Biochemistry of Russian wheat aphid resistance in wheat: Involvement of lipid-like products

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By

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PREFACE

The work presented here is a result of an original study conducted at the Department of Plant Sciences, University of the Free State, Bloemfontein. This research was done under the supervision of Prof. A.J. van der Westhuizen.

The Russian wheat aphid not only poses a serious threat to the South African wheat industry, but to wheat production in the rest of the world. Much has been learned about the defence mechanism of wheat against the Russian wheat aphid, but it is still unclear to what extend the lipoxygenase and the cyclooxygenase pathways are involved during the defence response. Both of these pathways are involved in the biosynthesis of lipid-like products. Many of these lipid-like products have been implicated in various forms of stresses and play a very critical role in the establishment of a successful resistance response. In this study I have aimed to describe the importance of the involvement of the lipoxygenase- and the cyclooxygenase pathways during the defence response of resistant wheat to the Russian wheat aphid.

I have not previously submitted this dissertation to any other universities/faculties. I therefore cede its copyright in favour of the University of the Free State.

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List of Abbreviations

amu	atomic mass units
AOS	active oxygen species
CAT	catalase
COX	cyclooxygenase
cv	cultivar
DN	*Diuraphis noxia*
EDTA	ethylenediaminetetra-acetate
et al	*et alii* (others)
h.p.i.	hours post infestation
HPOD	hydroperoxy octadecanoic acid
IR	infested resistant
IS	infested susceptible
JA	jasmonic acid
kDa	kilo Dalton
LOX	lipoxygenase
MDA	malondialdehyde
PGHS	prostaglandin endoperoxide H-synthases
PIOX	pathogen induced oxygenase
POD	peroxidase
R	resistant
RT	retention time
RWA	Russian wheat aphid
S	susceptible
SAR	systemic acquired resistance
SDS-PAGE	sodium dodecylsulphate-polyacrylamide gel electrophoresis
TBS	tris-buffered saline
TBST	tris buffered saline Tween-20
TMS	trimethylsilyl

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Chapter 1

INTRODUCTION

The Russian wheat aphid (RWA), *Diuraphis noxia* (Mordvilko), is of the most noxious pests of cereal crops throughout the world (Kovalev et al., 1991). It became a serious pest in South Africa in 1978 (Walters, 1984) and since then, it remained persistently a serious pest. In field experiments the RWA could account for up to 90% of crop losses, especially in the summer rainfall production region (Du Toit & Walters, 1984) of South Africa.

The RWA is endemic to southern Russia and countries bordering the Mediterranean Sea such as Iran and Afghanistan (Hewitt et al., 1984), where it has been a pest since 1912. Since then sporadic outbreaks of this pest have occurred in the former USSR. In the Crimea, this species has decreased crop yield by as much as 75%. In south central Turkey, heavy damages were reported in 1962 in wheat and barley crops (Burton & Webster, 1993). The aphids spread to Ethiopia and became a serious pest during the 1972/1973 wheat season (Haile, 1981). In 1978, the RWA was reported in South Africa (Walters, 1984) and two years later in Mexico (Gilchrist et al., 1984). The RWA was first noted in 1986 in the USA and spread quickly to all the major wheat producing areas and was soon afterwards detected in Canada (Jones et al., 1989 & Miller et al., 1994). Damage to wheat as far as Asia and countries surrounding the Mediterranean Sea was also reported (Marasas et al., 1997).

The aphid is small and lime-green coloured aphid with a distinctive football-shaped body. The legs, antennae and cornicles are short compared to most other aphids. Viewed from the side, the terminal segment of the abdomen has a supracaudal structure that appears as a double tail (Michaud & Sloderbeck, 2005). The aphids feed on wheat until the plant is mature and can often be found in developing heads. When wheat plants die in response to heavy aphid feeding, the third and forth instar aphids develops wings. The RWA reproduces asexually. All aphids are females, and each gives birth to live daughters carrying embryonic granddaughters. In Asia, the RWA may produce a sexual generation in the fall, with mated females laying eggs overwinter (Michaud & Sloderbeck, 2005).

They feed by probing their stylets intercellulaly until they reach the phloem (Fouché et al, 1984). The aphids prefer to feed on the basis of the leaves where they are also protected from their natural enemies. After several days of feeding, the chloroplast and the cell membranes become disrupted or disintegrated (Marasas, 1999). However, leaves of resistant wheat cultivars are able to maintain their

chlorophyll content at a relative stable level for much longer periods than susceptible ones (van der Westhuizen & Pretorius, 1995; Ni & Quisenberry, 2005). The elicitation of the defence mechanism still eludes us today, but it is believed that the RWA secretes a phytotoxin during feeding, which results in the early breakdown of chloroplasts in susceptible cultivars (Fouché et al., 1984; Burt & Burton, 1992).

Visible damage caused to the susceptible wheat leaves include longitudinal leaf chlorosis and leaf rolling. Infested resistant wheat leaves exhibit chlorotic spots compared to the streaks (white, yellow and purple to reddish-purple) formed on susceptible leaves and no leaf rolling occur in resistant wheat leaves. Growth is only slightly affected and the resistant wheat plant manages to survive the continuous presence of the aphids under confined conditions (Walters et al., 1980). Damage is greatest when crops start to ripen and this is concurrent with peak aphid numbers. The RWA also occurs on oats, rice, sorghum, brome, canary grass, wheat grass and other native grasses (Marasas et al., 1997).

Very little was known about the RWA and threshold values of insectisides to control it when reseach commenced in 1980. At this point in time insecticides registered for the control of other cereal aphids were ineffective (Du Toit & Walters, 1984). The use of insecticides began in 1983 (Marasas et al., 1997) and although it is expensive and also harmful to the environment, its use to control sporadic outbreaks of the aphid is continued to this day (Hayes, 1998). A certain level of financial resources and management skills are required to chemically control the RWA in an economically viable manner (Marasas, 1999).

Searching for the resistance genes for control of the RWA, researchers maintained that genetic resistance was more likely to be found in the primitive wheat species from Asia and the original distribution area of both wheat and the RWA. The spread of the RWA to the USA and Mexico during the 1980's intensified the search for resistance genes to the RWA. Research commenced in 1985 in South Africa when genetic resistance was found in bread-wheat lines (Marasas et al., 1997). These genes were introduced into lines with more acceptable agronomic characteristics by means of the backcrossing technique. Because RWA populations all over the world interact differently with resistant cultivars (Puturka et al., 1992), germplasm of South African RWA populations was screened to ensure that suitable types were used in the breeding programme (Marasas, 1999). The first resistant wheat cultivar, Tugela DN, was released in 1993 (Du Toit, 1988). Since then several

resistant cultivars have been released into the market. Most of these cultivars contain the same single dominant gene, Dn1, conferring the resistance (Marasas, 1999). Other resistant genes, like Dn2 and Dn5, have also been identified and incorporated into different wheat lines. The Small Grain Institute at Bethlehem, South Africa, developed several of the new RWA resistant cultivars e.g., 'Elands', with a much higher yield potential compared to 'Tugela DN', while also showing resistance towards various pathogens (Hayes, 1999). Plants resistant to the RWA are able to maintain their yield under infestation conditions whereas the susceptible lines show decreases in yield (Mornhinweg *et al.*, 2005). An alternative to breeding for possible genetic resistance, biological control of the aphid has also been considered. In the case of the RWA, biological control is understood as the use of living organisms to control the pest. Classically, biological control involves the importation and subsequent release of natural enemies of a pest. The RWA invaded South Africa without its natural enemies (Marasas, 1999). Indigenous enemies in South Africa, such as ladybirds, were unable to control the RWA (Aalbersberg et al., 1988; Prinsloo, 1990). A reason for this is that they are more polyphagous and not species-specific. Polyphagous natural enemies react to prey when present in fairly high numbers but the wheat crop is already damaged by the time the RWA population is sufficient for natural enemy aggression and the developmental rate of the natural enemies is slower than that of the RWA (Marasas, 1999). If natural enemies are imported from their countries of origin and released in South Africa, they could theoretically control the pest (Marasas et al., 1997). The first natural enemies (*Aphidius matricariae*) were imported from Turkey in 1988 (Marasas, 1999). Unfortunately no differences in the parasitization of RWA could be demonstrated in the field (Marasas, 1999). This was followed by several rounds of parasitoid releases (Marasas et al., 1997 & Tolmay et al., 1998). Evidence confirmed that the parasitoid was able to travel great distances, but could not be recovered consistently. This could either be a result of the host-specificity of the parasitoids, implying their inability to survive on other aphids during the summer season (Marasas, 1999).

Subsequently, the Small Grain Institute developed an integrated control program, which includes the use of several resistant cultivars together with the natural enemies of the RWA, in an attempt to alleviate the problem. Ladybirds and entomopathogenic fungi were also used to control the RWA (Marasas et al., 1997). Trials in 1993 demonstrated that when both plant resistance and biological control measures were used in the field, aphids could be controlled without the use of insecticides. It was also believed that this approach reduces the chance of a

resistance breaking biotype of the RWA to form (Marasas et al., 1997). Applying the integrated program requires no technical knowledge or equipment and is therefore suitable for both commercial and subsistence farmers. Hatting *et al*. (1999) reported that there is no advantage in spraying resistant wheat with insecticides to control the RWA. As a result of the implementation of these control strategies the RWA is contained and do not pose a serious threat to farmers anymore. However, the outbreak of a resistance breaking biotype in the USA (Jyoti *et al*., 2006 & Qureshi *et al.,* 2006) once again necessitated the use of insecticides to control the aphid. Further, new resistant wheat cultivars need to be developed to successfully implement biological control strategies and to reduce the chances for resistance breaking biotypes to appear. In this regard there were reports that a resistance breaking biotype was noticed in some of the wheat producing areas in South Africa (personal communication with Goddy Prinsloo, Small Grain Institute). An isolated occurrence of the RWA was reported in the winter grain region of the Western Cape during the 2004 season (personal communication with Vicky Tolmay, Small Grain Institute). The reason for the occurrence of RWA in the Western Cape was propably due to a warmer dryer winter period.

During the 2003 wheat season a new RWA biotype was identified in Colarado, USA (Jyoti *et al.*, 2006). This biotype was designated as "biotype 2" and caused extensive yield losses in Colorado, especially during the 2004/2005 season, in varieties that carry the original resistance to the RWA (designated as "biotype 1") (Collins *et al*., 2005). This recent identification of a RWA biotype that is virulent on current RWA-resistant cultivars necessitates the rapid identification of resistance to this new biotype. Genetic characterization has now become extremely critical to the identification of aphid resistant genes.

It is not surprising to find that a new resistance breaking biotype has evolved. Many greenbug biotypes have developed over the years that were able to overcome various resistant wheat and sorghum plants. The RWA being a world-wide pest and its ability to destroy crops have led to the planting of thousands of hectares wheat resistant to the RWA. This increase in the cultivation of resistant wheat could have initiated a strong selection pressure that favoured aphids with resistance breaking mutations (Michaud & Sloderbeck, 2005). This new biotype proves to be even more virulent which poses a great threat for wheat producing areas. The search for additional resistant genes will continue in the future. They will have to be characterized and their effects on the RWA assessed to identify alternative

resistance mechanisms. This would be crucial if further resistance-breaking biotypes should develop.

Understanding the underlying biochemical defence mechanism, has become now even more crucial than ever before. Moreover, the use of insecticides is becoming more and more undesirable since there is a lot of pressure on the agricultural community to do away with insecticides. The use of transgenic plants does not seem to be the answer to the problem either, for the fear of the continuous outbreak of new resistant breaking biotypes. Only once the biology of plant-microbe and plant-insect interactions are understood, scientists will be able to identify genes and ways that could be used to make plants more resistant to pests and pathogens without the fear of new biotypes evolving. To identify the biochemical mechanisms involved during a defence response will be a step closer to understand this complex plant-insect interaction. This could supply the rationale for future molecular biological studies.

The objectives of this study were to:

a) elevate the current understanding of the biochemical basis of aphid resistance in wheat,
b) identify novel defence compounds (e.g. fatty acids) that could be involved in the defence mechanism of wheat during the resistance mechanism and
c) identify biochemical pathway(s) that are induced in the resistance response of wheat.

It is envisioned that this knowledge might be very useful in future to invent novel control strategies.

CHAPTER 2

Literature Review

2.1 DEFENCE RESPONSES

Plants encounter a wide range of abiotic stresses, including drought, cold, and salt etc., and biotic stresses such as plant pathogen and insect attacks. To adapt to these stresses, plants use diverse and sophisticated signalling strategies for recognizing and responding to these stresses. The first step in activating a defence response is to perceive the stress and then to relay the received information through complex signal transduction pathways to the genes needed to be activated. The products of these defence genes are responsible for a successful defence mechanism (Gang *et al.*, 1999, García-Garrido & Ocampo, 2002).

Plants perceive the stresses in different ways, including by means of sensors, receptors, elevated calcium concentrations and changing membrane fluidity. Stress perception and signalling leads to biochemical reactions, metabolic adjustments and an altered physiological state. By doing this plants have evolved mechanisms by which they can increase their tolerance against these stress factors (Gang *et al.*, 1999, McDowell & Dangl, 2000). Consequently, the signalling pathways underlying plant adaptation is very complex. Knowledge about the signal transduction pathways and the genes they influence is essential to develop plants with properties of high tolerance against abiotic and biotic stresses.

There is a continuous struggle for survival between pathogens and their hosts. Over the millenniums, plants have adapted unique defence strategies to protect themselves against harmful herbivores and pathogens. It is speculated that many plant species evolved in a very hostile environment and adapted in order to survive. As food source leaves are very tempting for a wide range of animals, especially in dry areas, and plants had to adapt in order to protect themselves and minimize grazing by herbivores. Insects, in response, have developed ways to circumvent the defence mechanisms of the plant. The successful colonization of a plant by a pathogen leads to disease; the plant is said to be “susceptible” and the interaction is described as “compatible”. Effective resistance of the plant is expressed in the “incompatible” interaction and disease fails to develop (Slusarenko, 1996).

Plants developed a number of passive and active defence strategies. Passive defence mechanisms can be subdivided into morphological, structural,

anatomical and chemical factors. Anatomical features such as leaf and flower colour, presence of trichomes and the texture of the cuticle may cause insects to avoid a plant. Features such as secondary wall thickening, stellar structure and other aspects of basic structure may also occur. Structural and morphological structures are good barriers against grazers, but prove to be less effective against viruses, fungi, bacteria and nematodes (Lucas *et al*., 2000). Some plants contain significant amounts of preformed chemicals produced via secondary metabolism, which may include phenolics, terpenoids and steroids. Some preformed compounds are directly toxic, while others exist as conjugates such as glycosides, which are not toxic but, become toxic following the disruption of the conjugate (Baker *et al.*, 1997). These defence barriers need to be overcome before colonization and disease can set in.

In many plants a second line of defence is in place when the first line of defence (passive) becomes obsolete. This line of defence depends upon the successful recognition of the pathogen, or other intruders, by the plant (Baker *et al.*, 1997). After recognizing the invader, a signal continues along the signal-conducting pathway activating the defence genes to produce, through transcription, enzymes of the secondary metabolic pathway (Fig. 2.1), which will ultimately lead to the synthesis of phytoalexins, either located in the vicinity of the attack or systemically throughout the plant (Blechert *et al*., 1995). The plant's metabolism is subsequently reorganized to synthesize new enzymes and metabolites, that are channelled into newly activated biosynthetic pathways. Some of the newly synthesized plant enzymes, for example chitinase and ß-1,3-glucanase, can degrade pathogen cell walls and also considered to be antimicrobial (Slusarenko, 1996).

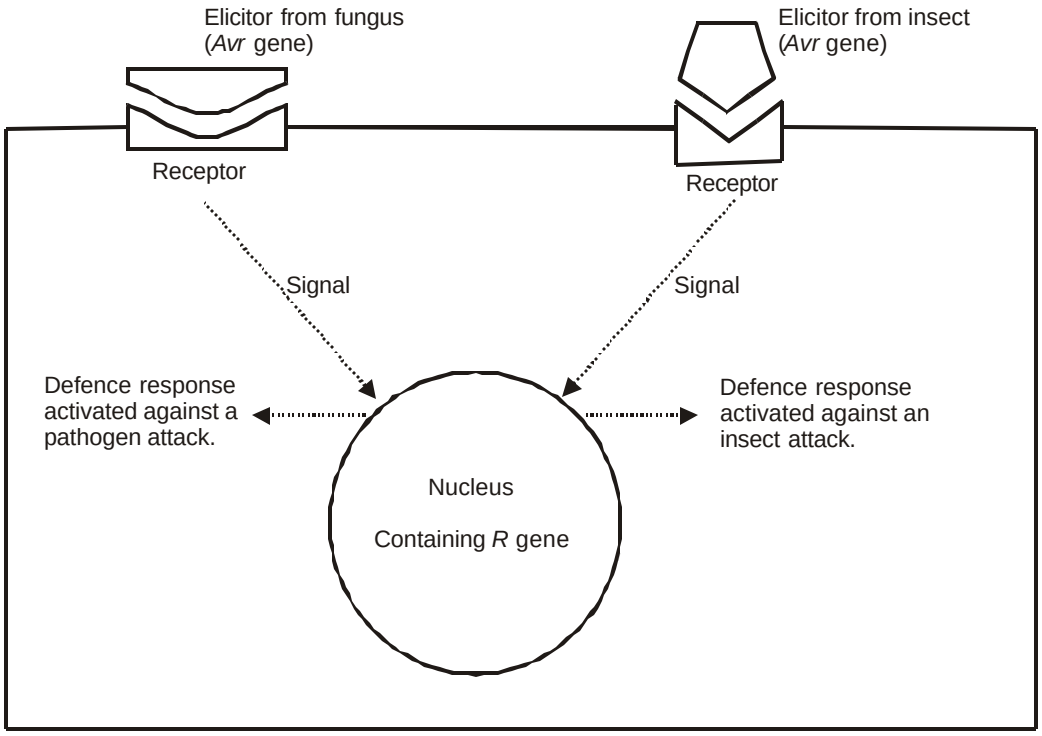


Figure 2.1 Perception and activation of defence responses after a pathogen or insect attack (Slusarenko, 1996).

