INVOLVEMENT OF LIPOXYGENASE IN RESISTANCE OF WHEAT AGAINST THE RUSSIAN WHEAT APHID.

E Swart

INVOLVEMENT OF LIPOXYGENASE IN RESISTANCE OF WHEAT AGAINST THE RUSSIAN WHEAT APHID.

By

Elsabè Swart

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Supervisor: Prof AJ van der Westhuizen

To my sister Izèlle Swart,
who was the sunshine in
many people's lives.

Preface

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The results presented here are original and have not been submitted to any other University.

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Abbreviations

µ	Micro
^{0}C	Degree celsius
ABA	Abscisic acid
cDNA	Complementary Deoxyribonucleic acid
cv	Cultivar
d.p.i.	Days post infestation
Dowex	Dowex 1x2 (200-400)
e.g.	*exempli gratia* (for example)
EDTA	Ethylenediaminetetraacetic acid
et al.	*et alia* (and others)
Fe	Iron
Fig.	Figure
g	Gram(s)
Glu	β-1,3-glucanases
h.p.i.	Hours post infestation
HPOD(s)	Fatty acid hydroperoxy derivative(s)
HR	Hypersensitive reaction
i.e.	*id est* (that is)
JA	Jasmonic acid
kDa	Kilodalton
LAR	Local acquired resistance
LOX(s)	Lipoxygenase(s)
m/v	Mass per volume
mg	Milligram(s)

M-JA	Methyl-jasmonate
ml	Millimetre(s)
mol	Mole
Mr	Molecular mass
mRNA	Messenger Ribonucleic acid
nm	Nanometre(s)
nPG	n-Propyl-gallate
PAL	Phenylalanine ammonia lyase
PC	Piroxicam
PI(s)	Protease inhibitor(s)
POD(s)	Peroxidase(s)
PR-proteins	Pathogenesis-related proteins
PVP	polyvinylpyrrolidone
RSA	Republic of South Africa
RWA(s)	Russian wheat aphid(s)
SA	Salicylic acid
SAR	Systemic acquired resistance
SHA	Salicylhydroxamic acid
T (Dn2)	Tugela with resistant gene (Dn2)
T (Dn5)	Tugela with resistant gene (Dn5)
T-DN	Tugela with resistant gene (Dn1)
Tween 20	Polyoxyethylene sorbitan monolaurate
v/v	Volume per volume

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Chapter 1

Introduction

In South Africa, wheat has been cultivated since the middle of the 17th century (Du Plessis 1993). Wheat and maize are the predominant crops in South Africa and account for 90% of the cultivated area. Agriculture is not only a major factor in rural economic growth and development, but also plays a distinctive role in broadening the economic and social options of the rural people, consequently improving the quality of life (Marasas *et al.* 1997).

Approximately 14% of the economically active population in the Republic of South Africa (RSA) is gainfully employed in agriculture. However, the true contributions of agriculture to the national economy are the following: it generates almost R43 billion a year and more than 10% of the gross domestic product in the RSA; more than 25% of employment in the RSA is sustained by agricultural activity, and more than 13 million people (~32% of the population) are dependent on rural agricultural production; the contribution of agriculture to net foreign exchange earnings has increased from 7.8% (1952) to 9.2% (1994); the agricultural sector generates 32% of the total input of the food manufacturing sector (Van Rooyen *et al.* 1996). The gross value of wheat in the RSA amounted to R1 354 million in 1994/95. Its contribution to the value of gross agricultural production was estimated at 3.59 - 6.3% over the past decade (1984-1994). These statistics clearly demonstrate the vital role of agriculture in the national economy and its role to broaden the economic and social options for all South Africans. It also emphasises the detrimental effect agricultural stress factors could have on the economy of the country (McIntosh *et al.* 1995). The RSA is the largest producer of wheat in Southern Africa and it includes winter rainfall areas (Western Cape) and summer rainfall areas

(Free State, Northern Cape, North Western and Northern Provinces) (Aalbersberg & Du Toit 1987). An important stress factor of wheat in South Africa is the Russian wheat aphid (RWA), *Diuraphis noxia* (Mordvilko), which is regarded as the most noxious pest of cereal crops in Southern Africa (Aalbersberg & Du Toit 1987).

The RWA was discovered in the RSA in 1978, while research on RWA control started at the Small Grain Institute (Bethlehem, RSA) during 1980 (Marasas *et al.* 1997). It is endemic to Southern Russia, and countries bordering the Mediterranean Sea, Iran and Afghanistan (Walters *et al.* 1980). After it had spred from Asia to Africa, it was recorded as a wheat pest in South Africa (Aalbersberg & Du Toit 1987, Du Toit 1986). By 1979, the RWA had spread throughout most of the Western Free State and Lesotho (Fig. 1.1) (Du Toit 1989).

In South Africa the yield losses caused by the RWA amounted to about R30 million for the year 1993 (Personal communication, Dr F du Toit, Pannar, Bloemfontein, RSA). The economic loss attributed to the RWA in the USA exceeded 890 million US dollar (1987 - 1993), with approximately 83 million US dollar being spent on control, and 349 million US dollar lost in production (Marasas *et al.* 1997).

Individual wheat yield losses of up to 90% were recorded under field conditions (Du Toit & Walters 1984), while on trial plots losses of 56.8% were recorded (Girma *et al.* 1993), and losses of 35-60% for winter wheat (Du Toit 1986), indicating losses in wheat yields of between 21-92%. The RWA can affect the food production capacity of South Africa,

and may have disastrous economical effects. Control of the RWA pest has become a major global concern (Marasas *et al.* 1997, Potgieter *et al.* 1991).

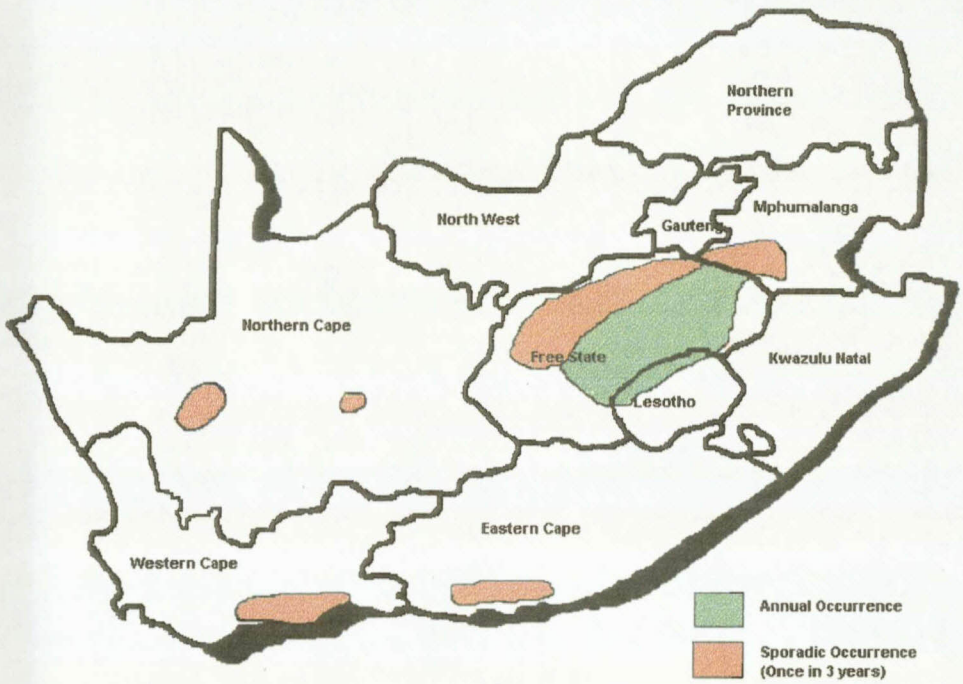

Figure 1.1 Area infested by the RWA in the RSA and Lesotho (Marasas *et al.* 1997).

Invading RWAs migrate upward on growing wheat plants and eventually colonise on the adaxial surface of the newest growth, in the axils of leaves or within rolled leaves (Du Toit 1986, Marasas *et al.* 1997). It has been suggested that feeding RWAs also secrete a phytotoxin in the plant while feeding (Du Toit & Aalbersberg 1980). Damage to wheat plants is thus caused directly by feeding and the effect of the phytotoxin injected during feeding (Du Toit 1986, Du Toit 1989, Du Toit & Aalbersberg 1980, Valiulis 1986). The

toxin or biochemical reaction that causes the damage has yet to be identified, though the effects are well known (Marasas *et al.* 1997). The damage inflicted by feeding RWAs on susceptible wheat cultivars results in the typical symptoms of susceptibility (Kindler *et al.* 1991).

Damage symptoms characteristic of RWA infestations in susceptible plants include longitudinal leaf streaking (longitudinal white, yellow, and purple streaks), inward curling of leaf edges (Du Toit 1986, Du Toit 1989, Gilchrist *et al.* 1984) and plant stunting (Kindler & Hamman 1996). Infestation also leads to a reduction in chlorophyll content (Kruger & Hewitt 1984) which, when combined with the rest of the characteristics, causes a considerable loss of photosynthetic effective leaf area on susceptible plants (Walters *et al.* 1980). Water imbalances also occur in host wheat plants which leads to a loss of turgor and growth reduction. The yield and quality of wheat (i.e. plant height, shoot weight, and number of spikes) are therefore significantly reduced. The yield per plant and protein content are also reduced (Girma *et al.* 1993). Resistant cultivars only exhibit chlorotic spots and no leaf rolling, and their growth is only slightly affected (Van der Westhuizen & Botha 1993). The resistant cultivars are colonised by the RWA in lower numbers than on the susceptible cultivars, they tend to stay green longer, and are less stressed (Marasas *et al.* 1997).

Currently, insecticides are widely used to control the pest. However, management by means of spraying insecticides and biological control (RWA predators) is complicated by the fact that the leaves are prevented from uncurling to expose the aphids to the predators

and insecticides (Valiulis 1986). The use of systemic insecticides is more effective, but this method again has its own disadvantages. Application of insecticides is expensive (Birch & Wratten 1984) and annually about R15 million is spent on chemical control of the aphid in South Africa (Cilliers *et al.* 1992). It also has a detrimental ecological impact on the environment as it is washed into the river systems and wetlands (Dreyer & Campbell 1987, Du Toit 1986).

Natural enemies such as ladybirds (*Coccinelidae*) and parasitic wasps also play an important role in restricting the RWA population (Walters *et al.* 1980). Four parasitic wasp species were imported from countries where the RWA originated, and were evaluated under South African conditions. Amongst them, *Aphelinus hordei* showed the best biological control potential. This wasp was first released in South Africa in 1993 in the Eastern Free State. Parasitising on 48 - 83% of the RWA was observed. Within one year they spred in a 30km radius from the site of release. Under laboratory conditions the wasps reduced the aphid population on resistant plants by 50% (Prinsloo 1995). Theoretically a combination of plant resistance and natural enemies would be able to reduce the aphid numbers below the threshold value, rendering other control measures unnecessary (Marasas *et al.* 1997).

The availability of resistant cultivars offers a positive alternative to the application of expensive insecticides (Barret 1996, Du Toit 1989). Research on the development of resistant wheat cultivars has become an urgent task for the South African wheat industry. Since 1984 increasing efforts have been made to find sources of resistance in wheat

cultivars to be utilised in breeding programmes (Du Toit 1988). Benefits of cultivating resistant cultivars include a yield advantage of the resistant over the susceptible cultivars, and cost savings on reduced chemical treatments (nett present value of R19-35.9 million when all savings are considered). It is estimated that resistant cultivars will yield 0.2 metric ton per hectare more than susceptible cultivars in the Eastern Free State, because of the RWA's influence on the crop yield in susceptible wheat. The better yield brought about by the resistant cultivars will amount to about R46-68 million more than that of the susceptible cultivars (Marasas *et al.* 1997).

The first genetic resistance to the RWA was identified in bread-wheat, *Triticum aestivum* L., in 1985 (Marasas *et al.* 1997). A number of *Triticum* genotypes from countries where the RWA originated, were then screened for resistance at the Small Grain Institute in Bethlehem, South Africa. Several resistant lines were identified in greenhouse tests and confirmed under field conditions (Du Toit 1987, 1988, 1990). Over the last few years, some resistance sources have also been identified in other countries (Martin & Harvey 1994, Quick *et al.* 1991, Smith *et al.* 1992). The backcrossing technique was followed to introduce resistance genes into wheat lines with more acceptable agronomic characteristics. The first resistant cultivar (Tugela-DN) to be released in the world, was released in 1992 by the ARC-SGI (Agricultural Research Council - Small Grain Institute) (Marasas *et al.* 1997). Studies on the inheritance of resistance indicated that resistance in each line was controlled in most instances by a single dominant gene. These genes were independently inherited and named (Dn1) to (Dn5) (Du Toit 1989, Du Toit *et al.* 1995, Nkongolo *et al.* 1991).

Currently the total wheat area of Central Free State, Eastern Free State, Qwa-Qwa and Thaba Nchu comprises of 78% susceptible and 22% resistant cultivars, of which Tugela-DN is the predominant resistant cultivar, with a potential yield of 6 metric tons per hectare. The practice of spraying insecticides has declined in the Eastern and Central Free State since resistant cultivars and treated seeds were released. From 1990 to 1996, the average area sprayed decreased by 71% (Marasas *et al.* 1997).

Although resistant cultivars have been released in South Africa, the possible development of new biotypes of the aphid in future necessitates faster development of new cultivars. To accomplish this, plant breeders urgently require molecular markers that could enable more effective and time-saving screening procedures. The identification of the resistance gene(s) is also important (Van der Westhuizen & Pretorius 1995). It is anticipated that knowledge of the resistance mechanism could contribute toward more directed breeding and could also contribute to finding biochemical / molecular markers of resistance which would help in developing more effective and shorter screening procedures. In addition, a better understanding of the biochemical defence mechanism can contribute to the development of new controlling measures.

The resistant Tugela wheat cultivars used in this study, contained different resistance genes, i.e. (Dn1), (Dn2) and (Dn5). They originated from crosses between resistant line PI 137739 (Dn1), PI 262660 (Dn2) and PI 294994 (Dn5) and local susceptible winter wheat cultivar Tugela (Cilliers *et al.* 1992, Du Toit 1989, Ma *et al.* 1998). The resistance

gene was bred into the genetic background of Tugela without losing any of the excellent grain production characteristics of the susceptible cultivars (Cilliers *et al.* 1992).

Most of the former research on the resistance of cereals to insects concentrated on physical barriers and deterrent chemical compounds. Studies on the physical barriers include epidermal wax composition (Corcuera 1993), type, density and length of epidermal hairs and/or hooks (Dixon 1985), degree of methylation and branching of intercellular pectin (Corcuera 1993). Structural adaptations, unfortunately, can only protect the plant to a certain extent and further defence occur through the manipulation of biochemical substances (Campbell *et al.* 1982, Chatters & Schlehuber 1951).

Research on the chemical resistance of plants to insects mainly involved secondary plant chemicals, protease inhibitors and nutritional and environmental factors (Concuera 1993, Dixon 1985, Niemeyer 1990, Niraz *et al.* 1985). All are part of the resistance mechanism, but the sequence of events to establish resistance, is still unknown. What exactly triggers the resistance response in wheat against the RWA? What happens after recognition? We aimed to obtain more information concerning the biochemical mechanism of resistance to understand the resistance response.

Chapter 2

Literature review

2.1 Plant defence

For survival, plants have to protect themselves against different biotic and abiotic stress conditions. Biotic enemies include fungal, bacterial and viral pathogens and herbivores. Abiotic stresses, on the other hand, include abnormal temperatures, drought, waterlogging nutrient deficiencies, etc. (Chessin & Zipf 1990). By deploying a wide range of defence mechanisms (Benhamou 1996, Pugin & Guern 1996), plants are able to defend themselves to various extents. These defence mechanisms include constitutive or induced, structural and biochemical defence mechanisms (Benhamou 1996, Dixon *et al.* 1994, Hammerschmidt & Schultz 1996).

Several studies indicated that there are resemblances between the resistance mechanism against herbivores (e.g. the RWA), and the resistance mechanism against pathogens (Botha *et al.* 1998, Hammerschmidt & Schultz 1996, Van der Westhuizen & Pretorius 1996, Van der Westhuizen *et al.* 1996, Van der Westhuizen *et al.* 1998a & b).

2.1.1 Constitutive defence

Plant defence against stress agents can be grouped into two categories, namely constitutive (preformed) and induced defences. Constitutive defence usually comprises of preformed toxic compounds and/or structural barriers, while structural barriers, such as the cuticle or periderm, are breached to allow infection to occur (Chessin & Zipf 1990, Hammerschmidt & Schultz 1996, Sticher *et al.* 1997).

2.1.1.1 Constitutive structural defence

Structural barriers against pathogens include waxes, hairs, thick cuticle, thickness and toughness of the outer wall of epidermal cells, structure of the stomata and closure condition, and reinforcement of cells (Benhamou 1996, Hammerschmidt & Schultz 1996).

Structural defences against insects and herbivores include general tissue toughness, deposition of silica, calcium carbonate, or lignin around vascular bundles or throughout tissues (Norris & Kogan 1980). Stem toughness is a heritable trait conferring protection of wheat plants against, e.g. the wheat stem sawfly (Wallace *et al.* 1973).

This type of structural defence also include leaf hairs and trichomes that have a heritable basis and can be shown to confer protection on their bearers. Density and type of granular trichomes are perhaps the best studied structural defence that are actually "physico-chemical" in nature because of its chemical contents. Various genotypes of tomato, tobacco, potato, and cotton produce large numbers of glandular trichomes that rupture when contacted by insects, producing a rapidly oxidised phenolic mixture that darkens and hardens upon exposure to air, immobilising even moderate-sized insects (Berenbaum *et al.* 1986).

2.1.1.2 Constitutive chemical defence

Most classes of natural plant products have at least some antimicrobial or antiherbivore activity, or both, at least *in vitro* (Deveral 1976, Hammerschmidt & Kuc 1995, Harborne 1993, Schonbeck & Schlosser 1976). The real impact of these constitutive defences is difficult to predict, because plants are simultaneously dealing with pathogens and

herbivores, and because herbivores are often infected by their own pathogens. To the extent that their modes of action are understood, some of these molecules can act against microbial and herbivore cells in common ways. Hence, it is difficult to determine which chemical may be primarily a defence against pathogens, and which against herbivores, because evidence suggests that many are both (Deveral 1976, Hammerschmidt & Kuc 1995, Harborne 1993, Karban & Myers 1989, Schonbeck & Schlosser 1976, Schultz & Keating 1991).

Constitutive chemical defences have one of several effects on herbivores. Among other, they may be anti-feedant, which provides the plant with the greatest potential protection, since damage may be prevented almost before it begins. They may be acutely toxic, which has the potential to stop damage quickly, or they may have multiple chronic toxicity. The last mentioned, offers the least benefit to the plant, since considerable consumption can still occur (Hammerschmidt & Schultz 1996).

One of the best studied examples of constitutive defence against herbivores is the production of furanocoumarins by *Pastinaca sativa* (wild parsnip). Resistance to the parsnip webworm (*Depressaria pastinacella*) is determined quantitatively by the concentration relationships among three furanocoumarins (Kuc 1984, Schultz & Keating 1991, Sinden *et al.* 1984) and nitrogen in flower head tissues (Berenbaum & Zangerl 1992).

Many of the steroid glucoalkaloids of Solanaceous species that have been implicated in disease resistance are O-glycosides of solanidine and tomatidine. Steroid glycoalkaloids are also toxic to some insects and not only to pathogens. Thus, it is possible that they may play a role in host plant defence against both insects and pathogens (Kuc 1984, Sinden *et al.* 1984). However, scientists are left to estimate how much resistance is constitutive, and how much is induced.

Substances formed in plant cells before (or after) pathogen infection, which may be detrimental to the pathogen, include inhibitors which are exudated through the cells' surfaces into the surrounding environment, pathogen penetration inhibitors which are present in plant cells before infection, and defence may be through a lack of essential factors. The lack of essential factors includes the lack of recognition between host and pathogen, the lack of recognition of host receptors and sensitive sites for toxins, and the lack of essential nutrients for the pathogen (Agrios 1988).

Specific recognition factors (e.g. oligosaccharides, polysaccharides, proteins or glycoproteins) must be recognised by the pathogen before successful infection can occur. Certain pathogens produce host specific toxins which bind with a specific receptor to produce disease symptoms. Certain pathogens can only form the necessary structures for infection if the plant provides certain growth factors. Plants unable to provide such growth factors cannot be infected by these pathogens and remain resistant to the toxin and develop no symptoms (Agrios 1988).

2.1.2 Induced defences

Induced defences include toxins and/or physical barriers that are only produced upon infection or attack. Biochemically induced defences in response to pathogens (Fig. 2.1) and herbivores can be classified into local or systemic defence/resistance responses (Chessin & Zipf 1990, Greenberg 1997, Hammerschmidt & Schultz 1996).

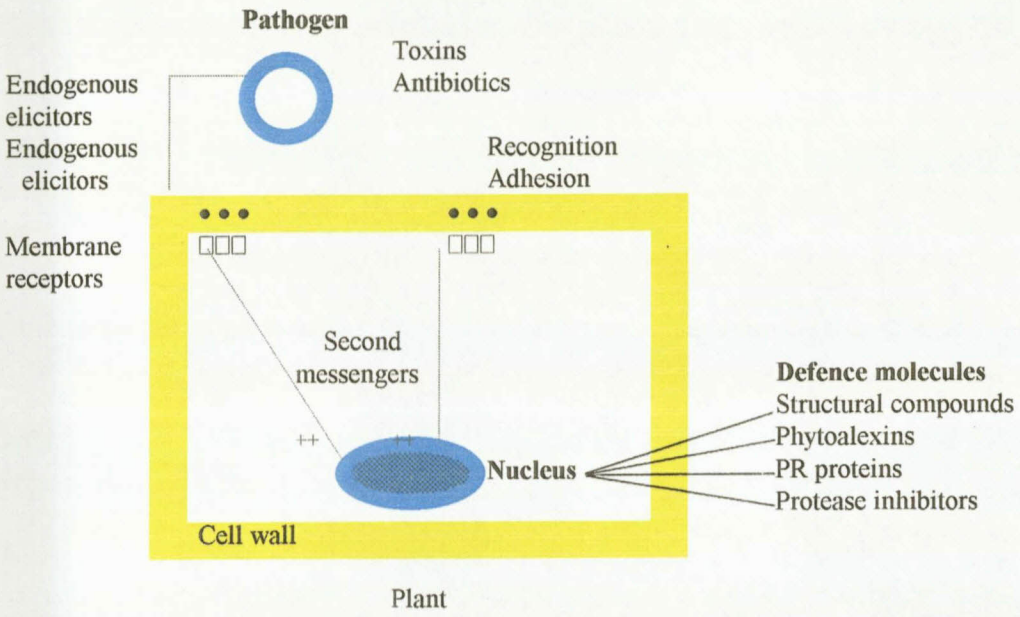

Figure 2.1 Chronological steps involved in the initiation of defence responses.

Signals and responses in plant-pathogen interactions. Upon recognition between both partners, the pathogen produces an array of metabolites, including endo-polygalacturonases, that contribute to the release of signalling pectic oligomers (the endogenous elicitors). Binding of these elicitors to specific membrane receptors causes membrane depolarization, leading to the activation of second messengers that transduce the signal to the nucleus. Defence genes, encoding structural compounds, enzymes of secondary metabolism pathogenesis-related (PR) proteins and protease inhibitors (PIs) are then triggered (Benhamou 1996). Among the PR-proteins, chitinases and β-1,3-glucanases may cause fungal cell wall hydrolysis (Slusarenko 1996), leading to the specific membrane receptors and trigger a cascade of events similar to that induced by the endogenous elicitors (Benhamou 1996).

Most of the induced defence responses also apply to the induced resistance response, and is part of the systemic defence response (Hammerschmidt & Schultz 1996). After the induction of the resistance response, protection against a later infection can be restricted to the site of primary infection or injury (local acquired resistance, LAR) or can encompass tissue of the plant that has not been treated (systemic acquired resistance, SAR) (Schaffrath *et al.* 1997, Sticher *et al.* 1997).

The hypersensitive reaction (HR) is involved in both induced defence and induced resistance responses. Plants that undergo a defence response that include the HR on one or few leaves, develop 'immunity' to many other pathogens in the leaves that have not previously been exposed to pathogens (Greenberg 1997).

2.1.3 Defence through induced resistance

Plants can activate protective mechanisms upon contact with invaders, and this is then called induced or acquired resistance. Acquired resistance develops after pathogen infection (Schaffrath *et al.* 1997) or insect infestation (Sticher *et al.* 1997), but also on application of chemical substances such as methyl jasmonate (Enyedi *et al.* 1992).

In most cases, the first inoculation leads to localised necrosis. In gene-for-gene resistance, a plant is either resistant or susceptible to certain races of a pathogen. The successful colonisation of a pathogen or pest leads to disease development, and the plant is called "susceptible" (compatible interaction) (Greenberg 1997, Slusarenko 1996). However, this

establishment of disease, or successful colonisation of parasites, is likely to be caused by delayed or diminished plant defence expression rather than by any absence or inactivation of defence mechanisms (Benhamou 1996, Greenberg 1997, Ocampo *et al.* 1986). Disease resistance can be defined as the ability of the plant to prevent or restrict, pathogen or pest development and multiplication (incompatible interaction) (Greenberg 1997, Slusarenko 1996).

Systemic acquired resistance (SAR) confers quantitative protection against a broad spectrum of micro-organisms and insects. Infection of hypersensitive tobacco with tobacco mosaic virus (TMV), induces systemic resistance against TMV, several viruses, fungi, bacteria and aphids (McIntyre *et al.* 1981, Ajlan & Potter 1992).

The time needed for the establishment of SAR depends on both the plant and type of inducing organism. The level of protection may vary depending on the organism used for the primary inoculation and particularly on the extent of the necrosis. Induction of resistance in parts remote from the site of primary inoculation is postulated to result from the translocation of a *hitherto* unknown systemic signal produced at the site of primary infection. This signal primes the plant against further pathogen attacks, probably by triggering a complex array of defence responses (Sticher *et al.* 1997).

Much interest has been directed toward understanding the sequence of molecular events leading to the establishment of multicomponent (chemical defence, structural defence, LAR and SAR) plant defence mechanisms (Benhamou 1996, Slusarenko 1996), but still

the exact mechanism is unknown. A model has been proposed for the sequence of events leading to the formation of defence and resistance products, called the "signal transduction pathway" (Fig. 2.2) (Benhamou 1996).

2.1.3.1 Local acquired resistance

Resistance to specific pathogens is often controlled by one or a few "major" genes in the host plant and a gene for avirulence in the pathogen (Deverall 1976). The interaction of the products of the resistant and avirulent genes allows the host plant to recognise the presence and identity of an attacking pathogen. Recognition then results in the expression of the various defence responses, but the membrane receptors involved in this recognition step are still unknown (Benhamou 1996).

Some of the earliest responses after recognition are membrane depolarisation, changes in membrane permeability, production of oxygen species ("oxidative burst") and increase in intracellular calcium concentrations. The so called 'oxidative burst' precedes cell death and is thought to trigger the HR (Benhamou 1996, Greenberg 1997). The HR is therefore often associated with the resistance response (Pugin & Guern 1996, Slusarenko 1996).

LAR in wheat against powdery mildew is accompanied by the accumulation of mRNA species, which encode for putative cell wall proteins, a traumatin-like protein, peroxidases (PODs), lipoxygenases (LOXs) and a cystein proteinase. The fact that in wheat the biological and chemical acquired resistance inducers result in the accumulation of non-

identical sets of transcripts, indicates the possibility of different signal transduction pathways for induced resistance (Schaffrath *et al.* 1997).

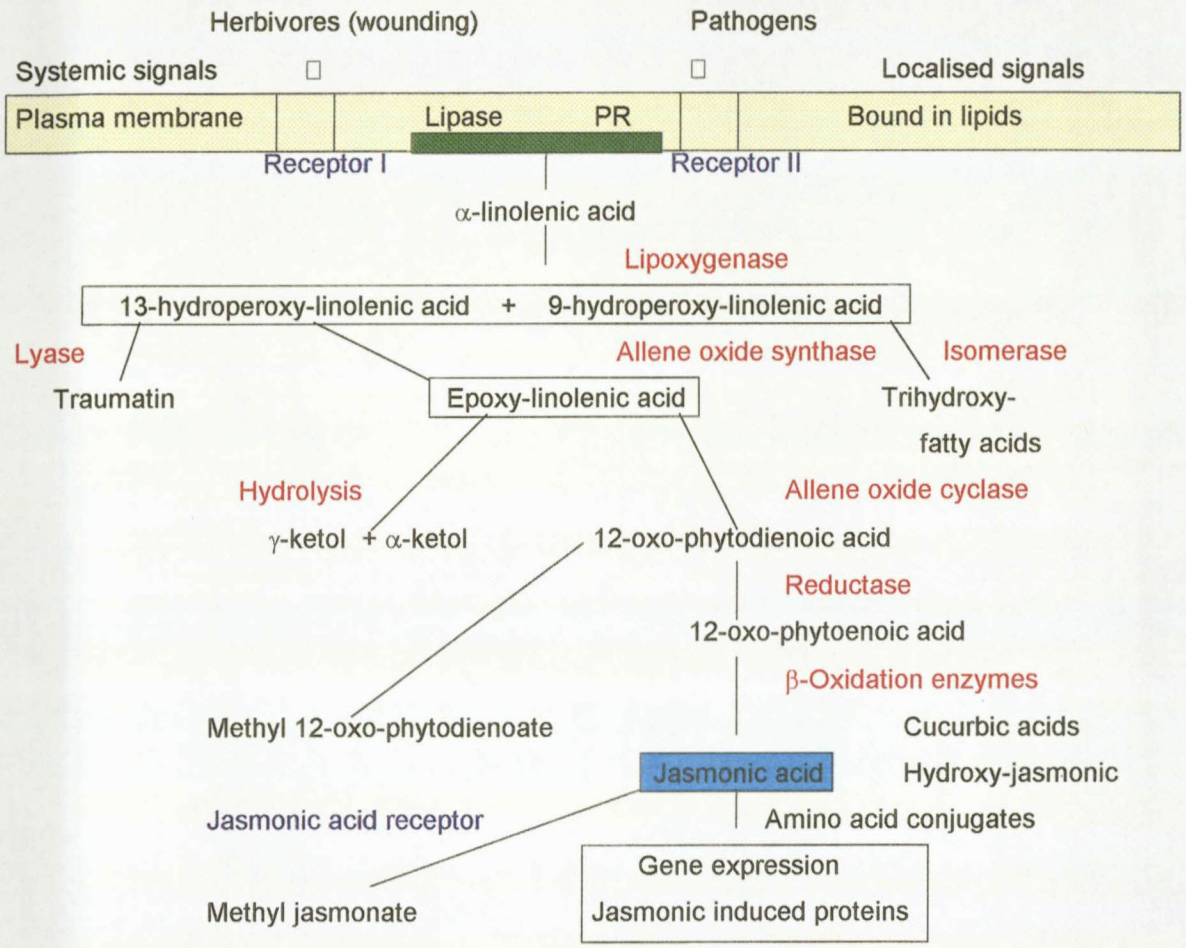

Figure 2.2 Proposed model for the signalling that leads to the expression of wound-inducible proteinase genes in tomato leaves. PR, pathogenesis related proteins (Rosahl 1996).

