the resistant line, as opposed to the susceptible one, is an indication of an early abortive penetration of infection structures. Abortion of infection structutes reduces the number of rust colonies that ultimately develop. Host resistance may be responsible for enhancing this abortive

penetration. No differences were, however, observed in the germination of spores, penetration of appressoria, vesicle size or shape between the susceptible and the resistant sunflower lines. These results were in agreement with those of Sood and Sackston (1970) who also found no differences in spore germination, appressorium penetration, vesicle shape or size between the susceptible and resistant sunflower lines.

Six or less haustorium mother cells were considered as early abortion of the fungus. This stage was significantly dependent on host genotype, period of infection as well as the interaction of the genotype and infection period. In the resistant line more HMCs were associated with necrosis as compared to the susceptible line. This may be an expression of host resistance response, manifested as host cell death in and/or around the infected cells. In an experiment by Sood and Sackston (1970) involving sunflower and rust, the number of collapsed host cells increased with time after inoculation and host cell collapse in the susceptible plants was not observed except under developing pustules.

A colony was considered ‘early aborted’ if up to five or six haustorium mother cells were formed and if infection hyphae were not intensively branched (Niks, 1982). Colonies with more than six haustorium mother cells were considered likely to sporulate and propagate the rust. The larger colony sizes in the susceptible than resistant line are an indication of a compatible interaction with the host. In such a susceptible interaction, the pathogen either overcomes defences of the host or evades recognition. In some instances, the pathogen may produce suppressors of host resistance. On the other hand the larger necrotic size in the resistant than susceptible line is an indication of host resistance response. In many incompatible host-pathogen interactions, the host responds by displaying the hypersensitive cell death around the pathogen. This delimits the source of food for the invading pathogen. Rust is an obligate parasite acquiring its nutrients from the host cells through a haustorium (Staples, 2001). Hypersensitive death of host cells cuts off the supply of nutrients to the fungus leading to its starvation. The hypersensitive response characterised by rapid death of invaded and neighbouring cells, resulting in macroscopic lesions at the infection sites (Tani *et al.*, 1974), is an active defence

system occurring in most higher plants in response to pathogens (Keen, 1990). The hypersensitivity index (Kloppers and Pretorius, 1995) greater than one (Table 3.1) emphasised the fact that at each infection site in resistant plants the necrotic area actually exceeded the mycelium dimensions.

The resistant line, PhRR 3, contains the resistance gene R_3, which is not present in the susceptible line, S37-388 (Putt and Sackston, 1963; Goulter, 1990). This gene confers resistance to sunflower rust in a gene-for-gene interaction (Crute and Pink, 1996) and may be responsible for the significant differences in the development of fungal infection structures between the two lines. The products of this gene are probably responsible for the biochemical events associated with resistance, which hinders pathogen development in the resistant cultivar.

Biochemical defence-related components of the rust resistance response

4.1 Introduction

The natural resistance of plants to disease-causing pathogens is dependent on the constitutive and active defence mechanisms. The latter relies on the recognition of the pathogen and subsequent signal transduction events leading to the induction of various defence responses. Resistance can be expressed locally around the infection site, or systemically in uninfected parts of the plant. The active oxygen species (AOS) are one of the earliest cues associated with the signalling of secondary defence responses. Local resistance is usually relayed through other signalling molecules to systemic uninfected parts of the plant. Salicylic acid (SA) is a likely endogenous molecule involved in the activation of systemic resistance (Sticher *et al.*, 1997; Yang *et al.*, 1997). The lipoxygenase (LOX) pathway involved in fatty acid biosynthesis also releases products such as jasmonates, which are involved in the signalling of defence responses (Penninckx *et al.*, 1996).

Information on the biochemical events related to rust resistance in sunflower is limited, and this study was undertaken in an attempt to increase the current understanding of the resistance response in this pathosystem.

In many plant-pathogen interactions, defence-related metabolites accumulate in the apoplast (Bowles, 1990). The defence response invariably begins with the activation of gene transcription in the host that leads to mRNA production and culminates in the formation of new proteins. The project was based on the premise that rust infection of sunflower will induce the synthesis of resistance related proteins and activation of certain enzyme activities, causing an inhibition of the development of rust disease. The aims of this study were therefore to:

- investigate the apoplastic protein profile of sunflower during rust infection,
- identify some of the synthesised proteins,
- investigate the changes in activity of certain resistance-related enzymes and
- investigate the possible involvement of hydrogen peroxide and salicylic acid in signalling defence related responses.

4.2 Materials and Methods

4.2.1 Biological material

Seeds of sunflower (*Helianthus annuus* L.) lines susceptible (S37-388) and resistant (PhRR3) to sunflower rust (*Puccinia helianthi* Schw.) (Kong *et al.*, 1999) were germinated and transplanted into cylindrical (4.2 L) pots (three seedlings/pot). The seedlings were cultivated in a sterilised soil/peat (3:1) mixture in a glasshouse at a temperature of 25 ± 3ºC. Daylight was supplemented with 120 µmol m^{-2} s^{-1} photosynthetically active radiation provided by cool white fluorescent tubes for 14 h each day. The plants were watered daily and fertilised with 100 ml of nutrient solution (2 g L^{-1}, w/v) containing N, P and K (6.5:2.7:13) and microelements per pot, three times per week.

Thirty days after sowing (three-leaf-pair growth stage) the plants were spray-inoculated with a *P. helianthi* (pathotype UVPhe2, Los *et al.*, 1995) spore suspension (9 x 10^5 spores ml^{-1}). Pathotype UVPhe 2 is virulent to the sunflower line S37-388 and avirulent to the line PhRR3 (Los *et al.*, 1995). Freshly harvested spores were suspended in sterile distilled water containing 0.05% (v/v) Tween 20. Control plants were sprayed with sterile distilled water containing 0.05% (v/v) Tween 20 only. The plants were left to dry at room temperature (2 h) and subsequently incubated in a dew chamber (>96% relative humidity, 21ºC) for 16 h in the dark. After a 2 h drying period at room temperature the plants were transferred to the glasshouse. A total of sixty plants (30 susceptible and 30 resistant) were used for each experiment.

Two leaves (3rd and 4th) were harvested from each plant in three different pots, all mixed and a certain leaf mass used for extractions. The leaves were harvested at 0,

3, 6, 9, 12 and 24 h after inoculation for the determination of NADPH-oxidase, superoxide dismutase and peroxidase activities as well as H_2O_2 content. In the case of phenylalanine ammonia-lyase and lipoxygenase activities, and SA content determinations, leaves were sampled at 0, 24, 48, 72, 96 and 144 h after infection. The collected leaves were immediately dipped in liquid nitrogen and stored at -20°C for extraction of enzymes, H_2O_2 and SA. The enzyme activities as well as H_2O_2 and SA contents were determined in triplicate and the entire experiments repeated. For collection of intercellular wash fluid (IWF), two leaves (3rd and 4th) were harvested from three different pots at each sampling time. The leaves were harvested at 0, 24, 48, 72, 96 and 144 h after infection.

4.2.2 Collection of intercellular wash fluid (IWF)

To get a representative sample at each sampling time, two leaves (3rd and 4th) from any one of the three plants growing in one pot were harvested. Leaves were cut from three different pots to get a total of six leaves. These leaves were combined and intercellular wash fluid extracted using a modified method of Jung et al. (1993). Leaves were rinsed in distilled water and vacuum infiltrated with 50 mM sodium acetate buffer, pH 5.2, containing 15 mM mercaptoethanol. The leaves were blot dried with paper and rolled between plastic sheets around a tube, which was then inserted into a centrifuge tube and centrifuged at 484 x g for 10 min at 4ºC. The entire procedure was repeated and the combined IWF used for SDS-PAGE, Western blots and β-1,3-glucanase, chitinase and peroxidase activity determinations.

4.2.3 Enzyme activities

4.2.3.1 β-1,3-glucanase (EC 3.2.1.39) activity

β-1,3-glucanase activity was assayed using a modified method of Fink et al. (1988). The assay mixture contained 10 µl enzyme extract, 250 µl laminarin (2 mg ml^{-1} water; Sigma), and 240 µl 50 mM sodium acetate buffer, pH 4.5. After incubation at 37°C for 10 min, 500 µl of Somogyi's reagent (1952) were added. The samples were then boiled at 100°C for 10 min. After cooling, 500 µl of Nelson's reagent (1944) were added. The absorbance of the samples was read at 540 nm against a

blank prepared in the same manner but lacking the enzyme extract. The amount of glucose in the samples was estimated from a prepared glucose standard curve and specific β-1,3-glucanase activity was expressed as mg glucose mg^{-1} protein min^{-1}. For each of the two independent experiments β-1,3-glucanase activity of the representative IWF sample was determined in triplicate.

4.2.3.2 Chitinase (EC 3.2.1.14) activity

Total chitinase activity determination was carried out using modified methods of Boller *et al.* (1983) and Legrand *et al.* (1987). The reaction mixture containing 3 mg chitin (Sigma), an aliquot of IWF and 50 mM sodium acetate buffer, pH 6.5, in a total volume of 500 µl was incubated at 37°C for 30 min. After centrifugation (10 390 x g, 3 min), 300 µl of the supernatant was incubated (37°C, 30 min) with 20 µl of cytohelicase [1.5% (w/v) from snail gut, Sigma] and 30 µl of 1 M potassium phosphate buffer, pH 7.1. The resulting N-acetylglucosamine (GlcNAc) was determined according to a modified procedure of Reissig *et al.* (1955). To 250 µl of the mixture 50 µl of 0.8 M potassium tetraborate buffer, pH 9.1, was added and heated (3 min) in a boiling water bath. After cooling, 1.5 ml of dilute 4-dimethylaminobenzaldehyde reagent (1:9 in glacial acetic acid) was added and the mixture incubated at 37°C for 20 min. The stock solution of the reagent contained 10% (w/v) 4-dimethylaminobenzaldehyde in a mixture of glacial acetic acid and HCl (10 N), (87.5:12.5, v/v).

The absorbance of N-acetylglucosamine was read at 585 nm against a blank prepared in the same manner, but lacking IWF. A standard curve relating GlcNAc to absorbance was used to estimate GlcNAc in the sample and specific enzyme activity was expressed as µmol GlcNAc mg^{-1}protein min^{-1}. For each of the two independent experiments chitinase activity of the representative IWF sample was determined in triplicate.

4.2.3.3 Peroxidase (EC 1.11.1.7) activity

Peroxidase activity was determined both extracellularly (IWF) and intracellularly. In the case of intracellular peroxidase determination, leaf tissue (0.5 g) was ground in liquid nitrogen in a pre-cooled pestle and mortar. The leaf powder was homogenised in 100 mM sodium acetate buffer, pH 5.5 (leaf tissue: buffer; 1:4), containing 10 mM mercaptoethanol, 2 mM EDTA and 2 mM phenylmethylsulfonyl fluoride (PMSF). The homogenate was centrifuged at 17 400 x g for 20 min at 4°C. The supernatant was used as the crude enzyme extract and enzyme activity was determined spectrophotometrically. The assay mixture contained 0.5% (v/v) guaiacol (Merck) in 40 mM potassium phosphate buffer, pH 5.5, 15 mM H_2O_2 and 10 µl enzyme extract (IWF or supernatant) in a total volume of 1 ml. The increase in absorbance was measured at 470 nm for a period of 180 s against a blank. The specific activity was calculated using the molar extinction coefficient ($\varepsilon = 2.66 \times 10^4$ mol^{-1} cm^{-1}) of tetraguaiacol and expressed as nmol tetraguaiacol mg^{-1} protein min^{-1}. For each of the two independent experiments peroxidase activity of the representative IWF sample was determined in triplicate.

4.2.3.4 NADPH oxidase (EC 1.6.3.1) activity

NADPH oxidase activity was carried out using a slightly modified method of Rao *et al.* (1996). Leaf tissue was ground in liquid nitrogen and extracted with 50 mM potassium phosphate buffer, pH 7.0 (leaf tissue: buffer, 1:4), containing 0.1% (v/v) Triton X-100, 10 mM ethylenediaminetetraacetic acid (EDTA), 1% (w/v) insoluble polyvinylpyrrolidone (PVP) and 0.004% (w/v) sodium metabisulfite. The homogenate was centrifuged at 17 400 x g for 15 min at 4°C. The supernatant was used as the crude enzyme extract and the reaction mixture contained 50 mM potassium phosphate buffer, pH 7.0 (800 µl), 150 µM NADPH (100 µl), 10 µM potassium cyanide (20 µl) and the enzyme extract (± 80 µl) in a total volume of 1 ml. The reaction proceeded at 25°C and the decrease in absorbance was measured against a blank at 340 nm over a period of 5 min. An enzyme unit was described as change in absorbance over time per µg protein.

4.2.3.5 Superoxide dismutase (SOD, EC 1.15.1.1) activity

Superoxide dismutase (SOD) activity was determined using modified methods of Beauchamp and Fridovich (1971) and Milosevic and Slusarenko (1996). Leaf tissue (0.5 g) was ground in liquid nitrogen in a pre-cooled pestle and mortar and homogenised in 50 mM potassium phosphate buffer, pH 7.0 (leaf tissue: buffer; 1:4), containing 0.1% (v/v) Triton X-100, 10 mM EDTA, 1% (w/v) insoluble PVP and 0.004% (w/v) sodium metabisulfite. The homogenate was centrifuged at 17 400 x g for 15 min at 4°C. The supernatant was used as the enzyme extract and the reaction mixture contained 50 mM potassium phosphate buffer, pH 7.8, 13 mM methionine, 75 µM nitroblue tetrazolium (Boehringer Mannheim GmbH), 2 µM riboflavin, 10 mM EDTA and 20 µl enzyme extract in a total volume of 1 ml. The cuvettes were placed 30 cm below fluorescent light tubes (2 x 20 W) in a box (100 x 30 x 15 cm) lined with aluminium foil. Switching on the light source initiated the reaction, which proceeded for 10 min. The absorbance of the samples was measured at 560 nm against a blank that had not been irradiated. A control made up of the reaction buffer only was also irradiated and its absorbance measured to give the maximum attainable value. A unit of enzyme activity was described as the amount of enzyme necessary to cause a 50% loss in nitroblue tetrazolium reduction (Milosevic and Slusarenko, 1996).

4.2.3.6 Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) activity

Phenylalanine ammonia-lyase (PAL) activity was determined spectrophotometrically according to Green *et al.* (1975) with some modifications. Leaf tissue (0.5 g) was ground in 0.1 M sodium borate buffer, pH 8.8, containing 1 mM EDTA (freshly added), 1 mM dithiothreitol, 50 mg polyvinylpyrrolidone, 25 mg Dowex (1 x 4, Cl^-; Sigma) and acid washed sea sand abrasive. The ratio of leaf tissue to buffer was 1:4. The extract was stirred for 15 min at 4ºC then centrifuged (23 700 x g, 10 min, 4ºC). The supernatant was used as an enzyme extract. The assay mixture consisted of an aliquot of enzyme extract, 0.1 M sodium borate buffer, pH 8.8 and 100 µl of 60 mM L-phenylalanine in a total volume of 1 ml. The change in absorbance was measured at 290 nm for 20 min at 40ºC. The amount of cinnamic acid liberated was calculated from a standard curve and PAL activity was expressed as µg cinnamic acid mg^{-1} protein min^{-1}.