Plant cells located at the site of infection or infestation can undergo a type of programmed cell death called the hypersensitive response (HR). The HR is associated with stopping the pathogen spread (Montellit *et al*., 2005). The HR is an active form of defence and can be activated by disease-resistance genes and can be activated as a general response as well. These genes enable the plant to detect and resist pathogens. The plant needs to recognize at least one molecule produced as a result of the invading pest to activate the HR. These molecules are called elicitors (Baker *et al*., 1997). Elicitors are low molecular weight compounds of either pathogen/insect or host origin that are able to induce defence responses in plant tissue. Many elicitors have been described, including polysaccharides, oligosaccharide fragments, proteins, glycoproteins and fatty acid derivatives (Dixon & Lamb, 1990). Elicitors derived from fungal plant pathogens induce defence responses normally associated with fungal infection. Enzymes such as chitinase, peroxidase and ß1,3-glucanase are induced and have a direct action on fungi (Benhamou, 1996; Gelli *et al*., 1997). Elicitors themselves, in the absence of the

living pests, are able to initiate the active defence response. Elicitors can be divided into race-specific and non-specific elicitors. Race-specific elicitors induce a response only in host cultivars. In this gene-for-gene resistance system (host incompatibility), a specific host *R* gene is needed to detect specific pathogen-derived components (products of *Avirulence* [*Avr*] genes) in much the same way in which animal adaptive immune systems are capable of recognizing foreign molecules (Taylor, 1998). A non-specific elicitor will induce a general defence mechanism leading to basic incompatibility. Perception of the elicitors takes place at specific elicitor-binding sites. Wounding by insects or microbial pathogen attack leads to an interaction of elicitors with receptors initiating the octadecanoic-based pathway from the C_{18} fatty acids (Vick & Zimmerman, 1984). Specific and non-specific elicitors trigger signal transduction cascades such as protein kinases, elements of the mitogen-activated protein (MAP) kinase pathway and protein phosphatases (Desikan *et al.,* 1990; Nürnberger, 1999) all of which are needed for the establishment of a successful defence response.

Of the earliest responses activated after host recognition are the oxidative burst and the opening of specific ion channels (Hammond-Kosack & Jones 1996). During the oxidative burst, there is a sudden increase in the generation of reactive oxygen species (ROS) (Alvarez *et al*, 1998; Hammond-Kosack & Jones, 1996; Wojtaszek, 1997). Reactive oxygen species (H_2O_2, OH^- and $O\cdot_2^-$) play a key role during defence. They can be generated by means of different mechanisms involving different enzymes such as oxalate oxidase using oxalic acid as substrate (Zhang *et al.*, 1995), cell wall peroxidases, the NADPH-oxidase complex (Desikan *et al*., 1996), the xanthine oxidase complex (Montalbini, 1992) and superoxide dismutase (SOD) (Liochev & Fridovich, 1994; Fridovich, 1995). This oxidative burst, which occurs in the cell wall, is thought to function as a signal for downstream defence responses and to participate directly in chemical reactions that strengthen the cell wall and attack pathogen surfaces, thereby limiting the progress of invasion (Cosgrove *et al.*, 2000). Since H_2O_2 has no unpaired electrons, it can easily cross biological membranes, which the charged O_2^- species can only do very slowly (Halliwell & Gutteridge, 1990). Both O_2^- and H_2O_2 are only moderately reactive. However, the cellular damage caused by ROS appears to be due to their conversion into more reactive species such as $OH^{\bullet}$ and $HO_2^{\bullet}$.

The large and rapid build-up of ROS intermediates exerts a severe oxidative stress on the affected cell and consequently counter measures should be taken to

alleviate the stress. Although high concentrations of ROS are very useful to kill pathogens and the affected plant cell (hypersensitive cell death), only a low dosage is needed for signalling and to activate the detoxification mechanism (Lamb & Dixon, 1997). The detoxification mechanism involves the induction of SOD, glutathione-*S*-transferase and the ascorbate cycle (Wojtaszek, 1997). The super oxide free radical, O_2^-, acts downstream of the membrane-associated reactions (Ligterink *et al.*, 1997) while H_2O_2 is able to diffuse much more easily through membranes and induces defence response in neighbouring unaffected cells.

Several roles for ROS in plant defence have been proposed. Hydrogen peroxide (H_2O_2) increases benzoic acid-2 hydroxylase (BA2-H) enzyme activity (Léon *et al.*, 1995), which is required for salicylic acid (SA) biosynthesis. Lipid peroxides are formed, because H_2O_2 stimulates SA acid accumulation (Léon *et al.*, 1995). Hydrogen peroxide is also toxic to microbes (Peng & Kuc, 1992) and contributes to structural reinforcement of plant cell walls during lignification (Bradley *et al.*, 1992; Bolwell *et al.*, 1995).

Another signal molecule that has been implicated in defence is nitric oxide (NO). This compound has previously been shown to serve as a key redox-active signal for the activation of various mammalian defence responses, including the inflammatory and innate immune responses (Schmidt & Walter, 1994; Stamler, 1994).

Further, signal transduction pathways regulate the inducible defence-related genes involving several regulators such as JA, ethylene and salicylic acid (SA) (Reymond & Farmer, 1998) as well as oxylipins (Hamberg & Gardner, 1992). Jasmonic acid (JA) is synthesized via the octadecanoid pathway from peroxidized linolenic acid (Hamberg & Gardner, 1992). Methyl-jasmonate (Me-JA), which is the volatile counterpart of JA, oxo-phytodienoic acid, the precursor Me-JA and dinor-oxo-phytodienoic acid, are all powerful cellular regulators in plant tissues (Weber *et al.*, 1997). Jasmonic acid and its volatile ester methyl-jasmonate are potent inducers of proteinase inhibitors (Farmer *et al.*, 1992; Ryan 1990) and of polyphenol oxidase and lipoxygenase (LOX) (Duffey & Stout, 1996).

Salicylic acid plays a central role as a signal molecule being involved in both local and systemic resistance (Durner *et al.*, 1997). Salicylic acid regulates the induction of the pathogenesis-related (PR) genes of which many exhibit antifungal

properties (Durner *et al.*,, 1997; Kombrink & Somssich, 1997). Inhibition of the SA signal pathway leads to susceptibility in plants towards pathogens (Delaney *et al.*, 1994). Salicylic acid does not induce resistance to insect herbivory (Karban & Baldwin, 1997), although certain feeding insects can induce SA. The RWA have the ability to induce SA and PR-proteins (Mohase & Van der Westhuizen, 2002), but do not induce proteinase inhibitors, which is typical of insect or a wounding response (Fidantsef *et al.*, 1999; Stout *et al.*, 1999). Feeding damage caused by sucking insects (aphids) triggers a signalling and a defence response similar to that of pathogens (Bostock, 1999). Tobacco and cucumber plants, infected with a pathogen, induce SA accumulation and this increase is correlated with SAR (Métraux *et al*., 1990; Malamy *et al*., 1990; Rasmussen *et al.*, 1991).

The most common expression of resistance in the plant is the hypersensitive response (HR). The HR is defined as the death of host cells within a few hours of pathogen perception (Agrios, 1988). The reaction is associated with the prevention of pathogen spread; since the plant behaves as though it was "more than usually sensitive" to the presence of the pathogen. This is described as "hypersensitive" and referred to as the "hypersensitive response" (Stackman, 1915). The expression of the HR can be diverse ranging from HR in a single cell to the spreading necrotic areas accompanying limited pathogen colonization (Holab *et* al., 1994; Hammond-Kosack & Jones, 1996). The localized cell death associated with the HR resembles animal programmed cell death and in both cases it prevents the pathogen from spreading to uninfected sites. In interactions where pathogens form intimate haustorial associations with host cells, plant cell death would deprive the pathogen of access to further nutrients (Hammond-Kosack & Jones, 1996). This is especially true for biotrophic fungal pathogens.

The term pathogenesis-related (PR) protein was first used to describe numerous extracellular proteins that accumulate in response to tobacco mosaic virus (TMV) infection of susceptible tobacco genotypes. During plant-pathogen interactions, different PR-genes which are associated with incompatibility are induced (Bol *et al.*, 1990; Bowles 1990; Linthurst, 1991). The definition of PR-proteins has been broadened to include intra- and extracellular proteins that accumulate in intact plant tissue or culture cells after pathogen infection or when treated with an elicitor (Bowles, 1990).

Increased synthesis of several PR-proteins in the inoculated leaves is associated with the HR (Kombrink & Somssich, 1997). PR-proteins accumulate in and around affected tissue and systemically activate the defence mechanism in neighbouring cells (Kombrink & Somssich, 1997). Many of these PR-proteins exhibit antimicrobial or antifungal activity and some are now known to be chitinases and ß-1,3-glucanases (Colligne *et al.*, 1993; Melchers *et al.*, 1994). Another group of PR-proteins is basic cysteine-rich thionins with known antimicrobial activity (Bohlmann, 1994).

Plants infected with pathogens and treated with elicitors reacted by the induction of chitinase activity (Bowls, 1990). Russian wheat aphids also have the ability to induce chitinase activity in resistant wheat plants (Van der Westhuizen *et al*., 1998b). There seems to be no clear role for chitinase during insect attack, but it does play a role when plants are attacked by pathogens (Boijsen *et al.,* 1993). Plants infested with aphid and subjected to mechanical wounding results in the expression of different chitinase isoenzymes (Zhang & Punja, 1994; Botha *et al.*, 1998).

Chitinase catalyses the hydrolysis of chitin (Mauch & Staehlin, 1989). This enzymatic degradation of chitin has been found in micro-organisms, plants and animals (Flach *et al*., 1992). It is difficult to attribute a specific role for chitinase since its substrate, chitin, does not occur in higher plants. It is believed that plants produce chitinase to protect themselves against chitin-containing parasites (Boller, 1995). Therefore it is believed that chitinase together with ß-1,3-glucanase act synergistically to inhibit fungal growth (Mauch *et al*., 1988). The role of these PR-proteins is to protect the host from invasion by fungal pathogens and they form an integral component of a general disease resistance mechanism.

Differences in defence responses towards insects and pathogens are noticed at the levels of gene expression, induced chemistry and host resistance to further challenge (Bostock & Stermer, 1989; Farmer, 1994). However, in plants there are some similarities between insect attack and pathogen infection. Both trigger oxidative reactions involving polyphenol oxidases and peroxidases, generate reactive oxygen species and induce LOX that participates in the peroxidation of membrane lipids and synthesis of signalling molecules (Blechert *et al.*, 1995).

Salicylic acid is a critical signal molecule when plants are infected with pathogens leading to local and systemic resistance (Durner *et al.*, 1997). Jasmonic acid (JA), also a signal molecule, induces local and systemic resistance when plants are under herbivore attack. Some evidence does exist that JA also play a role in signalling during pathogenesis (Dong, 1998; Pieterse & van Loon, 1999).

2.2 LIPOXYGENASE (LOX) PATHWAY

Much of the the focus of plant defence mechanisms has recently shifted towards the involvement of oxygenated fatty acids (oxylipins). In mammals, oxylipins are derived from the arachidonic acid (C_{20} fatty acid) cascade that plays an important role during inflammation and infection (Nicolaou *et al*., 1991). In plants, oxylipins are derived from C_{18} fatty acids like linolenic or linoleic acid (Figure 2.2) via the lipoxygenase pathway (Gardner, 1991; Vick, 1993).

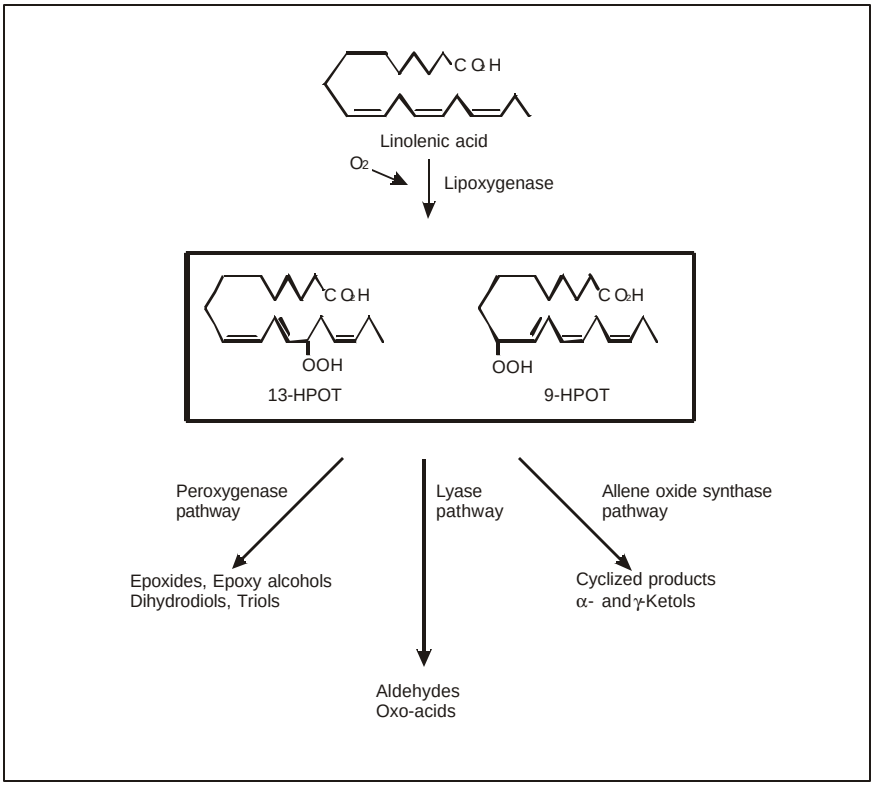


Figure 2.2 The lipoxygenase pathway. Oxidation of unsaturated C_{18} fatty acids by lipoxygenase forms into 9- and 13-hydroperoxides (HPOT) of linolenic- or linoleic acid (Bleé, 1998).

Plant lipoxygenases (LOXs) (linoleate: oxygen oxidoreductase, EC 1.13.11.12) constitute a large gene family of nonheme iron containing fatty acid dioxygenases, which are ubiquitous in plants and animals (Brash, 1999). Lipoxygenase catalyses the addition of molecular oxygen to fatty acids (Figure 2.2) containing a *cis, cis,*-1,4-pentadiene system to give an unsaturated fatty acid hydroperoxide (Hamberg & Samuelson 1967). LOX also catalyses the conversion of hydroperoxy lipids (Kuhn *et al*., 1990) and synthesizes epoxy leukotrines (Shimizu *et al*., 1984).

The initial step of enzymatic lipid peroxidation is the dioxygenation of polyunsaturated fatty acids (PUFAs) by LOX at either C-9 or at carbon atom C-13 respectively, yielding a 13- or 9-hydroperoxide (HPOT) (Figure 2.2) (Feussner & Wasternack, 2002). The terms 9-LOX and 13-LOX are used to describe the enzymes that generate predominantly 9- or 13-HPOT respectively. These fatty acid

HPOTs are substrates for other enzymatic systems that transform these highly reactive molecules into a series of oxylipins via the so-called 'LOX-pathway'. Understanding oxylipin biosynthesis we have to take note that it is organized into discrete 9-LOX and 13-LOX pathways, each of which is divided into several sub-branches (Fig. 2.3) (Howe & Schilmiller, 2002). The hydroperoxide lyase (HPLS) sub-pathway yields C6-aldehydes and 12-oxo-*trans*-9-dodecenoic acid, a precursor of traumatin (Vick & Zimmerman, 1987). An allene oxide synthase (AOS), dehydrates 13-HPOT into a chemically very unstable 12-oxo-phytodienoic acid (Hamberg, 1988), the precursor of jasmonic acid (JA), or it is hydrolyzed spontaneously to a– and ß-ketols. A third fate of these HPOT's is their reduction to their corresponding alcohols and further transformation by peroxgygenase (Bleé, 1998). Various enzymes are involved in these pathways and they include: peroxygenase (POX) or reductase leading to hydroxy PUFAs (HOD or HOT), LOX leading to keto PUFAs, divinyl ether synthase (DES) leading to vinyl ether-containing PUFAs, allene oxide synthase (AOS) leading to jasmonic acid and hydroperoxide lyase (HPLS) leading to ?-keto fatty acids and aldehydes by fragmentation of the fatty acid molecule (Blee, 1998). Allene oxide synthase, DES and HPLS are closely related to the P540 cytochrome family, designated CYP74 (Song *et al.*, 1993; Matsui *et al.*, 1996). In contast to "classical" P450 monooxygenases, CYP74 P450's do not require O_2 and an external redox partner for activity. A hydroperoxide group is used both as oxygen donor and as a source of reducing equivalents.

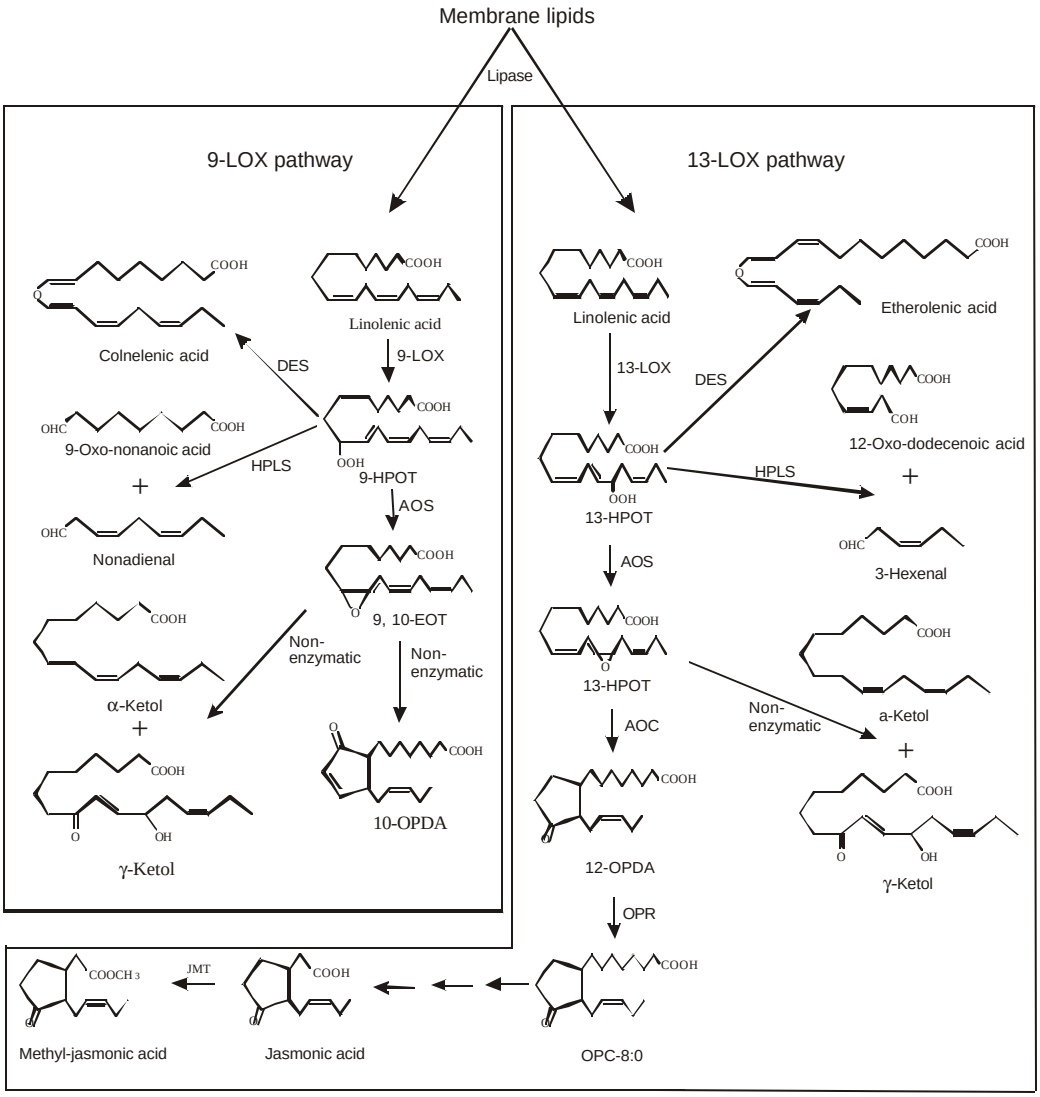


Figure 2.3 The 9-LOX and 13-LOX pathways for the metabolism of linolenic acid (Howe & Schimiller, 2002)

The AOS branch of the 13-LOX pathway transforms 13-HPOT to jasmonic acid (JA) and methyl-jasmonic acid (MeJA) as well as their metabolic precursor, 12-oxo-phytodienoic acid (12-OPDA) (Figure 2.3). Jasmonates play important roles in signalling and are able to regulate the expression of wound-induced proteinase inhibitor genes (Farmer & Ryan, 1990, 1992). It is very unlikely that JA is a solitary signal *in vivo*. The volatile counterpart MeJA, 12-oxo-phytodienoic acid and dinor-oxo-phytodienoic acid may be a very powerful cellular regulator in plant tissue (Weber *et al.*, 1997; Farmer *et al.*, 1998). The metabolism of linolenic hydroperoxide

by AOS also leads to the formation of ketols and cyclized compounds. a-Ketols are oxidized to hexanal and to a precursor of traumatic acid, known to act as an antifungal agent and as a wound hormone. The hemolytic cleavage of the hydroperoxide mediated by the heme of the AOS, results in an iron-oxo complex, which is the initial step in the formation of both epoxyalcohols and allene oxide (Bleé, 1998).