2.1.3.1.1 Hypersensitive reaction

Upon interactions with the environment (pathogen or insect), the infected plant cells often undergo rapid death (HR) that is accompanied by the induction of various local defence

responses. The cell death and collapse caused in the infected and few surrounding cells, results in the formation of dry lesions (Pennell & Lamb 1997).

In general, it is believed that the HR is triggered after recognition of the pathogen (or insect) by the host cell. Associated with the HR is the localised induction of an array of defences that include phytoalexin production, accumulation of active oxygen species, pathogenesis-related (PR) protein synthesis and cell wall modifications (Chessin & Zipf 1990, Hammerschmidt & Schultz 1996). Also, during the active HR cell death, oxygen and hydroperoxide species accumulate and lead to an elevation in cytosolic calcium cations which triggers a protein kinase-mediated cell death process. Attacks by virulent pathogens, which do not trigger the HR, result instead in disease development (Pennell & Lamb 1997).

The death of the plant cell itself may be an effective defence against obligate pathogens such as mildews and rusts that need a living plant cell for growth and reproduction. Fungal and bacterial pathogens within the area of operation of the HR, are isolated by the necrotic tissue and die off (Chessin & Zipf 1990, Ocampo *et al.* 1986, Slusarenko 1996, Sticher *et al.* 1997). In the case of virus diseases, the HR always result in formation of the so-called local lesions in which the virus may survive for considerable time (Enyedi *et al.* 1992).

In wheat rust interactions, LOXs appear to be involved in early events leading to the HR processes such as lignification and formation of callose (Ocampo *et al.* 1986).

2.1.3.1.2 Phytoalexin

The term phytoalexin is used to describe several diverse types of relatively small chemical compounds of plant origin that can deter a broad range of invaders. Phytoalexins are not found in healthy plant tisssues but are synthesised in infected tissues in response to chemical signals, like elicitors (Ryan 1987). Phytoalexins can kill or deter the invader, as well as the plant cells in the vicinity of invasion. This killing of cells results in small brown spots, referred to as small necrotic lesions, on the leaves. Thus, the plant sacrifices a small group of cells to save itself (Chrispeels & Sadava 1994).

2.1.3.1.3 Oxidases and activated oxygen

Active oxygen species such as hydrogen peroxide, hydroxyl radicals and superoxides, appear to be important factors in resistance to pathogens (Baker & Orlandi 1995, Mehdy 1994, Sutherland 1991). One of the earliest events in the HR is an increase in oxidative potential and production of active oxygen species (Keen & Littlefield 1979), the so-called "oxidative burst" (Baker & Orlandi 1995). Active oxygen species are believed to function in resistance (1) by increasing host cell wall resistance to hydrolytic enzymes by the crosslinking cell wall polymers (Stermer & Hammerschmidt 1987) (2) by acting as antimicrobial factors (Baker & Orlandi 1995) (3) and by acting as local signals involved in the induction of defence genes (Chen & Klessig 1991, Chen *et al.* 1993).

Induced defence reactions in plants usually include increases in oxidative enzymes such as peroxidases (PODs) (Siegel 1993) and polyphenoloxidases (PPOs) (Mayer 1987). Polymerisation of lignin precursors into lignin and crosslinking of hydroxyproline-rich glycoproteins in the cell wall are two possible functions for POD. In addition, cell wall-associated PODs are also involved in the production of the hydrogen peroxide (H_2O_2) needed for lignin formation and wall protein crosslinking. PODs, however, often increase in activity after many pathogenic and non-pathogenic stresses and may occur as numerous isozymes (Siegel 1993).

The phenolic contents of many leaves are exposed to oxidative conditions when cells are disrupted by herbivores. In the presence of air, PODs, phenoloxidases (POs) and other oxidative enzymes generate significant, localised oxidative transformations (Appel 1993). The consequences range from lignification, cell wall toughening and formation of polyphenolic polymers, to the creation of toxic or distasteful compounds (oxygen radicals) and quinones. This phenomenon has not been studied on a localised basis, although Felton *et al.* (1994a & b) produced evidence that such transformations occur in soybean leaves upon herbivore attack, including an 'oxidative burst' resembling the response to pathogens (Felton *et al.* 1994a &b).

2.1.3.1.4 Pathogenesis-related proteins and other proteins

Many plants respond to pathogenic infestation with an altered protein synthesis pattern (Cutt & Klessig 1992, Inbar *et al.* 1997, Stermer 1995). These proteins appear to be involved in at least three types of defence reactions, namely (a) direct attack of the

invading pathogen e.g. hydrolytic enzymes; (b) localisation of the pathogen at the site of infestation, e.g. enzymes involved in lignification; and (c) an adaptation of the host metabolism to the stress condition, e.g. superoxide-dismutase (Bol *et al.* 1990, Ocampo *et al.* 1986, Slusarenko 1996, Stermer 1995, Sticher *et al.* 1997). Amongst these synthesised proteins is a set of proteins termed pathogenesis-related (PR) proteins (Linthorst 1991). These proteins are a group of plant encoded proteins whose synthesis is not only induced by infestation with viroids, viruses, pathogens and insects, but also in response to chemically induced stress, and even in natural senescences (Bol 1988, Van der Westhuizen & Pretorius 1995, Van Loon 1985, 1989).

PR-proteins are characterised by (a) their acidic nature (Gininazzi *et al.* 1977, Van Loon 1976), (b) their resistance to the action of proteolytic enzymes of endogenous or exogenous origin (Stintzi *et al.* 1993, Van Loon 1982), (c) their location in compartments such as the vacuole, the cell wall and/or the apoplast (Payne *et al.* 1989), and (d) by their low molecular mass (8-50kD). With only a few exceptions (Stintzi *et al.* 1993), PR-proteins are all monomers. More recently, basic homologues to a number of acidic PR-proteins have been identified (Bol *et al.* 1990).

In recent years PR-proteins have been studied extensively in tobacco, because their induction is correlated to the acquisition of systemic resistance (Bol *et al.* 1990, Linthorst 1991). Five PR-protein groups (Table 2.1) have been identified in tobacco (Van Loon *et al.* 1987). Group 1 comprises of the PR-1 proteins of unknown function (Kessmann *et al.* 1994, Ryals *et al.* 1994). Group 2 contains PR-proteins with β-1,3-glucanase activity.

The cell wall degrading enzyme, chitinase, constitutes group 3 (Broglie *et al.* 1991, Cutt & Klessig 1992, Danhash *et al.* 1993, Stermer 1995, Sticher *et al.* 1997, Verburg & Huynh 1991). TMV infection was found to induce four tobacco chitinases, namely the acidic PR-proteins P and Q, and the basic PR-proteins Ch32 and Ch34 (Legrand *et al.* 1987). The low molecular weight proteins, classified as group 4, have been characterised in less detail. Group 5 consists of traumatin-like proteins and includes two, almost neutral, proteins named R and S (Bol *et al.* 1990).

Studies indicated that some of the PR-proteins are also induced by the Russian wheat aphid (RWA) after infestation, e.g. chitinases and β-1,3-glucanases (Botha *et al.* 1998, Van der Westhuizen *et al.* 1998a & b, Van der Westhuizen & Pretorius 1996).

2.1.3.2 Systemic acquired resistance

Challenging plants with pathogens that cause necrotic lesions often results in systemic acquired resistance (SAR) (Kessmann *et al.* 1994, Ryals *et al.* 1994, Hammerschmidt 1993, Hammerschmidt & Kuc 1995, Hammerschmidt & Smith 1996). These observations suggest that multiple defence mechanisms or a single mechanism with overlapping effects have been induced. The systemic mechanisms appear to be similar to localised resistance responses. Some may be activated as a result of the inducing inoculation, while others are rapidly induced only after a subsequent inoculation with a virulent pathogen (Dann & Deveral 1995, Elliston *et al.* 1977, Ryals *et al.* 1994, Stermer 1995).

Table 2.1 Pathogenesis-related proteins in tobacco (Fritig *et al.* 1989).

	Acidic isoforms		Basic isoforms		
Group	Name	Mr (kD)	Name	Mr (kD)	Function
1	1a	15.8	16 kD	16.0	Unknown
	1b	15.5			
	1c	15.6			
2a	2	39.7	Gluc.b	33.0	β-1,3-glucanase
	N	40.0			
	O	40.6			
	Q	36.0			
2b	Q	25.0			β-1,3-glucanase
3	P	27.5	Ch.32	32.0	Chitinases
	Q	28.5	Ch.34	34.0	
4	s1	14.5			Unknown
	r1	14.5			
	s2	13.0			
	r2	13.0			
5a	R	24.0	Osmotin	24.0	Traumatin-like proteins
	S	24.0			
5b			45 kD	45.0	Unknown

SAR was first reported in cucumber (Kuc *et al.* 1975). The inoculation of one leaf of a plant susceptible to the anthracnose fungus *Colletotrichum lagenarium* resulted in the systemic development of resistance to subsequent infection by the same pathogen. Similar studies showed that resistance could be induced by and against a number of cucumber pathogens (Hammerschmidt & Yang-Cashman 1995). The only common feature was that the effective resistance-inducing pathogens caused necrotic lesions.

Histological studies revealed that at least part of the resistance expressed against *Colletotrichum lagenarium* was based on a failure of the pathogen to infect the host tissue successfully. Although fungal conidia germinated and appresoria were formed, few were successful in penetrating the epidermal layers of plants with acquired resistance. No obvious host response was observed. Later it was found that a lignin-like polymer was deposited under many of the appressoria that did not penetrate, and concluded that this prevented the fungus from penetrating the host (Hammerschmidt & Kuc 1982). The structures that appear to block penetration of induced tissues contain callose (Schmele & Kauss 1990), a common defence-related polymeric cell wall glucan (Aist 1983) and silicon (Stein *et al.* 1993). Thus, it appears that at least part of the acquired resistance involve multiple cell wall modifications and could be considered to be a type of defensive redundancy.

How lignin and the other wall modifications function in this resistance response is not known, but it is likely that changes in the mechanical strength or ability to be enzymatically degraded may be involved (Ride 1978). Lignification may also slow the development of

hyphae that have successfully penetrated the outer epidermal wall by trapping it in the invaded cell (Hammerschmidt & Kuc 1982, Stein *et al.* 1993).

As a result of induced systemic resistance in cucumber, there is also an increase in an apoplastic chitinase (a PR-protein) (Mètraux *et al.* 1988) and a group of PODs (Hammerschmidt *et al.* 1982, Rasmussen *et al.* 1995, Smith & Hammerschmidt 1988). These enzymes are referred to as useful markers to indicate when SAR is developing. The actual purpose of the enhanced activity of these enzymes has yet to be determined conclusively (Hammerschmidt & Yang-Cashman 1995). LOX activity also increases in cucumbers expressing SAR (Avdiushko *et al.* 1993). This enzyme may be important in the generation of antifungal lipid peroxides or lipid oxidation products (Croft *et al.* 1993). Additionally, increased LOX activity may result in the synthesis of other signal molecules such as jasmonic acid (JA) (Farmer 1994).

Systemic induction of POD and LOX activities suggests that activated oxygen species may also be part of SAR expression. This is supported by induction of local and systemic resistance in potato foliage to *Phytophthora infestans*, which is accompanied by an increase in superoxide-generating activity and in superoxide dismutase, which may be involved in the conversion of superoxide into hydrogen peroxide (Chai & Doke 1987). It was suggested that the putative SAR signalling molecule, salicylate, inhibits catalase, permitting accumulation of hydrogen peroxide and other active-oxygen species which then act as second messengers (Chen &Klessig 1991, Chen *et al.* 1993). But, in tobacco leaves related to systemic resistance to blue mold (*Peronospora tabacina*), the systemic increase

in activated oxygen may also function in the strengthening of cell walls by crosslinking hydroproline-rich glycoproteins, after TMV infection (Ye *et al.* 1992).

In the herbivore/plant interaction, the systemic response is not as well studied as the systemic response against pathogens. Also, there is no well established model for herbivore/plant interactions as in the case of the "signal transduction pathway" model for pathogen/plant interactions. An alternative model, called the 'optimal defence theory', was formed. In this theory, it is suggested that plants respond to herbivores and wounding in an active, presumably adaptive, way (Rhoades 1985). Such a response would require the plant to recognise that it has been wounded and to organise and mobilise systemic changes that provide resistance. This model closely resembles the SAR resulting from pathogen attack (Hammerschmidt & Schultz 1996).

This means that during herbivore/plant interactions there must also be an elicitor present for recognition by the plant. Carefully plucking leaves at the petiole usually fails to elicit wound responses, while many wound responses, including increased phenolic metabolism, are supposed to be elicited by minor damage. Altered phenolic metabolism was observed in poplars receiving less than 5% leaf area removal by tearing, but no changes were observed in 'plucked' trees (Schultz & Bladwin 1982). Even less damage is needed to induce alkaloid synthesis in tobacco (Bladwin 1993). "Wound-induced genes" were expressed in response to squeezing poplar leaves with pliers (Parsons *et al.* 1989).

Some plant responses are elicited specifically by insects and many plant tissues release volatiles into the air when damaged. However, it was found that the suit of volatiles emitted by insect-damaged plants is distinct from the normal 'green leaf odour' released (Turlings *et al.* 1991). When various arthropods are the agents, or when insect regurgitant is added to an artificial wound, volatiles attractive to parasites or predators of the herbivores are enhanced in the emitted vapour. An elicitor in caterpillar oral secretions, e.g., can induce corn seedlings to limit chemical signals attractive to parasitic wasps, and several allochemicals can attract larval parasitoids, *Cotesia marginiventris* (Cresson), to the microhabitat of one of its hosts (Turlings *et al.* 1990, Turlings *et al.* 1991, Turlings *et al.* 1993). The widespread enzyme, β-glucosidase, has been identified as a potential elicitor of herbivore-induced plant odour that attracts host-searching parasitic wasps (Mattiacci *et al.* 1994). It is possible that some of the observed reactions are actually components of the classical HR or SAR. Hartley and Lawton (1991) reported greater up-regulation of phenylalanine ammonia lyase (PAL) in birch leaves adjacent to leaves damaged by insects or scissors than in leaves adjacent to leaves wounded with sterilised scissors (Hartley & Lawton 1991).

Only one wound response has been described in as detailed fashion as SAR against pathogens. Many plants synthesise protease inhibitors (PIs) in response to wounding. In tomato, transcription of PI genes is accomplished *via* a complex web of elicitors and signals involving pectic fragments, up-regulation of LOX, accumulation of JA (Nicholson & Hammerschmidt 1992), and enhanced oxidative activity throughout the plant (Farmer 1994). Accumulation of JA or its methyl ester (M-JA) (Rosenthall & Berenbaum 1992) is

related (as cause or effect) to expression of a gene coding for a small protein, "prosystemin", which is enzymatically degraded to a small peptide, "systemin" (Farmer 1994, Hammerschmidt 1993). Systemin is mobile in the vascular system and appears responsible for eventual increased expression of PI genes and increased PI concentrations. Systemin is the only protein identified as a systemic signal in plants. Its role in PI induction seems clear, but early events are less clear. Neither oligosaccharides nor JA appear to be very mobile in vascular tissues, and regulation of the LOX pathway, that leads to JA production, is complex with several other products of unknown activity. All the steps leading to production of prosystemin are not clearly defined. Overall, this response resembles SAR in many ways, and may have several biochemical steps in common with it (Hammerschmidt & Schultz 1996).

Several biochemical phenomena appear common to wound / herbivore responses. Not only is systemic signalling a common theme, but a limited set of signals [e.g. jasmonates, ethylene, abscisic acid (ABA)] is common to many systems (Hammerschmidt 1993). As might be expected in any gene regulation phenomenon, cell membrane depolarisation and rapid cation (Ca^{+2}) fluxes are observed frequently, together with "oxidative burst" and up-regulation of LOX, POD, PO, PPO and various phenylpropanoid enzymes. Many of these mechanisms are also central to SAR. The functional similarities lead us to anticipate potential linked consequences of plant responses to either microbes or herbivores (Hammerschmidt & Schultz 1996).

The cucumber system illustrates the uncertainty about the causes, mechanisms and consequences of multiple responses. The interaction of cucumber with pathogens comprises one of the best studied SAR systems (Hammerschmidt & Yang-Cashman 1995), and its cucurbitacin induction is one of the oldest and most intensively studied responses to wounding (Tallamy & Raupp 1991, Tallamy 1985). Other common anti-herbivore responses such as PIs, LOXs, saponin production, and "oxidative bursts" are also present, but relatively unstudied. It is not clear whether wound-induced increases in cucurbitacins are systemic (Tallamy & McCloud 1991).

Systemic plant responses to herbivores probably share many steps with SAR involving pathogens, but too few systems have been studied in enough detail to understand the relationships between them. Systemic wound responses are syncronised by signals, as in SAR against pathogens. Similar pathways can yield overlapping results, and that is why many secondary metabolites induced by herbivory are also antimicrobial, and elicitors for wound responses are generally unknown. It is not clear how many "wound- or herbivore-induced" responses may actually be elicited by microbial products, or whether all of these responses benefit the plant. Since they may influence microbial and other agents controlling herbivores, wound responses may actually benefit herbivores more than plants (Hunter & Schultz 1993, Schultz & Keating 1991).

2.1.3.3 Russian wheat aphid/wheat interactions

The Russian wheat aphid (RWA) induces the accumulation of specific proteins in wheat (Tugela cv) upon infestation. This induced response occurs selectively in the resistant

lines (Tugela-DN). Several of these induced proteins related to PR-proteins, e.g. β-1,3-glucanase, chitinase, PR-4 family and other enzymes like POD, are also induced. These results, together with other research, indicate that there are resemblances between pathogenesis and responses to RWA-infestation (Van der Westhuizen & Pretorius 1996, Van der Westhuizen *et al.* 1996).

β-1,3-Glucanases, chitinases and PODs are induced within 48 hours after RWA infestation in resistant cultivars. These induced enzyme activities, closely resemble defence responses during pathogenesis and seem to be part of a general defence response like the HR, which confers resistance to the RWA (Botha *et al.* 1998, Van der Westhuizen *et al.* 1998a & b).

2.2 Elicitors and systemic signals

The fact that localised injury or pathogen attack can result in systemic changes in resistance (to herbivores and pathogens), indicates that a signal must be generated at the site of the initial injury or infection, from where it is then translocated through the plant. Induction of resistance, both above and below the inoculation or wounding site, suggests that the signal is translocated in the phloem (Appel & Martin 1992, Bladwin 1993).

2.2.1 Types and production

The term elicitor can be confusing, as it is used to describe agents that induce any defence response, from cellular changes (such as the HR) to molecular changes (such as transcriptional activation of defence response genes) (Dixon *et al.* 1994). Biotic elicitors usually refer to macromolecules, originating either from the host plant (endogenous

elicitors) or from the plant pathogen (exogenous elicitors) (Benhamou 1996, Dixon *et al.* 1994). These biotic elicitors are capable of inducing structural and/or biochemical responses which are associated with the expression of plant disease resistance (Dixon *et al.* 1994, Pugin & Guern 1996). Some biotic elicitors, are oligosaccharides, glycoproteins, peptides, and phospholipids (Benhamou 1996). Other known biotic elicitors are e.g. polygalacturonides, β-glucans, chitosan, and some types of lipids (Dixon *et al.* 1994). Smaller molecules, such as arachidonic acid, JA, salicylic acid (SA) (Enyedi *et al.* 1992) and abscisic acid (ABA) are not derived from the cleavage of more complex structural molecules (Benhamou 1996), but, nevertheless, can elicit pathogen defence processes (Sticher *et al.* 1997).

2.2.1.1 Jasmonates (also see section 2.3.6.1)

Downstream products of the LOX action (Farmer 1994) are jasmonates, which include JA (Nicholson & Hammerschmidt 1992) and M-JA (Rosenthall & Berenbaum 1992), and traumatin (Marquies 1991).

JA is probably ubiquitous in higher plants and can induce many biochemical and physiological changes (Farmer 1994, Sembolner & Parthier 1993). JA is a wound-induced signal (Farmer 1994), which is transported through the phloem (Enyedi *et al.* 1992), that up-regulates LOX, and then in turn increases the production of JA as well as that of several other products in the same pathway (Sembolner & Parthier 1993). SA and JA are believed to interact in an antagonistic fashion to elicit the resistance response (Farmer 1994).

2.3 The role of lipoxygenase in development and resistance responses in plants

The presence of an enzyme activity in plants, termed 'lipoxidase', that was able to catalyse the oxidation of fatty acids was first reported almost 60 years ago. 'Carotene oxidase', that was associated with the degradation of carotenoids, was found to be the same enzyme. The name 'lipoxygenase' has since been used when referring to this enzyme. Although the existence of lipoxygenases (LOXs) has been known for many years, only recently has there been a better understanding of this enzyme's activity (Siedow 1991).

LOXs (linoleate: oxygen oxidoreductase, E.C. 1.13.11.12) are non-heme iron containing dioxygenases which catalyse the formation of hydroperoxy derivatives of polyunsaturated fatty acids containing a *cis*-, *cis*-1,4-pentadiene structures (Chamulitrat *et al.* 1991, Galliard & Chan 1980, Slusarenko 1996). These fatty acid hydroperoxy derivatives (HPODs) are then further metabolised to compounds with different biological activities. These compounds play important roles in plant growth, development and defence reactions. The exact role of LOXs in plants is still uncertain. In animals, however, it is well established that the products of several different mammalian LOXs (using arachidonic acid mainly as substrate [Rosahl 1996]) are the primary metabolites on pathways that lead to the formation of important regulatory molecules in inflammatory responses, leukotrines and lipoxins. Since some LOX pathway products are structurally similar to important mammalian signal substances, for example leukotrines and prostaglandins, it is tempting to

speculate that they may play analogous roles in signal transduction in plant disease (Slusarenko 1996).

2.3.1 Occurrence and distribution of lipoxygenases in plants

LOX activity has been reported in a wide range of organisms (Galliard & Chan 1980), including more than sixty higher plant species, eucaryotic algae, baker's yeast and other fungi (Siedow 1991).

LOXs are also widely distributed in various organs of the plant, and are found in the outer regions of organs such as hypocotyls, sepals, and pericarp (Slusarenko 1996). Its isoforms occur in most plant cells, but the tissue-specific expression levels of LOX within a plant can vary substantially (Galliard & Chan 1980), depending on developmental and environmental conditions. Young and expanding tissues (Douillard & Bergeron 1981), as well as senescing tissues (Rosahl 1996, Ocampo *et al.* 1986) appear to have high LOX activity levels. High LOX activity levels are also present in soybean leaves and potato tubers, in which the 94 kDa vegetative storage protein was later identified as a LOX protein (Rosahl 1996).

Subcellularly, LOX isoforms predominantly occur in the cytosol, chloroplasts (in wheat leaves mainly in the chloroplast lamellae [Douillard & Bergeron 1981]), mitochondria, vacuoles, nuclei, lipid bodies and in association with microsomal and plasma membranes (Douillard & Bergeron 1981, Galliard & Chan 1980, Rosahl 1996). It is difficult to assign a general role for LOX, because the LOX isoforms are widely distributed within a cell.

One should rather consider that different isoforms might exert distinct functions in plant growth and development (Rosahl 1996).

2.3.2 Characteristics and structural features

Most information on the structure was derived from soybean seed (*Glycine max*) LOXs (LOX-1, -2, -3a and -3b), which are soluble proteins consisting of a single polypeptide chain. Various other plants contain LOXs with a rather conserved protein size of 95 kDa (Rosahl 1996, Siedow 1991), while soybean LOX-1 is 94 kDa (Gardner 1996). Exceptions have been reported on molecular weights ranging from 72 - 108 kDa for LOX isolated from peas (Siedow 1991). Up to four isoenzymes have been found in wheat (Galliard & Chan 1980). The three soybean isozymes differ with respect to each other, and the characteristics are indicated in table 2.2.

Table 2.2 Comparison of the soybean LOX isoenzymes (Galliard & Chan 1980, Gardner 1996, Siedow 1991).

	LOX-1	LOX-2	LOX-3
pI(Isoelectric points)	5.68	6.25	6.15
pH optimum	9	6.5	7
9/13 HPOD ratio	5 : 95	50 : 50	
Iron content	1 atom /mol	1 atom /mol	
Molecular mass -according to cDNA	94	97	96.5

Previous studies indicated that maximal LOX activity for most plant species tested, occurred at pH7, with the exception of a soybean cultivar where a pH of 5.5 was recorded. Subsequently, most measurements of plant activity are made at pH7 (Rosahl 1996). Some scientists classify LOX isozymes into two classes, namely those with optimum activity at relative high pH of 8-9 (type-1) and those most active at near neutral pH (type-2), to make classification easier (Slusarenko 1996).

Furthermore, comparisons of deduced amino acid sequences from cDNA and genomic clones of different plants showed a high overall homology between LOXs. Only the N-terminal sequences seem specific for each isoform, while the rest of the amino acids exhibit a high degree of similarity (Rosahl 1996, Siedow 1991).

2.3.3 The lipoxygenase pathway

LOX catalyses the stereospecific dioxygenation of polyunsaturated fatty acids by incorporating molecular oxygen (O_2), which results in the formation of HPODs (Galliard & Chan 1980, Kuo *et al.* 1997, Mauch *et al.* 1997, Rosahl 1996). The single non-heme iron cofactor exists either as Fe(II) (the catalytically inactive form) or as Fe(III) (the active form of the enzyme) (Galliard & Chan 1980). The reaction starts with the binding of the unsaturated fatty acid to the active site of LOX, resulting in the formation of HPODs of the fatty acid (Rosahl 1996).

The levels of LOX activity vary among the various host plants tested from 5 to 1458 nmol HPOD min^{-1} g^{-1} fresh mass (Rosahl 1996). Well over one hundred products from LOX-

generated hydroperoxides of linoleic acid alone have been described (Gardner 1996). Products formed during LOX pathway may have multiple functions, where the physiological function in plants could be for growth and development, and for the plant response to pathogen infection and wounding (Siedow 1991).

In plants, linoleic acid and linolenic acid are the major polyunsatturated fatty acids found in plant membrane phospholipids (Felton *et al.* 1994a & b, Rosahl 1996, Slusarenko 1996). Oxygen binds either to the 9-/13-C of linolenic acid or linoleic acid, to form either 9- or 13-hydroperoxylinoleic or -linolenic acid (Fig. 2.3) (Luning *et al.* 1995, Siedow 1991). The prevalent plant polyunsaturated fatty acids, linoleic acid and linolenic acid, are thus oxygenated into (13S, 9Z, 11E)-13-hydroperoxy-9,11-octadecadienoic acid (13S-HPODE) and (13S, 9Z, 11E, 15Z)-13-hydroperoxy-9,11,15-octadecatrienoic acids (13S-HPOTE), respectively (Galliard & Chan 1980, Gardner 1996). The 9- to 13-hydroperoxides ratio also differs between various plant species (Siedow 1991).

The mechanism of LOX reactions were extensively studied using soybean LOX-1 and qualitatively similar behaviour occurred in LOX-2 and LOX-3 reactions (Galliard & Chan 1980, Rosahl 1996). LOX-1 almost exclusively forms 13-HPODE (hydroperoxy linoleic acid) from linoleic acid, while LOX-2 & -3 give roughly equal amounts of 9- & -13-HPODE (Siedow 1991).

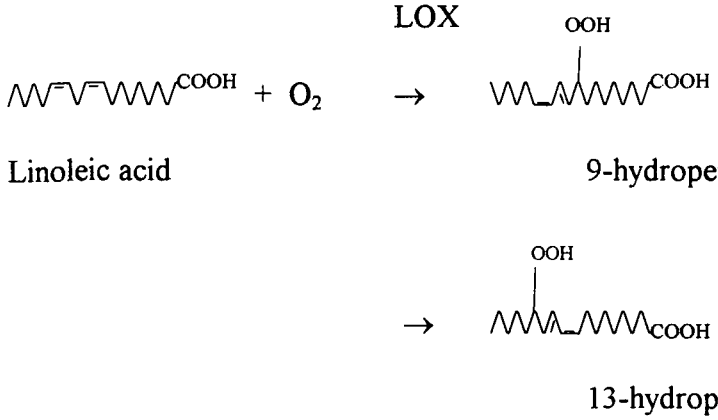

Figure 2.3 The primary reaction catalysed by LOX using linoleic acid as a substrate, indicating the two possible reaction products (Siedow 1991).

The HPODs of linoleic acid and linolenic acid are further metabolised in plants by different enzymes, including hydroperoxide lyase, dehydrase, and peroxygenase (Rosahl 1996). Hydroperoxide lyase acts on 13-HPOTE to form traumatin (a wound hormone), while hydroperoxide cyclase reacts with 13-HPOTE to form JA (a plant growth inhibitor) (Fig. 2.4) (Siedow 1991). It is also believed that LOX might be indirectly involved in the biosynthesis of abscicic acid (ABA) (Mauch *et al.* 1997).

A cyclic fatty acid derivative of 13-HPOTE whose synthesis also involves an epoxy intermediate, requires the presence of a allene oxide synthase. The cyclisation of the allene oxide 12,13-epoxy-linolenic acid is catalysed by allene oxide cyclase. 12-Oxo-phytodienoic acid is a precursor for the synthesis of JA. It is first reduced by 12-oxo-phytodienoic acid-reductase and the resulting 3-oxo-2-(2'-pentenyl) cyclopentaneoctanoic acid is subsequently shortened by three cycles of β-oxidation to the 12-carbon product JA, 3-oxo-2-(2'-pentenyl) cyclopentaneacetic acid. JA has phytohormone-like activities and is

involved in the regulation of developmental processes as well as in the plant's response to wounding and pathogens (Rosahl 1996).