4.2.3.7 Lipoxygenase (LOX, EC 1.13.11.12) activity

Lipoxygenase (LOX) activity was determined spectrophotometrically by measuring the increase in extinction caused by the formation of conjugated dienes. Linoleic acid was used as the substrate. Leaf tissue (0.5 g) was ground together with 20 mg insoluble polyvinylpyrrolidone and 50 mg acid-washed sea-sand in a pre-cooled pestle and mortar. The ground tissue was homogenised in 0.1 M potassium phosphate buffer, pH 7.5, containing 1 mM EDTA (leaf tissue: buffer, 1:4). The extract was shaken for 15 s and centrifuged for 8 min at 23 700 x g at 4°C. The supernatant was used as the enzyme extract. The reaction mixture contained 0.1 M sodium citrate phosphate buffer, pH 6.2, 2.5 mM linoleic acid and the enzyme extract in a total volume of 1.2 ml. The change in absorbance was measured against a blank at 234 nm for 10 min at 30°C. The specific LOX activity was calculated using the molar extinction coefficient of the 9-hydroperoxy fatty acids (HPOD) formed, $\varepsilon = 2.5 \times 10^4$ mol^{-1} cm^{-1} and expressed as nmol HPOD mg^{-1} protein min^{-1}.

4.2.4 Metabolite levels

4.2.4.1 Hydrogen peroxide content

Hydrogen peroxide content in leaf tissue was determined using a method adapted from Ferguson *et al.* (1982). Leaf tissue (1 g) was ground to a fine powder in liquid nitrogen using a pre-cooled pestle and mortar. The ground tissue was then extracted in 5 ml ice-cold acetone and centrifuged at 1480 x g for 20 min at 4°C. To 4.8 ml of the supernatant 0.5 ml $TiCl_4$ (Merck) in HCl (20% v/v $TiCl_4$ in 32% HCl) was added. The mixture was set shaking (3 min), and 3.5 ml of 1/5 strength NH_4OH (25% NH_3) were added drop wise with thorough mixing. The sample was then centrifuged (1480 x g, 15 min, 4°C) and the supernatant discarded. The precipitate was washed repeatedly with 10 ml volumes of ice-cold acetone until the supernatant was colourless. The precipitate was solubilised in 10 ml 2 N H_2SO_4 and made up to 20 ml with 2 N H_2SO_4. The samples were filtered on a Whatman no. 40 ashless filter paper and measurements of absorbance were taken against a blank that had also been reacted with $TiCl_4$, at 415 nm.

Hydrogen peroxide content was estimated from a standard curve relating known concentrations of H_2O_2 to absorbance at 415 nm. In preparing the standard curve, instead of adding 0.5 ml $TiCl_4$ to the supernatant as in extracting H_2O_2 from the sample, it was added to 4.8 ml of different H_2O_2 concentration solutions. The mixture was set shaking and 3.5 ml of 1/5 strength NH_4OH (25% NH_3) added drop wise while mixing. The mixture was centrifuged, washed, solubilised and filtered as above. The absorbance of the samples was measured at 415 nm against a blank carried through the same procedure.

4.2.4.2 Salicylic acid (SA) extraction and determination

Total SA (free and sugar-conjugated forms) was extracted from infected and non-infected susceptible and resistant sunflower plants using a modified method of Tuula *et al*. (1994). Leaf tissue (0.5g) was ground to a fine powder in liquid nitrogen and through intermittent centrifugation (13 000 x g, 20 min, 4ºC), was extracted twice with 1 ml 80% (v/v) ethanol. The combined supernatant was kept at -20ºC for 1 h (for complete sedimentation) then centrifuged (13 000 x g, 10 min, 4ºC). The supernatant was concentrated under reduced pressure in a rotavapor to a tenth of the original volume and adjusted to 4 ml with 2% (w/v) metaphosphoric acid in double distilled water. This extract was then partitioned three times with ethyl acetate (1:1). The combined ethyl acetate extract was evaporated to dryness under reduced pressure (45ºC). The remaining aqueous phase was adjusted to 1 M HCl and heated (80ºC) in a sealed test tube for 1 h, then cooled and partitioned three times with ethyl acetate (1:1). The combined ethyl acetate extract was dried under reduced pressure (45ºC). The dried residues originating from the unhydrolysed and acid hydrolysed samples were redissolved together in 3 ml 70% (v/v) methanol and passed through a C-18 Sep Pak cartridge (Waters Corporation, Milford, Massachusetts, USA) that had been pre-equilibrated with 5 ml 70% (v/v) methanol. Salicylic acid was eluted with 6 ml 70% (v/v) methanol. The eluate was dried under reduced pressure (45ºC) and redissolved in 400 µl of the HPLC mobile phase.

Salicylic acid was analysed by HPLC. Representative samples (20 µl) were run through a C-18 reverse phase column at a flow rate of 1 ml min^{-1} at ambient

temperature. Detection of SA with a retention time of 25 min was by means of a UV-detector at 240 nm. Identity of SA peaks from representative samples was confirmed by comparison of absorption spectra with that of an authentic SA standard. Salicylic acid peaks were further confirmed by running an internal SA standard along with some of the representative samples. Salicylic acid content of two independent experiments was determined in triplicate.

4.2.5 Protein concentration

The protein concentration was determined according to the method of Bradford (1976) using γ-globulin as the standard.

4.2.6 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (PAGE) was performed according to Laemmli (1970) using a 12% separating gel and a 6% stacking gel. Wells were loaded with 30 µg protein from IWF of susceptible and resistant, infected and uninfected plants. The gels were silver stained using the method of Morrisey (1981).

4.2.7 Immunoblotting (Western Blot)

Immediately after electrophoresis the polypeptides were transferred for 1 h onto a hybond C nitrocellulose membrane using a BioRad wet electrophoretic transfer cell. The transfer buffer contained 25 mM Tris (pH 8.3), 192 mM glycine and 20% (v/v) methanol. After the transfer, the membrane was stained in Ponceau S (Amersham Biosciences) for 2 min to verify protein transfer and destained with several changes of distilled water. All the active sites on the membrane were then blocked for 1 h in 8% (w/v) fat-free milk powder (Elite) in Tris buffered saline (10 mM Tris-HCl, pH 7.9, 1.0 M NaCl) containing 0.1% (v/v) Tween-20 (TBST). The membranes were then incubated in the primary antibody (anti-wheat β-1,3-glucanase) diluted 1:8500 in TBST containing 4% (w/v) fat-free milk powder, for 1 h. Subsequent to thorough rinsing, the membranes were incubated for another 1 h in the secondary antibody, anti-rabbit IgG, horseradish peroxidase linked whole antibody from donkey (Amersham) at a dilution of 1:2000 in TBST. The membranes were then thoroughly rinsed in TBST and TBST containing 0.1% (w/v) SDS, then reacted with enhanced

chemiluminescence (ECL) detection reagents according to the manufacturer (Amersham Pharmacia Biotech). An autoradiography film was placed on top of membranes sealed in a transparent plastic bag in a film cassette. The membranes were exposed for 2-12 h. The film was developed in normal developing solutions (Kodak) and further rinsed in tap water and air-dried.

4.2.8 Sample preparation for transmission electron microscopy (TEM)

4.2.8.1 Fixation of leaf material

Three leaf pieces of both susceptible and resistant infected and uninfected plants at 144 h after infection were used. Leaf sections (1 cm^2) were cut from between the veins. Sections were immediately fixed in 3% (v/v) glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4, for 1 h at ambient temperature with constant agitation. Sections were then washed in a phosphate buffer (5 x) followed by distilled water (3 x), then dehydrated in an ethanol series (25, 50, 75 and 95%, v/v) for 30 min in each concentration. The samples were further dehydrated for 19 h in 100% ethanol followed by another two changes of 12 h each in the same medium. Subsequently, the sections were infiltrated with 50% LR-white acrylic resin (London resin company, Berkshire, England) in ethanol for 60 min and 3 x 8 h changes in 100% LR-white and polymerised for 45 h at 50°C.

4.2.8.2 Ultra thin sections

Leaf pieces were cut into ultra thin sections using an LKB III ultramicrotome and collected on 3.05 mm diameter, 400 mesh nickel grids.

4.2.9 Immunolocalization

Grids bearing ultra thin sections were placed vertically in wells of a grid box filled with phosphate buffered saline containing 0.5% (v/v) Tween-20 (PBST) and 1% (w/v) bovine serum albumin (BSA) twice for 15 min each. This blocked all the unwanted active sites. Control samples were treated with the pre-immune rabbit serum and the test samples probed with the primary antibody, rabbit anti β-1,3-glucanase (1:250) overnight with constant agitation. The grids were then rinsed (3 x 5 min) in PBST containing BSA followed by the incubation in immunogold-labelled secondary antibody, EM goat anti-rabbit IgG: 15 nm (Agar Scientific, Essex, England) for 1 h at room temperature. Grids were then rinsed (3 x 5 min) in PBST containing BSA followed by double distilled water (3 x 5 min). Samples were finally stained with 6% uranyl acetate and lead citrate (Reynolds, 1963) and observed under a Philips CM 100 transmission electron microscope at 60 kV.

4.2.10 Quantification of labelling

The density of immunogold-labelling in leaf sections was compared by determining the number of gold particles per μm^2 on the cell wall and chloroplasts on four micrographs. Density was expressed as the mean number of gold particles per µm.

4.3 Results

4.3.1 Apoplastic protein composition

Extracellular proteins from rust infected and uninfected susceptible and resistant plants were separated on SDS-PAGE gels. According to the polypeptide profiles the accumulation of at least five polypeptides with molecular masses 50, 43, 34, 32 and 27 kDa, were induced during rust infection in the resistant plants (Fig. 4.1). The accumulation of polypeptides with molecular masses 44 and 32 kDa particularly increased as infection proceeded. A polypeptide with a molecular mass of 27 kDa was observed only at a later stage of infection (96 and 144 hpi) in the resistant plants. In the infected susceptible plants accumulation of the polypeptides with molecular masses between 50 and 32 kDa was observed in the late stages of infection (Fig. 4.1).

4.3.2 Apoplastic β-1,3-glucanase activity

Rust infection differentially induced β-1,3-glucanase activity to higher levels in resistant than susceptible plants as infection proceeded (Fig. 4.2). The activity was 1.9 times higher in infected resistant plants than the uninfected resistant plants at 144 hpi, at which time the degree of induction in the infected susceptible plants was 1.1-fold (experiment 2).

4.3.3 Western blot analysis of apoplastic proteins

According to the Western blots in Fig 4.3, β-1,3-glucanases with molecular masses between 34 and 42 kDa were induced to a higher level in the resistant plants during sunflower-rust interaction. In the late stages of infection (144 hpi) a β-1,3-glucanase isoenzyme with a molecular mass of 30.2 kDa accumulated in the infected resistant plants. This isoenzyme was not induced in the susceptible plants.

4.3.4 Localisation of β-1,3-glucanases

β-1,3-glucanases cross-reacting with wheat-anti β-1,3-glucanase were localised using the immunogold labelling technique. Substitution of wheat-anti β-1,3-glucanase with rabbit pre-immune serum yielded a very low number of gold particles on the cell walls of infected resistant (23% higher than in uninfected resistant plants) plants (Figs. 4.4a and 4.4b). In the susceptible plants no labelling was observed along the cell walls (Figs. 4.4a and 4.4b). Unspecific labelling occurred in the chloroplasts of both uninfected and infected susceptible and resistant plants (Fig. 4.4a). The results depicted in Figs. 4.4a and 4.4b indicate that more labelling occurred on the cell walls of infected resistant plants (4.5-fold higher than in the susceptible plants). In the susceptible plants, no labelling or much less than in the resistant plants was observed.

4.3.5 Apoplastic chitinase activity

Rust infection also induced apoplastic chitinase activity to much higher levels in resistant than susceptible plants as infection proceeded (Fig. 4.5). A 24-fold increase was reached in infected resistant plants 144 h after infection. In the

susceptible plants a slight induction, even though not significant, was observed from 96 h after infection.

4.3.6 Apoplastic peroxidase activity

The extracellular peroxidase activity was induced much earlier to higher levels in resistant than susceptible plants during rust infection. Induction began 24 hpi in the resistant plants whereas in the susceptible plants it was much delayed (Fig. 4.6).

4.3.7 Phenylalanine ammonia-lyase (PAL) activity

Phenylalanine ammonia-lyase (PAL) activity was differentially induced to a higher level in the resistant than susceptible plants, and peak activity in the first experiment (3.7-fold increase) was reached 48 h after infection (Fig. 4.7A). In the second experiment induced peak activity (2.1-fold increase) occurred 72 h after infection.

4.3.8 Salicylic acid (SA) content

Rust infection induced an increase in the SA content of the resistant plants (Fig. 4.8). Salicylic acid content began to rise sharply 16 h after infection and reached a maximum level 96 h after infection, which represented a 29-fold increase in the first (Fig. 4.8A) experiment. In the susceptible plants a slight increase in SA content was delayed, occurring from 24 h after infection in the first experiment and 96 h after infection in the second experiment (Figs. 4.8A and B).

4.3.9 Lipoxygenase (LOX) activity

Rust infection differentially induced LOX activity to much higher levels in resistant than susceptible plants. The increase began 24 h after infection and reached a peak 72 h after infection (Fig. 4.9). Peak activity represented a 2.2-fold and a 2.1-fold increase in the first (Fig. 4.9A) and second (Fig. 4.9B) experiments respectively. Induction in susceptible plants was not only to a lower level than in the resistant plants, but also much more gradual.

4.3.10 Hydrogen peroxide content

Rust infection differentially induced a higher H_2O_2 outburst in resistant than susceptible plants (Fig. 4.10). The H_2O_2 level began to rise sharply as early as 3 h after infection and peaked (2.9-fold) 9 h after infection (Fig. 4.10A). These results were confirmed in the second experiment where a 1.7-fold increase was obtained during the outbreak (Fig. 4.10B).

4.3.11 NADPH oxidase activity

A transient sharp increase in NADPH oxidase activity was selectively induced in the resistant plants by rust infection. The selective induction began immediately upon infection and peaked at 9 h after infection. At this point the degree of induction in the resistant plants was 21-fold (Fig. 4.11A) and 24-fold (Fig. 4.11B) for the two experiments respectively. In the susceptible plants there was no significant induction of NADPH oxidase activity during this early post infection period even though a slightly increased activity occurred 24 h after infection in both experiments (Fig. 4.11A and B).

4.3.12 Superoxide dismutase (SOD) activity

The SOD activity was differentially induced to a higher level in resistant than susceptible plants by rust infection (Fig. 4.12). In the resistant plants a sharp rise in SOD activity was observed within 3 h after infection, which peaked 6 h after infection. In the susceptible plants, induced SOD activity was delayed and to a smaller magnitude, especially in the first experiment (Fig. 4.12A).

4.3.13 Total peroxidase activity

Rust infection selectively induced a sharp transient increase in total peroxidase activity of the resistant sunflower line (Fig. 4.13). The increase began immediately after infection and reached a climax 6 h after infection, representing a 5.8-fold increase (Fig. 4.13A). In the susceptible sunflower line, there was no induction at any time.