The HPLS branch of the 13-LOX pathway directs the formation of C_6-aldehydes, hexenal and C_{12} ? -keto fatty acids. The lyases involved can be roughly classified into two groups, according to their substrate specificity. The one group cleaves the 13-hydroperoxy derivatives of linoleic- or linolenic acid to form 3(Z)-hexenal and 3(Z)-hexanol. The other enzyme cleaves the 9-hydroxy yielding 3(Z),6(Z)-nonadienal and 3(Z)-nonenal respectively. *Trans*-2-hexenal is formed upon numerous incompatible interactions such as pathogen infestation, wounding, pests and diseases (Croft *et al.* 1993). Besides for C_6 aldehydes to be important volatile constituents of fruit, vegetables and green leaves, the C_6 aldehydes derived from 13-HPLS are important in defence against microbial pathogens and insects (Vancanneyt *et al.*, 2001). Physical and biological injuries to fresh fruit and vegetables result in the rapid formation of C_6- and C_9-aldehydes and their corresponding alcohol derivatives, can attract and/or repels insects, inhibit seed germination, exhibit antiprotozoal-, bactericidal- and antifungal activity. Aldehydes formed from the cleavage of 9-hydroperoxy linolenic acid i.e. 3(Z) and 2(E)-nonenal, were found to be more toxic than hexenals, but less effective due to their low volatility. Enzymatic cleavage of 13-HPOT also leads to the formation of 12-oxo-10(E)-dodecenoic acid, the active component of traumatin (Zimmerman & Coudron, 1979). This hormone triggers cell division near the wounding site leading to the development of a protective callus around it. Aldehydes and traumatin result from the a-cleavage of fatty acid hydroperoxides, catalyzed by HPLS (Bleé, 1998). Metabolism of 13-HPOT by DES gives rise to divinyl ether fatty acids such as etherolenic acid.

The peroxygenase pathway catalyzes (peroxygenase) the epoxidation of double bonds in fatty acids and the stereo controlled hydrolysis of such epoxides into their corresponding diols (epoxide hydrolyse). Peroxygenase exclusively mediates the heterolytic cleavage of the hydroperoxide, yielding the corresponding alcohol and a ferryl-oxo complex. When starting with a 13-hydroperoxide, the ferryl-oxo complex intermediately epoxidizes the more reactive nonconjugated double bonds either before it diffuses out of the active site (intermolecular mechanism), or

after its reassociation with the active site (intermolecular oxygen transfer). The biosynthesis of cutin monomers of the C_{18} family has been shown to possibly involve the peroxygenase pathway. The latter leads to an array of hydroxy and epoxy derivatives which can inhibit conidial germination, inhibit growth of germ tubes and inhibit the formation of appressoria of rice blast fungus (Bleé, 1998).

2.3 LOX AND SIGNALLING

The lipid based signalling pathway is composed of at least four structurally different types of compounds: (a) acylic fatty acids and functionalised derivatives, (b) cyclopentanoid C_{18} fatty acids, (c) cyclopentanoid C_{12} fatty acids such as epi-JA and JA and (d) amino acid conjugates (Wasternack *et al.*, 1998).

JA and its methyl ester (MeJA) are by far the most studied fatty acid derived signal in plants and it is now known to be crucial for plant stress responses, anther dehiscence and pollen development. In addition to jasmonates, plants can synthesize a huge range of oxylipins and many of them display some biological activity (Weber, 2002). Barley leaves treated with salicylic acid (SA) accumulated a 13(*S*)-hydroxy octadecatrienoic acid (13-HOTrE) (Weichart *et al.*, 1999). Salicylic acid is a key signal molecule in the defence response of plants against pathogens known for inducing pathogen-related proteins (PR1b). 13(*S*)-Hydroxy octadecatrienoic also manages to induce the PR1b protein in barley leaves after treatment, suggesting that SA treatment in barley leaves might be mediated by 13-HOTrE (Weichart *et al.*, 1999).

Arabidopsis plants exposed to wounding and infected with a pathogen accumulated 9-keto-octadecadienoic acid (9-KODE) (Vollenweider *et al.*, 2000). Keto-octadecadienoic acids have the ability to induce the gene that encodes for glutathione-*S*-transferase (*GST1*), which is involved in antioxidant defence (Vanacker *et al.,* 1998), and also induces cell death (Vollenweider *et al.*, 2000). Hydroperoxy, hydroxy and keto fatty acids accumulate during pathogenesis, being formed via the LOX-pathway. The formation of hydroperoxides is necessary for the development of hypersensitive cell death in tobacco (Rustérucci *et al.,* 1999). A second enzymatic reaction has been discovered in plants that synthesise fatty acid hydroperoxides via a-dioxygenase (a-DOX) (Hamberg *et al*., 1999). a-Dioxygenase is strongly induced in

Arabidopsis plants after infection with *Pseudomonas syringae*. Lipids derived from a-DOX are believed to protect the plant against oxidative stress and cell death (Ponce de Leon *et al.*, 2002).

An end product of the LOX-pathway, 4-hydroxy-2-nonenal (HNE), exhibits a large range of biological activities including the inhibition of proteins and DNA synthesis, inactivation of enzymes, stimulation of phospholipase C and reduction of gap-junction communication (Esterbauer *et al.*, 1991). 4-Hydroxy-2-nonenal can be produced from arachidonic acid, linoleic acid or their hydroperoxides in relatively large amounts and is largely responsible for the cytopathological effects observed during oxidative stress *in vivo* (Esterbauer *et al.*, 1991).

Plants can perceive and respond to signals differently generated during wounding and pathogenesis. Many of the biochemical barriers that are formed during a wound response are different from those generated during pathogenesis (Bostock & Stermer, 1989). In solanaceous plants there is a rapid redirection of isoprenoid biosynthetic pathway from antimicrobial steroid derivatives towards sesquiterpenoid phytoalexins when wounded tissues are exposed to elicitors or isolates of pathogens that indicate a hypersensitive response (Bostock & Stermer, 1989; Tjamos & Kuc, 1982). The induction of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR) is essential for the synthesis of steroid derivatives and sesquiterpenoid phytoalexins in solanaceous plants following mechanical injury or pathogen infection (Choi *et al.*, 1994). *Solanum tuberosum* treated with fungal elicitors and MeJA induced different sets of HMGR genes (Choi *et al.*, 1994). This indicates that the isoprenoid pathway is regulated by different lipid derived signals that can be present in certain plant/pathogen interactions. The response observed during hypersensitivity is not simply an enhancement of wound responses, but rather induction of different cellular programs.

2.4 INVOLVEMENT OF VOLATILE COMPOUNDS IN PLANT DEFENSE MECHANISMS.

Plants may respond to insect feeding by releasing chemical cues into the air, which may serve as signals for the herbivore's natural enemy (Paré & Tumlinson, 1997). These cues guide the host-seeking parasite or predator to insect-damaged

plants. Once a parasitic wasp has located its host, she lays her eggs in her host, which leads to the shortening of the hosts' life and terminates its reproductive cycle while propagating the wasp's own species (Tumlinson *et al.*, 1993). Many of the volatiles being released include the acrylic terpenes (*E*, *E*)-a-farnesene, (*E*)-ß-farnesene, (*E*)-ß-ocimene, linalool, (*E*)-4,8-dimethyl-1,3,7-nonatriene and (*E*, *E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene as well as the shikimate pathway product, indole (Paré & Tumlinson, 1997).

The signal transduction pathway initiating biosynthesis may involve the activation of the octadecanoid pathway. Lima bean possesses at least two different biological active signals that trigger different biosynthetic activities. Early intermediates of the pathway especially 12-oxo-phytodienoic acid (PDA) are able to induce the biosynthesis of the diterpenoid-derived 4,8,12-trimethyltrideca-1,3,7,11-tetraene. JA, which is the last compound in the sequence does not have the ability to induce diterprenoid-derived compounds, but is very effective at triggering the biosynthesis of other volatiles (Koch *et al.,* 1999).

Airborne signals such as methyl salicylate and methyl jasmonate may function as signals for neighbouring uninfested plants by activating defence-related genes (Farmer & Ryan, 1990; Shulaev *et al.*, 1997). The volatile compounds released from a specific plant after damage by different herbivores or microorganisms may differ in their quantities and quantitative composition (Takabayashi *et al.*, 1996; De Moraes *et al.*, 1998).

2.5 MODIFICATIONS OF PROTEINS BY POLYUNSATURATED FATTY ACID PEROXIDATION PRODUCTS.

The oxidation of proteins by the ascorbate/ion system is enhanced in the presence of polyunsaturated fatty acids and, methyl esters while the ability of these lipids to stimulate protein carbonyl formation is strongly dependent upon unsaturation (Hanne *et al.*, 1999). Saturated and unsaturated fatty acids have no detectable effect on carbonyl formation, whereas the ability of polyunsaturated fatty acids to promote carbonyl formation increases in the order linoleate < linolenate < arachidonidonate (Hanne *et al.*, 1999).

The metal-catalyzed oxidation of polyunsaturated fatty acids leads to the formation of several products that have shown to form carbonyl derivatives with proteins. These compounds have been identified to all play an important role during the defence mechanism of plants. These compounds include: (1) malonyl-dialdehyde (MDA), which react with lysine residues of proteins to form stable carbonyl derivatives (Burcham & Kuhan, 1996), (2) a-, ß-unsaturated aldehydes, such as 4-hydroxy-2-nonenal, which can undergo Michael addition-type reactions with the aminogroup of lysine residues, the sulfhydryl group of cysteine residues and imidazole group of histidine (Brunner *et al.*,1995; Sayre *et al.*, 1993) and (3) lipid peroxides, which can undergo metal ion-catalyzed conversion to alkoxyl and peroxyl radicals that can react directly with side chains of some amino acid *residues to* form carbonyl derivatives, by mechanisms analogous to those obtained with hydrogen peroxide (Kato *et al.*, 1992).

2.6 PATHOGEN INDUCED OXYGENASES (PIOX)

A 75 kDa protein was found to accumulate in tobacco leaves in response to bacterial infection. This protein, as well as a protein found in *Arabidopsis* showed a 75% homology in amino acid sequence, were expressed in insect cells and found to cause the uptake of molecular oxygen in the presence polyunsaturated fatty acids such as linolenic, linoleic acid arachidonic acid (Sanz *et al.*, 1998). The tobacco enzyme was called “pathogen-induced oxygenase” (PIOX). The expression of the oxygenase protein (PIOX) from tobacco, was induced significantly earlier than the expression of defence related genes; *PR-1* and gn2 (encoding a basic ß-1,3-glucanase) genes (Sanz *et el.*, 1998). The induction of PIOX was not only the result of a fungus infection, but also as a result of bacterial infection (Sanz *et el.*, 1998).

Pathogen-induced oxygenase showed a significant homology to prostaglandin-endoperoxide H synthase-1 and –2 present in animal tissue (Sanz *et al.*, 1998). Endoperoxide synthases are dual function enzymes possessing cyclooxygenase and peroxidase activities (Smith, 1996). The recombinant PIOX protein from tobacco and *Arabidopsis* possesses oxygenase activity towards several polyunsaturated fatty acids, but no peroxidase activity could be demonstrated (Sanz

et al., 1998). In the presence of linolenic acid the oxygenase protein from tobacco and its homologue from *Arabidopsis* formed a C_{17} unsaturated aldehyde, 8(*z*), 11(*z*), 14(*z*)-heptadecatrienal (Hamberg *et al.*, 1999). Other fatty acids such as linoleic and palmitic acid can be metabolized in the same way. Linoleic acids lead to the formation of 8(z), 11(z)-heptadecadienal, 2-hydroxy-9(z), 12(z)-octadecadienoic acid and 8(Z), 11(z)-heptadecadienoic acid. Palmitic acid as substrate leads to the formation of pentadecanal 2-hydroxyhexadecanoic acid and pentadecanoic acid (Hamberg *et al.*, 1999). These product profiles are the same as for the product profile found in plants where a-oxidation occurs.

Cyclooxygenase (COX) is a key enzyme in the production of lipid-derived signal molecules that regulate diverse cellular processes in vertebrates including the immune response (Serhan *et al.*, 1996). Cyclooxygenase belongs to prostaglandin endoperoxide H synthases (PGHSs), which catalyze the conversion of arachidonic acid and oxygen to prostaglandins (Fig. 2.4), the committed step in prostanoid biosynthesis (Smith, 1996). Before 1991, only one PGHS has been described, the enzyme now called PGHS-1 or COX-1. A new second PGHS (PHGS-2 or COX-2) has been also described with a similar structure to COX-1, but it differs in its pattern of expression and biology (Smith, 1996). Cyclooxygenase-1 and -2 are often expressed in the same cell and may act as parts of a separate prostanoid biosynthetic pathway.

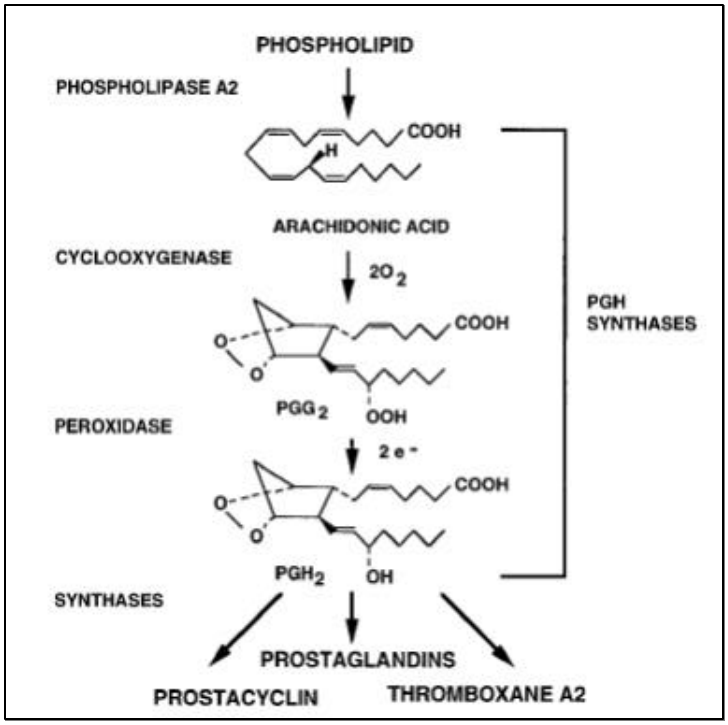


Figure 2.4 Prostanoid biosynthetic pathway (vertebrate), (Smith, 1996).

Although beta-oxidation is the major route for fatty acid degradation, fatty acids can also be subjected to alpha- and omega-oxidation. In alpha-oxidation, a fatty acid is oxidized at the alpha-position (C-2) to give rise to a 2-hydroperoxy fatty acid. 2-Hydroperoxy fatty acids are chemically unstable and are quickly converted to chain-shortened (by one carbon) fatty aldehydes through decarboxylation, or to 2-hydroxy fatty acids. The chain-shortened fatty aldehydes are further oxidized to free fatty acids, which can enter the next round of alpha-oxidation. The conversion of the intermediate 2(R)-hydroperoxy fatty acids to 2(R)-hydroxy fatty acids may be spontaneous or catalyzed by a peroxidase (Hamberg, 2000). The 70 kDa subunit of pea alpha-dioxygenase was shown possess such a peroxidase activity (Saffert *et al*., 2000). Similarly, the conversion of the chain-shortened aldehydes to corresponding fatty acids may be spontaneous or catalyzed by a NAD^+ oxidoreductase. The 50 kDa subunit of pea alpha-dioxygenase was shown to have an aldehyde dehydrogenase activity with NAD^+. Both polysaturated and unsaturated fatty acids are effective substrates in the pathway. Among the tested ones are laurate, palmitate, stearate, oleate, linoleate, linolenate, and arachidonate.

In mammals, alpha-oxidation is important to degrade beta-methyl branched fatty acids which cannot be degraded through beta-oxidation. The physiological role

of fatty acid alpha-oxidation in plants (Fig. 2.5) is not clear despite of its expected role in chlorophyll degradation. It has been suggested that the pathway may be related to seed germination (in the case of pea where alpha-dioxygenase is induced only during seed germination) (Saffert *et al*., 2000) and plant response to wounding and plant-pathogen interactions [(in the case of tobacco and *Arabidopsis* where alpha-dioxygenase is induced upon pathogen attack (Hamberg *et al.*, 2003; De Leon *et al*., 2002)].