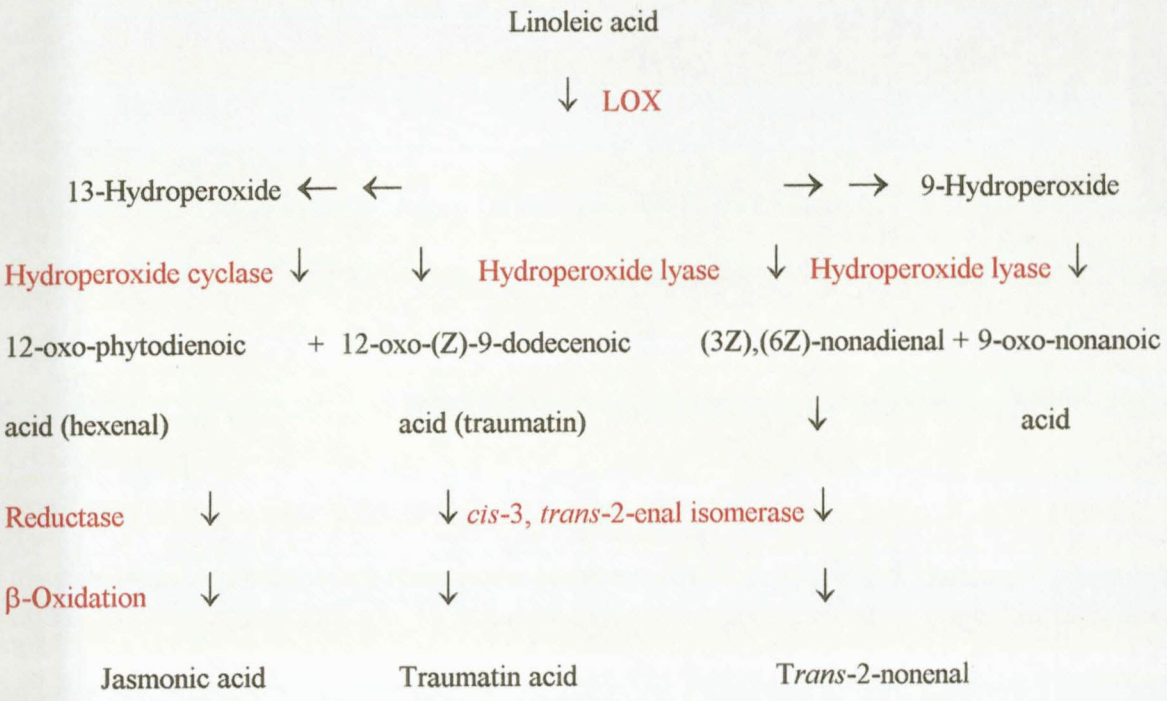

Figure 2.4 The LOX pathway.

The LOX-pathway for the biosynthesis of JA and traumatin acid from the LOX product, 13-hydroperoxylinolenic acid (Galliard & Chan 1980, Siedow 1991).

Further modifications of hydroperoxides of polyunsaturated fatty acids include the epoxidation by epoxygenase or peroxygenase, yielding epoxy fatty acids which can be converted to vicinal dihydroxy acids by the action of epoxide hydrolases. Epoxy and hydroxy fatty acids have been shown to possess antimicrobial activity (Rosahl 1996).

Volatile aldehydes and oxo-acids are produced by the action of hydroperoxide lyase, from 13-HPOTE as substrate, while the 9-HPOTE is converted to *cis*-3,*cis*-6-nonadienal and 9-oxo-nonanoic acid. These compounds give rise to the characteristic flavour and odour of fruits and leaves (Rosahl 1996).

Other metabolites derived from lipid hydroperoxides are α- and γ-ketols whose formation is attributed to the action of hydroperoxide dehydrase. Since allene oxides were identified as unstable intermediates in the synthesis of these ketols, the enzyme was also called 'allene oxide synthase'. Hydroperoxide dehydrases convert 13-HPODE and 13-HPOTE to the respective allene oxides, which yield ketol or cyclopentenone derivatives by either hydrolysis or intramolecular cyclisation by enzyme action (Rosahl 1996).

2.3.3.1 Regulation of lipoxygenase activity

LOX levels are regulated by various compounds such as JA and its methyl ester (M-JA) which induce LOX gene expression in soybean, potato and barley. Furthermore, nitrogen, wounding, drought stress, ABA and auxin also play a role in regulating LOX levels (Rosahl 1996). Numerous antioxidants (Siedow 1991), and some alkaloids inhibit LOX activity (Misík *et al.* 1995), while 0.2mM Ca^{2+} stimulate LOX activity of sea algae (Kuo *et al.* 1997). In contrast Swammy & Sunguna (1992) and Galliard & Chan (1980) reported that $CaCl_2$ and divalent cations (especially Ca^{2+}) inhibit LOX activity. However, no consistent picture emerged, therefore these results may be due to secondary effects or substrate interactions (Galliard & Chan 1980).

2.3.3.2 Lipoxygenase inhibitors

Piroxicam (PC), a LOX inhibitor, reduces the amount of electrolyte leakage of cations (to stimulate cellular breakdown - HR). Given the lack of knowledge of how specific the inhibitor PC is in plants, it is difficult to draw many meaningful conclusions from these observations (Peever & Higgins 1989, Siedow 1991).

Salicylhydroxamic acid (SHA) is known as a LOX inhibitor (Parrish & Leopold 1978, Preisig & Kuc 1987, Stelzig *et al.* 1983), and prevents accumulation of sesquiterpenes (Preisig & Kuc 1987, Rosahl 1996). It is also known to inhibit wound induced gene expression by blocking JA synthesis (Slusarenko 1996) and oxidoreductase activity (e.g. LOX and alternative oxidase of cyanide resistant respiration). SHA is a reversible inhibitor (Preisig & Kuc 1987) and only seems to delay the HR (Chen & Heath 1994). SHA is not specific for LOX and also inhibits POD activity (Beckman & Ingram 1994). Kinetin (a synthetic cytokinin) seems to mimic the action of SHA (Beckman & Ingram 1994, Swammy & Sunguna 1992).

n-Propyl gallate (nPG) is also known to be a LOX inhibitor (Krens *et al.* 1994, Luning *et al.* 1995, Parrish & Leopold 1978), but has been applied with limited success.

2.3.4 Role of lipoxygenase in growth and development

Despite detailed knowledge on structural and enzymatic features of LOXs, the physiological functions in plants have yet to be elucidated. There appears to be a correlation between increases in LOX proteins and transcripts, and the onset of specific

developmental processes (Rosahl 1996, Siedow 1991). In the case of plant growth and development, there is a correlation between the amount of LOX activity found in a given plant tissue and its rate of elongation. High LOX levels are found in rapid growing tissues (Mauch *et al.* 1997, Siedow 1991, Slusarenko 1996).

It was also suggested that LOX plays a possible role in seed germination (Galliard & Chan 1980, Mauch *et al.* 1997) and senescence (Galliard & Chan 1980, Krens *et al.* 1994, Mauch *et al.* 1997). Superoxide anions and ethylene are possibly the products that are involved in senescence (Ocampo *et al.* 1986).

The LOX activity level increases in maturing soybean seeds. After germination, soybean LOX activity decreases over the first 24 hours, followed by an increase in activity again. In cotyledons of soybeans a continuous loss of both the LOX protein and activity was found over a 5-9 day period following imbibition. The reported increase in LOX activity after germination may be due to the appearance of distinct new isozymes in the emerging soybean hypocotyl and radicle, whereafter LOX activity increases in the expanding hypocotyl / radicle. The role of LOX, or specifically the products of the LOX reaction, in such tissues is not presently understood (Siedow 1991).

That LOX plays a role in plant senescence is probably the oldest of the current existing theories about its function. The reason for this is obvious, because senescence is essentially a degradative process, which includes the loss of membrane integrity. Several

studies have indicated that an increase in LOX activity is a common feature of senescing plant tissues (Siedow 1991).

2.3.5 Possible function of lipoxygenase in environmental stress responses and resistance

Membrane-associated LOX may particularly play a role in the degradation of plant membranes during senescence (Slusarenko 1996, Swammy & Sunguna 1992), but also in plant stress responses to wounding, pathogen infection and pest infestation (Galliard & Chan 1980, Krens *et al.* 1994, Mauch *et al.* 1997, Ocampo *et al.* 1986, Rosahl 1996). This also strengthens the case that LOX may have a role in the HR (Slusarenko 1996).

The products of LOX (e.g. traumatin, JA, oxylipids and volatile aldehydes) are thought to play a role in signal transduction of wound responses, as antimicrobial substances in host-pathogen interactions, as regulators of growth and development, and as aromatic compounds that affect food quality (Luning *et al.* 1995, Rosahl 1996). Felton *et al.* (1994a &b) suggested that LOX may also be an important mediator of both insect and phytopathogen resistance by producing reactive oxygen species and other oxidants. The oxidative status of host-plant tissues may undergo a rapid shift from a reduced state to a more oxidative state in response to invading pests and pathogens. These oxidative shifts result from increased activities of oxidative enzymes such as LOX, POD and polyphenol oxidase (Felton *et al.* 1994a, Hildebrand *et al.* 1988). However, analysis of the function of LOX in plants is complicated due to the presence of multiple isoforms and their

heterogeneous tissue-specificity and developmentally regulated expression patterns (Rosahl 1996).

2.3.5.1 Wounding

Plants respond to wounding by a variety of defence mechanisms, including the initiation of processes leading to wound healing (Siedow 1991). LOX is induced by wounding of any type of external biological agent, like wind (Siedow 1991), touch (Mauch *et al.* 1997) and insects (Felton *et al.*1994a & b). The production of HPOD and its accompanying decomposition products, could help stimulate the breakdown of cellular membranes leading to a localised cell death, commonly associated with the HR. Hydroperoxy radicals could also help to promote the lignification process associated with the HR (Rosahl 1996).

The oxidative stress conditions (causing membrane degradation) may be the result of not only LOX and other enzyme activities, but also of biochemical changes in the redox status of the host plant. The reactive products of the oxidative enzymes (e.g. LOX & POD) may directly or indirectly influence feeding insects. It was proposed that the products of lipid peroxidation might be toxic and function in antibiosis-based resistance (Felton *et al.* 1994b). Therefore, LOXs may function as plant defence proteins by affecting insect growth and development in a variety of direct and indirect manners. There are at least three potential roles for oxidative stress in antiherbivore defence, namely the direct oxidative injury to the herbivore, indirect injury to the herbivore through oxidative damage to dietary lipids, proteins, vitamins, antioxidants, etc., and signal transduction to elicit plant defensive systems (Felton *et al.* 1994a & b, Siedow 1991).

Traumatic acid (*trans*-2-dodecenedioic acid) was first isolated from mesocarp of wounded bean plants as the wound hormone that enhances cell proliferation at the wound site. It was also able to induce wound periderm formation in potato tuber discs. Furthermore, abscission of cotyledonary petioles of cotton explants induced by traumatic acid was accompanied by proliferation of cells in the protective layer after separation, leading to callus formation. The increase of this ω-keto fatty acid, traumatin, after wounding in some plant species and its effect on cell proliferation at wound sites, suggest that traumatin acts as a signal for wound healing (Rosahl 1996). Another product of the LOX pathway involved in the plant's response to wounding, is JA, as well as its derivatives. Like traumatin, JA is also synthesised from 13-HPOTE and plays a role as a plant growth regulator with various physiological activities (Mauch *et al.* 1997).

The rapid increase in JA levels after wounding of plant tissue, which can also be prevented by LOX inhibitors, is at least partly due to *in vivo* synthesis of JA. In various plants, LOX activity and mRNA accumulation are also induced by wounding, but this is a rather late increase and cannot account for the rapid *in vivo* synthesis of JA. Therefore, LOX isoenzymes that are constitutively expressed in leaves are more likely to be responsible for the rapid synthesis of JA after wounding (Rosahl 1996).

A function of the JA in the expression of wound response induction, is the accumulation of the PIs I and II. Accumulation of these proteins is systemically induced by insect chewing, mechanical wounding and oligouronide treatment. Exogenously applied M-JA

also induces the synthesis of PIs in unwounded plants. A model of the signal transduction events after perception of the wound signal to activation of wound-induced genes was proposed (See Fig. 2.2). According to this model, perception of specific stimuli like wounding or oligouronides leads to the activation of a membrane-bound phospholipase which release linolenic acid from the plasma membrane. Hydroperoxydation of linolenic acid by LOX yields the precursor for the synthesis of JA, which induces the expression of a number of genes whose products are possibly involved in defence reactions. Thus, the LOX pathway is also important in the induction of PI genes involved in the wounding response (Rosahl 1996).

2.3.5.2 Pathogen attack

When the plant successfully recognises a pathogen attack, defence reactions are activated (signal transduction pathway). The plant's defence response to pathogen attack comprises of the activation of various defence genes (Slusarenko 1996), synthesis of antimicrobial compounds, and often hypersensitive cell death (HR) (Rosahl 1996). Defence genes will also be responsible for antimicrobial compounds e.g., those that might be linked to the development of resistance (Slusarenko 1996).

During interactions of pathogens with their hosts, changes in LOX activity have been observed, indicating that the enzyme may be involved in the plant's response to infection (Rosahl 1996, Siedow 1991). In particular, since LOX activity increases specifically during incompatible interactions in several plant-pathogen systems, it has been argued that LOXs play a role in the development of resistance *via* the HR (Slusarenko 1996). LOX

responds differentially to various pathogen infections (rust, fungal or bacterial) between resistant and susceptible plant lines (tomato, wheat or rice) (Siedow 1991, Slusarenko 1996). The *in vivo* synthesis of two LOX isozymes during the incompatible interaction of oats and the crown rust fungus, *Puccinia coronata* f.sp. *avenae*, suggested a relationship between the presence of the LOX isozymes and resistance (Rosahl 1996).

The HR in incompatible (resistant) rust/wheat interactions is characterised by an increase in LOX activity, as early as 28 hours after penetration of the pathogen. Injection of a specific elicitor preparation from *Cladosorium fulvum* increased LOX activity in tomato leaves of resistant, but not a susceptible cultivar. LOX activity also increased following treatment of wheat leaves with an elicitor fraction from germ tubes of *Puccinia graminis tritici* (Ocampo *et al.* 1986). The fact that LOX activity increased during the HR of wheat leaves infected with avirulent races of rust fungi suggests a direct involvement of LOX in the sequence of events leading to cell death.

LOX activity is also selectively induced only in resistant plants upon fungal (in rice plants infected with the fungus *Magnaporthe grisea*) and bacterial infection (infection of French bean leaves with *Pseudomonas syringae* pv. *phaseolicola*). Induced LOX isozymes are probably located in the chloroplasts. This raises a question as to whether LOXs might have a function in the development of the HR and thus might be involved in conferring resistance. LOXs might initiate or contribute to membrane damage during the HR due to their ability to peroxidise lipids and to generate reactive oxygen species (such as H_2O_2, superoxide anions and hydroxyl radicals [Felton *et al.* 1994a]). LOX might however just act as a scavenger of potentially toxic free fatty acids. The production of several

antimicrobial substances also proceeds *via* the LOX pathway, e.g. H_2O_2 (Rosahl 1996, Siedow 1991).

It has been speculated that it is the lipid-derived signals that discriminate between wound- and pathogen-responsive isoprenoid pathways in plants. JA and M-JA modulate wound-inducible genes, through the signal transduction pathway, in plant defence responses to insects. In Solanaceous plants, there is a redirection of isoprenoid biosynthetic pathways toward sesquiterpenoid phytoalexins when wounded tissues are exposed to elicitors or isolates of pathogens that induce a HR. The changes in the levels of these compounds are correlated with changes in the activities of 3-hydroxy-3-methylglutaryl-coenzyme A reductase and of subsequent enzymes in the pathways leading to their synthesis. The cellular signals that control the expression of these and other genes that encode enzymes involved in wound- and pathogen-responsive isoprenoid pathways are still poorly understood (Choi *et al.*1994).

2.3.5.3 Contribution to membrane damage during the hypersensitive reaction

The HR is considered to be one of the mechanisms of resistance of plants against pathogens. Early physiological changes during the hypersensitive death include irreversible membrane damage (Ocampo *et al.* 1986) which is supposed to be due to alterations of membrane lipids (the peroxidation of polyunsaturated fatty acids of membranes) (Ocampo *et al.* 1986, Rosahl 1996, Siedow 1991, Slusarenko 1996).

Lipid peroxidation can be triggered by active oxygen species or is suggested to occur through enzymatic action of LOX. The rapid and transient generation of reactive oxygen species, the oxidative burst, is characteristic of the plant's early response to pathogens or elicitors and is assumed to be involved in mediating the hypersensitive cell death (Ocampo *et al.* 1986, Slusarenko 1996). H_2O_2 is involved in the fortification of cell walls (oxidative crosslinking of cell wall components), but also acts as a local trigger for programmed cell death of infected cells, and as a diffusible signal for the induction of defence genes in neighbouring cells (Rosahl 1996).

The generation of active oxygen species in pathogen/plant interactions, leading to resistance, has been observed in many systems. In potato e.g., membrane fractions from tubers undergoing a hypersensitive response induced by incompatible races of *Phytophthora infestans*, produced superoxide anions. The crucial role of superoxide radicals was demonstrated by studies showing that application of superoxide dismutase to cucumber cotyledons infiltrated with avirulent strains of *Pseudomonas syringae* reduced lipid peroxidation and thus membrane alterations. Moreover, cellular antioxidants decreased electrolyte leakage and delayed the development of the HR of tobacco inoculated with *Pseudomonas syringae* (Rosahl 1996).

The initiation of lipid peroxidation has also been proposed to be mediated enzymatically by LOX due to the concomitant increase in LOX activity and membrane lipid peroxidation during the early stages of the HR (Slusarenko 1996). In addition, the LOX-mediated hydroperoxidation of polyunsaturated fatty acids produces active oxygen or superoxide

radicals. Therefore, LOX has been envisaged to contribute to enzyme-independent lipid peroxidation by its ability to produce active oxygen species. Although it is not clear whether LOX is indeed involved in the initiation of lipid peroxidation, or is induced merely as a response to radical-induced membrane alterations, LOX appears to be able to further contribute to membrane damage (Rosahl 1996, Slusarenko 1996).

2.3.5.4 Synthesis of anti-microbial and anti-herbivore substances

Another aspect of the role of LOX in plant defence against pathogens lies in the antimicrobial nature of many products of the LOX pathway (Siedow 1991).

The primary products of the LOX reaction (e.g. 9- and 13-HPODE) on polyunsaturated fatty acids, show antifungal activity. Mono- and trihydroxy derivatives of linolenic acid that arise by lipid hydroperoxide-decomposing activities are active against pathogens at even lower concentrations than the hydroperoxy fatty acids. Other metabolites derived from the LOX pathway are reduced derivatives, and antimicrobial activity has been demonstrated at low concentrations (*trans*-2-hexenal) and higher concentrations (*cis*-3-hexenol). These compounds arise from 13-HPOTE and are produced as volatiles in significantly higher amounts during infection (Rosahl 1996).

Resistance of soybean against the fungus *Aspergillus flavus,* requires a functional LOX pathway. Application of LOX substrates to soybean cotyledons resulted in efficient inhibition of spore germination that could be partially reversed by LOX inhibitors. The active compound was identified as hexenal that acts as an antifungal substance. Its activity

towards other bacteria, fungi and arthropods has also been described (Farmer 1994). In addition, hexenal and hexanol are components of a mixture of volatiles produced by corn seedlings after insect chewing that have been suggested to serve the secondary function of attracting natural enemies of herbivores (Rosahl 1996).

JA itself has also been identified as an antifungal compound in rice. *In vitro*, both the discharge of zoospores from sporangia of *Phytophthora infestans* and the growth of mycelia in liquid medium were inhibited in a dose-dependent manner by JA and M-JA (Cohen *et al.* 1993). Schweizer *et al.* (1993) suggested that JA does not act as a general toxin, but rather exhibits an antifungal activity by inhibiting specific fungal differentiation processes.

2.3.5.5 Synthesis of signal molecules

The observation that endogenous levels of JA increase in cell cultures (of mono- and dicotyledonous plants) after elicitation by a yeast cell wall preparation, suggested that the lipid-based signal transduction pathway may be applicable against insects and plant-pathogen interactions (Farmer and Ryan 1992).

Both mono- and dicotyledonous plants responded to elicitation by the yeast cell wall preparation with a transient increase in JA levels. Furthermore, the level of linolenic acid was increased, possibly due to its release from lipids after elicitation. Jasmonates were shown to induce the accumulation of secondary metabolites and of mRNA encoding phenylalanine ammonia lyase (PAL). In parsley cells, 12-oxo-phytodienoic acid induced

the synthesis of the flavonoid apiin and accumulation of transcripts encoding enzymes of the secondary phenolic metabolism like PAL, 4-coumarate: CoA ligase and chalcone synthase as well as phytoalexin synthesis. Accordingly, it has been postulated that JA might function as an intracellular signal molecule in elicitation (Mauch *et al.* 1997, Rosahl 1996, Siedow 1991).

The rather late increase in induced LOX activity in tobacco cell cultures by glycopeptide elicitors from *Phytophthora parasitica* var. *nicotianae*, as well as the predominant production of 9-HPOTE, argues against the possible role that this induced LOX synthesises the elicitor-induced JA (Rosahl 1996). Also, according to Bohland *et al.* (1997), LOX preparations from wheat (treated with elicitors like chitin and a rust fungus elicitor) converted linolenic acid to 9-HPOTE, and not 13-HPOTE which is the precursor of JA. It is argued that a study of the activation of pre-existing LOXs, which are more likely responsible for the synthesis of JA after elicitation (as was the case in wounding studies), might elucidate the role of LOXs in the process of elicitor-induced gene expression (Bohland *et al.* 1997).

Interestingly, the plasma membrane-associated LOX in soybean appears to be stimulated by low levels of H_2O_2. This suggests that the elicitor-induced oxidative burst might directly induce the synthesis of a signal molecule by activating the LOX enzyme, thus linking components of the different signal transduction pathways that activate sets of defence genes (Farmer & Ryan 1992). It seems as if fatty acids can replace H_2O_2 as a co-substrate in some peroxidase-catalysed reactions (Slusarenko 1996).

In plants, a functional significance of jasmonates in the signal transduction leading to resistance against pathogens has not been demonstrated. Chemically induced or genetically based resistance of barley plants against powdery mildew is not correlated with higher levels of jasmonates. In potato, jasmonates do not induce a HR in aged tuber disks, possibly because there are two distinct arachidonic acid- and jasmonate-response pathways. Therefore, it has been speculated that jasmonates might exert an alternative function in signalling by conditioning plant tissue for optimal response to elicitors or pathogens (Rosahl 1996).

2.3.5.6 Metabolism of fatty acid elicitors

The discovery that the LOX substrates arachidonic acid and eicosapentaenoic acid act as elicitors of the HR, led to the postulation of another direct involvement of LOX in the signal exchange during pathogenesis. A possible involvement of LOX in mediating this response was postulated, based on the finding that the LOX inhibitor [i.e. salicylhydroxamic acid (SHA)] prevents the accumulation of sesquiterpenes elicited by arachidonic acid. Metabolites of arachidonic acid are rapidly produced by LOX catalysed reactions after application of the elicitor. However, there are conflicting data on the elicitation capabilities of these hydroperoxy-eicosatetraenoic acids. A reduction in the amount of HPOD from the substrate arachidonic acid was observed, suggesting that the metabolism of arachidonic acid by LOX might not necessarily be for elicitor activity. In contrast, 5-hydroperoxyeicosatetraenoic acid was at lower concentrations than

arachidonic acid itself. Thus, it is not clear whether LOXs are indeed necessary for elicitor activity of arachidonic acid (Rosahl 1996).

2.3.6. Products of lipoxygenase activity affecting the plant/pathogen interactions

In many plant species, compounds such as JA, traumatic acid, or ABA have been demonstrated to have dramatic effects on plant growth and development (Mauch *et al.* 1997).

2.3.6.1 Jasmonic acid

JA [3-oxo-2-(2 -*cis*-pentenyl)-cyclopentane-1-acetic acid] is synthesised from linolenic / linoleic acid (Dixon *et al.* 1994, Sticher *et al.* 1997). In a LOX catalysed reaction linolenic acid is converted to a 13-HPOD derivative which is subsequently converted to 12, 13-epoxy-linolenic acid, ultimately forming JA (See LOX pathway Fig. 2.4) (Felton *et al.* 1994b, Sticher *et al.* 1997, Slusarenko 1996). Therefore, production of JA is regulated by the availability of the initial substrate linoleic acid (Dixon *et al.* 1994).

Jasmonates occur through the entire plant, with the highest activity in growing tissues (growing point, ripening fruits, etc.). Jasmonates exhibit many of the characteristics of a plant growth regulator hormone (Dixon *et al.* 1994, Slusarenko 1996), meaning it is active at concentrations of 10ng to 3μg per gram fresh mass (although induced levels were found to be up to 38mg g^{-1} fresh mass). Low concentrations of JA (10^{-4} - 10^{-6}M) exhibited

inhibiting properties (Herrmann *et al.* 1987, Meyer *et al.* 1984, Parthier 1991, Ravnikar *et al.* 1992).

Jasmonates are best known as growth inhibitors that inhibit seedling growth and stimulate ageing of leaves. The jasmonate-induced leaf ageing is associated with the degradation of chlorophyll, and the degradation of rubisco (RuBPCase), increase in protease and POD activities, and reduction of photosynthetic activities. Other physiological processes affected by JA(s) include root growth, tuber formation, tendril coiling and stomatal opening (Mauch. *et al.* 1997, Sticher *et al.* 1997).

As mentioned, JA and M-JA are possible signalling molecules, by inducing the synthesis of several plant defence proteins (or chemicals) which accumulate in the nucleus, cytosol, chloroplasts and vacuoles, but not in the mitochondria (Rosahl 1996). These defence proteins include PIs, chitinase (CHT), POD, LOX, PAL, and alkaloids (Dixon *et al.* 1994, Slusarenko 1996).

Endogenous JA-levels are increased by external stimuli, like wounding (Dixon *et al.* 1994, Mauch *et al.* 1997, Sticher *et al.* 1997), mechanical forces and osmotic stress. Wounding actually induces local and systemic accumulation of JA and M-JA, therefore JA and M-JA might also act as secondary messengers in SAR (Sticher *et al.* 1997). M-JA is believed to be actually more reactive than JA, probably because it is more volatile. It has also been speculated that M-JA is involved in wound-induced signalling, while JA is involved in pathogen-induced resistance. Therefore, two distinct signalling pathways are operating: one for wound responses and the other for pathogen response (Slusarenko 1996). There

is, however, controversy as to whether JA and M-JA do induce host defence responses and if they can be associated with resistance. Some scientists also indicated that JA might not be derived from the LOX pathway (in plant resistance) (Slusarenko 1996).

JA affects its own biosynthesis, because low concentrations of gaseous M-JA induced accumulation of a 94 kDa storage protein, believed to be a LOX. Therefore, M-JA seems to induce the accumulation of LOX (Felton *et al.* 1994b, Mauch *et al.* 1997). JA might be a positive regulator of its own synthesis and JA induced LOX might be part of a signal amplification mechanism, either by a negative feedback regulation mechanism or the encoded proteins do not feed into the pathway leading to jasmonate production (Mauch *et al.* 1997). JA action can be reversed or counteracted by cytokinins and salicylic acid (SA) (Sticher *et al.* 1997). Interactions with other hormones are not yet known.

2.3.6.2 Traumatin

Another compound in the linolenate cascade, formed by LOX activity, is the wound hormone, traumatin. This compound has been reported to mimic the physiological effects seen upon wounding of plant tissues, including cell division and subsequent callus formation in cucumber hypocotyl assays. Traumatin can readily be oxidised non-enzymatically to traumatic acid by oxidation of the aldehyde. It appears that both of these compounds are involved in plant responses to wounding (Siedow 1991). Some precursors of traumatin, existing as volatile aldehydes, are also formed in the linolenate cascade, and might also be physiologically active in plants (Slusarenko 1996). The possibility that traumatic acid and/or traumatin are primary agents of signal transduction in plant wound

responses, needs clarification. It is uncertain how traumatic acid or traumatin might be associated with any role played by LOX in rapidly growing tissues (Siedow 1991).

2.3.6.3 Abscisic acid

ABA has regulatory roles in physiological processes, like stomatal closure, bud dormancy, seed dormancy, seed development and germination, abscission (Moore 1989), shoot growth inhibition, root growth stimulation (Creelman *et al.* 1992a), and several resistance responses (Veisz *et al.* 1996).

Little is known about the biosynthetic pathway of ABA, except that it is ultimately derived from mevalonic acid (Creelman *et al.* 1992a). There is, however, evidence that the plant growth regulatory substance, ABA, might be synthesised from xanthoxin (Slusarenko 1996). LOX is therefore indirectly involved in the synthesis of ABA from linoleate *via* the LOX product, xanthoxin (Creelman *et al.* 1992a & b, Slusarenko 1996).

LOX inhibitors (5,8,11-eicosatriynoic acid, nordihydroguaiaretic and naproxen) inhibited the accumulation of stress-induced ABA in soybean seedlings, *Glycine max* L. (Creelman *et al.* 1992a, Slusarenko 1996). These results suggested that the *in vivo* oxidative reaction involved in ABA biosynthesis required the activity of a non-heme oxygenase having LOX-like properties (Fig. 2.5) (Creelman *et al.* 1992a & b). It has also been said that ABA induces membrane depolarisation (Pugin & Guern 1996), and this is also a possible function of LOX.

In host-pathogen interactions, raised ABA levels sometimes correlated with resistance, although there are exceptions. Low temperatures induced ABA in several wheat species, causing the freezing resistance to increase (Veisz *et al.* 1996). It was also reported that ABA enhanced phytoalexin accumulation in *Phaseolus vulgaris* cell suspension cultures. Thus, induced ABA content has been shown to be associated with physiological induced resistance of *Phaseolus vulgaris* to the pathogenic fungus *Colletotrichum lindemuthianum.* In this regard, it has been observed that ABA levels also increased concurrently with local necrosis (Slusarenko 1996). Therefore, since LOX increases in SAR, and ABA synthesis might depend on LOX, this might explain the importance of LOX in acquired resistance.