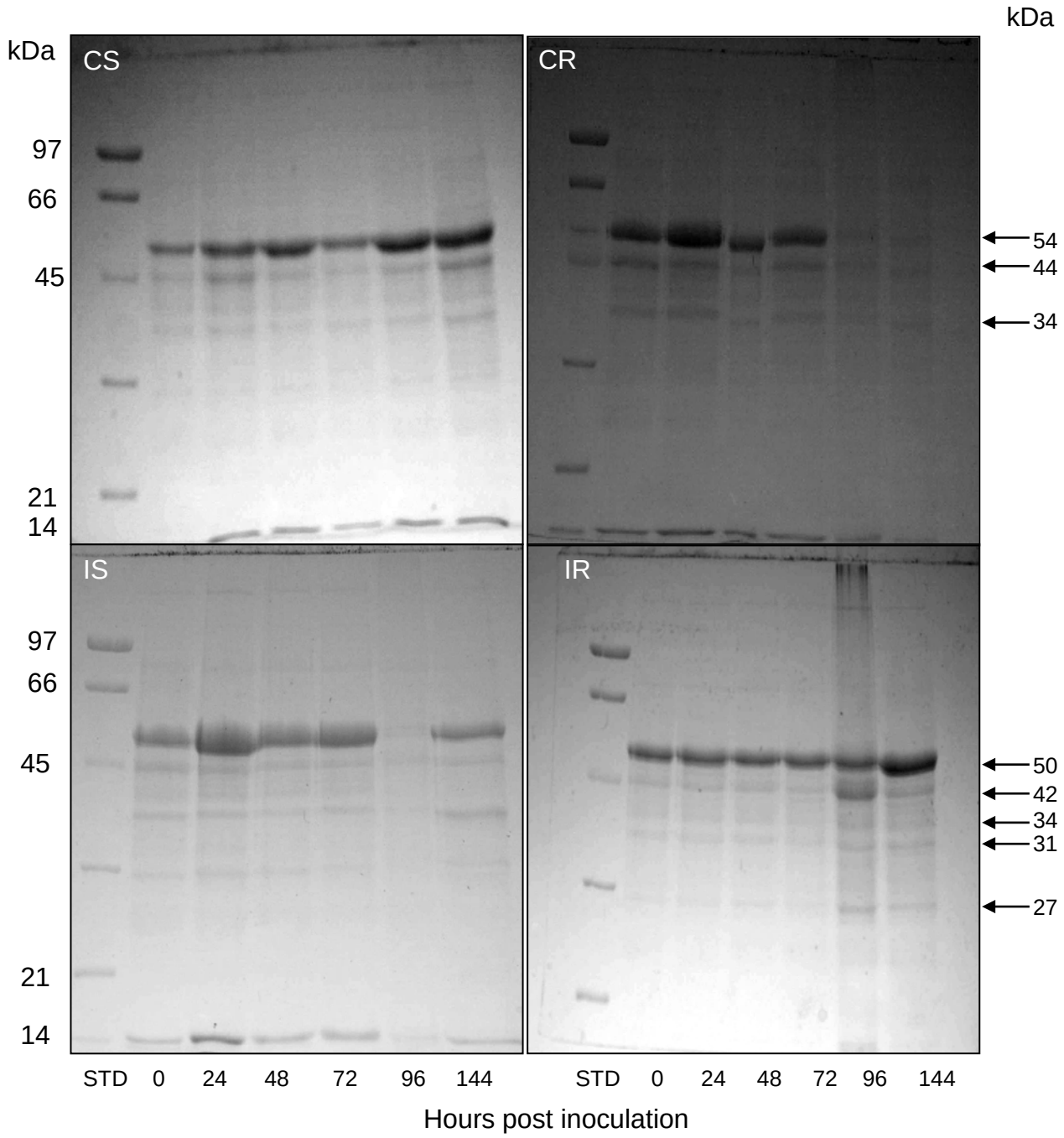

Fig. 4.1: Polypeptide profiles of IWF proteins from uninoculated susceptible, S37-388 (CS) and resistant, PhRR3 (CR), and inoculated (*Puccinia helianthi* Schw., pathotype UVPhe 2) susceptible (IS) and resistant (IR) sunflower plants, STD: Low molecular weight protein standards.

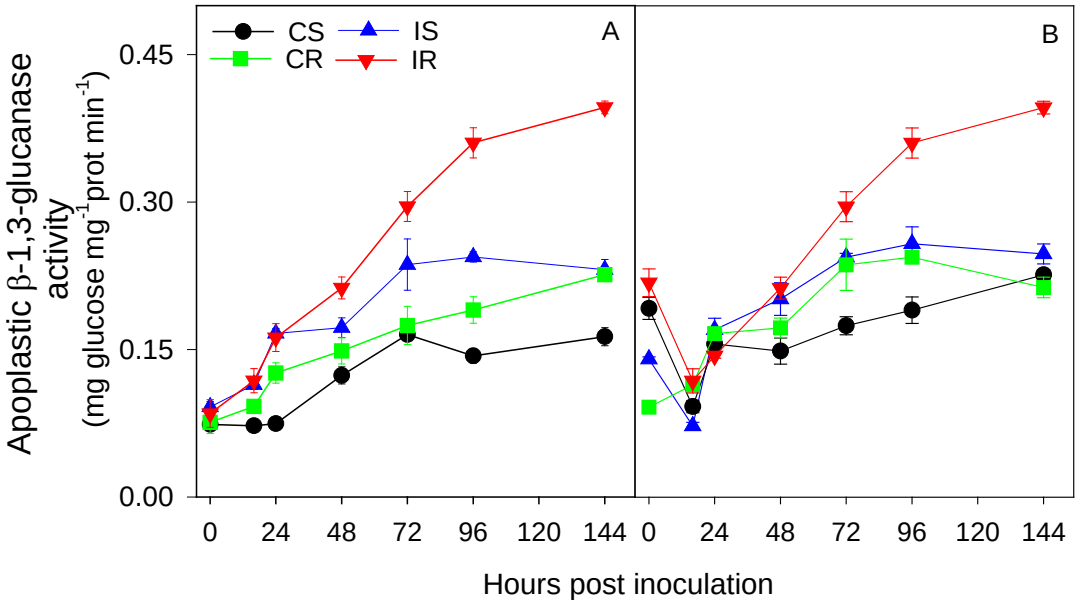

Fig. 4.2: Effect of rust (*Puccinia helianthi* Schw., pathotype UVPhe 2) infection on β-1,3-glucanase activity of susceptible (S37-388) and resistant (PhRR3) sunflower plants. CS, control susceptible; IS, inoculated susceptible; CR, control resistant; IR, inoculated resistant. A and B denote two independent experiments. The values are means ± SD (n=3).

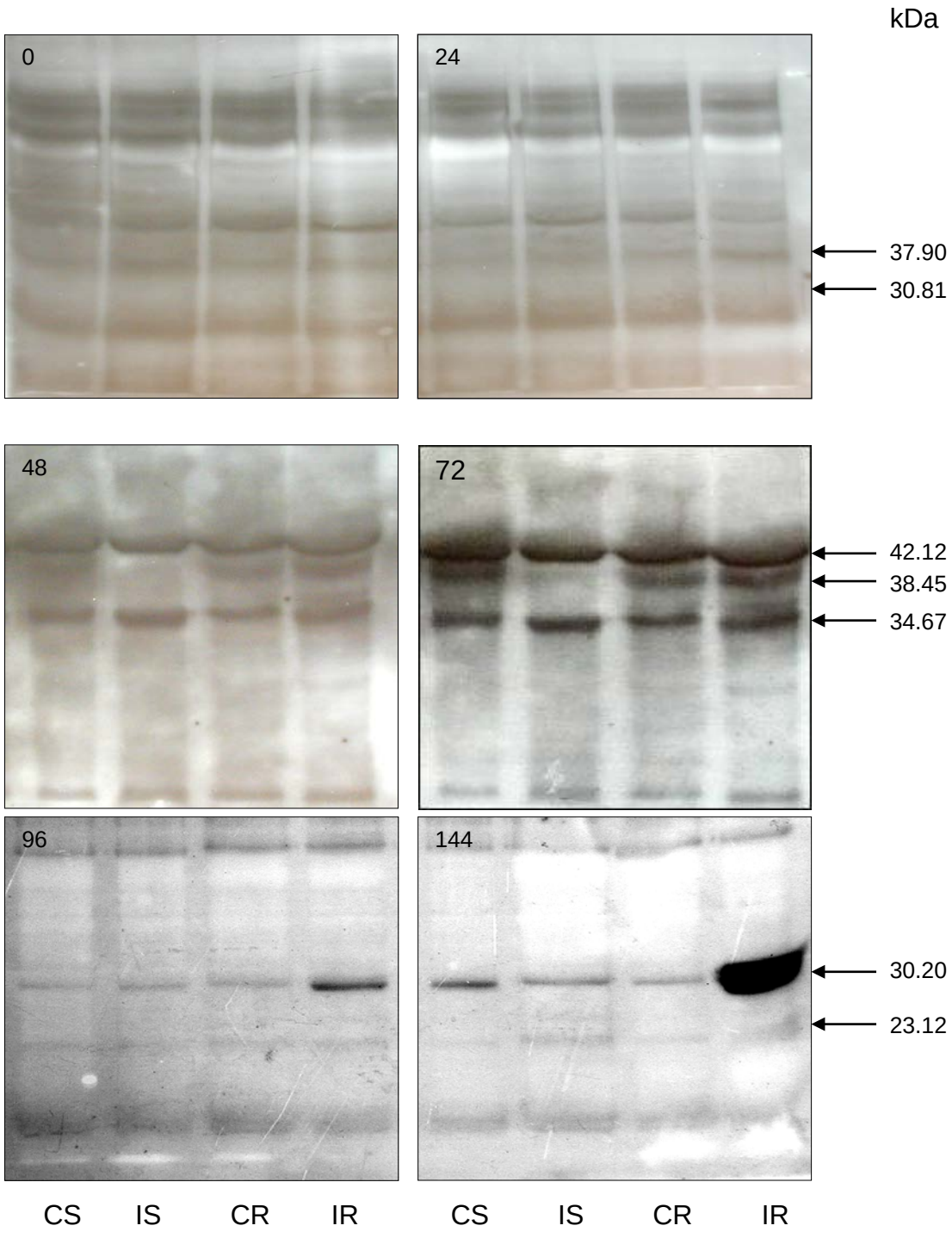

Fig. 4.3: Western blots of intercellular proteins (80 µg) collected from uninfected (C) and rust (*Puccinia helianthi* Schw., pathotype UVPhe 2)-infected (I) susceptible, S37-388 (S) and resistant, PhRR3 (R) sunflower plants after different infection periods. Blots were probed with anti-wheat β-1,3-glucanase. Hours after infection are indicated in the figures.

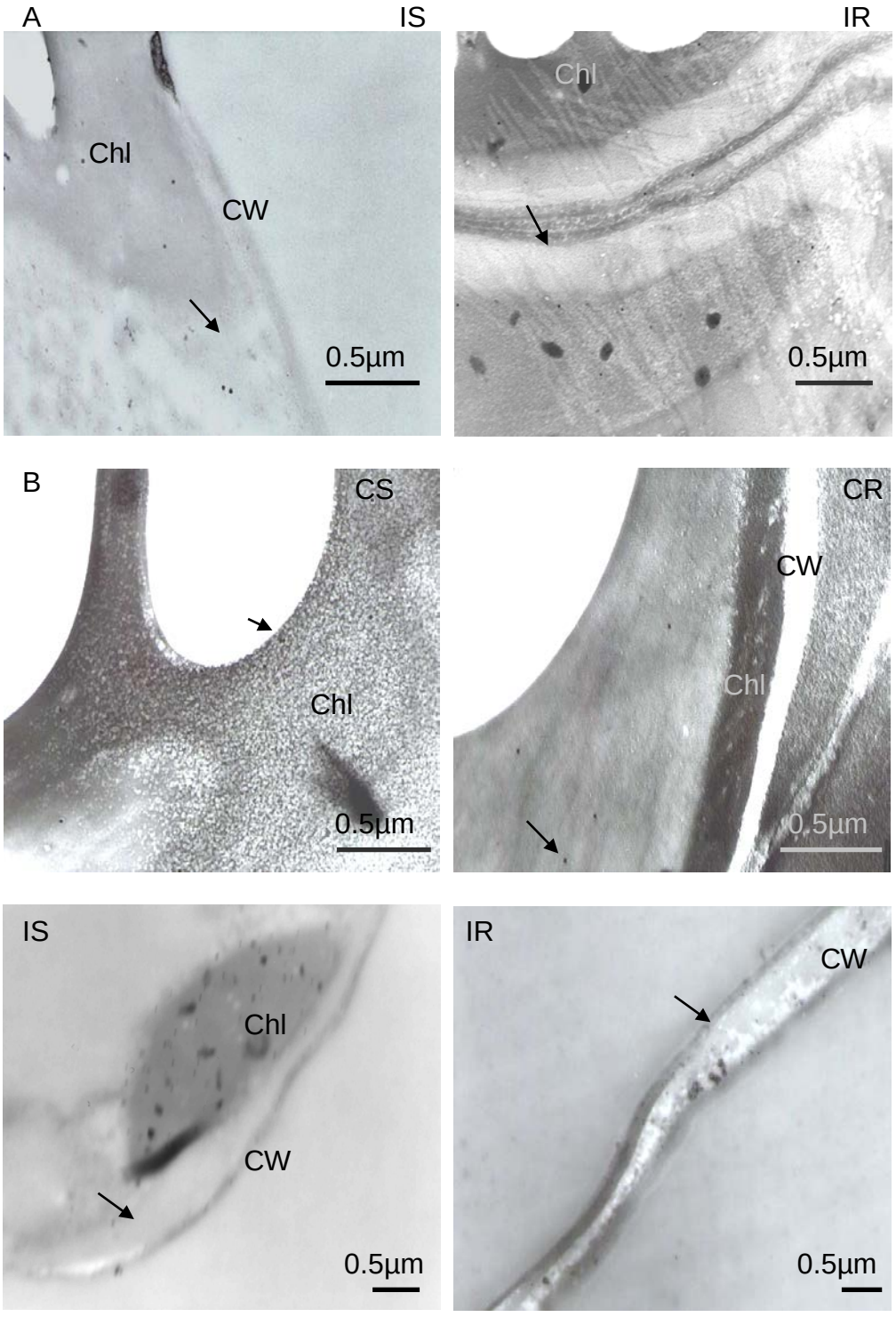

Fig. 4.4a: Immunocytochemical localisation of β-1,3-glucanase in leaf cells of susceptible (S37-388) and resistant (PhRR3) plants using anti-wheat β-1,3-glucanase antibodies. A; Leaf sections treated with pre-immune serum, B: Sections treated with anti-wheat β-1,3-glucanase antibodies. Leaf tissue was sampled 144 h after infection. CS, control susceptible; CR, control resistant; IS, infected susceptible; IR, infected resistant. Chl = chloroplast, CW = cell wall.

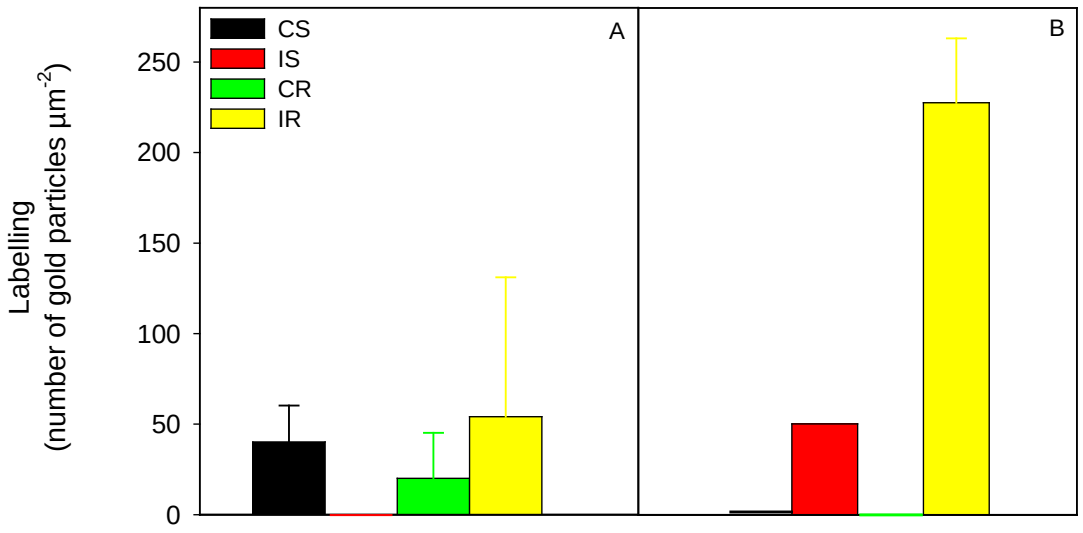

Fig. 4.4b: Immunolocalisation of β-1,3-glucanase in leaf cells of rust (*Puccinia helianthi* Schw., pathotype UVPhe 2)-infected (I) and uninfected (C) susceptible, S37-388 (S) and resistant, PhRR3 (R) sunflower plants. A: Pre-immune serum, B: Anti-wheat β-1,3-glucanase antibodies. Leaf tissue was sampled 144 h after infection. The values are means ± SD (n=4).