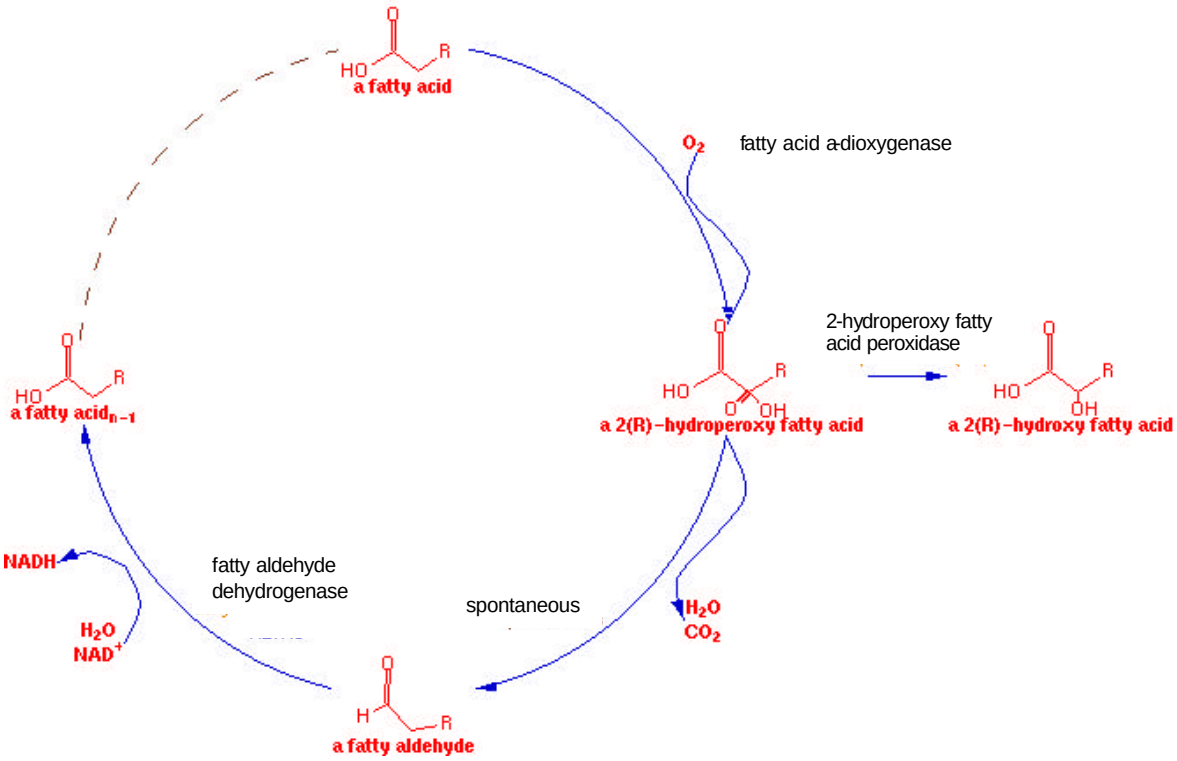


Figure 2. 5 Alpha-oxidation of fatty acids in plants.

CHAPTER 3

Material & Methods

3.1 PLANT MATERIAL

Wheat plants (*Triticum aestivum* L.) resistant to the RWA [*Diuraphis noxia* (Mordvilko)] cv. 'Tugela DN' (PI137739/5; Du Toit, 1989), 'Gariep', 'Betta DN' and near isogenic susceptible ones (cv. 'Tugela', 'Molopo', 'Betta') were grown under controlled conditions in a glasshouse at day and night temperatures of 25ºC and 20ºC respectively. Plants were infested on the two leaf growth stage by spreading the aphids over the plants, about 25 aphids per plant.

Leaves were collected at specific time intervals after infestation. All the leaves of the plant were harvested, the aphids were removed and the leaves immediately frozen in liquid nitrogen and stored at –20°C for subsequent analyses.

3.2 EXTRACTION PROCEDURE OF ENZYMES

Extraction of enzymes was performed according to Rao *et al.*, (1997). One gram of frozen leaf tissue was ground in 10 ml of 100 mM potassium phosphate buffer (pH 7.5) containing 100 mg acid washed sand, 1 mM ethylenediaminetetra-acetic acid (EDTA) and 1% (m/v) polyvinylpyrrolidone (PVP). After centrifugation, (25 000 g, 20 min) the supernatant was used for LOX and POD assays.

3.3 DETERMINATION OF PROTEIN CONCENTRATION

The protein concentration was determined using a Biorad Microplate Reader model 3550 at 595 nm (Rybutt & Parish, 1982). The protein content was determined using the dye-binding assay technique (Bradford, 1976). The assay mixture consisted of 150 µl distilled water, 40 µl BioRAD and 10µl enzyme extract and for the protein standard 10 µl of a 0.5 µg?µl^{-1} ?-globulin solution was used.

3.4 LIPOXYGENASE (LOX) ASSAY

The assay was done according to the methods of Grossmann & Zakut (1997) and Ocampo *et al.* (1986). The LOX reaction mixture consisted of 1 ml 0.1 M sodium citrate phosphate buffer (pH 6.2), 50 µl of enzyme extract and 150 µl of 2.5 mM linoleic acid. The change in absorbance was measured at 234 nm for 15 min at 30°C with a double beam spectro photometer equipped with a temperature controlled water bath. Lipoxygenase was expressed as nmol HPOD mg^{-1} protein min^{-1}.

Preparation of the linoleic acid substrate (2.5 mM linoleic acid in 0.15% Tween 20). Linoleic acid (400 µl), 768 µl Tween 20 and 40ml methanol were added into a round bottomed flask and subjected to rotary evaporation at 60°C until dry. The residue was redissolved in 500 ml 0.05 M sodium phosphate buffer (pH 9). The entire volume was divided into 5 ml aliquots and stored in air tight bottles at -20°C. During transfer to the air tight bottles, nitrogen gas was bubbled through the content of round-bottomed flask and into the small bottles before adding the aliquots. The substrate was used once and stored on ice during experiments.

3.5 DETERMINATION OF LIPID PEROXIDATION

Lipid peroxidation was determined by measuring the malondialdehyde (MDA) content. Plant tissue was homogenized with the aid of inert sand in 2.5 ml (per g fresh mass) of ice-cold 0.1% (m/v) trichloroacetic acid (TCA), 1% (v/v) Triton X-100 and 0.01% (v/v) butylhydroxytoluene (BHT). Triton X-100 destroys the membranes and the BHT is a synthetic antioxidant. After centrifugation (5 min, 1 000 g), 4 ml of 20% (m/v) TCA containing 0.5% (v/v) TBA (2-thiobarbituric acid) was added to 1 ml aliquot of the supernatant. The mixture was heated at 95°C for 20 min, cooled down on ice and centrifuged for 5 min at 1 000 g. The absorbance was measured at 532 and 660 nm (Yamamoto *et al.*, 2001; Rustérucci *et* al., 1996).

3.6 INDOMETHACIN INHIBITION

Indomethacin is an inhibitor of prostanoid biosynthesis (Attiga *et al*., 2000). After 72 hours of infestation, plants were cut off just above the ground and placed in a solution of 20 μg ml^{-1} indomethacin. Excised plants were left in the solution (50 ml) for 2 hours, transferred to a beaker containing pure water (50 ml), and left for another 2 hours where after LOX and POD activities were determined. In addition indomethacin solution 40 μg ml^{-1}, was applied as a soil drench (500 ml), 24 hours before sampling. The *in vitro* effect of indomethacin inhibition on enzyme activity was also tested for. A 20 and 40 μg ml^{-1} indomethacin solution was used *in vitro* respectively when LOX and POD activities were measured spectrophotometrically.

3.7 PEROXIDASE (POD) ASSAY

Frozen leaf tissue was ground in liquid nitrogen and subsequently extracted in 3 ml extraction buffer [100 mM Na-acetate (pH 5.5), 10 mM mercaptoethanol, 2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride (PMSF)]. The homogenate was centrifuged (12 000 g, 20 min) at 2°C.

The peroxidase assay mixture consisted of 40 mM potassium phosphate buffer (pH5.5), 5 mM guaiacol and 8.2 mM H_2O_2. The change in absorbance was measured at 470 nm for 180 seconds at 30°C (Hitachi U-2000 double-beam spectrophotometer) (Zieslin and Ben-Zaken; 1991). Peroxidase activity was expressed as nmol tetraguaiacol mg^{-1} protein min^{-1}.

3.8 SDS-PAGE AND IMMUNOBLOTTING

Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on a 10% (m/v) separating gel in a Mini Protein II, BioRAD gel system according to Laemmli (1970) at 200V constant voltage for 60 min. The separating gel consisted of 4.86 ml 2x-distilled water, 2.5 ml 1.5 M Tris-HCl (pH 8.8),

100 µl 10% (w/v) SDS, 3.3 ml (30% stock) acryamide:bisacrylamide, 50 µl 10% (w/v) ammonium persulfate and 5 µl *N,N,N',N'*-tetramethylenediamine (TEMED). The stacking gel consisted of (30% m/v stock) acrylamide:bisacrylamide, 0.5 M Tris-HCl (pH 8.6), 2.7 ml 2x-distilled water, 10% (w/v) SDS and 10% (v/v) TEMED.

Electroblotting onto a nitrocellulose membrane was performed for 45 min in a semi-wet transfer unit (BioRAD) at a constant current of 350 mA in a transfer buffer (25 mM Tris, 192 mM glycine and 20% (v/v) methanol, pH 8.3). Afterwards the nitrocellulose membranes (Hybond-C, extra membrane, Amersham Biosciences) were blocked by incubating it in 8% (m/v) skimmed milk in TBS (10 mM Tris-HCl, pH 8.3 and 1.5 M NaCl) at room temperature for 45 min. Incubation with anti-bean LOX (gift from Ivo Feussner*) and anti-tobacco PIOX (gift of Carmen Castresana*) antibodies in blocking solution (1:2000) was carried out for 1 h at room temperature. After 3x 10 min washes in TBST (TBS containing 0.1% Tween-20) the nitrocellulose membranes were incubated in alkine phosphatase-conjugated secondary antibodies (anti-chicken IgG, 1:9 500 in 4% (m/v) skimmed milk) for 1h at room temperature. Consequently the membranes were washed 3x in TBST and 0.05% (w/v) SDS, 2x in TBST and 1x in TBS; for 5 min each. Wide-range protein molecular mass markers (7200 – 206000 Da) from BioRad were used to estimate the molecular masses of detected proteins on blots and gels.

*[The anti-bean LOX antibodies was a gift of Ivo Feussner at the Dept of Plant Biochemistry, Institute of Plant Bio Sciences, University of Göttingen, Germany (Göbel *et al.*, 2001) and the anti-tobacco PIOX antibodies was a gift of Carmen Castresana; Centro Nacional de Biotecnologia, Campusde Universidad Autonoma Cantoblanco; Sanz *et al.*, 1998)]

3.9 EXTRACTION AND ANALYSIS OF LIPID-LIKE PRODUCTS

One gram of frozen leaf tissue was ground to a fine powder in liquid nitrogen. Immediately after the addition of 4 ml of ice-cold methanol, the mixture was homogenized with a Polytron homogeniser for 1 min on ice. The homogenized samples were shaken in their tubes for 3 hours at 4°C. The samples were then centrifuged at 15 000 g for 10 min at 4°C. Ice-cold distilled water (1.5 ml), was added

to the supernatants and passed through a C_{18}-Sep-Pack Plus (Separations) cartridge. The cartridge was prewashed with methanol followed by distilled water. The column was washed afterwards with methanol / water (70:30, v/v) and the eluates were combined. The combined eluate was concentrated to less than 1 ml under reduced pressure at 40°C in a rotary evaporator. The volume was adjusted to 5 ml with water and acidified with 300 µl of formic acid. The lipids were extracted with chloroform. The chloroform phase was dried over anhydrous $MgSO_4$ whereafter the chloroform was evaporated with slow blowing nitrogen gas. Hydroxy fatty acids were methylated with 450 ml diazomethane and after 1 hour, the diazomethane was evaporated under a stream of nitrogen gas. The residue was dissolved in 100 µl pyridine and silylated with 100 µl bis(trimethylsilyl)trifluoracetamide (TMS). After 15 min the TMS was dried under a stream of N_2. The dried residues were dissolved in 10 ml hexane and stored at -80°C. Fatty acids were analysed by gas chromatography-mass spectrometry (Weber *et al.,* 1997).

3.10 GAS LIQUID CHROMATOGRAPHY – MASS SPECTROMETRY (GC-MS)

Fatty acids were analysed using GC-MS. A Hewlett Packard 5890 gas chromatograph equipped with a Hewlett Packard 5970 MSD. The gas chromatograph was fitted with a HP-1 30 m fused silica capillary, 0.25 mm i.d. 0.25 µm coating. Helium was used as carrier gas at 154 psi head pressure. Injector temperature was at 230ºC, initial column temperature was 140ºC and final column temperature was 300ºC. The ramp rate was 5ºC.min^{-1} and the final time 40 min. One µl aliquots of the sample were injected. The mass spectrometer parameters were as follow: EMV 1447, Amu Gain 619, Scan 50 to 650 amu, GC-MS interphase temperature 280ºC (Kock *et al*., 1997)

CHAPTER 4

Results

4.1 THE EFFECT OF RWA INFESTATION ON LOX ACTIVITY AND PROTEIN EXPRESSION.

Changes in LOX activity was measured over time in infested and uninfested wheat cultivars; expressing different levels of resistance to the RWA. Lipoxygenase activities were selectively induced in all three resistant cultivars; Gariep (Fig . 4.1), Betta DN (Fig. 4.2) and Tugela DN (Fig. 4.3). The degree of induction was the highest in Tugela DN (Fig. 4.3) followed by Betta DN (Fig. 4.2) and Gariep (Fig . 4.1). After 120 hours of infestation in the duplicate experiments, the level of LOX activity was twice as high in Tugela DN (Fig. 4.3 b) as in Gariep (Fig. 4.3 b).

Again, in the second experiment the level of LOX activity, at 120 hours post infestation in the infested resistant Tugela DN (Fig. 4.3b), was 11 fold higher than in the infested susceptible Tugela wheat. There were also no differences in the expression of LOX activity in the uninfested resistant and susceptible wheat plants, and the levels remained relatively unchanged during the experiment. The level of LOX expression in the resistant Gariep (Fig. 4.1) and Betta DN (Fig. 4.2) were respectively 5 and 4 fold higher in the infested resistant than in the infested susceptible wheat cultivars. Very little induction of LOX activities occurred in susceptible cultivars during infestation (Fig's 4.1, 4.2 & 4.3). The increase in LOX activity observed in the resistant cultivars resulted from RWA infestation. In a second experiment, similar trends in LOX expression were observed (Figure 4. 1b, Figure 4. 2b, 4.3b).

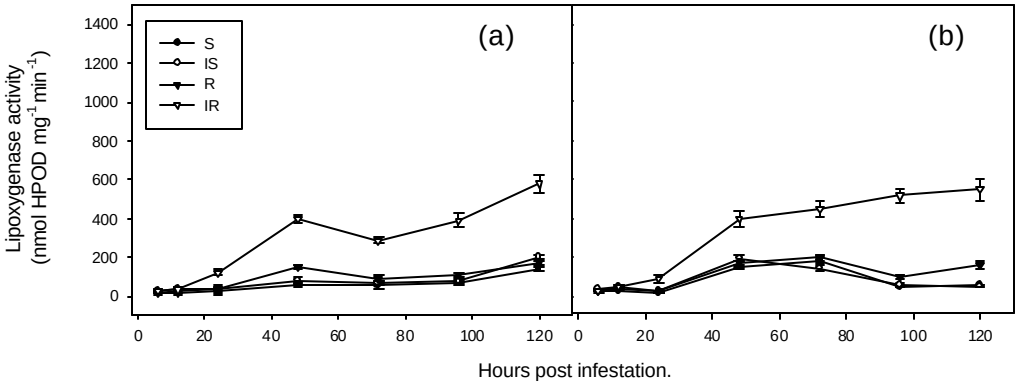


Figure 4. 1 Time course of LOX activity after RWA infestation (I) of susceptible (S), Molopo and the resistant (R) Gariep wheat cultivars; (a) and (b) indicate duplicate experiments. Error bars indicate standard deviation, n = 3; HPOD = hydroperoxy octadecadienoic acid.

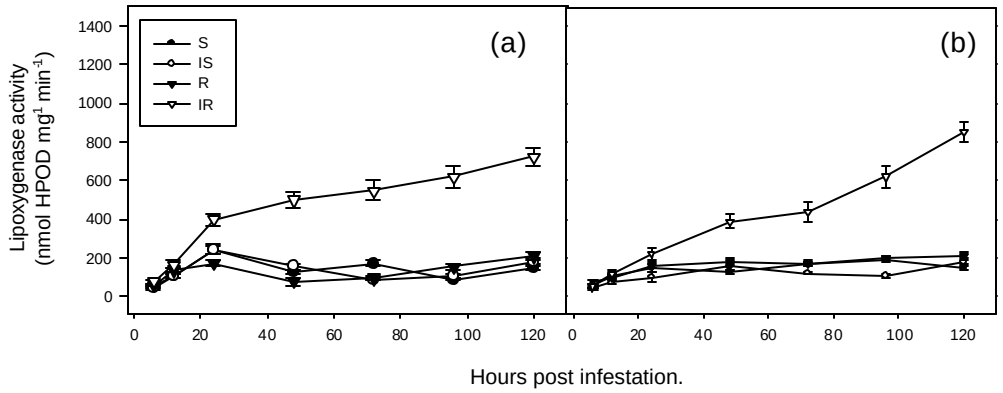


Figure 4. 2 Time course of LOX activity after RWA infestation (I) of susceptible (S), Betta and the resistant (R) Betta DN wheat cultivars; (a) and (b) indicate duplicate experiments. Error bars indicate standard deviation, n = 3; HPOD = hydroperoxy octadecadienoic acid.