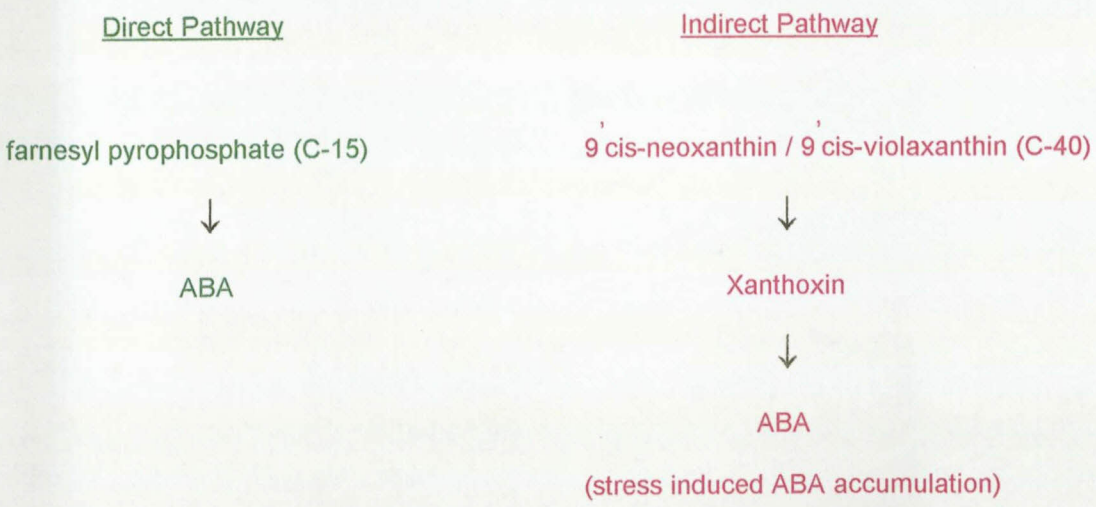

Figure 2.5 Direct and indirect pathways for biosynthesis of ABA (Creelman *et al.* 1992a, Creelman & Zeevaart 1984).

In this study our main aim was to investigate the possible role of LOX in the resistance mechanism of wheat against the RWA. A key question was also whether possible LOX

involvement would apply to different RWA resistance genes. These results might give us insight in the signalling events leading to the induction of the secondary defence products, of which some were identified during previous studies (Van der Westhuizen & Pretorius 1996, Van der Westhuizen *et al.* 1996, Van der Westhuizen *et al.* 1998a & b).

Studies indicated that the HR is involved in the resistance response of wheat against the RWA, which include the induction PR-proteins (chitinase and β-1,3-glucanase), POD, phenolics, increased respiration rates, etc. (Botha *et al.* 1998, Van der Westhuizen & Pretorius 1996, Van der Westhuizen *et al.* 1996, Van der Westhuizen *et al.* 1998a & b). These "downstream" products were selectively induced in resistant wheat cultivars to much higher levels than in susceptible cultivars, which indicated their involvement in the resistance mechanism - although it is obvious that "upstream" events are responsible for the differential induction of these possible defence (resistance) related products. The primary causes of resistance or susceptibility have therefore to be sought in upstream signalling and eliciting events, and this is where LOX is thought to play a role.

In cell cultures of both mono- and dicotyledonous plants, stressed with yeast cell wall preparations, products of LOX activity (JA) were found to be involved in signalling events eliciting accumulation of secondary metabolites (PAL, POD, etc.) (Farmer & Ryan 1992, Dixon *et al.* 1994, Slusarenko 1996). By using LOX inhibitors, we tried to establish whether LOX activity had any influence on the RWA's induced secondary metabolites ("downstream" metabolites like POD and β-1,3-glucanase) in wheat. We also tried to determine which of the end products (JA and ABA) in the LOX pathway might be the

signal molecule responsible for eliciting "downstream" events. Hopefully these results will help us to understand the events of the signal transduction pathway leading to the resistance response in wheat against the RWA.

Chapter 3

Materials & Methods

3.1 Materials and methods

3.1.1 Biological material

Russian wheat aphids (RWAs) (*Diuraphis noxia* Mordvilko) and wheat (*Triticum aestivum* L.) cultivars, resistant and susceptible to the RWA, were supplied by the Small Grain Institute, Bethlehem, South Africa. Resistant cvv Tugela (Dn1) (PI137739), Tugela (Dn2) (PI262660), Tugela (Dn5) (PI294994) (Cilliers *et al.* 1992, Du Toit 1989, Ma *et al.* 1998), and the susceptible cv Tugela (a winter wheat) were grown in a greenhouse at ~20^{0}C during the night and ~25^{0}C during the day (± 2^{0}C).

Seeds were planted in 10 cm deep rectangular buckets, containing a mixture of sandy and loam soil, that were watered every second day.

For infestation, approximately 100 aphids of various instars were gently brushed off the culture plants and transferred to a glass vial. Seedlings were infested at a two leaf stage (10-14 days after planting), by evenly scattering them from the vial over ~80 plants (in ratio plant:aphid of 1:~10). Additionally, infested leaf pieces were also placed on top of the seedlings to ensure a high infestation. All buckets were covered with sterilised nets. A one hour period was given for the aphids to settle on the seedlings, after which the infestation time lapse started (h.p.i. / d.p.i.).

To study the local and the systemic accumulation of lipoxygenase (LOX), Tugela DN plants were infested with RWAs at the third leaf stage in the following way:

About 20 aphids were enclosed in a small cage, and forced to feed on the second leaf of each plant. Second leaves as well as the remaining leaves of infested and uninfested plants were harvested separately 8 d.p.i. for assay purposes.

Initially only second leaves and/or the whole seedlings (except for roots) were harvested at time intervals 2, 10, and 14 d.p.i. for the preliminary investigations to investigate the method for LOX determination. For all other experiments that followed, the whole seedlings were harvested.

For time studies, seedlings were harvested at indicated times after infestation (h.p.i.). Harvested leaves were cut into 2cm pieces, weighed and frozen in liquid nitrogen in the shortest time possible to eliminate a wounding effect. Frozen leaves were stored at -20^{0}C for further assay purposes for all experiments.

3.1.2 Chemicals

Linoleic acid, salicylhydroxamic acid (N,2-dihydroxybenzamide), n-propyl gallate (3,4,5-trihydroxybenzoic acid n-propyl ester), piroxicam (4-hydroxy-2-methyl-3-[pyrid-2-ylcarbamoyl]-2H-1,2-benzothiozine 1,1-dioxide), and jasmonic acid were purchased from Sigma*. All other chemicals used, were of analytical grade.

3.2.1 Lipoxygenase extraction

The extraction buffer consisted of 1,4-dithiothreitol (0.1542mg ml^{-1}) and 1mM EDTA in 0.1M sodium borate buffer (pH8.8). A quantity of Dowex (Dowex 1x2 [200-400] anion-

*Sigma Chemical Company, St. Louis, Missouri, USA

exchange resin) was regenerated as follows: Dowex (~5g) was washed with distilled water, whereafter it was boiled at 100^{0}C for 30min. Washed Dowex was then stirred in 0.5M HCl (30min.), followed by 0.5M NaOH (30min.) and lastly in 0.5M NaCl (30min.). After each step, the Dowex was washed thoroughly with 2x distilled water. Regenerated Dowex was then stored in 0.1M sodium borate buffer (pH8.8) until needed for further use in making crude enzyme extracts.

A quantity of the regenerated Dowex (~500g for ~8g of plant material) was further prepared by first filtering it under suction through a sintered glass funnel. Filtered Dowex was resuspended and stirred in a portion of extraction buffer (15 min.), followed by suction filtering again. This pretreated Dowex was used in the extraction procedure.

Frozen leaf pieces were ground in cold extraction buffer (ratio 1:18), containing freshly pretreated Dowex anion exchange resin, insoluble PVP and acid washed sand (100mg, 100mg and 400mg respectively for each ~0.7g plant material) in a precooled mortar (Moerschbacher 1988). The crude extract was centrifuged at 15 000*g* at 0^{0}C (20 min.) and the clear supernatant used for further assays.

3.2.1.1 Assay of lipoxygenase activity

LOX activity was determined according to Moerschbacher (1988). All assays were carried out at 30^{0}C in a total volume of 1150µl reaction solution, containing 0.1M citrate buffer (pH6.2), 0.22mM linoleic acid, and 50µl crude LOX extract. The oxidation of

linoleic acid was followed spectrophotometrically at 234nm as an indication of LOX activity. LOX activity was expressed as nmole HPOD mg^{-1}protein $min.^{-1}$.

3.2.1.2 Lipoxygenase characterisation

For all characterisation studies fresh Tugela DN leaves (three leaf stage: 10 d.p.i) were used. The same extraction procedure was followed as described in section 3.2.1.

To determine the effect of cold storage on the stability of a crude LOX extract, one extract was stored in 10% (v/v) glycerol at -70^0C and another without glycerol at -20^0C. Lastly, a crude LOX extract was kept on ice. LOX activity was determined at different time intervals of storage.

Citrate buffer (0.1M) at pH's 4.4, 5.2, 5.6, 6.0, 6.2, 6.4, and 6.8 was used to determine the pH for optimum LOX activity.

Two concentrations (1.0M and 0.5M) $MgCl_2$, $CaCl_2$ and KCl (100µl in a total reaction solution volume of 1150µl) were used to determine the effect of divalent (Mg^{+2} and Ca^{+2}) and monovalent (K^+) cations on LOX activity.

LOX activity was determined at different temperatures to establish the optimum temperature for the LOX catalysed reaction *in vitro.*

To determine the Km-value of LOX, the activity was determined at different linoleic acid concentrations (0.2mM, 0.4mM, 0.8mM, 1.6mM, 2.5mM, 3.1mM, and 6.3mM). An Eadie-Hofstee graph (Mathews & Van Holde, 1990) was plotted, and the slope was used to calculate Km.

3.2.2 Assay of peroxidase activity

POD activity was determined as described by Moerschbacher (1988). The POD crude extract was prepared as described in section 3.2.1. The reaction is based on the use of guaiacol as a substrate in the presence of H_2O_2. Assays were carried out at 30^0C. The assay solution consisted of 1ml 0.1M potassium phosphate buffer containing 18mM guaiacol (pH5.8), 2.5% (v/v) H_2O_2, and 10µl crude extract. The rate at which absorption changed at 470nm was obtained ($\Delta A\ min^{-1}$), and the linear part of the curve was used to calculate the enzyme activity. Peroxidase activity was expressed as nmole tetraguaiacol mg^{-1} protein $min.^{-1}$.

3.2.3 Assay of β-1,3-glucanase activity

The activity of β-1,3-glucanase was assayed using the method described by Fink *et al.* (1988). The assay mixture contained 250µl laminarin (2mg ml^{-1} distilled water), 50mM sodium acetate buffer (pH4.5) and 10µl enzyme extract (see section 3.2.1) in a total volume of 500µl. After a 10 minute incubation period at 37^0C, 0.5ml of Somogyi (1952) reagent was added and the mixture was heated at 100^0C for 10 min. A Nelson (1944) reagent (0.5ml) was added after cooling the reaction solution (5min. in cold water). The optical density was measured at 540nm.

A standard curve, relating the absorbance (A_{540nm}) to glucose concentration, was used to calculate the enzyme activity. β-1,3-Glucanase activity was expressed as mg glucose mg^{-1} protein $min.^{-1}$.

3.2.4 Protein determination

Protein content was determined using the dye-binding assay technique (Bradford 1976) with bovine γ-globulin as standard. The Bio-Rad Model 3550 Microplate Reader was used for this purpose as described by Rybutt and Parish (1982).

3.3.1 In vitro inhibition of lipoxygenase, peroxidase and β-1,3-glucanase activities

Infested Tugela DN wheat leaves (three leaf stage; 10 d.p.i.) were used for inhibition determinations. The same extraction procedure was followed as described in section 3.2.1, but the assay mixture additionally contained 10µl of stock inhibitor solution. Inhibitors, piroxycam (PC), salicylhydroxamic acid (SHA) and n-propyl gallate (n-PG) used, were dissolved in sodium chloride solution (NaCl was in a 1:1 molar ratio relationship to the OH-groups of the inhibitor). Stock solutions of PC used were 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 2.0, 4.0, 5.0 and 10.0mM, while those of SHA and n-PG were 0.63, 6.25, 12.5, 25.0, 50.0, 100.0 and 200.0mM. LOX, POD and β-1,3-glucanase activities were determined according to sections 3.2.1.1, 3.2.2 and 3.2.3 respectively.

3.3.2 In vivo inhibition of lipoxygenase, peroxidase and β-1,3-glucanase activities

Infested seedlings (three leaf stage; 10 d.p.i.) were cut off just above the soil level and placed in water whereafter 3mm stem pieces were cut off under water. These plants were then transferred to different inhibitor solutions (dissolved as described in section 3.3.1), placed under a light (100W tungsten bulb) and left in a well ventilated room for 2 hours. Inhibitor solutions used (2ml total volume) were: 1.0, 2.0, 4.0, 5.0, 6.0, 8.0, 10.0 and 20.0mM PC; 10.0, 20.0, 40.0, 50.0, 60.0, 80.0, 100.0, 120.0, 150.0 and 200.0mM SHA; and 10.0, 20.0, 50.0, 60.0, 100.0, 120.0, 150.0 and 200.0mM n-PG. Subsequently, wheat plants were removed from inhibitor solutions, weighed, frozen in liquid nitrogen and stored at -20^{0}C. LOX, POD and β-1,3-glucanase assays were done according to procedures described in sections 3.2.1.1, 3.2.2 and 3.2.3.

To determine the influence of LOX activity inhibition on *in vivo* JA concentrations, cut off seedlings were placed in 20mM PC under the same conditions as described above. Freezing procedures were done as described in section 3.4.1, while JA was extracted according to the described procedure in section 3.4.2.

3.4.1 Extraction of jasmonic acid and abscisic acid

Infestation and harvesting procedures were done as described in sections 3.1.2.2 and 3.1.2.3 respectively. Leaf extracts were made according to Wasternack *et al* (1995). Frozen leaf segments were ground into a pulp in 70% (v/v) aqueous methanol (in a ratio fresh mass : volume methanol of 1 : 30) and shaken for 4 hours at 4^{0}C. The pulp was

then centrifuged (10 000*g*) for 10min at 0^0C. Supernatants were passed through C_{18}-reversed phase cartridges equilibrated with 70% methanol, and the eluate was concentrated to dryness by vacuum evaporation. The residues were resuspended in 30% (v/v) aqueous methanol, and pH adjusted to pH3 with acetic acid. This acidified solution was passed through a C_{18}-cartridge equilibrated with acidified 30% methanol (pH 3.0). The acidic compounds were eluted with a mixture of methanol and ethyl acetate (1:1) whereafter the eluate was loaded onto the HPLC. Control experiments with abscisic acid (ABA) and jasmonic acid (JA) standards confirmed the reliability of this method.

3.4.2 Detection of jasmonic acid and abscisic acid by HPLC

Reversed phase chromatography was used as described by Meyer *et al.* (1984). A Spectra System (P4000) HPLC equiped with a spectrophotometrical (UV100) detector was used. A Luna column (250 x 4.6mm) packed with Luna 5µ C18 reverse phase medium was eluted with 70% (v/v) methanol containing 0.1% (v/v) phosphoric acid at 25^0C. JA was separated at a flow rate of 0.6ml $min.^{-1}$ and monitored at 206nm, while ABA was separated at a flow rate of 0.5ml $min.^{-1}$ and monitored at 210nm.

3.4.3 <u>In vitro</u> effect of jasmonic acid and abscisic acid on lipoxygenase, peroxidase and β-1,3-glucanase activities

Assay procedures for LOX, POD and β-1,3-glucanase activities were performed as described in sections 3.2.1.1, 3.2.2 and 3.2.3 respectively, but JA or ABA (10µl of JA or ABA stock solutions) was added to the assay mixture while keeping the total reaction

volume constant. JA stock solutions were 0.099, 0.99, 9.9, and 99mM and ABA stock solutions were 0.0004, 0.004, 0.04, 0.4 and 4.0mg ml^{-1}.

3.4.4 In vivo effect of jasmonic acid on lipoxygenase, peroxidase and β-1,3-glucanase activities

Tugela and Tugela DN plants (three leaf stage, 10 d.p.i.) were intercellulary injected with JA (stock solutions of 1.0, 2.0, 5.0, 10.0 and 20.0μM JA) in the second leaf. Injections were done with an adjustible needle syringe. The needle's length was adjusted beforehand to ensure intercellular injection (See Fig. 3).

A constant volume JA sample was injected in each leaf by limiting the intracellular flow movement to a length of 6.5cm.

The injected leaf pieces (6.5cm) were harvested 48 hours post injection, weighed (0.2-0.4g fresh mass) and frozen in liquid nitrogen, whereafter it was stored at -20^{0}C. LOX, POD and β-1,3-glucanase assays proceeded as described in sections 3.2.1.1, 3.2.2 and 3.2.3 respectively.

3.5 Processing of results

Experiments were repeated at least twice and determinations were done in triplicate (n=3). The plotted values represented the average of the triplicate values. Standard deviations were calculated (n=3).

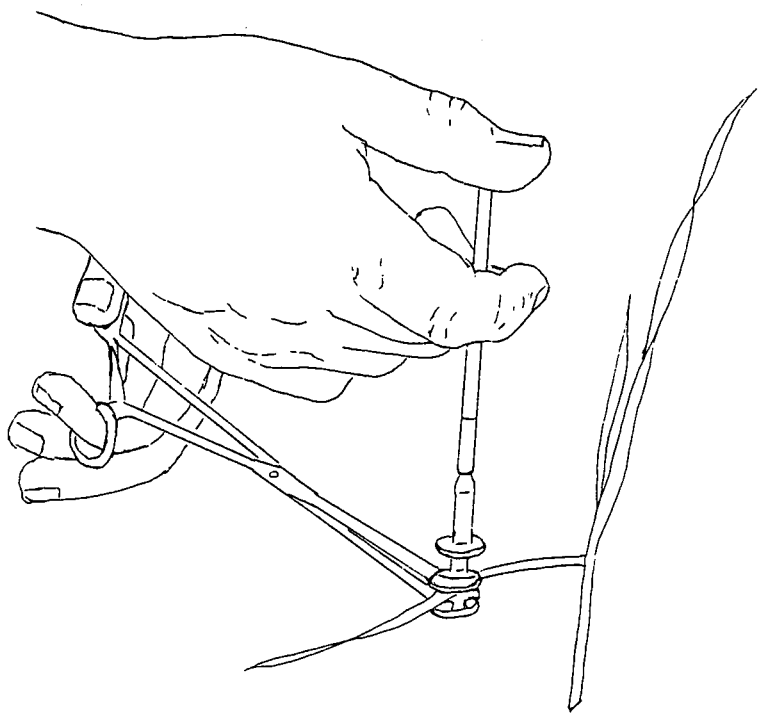

Figure 3 Apparatus used for intercellular injection of plants.

Chapter 4

Results

4.1 Preliminary investigations to optimise the method for lipoxygenase determination

4.1.1 Plant samples for enzyme extract

To establish which part of the plant should be harvested to obtain maximum LOX activity, the activity was followed as infestation proceeded in the second leaves and the entire plants of uninfested and infested susceptible (Tugela) and resistant (Tugela-DN) cultivars. However, LOX extracts from entire seedlings had much higher LOX activity than extracts from second leaves (Fig. 4.1).

It was found that LOX activity was selectively induced in the resistant wheat cultivar, Tugela-DN, after RWA infestation. The LOX activity in uninfested resistant, susceptible and infested susceptible cultivars remained low (25-75 nmol HPOD mg^{-1} prot. $min.^{-1}$), while LOX activity was induced up to ~375nmol HPOD mg^{-1} prot. $min.^{-1}$ in the entire seedling sample and up to ~160nmol HPOD mg^{-1} prot. $min.^{-1}$ in the second leaf sample (Fig. 4.1).

4.1.2. Effect of freezing plant material and cold storage of plant extracts on lipoxygenase activity

Plant material that was frozen in liquid nitrogen, before enzyme extractions were made, showed a 67% reduction in activity in comparison to the unfrozen samples. Crude LOX extracts which were not preserved in glycerol, showed a total loss in LOX activity after it

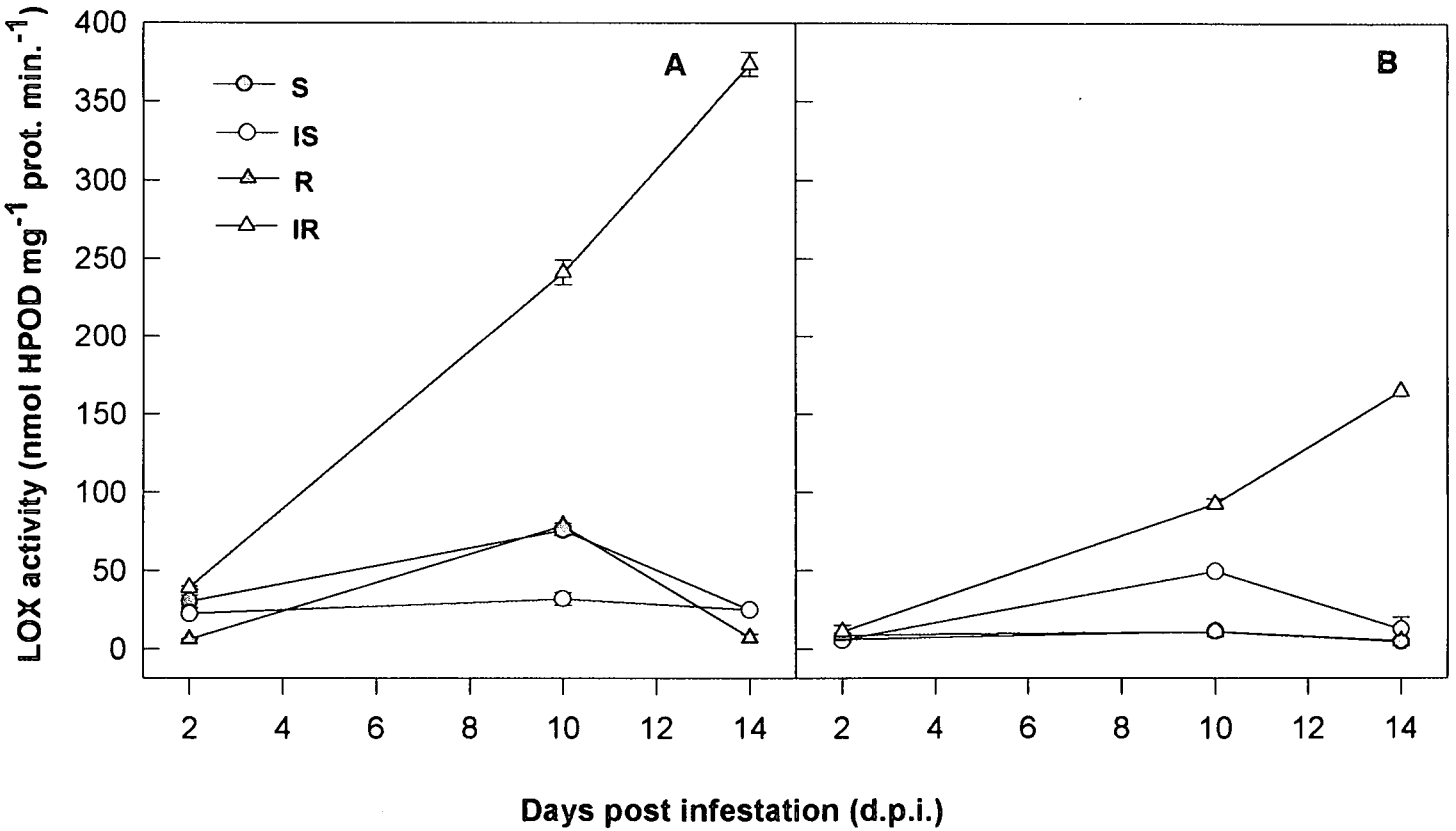

Fig. 4.1. Effect of RWA infestation on LOX activity of the entire plant (A) and of the second leaf (B) as infestation proceeded. (S, uninfested susceptible Tugela; IS, infested susceptible Tugela; R, uninfested resistant Tugela-DN; IR, infested resistant Tugela-DN; HPOD, linoleic acid hydroperoxy derivative). Values are means ± standard deviation (SD) (n=3).

was stored at -20^{0}C. LOX extracts frozen in 10% glycerol, showed a ~40% activity loss in comparison to the fresh extracts which were not frozen after extraction (Fig. 4.2a).

The 'half-life' of LOX extracts were determined by measuring the activity over a time period of 48 hours at various time intervals, while it was kept at 0^{0}C. This was done to determine the time period in which assays should be completed. Both crude extracts (frozen and unfrozen) showed a decrease in LOX activity of nearly 60% (decreased from ~3000nmol HPOD mg^{-1} prot. min.$^{-1}$ to ~1250nmol HPOD mg^{-1} prot. min.$^{-1}$) within 2 hours after extraction, whereafter the activity decreased more gradually (Fig. 4.2b).

4.1.3 Partial characterisation of lipoxygenase

4.1.3.1 pH Optimum for lipoxygenase activity

The optimum LOX activity was recorded at a pH of 5.6. More than 80% of the maximum activity was maintained at a pH interval between 5.2 and 6.4 (Fig. 4.3a).

4.1.3.2 Effect of substrate concentration on the rate of the lipoxygenase catalysed reaction

To determine whether linoleic acid was a suitable substrate for further studies, the LOX affinity for linoleic acid had to be determined. LOX's Km-value, when using linoleic acid as substrate, was ~5.8 x 10^{-4}M, with a V_{max} of 370nmol HPOD mg^{-1} prot. min.$^{-1}$ (~791nmol HPOD g^{-1} fresh mass min.$^{-1}$) (Fig. 4.3b).

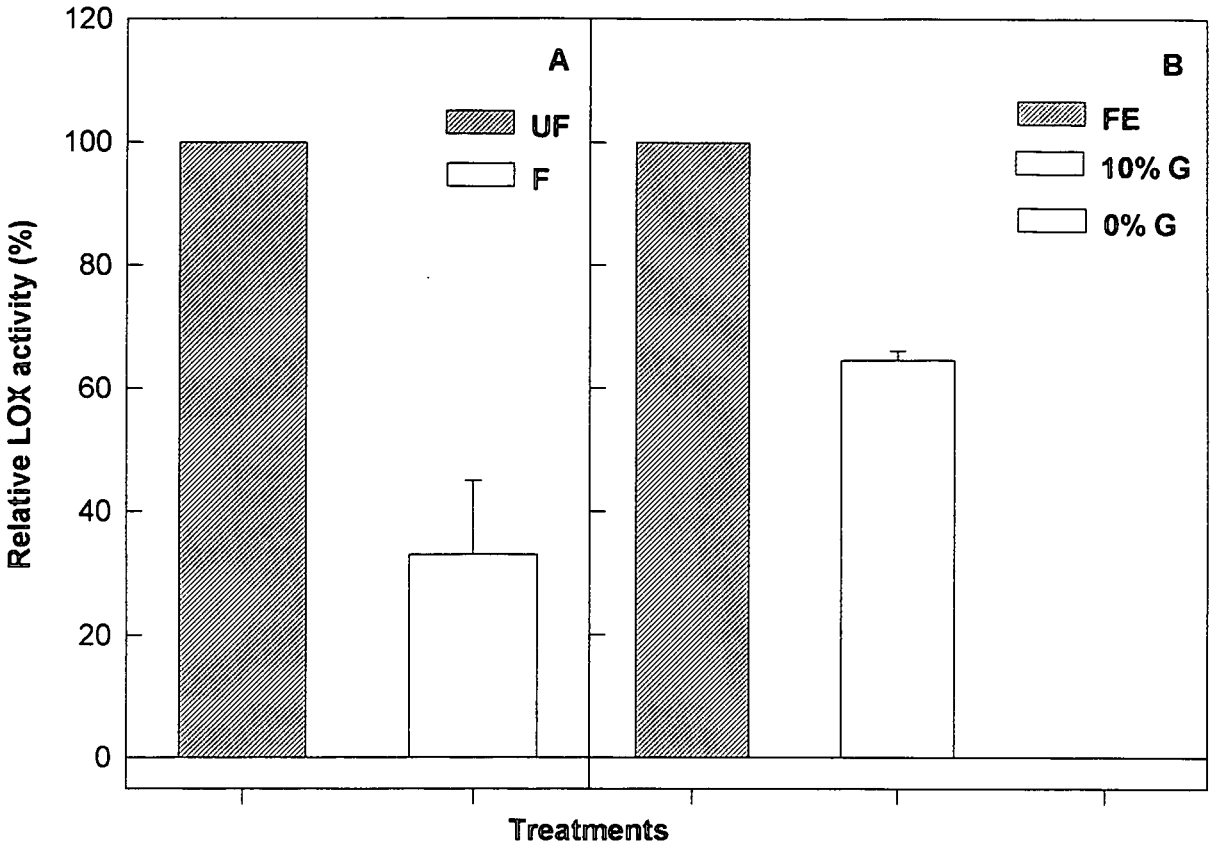

Fig. 4.2a. Effect of freezing plant material in liquid nitrogen (A) and cold storage of plant extract (-20^{0}C) in glycerol (B) on LOX activity of infested (10 d.p.i.) Tugela-DN plants. (UF, unfrozen leaves; F, frozen leaves; FE, fresh LOX extract; 10% G, LOX extract in 10% glycerol; 0% G, LOX extract in 0% glycerol) Activities of untreated samples were taken as 100% and the activities of treated samples were expressed as fractions (%) thereof. Values are means ± standard deviation (SD) (n=3).

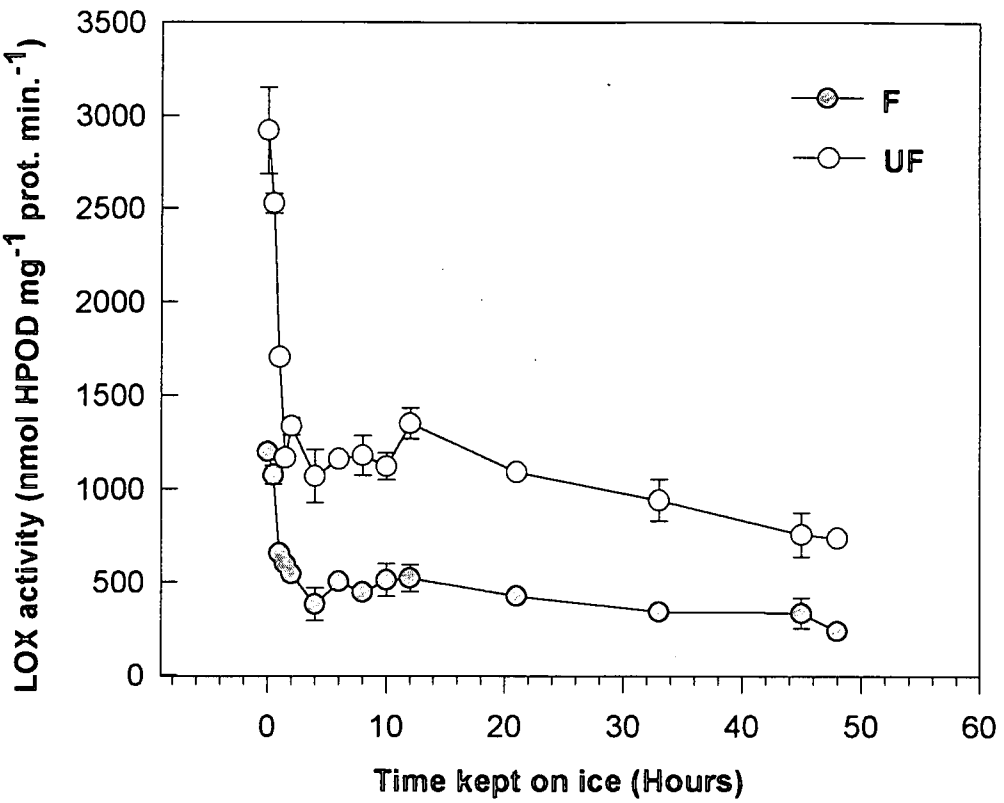

Fig. 4.2b. Effect of storage at 0°C on the LOX activity of an extract from fresh leaves (UF) and from leaves frozen in liquid nitrogen (F) of infested (10 d.p.i) Tugela-DN plants. (HPOD, linoleic acid hydroperoxy derivatives) Values are means ± standard deviation (SD) (n=3).