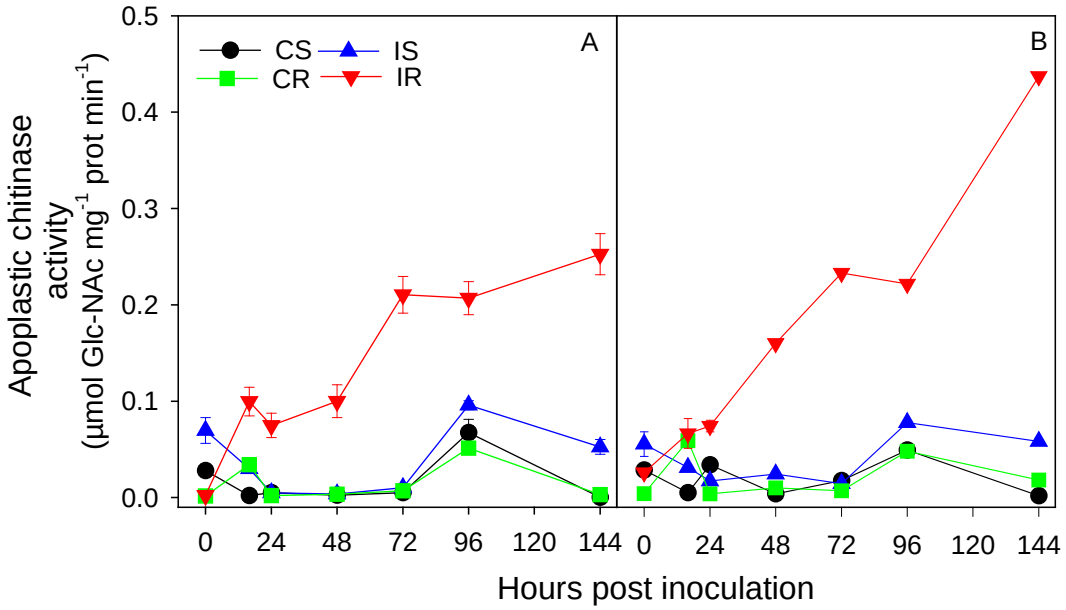

Fig. 4.5: Effect of rust (*Puccinia helianthi* Schw., pathotype UVPhe 2) infection on chitinase activity of susceptible (S37-388) and resistant (PhRR3) sunflower plants. CS, control susceptible; IS, inoculated susceptible; CR, control resistant; IR, inoculated resistant. A and B denote two independent experiments. The values are means ± SD (n=3).

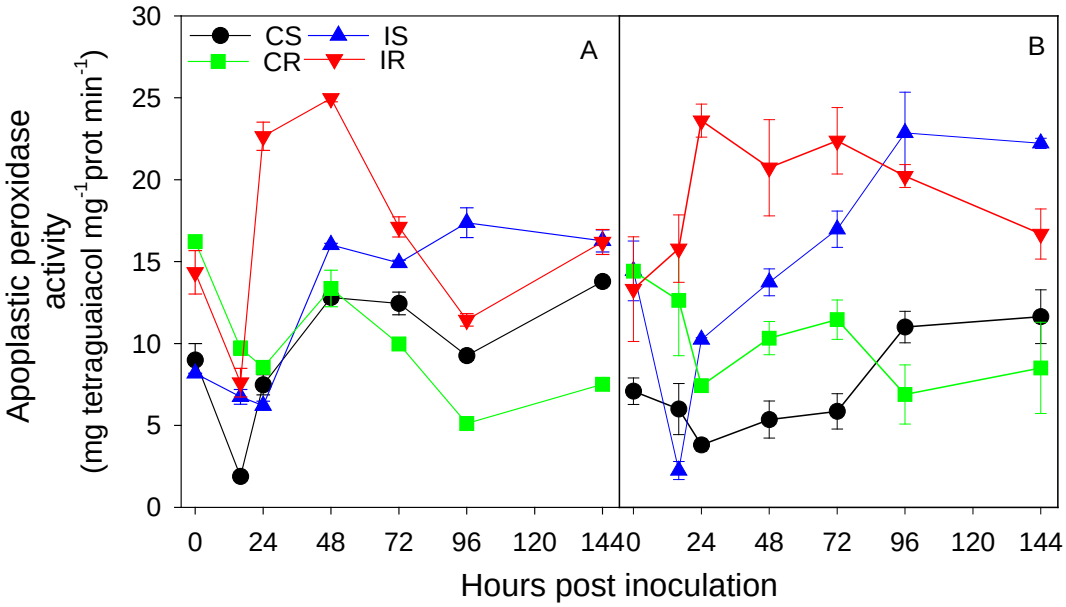

Fig. 4.6: Effect of rust (*Puccinia helianthi* Schw., pathotype UVPhe 2) infection on apoplastic peroxidase activity of susceptible (S37-388) and resistant (PhRR3) sunflower plants. CS, control susceptible; IS, inoculated susceptible; CR, control resistant; IR, inoculated resistant. A and B denote two independent experiments. The values are means ± SD (n=3).

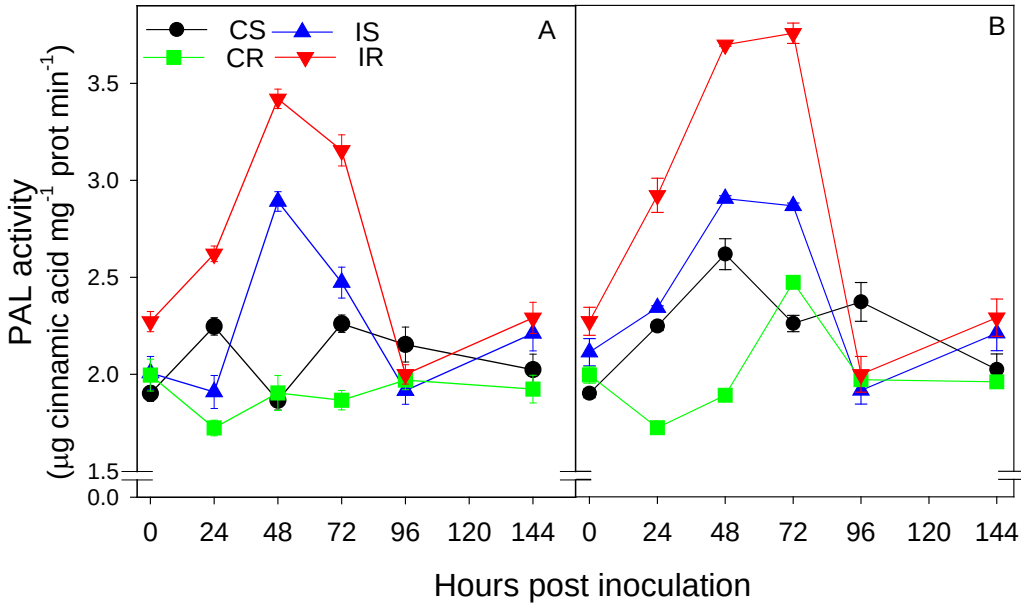

Fig 4.7: Effect of rust (*Puccinia helianthi* Schw., pathotype UVPhe 2) infection on PAL activity of susceptible (S37-388) and resistant (PhRR3) sunflower plants. CS, control susceptible; IS, inoculated susceptible; CR, control resistant; IR, inoculated resistant. A and B denote two independent experiments. The values are means ± SD (n=3).

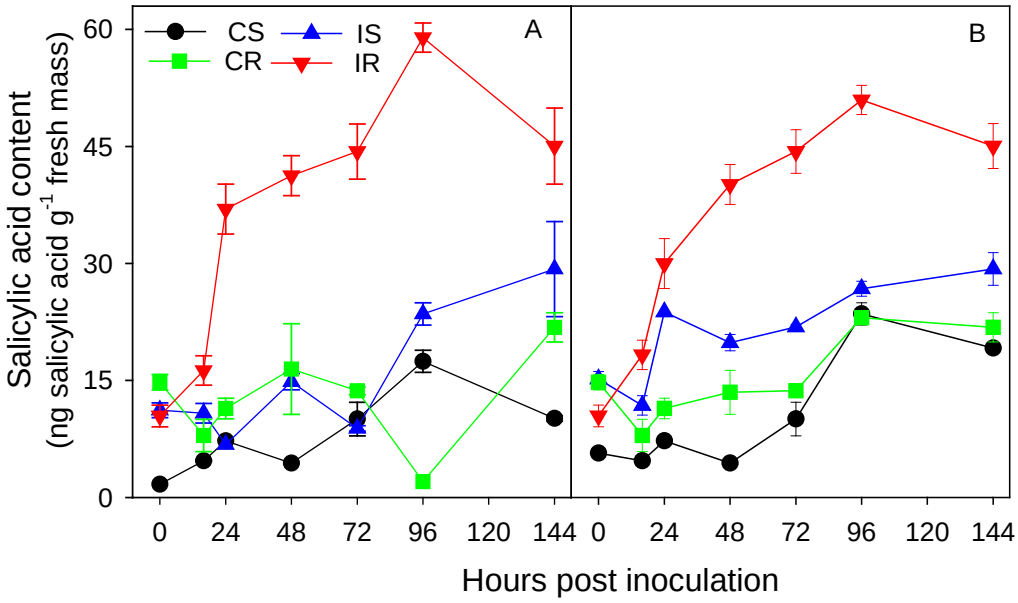

Fig 4.8: Effect of rust (*Puccinia helianthi* Schw., pathotype UVPhe 2) infection on the SA content in susceptible (S37-388) and resistant (PhRR3) sunflower plants. CS, control susceptible; IS, inoculated susceptible; CR, control resistant; IR, inoculated resistant. A and B denote two independent experiments. The values are means ± SD (n=3).

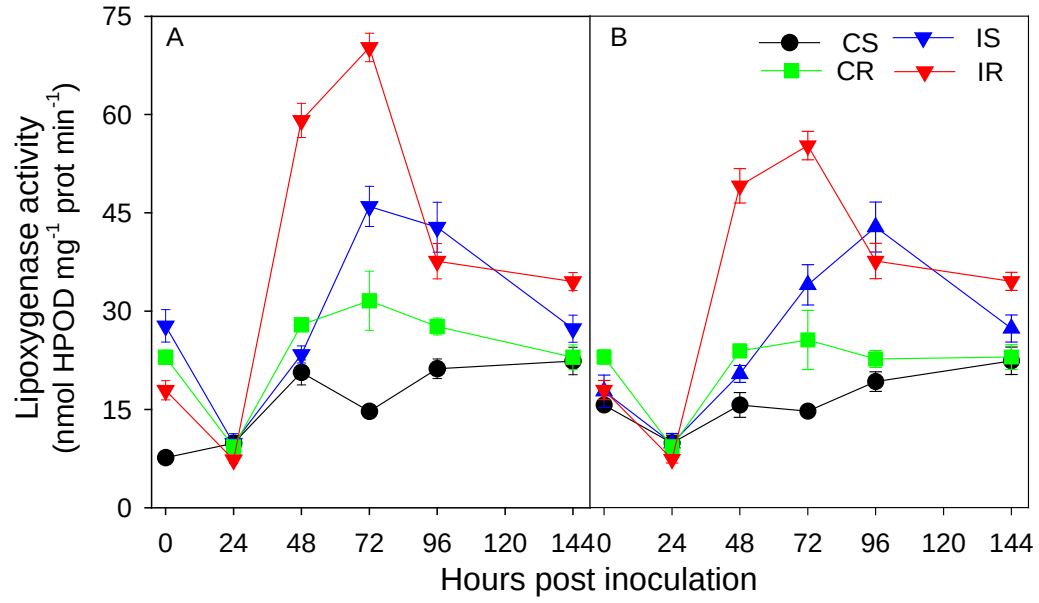

Fig 4.9: Effect of rust (*Puccinia helianthi* Schw., pathotype UVPhe 2) infection on LOX activity of susceptible (S37-388) and resistant (PhRR3) sunflower plants. CS, control susceptible; IS, inoculated susceptible; CR, control resistant; IR, inoculated resistant. A and B denote two independent experiments. The values are means ± SD (n=3).

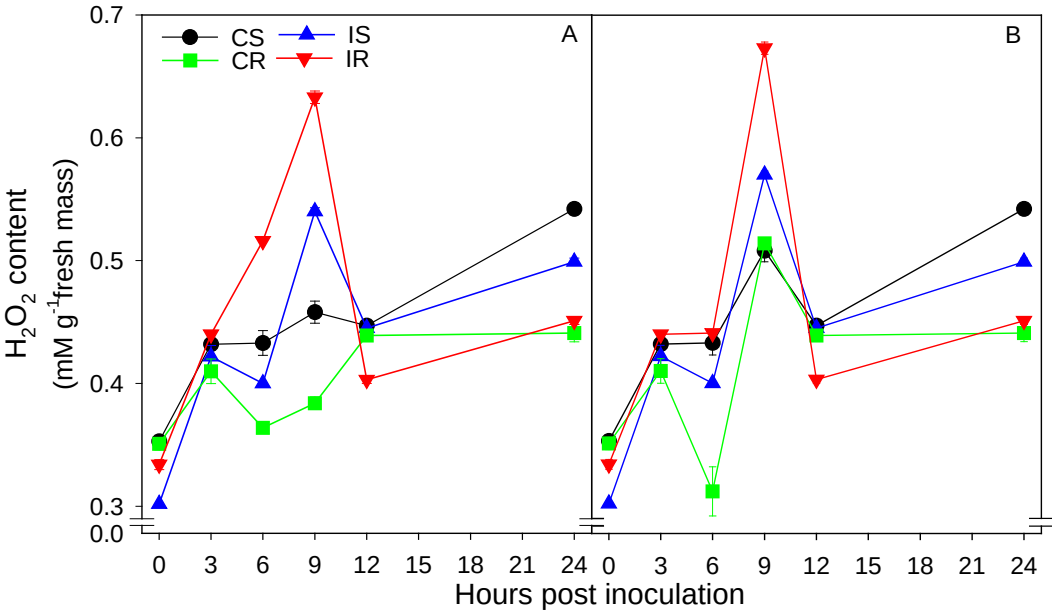

Fig. 4.10: Effect of rust (*Puccinia helianthi* Schw., pathotype UVPhe 2) infection on H_2O_2 content of susceptible (S37-388) and resistant (PhRR3) sunflower plants. CS, control susceptible; IS, inoculated susceptible; CR, control resistant; IR, inoculated resistant. A and B denote two independent experiments. The values are means ± SD (n=3).