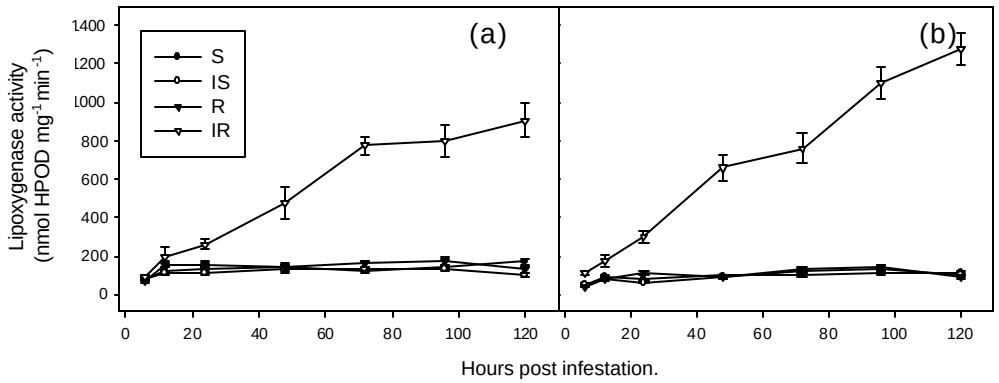


Figure 4. 3 Time course of LOX activity after RWA infestation (I) of susceptible (S), Tugela and the resistant (R) Tugela DN wheat cultivars; (a) and (b) indicate duplicate experiments. Error bars indicate standard deviation, n = 3; HPOD = hydroperoxy octadecadienoic acid.

The expression of a LOX protein was investigated in infested and uninfested susceptible (Tugela) and resistant (Tugela DN) wheat. A ~78 kDa LOX protein was selectively induced, to a much higher level in resistant than in susceptible wheat during infestation (Fig. 4.4a). This induction started after 12 hours of infestation and increased gradually as infestation proceeded (Fig. 4.4b). This LOX protein was weakly expressed in the uninfested resistant and susceptible wheat cultivars, although a somewhat higher level of expression was observed in the uninfested resistant than uninfested susceptible wheat plants (Fig. 4.4a). It is also clear that a slight induction occurred in the susceptible wheat after infestation.

The LOX antibodies also cross-reacted with a 76 kDa protein, especially strongly in the susceptible cultivar. A somewhat lower expression of this protein occurred after infestation of the susceptible cultivar.

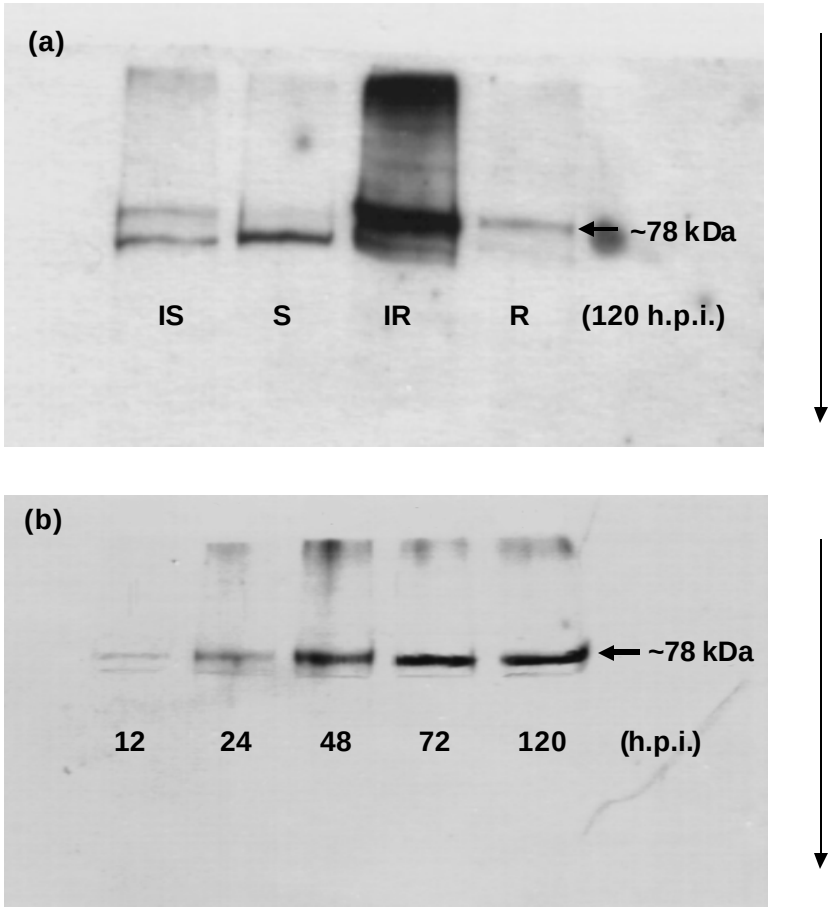


Figure 4. 4 Western blot analyses of the effect of RWA infestation (I) on LOX protein expression in (a) after 120 hours of infestation in the susceptible (S), Tugela and the resistant (R) Tugela DN wheat cultivars and (b) a time course of expression of LOX protein in the infested resistant, Tugela DN wheat; h.p.i. – hours after infestation; ? - indicate the direction of migration.

4.2 THE EFFECT OF RWA INFESTATION ON THE EXPRESSION OF AN OXYGENASE PROTEIN.

The COX antibodies cross-reacted with at least 2 proteins in the resistant and susceptible cultivars. Of these two the 80 kDa protein was differentially expressed to higher levels in the two cultivars after infestation. Induction was much higher in the resistant than the susceptible cultivar (Fig. 4.5a). An increase in the expression of the oxygenase protein could be observed from 6 hours after infestation onwards in the infested resistant plants (Fig. 4.5b).

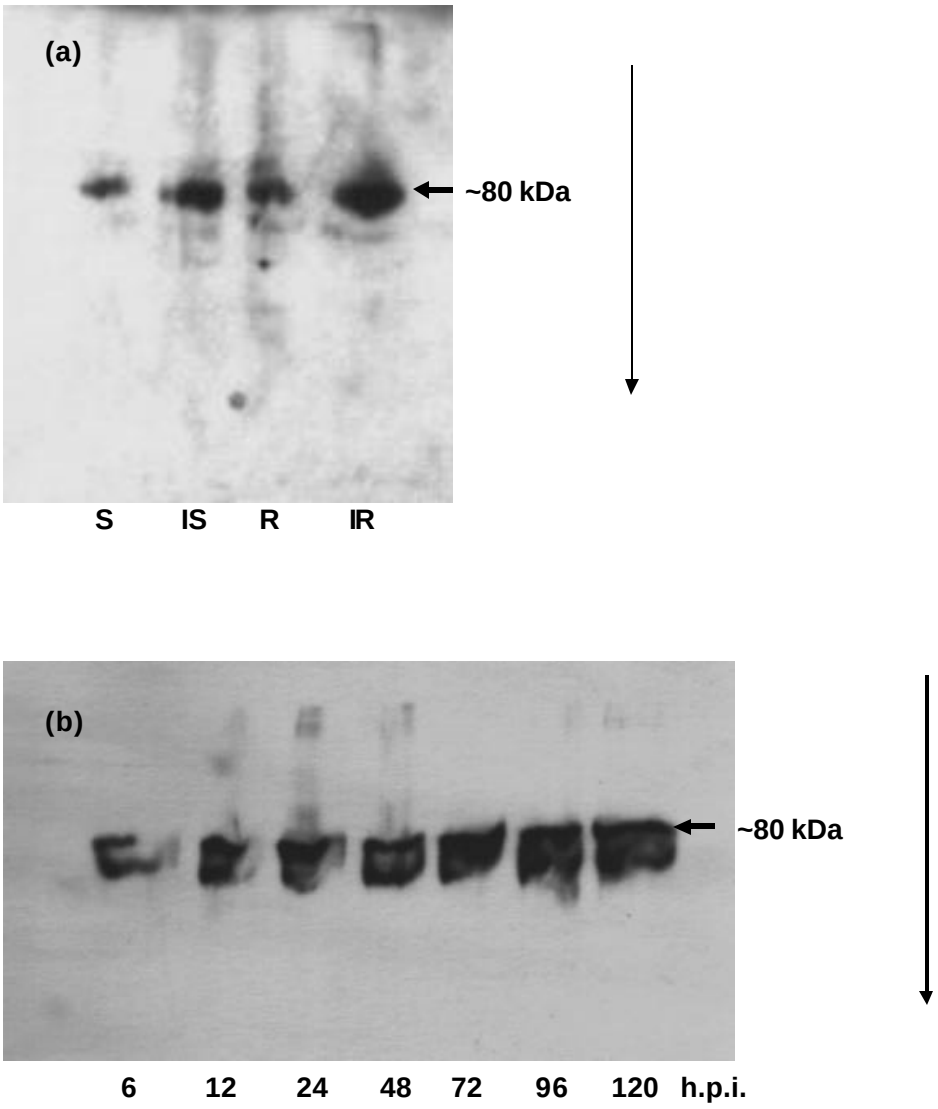


Figure 4. 5 Western blot analyses of the effect of RWA infestation on the expression of an oxygenase protein in (a) susceptible (S), Tugela and the resistant (R), Tugela DN, wheat cultivars and (b) time course of the expression of the oxygenase protein in the infested resistant wheat; h.p.i. – hours after infestation ; ? - indicate the direction of migration.

4.3 THE EFFECT OF RWA INFESTATION ON LIPID PEROXIDATION.

Lipid peroxidation was selectively induced in the infested resistant wheat (Fig. 4.6). The induction occurred already at 12 hours after infestation and peroxidation increased as the infestation proceeded. Lipid peroxidation remained relatively unaffected in the infested susceptible wheat. The level of peroxidation was two fold higher in the infested resistant wheat than in the uninfested resistant wheat (Fig. 4.6).

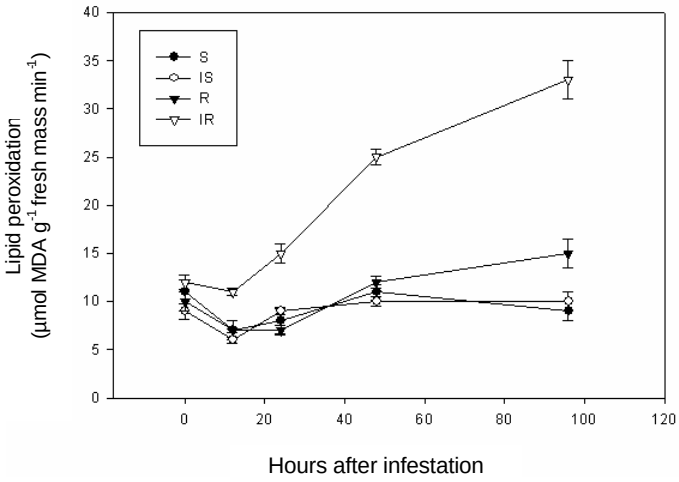


Figure 4. 6 The effect of RWA infestation (I) in lipid peroxidation of the susceptible (S), Tugela and the resistant (R) Tugela DN, wheat cultivars. Error bars indicate standard deviation, n = 5. MDA: malondialdehyde.

4.4 THE EFFECT OF PROTOSTANOID SYNTHESIS INHIBITION ON LOX AND PEROXIDASE (POD) ACTIVITIES.

The *in vivo* and *in vitro* effects of indomethacin inhibition of prostanoid synthesis were studied in infested resistant and susceptible wheat. The effect of indomethacin given as a soil drench and fed to excised plants (placed into an indomethacin solution) was studied after infestation with the RWA. Lipoxygenase and peroxidase (POD) activities were determined for signs of inhibition of the resistance response as a result of protostanoid inhibition.

In vitro application of indomethacin did not affect the LOX (Fig. 4.7) and POD (Fig. 4.8) activities. Inhibition of LOX (Fig. 4.7a) and POD (Fig. 4.8a) activities was observed after cut plants, taken at 72 hours after infestation, were placed in the indomethacin solution. Peroxidase activity declined by 9% and LOX by 40%. A reduction in LOX (33%) (Fig. 4.7b) and POD, (15%) (Fig. 4.8b) activities were also detected after the indomethacin was applied as a soil drench.

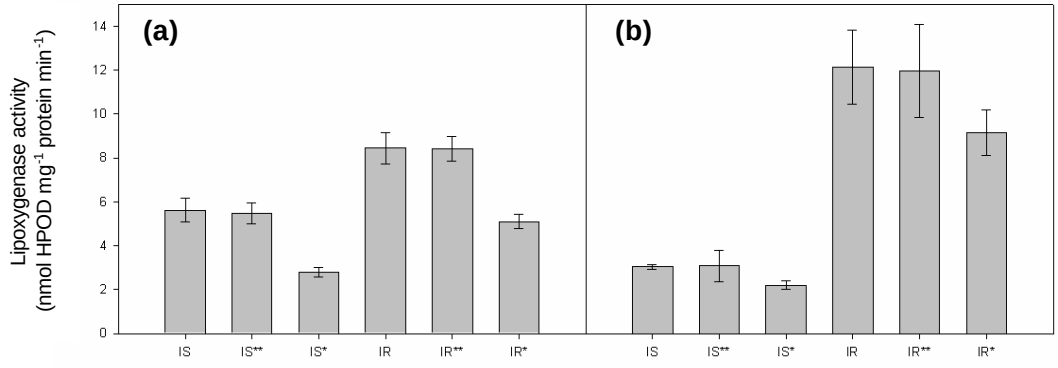


Figure 4. 7 The *in vivo* (*) and *in vitro* (**) effect of indomethacin on LOX activity in infested (I), susceptible (S) and resistant (R) wheat after infestation with the RWA. Excised (a) plants were placed in indomethacin solution and (b) indomethacin was applied as a soil drench. Error bars indicate standard deviation, n = 3.

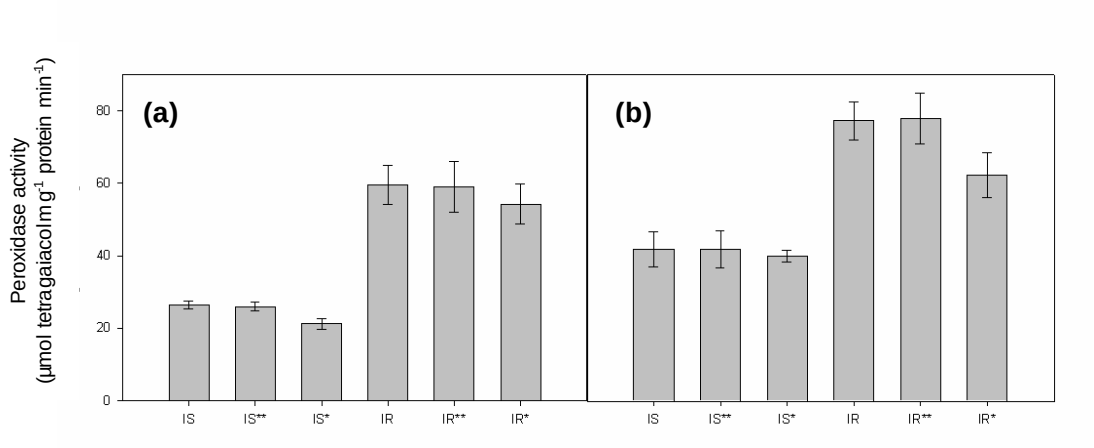


Figure 4. 8 The *in vivo* (*) and *in vitro* (**) effects of indomethacin on POD activity in infested (I), susceptible (S) and resistant (R) wheat. (a) Excised leaves were placed in indomethacin solution and (b) indomethacin was applied as a soil drench. Error bars indicate standard deviation, n = 3.

4.5 GC/MS ANALYSES OF LIPID-LIKE PRODUCTS

Lipid-like products were extracted from RWA infested and uninfested wheat leaves of susceptible (Tugela) and resistant (Tugela DN) cultivars and analyzed by means of GC/MS. According to the peaks in the chromatograms (Fig's 4.10, 4.12, 4.14) it is clear that infested resistant wheat selectively expressed certain products (indicated by retention times).

As early as 1 hour after infestation a single lipid (Rt = 33.31) could be identified to be differentially induced to higher levels in infested resistant wheat than uninfested resistant wheat (Fig. 4.10B). This peak was not induced in the susceptible wheat on infestation (Fig. 4.9B) and also not in the uninfested susceptible controls (Fig. 4.9A). As infestation progressed towards 48 hours more lipids could be identified to have been selectively induced after infestation (Fig. 4.12B). A total of 12 selectively induced lipids were identified in the infested resistant wheat. These lipids were either weakly expressed or not expressed at all in the infested susceptible (Fig. 4.11B) and the uninfested controls (Fig. 4.11A and Fig. 4.12 A).

After 96 hours of infestation (Fig. 4.14B) it is clear that some lipid products were expressed to much higher quantities in the infested resistant wheat than in the uninfested resistant wheat (Fig. 4.14A). In total 14 lipid products were identified to be selectively induced after 96 hours of infestation. The lipid products being selectively formed in the infested resistant wheat would seem to play a role in the resistance response of Tugela DN contributing to resistance. The next step was to identify these products.

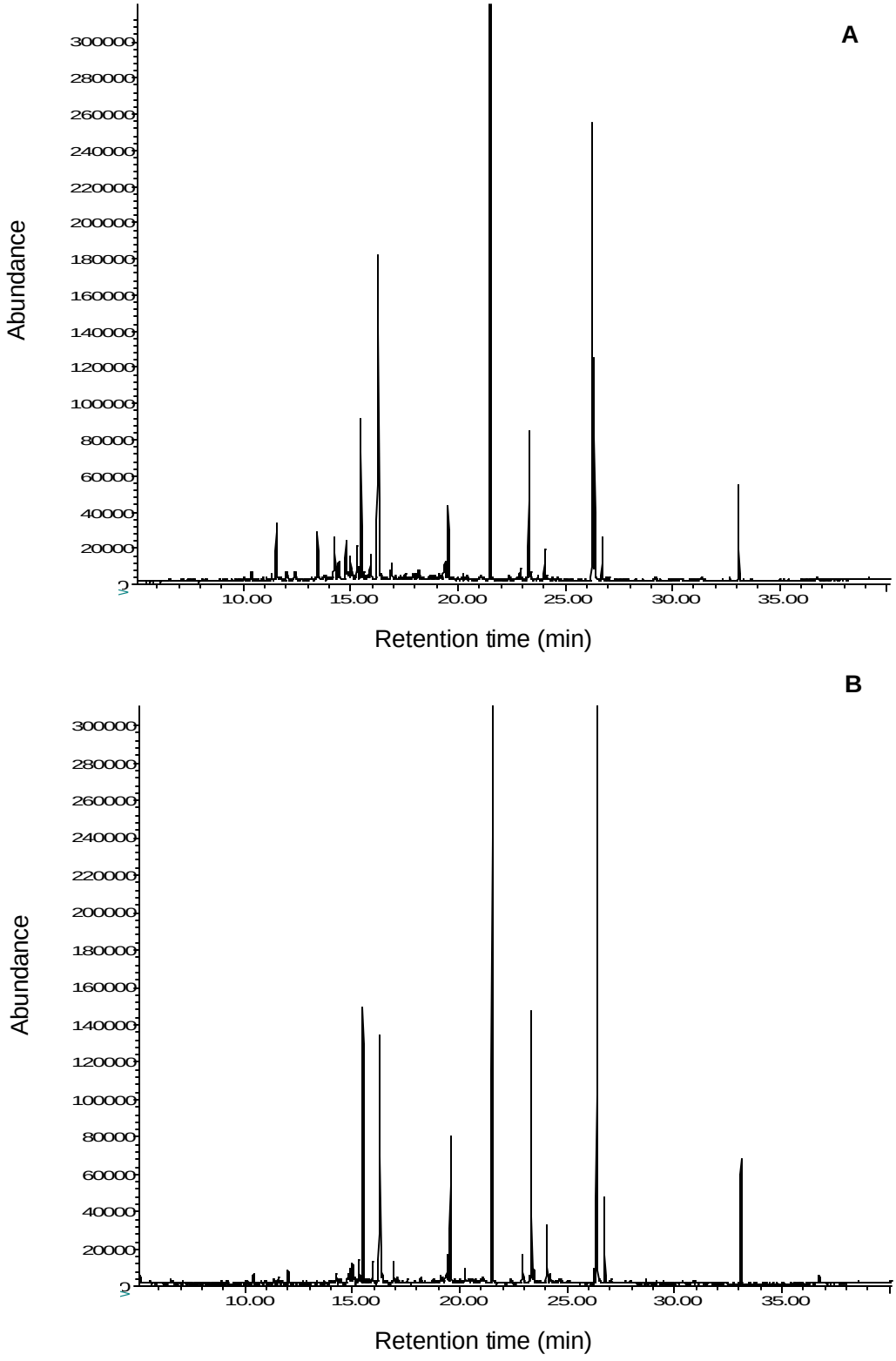


Figure 4.9 GLC Chromatograms of lipid-like products of uninfested (A) and infested (B) susceptible (Tugela) wheat, 1 hour after infestation.