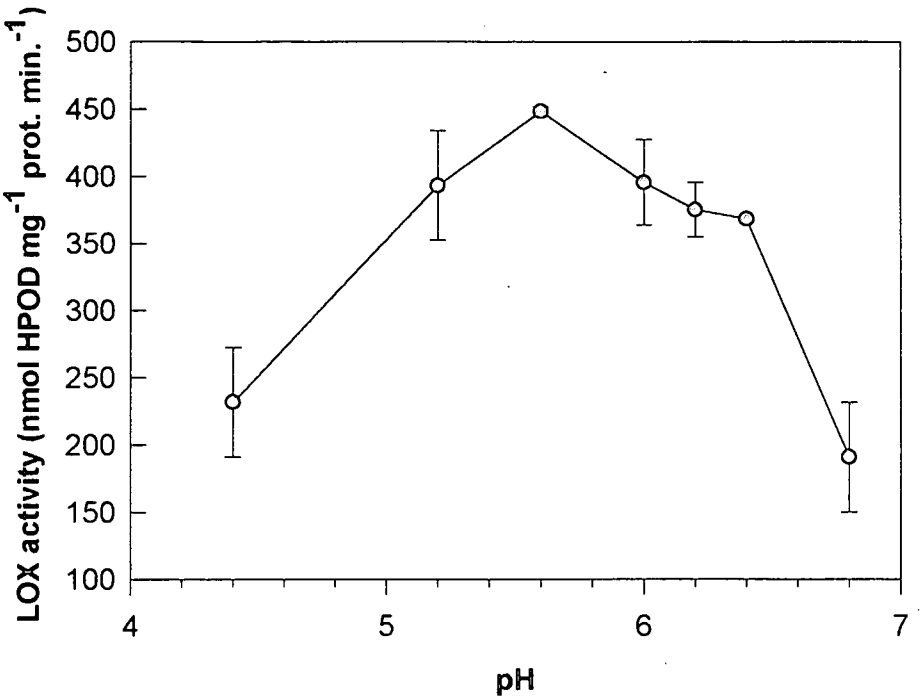

Fig. 4.3a. Effect of pH on LOX activity of a leaf extract from Tugela-DN (10 d.p.i.). (HPOD, linoleic acid hydroperoxy derivative) Values are means ± standard deviation (SD) (n=3).

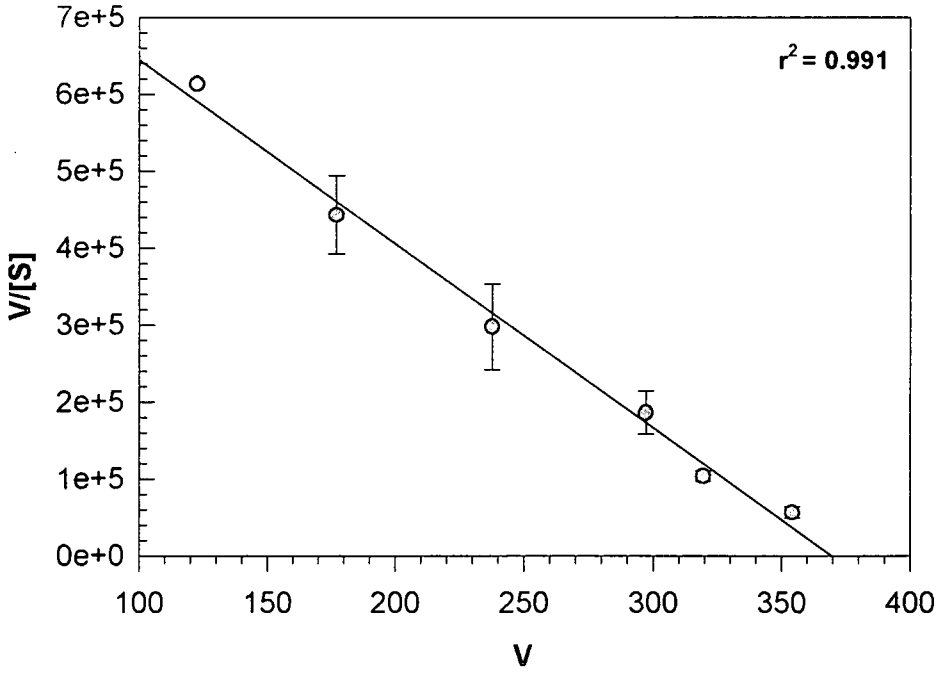

Fig. 4.3b. Eadie-Hofstee curve of the effect of substrate concentration [S] on the rate (V) of LOX (crude extract from Tugela-DN, 10 d.p.i.) catalysed reaction. Values are means ± standard deviation (SD) (n=3).

4.1.3.3 Optimum temperature for the lipoxygenase activity

Optimum LOX activity was recorded at 40^0C. Between temperatures 30^0C and 40^0C, an activity of 95-100% was maintained (Fig. 4.3c).

4.1.3.4 Effect of cations on the lipoxygenase activity in vitro

Cations, 4.3mM Mg^{2+} and 8.6mM K^+, did not affect LOX activity, while 8.6mM Mg^{2+} and 4.3mM K^+ seemed slightly inhibitory. Ca^{2+} was the most inhibitory, inhibiting LOX activity between 20% and 35% (150 and 220nmol HPOD mg^{-1} prot. $min.^{-1}$) at concentrations of 8.6 and 4.3mM respectively (Fig. 4.3d).

4.2 Effect of Russian wheat aphid infestation on peroxidase activity of wheat cultivars

POD activity remained relatively low for uninfested resistant and susceptible, and infested susceptible wheat cultivars. Infested resistant Tugela-DN was selectively induced at 48 h.p.i., reaching five times the initial activity at 288 h.p.i. (Fig. 4.5b).

4.3 Effect of Russian wheat aphid infestation on lipoxygenase activity of wheat cultivars containing different resistant genes

As a result of our findings in preliminary studies (Fig. 4.1), LOX activities were determined over a prolonged infestation period. LOX activity was determined in extracts from RWA infested and uninfested, resistant (Tugela-DN) and susceptible (Tugela) wheat cultivars at post infestation time intervals as indicated. Except for reductions in

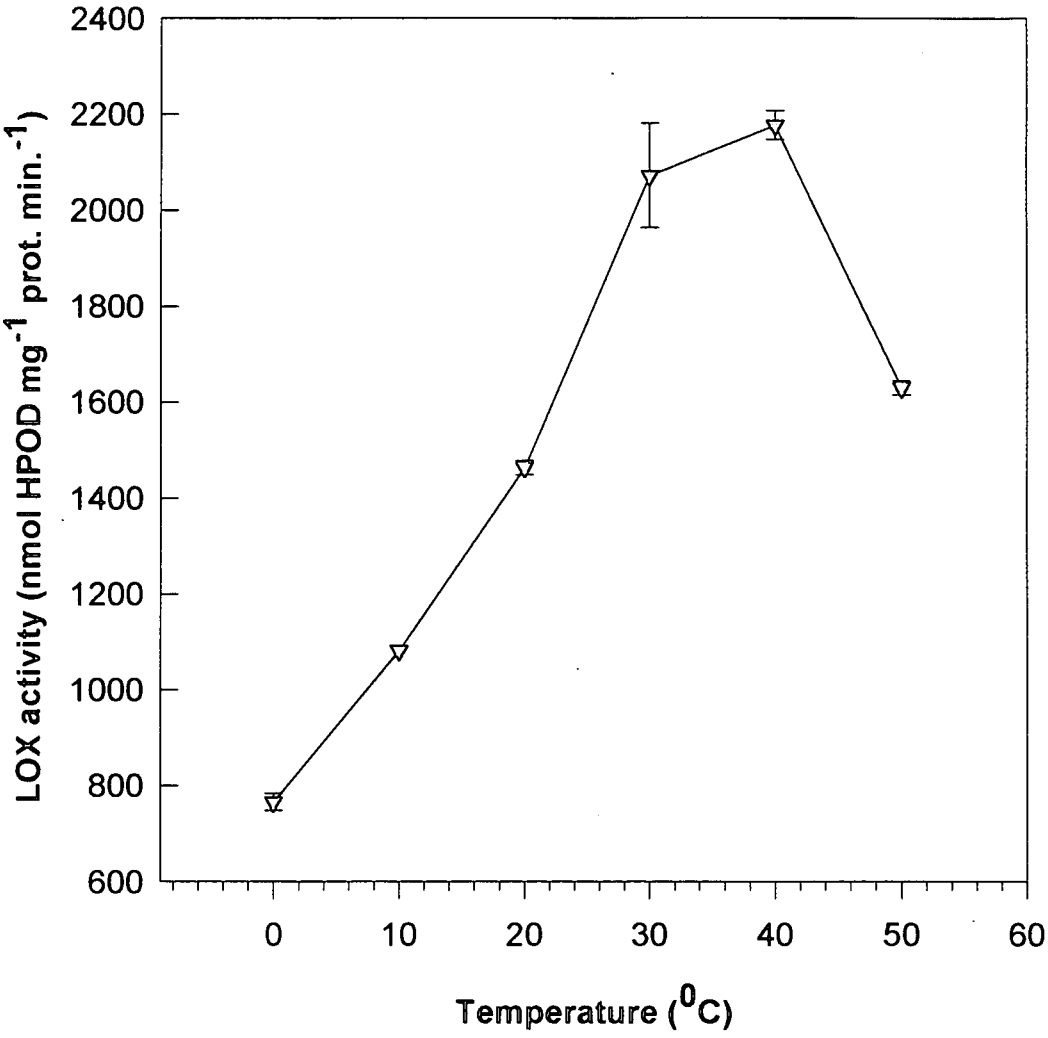

Fig. 4.3c. Effect of temperature of the test solution on the LOX activity of a crude extract from Tugela-DN (10 d.p.i.). (HPOD, linoleic acid hyro-peroxy derivative) Values are means ± standard deviation (SD) (n=3).

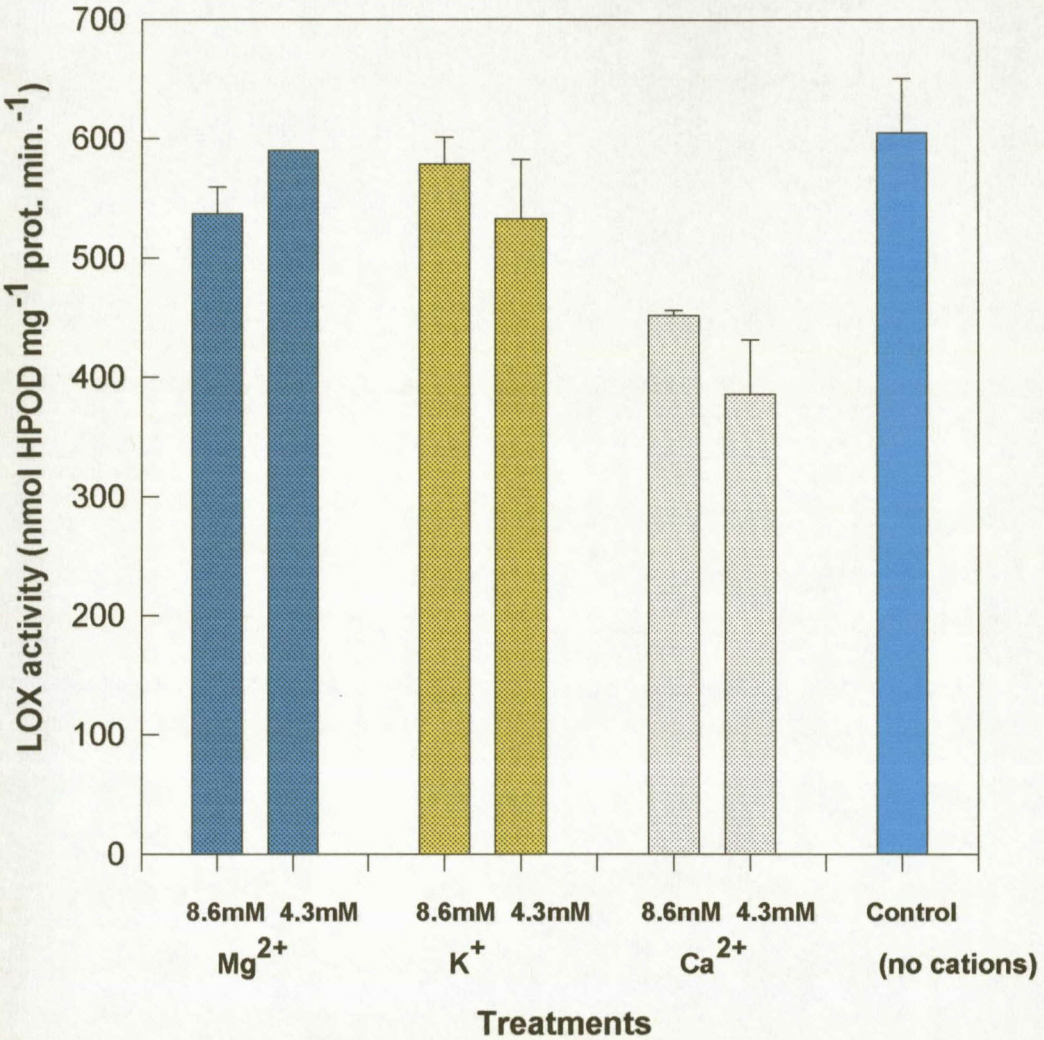

Fig. 4.3d. Effect of cations on LOX activity *in vitro.* (HPOD, linoleic acid hydroperoxy derivative) Values are means ± standard deviation (SD) (n=3).

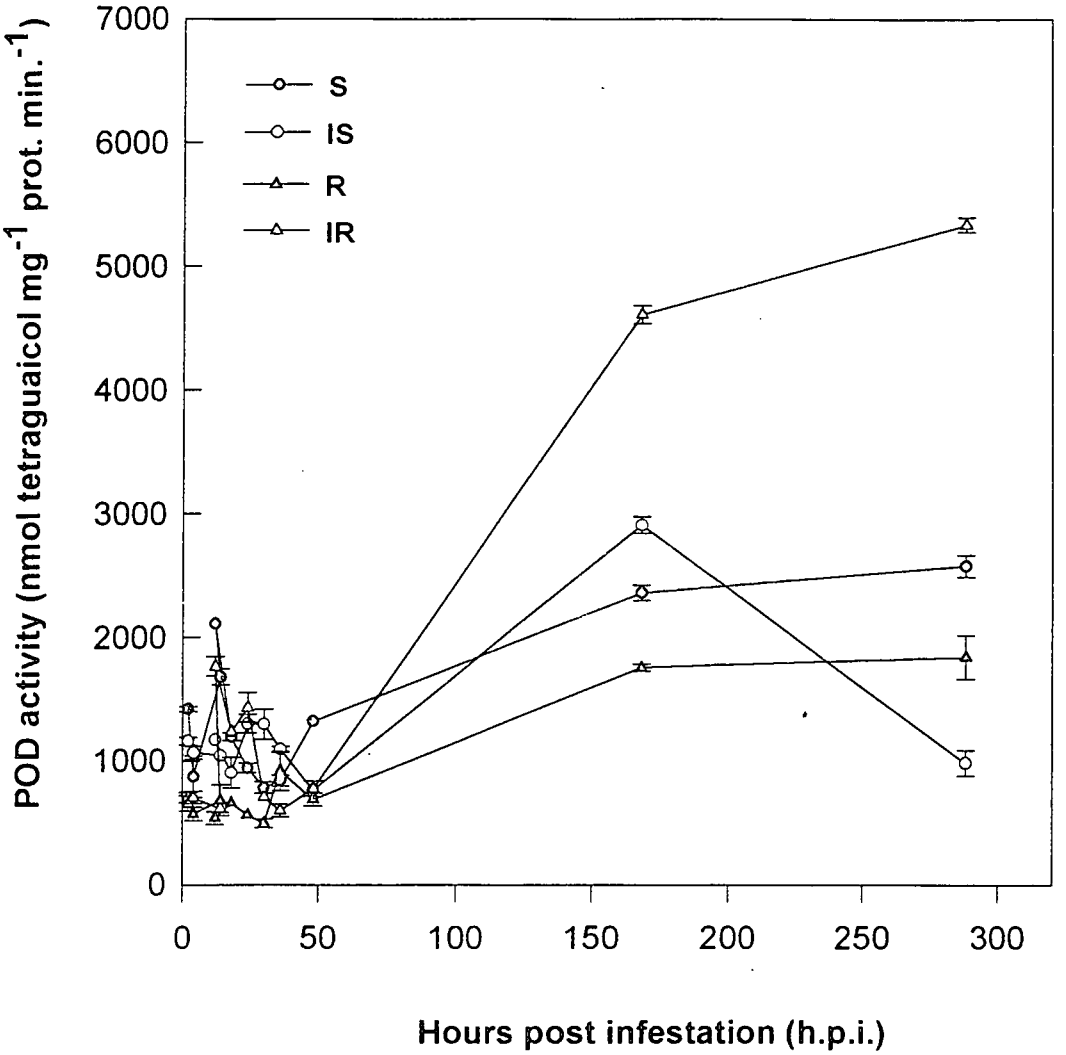

Fig. 4.5b. Effect of RWA infestation on POD activity of resistant (Tugela-DN) and susceptible (Tugela) wheat cultivars. (S, uninfested susceptible Tugela; IS, infested susceptible Tugela; R, uninfested resistant Tugela-DN; IR, infested resistant Tugela-DN) Values are means ± standard deviation (SD) (n=3).

LOX activity after the 12 h.p.i. period, the activity remained relatively constant at a low level in uninfested resistant and susceptible wheat, as well as in the infested susceptible

wheat cultivar. The initial decrease in LOX activity (12 h.p.i.) was followed by an selective increase in infested resistant Tugela-DN (48 h.p.i.), reaching about five times (400% increase) that of the initial activity (Fig. 4.4).

As a result of the possible peak LOX activity during 12 h.p.i., we performed an even more detailed time study. During this time study, different resistant cultivars were also compared, namely Tugela containing Dn1, Dn2 and Dn5 resistance genes (Fig. 4.5a).

LOX activity initially decreased (2 h.p.i. - 36 h.p.i.) in uninfested resistant (Tugela-DN) and susceptible (Tugela), and infested susceptible wheat cultivars, whereafter it remained relatively unchanged at a low activity level. The infested resistant Tugela-DN, on the other hand, showed an earlier 'spike' peak at 7 h.p.i. and peaked to a much higher level after 48 hours. The earlier peak reached an activity nearly twice (100% induced) that of the initial activity, while the second peak reached nearly five times (400% induced) its initial activity (Fig. 4.5a).

Tugela (Dn5) was the only infested resistant cultivar that did not show an earlier LOX induction at 7 h.p.i., while Tugela (Dn1) and (Dn2) both showed earlier peaks. The first peak was slightly more induced in (Dn1) (nearly 200nmol HPOD mg^{-1} prot. $min.^{-1}$) than in (Dn2) (nearly 170nmol HPOD mg^{-1} prot. $min.^{-1}$) (Fig. 4.5a).

By comparing Tugela (Dn1), (Dn2) and (Dn5)'s secondary peaks (48 h.p.i.), it was found that in all three resistant cultivars' LOX activity was selectively induced upon RWA infestation. There was, however, differences between these induced responses. LOX activity was most effectively induced in Tugela (Dn1) (at 48 h.p.i.), while it was most uneffectively induced in Tugela (Dn5). LOX activity was not induced at 48 h.p.i., but at

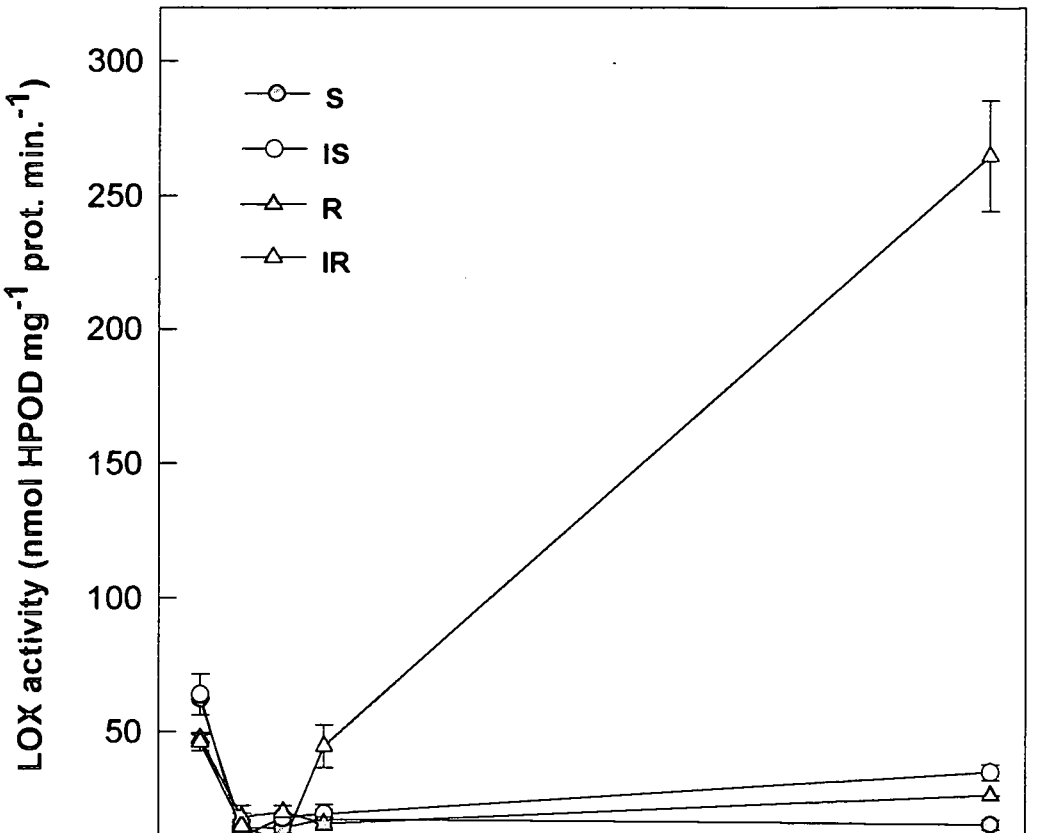

Fig. 4.4. Effect of RWA infestation on the LOX activity of resistant (Tugela-DN) and susceptible (Tugela) wheat cultivars. (S, uninfested susceptible Tugela; IS, infested susceptible Tugela; R, uninfested resistant Tugela-DN; IR, infested resistant Tugela-DN; HPOD, linoleic acid hydro-peroxy derivative) Values are means ± standard deviation (SD) (n=3).

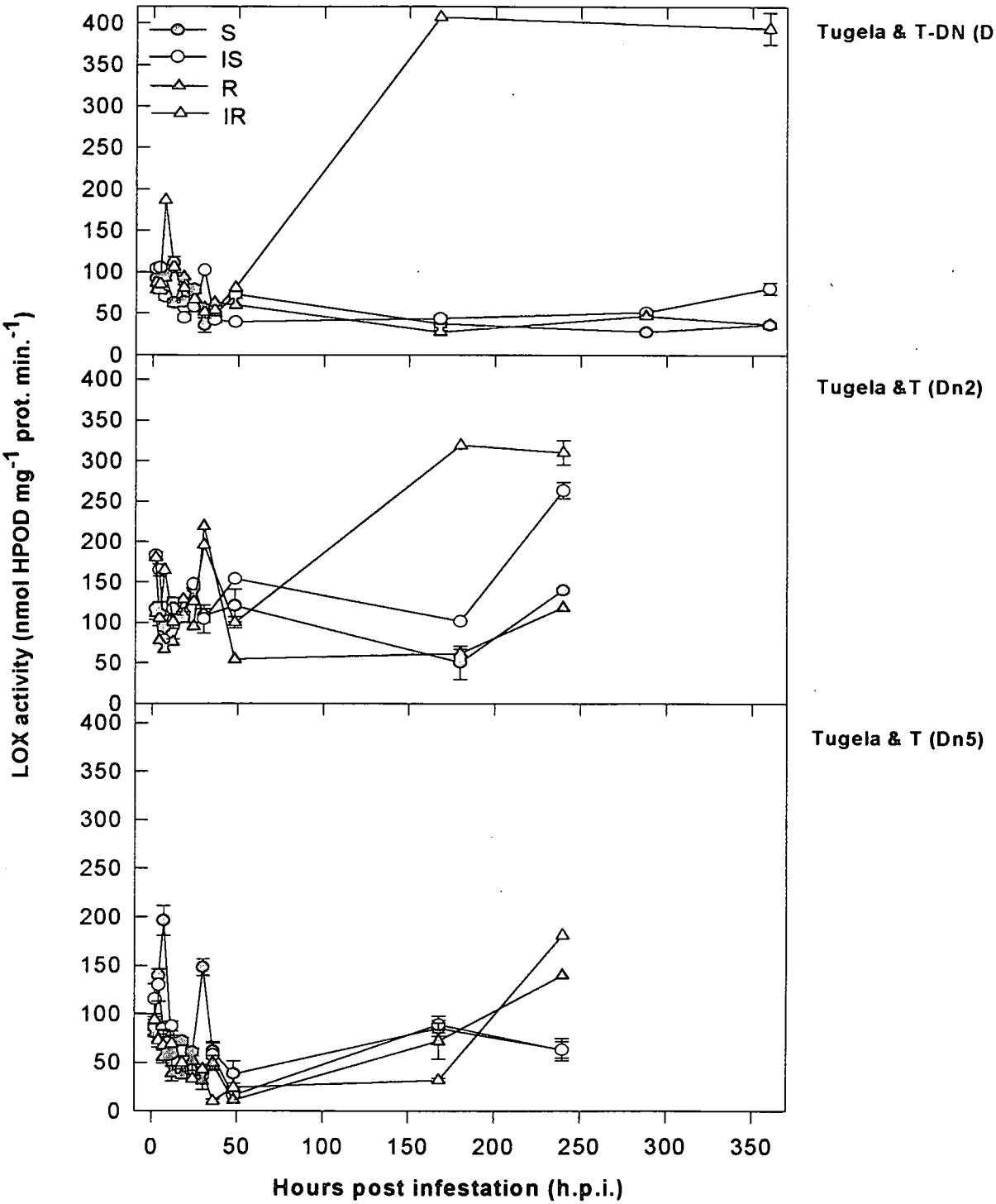

Fig. 4.5a. Effect of RWA infestation on the LOX activity of resistant (Tugela DN) and susceptible (Tugela) wheat cultivars. (S, uninfested susceptible Tugela; IS, infested susceptible Tugela; R, uninfested resistant Tugela-DN; IR, infested resistant Tugela-DN; HPOD, linoleic acid hydroperoxy derivative) Values are means ± standard deviation (SD) (n=3).

168 h.p.i. in Tugela (Dn5). This was the time at which maximum LOX activity was detected in Tugela-DN. LOX activity was ~25% higher induced in Tugela (Dn1) than in (Dn2) at 168 h.p.i. Tugela (Dn5) showed the latest and weakest LOX induction at 168 h.p.i., whereafter the activity increased to a level half that of (Dn1) at 240 h.p.i. (Fig. 4.5a).

4.4 Systemical spread of lipoxygenase activity

LOX activity was locally and systemically induced in wheat after RWA infestation. Uninfested resistant (Tugela-DN) and susceptible (Tugela) wheat showed a lower LOX activity than infested resistant and susceptible Tugela cultivars, in both the second and third leaves. RWA infestation induced a ~16% increase in LOX activity locally in the infested second leaf of susceptible plants. In the remote third leaf (of the infested susceptible plant) the LOX activity was only slightly induced in comparison with the third leaf of uninfested plants. In infested resistant Tugela-DN (second leaf), LOX activity was induced ~600% (seven times that of the uninfested sample) locally in comparison to the uninfested second leaf. The LOX activity in the uninfested resistant cultivar was initially ~5nmol HPOD mg^{-1} prot. $min.^{-1}$ whereafter it was induced to ~35nmol HPOD mg^{-1} prot. $min.^{-1}$. In the remote third leaf of the infested resistant cultivar, LOX activity was ~400% (five times) higher in comparison with the uninfested third leaf. The LOX activity increased from ~9 to 48nmol mg^{-1} prot. $min.^{-1}$ (Fig. 4.6).