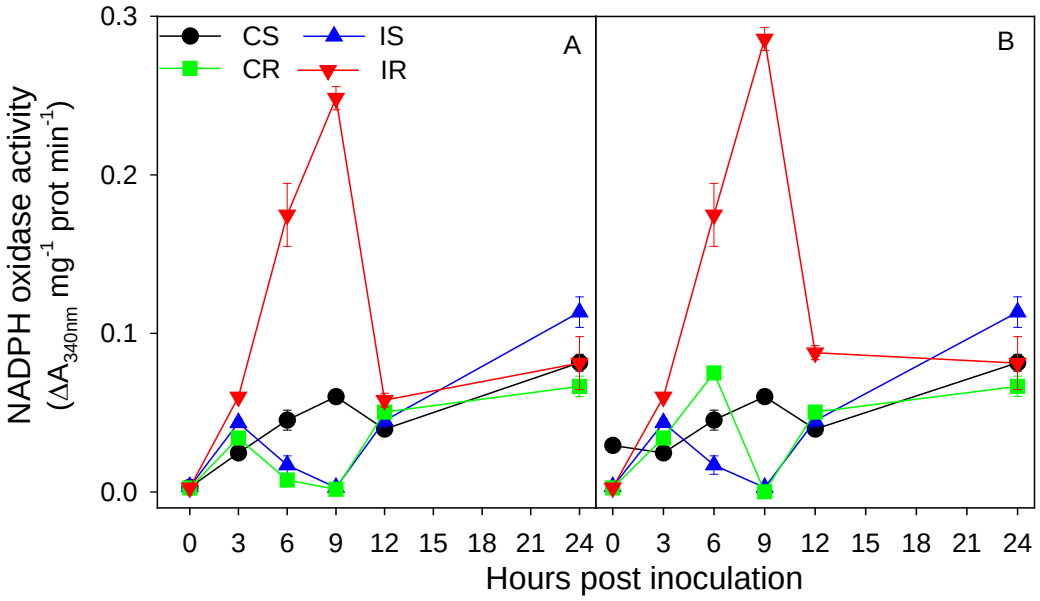

Fig. 4.11: Effect of rust (*Puccinia helianthi* Schw., pathotype UVPhe 2) infection on NADPH oxidase activity of susceptible (S37-388) and resistant (PhRR3) sunflower plants. CS, control susceptible; IS, inoculated susceptible; CR, control resistant; IR, inoculated resistant. A and B denote two independent experiments. The values are means ± SD (n=3).

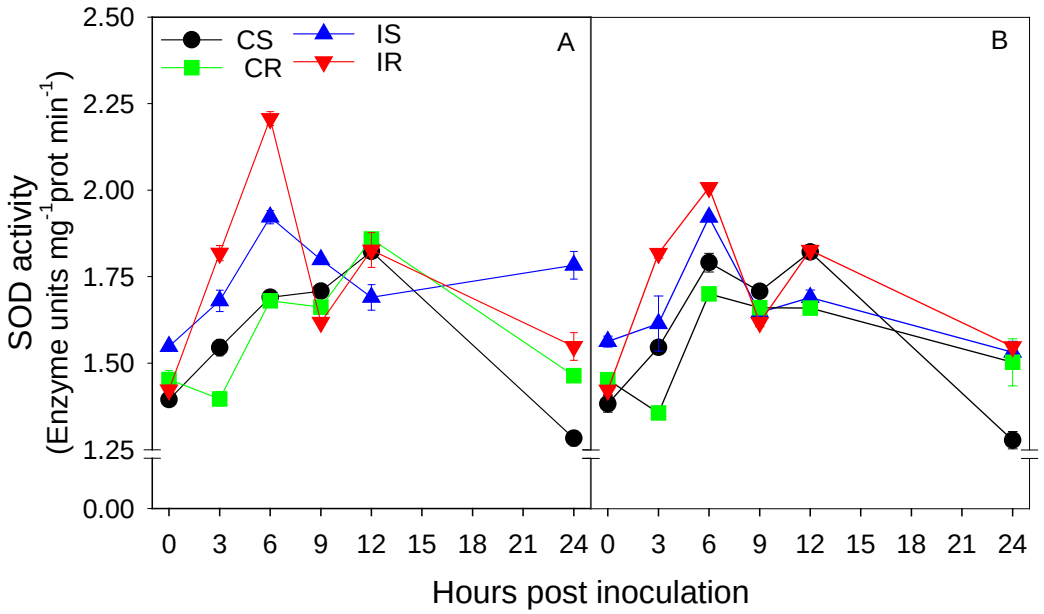

Fig. 4.12: Effect of rust (*Puccinia helianthi* Schw., pathotype UVPhe 2) infection on SOD activity of susceptible (S37-388) and resistant (PhRR3) sunflower plants. CS, control susceptible; IS, inoculated susceptible; CR, control resistant; IR, inoculated resistant. A and B denote two independent experiments. The values are means ± SD (n=3).

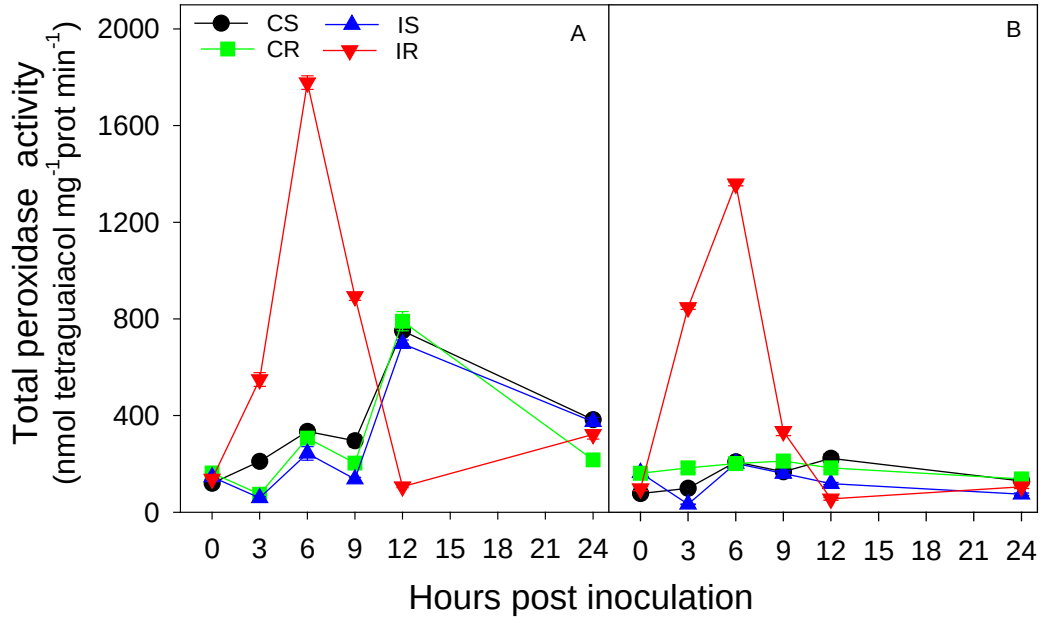

Fig. 4.13: Effect of rust (*Puccinia helianthi* Schw., pathotype UVPhe 2) infection on total peroxidase activity of susceptible (S37-388) and resistant (PhRR3) sunflower plants. CS, control susceptible; IS, inoculated susceptible; CR, control resistant; IR, inoculated resistant. A and B denote two independent experiments. The values are means ± SD (n=3).

4.4 Discussion

A proper understanding of the induced biochemical events during the resistance responses in plant-pathogen interactions is crucial in manipulating plants for improved resistance.

The polypeptide profiles are images of protein compositions and when related to the resistance response could reveal proteins involved in rust resistance. The accumulation of some polypeptides in the apoplast of infected resistant plants (Fig. 4.1) is a good indication that these proteins might be involved in sunflower rust resistance. Apoplastic proteins induced by pathogen infection belong to the group of pathogenesis related (PR) proteins (Stintzi *et al*., 1993). The molecular masses of these proteins can give a rough indication of the nature of the proteins, but if antibodies against all the PR proteins were available, we could have identified the rust-induced proteins.

The accumulation of distinct polypeptides in the apoplast during infection coincided with the increase in activity of some anti-microbial PR proteins. β-1,3-glucanase is one of the PR proteins with anti-microbial activity and its selective induction in the apoplast of resistant plants (Fig. 4.2) is an indication that it plays a role in rust resistance. This enzyme can function alone or more effectively in synergy with chitinases in the resistance response (Boller, 1993). The higher apoplastic chitinase activity found in the resistant opposed to susceptible plants (Fig. 4.5) also points to its association with the rust resistance response.

The high apoplastic activities of β-1,3-glucanases and chitinases in the resistant plants may be especially important in the resistance response against rust. During disease development, our results have indicated that vesicles, from which infection hyphae develop, as well as the haustorium mother cells (HMCs), develop in the apoplast. This should therefore be the prime line of defence. The apoplast includes the region along the cell walls where most defence related metabolites including PR proteins accumulate (Bowles, 1990). The induced apoplastic β-1,3-glucanase and chitinase activities may directly

hydrolyse the fungal cell walls of the fungal infection structures ramifying the intercellular spaces and halt any further disease progression. Sunflower rust is a basiodiomycete with cell walls composed of glucan and chitin units (Wessels and Sietsma, 1981), which are substrates for β-1,3-glucanases and chitinases, respectively. In addition, the action of these enzymes on the fungal cell walls may lead to the release oligosaccharide elicitors which further sustain or amplify defence responses as described by Kendra *et al.* (1989) and Hahn *et al.* (1993). As the apoplastic β-1,3-glucanase and chitinase activities increased there was a coinciding decline in the number of branching hyphae, HMCs and colony sizes formed in the resistant plants. An increase in activities of these enzymes is therefore an attribute of resistance.

The identity of apoplastic β-1,3-glucanases was confirmed by the Western blots (Fig. 4.3). This showed that increase in activity as infection proceeded (Fig. 4.2) is related to increased protein levels in the apoplast. β-1,3-glucanases were also localised along mesophyll cell walls in the resistant plants (Figs. 4.4a and b). This deployment of the enzyme in the cell walls during infection may be to empower the plant with defence arsenals to combat the invading pathogen. Unspecific β-1,3-glucanase labelling with anti-β-1,3-glucanase antibodies found in the chloroplasts of both susceptible and resistant plants (Fig. 4.4a) may not be an integral part of induced resistance responses. Nevertheless, this unspecific labelling requires further investigation since Hu and Rijkenberg (1998) used the same antibodies but did not find any labelling in the chloroplasts of *Puccinia recondita* f. sp *tritici*-infected wheat plants.

Our results are in agreement with reports from other studies. Increased β-1,3-glucanase and chitinase activities have been induced by a fungal elicitor from *Phytophthora megasperma* (Siefert and Grossmann, 1997), *Plasmopara halstedii* infection (Cachinero *et al.*, 1996), acetylsalicylic acid treatment (Jung *et al.*, 1993), BTH treatment (Sauerborn *et al.*, 2002) and other chemicals (Jung *et al.*, 1995).

The understanding of the role of PR proteins in antimicrobial activity is of crucial importance to manipulate plants for improved resistance against fungal pathogens. Currently, breeding of sunflower for disease resistance depends mainly on traditional breeding methods and subsequent selection for desired traits in the progeny. Although this approach is generally successful, the deployment of R genes may lead to a boom-and-bust scenario where the popularity of new resistant cultivars increases the selection pressure against the matching avirulence genes in the pathogen population (Pink, 2002). The importance of alternative ways to enhance disease resistance in plants is therefore obvious. One approach has been to enhance the expression of inherent antimicrobial metabolites in plants or to transform plants to constitutively express genes encoding PR proteins such as glucanases and chitinases.

Even though gene efficacy in these transgenic plants has often been acceptable, commercialisation has been impossible due to detrimental effects on plant growth, development and crop yield. Different strategies have come forward to control diseases in plants and one approach concentrates on the direct weakening of the pathogen. Through DNA shuffling techniques, various forms of chitinase with enhanced activity can be deployed with antimicrobial proteins. Chitinase activity slows hyphal tip growth and increases the efficacy of other antimicrobial proteins (Hammond-Kosack and Parker, 2003).

In addition to β-1,3-glucanases and chitinases, peroxidases (PR-9) have also been associated with rust resistance in sunflower. Induced increase in apoplastic peroxidase activity in the resistant plants (Fig. 4.6) may be involved in reactions that reinforce the cell walls to resist penetration by rust HMCs. Extracellular peroxidases are involved in the catalysis of reactions that fortify plant cell walls (Kolattukudy *et al*., 1992). These include incorporation of phenolics into cell walls, lignification and suberisation of cell walls. Induced apoplastic peroxidase activity in the resistant plants could also be involved in diverse reactions that collectively take part in rust resistance response. Peroxidases also take part in the production of active oxygen species (AOS)

during plant-pathogen interactions (Peng and Kuc, 1992; Bolwell and Wojtaszek, 1997), which are associated with signalling events in resistance responses.

The induction of PR proteins is one of the secondary events in resistance responses and possible events leading to their activation were investigated. The very early-induced peak level of H_2O_2 in the infected resistant plants (Fig. 4.10) proves the existence of AOS in sunflower-rust interaction. Hydrogen peroxide is a member of the AOS, which also includes superoxide anions and hydroxyl radicals. They are reactive intermediates in the reduction of O_2 (Bolwell and Wojtaszek, 1997).

The release of AOS known as the oxidative burst is a common phenomenon prior to the HR in most plant-pathogen interactions (Mehdy, 1994). Hydrogen peroxide has been shown to act as a signal in the induction of cell death and defence gene expression (Desikan *et al.*, 1998, 2000). In addition, H_2O_2 can directly kill pathogens (Peng and Kuc, 1992) and further mediate the cross-linking of specific structural proteins in plant cell walls (Bradley *et al.*, 1992). This reinforces the rigidity of the cell walls to deter pathogen penetration. The fact that the H_2O_2 outburst occurred very early (9 h) after infection strongly suggests a signalling role in rust resistance.

Peak activities of NADPH oxidase (Fig. 4.11) and SOD (Fig. 4.12) accompanied the induced H_2O_2 outbreak. Our results are in agreement with those of Doke (1985), who showed that NADPH oxidase activity increased in potato tubers infected with avirulent races of *Phytophthora infestans.* Keller *et al.* (1998) later proposed that plants have an NADPH oxidase complex similar to that in animal cells but with distinct regulatory mechanisms.

Superoxide dismutase (SOD), as NADPH oxidase, catalyses the generation of H_2O_2 during pathogen-induced oxidative burst (Desikan *et al.* 1996; Lamb and Dixon, 997). Multi enzymic forms of SOD with different locations exist in plants (Fridovich, 1986). Our results show an increase in total SOD activity of the resistant plants. In other studies, rust induced an increase in Cu/Zn SOD of

bean plants (Buonaurio *et al.*, 1987) while in tobacco-TMV interaction the activities of both Cu/Zn and Mn SODs were induced (Montalbini and Buonaurio, 1986).

Induced total peroxidase activity of the resistant plants (Fig. 4.13) peaked before the release of AOS. Peroxidases have been proposed as sources of AOS during plant-pathogen interactions (Peng and Kuc, 1992; Blee *et al.*, 2001; Bolwell *et al.*, 2002). Increased peroxidase activity in the resistant plants indicates that peroxidases may play a role as sources of AOS in sunflower-rust interaction.

During the early hours of infection, when the oxidative burst was released, there were no significant differences in the numbers of infection structures between the susceptible and resistant plants. At this stage, rust had developed only germ tubes and appressoria, which had formed on the leaf surface. The absence of differences in the development of infection structures in both susceptible and resistant cultivars may be an indication that the secondary defences mediated by the AOS are responsible for debilitating the fungus once it has entered the leaf tissue.

Although H_2O_2 is a diffusible molecule, its half-life basically excludes it from being a mobile signal for defence reactions in systemic tissues. Van Camp *et al.* (1998) proposed a model where H_2O_2 and SA, a signal molecule involved in SAR (Gaffney *et al.*, 1993), operate in harmony as a self-amplifying system. According to this model H_2O_2 induces SA accumulation (Chamnongpol *et al.*, 1998; Léon *et al.*, 1995) and SA also enhances H_2O_2 accumulation (Chen *et al.*, 1993b; Rao *et al.*, 1997). This self-amplification loop may generate H_2O_2 microbursts that intensify and spread the H_2O_2 signal required for oxidative cell death associated with HR and establishment of SAR (Alvarez *et al.*, 1998; Van Camp *et al.*, 1998).