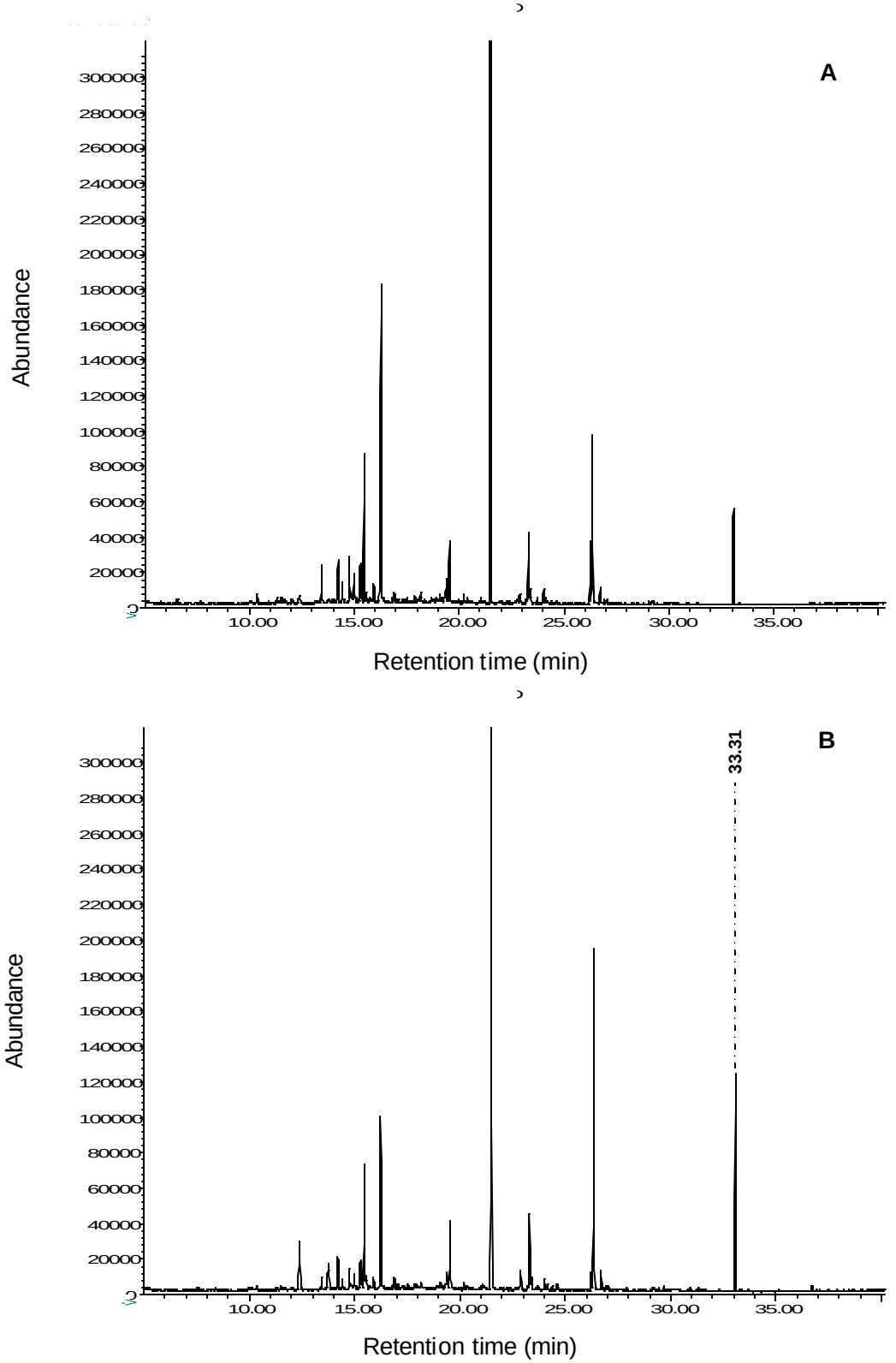


Figure 4.10 GLC chromatograms of lipid-like products of uninfested (A) and infested (B) resistant (Tugela DN) wheat, 1 hour after infestation.

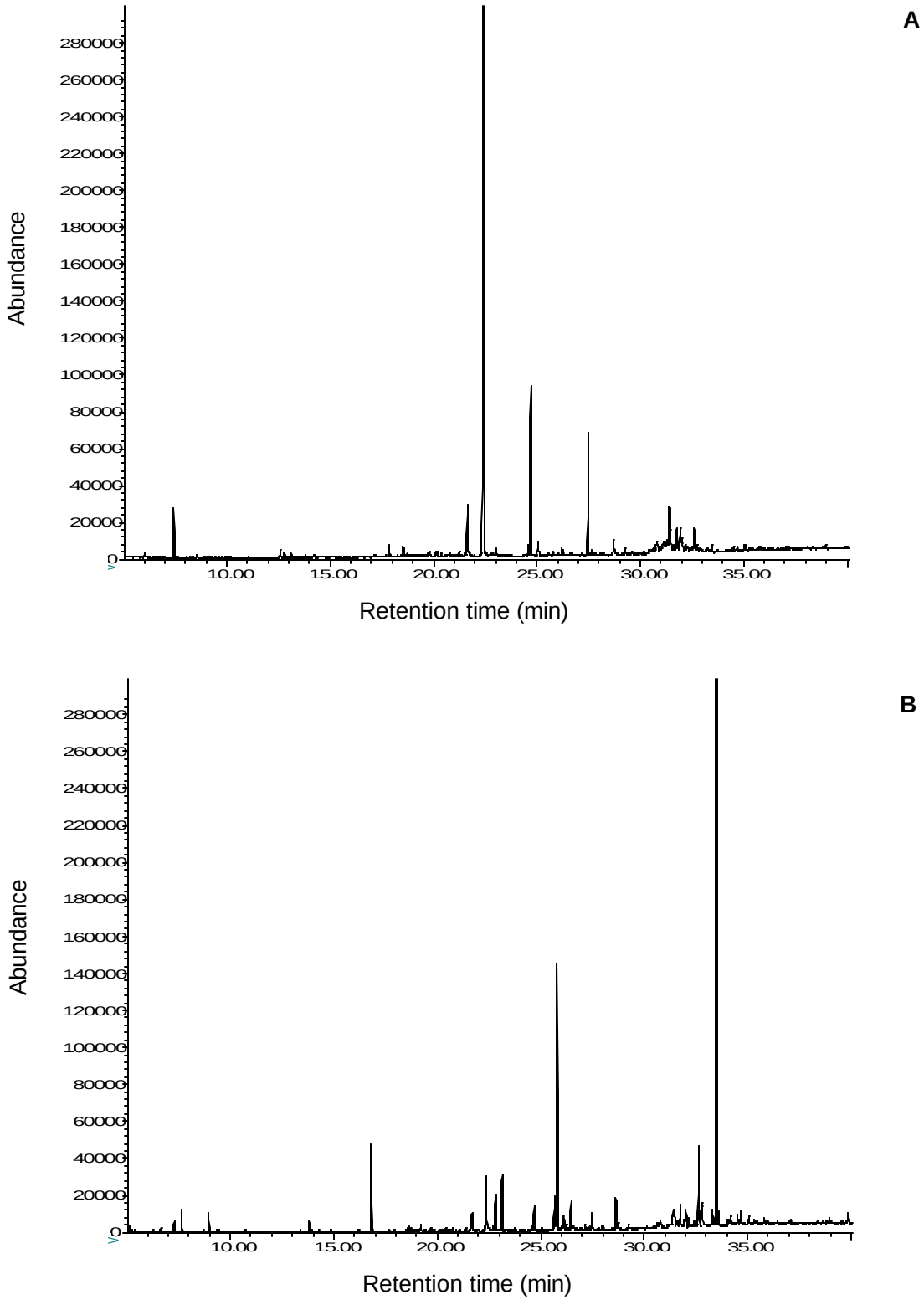


Figure 4.11 GLC chromatograms of lipid-like products of uninfested (A) and infested (B) susceptible (Tugela) wheat, 48 hours after infestation.

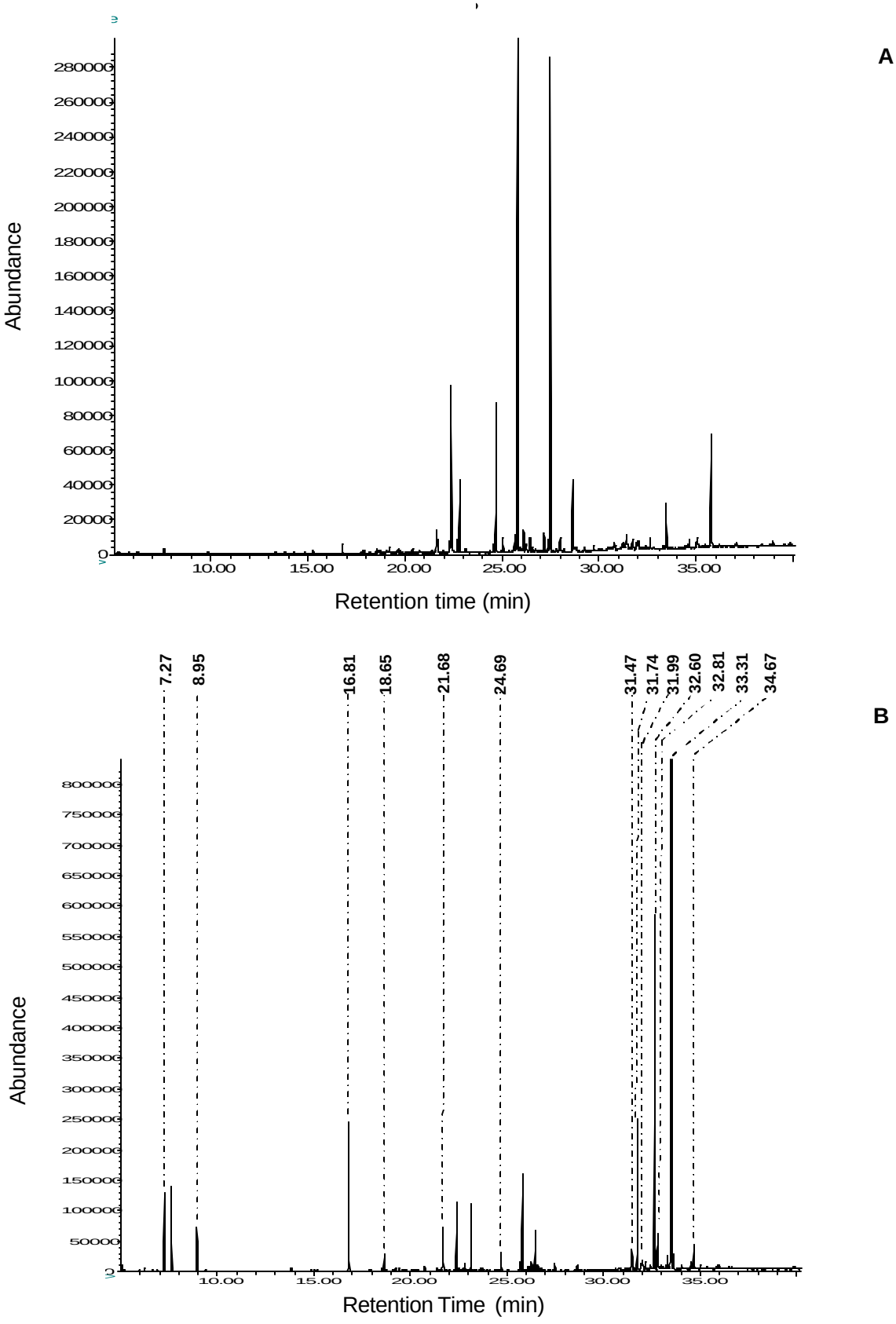


Figure 4.12 GLC chromatograms of lipid-like products of uninfested (A) and infested (B) resistant (Tugela DN) wheat, 48 hours after infestation.

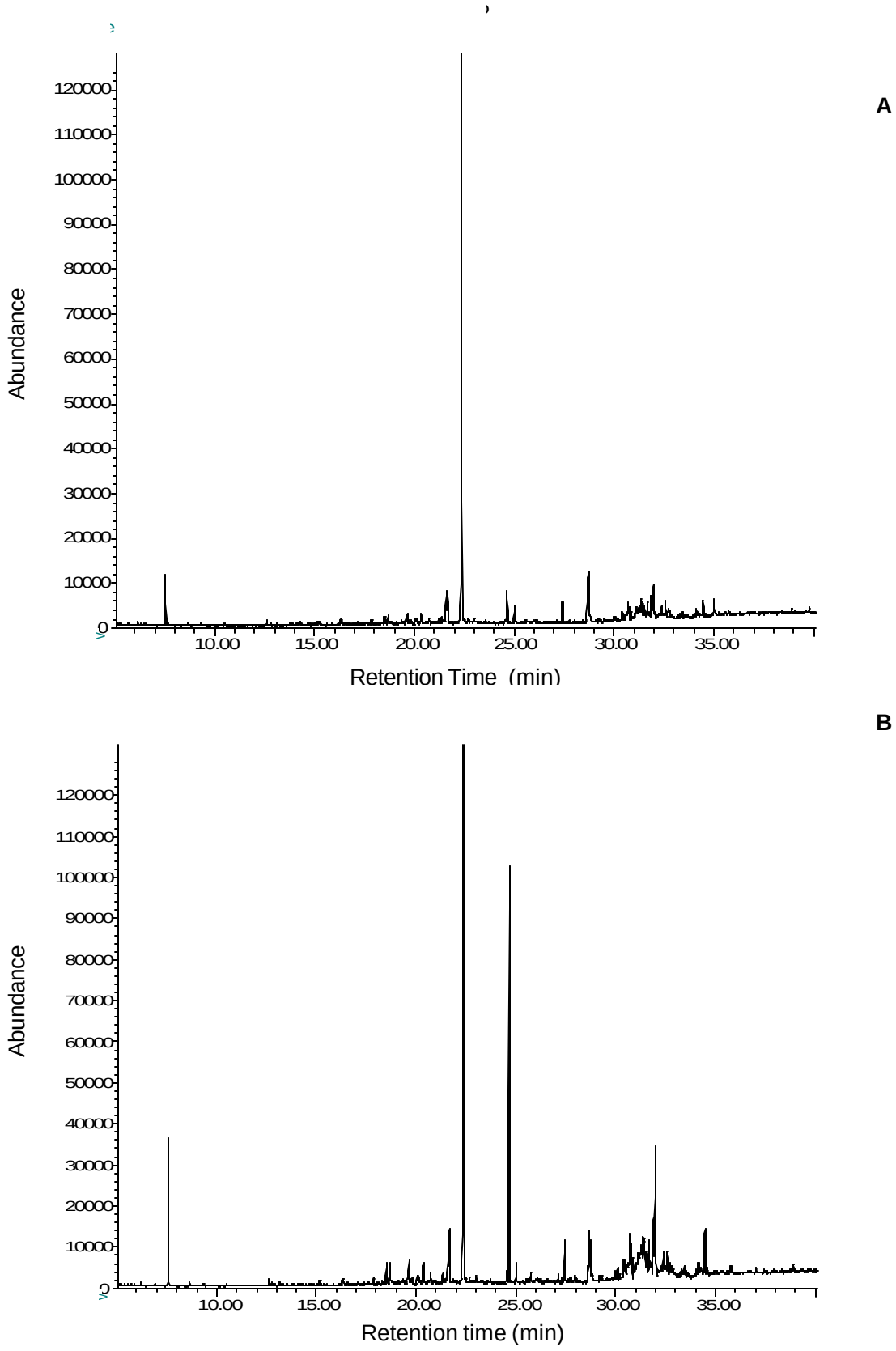


Figure 4.13 GLC chromatograms of lipid-like products of uninfested (A) and infested (B) susceptible (Tugela) wheat, 96 hours after infestation.