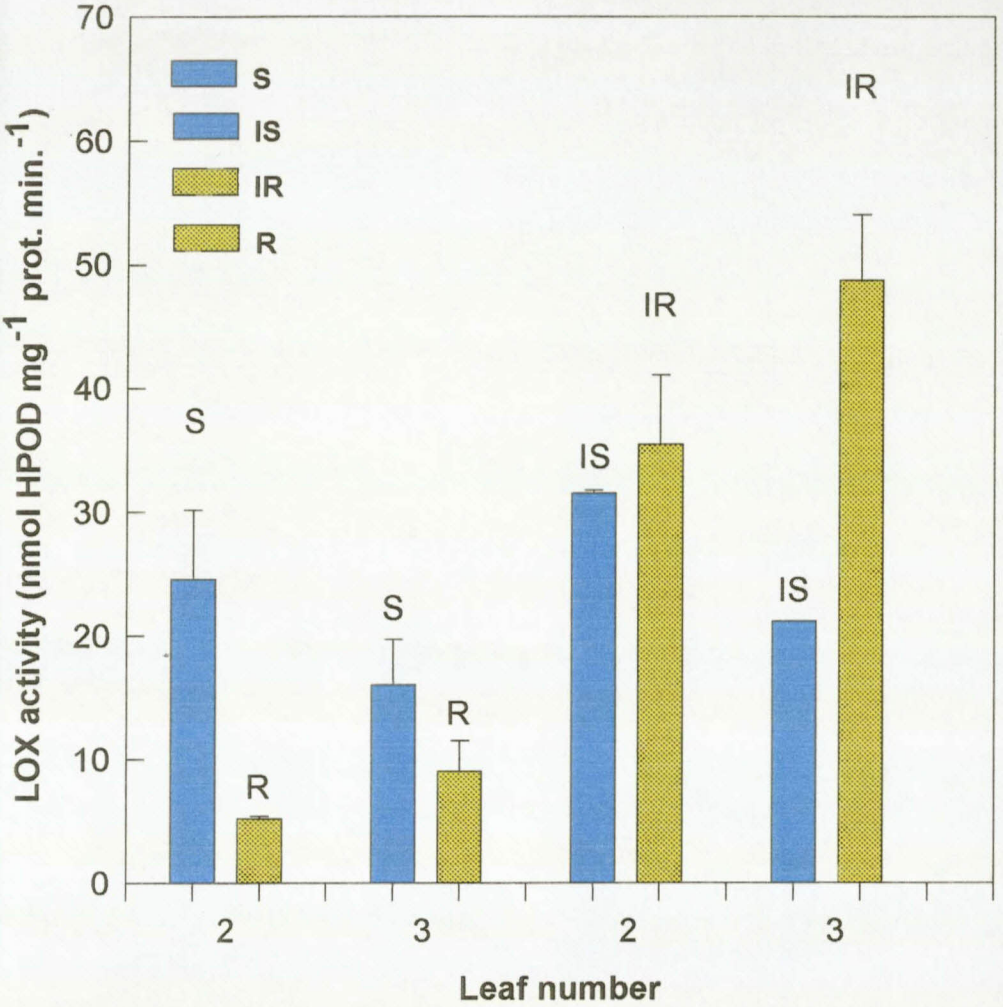

Fig. 4.6. LOX activity in the second (2) and third (3) leaves 8 days post localised infestation of the second leaf. (S, uninfested susceptible Tugela; IS, infested susceptible Tugela; R, uninfested resistant Tugela-DN; IR, infested resistant Tugela-DN; HPOD, linoleic acid hydroperoxy derivative) Values are means ± standard deviation (SD) (n=3).

4.5 In vitro and in vivo effect of lipoxygenase inhibitors on lipoxygenase, peroxidase and β-1,3-glucanase activities

Piroxicam (PC) inhibited LOX activity 100% at concentrations higher than 34.8μM, had no effect on the POD activity, and stimulated β-1,3-glucanase activity at concentrations higher than 7.6μM *in vitro* (Fig. 4.7a).

Salicylhydroxamic acid (SHA) inhibited LOX activity by 100% at concentrations higher than 434.8μM, inhibited POD activity by 80% at a concentration of 1941.7μM, and stimulated β-1,3-glucanase activity with increasing SHA concentration *in vitro* up to 750μl SHA (Fig. 4.7b).

n-Propyl gallate (nPG) inhibited LOX activity by 100% at concentrations higher than 869.6μM, inhibited POD activity up to 80% at a concentration of 1941.7μM and activated β-1,3-glucanase activity with increasing nPG concentrations *in vitro* (Fig. 4.7c).

To confirm that the inhibitor solution (namely PC) was effectively absorbed by the excised seedlings, the % LOX inhibition was determined in various parts of the seedlings. PC inhibited LOX activity of the entire excised seedling by ~65% at both concentrations of 10 and 20mM *in vivo.* In the lower part (first 7cm from excised side) of the seedlings, LOX activity was inhibited by 80% in a 10mM PC solution. In the top part of the seedling, LOX activity was inhibited ~65% *in vivo* (Fig. 4.8).

The maximum *in vivo* LOX inhibition caused by PC, was ~65% at concentrations 10 and 20mM. At a lower concentration range (4-5mM), PC caused an average *in vivo* inhibition of ~40% of LOX activity, ~20% for POD activity and ~50% for β-1,3-

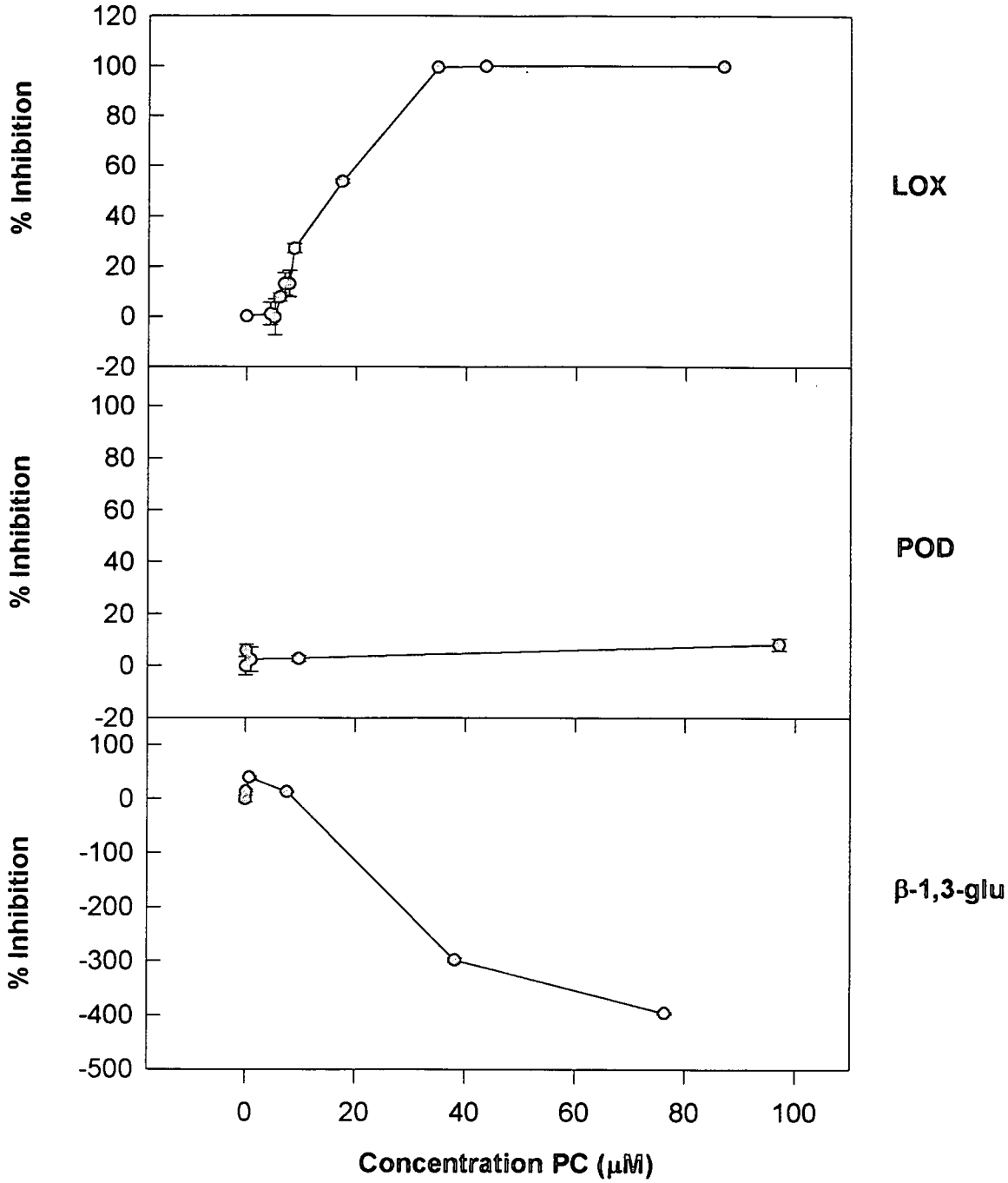

Fig. 4.7a. The *in vitro* effect of the lipoxygenase (LOX) inhibitor piroxicam (PC), on the LOX, peroxidase (POD) and β-1,3-glucanase (β-1,3-glu) activities of an extract from infested resistant Tugela-DN (10 d.p.i.). Values are means ± standard deviation (SD) (n=3).

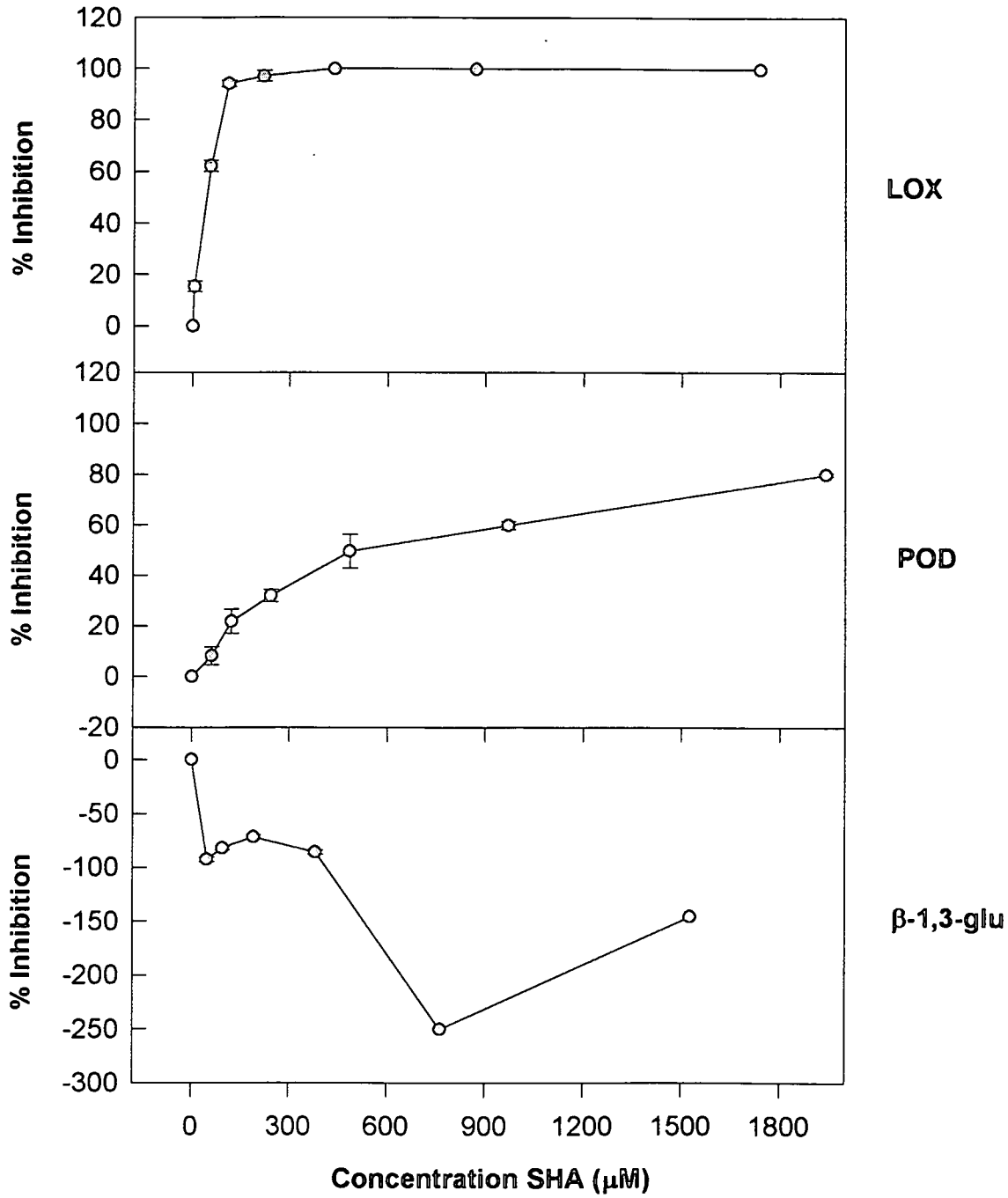

Fig. 4.7b. The *in vitro* effect of the lipoxygenase (LOX) inhibitor, salicylhydroxamic acid (SHA), on the LOX, peroxidase (POD) and β-1,3-glucanase (β-1,3-glu) activities of an extract from infested Tugela-DN (10 d.p.i.). Values are means ± standard deviation (SD) (n=3).

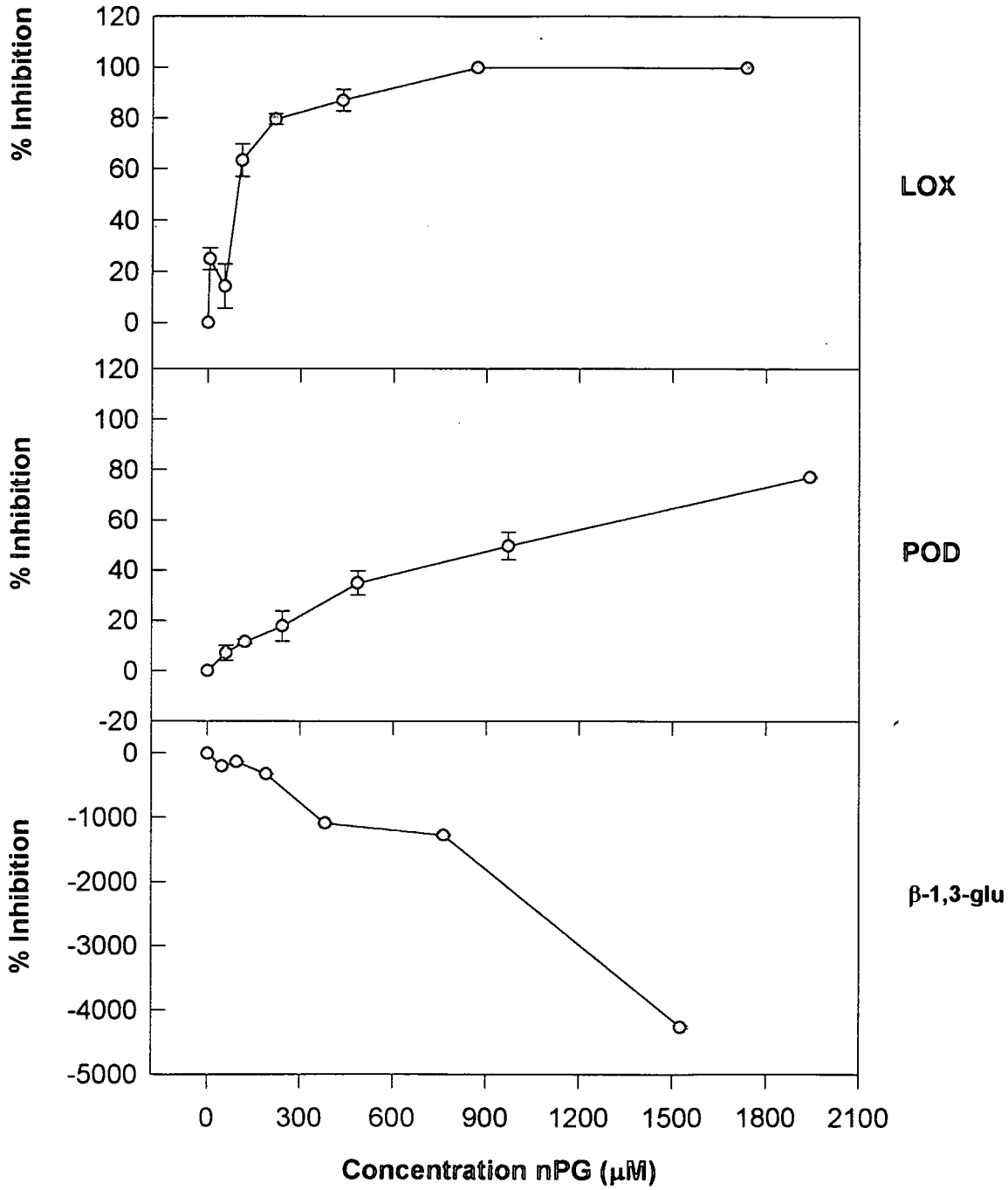

Fig. 4.7c. The *in vitro* effect of the lipoxygenase (LOX) inhibitor, n-propyl gallate (nPG), on the LOX, peroxidase (POD) and β1,3-glucanase (1,3-glu) activities of an extract from infested Tugela-DN (10 d.p.i.). Values are means ± standard deviation (SD) (n=3).

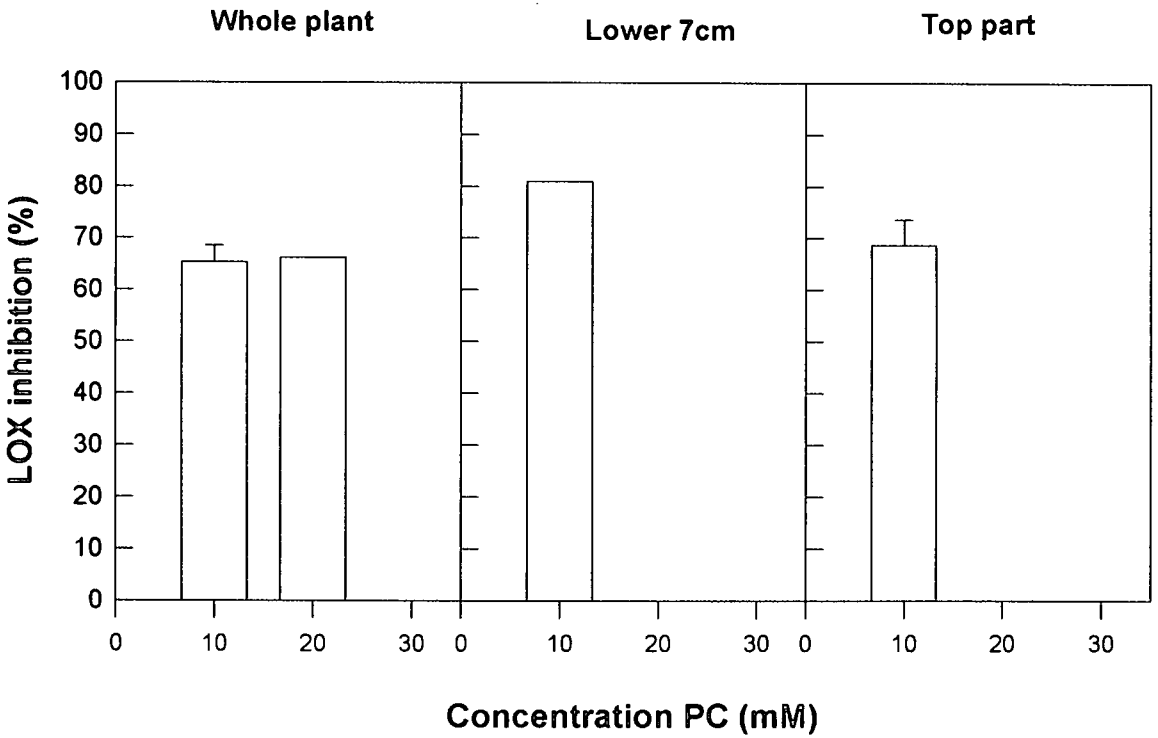

Fig. 4.8. Distribution of piroxicam (PC) in the excised plant inserted in a PC containing solution measured by effect on LOX activity. Values are means ± standard deviation (SD) (n=3).

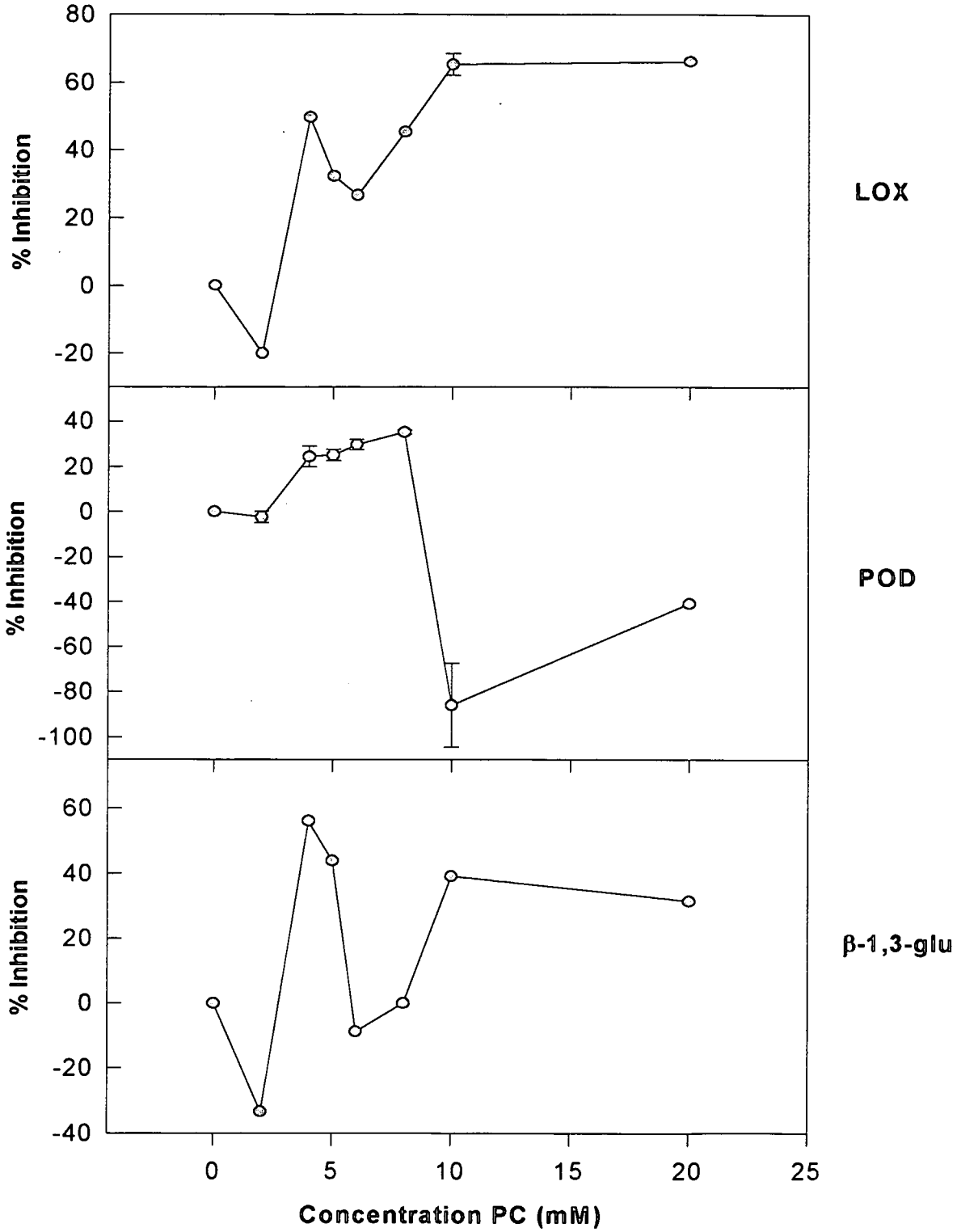

Fig. 4.9a. The *in vivo* effect of applied lipoxygenase (LOX) inhibitor, piroxicam (PC), on the LOX, peroxidase (POD) and β-1,3-glucanase (β-1,3-glu) activities of infested Tugela-DN plants (10 d.p.i.) Values are means ± standard deviation (SD) (n=3).

glucanase activity. Concentrations higher than 8mM had a stimulating effect on POD activity (Fig. 4.9a).

The maximum *in vivo* inhibition SHA caused to LOX activity, was ~50% at concentrations of 50-80mM and 150mM. The maximum *in vivo* inhibition for POD and β-1,3-glucanase activities were obtained at a concentration ranging from 150 to 200mM. Concentrations lower than 60mM (up to 40mM) tended to be stimulatory for POD activity (Fig. 4.9b).

Concentrations lower than 50mM nPG had no, or stimulatory effects on LOX activity *in vivo*, while the maximum inhibition of ~35% was obtained at a concentration of 150mM. A maximum inhibition of ~60% occurred at a concentration of 50mM for POD. At a concentration of 100mM, nPG inhibited LOX and POD activity by ~30%. All nPG concentrations tested, stimulated β-1,3-glucanase activity (Fig. 4.9c).

4.6 Jasmonic acid and abscisic acid levels in uninfested and infested, resistant and susceptible wheat cultivars

RWA infestation had no differential effect on JA levels between resistant (Tugela-DN) and susceptible (Tugela) cultivars. During the period prior to 48 h.p.i., peak JA concentrations were reached in both susceptible and resistant cultivars. The JA levels of infested and uninfested resistant and susceptible plants started to gradually increase as infestation proceeded beyond 48 hours. In susceptible cultivars (infested and uninfested), a somewhat higher level of JA was finally reached (240 h.p.i.) (Fig. 4.10a).

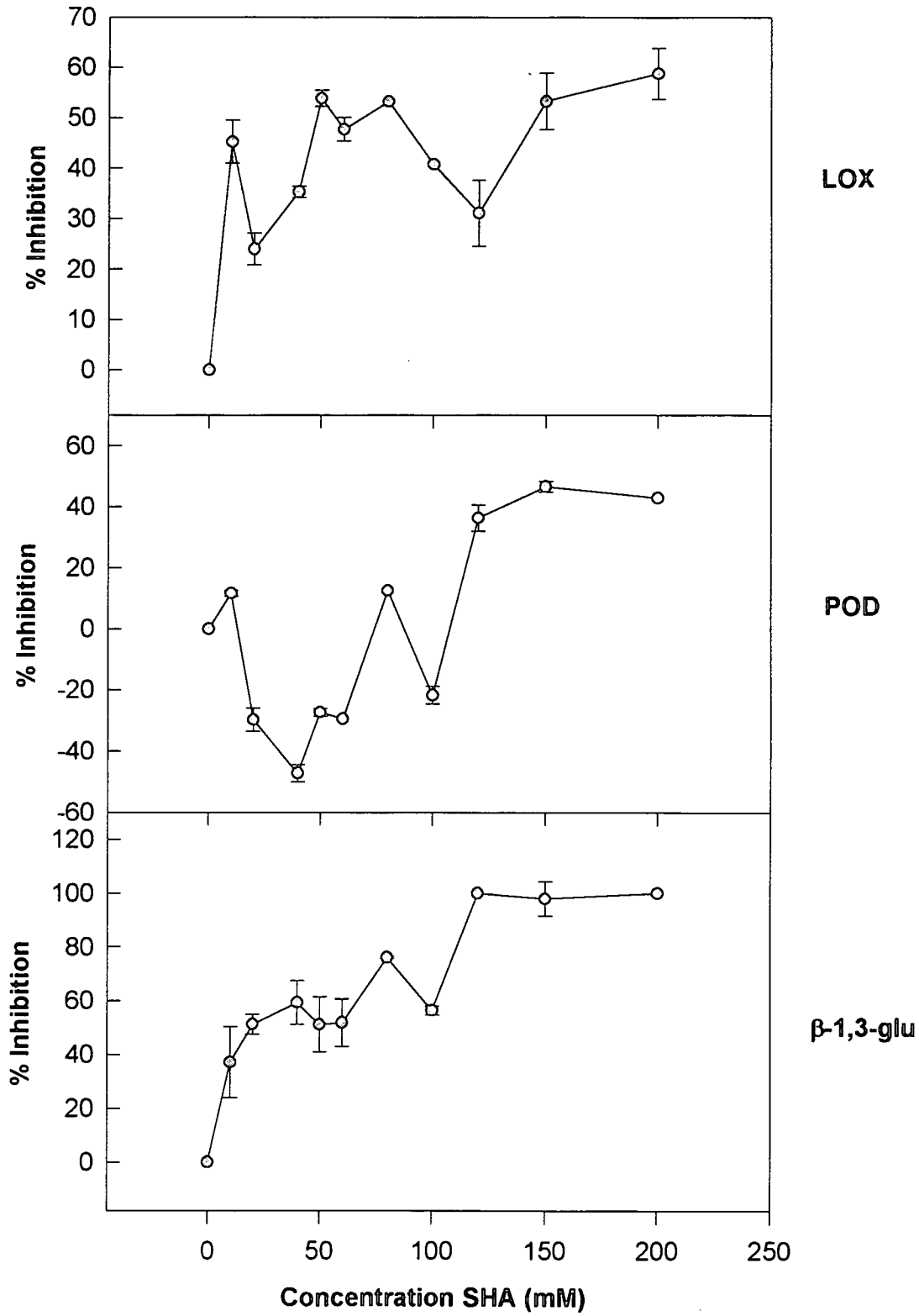

Fig. 4.9b. The *in vivo* effect of applied lipoxygenase (LOX) inhibitor, salicylhydroxamic acid (SHA), on the LOX, POD and β-1,3-glucanase (β-1,3-glu) activities of infested Tugela-DN plants (10 d.p.i.). Values are means ± standard deviation (SD) (n=3).

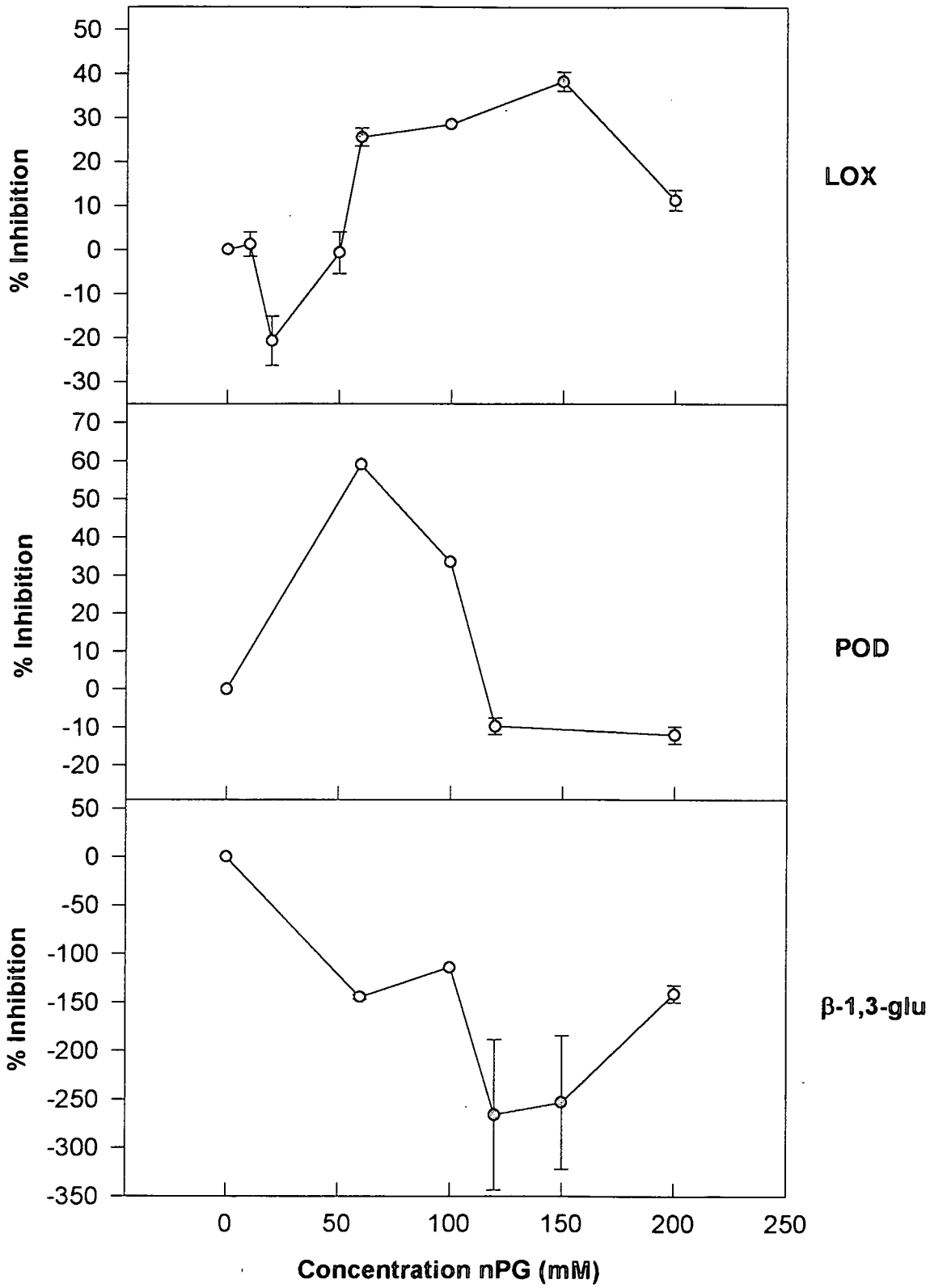

Fig. 4.9c. The *in vivo* effect of applied lipoxygenase (LOX) inhibitor, n-propyl gallate (nPG), on the LOX, peroxidase (POD) and β-1,3-glucanase (β-1,3-glu) activities of infested Tugela-DN plants (10 d.p.i.). Values are means ± standard deviation (SD) (n=3).