The selective accumulation of SA in the resistant plants (Fig. 4.8) provides evidence that it plays a role in the resistance mechanism of sunflower against

rust. The fact that its levels increased later than the release of AOS proposes a late signalling and/or the sustaining and amplification of the defence response. Our results agreed with those of Malamy *et al.* (1990) who reported pathogen-induced accumulation of SA in tobacco. High levels of SA were also induced by pathogens in cucumber (Metraux *et al.*, 1990) and *Arabidopsis thaliana* (Uknes *et al.*, 1993). In addition, SA accumulation has been implicated in the induction of SAR marker proteins such as PR 1 and enhanced resistance to pathogens (Yalpani *et al.*, 1991).

Salicylic acid accumulation occurred concomitant to increases in PAL activity (Fig. 4.7). Phenylalanine ammonia-lyase is the key enzyme in the phenylpropanoid pathway (Hahlbrock and Scheel, 1989). This is the pathway from which various phenolic acids are synthesised. Different phenolics play diverse roles in defence responses. Some phenolic acids are classified as phytoalexins and play defence related roles in plant-pathogen interactions. In soybean plants infected with *Pseudomonas syringae* pv *glycinea*, for instance, Keen *et al.* (1983) gave evidence that resistance to infection was associated with glyceollin production. The increase in PAL activity in the resistant plants symbolises the possible association of phytoalexins with rust resistance. Some phenolics such as SA have been postulated as key signals in the induction of defence responses and systemic acquired resistance (Yalpani *et al.*, 1991).

Even though no reports exist on rust-induced PAL activity in sunflower, the increased activity in the resistant plants clearly showed its involvement in rust resistance. Tena and López-Valbuena (1983) have rather supplied evidence for PAL activity in sunflower induced by oomycetous *Plasmopara halstedii*. Pathogen-induced PAL activity in incompatible plant-pathogen interactions has also been found in wheat (Thorpe and Hall, 1984) and bean-*Sclerotinia sclerotiorum* interaction (Miklas *et al.*, 1993). Since PAL is a key enzyme in the synthesis of phenolic compounds including SA, phytoalexins and prerequisites for lignin biosynthesis (Henderson and Friend, 1979; Hahlbrock and Scheel, 1989), its induction in sunflower may be to contribute to the synthesis of various components of the resistance response.

Salicylic acid is not the only molecule involved in signalling defence reactions. Signal molecules also arise from fatty acid biosynthesis in the lipoxygenase pathway where lipoxygenase (LOX) is the key enzyme (Gardner, 1991). The induced increased LOX activity in the resistant plants (Fig. 4.9) was a proof of its association with rust resistance response. Our results are in agreement with those of Ocampo *et al.* (1986) who reported increased LOX activity in wheat induced by the rust fungus *Puccinia graminis tritici.* Increased LOX activity following pathogen infection has also been reported in coffee (Rojas *et al.*, 1993), bean (Croft *et al.*, 1990), pepper (Buonaurio and Servili, 1999), oats (Yamamoto and Tani, 1986) and rice (Ohta *et al.*, 1991).

Several roles of LOX in incompatible plant-pathogen interactions have been suggested. Hydroperoxides and free radicals formed through LOX activity are involved in localised cell death observed during the HR (Croft *et al.*, 1993). Signal molecules such as jasmonic acid and methyl jasmonate also formed via LOX activity can trigger defence gene expression and amplify the initial defence response (Epple *et al.*, 1995; Penninckx *et al.*, 1998). Derivatives of hydroperoxides from LOX activity such as antifungal 2-*trans*-hexenal (Schauenstein *et al*., 1977) constitute a direct defence against pathogen attack (Croft *et al.*, 1993). The products of LOX also interact with other metabolites during the production of AOS, signal molecules, and in host membrane damage during the HR (Feussner *et al.*, 1997). Synergistic effects of jasmonic acid and ethylene on SA-inducible responses have been reported (Xu *et al.*, 1994).

Salicylic acid can interfere with fatty acid metabolism by inducing LOX activity, thus enhancing synthesis of products such as jasmonic acid. Jasmonates are involved in the inhibition of pathogen growth, induction of phytoalexin and cell wall synthesis as well as signalling (Feussner *et al.*, 1997). In cucumber, treatment of leaves with SA or its functional analogue 2,6-dichloroisonicotinic acid induced increased LOX activity (Feussner *et al.*, 1997). In sunflower-rust resistance, induced SA accumulation (Fig. 4.8) and LOX activity (Fig. 4.9) could be an indication that SA and LOX products may interact in rust

resistance mechanism. It may also symbolise the fact that the induced resistance response is in fact a collective effort from diverse biochemical pathways.

As high levels of SA, PAL and LOX activities were realised, the development of infection structures on the resistant plants was considerably retarded. The number of ASSVs and HMCs were significantly lower than in the susceptible plants and the colonies formed were much reduced in size. This may suggest that defence responses, which hampered rust progression, were activated and we speculate that phytoalexins and the products of LOX activity also played a role.

Availability of information on the signalling events associated with pathogen resistance opens up possibilities for manipulating plants for improved resistance. One approach for instance, goes further downstream of R genes and introduces a hydrogen peroxide-generating system into transgenic plants. Expression of the glucose oxidase gene leads to hydrogen peroxide generation and significantly enhanced disease resistance (Wu *et al.*, 1995). The fact that SA can induce defence responses has also led to the development of plant activators, which are functional analogues of SA such as benzothiadiazole (BTH). Treatment of disease-susceptible plants with BTH induces resistance responses (Friedrich *et al.*, 1996).

The components of the resistance mechanism found in this study all form part of the HR and this indicates the form of rust resistance in sunflower. The induced biochemical responses such as induction of activities of PR proteins directly correlated with the reduction of fungal infection structures and development of host cell necrosis. The induced enzyme activities and accumulated metabolites might have a direct lysing effect on fungal structures and/or an indirect effect that enhanced the overall resistance response of sunflower. This response enabled sunflower to reduce the spread of sunflower rust. An understanding of the resistance mechanism of sunflower-rust interaction can provide a base from which one, using molecular and

biotechnology techniques, can manipulate the defence related events to achieve broad durable resistance.

Benzothiadiazole (BTH) and the induction of resistance responses in sunflower

5.1 Introduction

Systemic acquired resistance (SAR) is an active defence mechanism that plays a pivotal role in disease resistance (Delaney *et al.*, 1994). It is induced by pathogens in incompatible plant-pathogen interactions. It spreads throughout the entire plant and can persist for several weeks following initial infection. It protects the plant not only against the inducing organism, but a broad spectrum of pathogens (Ryals *et al.*, 1994). Biochemical studies have shown that SAR is associated with the accumulation of some pathogenesis-related (PR) proteins including β-1,3-glucanases and chitinases (Kessmann *et al.*, 1994).

Induction of the resistance response that mimics SAR can also be induced by chemicals referred to as plant activators, such as salicylic acid (SA), 2,6-dichloroisonicotinic acid (INA) and benzothiadiazole (BTH, Fig. 5.1) (Friedrich *et al.*, 1996; Ryals *et al.*, 1996; Sticher *et al.*, 1997). Salicylic acid and INA are potent inducers of SAR, but crop intolerance has prevented their development as plant protection compounds (Friedrich *et al*., 1996). Benzothiadiazole, a functional analogue of SA, is currently being investigated as a plant protection agent. It has no antifungal activity and has induced SAR in a wide variety of plants (Norvatis Crop Protection, 1997). Benzothiadiazole is supplied under the trade name Bion® (Syngenta, Basel, Switzerland) as water-dispersible granules containing 50% active ingredient. The wettable powder used in BTH formulation did not induce SAR gene expression or resistance in tobacco (Ryals *et al.*, 1996).

The main objective was therefore to investigate the effect of BTH on resistance-related biochemical responses of sunflower against rust and disease severity. Enzyme activities of the apoplastic PR-proteins, β-1,3-glucanase and peroxidase, were used as indicators of the resistance response. This would give an insight into the possibility of using BTH as a control agent for rust in sunflower.

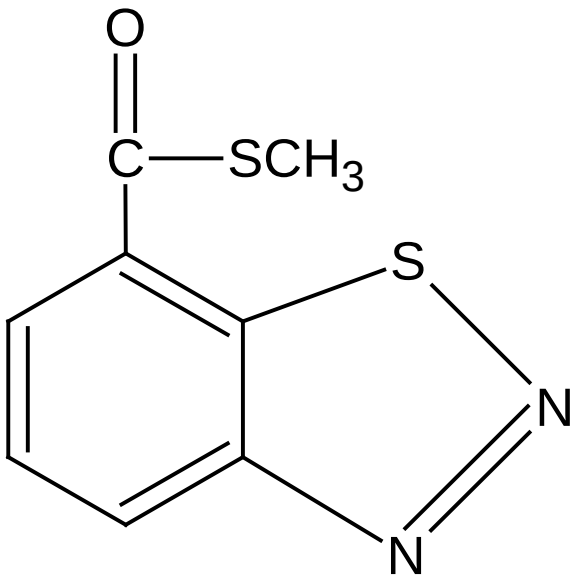

Fig. 5.1: Chemical structure of benzo (1,2,3) thiadiazole-7-carbothioic acid-S-methyl ester (BTH)

5.2 Materials and Methods

5.2.1 Biological material

Sunflower (*Helianthus annuus* L.) seeds that are susceptible (S37-388) to sunflower rust (*Puccinia helianthi* pathotype UVPhe 2) were germinated and transplanted into 15 x 30 cm pots (three seedlings/pot). The seedlings were cultivated in a sterilised soil/peat (3:1) mixture in a glasshouse at a temperature of 25 ± 3ºC. Daylight was supplemented with 120 µmol m^{-2} s^{-1} photosynthetic active radiation provided by cool white fluorescent tubes for 14 h each day. The plants were watered daily and fertilised (100 ml/pot) with a hydroponics nutrient solution (2 g L^{-1}) containing N, P and K (6.5: 2.7:13) plus microelements, three times per week.

5.2.2 Benzothiadiazole (BTH) treatment of plants

5.2.2.1 Dose response

Three different BTH concentrations, 0.015 g L^{-1}, 0.03 g L^{-1} and 0.045 g L^{-1} were applied on susceptible sunflower (S37-388) plants as foliar sprays until runoff. Sixteen pots, each containing three plants, were used. One set of four pots sprayed with distilled, sterile water served as control. The other ste of four pots each, were each sprayed with 0.015 g L^{-1}, 0.03 g L^{-1} and 0.045 g L^{-1} BTH respectively. Four leaves at the same position on each plant were harvested from three different pots

at each sampling time per treatment. The leaves were harvested 5, 6, 7, 8 and 9 days after BTH treatment and used for collection of apoplastic fluid. Seven days were taken as the optimum lag period for BTH activity in sunflower. An intermediate concentration of 0.03 g L^{-1} BTH was used in subsequent experiments.

5.2.2.2 Rust inoculation

A total of eight pots, each containing three plants were used. Four pots were treated treated with sterile distilled water as controls, and after 7 days two of these pots were inocultated with a rust (*Puccinia helianthi* Schw., pathotype UVPhe 2, Los *et al.*, 1995) suspension (9.3 X 10^5 spores ml^{-1}). The other set of four pots was treated with 0.03 g L^{-1} BTH and seven days later two of these pots were inoculated with a rust suspension (9.3 X 10^5 spores ml^{-1}). Four leaves (3^{rd} and 4^{th}) were harvested from three different pots at each sampling time. The leaves were harvested after 0, 24, 48, 72, 96, and 144 hpi.

5.2.3 Collection of intercellular wash fluid (IWF)

The IWF was collected as described in section 4.2.2 and IWF was used as an enzyme extract for β-1,3-glucanase and peroxidase activity determinations.

5.2.4 Enzyme activities

5.2.4.1 β-1,3-glucanase (EC 3.2.1.39) activity

β-1,3-glucanase activity was determined as described in section 4.2.3.1. For each of the two independent experiments β-1,3-glucanase activity of the representative IWF was determined in triplicate.

5.2.4.2 Peroxidase (EC 1.11.1.7) activity

Peroxidase activity was determined as described in section 4.2.3.3. For each of the two independent experiments peroxidase activity of the representative IWF sample was determined in triplicate.

5.2.5 Protein concentration

The protein concentration was determined in triplicate according to the method of Bradford (1976) using γ-globulin as standard.

5.2.6 Disease rating

Five leaves were randomly selected from five different plants that were either treated or not treated with BTH prior to infection. After 15 days of infection the area of the leaf covered by pustules and chlorosis was estimated using WinDIAS Colour image analysis software programme (Delta-T Devices, Cambridge, England).

5.3 Results

5.3.1 Benzothiadiazole (BTH) dose and lag time response

Benzothiadiazole induced defence related reactions in sunflower rust susceptible plants. In the case of β-1,3-glucanase a concentration of 0.045 g L^{-1} BTH induced the highest level of activity (Fig. 5.2). The degree of induction directly correlated with the concentration of BTH sprayed on the leaves. A significant ($P<0.05$) increase in induced activity began 6 days after BTH treatment and reached a maximum on the 8^{th} day (Fig. 5.2). Peroxidase activity also gradually increased with increasing BTH concentrations (Fig. 5.3). A significant ($P<0.05$) increase in induced peroxidase activity was observed 7 days after BTH treatment and it reached a maximum 8 to 9 days after the BTH treatment.

5.3.2 Benzothiadiazole (BTH) and rust induced β-1,3-glucanase activity

Benzothiadiazole treatment (0.03 g L^{-1}) alone induced an increase in β-1,3-glucanase activity of susceptible plants, 76% at 72 hpi (Fig. 5.4i). In addition, BTH treatment followed by rust infection induced an extra increase (38% higher at 72 hpi) in β-1,3-glucanase activity (Fig. 5.4ii). At 72 hpi the activity was 6.4-fold higher in infected BTH treated plants than in uninfected untreated plants (control) compared to the 4-fold increase in uninfected BTH treated plants (Fig. 5.4 insert, experiment 1 and 2). In untreated plants, rust induced activity occurred very late after infection (96 hpi). The overall effect of BTH and rust infection on β-1,3-glucanase activity followed a similar pattern in the two independent experiments.

5.3.3 Benzothiadiazole (BTH) and rust induced peroxidase activity

A concentration of 0.03 g L^{-1} BTH induced an increase in peroxidase activity of rust susceptible plants, which peaked at 96 hpi (Fig. 5.5). In infected pre-treated plants induced peroxidase activity was about the same as in plants treated with BTH only

(Fig. 5.5 insert). The effect of BTH and rust infection was not significantly different in the two independent experiments.

5.3.4 Benzothiadiazole (BTH) and disease severity

Table 5.1 shows that the disease-affected leaf area of BTH treated rust susceptible plants were approximately 30% less than in leaves of untreated infected susceptible plants.