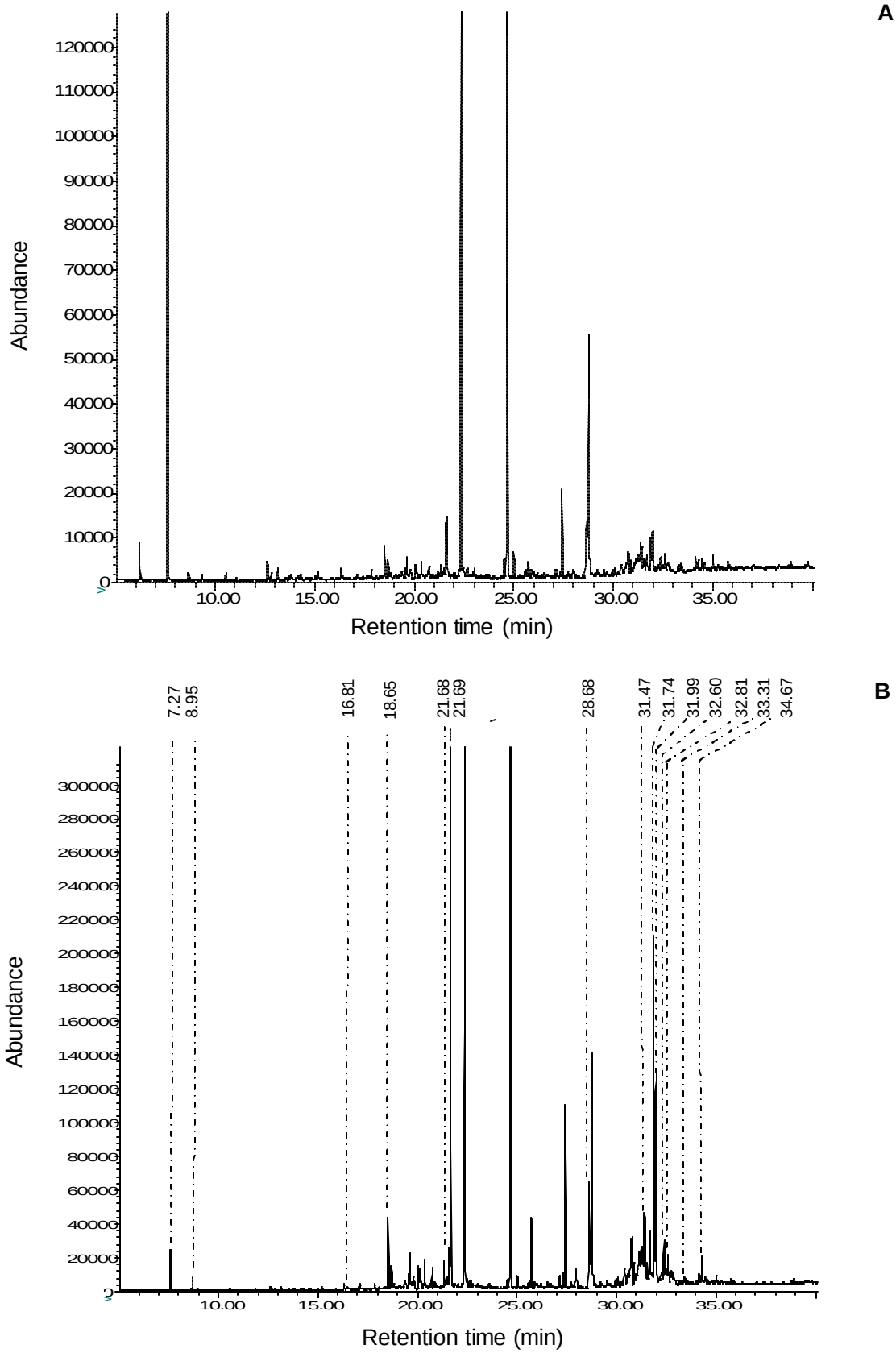


Figure 4.14 GLC chromatograms of lipid-like products of uninfested (A) and infested (B) resistant (Tugela DN) wheat, 96 hours after infestation.

4.6 IDENTIFYING DIFFERENT LIPID-LIKE PRODUCTS.

Though it was difficult to identify each product on the chromatogram, fragments that indicate the presence of oxylipins were identified and searched for in the mass spectra of peaks. Extracts were methylated as well as silinated before GC/MS analysis. Subsequently, the presence of fatty acids was identified by the following fragmentation pattern: m/z 59 and 73. Silization of the hydroxyl groups of fatty acids had the following fragmentation patterns: m/z = 175, 189, 203, 217, 277, 291. Oxygenated fatty acids were identified by the fragmentation patterns: m/z = 243, 271, 341.

4.6.1 OXO (KETO) FATTY ACIDS

The key diagnostic ions in mass spectra of methyl ester derivatives of oxo (keto) fatty acids are formed by cleavage of both *alpha* and *beta* to the oxo group (Ryhage & Stenhagen, 1960; Kenner & Stenhagen, 1964). An oxo group is indicated by a gap of 28 atomic mass units (amu) for the loss of the C=O unit (*alpha* cleavage), followed by loss of successive methylene groups. The ions are formed by *beta* cleavage to the oxo group that tends to be especially abundant, and that of higher mass is frequently odd-numbered, presumably, because it is protonated. However, there are also distinctive ions that serve to locate the double bond by the gap of 26 amu.

Compared to the infested susceptible wheat a total of seven keto-fatty acids (Rt = 31.47, 31.74, 31.99, 32.60, 32.81, 33.31 & 34.67) were selectively induced in the infested resistant (Tugela DN) wheat. All the keto fatty acids identified, had a similar core structure as shown below in Fig. 4.15.

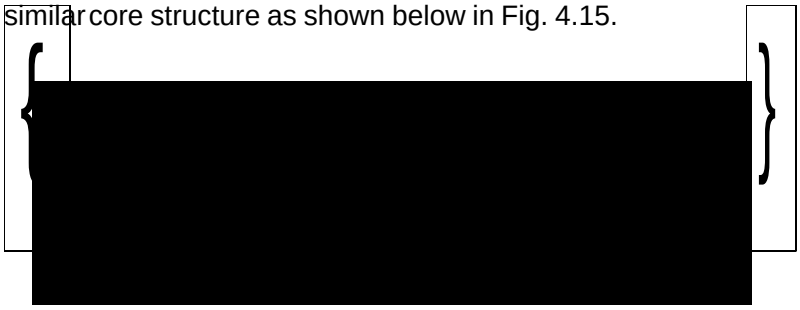


Figure 4. 15 A fatty acid fragment indicating the core structure of keto-fatty acids. m/z = 59, 73, 217, 243, 271, 341.

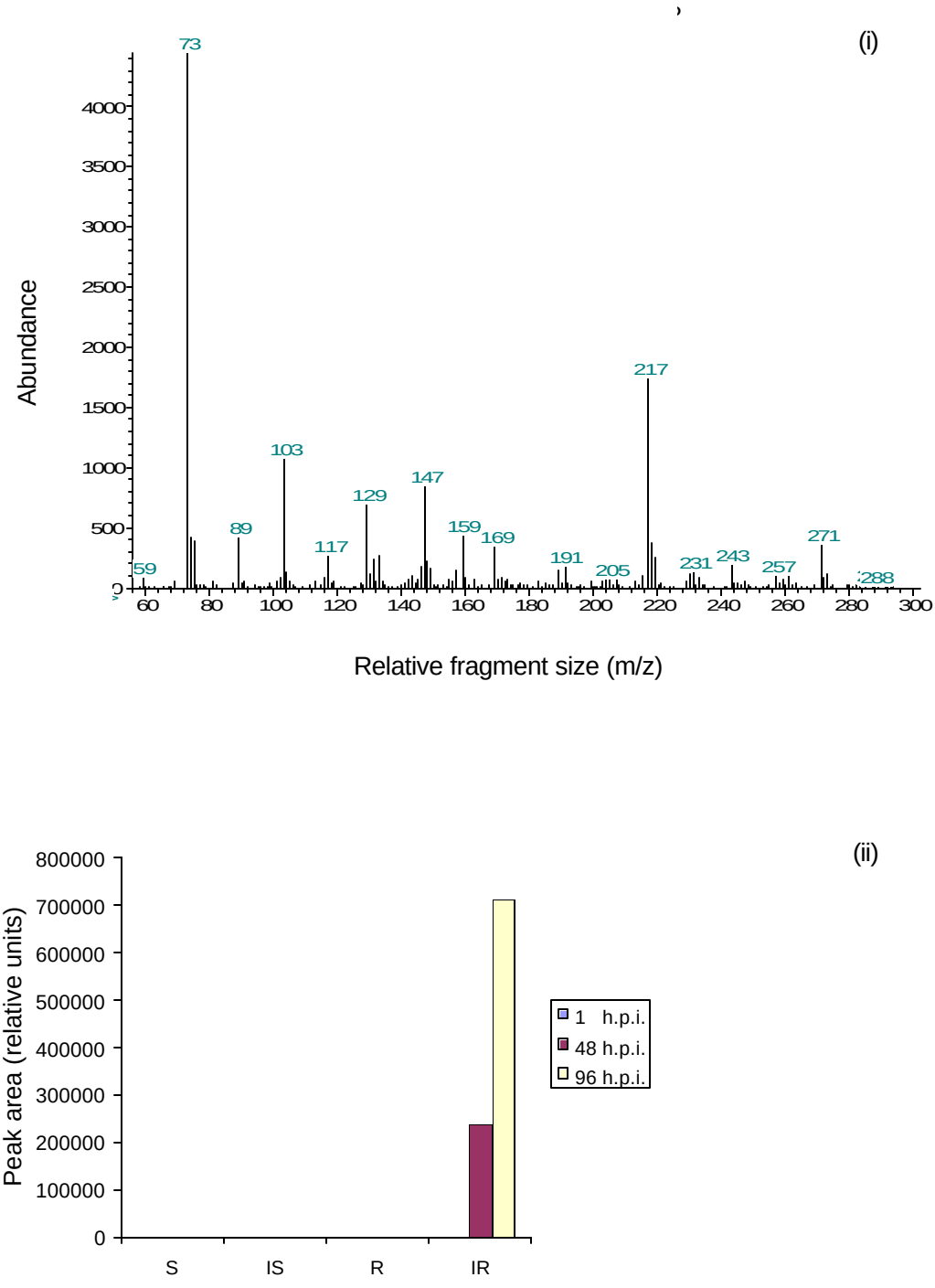


Figure 4.16 A Mass spectrum (i) and expression level (ii) of a keto fatty acid (Rt = 31.47) in the infested (I) susceptible (S), Tugela and resistant (R), Tugela DN wheat 1, 48 and 96 hours post infestation (h.p.i.). Spike numbers on the mass spectrum refer to the m/z values of the fragments.

A keto fatty acid (Rt = 31.47) was selectively induced in the infested resistant wheat. This fatty acid was detected at 48 hours after infestation. It was induced in the resistant wheat only after infestation and not detected in the infested susceptible wheat and the uninfested controls (Fig. 4.16 A).

Another keto fatty acid (Rt = 31.74) was differentially induced to much higher levels in infested resistant than in infested susceptible wheat. The expression was evident 48 h.p.i. and the levels increased as infestation proceeded. A 4.5 fold and an 8.5 fold induction occurred 48 and 96 h.p.i. respectively. Levels were low in uninfested susceptible and resistant wheat (Fig. 4.16 B).

An existing keto fatty acid (Rt = 31.99) was induced to much higher levels in resistant than in susceptible wheat from 48 h.p.i. onwards (Fig. 4.16 C). It was induced 11 and 27.5 fold 48 and 96 h.p.i. respectively. It seems to occur at higher levels, 1 h.p.i., in susceptible plants than in resistant plants. Otherwise induction in susceptible plants was negligible compared to resistant plants. It should be noted that this keto fatty acid occurred in relative large amounts compared to the other keto fatty acids.

According to Fig. 4.16 D the keto fatty acid (Rt = 32.6) was differentially induced to much higher levels in resistant than susceptible plants. Induction peaked 48 h.p.i. in both resistant and susceptible plants at which stage it reached a level 12 fold higher in resistant than susceptible plants. The levels were low in control plants, though higher in resistant than susceptible plants 96 h.p.i.

After 48 hours of infestation a keto fatty acid (Rt = 32.81) was selectively induced in the infested resistant wheat (Fig. 4.16 E). This represents a 5.5 fold increase compared to the infested susceptible wheat. The level of the fatty acid decreased 96 h.p.i. This fatty acid could be detected in all of the samples. At 1 h.p.i. the levels were the same in all of the samples, but increased in the infested resistant wheat to a maximum level 48 h.p.i.

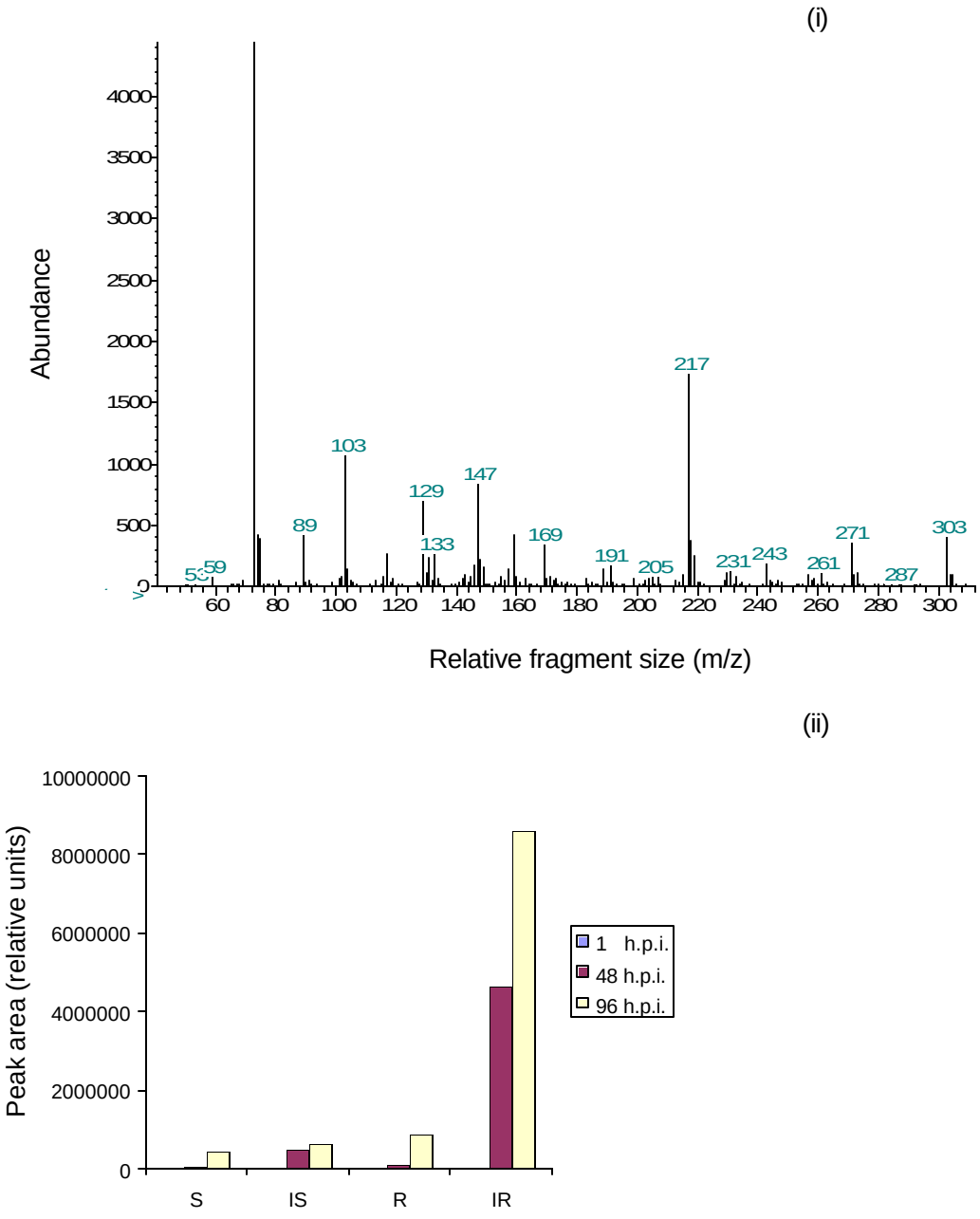


Figure 4.16 B Mass spectrum (i) and expression level (ii) of a keto fatty acid (Rt = 31.74) in the infested (I) susceptible (S), Tugela and resistant (R), Tugela DN wheat 1, 48 and 96 hours post infestation (h.p.i.). Spike numbers on the mass spectrum refer to the m/z values of the fragments.

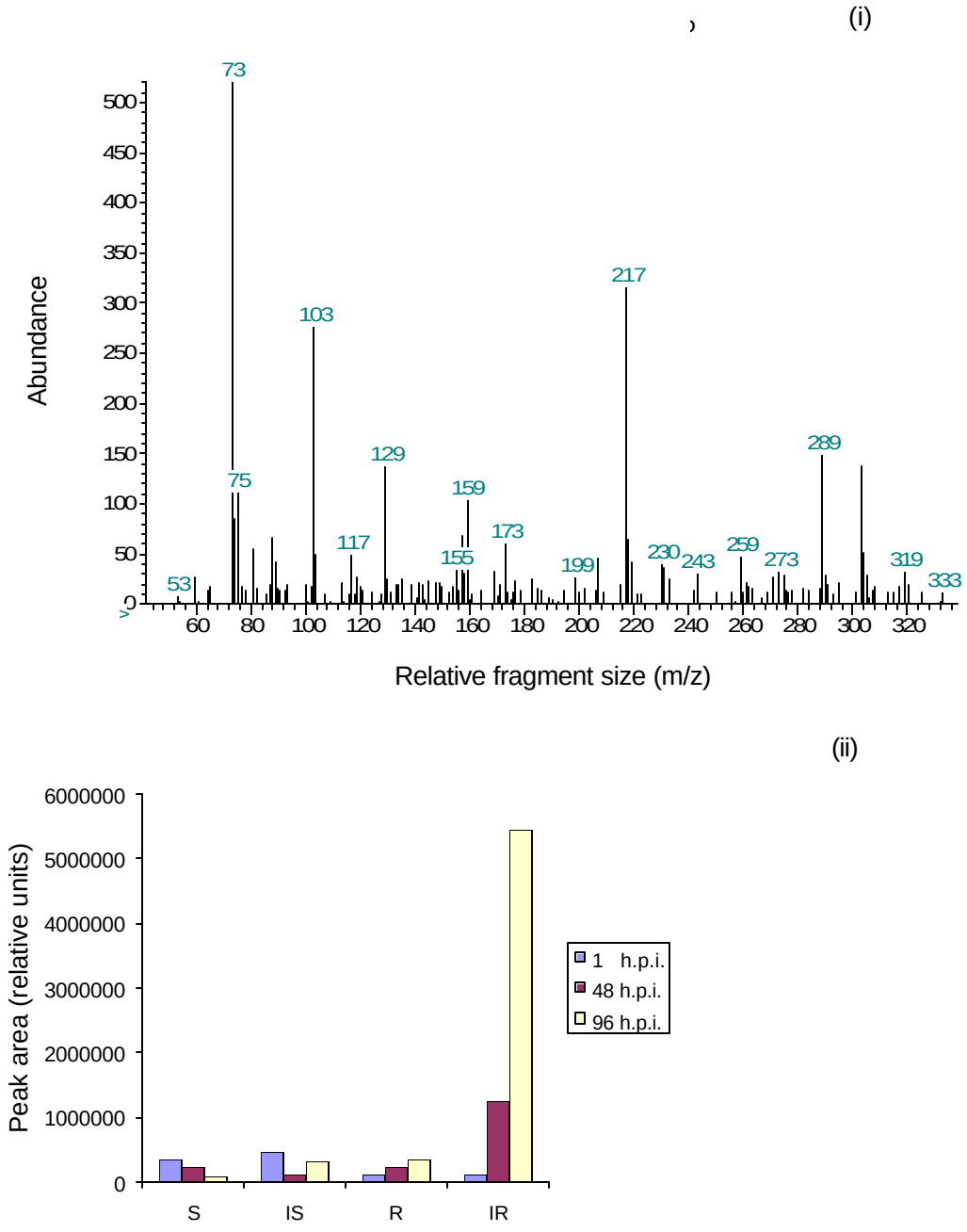


Figure 4.16 C Mass spectrum (i) and expression level (ii) of a keto fatty acid (Rt = 31.99) in the infested (I) susceptible (S), Tugela and resistant (R), Tugela DN wheat 1, 48 and 96 hours post infestation (h.p.i.). Spike numbers on the mass spectrum refer to the m/z values of the fragments.