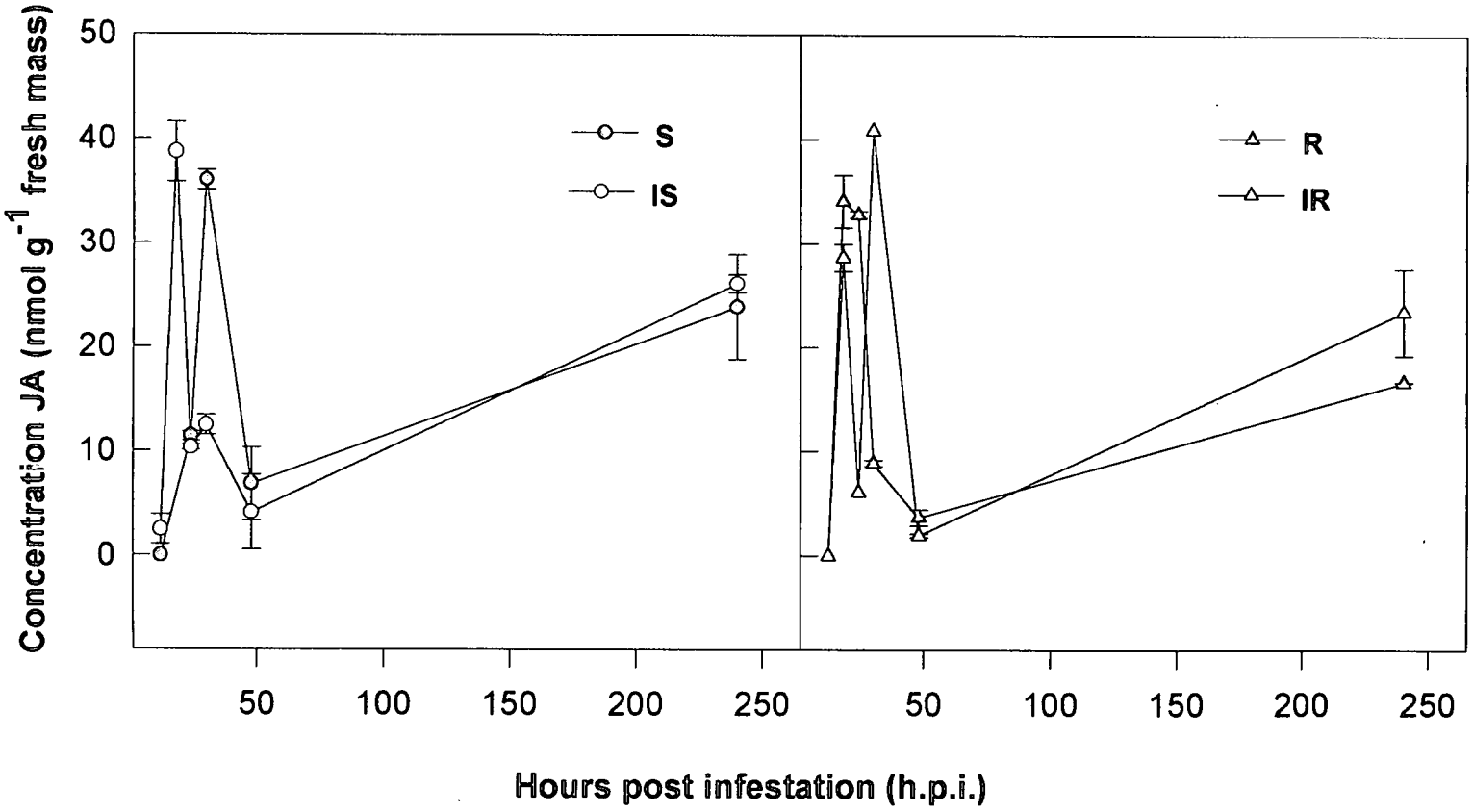

Fig. 4.10a. The effect of RWA infestation on the JA content of resistant and susceptible wheat cultivars. (S, uninfested susceptible Tugela; IS, infested susceptible; R, uninfested resistant Tugela-DN; IR, infested resistant Tugela-DN;) Values are means ± standard deviation (SD) (n=3).

RWA infestation had no differential effect on ABA levels between resistant (Tugela-DN) and susceptible (Tugela) cultivars. During the period prior to 48 h.p.i., peak ABA concentrations were reached in both resistant and susceptible cultivars, with the average ABA concentration being slightly lower in uninfested resistant and infested resistant cultivars. The ABA concentration of infested and uninfested resistant and uninfested susceptible wheat tended to gradually increase as infestation proceeded after 240 h.p.i. In uninfested susceptible cultivars, a somewhat higher ABA concentration (~50% higher) was finally reached at 360 h.p.i., than in uninfested resistant, infested resistant and infested susceptible cultivars. The ABA levels reached in infested resistant and infested susceptible cultivars, were slightly below (an average of ~20% below) that of the uninfested resistant and susceptible cultivars at 350 h.p.i. (Fig. 4.10b)

4.7 Effect of intercellularly injected jasmonic acid on lipoxygenase, peroxidase and β-1,3-glucanase activities

In general, applied JA had an inducing effect on the LOX activity, with a maximum induction at 20μM (Fig. 4.11).

POD activity was slightly induced (20%) at a concentration of 2μM, but declined nearly to its initial activity thereafter (Fig. 4.11).

JA (1μM) maximally induced β-1,3-glucanase activity. This inducing effect decreased with increasing JA concentration up to 10μM. β-1,3-glucanase inhibition tended to be reversed to activation again at a concentration of 20μM (Fig. 4.11).

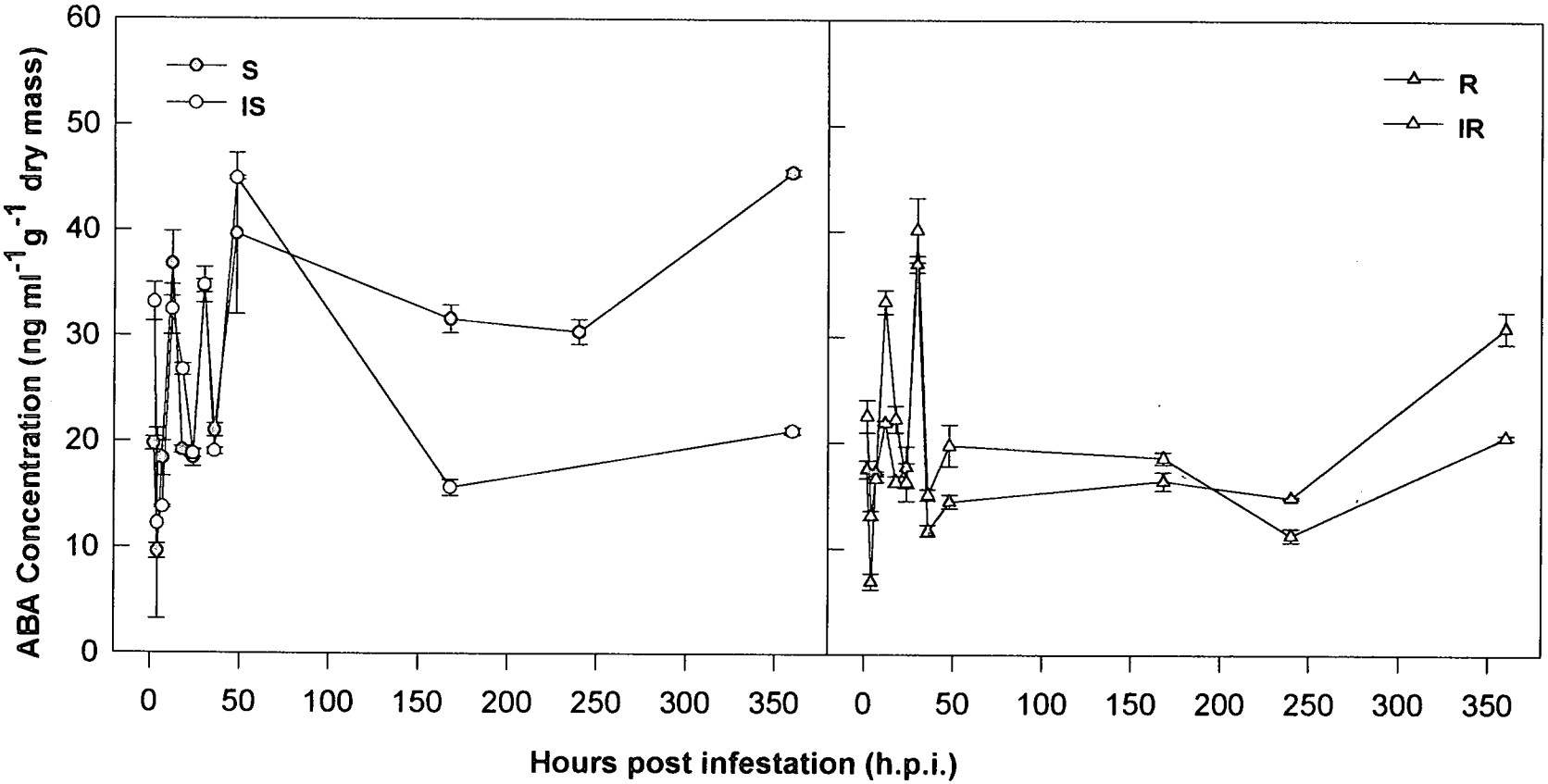

Fig. 4.10b. The effect of RWA infestation on the ABA content of resistant and susceptible wheat cultivars. (S, uninfested susceptible Tugela; IS, infested susceptible Tugela; R, uninfested resistant Tugela-DN; IR, infested resistant Tugela-DN) Values are means ± standard deviation (SD) (n=3).

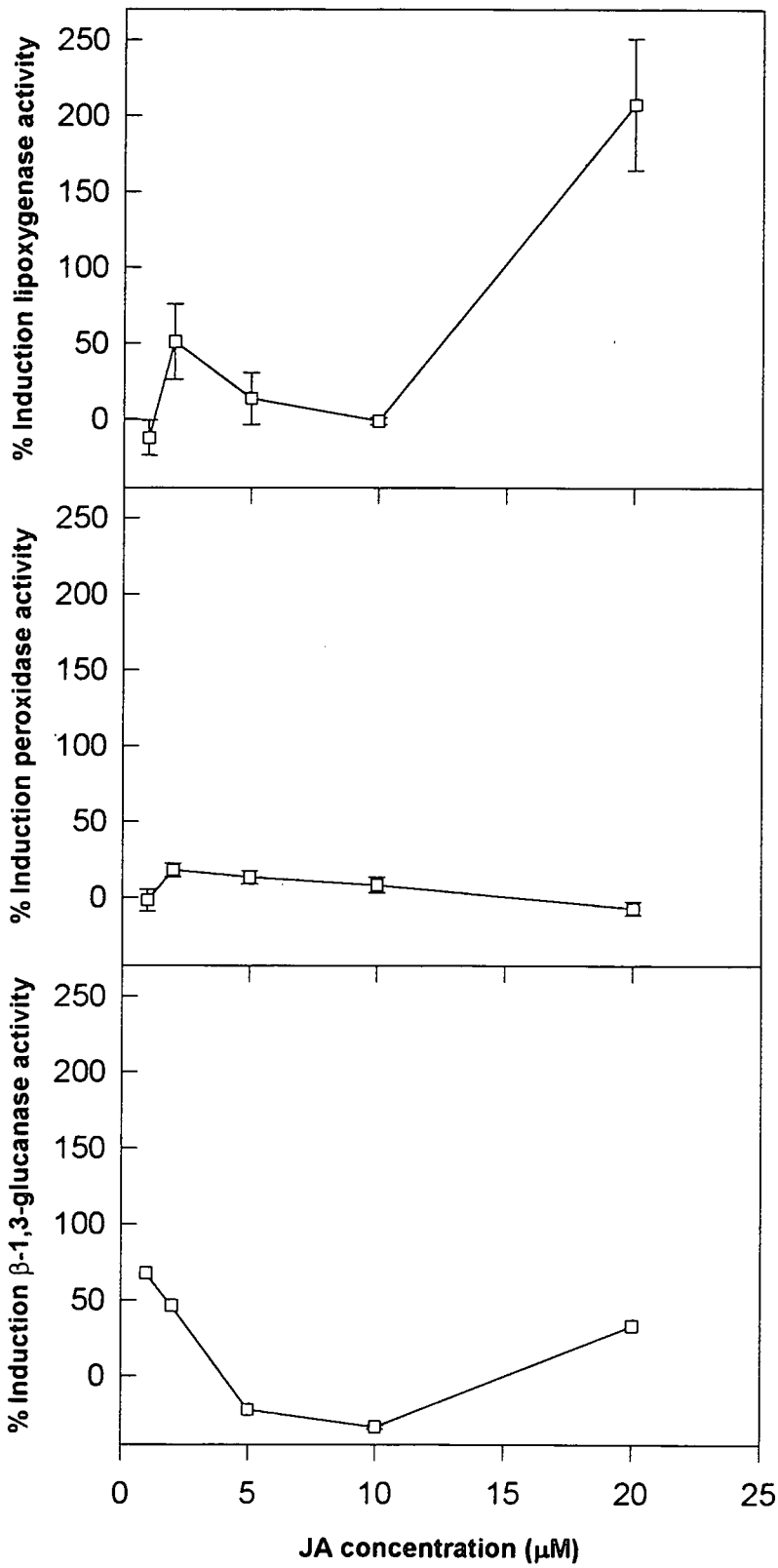

Fig. 4.11. Effect of intercellularly injected jasmonic acid (JA) on lipoxygenase (LOX), peroxidase (POD) and β-1,3-glucanase (β-1, 3-glu) activities of resistant Tugela-DN plants. (R, resistant Tugela-DN) Values are means ± standard deviation (SD) (n=3). Samples of untreated samples were taken as 100% and the activities of treated samples were expressed as fractions (%) thereof.

4.8 <u>In vivo</u> effect of lipoxygenase inhibition, by piroxicam, on jasmonic acid levels

PC (20mM), applied to resistant Tugela-DN, slightly induced JA synthesis. Uninfested resistant Tugela-DN treated with PC, had a ~20% higher JA concentration (~5nmol JA g^{-1} fresh mass) than uninfested resistant Tugela-DN that was not treated with PC. Infested resistant wheat treated with PC had ~20nmol JA g^{-1} fresh mass more (~65% JA induction) than the infested resistant wheat not treated with PC (Fig. 4.12).

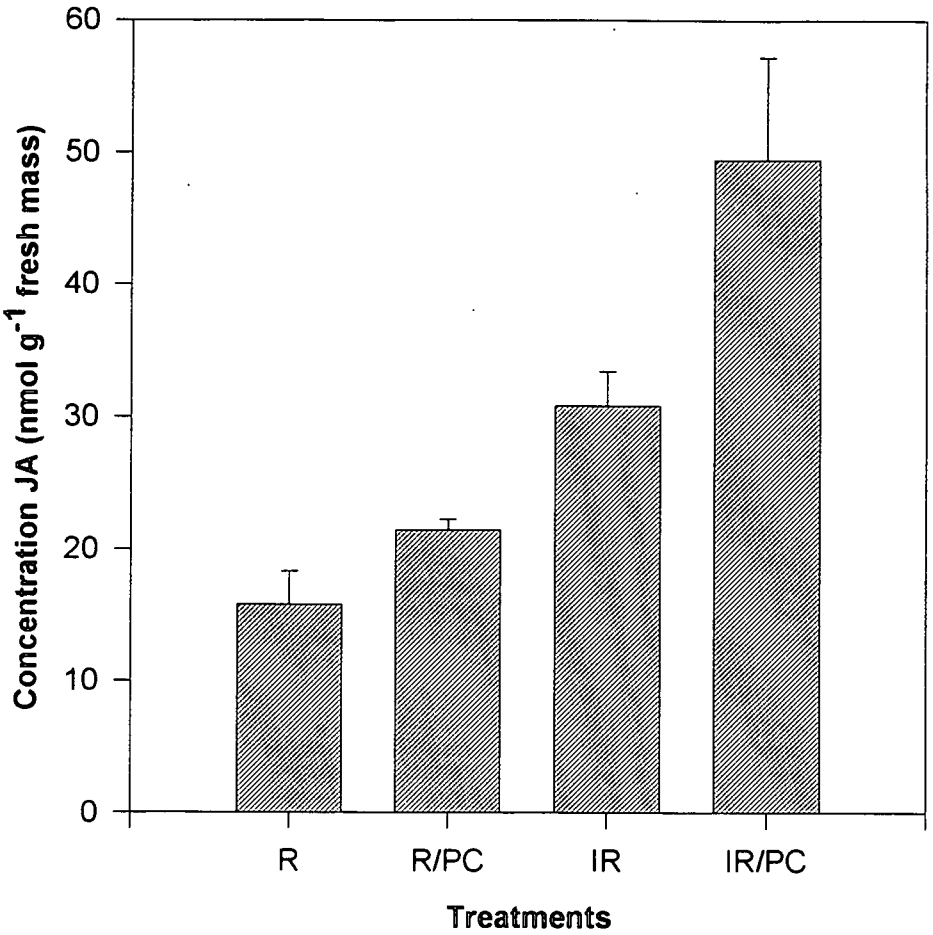

Fig. 4.12 Effect of a lipoxygenase (LOX) inhibitor, piroxicam (PC), on jasmonic acid (JA) content *in vivo*. (R, uninfested resistant Tugela-DN; R/PC, uninfested resistant Tugela-DN treated with PC; IR, infested resistant Tugela-DN; IR/PC infested resistant Tugela-DN treated with PC) Values are means ± standard deviation (SD) (n=3).

Chapter 5

Discussion

In animals it has been well established that the products of several different mammalian lipoxygenases (LOXs) are the primary metabolites in pathways that lead to the formation of important regulatory molecules in inflammatory responses, leukotrines and lipoxins (Rosahl 1996, Siedow 1991). In plants, however, little is known with certainty about the role of LOX in any plant cell, while the diversity of iso-enzymes and the subcellular distribution suggest multiple functions (Bohland *et al.* 1997, Rosahl 1996, Siedow 1991). Nonetheless, various research reports indicate that plant LOXs may play an important role in the plant's defence and resistance responses (Choi *et al.* 1994, Felton *et al.* 1994a & b, Ocampo *et al.* 1986, Rosahl 1996, Slusarenko 1996).

The downstream resistance responses in the Russian wheat aphid/wheat interaction, have been elucidated to a great extent. Various resemblances were found between the resistance responses against pathogens and against the RWA (Van der Westhuizen *et al.* 1998a & b, Van der Westhuizen & Pretorius 1995, Van der Westhuizen & Pretorius 1996, Van der Westhuizen & Botha 1993, Van der Westhuizen *et al.* 1995). In an attempt to unravel more of the RWA resistance response mechanism, we investigated the possible upstream role of LOX in the RWA induced resistance response in wheat.

It was important to adopt harvest and storage procedures for maximal LOX recovery. Freezing of plant samples in liquid nitrogen, directly after harvesting, caused a 67% loss in LOX activity compared to LOX activity in fresh unfrozen samples (Fig. 4.2a). We found that LOX extracts stored at -20^0C, showed a complete loss in activity (Fig. 4.2a). Storage of extracts in 10% glycerol, to prevent freezing during storage at -20^0C, resulted in a 40% activity loss within 24h (Fig. 4.2a). Furthermore, extracts kept on ice (0^0C) lost 60% activity within 2h after extraction (Fig. 4.2b). These results were supported by Oomah *et al.* (1997), who also found that flax seed LOX was very labile with $T_{1/2}$ = 4h at 4^0C.

The induced LOX activity was much more prominently expressed in the complete seedling sample than in the second leaf sample (Fig. 4.1). Thus, based on the preceding results as well as the harvesting results (Fig. 4.1), the entire above ground parts of the

plant were harvested to recover maximum LOX activity. Due to the time limits it was impractical to make extracts of fresh plant material during time course studies. Plant material was therefore frozen in liquid nitrogen directly after harvesting and stored at -20^{0}C until extracts were made. Extracts were then immediately assayed after extraction, in an effort to complete assays before any notable activity loss occurred.

The RWA induced LOX activity was also partially characterised and optimum conditions for enzyme assays were determined. It was established that the pH for optimum LOX activity in extracts from infested resistant wheat was 5.6, while more than 80% of the maximum activity was maintained between pH5.2 and 6.4 (Fig. 4.3a). This corroborated the optimum pH found for induced LOX activity in wheat (pH5.5-6.0) upon treatment with rust fungus elicitor, chitin, oligosaccharides, chitosan and M-JA. This activity resulted mainly from a 92-103kDa LOX isoenzyme which favours the production of the 9-HPOD intermediate (Bohland *et al.* 1997). In contrast to our results, soybean LOX-1, e.g., had a optimum pH of 9 (Galliard & Chan 1980), predominantly catalysing the 13-HPOD from the substrate linoleic acid (Siedow 1991).

By means of the Eadie-Hofstee graph, we established that RWA-induced wheat LOX has a high affinity for linoleic acids, with a Km-value of ~5.8 x 10^{-4}M (Fig. 4.3b). Soybean LOX-1 had a Km-value of 2 x 10^{-5}M for linoleic acid, revealing a higher affinity for linoleic acid than for linolenic acid (Galliard & Chan 1980). Shiiba *et al.* (1991) also found that wheat germ LOXs had a higher affinity for linoleic acid than for linolenic acid. Linolenic and linoleic acids are the two main LOX substrates in higher plants, known to be catalysed by LOXs into polyunsaturated fatty acid hydroperoxides (9- & 13-HPOD) (Shiiba *et al.* 1991, Shibata & Axelrod 1995).

The cations, K^+ and Mg^{2+}, had virtually no effect on LOX activity, while Ca^{2+} was slightly inhibitory (20-35% inhibitory) (Fig. 4.3d). The inhibitory effect Ca^{2+} had on LOX confirmed the findings that $CaCl_2$ inhibited LOX activity during senescence in *Vigna unguiculata* (Swammy & Sunguna 1992) and the findings of Galliard & Chan

(1980) that Ca^{2+} inhibits LOX activity. LOXs therefore lack cofactors other than its nonheme iron (Galliard & Chan 1980).

LOX activity assays were determined at pH 6.2, as reported for wheat by Bohland *et al.* (1997) and Moerschbacher (1988). This is well within the pH range for maximal activity (Fig. 4.3a). Linoleic acid was used as substrate, due to LOX's high affinity for it (Fig. 4.3b), and due to the fact it was the most used substrate in assay studies (Galliard & Chan 1980, Gardner 1996, Oomah *et al.* 1997, Slusarenko 1996). Bohland *et al.* (1997) also used linoleic acid specifically for wheat assays. The reaction mixture for LOX activity assays contained no K^{+}, Mg^{2+} or Ca^{2+} as possible cofactors due to their non-stimulating effect (Fig. 4.3d). The optimum temperature for maximum LOX activity was found to be $40^{0}C$ (Fig. 4.3c), but according to our preceding findings of LOX being highly unstable, we were discouraged to use this temperature. Various scientists indicated that a temperature of $30^{0}C$ was the most suitable for assay purposes (Bohland *et al.* 1997, Loers & Grambow 1998, Moerschbacher 1988, Swammy & Sunguna 1992). The LOX activity at $30^{0}C$ agreed with 95% of the maximum activity (Fig. 4.3c).

LOX activity was selectively induced within 48h.p.i. in resistant Tugela-DN (Dn1) and Tugela (Dn2), and within 176h.p.i. in Tugela (Dn5), while it remained relatively low in uninfested resistant and susceptible, and infested susceptible wheat. LOX activity was slightly higher in infested susceptible wheat than in uninfested susceptible wheat, although still much lower than the induced LOX activity in infested resistant wheat. From these results it is clear that LOX was more prominently and rapidly induced in Tugela-DN, than in the other resistant cultivars containing the (Dn2) and (Dn5) resistant genes (Fig. 4.5a). Tugela-DN was also found to be most resistant against the RWA during field experiments (Van der Westhuizen & Botha 1993, Van der Westhuizen personal communication[1]). The resistant gene incorporated (into the same genetic background) appears to play an important role in the level of induction of LOX activity which roughly corresponded to the level of resistance. One could also speculate that

[1] Prof. AJ Van der Westhuizen, UOFS, Bloemfontein, South Africa.

Tugela-DN was more resistant than Tugela (Dn5) due to the fact that it responded earlier to RWA invasion.

Our results are supported by various reports, indicating that LOX might play a role in the defence and resistance responses against pathogens and insects (Creelman *et al.* 1992b, Croft *et al.* 1993, Mauch *et al.* 1997, Ocampo *et al.* 1986, Shibata & Axelrod 1995). Felton *et al.* (1994a) found that the corn earworm (*Helicoverpa zea*) induced LOX activity in soybean and redbeans during their resistance responses. The bacterium *Pseudomonas syringae* pv *phaseolicola* induced LOX activity to higher levels in French bean plants (*Phaseolus vulgaris*) undergoing the HR (Croft *et al.* 1990). *Phytophthora parasitica nicotianae* (Ppn) also induced LOX activity in resistant tobacco (*Nicotiana tabacum* L.) (Vèronèsi *et al.* 1996). The LAR response in wheat against powdery mildew was also accompanied by the accumulation of mRNA species, which encoded for putative cell wall proteins, a traumatin-like protein, PODs, LOXs and a cystein proteinase (Schaffrath *et al.* 1997). Therefore, supporting the suggestion that RWA infestation induce local acquired resistance (LAR) in wheat, and that LOX plays a role in the RWA resistance response.

Although not directly comparable, the genetic background into which the Dn1 resistant gene was bred (cv Molopo and Betta), also appeared to affect the level of resistance and the induction of some PR-proteins (β-1,3-glucanase and chitinase activities) (Van der Westhuizen *et al.* 1996, Van der Westhuizen *et al.* 1998a & b, Botha *et al.* 1998). Western blot analyses proved that this increase in PR-protein enzyme activities were due to increased protein levels, and not due to enzyme activation (Van der Westhuizen & Pretorius 1996). Therefore, it appears that both the origin of the resistance gene incorporated and the genetic background into which the resistant gene was incorporated, play a role in the level of the plant's resistance response.

According to our results, LOX activity was induced at two time intervals [in Tugela-DN an Tugela (Dn2)] at 7 and 48h.p.i. (Fig. 4.5a). The initial LOX activity peaks (7h.p.i.), although inconsistant in the sense that it was not observed to be selectively induced in

resistant Tugela containing the resistance (Dn2) and (Dn5) genes, could be an indication of the involvement of LOX in early signal transduction events. Several LOX pathway products have been suggested to act as intracellular signal molecules, eliciting 'downstream' defence responses (Mauch *et al.* 1997, Siedow 1991) and inducing defence genes (Farmer & Ryan 1992).

According to Blèe (1998), JA is a typical example of an intracellular signal molecule (jasmonate-based signalling pathway). The *de novo* synthesised JA (upon wounding) is known to trigger a rapid and transient expression of individual LOX genes, yielding the formation of signal molecules (i.e. jasmonate and volatile aldehydes) which in turn activates locally and systemically 'downstream' responsive genes. These responsive 'downstream' genes include enzyme inhibitors, phenylalanine ammonia-lyase, chalcone synthase, berberine-bridge enzyme, PR-proteins 1 & 5, apoxide hydrolase, glucanase, chitinase, and POD (Blèe 1998).

Interestingly enough, it has previously been documented that treatment of tobacco cell suspensions, with a HR-causing bacteria (*Pseudomonas syringae* pv *syringae*), resulted in an initial rapid oxidative burst 0-1h after treatment. A second, prolonged increase in active oxygen species (3-6h) was observed in cells treated with HR-causing bacteria (*Pseudomonas syringae* pv *syringae*), but not in cells treated with non-HR-causing transposon mutant bacteria of *Pseudomonas syringae* pv *phasiollicola* (Mehdy 1994).

The oxidative burst, one of the earliest events in the HR (Keen & Littlefield 1979), is known to be part of the defence mechanisms against pathogens and insects (Dixon *et al.* 1994, Farmer & Ryan 1992, Mehdy 1994, Pennel & Lamb 1997). The oxidative status shift is caused by the increase of oxidative enzymes, such as LOX, POD, polyphenol oxidase (Felton *et al.* 1994a) and phenoloxidases (Appel 1993), which produce oxygen species and reactive oxidants during defence responses (Felton *et al* 1994a). Also, it is caused by a loss of chemical antioxidants (like carotenoids, ascorbate, glutathione and related thiols) and/or a decrease in antioxidant enzymes such as catalase, glutathione reductase and superoxide dismutase (Felton *et al.* 1994a). During the peroxidation of

membrane lipids (HR), oxygen specie-like singlet oxygen or superoxide radicals are also produced (Farmer & Ryan 1992, Felton *et al.* 1994a, Pennel & Lamb 1997). Thus, LOX could also act as an important mediator of resistance against pathogens and insects through the oxidative burst (Felton *et al.* 1994a). LOX (Fig. 4.4 & 4.5a) and POD (Fig. 4.5b) (Van der Westhuizen *et al.* 1998a & b) were selectively induced upon RWA infestation. Therefore, increased LOX and POD activities could contribute to the oxidative burst in wheat against the RWA, through the accumulation of active oxygen species such as $O^{\cdot}_2$ and H_2O_2 (Pennel & Lamb 1997).