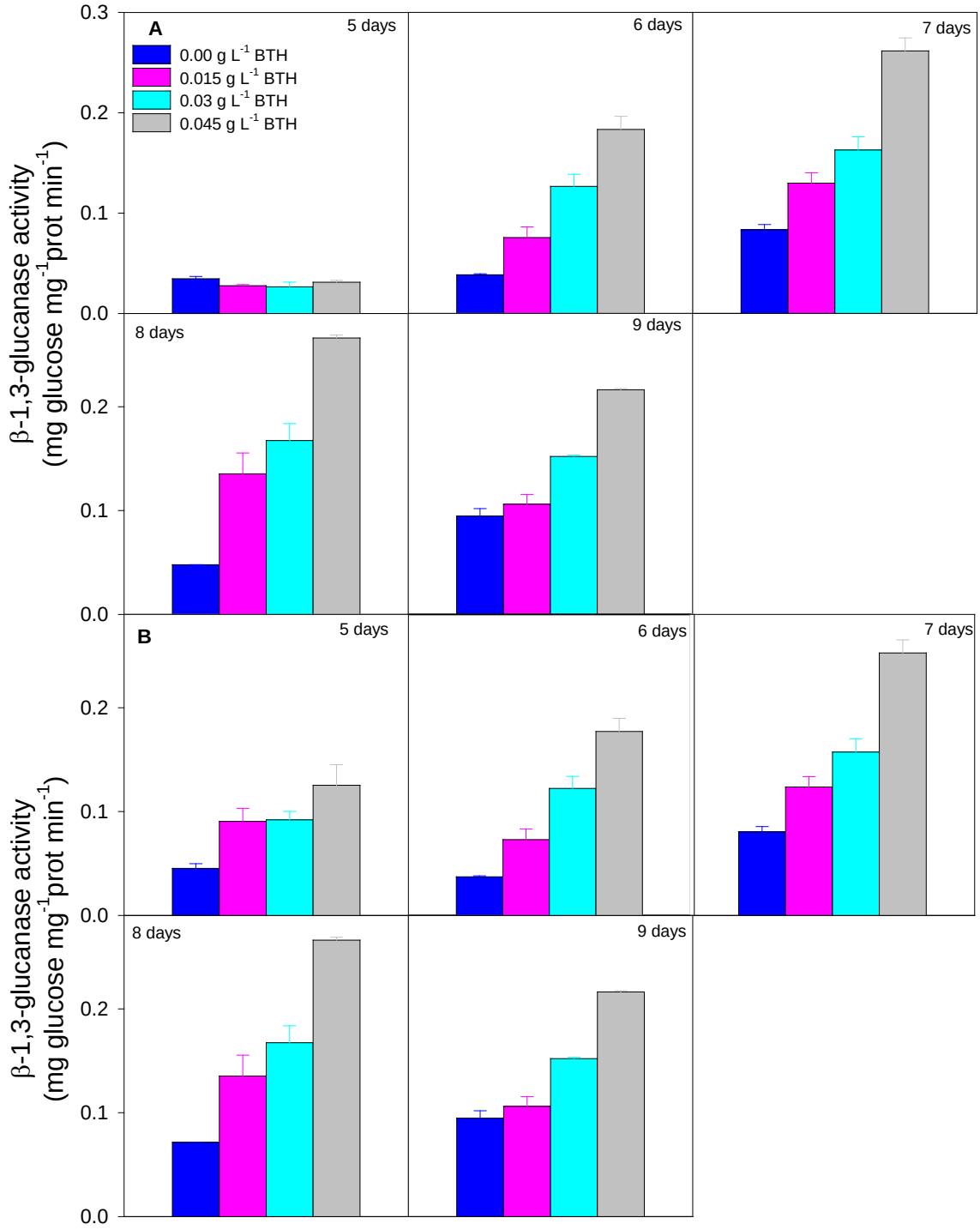

Fig. 5.2: Dose and lag time response of β-1,3-glucanase activity in susceptible (S37-388) sunflower plants during BTH treatment. Days after treatment are indicated in the figures. A and B denote two independent experiments. The values are means ± SD (n=3).

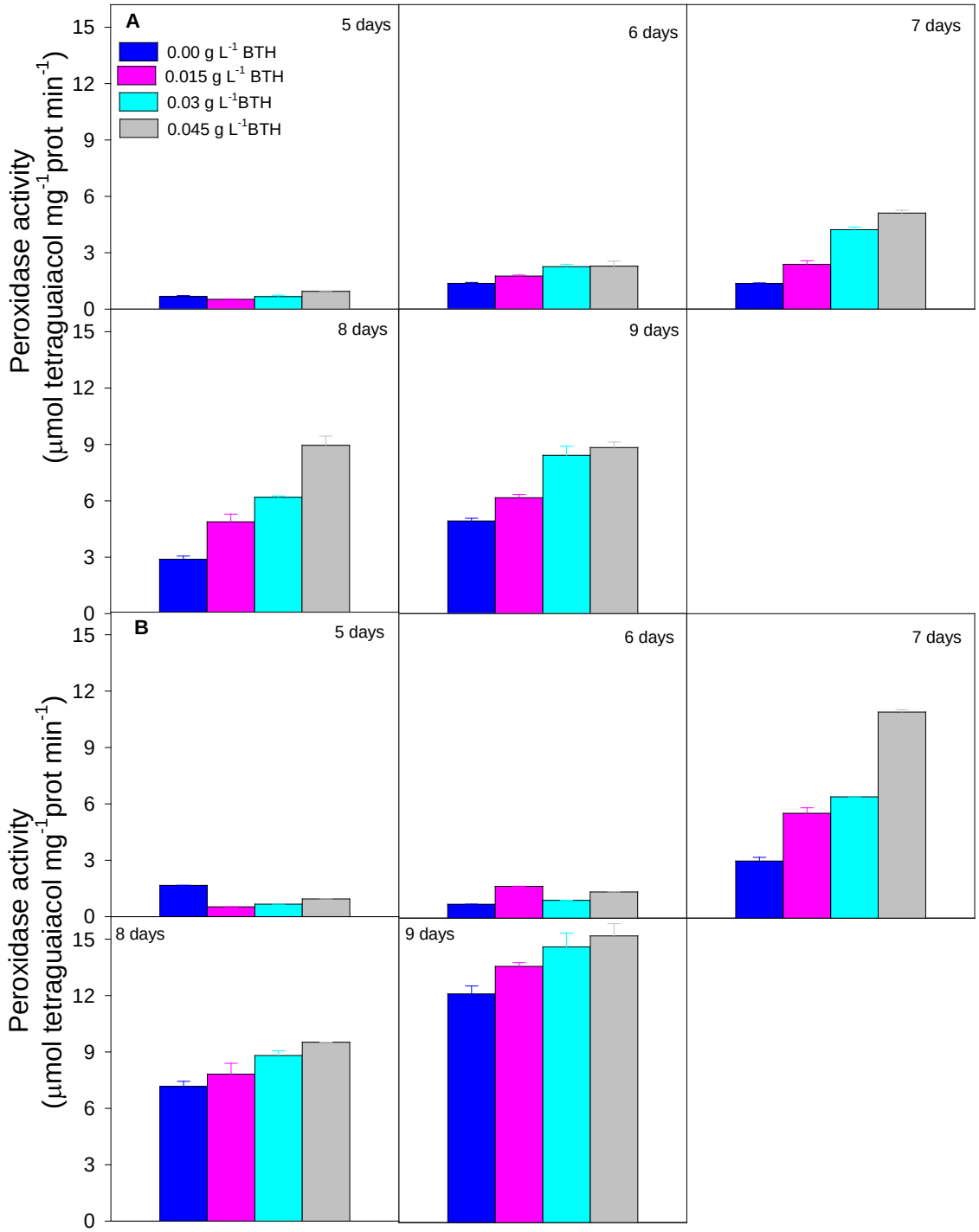

Fig. 5.3: Dose and lag time response of peroxidase activity in susceptible (S37-388) sunflower plants during BTH treatment. Days after treatment are indicated in the figures. A and B denote two independent experiments. The values are means ± SD (n=3).

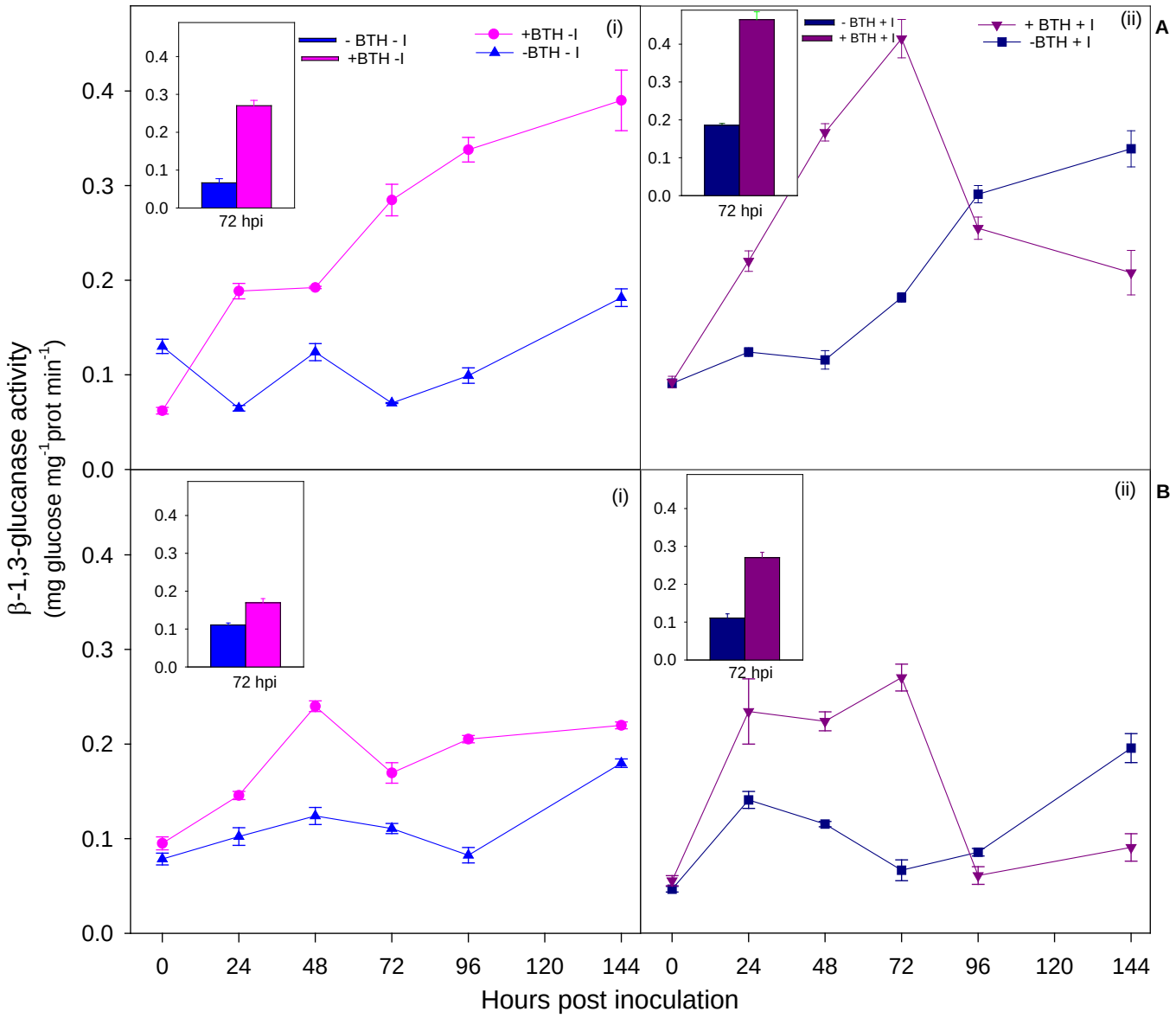

Fig. 5.4: Effect of rust (*Puccinia helianthi* Schw., pathotype UVPhe 2) infection (I) on the β-1,3-glucanase activity of susceptible (S37-388) sunflower plants pre-treated with 0.03 g L^{-1} BTH. The insert depicts the effect 72 hpi. (i): Uninfected plants; (ii): Infected plants. A and B denote two independent experiments. The values are means ± SD (n=3).

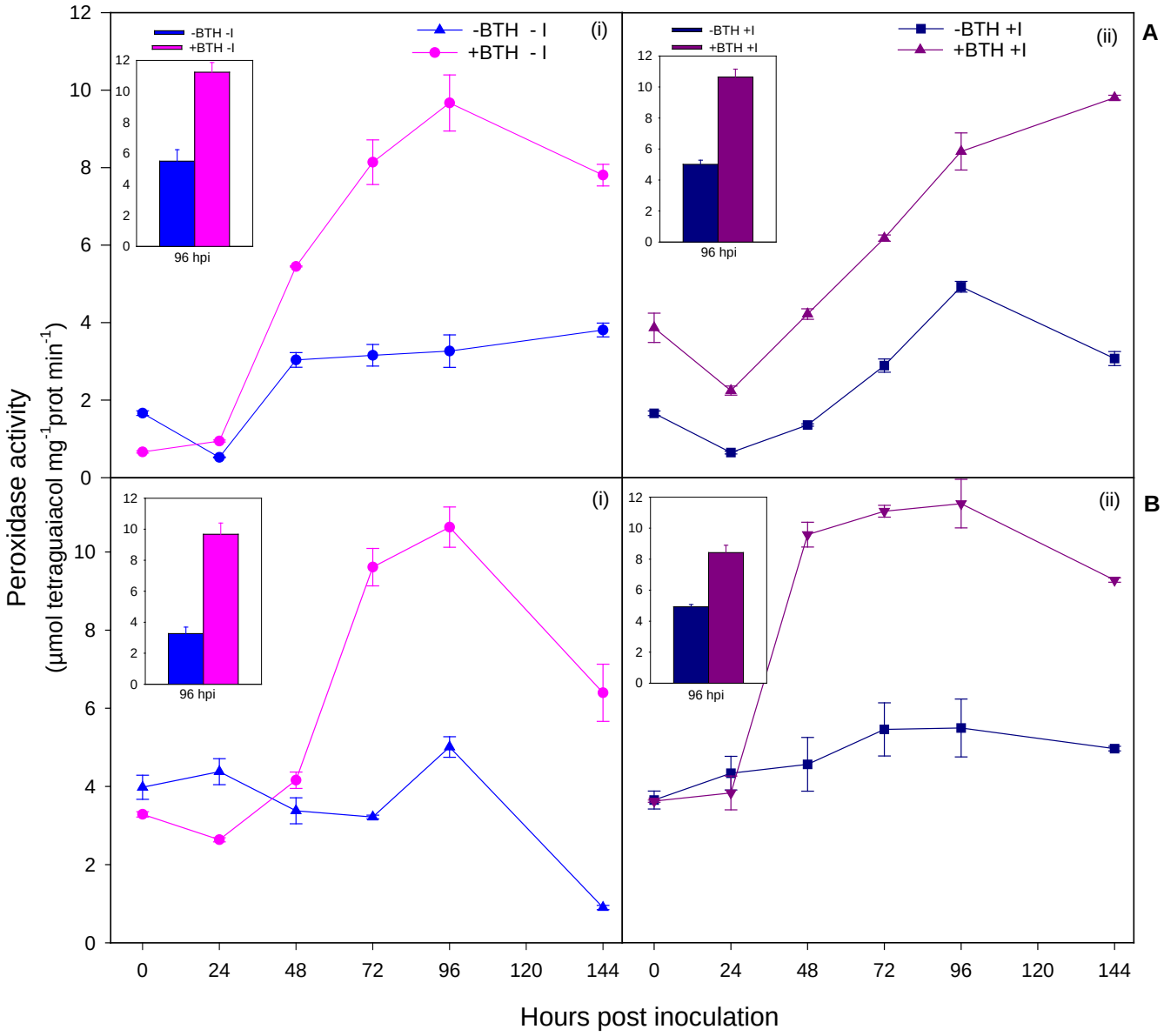

Fig. 5.5: Effect of rust (*Puccinia helianthi* Schw., pathotype UVPhe 2) infection (I) on the peroxidase activity of susceptible (S37-388) sunflower plants pre-treated with 0.03 g L^{-1} BTH. The insert depicts the effect 96 hpi. (i): Uninfected plants; (ii): Infected plants. A and B denote two independent experiments. The values are means ± SD (n=3).

Table 5.1: Effect of BTH (0.03 g L^{-1}) treatment on rust (*Puccinia helianthi* Schw., Pathotype UVPhe 2) development in susceptible sunflower plants. The values are means ± SD (n=5).

Treatment	Leaf area covered by pustules (%)	Total diseased leaf area (Pustules + Necrosis) (%)
-BTH + infection	61.76 ± 5.27	74.72 ± 5.58
+BTH + infection	35.95 ± 6.99	52.08 ± 2.39

5.4 Discussion

Systemic acquired resistance (SAR) is a resistance response induced by most pathogens in incompatible plant-pathogen interactions. It is associated with the expression of gene families, which comprise many of the genes encoding PR proteins (Ward *et al.*, 1991). Previously, (chapter 4), the PR-proteins, β-1,3-glucanases and peroxidases have been implicated in the resistance response of sunflower against rust (Figs. 4.2 and 4.6). In addition, results of the current study demonstrated the involvement of SA in the resistance response of sunflower (Fig. 4.8). Salicylic acid is regarded as a likely endogenous signal molecule in the establishment of SAR (Gaffney *et al.*, 1993). These findings prompted us to investigate the effect of BTH, a functional analogue of SA, on the resistance response of sunflower. β-1,3-glucanase and peroxidase activities were used as measures of the resistance response. According to Friedrich *et al.* (1996), BTH activates SAR at a step downstream of or at the same site as SA. Benzothiadiazole is a plant activator that was developed as an agrochemical for protection of rice plants against various diseases including the rice blast (Schweizer *et al.*, 1999). Since 1996, it has been used in Germany for the control of powdery mildew in wheat (Stadnik and Buchenauer, 2000).

Figures 5.2 and 5.3 illustrated that BTH induced the activities of the two PR proteins, β-1,3-glucanase and peroxidase. The increased activities of β-1,3-glucanase and peroxidase observed in plants from 7 days onwards after BTH treatment confirms the need for a lag period between application of the plant activator and the establishment

of protection, as emphasised by Friedrich *et al.* (1996) and Kessmann *et al.* (1996). This probably enables the plant to absorb and translocate BTH, which eventually induces synthesis of defence related proteins enabling the plant to acquire a certain level of resistance.

The degree of the induced resistance response in terms of β-1,3-glucanase and peroxidase activities in BTH treated plants (Figs. 5.2 and 5.3) was dose-dependent. The highest concentration (0.045 g L^{-1}) used induced the highest resistance response whereas the lowest concentration (0.015 g L^{-1}) induced the lowest resistance response. Our results were in agreement with those of Tosi *et al.* (1999) and Sauerborn *et al.* (2002) who reported a BTH-induced dose-dependent resistance response in sunflower against downy mildew and *Orobanche cumana,* respectively. In our results the high β-1,3-glucanase and peroxidase activities induced by the highest BTH concentration used may be an indication that BTH primed the plants for resistance against disease.

Benzothiadiazole treatment followed by infection induced an intensified increase in the enzyme activity of apoplastic of β-1,3-glucanase (Fig. 5.4). Our results were corroborated by the findings of Suo and Leung (2001). They found that BTH treatment resulted in increased activities of apoplastic β-1,3-glucanase and chitinase in rose plants. They reported that infection of BTH-treated rose plants by *Diplocarpon rosae* induced a much stronger and rapid increase in enzyme activities than BTH treatment alone. In cauliflower BTH induced a decrease in sporulation of *Peronospora parasitica*. This was associated with enhanced β-1,3-glucanase and chitinase activities, which increased further upon infection (Godard *et al.*, 1999; Ziadi *et al.*, 2001). In studies on green beans (Siegrist *et al.*, 1997) and tomatoes (Inbar *et al.*, 1998) it was also found that BTH induced increased β-1,3-glucanase and chitinase activities. Furthermore, Burketová *et al.* (1999) reported induced synthesis of apoplastic β-1,3-glucanases and chitinases in BTH treated sugar beet. All these results indicate that BTH has the potential to induce some components of the resistance response as also shown by our results.

Benzothiadiazole treatment of sunflower plants prior to infection reduced disease severity in rust susceptible plants. This was indicated by the reduction in the number of pustules on infected leaves as well as the total diseased leaf area on plants pre-treated with BTH (Table 5.1). This reduction in disease severity was associated with increased defence related reactions such as β-1,3-glucanase and peroxidase activities in BTH-treated plants (Figs. 5.4 and 5.5). Our results are in agreement with those of Prats *et al.* (2002) who reported that BTH treatment induced rust resistance in sunflower. This was characterized by reduced infection frequency due to a decline in germination and appressorium formation that was associated with an increase in the amount of accumulated coumarins and other phenolic compounds. Benzothiadiazole also provided protection against *Plasmopara helianthi* in sunflower (Tosi *et al.*, 1999). In tomato, BTH treatment reduced the rate and extent of host colonization by *Fusarium oxysporum* f. sp. *radicis-lycopersici* (Benhamou and Bélanger, 1998).

The results indicate that BTH treatment can induce the resistance mechanism in rust-susceptible sunflower plants as shown by activation of resistance response related β-1,3-glucanase and peroxidase activities. The marked reduction in disease symptoms further proved the ability of BTH to induce resistance in sunflower. Such information should be valuable in the search for a holistic approach to control disease incorporating both the inherent plant responses as well as those induced by chemicals. In *Arabidopsis* transgenic plants overexpressing the NPR1 signalling protein for instance, there is an enhanced response to BTH treatment and a higher efficacy of some three fungicide treatments (Hammond-Kosack and Parker, 2003).

Concluding remarks:

The effective control of sunflower disease is essential to minimise yield losses. There is, therefore, an urgent need for more environmentally friendly and cost-effective control measures than the commonly used chemical fungicides. Alternatively, the plant's own defence mechanisms can be harnessed to control diseases. In this regard, genetic resistance offers a harmless and relatively inexpensive alternative. Recently, new avenues for devising and exploiting resistance have been propagated. A better understanding of the sunflower rust resistance mechanism will undoubtedly contribute to improved resistance and the development of new and effective disease control strategies.

This study therefore focused on the induced biochemical reactions associated with resistance in sunflower against rust. An understanding of the resistance response requires some knowledge of the biochemical events that are induced during plant-pathogen interactions. In this study evidence was provided of the involvement of the PR proteins β-1,3-glucanase, chitinase and peroxidase in the resistance response. It is suggested that their elevated activities probably played a role in the retardation of the development of rust infection structures in the resistant plants. Induction of PR proteins was preceded by the oxidative burst, and accompanied by a high activity of PAL, a key enzyme in phenolic acid synthesis. The phenolics are involved in diverse defence reactions including signalling, where SA, which increased concomitant to PAL activity, plays a primary role. Benzothiadiazole, a functional analogue of SA, acted as an effective inducer of the resistance response and consequent resistance to rust in otherwise susceptible plants. The information gathered would add to the understanding of rust resistance, which has not been well studied in this pathosystem. This knowledge base paves the way to capitalise on previous applications (sometimes putative) involving similar defence components.

Many such applications including putative ones are described. Currently, breeding of sunflower for disease resistance depends mainly on traditional methods and

subsequent selection for desired traits in the progeny. Although this approach is generally successful, various other ways have been devised or envisaged using information emanating from our existing knowledge base, to enhance disease resistance in plants. One approach has been to enhance the expression of inherent antimicrobial metabolites in plants or to transform plants to constitutively express genes encoding PR proteins such as glucanases and chitinases. Through recombinant DNA techniques, various forms of chitinase with enhanced activity can be deployed with other proteins conferring antimicrobial activity. Chitinase activity slows hyphal tip growth and increases the efficacy of other antimicrobial proteins (Hammond-Kosack and Parker, 2003).

Signal molecules in plant-pathogen interactions represent potential switches for activating the resistance response. Introduction of glucose oxidase gene, which generates hydrogen peroxide in transgenic plants leads to elevated disease resistance (Wu *et al.*, 1995). This approach uses the fact that the oxidative burst plays a role in disease resistance responses. Information that endogenous SA acts as a signal in resistance responses has led to the development of plant activators, which can induce resistance responses in plants. Benzothiadiazole is one plant activator currently used as a plant protector (Schweizer *et al.*, 1999; Stadnik and Buchenauer, 2000).

Biotechnology approaches have now shifted emphasis towards marker-assisted breeding and the construction of vectors containing highly regulated transgenes that confer resistance in various ways (Hammond-Kosack and Parker, 2003). Resistance markers are needed in this endeavour, and information from our knowledge base could aid in the identification of such markers.

Integrated disease management is now considered the best control strategy for most plants. *Arabidopsis* plants oveexpressing the signalling protein NPR 1 show an enhanced response to the plant activator BTH, which also increased the efficacy of three fungicide treatments (Friedrich *et al.*, 2001). Low doses of chemical plant activators and/or fungicides applied to plants expressing the ‘master’ protein regulators may enhance defences to commercially acceptable levels (Hammond-

Kosack and Parker, 2003). Such information is especially important since producers are seeking ways of reducing input costs in farming. On the other hand consumers are also concerned about the environmental and health impact of pesticide use and demand minimal application of these chemicals (Pink, 2002).

The results of this study have elucidated some biochemical events related to the resistance response in sunflower, and this has satisfied our main objective in this study, to gain a better understanding of the resistance mechanism in the sunflower-rust interaction. Information emanating from this study, coupled to molecular and biotechnology-based techniques, should contribute in the challenge to incorporate broad-spectrum durable resistance into a seed.

Summary

The biochemical resistance response of sunflower (*Helianthus annuus* L.) to rust (*Puccinia helianthi* Schw., pathotype UVPhe 2) was investigated in a comparative study using susceptible (S37-388) and resistant (PhRR3) sunflower cultivars and related to rust development. In addition, the potential of a plant activator, benzothiadiazole (BTH), to induce rust resistance in susceptible sunflower was investigated.

Rust infection induced higher enzyme activities of the apoplastic pathogenesis-related (PR) proteins, β-1,3-glucanase, chitinase and peroxidase in the resistant than susceptible plants. The accumulation of β-1,3-glucanase isoenzymes was confirmed by Western blots. According to the labelling data of the immunogold localisation studies, β-1,3-glucanases were concentrated in the mesophyll cell walls during the rust resistance response. As the enzyme activities of the PR proteins were induced, the development of infection structures in the resistant plants was retarded, i.e. the number of vesicles, infection hyphae and haustorium mother cells.

An early transient accumulation of H_2O_2 in the infected resistant plants is proof of the involvement of active oxygen species (AOS) in the resistance response. Active oxygen species' burst correlated with an early induction (after 3 h of infection) of the activities of AOS-generating enzymes, NADPH oxidase, superoxide dismutase (SOD) and the NADH-dependent peroxidase.

The phenolic, salicylic acid (SA), has been implicated in the resistance response as a potential signal molecule. The increase in SA content occurred with a concomitant increase in the activity of phenylalanine ammonia-lyase (PAL), a key enzyme in the synthesis of phenolic compounds. Induction of the activity of lipoxygenase, a key enzyme in lipid biosynthesis, in infected resistant plants, suggests that lipid-derived signals may play a role in the rust resistance response.

As rust infection progressed, the number of aborted substomatal vesicles increased in resistant than susceptible plants. The number of infection hyphae, haustorium mother cells and rust colonies, which all favoured disease expression, were lower in the resistant than susceptible plants. Furthermore, in the resistant cultivar, haustorium mother cells were more often associated with host cell necrosis. The hypersensitivity index exceeded one, 96 hours after infection, indicating that the resistant plants were exhibiting the hypersensitive reaction (HR).

Since it was found that SA is a possible signal for rust resistance reactions, the effectiveness of BTH, a functional analogue of SA, to induce the resistance response was investigated. This was measured in terms of activities of defence-related enzymes, β-1,3-glucanase and peroxidase. Benzothiadiazole induced an increase in the activities of these enzymes and further reduced disease symptoms by about 30%.

In this study some of the biochemical components of the rust resistance response in sunflower have been identified and related to the development of rust infection structures. Furthermore, the potential of BTH as a plant activator in the control of rust diseases in sunflower has been demonstrated. The information gathered in this study contributes to a better understanding of the mechanisms in sunflower involved in rust resistance, which eventually could help to manipulate sunflower for improved resistance.

Key words: Active oxygen species, chitinase, defence reactions/mechanisms, β-1,3-glucanase, hydrogen peroxide, lipoxygenase, NADPH oxidase, pathogenesis related proteins, peroxidase, phenylalanine ammonia-lyase, salicylic acid, signalling, sunflower, sunflower rust, superoxide dismutase.

Die biochemiese weerstandsrespons van sonneblom (*Helianthus annuus* L.) teen roes (*Puccinia helianthi* Schw.) is in 'n vergelykende studie in vatbare (S37-388) en weestandbiedende (PhRR3) kultivars ondersoek en met roesontwikkeling gekorrelleer. Hierbenewens is die potensiaal van 'n plantaktiveerder bensotiadiasool (BTH), om roesweerstand in vatbare sonneblom te induseer, ondersoek.

Roesinfeksie het hoër ensiemaktiwiteite van die apoplatiese patogeneseverwante (PR) proteïene, β-1,3-glukanase, chitinase en peroksidase in weerstandbiedende as vatbare plante geïnduseer. Die akkumulering van β-1,3-glukanase-isoensieme is met Westernkladanalise bevestig. Volgens die etiketteringsdata van die immunogoudlokaliseringstudies was β-1,3-glukanases in die mesofilselwande tydens die roesweerstandsrespons gekonsentreer. Die geïnduseerde PR-proteïenensiemaktiwiteite het met vertraging in die ontwikkeling van infeksiestrukture in weerstandbiedende plante gepaard gegaan. Dit het die aantal visikels, infeksiehifes en houstoriummoederselle behels.

'n Vroeë kortstondige H_2O_2-akkumulering in geïnfekteerde weerstandbiedende plante is bewys van die betrokkenheid van aktiewe suurstofspesies (AOS) by die weerstandsrespons. Die AOS-uitbarsting het met die vroeë induksie (na 3 ure infeksie) van die aktiwiteit van AOS-opwekkingsensieme, NADPH-oksidase, superoksieddismutase (SOD) en die NADH-afhanklike peroksidase gekorreleer.

Die fenol, salisielsuur (SA), is as 'n moontlike seinmolekuul by die weerstandsrespons betrek. Die toename in SA-inhoud het met in toename in die aktiwiteit van fenielalanienammoniakliase (PAL), 'n sleutelensiem in die sintese van fenoliese verbindings, gepaard gegaan. Induksie van die aktiwiteit van lipoksigenase, 'n sleutelensiem in lipiedbiosintese, dui daarop dat lipiedverwante seine moontlik 'n rol by die roesweerstandsrespons speel.

Soos roesinfeksie vorder, het die aantal geaborteerde substomatale vesikels meer in weerstandbiedende as in vatbare plante geword. Die aantal infeksiehifes, houstoriummoederselle en roeskolonies, wat almal siekte-ontwikkeling bevorder, was minder in weerstandbiedende as vatbare plante. Boonop was die houstoriummoederselle meer dikwels met gasheerselnekrose in die weerstandbiedende kultivar geassosieer. Die hipersensitiwiteitsindeks was 96 uur na infeksie hoër as een in weerstandbiedende plante wat ’n aanduiding van die hipersensitiewe reaksie (HR) in aksie is.

Omdat bevind is dat SA ’n moontlike sein vir roesweerstandsreaksie is, is die doeltreffendheid van BTH, ’n funksionele analoog van SA, om die weerstandsrespons te induseer, ondersoek. Dit is ’n terme van die aktiwiteite van die verdedigingsverwante ensieme, β-1,3-glukanase en peroksidase gemeet. Bensotiadiasool het ’n toename in die aktiwiteite van hierdie ensieme geïnduseer en het boonop die siektesimptome met 30% verminder.

In hierdie studie is sommige van die biochemiese komponente van die roesweerstandsrespons in sonneblom geïdentifiseer en in verband gebring met die ontwikkeling van roesinfeksiestrukture. Verder is die potensiaal van BTH as plantaktiveerder vir die beheer van sonneblomroessiektes aangetoon. Die inligting ingewin in hierdie studie dra by tot ’n beter begrip van die meganismes in sonneblom betrokke by roesweerstand wat uiteindelik sal help om sonneblom vir beter weerstand te manipuleer.

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