The oxidative burst can cause direct or indirect damage to insects, through impairing insect growth, damaging essential nutrients like amino acids, carotenoids and other dietary lipids, or through acting as feeding repellents. Felton *et al.* (1994a) found that spidermite infestation of resistant soybeans induced lipid peroxidation and a loss of carotenoids. Also, the midgut epithelium tissue of the corn earworm (*Helicoverpa zea*) feeding on resistant soybeans, was oxidatively damaged (Felton *et al.* 1994a).

Loers and Grambow (1998) found that during the HR of an incompatible wheat/pathogen (*Triticum aestivum*/black rust glycoprotein elicitor) interaction, LOX and POD activities were induced at the same time post infection. *In vitro* studies revealed that LOX possibly acts as an electron acceptor and POD as the electron donor during the HR, coupling LOX and POD catalytic reactions. The relevance whether there is a reaction between POD and lipidperoxide *in vivo*, is uncertain (Loers & Grambow 1998).

Another possible function of LOX in the resistance response against the RWA, could be the peroxidation of membrane lipids, causing irreversible membrane damage during the hypersensitive cell death (Bohland *et al.* 1997, Croft *et al.* 1990, Farmer & Ryan 1992, Gardener 1996, Siedow 1991). In compatible wheat/black stemrust interactions, a correlation between the LOX activity increase and the HR was found (Bohland *et al.* 1997), suggesting that LOX could be a mediator of the eliciting reactions (Ocampo *et al.* 1986). Also, during the SAR response in wheat against *Erysiphe gramminis* f.sp. *tritici*, the induced LOX activity correlated with the onset of the resistance response (Bohland *et*

al. 1997). A differential induction of LOX in resistant and susceptible wheat, inoculated with *Puccinia gramminis tritici*, was also accompanied by an early and rapid hypersensitive cell death in resistant wheat (Ocampo *et al.* 1986), supporting the hypothesis that LOX play a role in the development of resistance *via* the HR (Delaney 1997, Farmer & Ryan 1992).

The locally and systemically accumulation of PR-proteins, chitinase and β-1,3-glucanase (48 h.p.i.), resembled the defence responses during pathogenesis and seemed to be part of the HR, which confers resistance to the RWA in wheat (Van der Westhuizen *et al.* 1998a & b, Van der Westhuizen *et al.* 1996, Van der Westhuizen & Botha 1993). RWA infestation (in resistant wheat) also resulted in necrotic lesions at the site where hypersensitive cell death was induced (Botha *et al* 1998, Van der Westhuizen *et al.* 1998a & b). The differential induction of LOX activity (Fig. 4.4) corresponded with the induction of the HR during the resistance response of wheat against the RWA (Van der Westhuizen *et al.* 1996, Van der Westhuizen *et al.* 1998a & b, Van der Westhuizen & Botha 1993), supporting the role of LOX in the development of resistance *via* the HR. This was further supported by Mehdy (1994), who found that tobacco, infested with *Pseudomonas syringae* pv *syringae*, exhibited a HR that was accompanied by a O_2-generation and a lipid peroxidation increase.

LOX can also produce endogenous signal molecules or regulatory molecules (Farmer & Ryan 1992, Gardner 1996, Heitz *et al.* 1997, Oohmah *et al.* 1997, Siedow 1991) during the RWA resistance response. It has been stated that the activation of the intracellular signaling cascade during defence responses against pathogens and wounding, involved a lipid derived pathway (LOX), called the octadecanoid pathway (Heitz *et al.* 1997). It is also known that the local events in the immediate zone of the RWA stylet entry, trigger systemic events (Van der Westhuizen *et al.* 1998a & b, Van Der Westhuizen & Pretorius 1996). Apparently these systemic events include the systemic induction of LOX (Fig. 4.6), POD, chitinase and β-1,3-glucanase activities (Van der Westhuizen *et al.* 1998a & b). In addition, results of this study confirmed the systemic induction of LOX activity by RWA infestation (Fig. 4.6).

Enyedi *et al.* (1992) reported that the SAR response usually develops after the appearance of necrotic lesions and the HR. It is also associated with the local and systemic induction of PR-proteins (chitinase and β-1,3-glucanase e.g.) (Enyedi *et al.* 1992). During the RWA resistance response, necrotic lesions formed, the HR took place and PR-proteins accumulated locally and systemically (Van der Westhuizen *et al.* 1998 a & b, Van der Westhuizen & Pretorius 1996, Van der Westhuizen *et al.* 1996, Van der Westhuizen & Botha 1993), suggesting the involvement of a systemic acquired resistance (SAR) response. In addition, the correlation between LOX activity and the onset of SAR was also reported elsewhere. During the SAR response in wheat against *Erysiphe gramminis*, it was found that the induction of LOX activity correlated with the onset of its resistance response (Bohland *et al.* 1997). LOX activity was also systemically induced in immunised (by necrotic lesion causing pathogens) cucumber (Avdiushko *et al.* 1993), rice and tobacco (Slusarenko 1996). Kessmann *et al.* (1994) even described LOX as a "molecular marker for the immunised state" in rice (Kessmann *et al.* 1994). When potato tubers were treated with LOX inhibitors, the induction of the SAR response was suppressed, supporting LOXs' role in the SAR response (Slusarenko 1996).

Although the precise role for LOX in the SAR response is unclear, there is the potential for it to act in the three spheres mentioned previously: (1) the production of active oxygen species (oxidative burst), (2) the production of signal substances, (3) or contributing to membrane damage (HR). Thus, it could be argued that LOX contributes to the wheat's state of sensitised anticipation that helps spread its response against the RWA. The systemic induction of POD and LOX activities suggests that activated oxygen species may be part of the SAR expression. This is supported by the induction of local and systemic resistance in potato foliage during *Phytophthora infestans* infection. These resistance responses were accompanied by an increase in superoxide-generating activity and in superoxide dismutase activity, which may be involved in the conversion of superoxide into hydrogen peroxide (Choi *et al.* 1994).

In an attempt to determine whether LOX plays an 'upstream' role in the SAR response against the RWA *in vivo*, LOX inhibitory studies were done. The potential inhibitory effect of three LOX inhibitors, piroxicam (PC), salicylhydroxamic acid (SHA) and n-propyl gallate (nPG), were first tested *in vitro* on LOX, POD and β-1,3-glucanase activities (Figs. 4.7a-c) to determine the most suitable inhibitor to use.

LOX inhibitors have been used widely, trying to establish the role of LOX in defence responses. Peever and Higgins (1989) used PC to confirm involvement of LOX in membrane depolarisation, suggesting that PC inhibits electron leakage (Siedow 1991). The inhibition of LOX activity by SHA, indicated that LOX was needed for wound induced JA synthesis (Slusarenko 1996). SHA was also used in arachidonic acid-stressed potato tuber disks to confirm that an unsaturated fatty acid elicits phytoalexin synthesis (Stelzig *et al.* 1983). SHA delayed the HR in potato (*Solanum tuberosum*), caused by arachidonic acid or poly-L-lysine (Preisig & Kuc 1987). SHA delayed the HR in resistant cowpea cv. (Dixie Cream), induced by the monokaryon of the cowpea rust fungus or the monokaryon derived cultivar-specific elicitor of necrosis (Chen & Heath 1994), confirming the role of LOX in the HR in these interactions. It was found however, that SHA was a reversible inhibitor, indicating that its inhibitory effect can be reversed (Preisig & Kuc 1987). Soybean seed (*Glycine max* var. Wayne) LOX was used to confirm that LOX uses molecular oxygen during catalytic reactions. When LOX was inhibited by SHA, the oxygen concentration was unaffected (Parrish & Leopold 1978). The involvement of LOX in the production of ethylene in sugarbeet (*Beta vulgaris*) was confirmed by the inhibition of LOX with nPG (Krens *et al.* 1994). Inhibition studies, using nPG, proved that LOX also plays a role in the volatile composition of homogenates in green and red bell peppers (Luning *et al* 1995).

The most suitable inhibitor for our purpose would be the one that inhibits LOX activity with no inhibition effect on POD or β-1,3-glucanase activities. According to Figures 4.7a, 4.7b and 4.7c, PC complied with this prerequisite. PC concentrations of 34.8μM (4mM stock solution) and higher, inhibited LOX activity (100%) only, while POD activity was relatively unaffected. Interestingly, PC actually stimulated β-1,3-glucanase

activity (Fig. 4.7a). *In vivo* studies showed that 4mM PC e.g. inhibited LOX, POD and β-1,3-glucanase activities (Fig. 4.9a). The reason for the stimulating effect of higher (higher than 8mM) PC concentrations on POD and β-1,3-glucanase activities *in vivo*, is unknown at this stage. A similar response was also found with nPG at higher concentrations (Fig. 4.8c). *In vitro* studies revealed that SHA is also an inhibitor of POD activity. Our results were supported by Beckman & Ingram (1994) who also found that SHA inhibited POD activity. LOX and β-1,3-glucanase activities were inhibited *in vivo* in a concentration relation manner, upon SHA treatment (Fig. 4.8b).

According to our results, it appears that the *in vivo* inhibition of LOX activity inhibited 'downstream' defence reactions, which include POD and β-1,3-glucanase activities, which strongly indicates the involvement of LOX in the induction of 'downstream' defence reactions.

Plants rely on transmissible signal molecules to activate resistance mechanisms, which result in the SAR response (Enyedi *et al.* 1992). Various LOX pathway products, of which JA is one, has been suggested as possible signal molecules, being induced endogenously upon wounding (Heitz *et al.* 1997) and certain pathogen infections (Pieterse & Van Loon 1999). LOX might therefore be involved in the signaling *via* JA and / or other fatty acid derived products, influencing the production of 'downstream' defence products, as well as inducing systemic defence responses.

Examples of endogenous molecular signals associated with the signalling pathway (and SAR in some instances) include e.g. IAA (indolyl-3-acetic acid), ABA, M-JA, JA, SA, systemin and ethylene (Enyedi *et al.* 1992, Heitz *et al.* 1997). Of the presently known plant fatty acid signals, JA has been studied most extensively (Blèe 1998, Farmer 1994). We decided to investigate the probability of JA being the possible endogenous signal molecule in the signaling pathway, leading to the induction of various defence responses in wheat against the RWA. The reasons for our decision were the following: (1) JA is known as a possible endogenous signal molecule in defence responses against pathogens and insects (Blechert *et al.* 1995, Farmer 1994, Farmer & Ryan 1992, Pieterse & Van

Loon 1999, Sano *et al.* 1996), (2) JA is known to be transported through the phloem (Enyedi *et al.* 1992), and (3) because JA is synthesised *via* the LOX pathway (Blèe 1998, Rosahl 1995).

JA peak concentrations were obtained within 48h in infested and uninfested resistant and susceptible wheat, whereafter it increased gradually in infested and uninfested resistant and susceptible wheat (Fig. 4.11a). It is obvious that the selective induction in LOX activity in infested resistant wheat (Fig. 4.4) did not correlate with a selective increase in JA concentration (Fig. 4.4).

Jasmonate accumulation has been detected in a wide variety of plant species, induced by insect wounding, microbial pathogen attack and elicitation or mechanical stimulation (Blèe 1998, Creelman *et al.* 1992b, Gundlach *et al.* 1992, Rickauer *et al* 1997, Rosahl 1996, Vèronèsi *et al.* 1996, Wasternack & Parthier 1997). According to Farmer (1994), induced jasmonate profiles fall between two extreme peak profiles, namely the 'spike' and 'step' profiles. The 'spike'-JA profile corresponded, in his report, with that of elicitor or pathogen treatments, while 'step'-like profiles are apparently associated with wound responses, as in wounded soybean hypocotyl tissues (Farmer 1994). Both cell suspensions cultures of snakeroot (*Rauvolfia*) (Farmer 1994) and *Agrostis tenius* (Mueller *et al.* 1993), treated with a yeast cell wall elicitor, induced 'spike'-JA profiles (Farmer 1994, Mueller *et al.* 1993). The 'spike'-JA profile corresponded with our JA profiles prior to the 48h infestation period, although we did not find a selective induction of JA in infested resistant Tugela-DN (Fig. 4.11a). It is therefore tempting to speculate that these results could contribute to the speculation that the RWA resistance response resembles that of pathogenesis (also found by Botha *et al.* 1998). But, by comparing the results of Mueller *et al.* (1993) and Blechert *et al.* (1995) with the above mentioned peaks (Fig. 4.11a) and Farmer's (1994) discriptions, one would rather suggest that particular jasmonate profile-shapes are associated with particular defence responses. That means that specific types of defence responses results in specific JA profiles, which can be associated with that defence response in future. Also, JA probably did not play a role in the induced RWA resistance response in wheat.

In addition, during the resistance response of barley against powdery mildew fungus (*Erysiphe graminis* f. sp. *hordei*), JA did not accumulate, suggesting that this resistance response bypassed the JA as signal (Feussner *et al.* 1995). Hause *et al.* (1997), also found no enhanced JA levels in barley leaves, infected with powdery mildew fungus *Egh (Erysiphe graminis* f.sp. *vulgare)*. He accordingly suggested that jasmonate did not act as the signal molecule during these compatible and incompatible interactions (Hause *et al.* 1997), confirming the results of Feussner *et al.* (1995). The absence of JA accumulation were also found in rice/rice blast fungus (*Magnaporthe grisea*) interaction (Bohland *et al.* 1997).

It has been well documented that JA is biosynthesised *via* the LOX pathway (Bohland *et al.* 1997, Croft *et al* 1993, Enyedi *et al.* 1992, Mauch *et al.* 1997, Rickauer *et al.* 1997, Rosahl 1995, Vick & Zimmerman 1983) and that some LOX isoforms have been closely related to the *de novo* synthesis of JA under stress conditions (Blèe 1998). LOX catalyses the oxygenation of unsaturated fatty acids into the 9- and 13-HPODs (Feussner *et al.* 1995, Rosahl 1995, Siedow 1991). Depending on the source of LOX and the invader, the ratio of 9-HPOD:13-HPOD varies (Croft *et al.* 1993, Rosahl 1995). Due to the fact that JA is specifically biosynthesised from the 13-HPOD (Farmer & Ryan 1992, Oomah *et al.* 1997, Rosahl 1996, Vick & Zimmerman 1983), and that there was no correlation between JA levels and induced LOX activity in RWA infested wheat (Fig. 4.4 & 4.11a), one could expect that the 9-HPOD pathway was favoured during the RWA resistance repsonse. In contrast, treatment of tobacco cell suspensions with an elicitor from *Phytophthora parasitica* var. *nicotianae*, induced JA accumulation. But, when LOX activity was inhibited with ETYA (eicosatetraynoic acid), JA did not accumulate (Rickauer *et al.* 1997).

The predominant production of 9-HPOD, has been reported in various defence responses, e.g. in wheat (Bohland *et al.* 1997, Kühn *et al.* 1985), potatoes (Choi *et al.* 1994, Gardner 1996,) and tomatoes (Gardner 1996), supporting the possibility that the 9-HPOD pathway is followed during the RWA/wheat interaction. This was supported by our inhibitory

studies, which indicated that *in vivo* inhibition of LOX (with PC), does not inhibit JA biosynthesis (Fig. 4.13). According to Blèe (1998), LOX isoforms induced in tomato leaves infected by powdery mildew and parsley cell cultures, rice seed or wheat seedlings treated with elicitors, produced mainly 9-HPOD, arguing against the role of these LOXs in the synthesis of elicitor-induced JA. This hypothesis was strongly supported by an experiment where transgenic potato plants were used. Transgenic potato plants, originating from an incompatible race, that was impaired with this particular LOX expression, were found to turn sensitive to infection (Blèe 1998). Rosahl (1996) reported that tobacco cell cultures treated with glycoprotein elicitors from *Phytophthora parasitica* var. *nicotinae*, induced LOX activity favouring the 9-HPOD, which is not the precursor for JA biosynthesis. Similar results were also found in barley leaves infected with powdery mildew *Erysiphe graminis* f.sp. *hordei* (Rosahl 1996). Taking into consideration the selective (after 48 hours of RWA infestation) increase in LOX activity (Fig. 4.4) in comparison to the non-selective increase in JA (Fig. 4.11a), as well as the possible predominant expression of 9-HPOD (referring to Fig. 4.11a), we argued against the role of the RWA induced LOXs in the synthesis of JA.

In wheat, treated with elicitors (e.g. rust fungus elicitor, chitin and M-JA), the induced LOX isoforms favoured the production of 9-HPOD. Partial characterisation of these induced LOX isoforms (mostly responsible for the induced activity) showed that their molecular masses ranged from 92 to 103 kDa. These induced LOX isoforms were also most reactive at pH 5.5-6.0 (Bohland *et al.* 1997). According to Fig. 4.3a, the RWA induced LOX isoforms correlated with those found by Bohland *et al.* (1997), in regard to its optimum pH ranges, supporting our suggestion that the RWA induced LOX isoforms favour the 9-HPOD pathway.

Possible products to be derived from the 9-HPOD, include e.g. ketols, cyclopentane, (3Z, 6Z)-nonadienal and (3Z)-nonenal (Blèe 1998). According to Blèe (1998) the aldehydes produced from 9-HPOD, were found to be more toxic than hexanals, but less effective due to their lower activity, indicating that: (1) most of these aldehydes are long-lived, (2) diffuse from their sites of production to reach extra-cellular targets (3) are active

compounds either as volatile molecules or as cellular constituents. However, these aldehydes can be transformed further into potentially cytotoxic and mutagenetic compounds. One such a metabolite example is 4-hydroxy-2-nonenal, which could act as a self-defence component against pests, and as a regulatory substance in plants (Blèe 1998).

We concluded that JA was most probably not the signalling molecule in the RWA/wheat interaction, eliciting defence responses, as was found to be the case for various other defence responses (Choi *et al.* 1994, Pieterse & Van Loon 1999, Sano *et al.* 1996). Another molecule possibly acted as the signalling molecule in the RWA resistance response. This suggestion was supported by findings of Choi *et al.* (1994) and Sano *et al.* (1996), who reported that other signalling pathways, involving salicylic acid (SA) and ethylene (Choi *et al.* 1994, Kessman *et al.* 1994, Pieterse & Van Loon 1999, Rickauer *et al.* 1997, Sano *et al.* 1996), were induced in tomato, potato and cucumber, and tobacco respectively, in response to pathogen attack. Bohland *et al.* (1997) also reported that JA did not act as signal molecule in the defence response in the rice/rice blast fungus interaction. Plants respond to wounding and pathogens, using distinct signalling pathways (Choi *et al.* 1994, Sano *et al.* 1996).

Generally, wound signals are transmitted by JA (Sano *et al.* 1996), inducing basic PR-proteins like proteinase inhibitors (PIs) (Enyedi *et al.* 1992, Pieterse & Van Loon 1999, Sano *et al.* 1996), while pathogenic signals cause accumulation of SA (Sano *et al.* 1996), inducing acidic PR-proteins like chitinase and glucanase (Pieterse & Van Loon 1999, Sano *et al.* 1996). By using transgenic (*rgp*1) tobacco plants (*Nicotiana tabacum* cv. Xanthi), Sano *et al.* (1996) found that SA induced acidic PR-proteins, while JA induced basic PR-proteins (Sano *et al.* 1996). The systemic accumulation of SA has also been reported by Choi *et al.* (1994) in tobacco and cucumber after pathogen inoculation, inducing the SAR response (Choi *et al.* 1994). Pieterse and Van Loon (1999) found that in transgenic plants, expressing the bacterial *nahG* gene (which encodes salicylate hydroxylase that inactivates the accumulation of SA), did not induce the SAR response to develop. This suggests that SA is required (SA-dependant) for expression of the SAR in

plants with the SAR-dependant pathway (Pieterse & Van Loon 1999). SA also reduced symptoms of TMV (tobacco mosaic virus) inoculation of both TMV-susceptible tobacco cv. Samsun nn and TMV-resistant tobacco cv. Samsun, probably through activating PR-protein genes (like glucanase and chitinase) and POD genes (Enyedi *et al.* 1992). Accordingly, these results also supported the suggestion that SA induce the SAR response, as was found by Kessman *et al.* (1994) and Enyedi *et al.* (1992).

This proposed role of SA as signalling molecule was supported by the findings of Mohase (1998), who found that SA accumulated differentially upon RWA infestation in wheat. Also, acidic PR-proteins were induced in the RWA/wheat interaction (Van der Westhuizen *et al.* 1998a & b), supporting the proposed SA signalling pathway in the RWA resistance response even further. It is interesting to note that SA derivatives inhibit the biosynthesis of JA and M-JA (Kessman *et al.* 1994, Sano *et al.* 1996). SA inhibited the expression of wound-induced gene expression through the suppression of JA biosynthesis (Kessman *et al.* 1994).

JA has been regarded as a positive regulator of its own biosynthesis, due to the fact that the LOXs responsible for JA formation are strongly induced by the application of JA and its methyl ester (M-JA) (Blèe 1998). The exogenous application of M-JA (250μM M-JA gas) to soybean leaves, led to the accumulation of a storage protein believed to be a LOX (Enyedi *et al.* 1992). LOX was also induced in barley upon JA treatment (Wasternack & Parthier 1997). Thaler *et al.* (1996) found that exogenous application of JA (10mM JA sprayed onto the leaves) to tomato (*Lycopersicon esculentum*) leaves, induced LOX and POD activities. In barley, parsley and transgenic tobacco JA also induced PR-proteins (Wasternack & Parthier 1997).

Similar results were found when JA was intercellularly injected into wheat leaves. JA (2μM) induced LOX, β-1,3-glucanase and POD activities by 50%, 50% and 20% respectively (Fig. 4.12). Our results were supported by Blèe (1998) and Thaler *et al.* (1996) who documented that JA induces LOX, POD, β-1,3-glucanase, chitinase, and PR-proteins 1 & 5. It can be expected, however, that higher concentrations of JA (>2μM)

would have different effects on POD and β-1,3-glucanase activities, as JA is regarded as a phytohormone (Rickauer *et al.* 1997). It is therefore apparent that JA can induce several defence responses when induced.

In addition, we determined the effect of RWA infestation on abscicic acid (ABA) concentrations in susceptible and resistant wheat. Although it has been reported that ABA could be biosynthesised indirectly through the LOX pathway (*via* xanthoxins) (Giraudat *et al.* 1994, Slusarenko 1996), no correlation was found between the induced LOX activity (Fig. 4.4) and the *in vivo* ABA concentrations (Fig. 4.11b) during RWA infestation studies. Therefore, LOX do not facilitate the selective accumulation of ABA in wheat upon RWA infestation. It is interesting to note that Giraudat *et al.* (1994) documented that JA synthesis is ABA-dependent and that ABA controls the early induction of one of the JA biosynthetic enzymes. One wonders whether the 'lack' of JA accumulation was caused by a lack of ABA during the RWA/wheat interaction.

To conclude: The selective local and systemic induction of LOX activity in resistant wheat, strongly indicates the involvement of LOX in the RWA/wheat interaction. The 'upstream' role of LOX was substantiated by inhibitory studies, revealing that the inhibition of LOX caused inhibition of 'downstream' POD and β-1,3-glucanase activities. Also, the selective induction of LOX activity is not to produce JA signalling molecule accumulation, nor to produce ABA accumulation. This was further substantiated by the *in vivo* inhibition of LOX activity, whereafter JA concentrations were not inhibited. Therefore, the 9-HPOD pathway is most probably induced upon RWA infestation, producing other unknown products and/ signal molecules.

Summary

The upstream role of lipoxygenase (linoleate: oxygen reductase, E.C. 1.13.11.12) in the induced defence response against the Russian wheat aphid (*Diuraphis noxia*) was studied in resistant wheat (*Triticum aestivum* L.) cultivars Tugela-DN (Dn1), Tugela (Dn2) and Tugela (Dn5) and the near isogenic susceptible cultivar, Tugela.

The RWA induced LOX was partially characterised. The pH for optimum LOX activity was 5.6 and it had no requirement for cofactors such as K^+, Mg^{2+} or Ca^{2+}. The enzyme also had a high affinity for linoleic acid, with a Km-value of ~5.8 x 10^{-4}M.

LOX activity was selectively induced in infested resistant Tugela cultivars containing the (Dn1), (Dn2) and, to a lesser extent, (Dn5) resistant genes. The differential induction of LOX activity corresponded with the induction of the hypersensitive response during the resistance response of Tugela-DN against the RWA. The incorporated resistant gene affected the level of induced LOX activity, which roughly corresponded with the cultivars' levels of resistance. Localised RWA infestation led to a systemic increase in LOX activity. All indications were that the induced LOX activity was involved in the resistance response in wheat against the RWA. Inhibitory studies were performed with LOX inhibitors, piroxicam, salicylhydroxamic acid and n-propyl gallate, to shed light on the possible upstream role of LOX. Piroxicam was the most suitable inhibitor due to the fact that it only inhibited LOX activity *in vitro*. Inhibition of LOX activity *in vivo* by piroxicam, led to the inhibition of 'downstream' defence responses such as peroxidase and β-1,3-glucanase activities. The selective increase in LOX activity did not correlate with a selective increase in jasmonic acid concentrations. The effect of intercellular injected JA on LOX, peroxidase and β-1,3-glucanase activities, indicated that jasmonic acid was not the primary product of the RWA induced LOX activity and that it was not the signal molecule in the RWA/wheat resistance response. The products of the RWA induced LOXs appeared to be derived from the 9-hydroperoxide derivative pathway, rather than the 13-hydroperoxide derivative (jasmonic acid precursor) pathway. Neither was abscicic acid a product of the selective increased LOX activity, nor playing a role in the RWA resistance response in wheat. Other studies in our laboratory showed that salicylic

acid is probably the signal molecule, activating defence genes after elicitation. It is evident that the RWA resistance response corresponded with that of pathogenesis, and that each defence response follows its own distinctive defence cascade.

Key words: Wheat (*Triticum aestivum* L.), Russian wheat aphid (*Diuraphis noxia*), lipoxygenase, resistance, resistance genes, systemic, inhibitors, signalling, jasmonic acid, abscisic acid, salicylic acid.

Opsomming

Die 'stroom-op' rol van lipoksigenase (linoleaat: suurstof reduktase, E.C. 1.13.11.12) in die geïnduseerde weerstandsreaksie teen die Russiese koringluis (*Diuraphis noxia)* was in weerstandbiedende koring (*Triticum aestivum* L.) kultivars, Tugela-DN (Dn1), Tugela (Dn2) en Tugela (Dn5) en die naby-genetiese vatbare kultivar, Tugela ondersoek.

Gedeeltelike karakterisering van die Russiese koringluis (RKL) geïnduseerde lipoksigenase het getoon dat die optimum pH vir maksimale lipoksigenase-aktiwiteit, 5.6 was. Die geïnduseerde lipoksigenase het geen behoefte aan ko-faktore soos K^+, Mg^{2+} en Ca^{2+} gehad nie en dit het 'n hoë affiniteit vir linoleïensuur getoon, met 'n Km-waarde van ~5.8×10^{-4}M.

Lipoksigenase-aktiwiteit was selektief geïnduseer in geïnfesteerde weerstandbiedende Tugela kultivars bevattende weerstandsgene (Dn1), (Dn2) en, tot 'n mindere mate, (Dn5). Die differensiële induksie van lipoksigenase-aktiwiteit het met die induksie van die hipersensitiewe-reaksie in weerstandbiedende koring Tugela-DN tydens RKL infestering gekorrelleer. Die geïnkorporeerde weerstandsgene het die vlak van geïnduseerde lipoksigenase-aktiwiteit beïnvloed. Die lipoksigenase-aktiwiteitsvlak het ook rofweg met die weerstandbiedendheid van die kultivars ooreengestem. Gelokaliseerde RKL infestering het tot die lokale en sistematiese induksie van lipoksigenase-aktiwiteit gelei. Alles dui daarop dat die geïnduseerde lipoksigenase-aktiwiteit in die weerstandsrespons teen die RKL in koring betrokke is. Inhibisie-ondersoeke was met behulp van lipoksigenase-remstowwe, piroksikam, salisielhidroksaamsuur en n-propielgallaat gedoen, om lipoksigenase se 'stroom-op' rol tydens die RKL-weerstandsreaksie te bepaal. Piroksikam was die geskikste remstof, aangesien dit alleenlik lipoksigenase-aktiwiteit *in vitro* gerem het. Die *in vivo* remming van lipoksigenase-aktiwiteit, het gelei tot die 'stroom-af' remming van peroksidase- en β-1,3-glukanase-aktiwiteite. Die selektiewe toename in lipoksigenase-aktiwiteit het nie met die selektiewe toename in jasmoonsuur-konsentrasies *in vivo* gepaard gegaan nie. Die aktiverende invloed van jasmoonsuur, intersellulêr toegedien, op lipoksigenase-, peroksidase- en β-1,3-glukanase-

aktiwiteite, bevestig dat jasmoonsuur nie die produk van die RKL geïnduseerde lipoksigenase-aktiwiteit was nie, en dat dit nie opgetree het as die seinmolekuul tydens die RKL-weerstandsreaksie in die koring nie. Die RKL geïnduseerde lipoksigenase-aktiwiteit se produkte blyk vanaf die 9-hidroperoksied-derivaat gesintetiseer te wees, en nie vanaf die 13-hidroperoksiedderivaat (jasmoonsuur voorloper) nie. Soortgelyk was absisiensuur (ABA) ook nie 'n produk van die selektief geïnduseerde lipoksigenase-aktiwiteit nie, en het dit ook nie 'n rol in die RKL-weerstandsreaksie in koring gespeel nie. Ander studies in ons laboratorium het getoon dat salisielsuur moontlik die seinmolekuul is wat verdedigingsgene aktiveer na elisitering. Dit is duidelik dat die RKL-weerstandsrespons ooreenstem met die van patogenese, en dat elke verdedigingsreaksie sy eie unieke verdedigings-sein-kaskade meganisme volg.

Sleutelwoorde: Koring (*Triticum aestivum* L.), Russiese koringluis (*Diuraphis noxia*), lipoksigenase, weerstandbiedendheid, weerstandsgene, sistemies, remstowwe, seinmeganisme, jasmoonsuur, absisiensuur, salisielsuur.

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