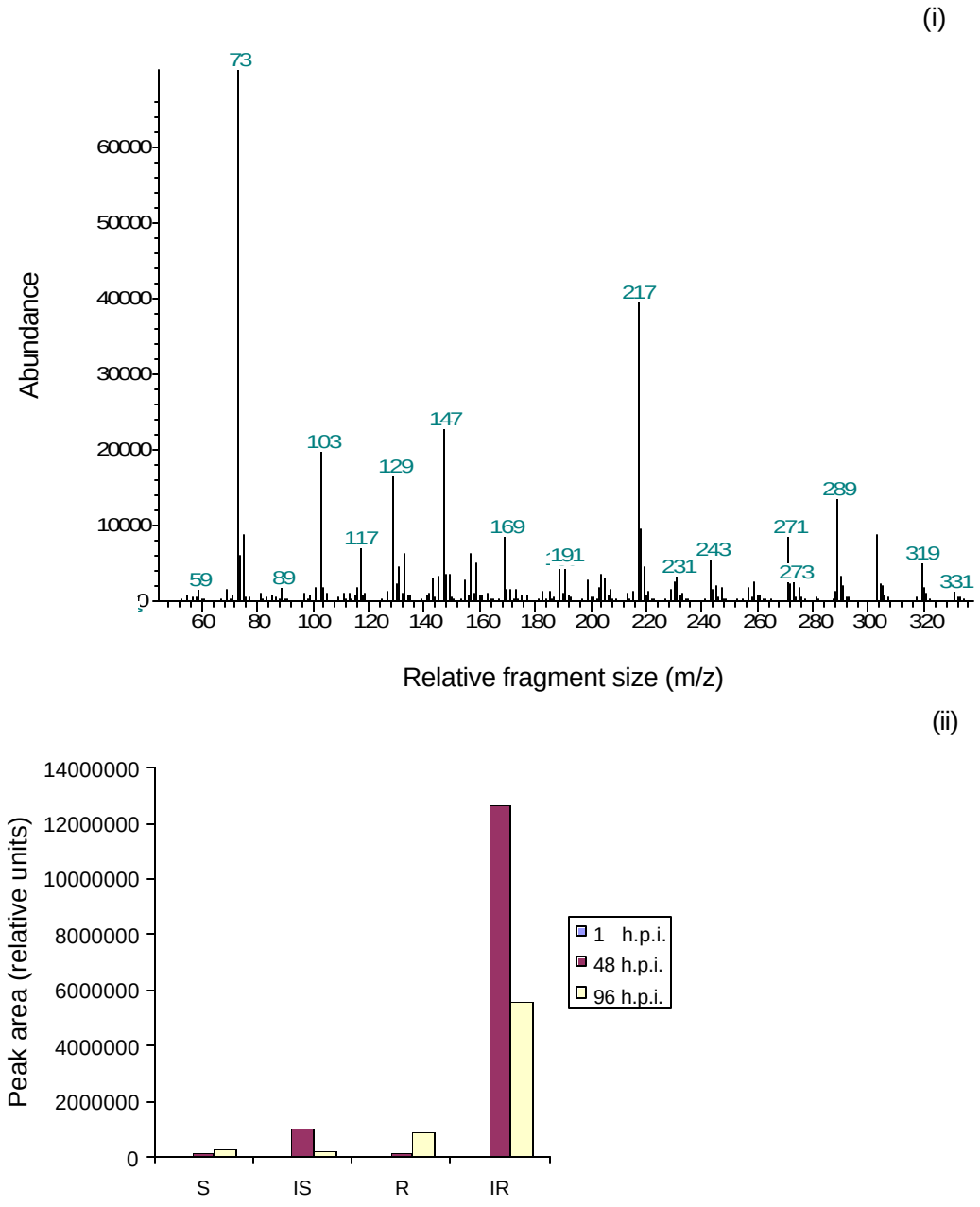


Figure 4.16 D Mass spectrum (i) and expression level (ii) of a keto fatty acid (Rt = 32.60) in the infested (I) susceptible (S), Tugela and resistant (R), Tugela DN wheat 1, 48 and 96 hours post infestation (h.p.i.). Spike numbers on the mass spectrum refer to the m/z values of the fragments.

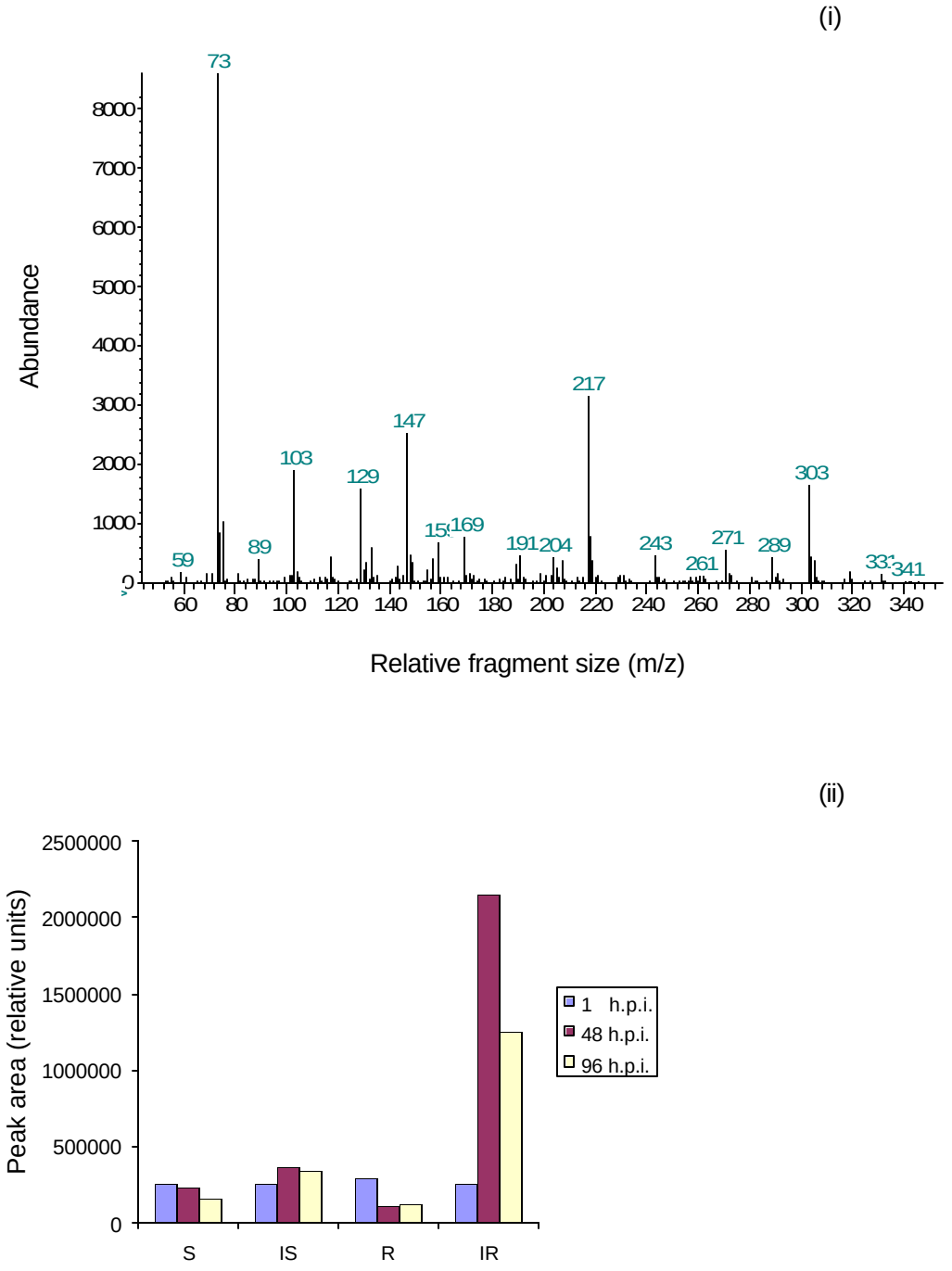


Figure 4.16 E Mass spectrum (i) and expression level (ii) of a keto fatty acid (Rt = 32.81) in the infested (I) susceptible (S), Tugela and resistant (R), Tugela DN wheat 1, 48 and 96 hours post infestation (h.p.i.). Spike numbers on the mass spectrum refer to the m/z values of the fragments.

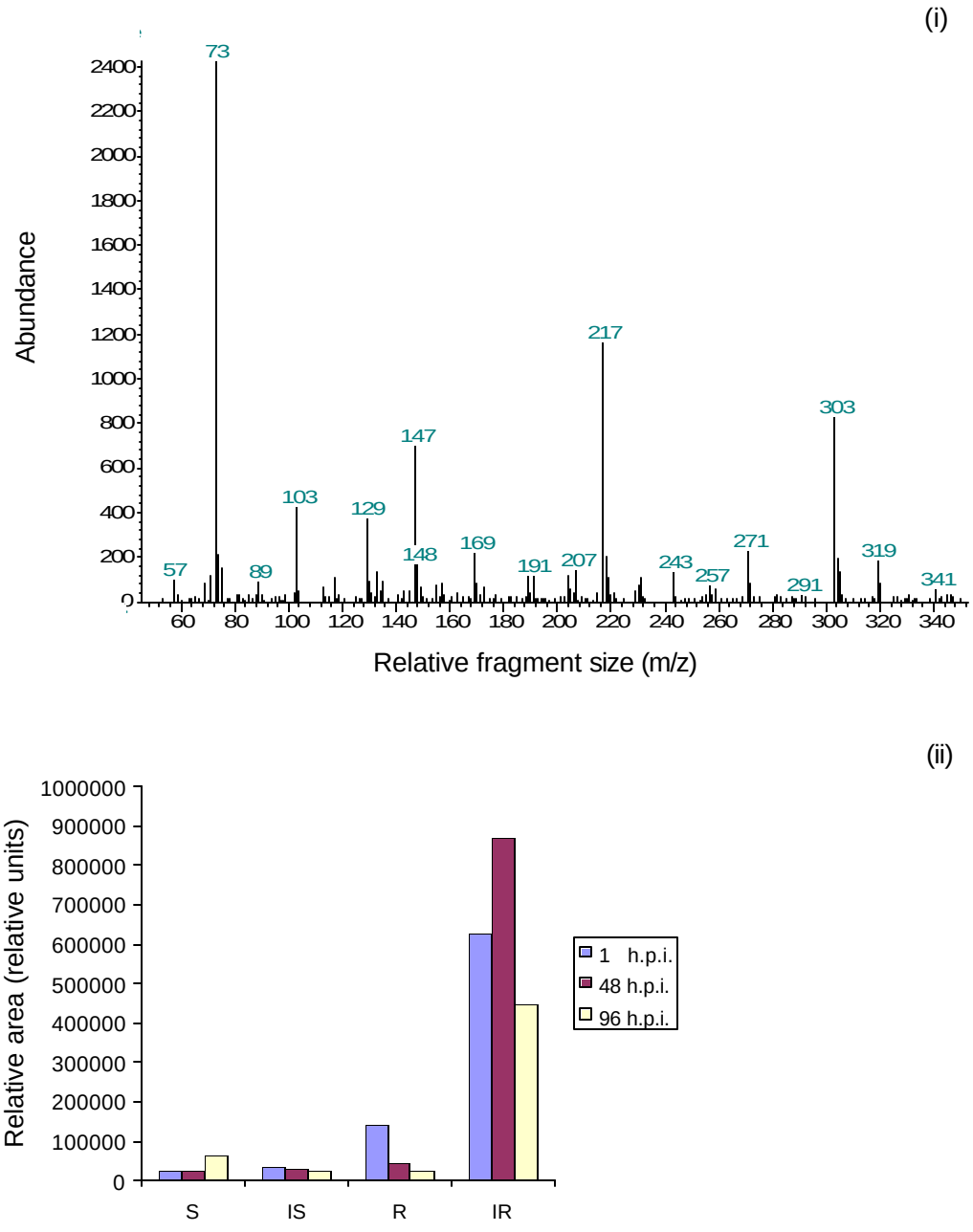


Figure 4.16 F Mass spectrum (i) and expression level (ii) of a keto fatty acid (Rt = 33.31) in the infested (I) susceptible (S), Tugela and resistant (R), Tugela DN wheat 1, 48 and 96 hours post infestation (h.p.i.). Spike numbers on the mass spectrum refer to the m/z values of the fragments.

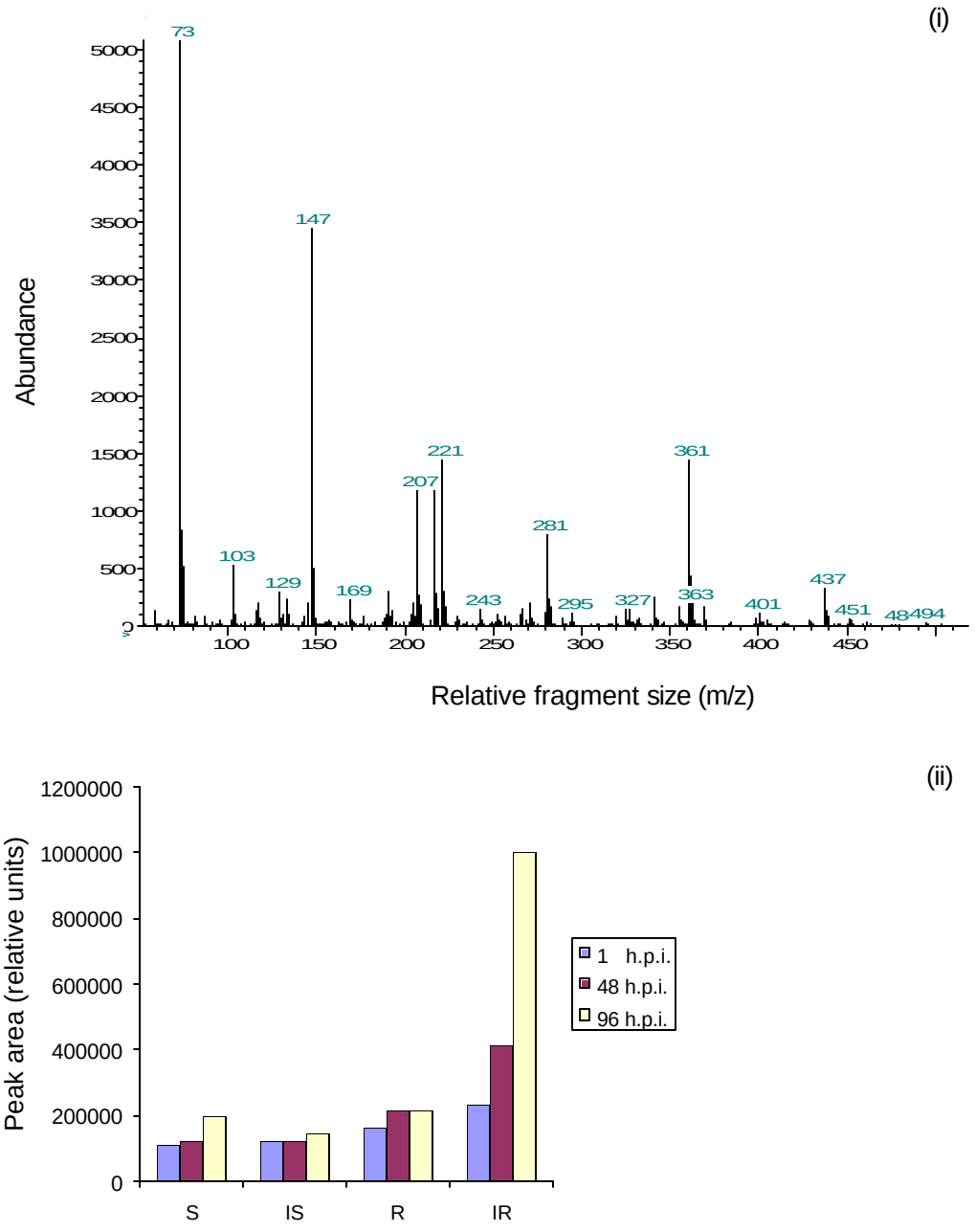


Figure 4.16 G Mass spectrum (i) and expression level (ii) of a keto fatty acid (Rt = 34.67) in the infested (I) susceptible (S), Tugela and resistant (R), Tugela DN wheat 1, 48 and 96 hours post infestation (h.p.i.). Spike numbers on the mass spectrum refer to the m/z values of the fragments.

In contrary to the other keto fatty acids, which were mostly induced 48 h.p.i., this fatty acid was differentially induced already 1 h.p.i. in the infested resistant compared to no induction in infested susceptible wheat where actually no induction occurred. The level peaked at 48 h.p.i. and declined to 96 h.p.i. The peak level represents an 18 fold induction. The uninfested resistant wheat contained more of this fatty acid than the uninfested susceptible wheat (Fig. 4.16 F).

According to Fig 4.16 G keto fatty acid (Rt = 34.67) occurred universally in susceptible and resistant plants. No induction was found in susceptible after infestation. However, in resistant plants it was gradually induced as infestation proceeded. Maximum induction at 96 h.p.i. represents a 10 fold induction.

4.6.2 HYDROXYL FATTY ACIDS

Trimethylsilyl (TMS) ethers of hydroxy esters are often prepared to reduce the polarity of the compounds and facilitate GC analysis. Silylated hydroxyl esters were identified by the ions at m/z = 175, 217. The keto fatty acids identified also had silylated hydroxyl groups. The hydroxyl fatty acids differ from the keto fatty acids since they did not show any fragmentation patterns that would lead us to believe that they are keto fatty acids. The hydroxyl fatty acids contain carboxyl and hydroxyl groups. In these examples the hydroxyl group are located on the third and sixth carbon (Fig. 4.17). Four hydroxy fatty acids was found with Rt values of 8.95, 21.68, 24.69 and 28.68.

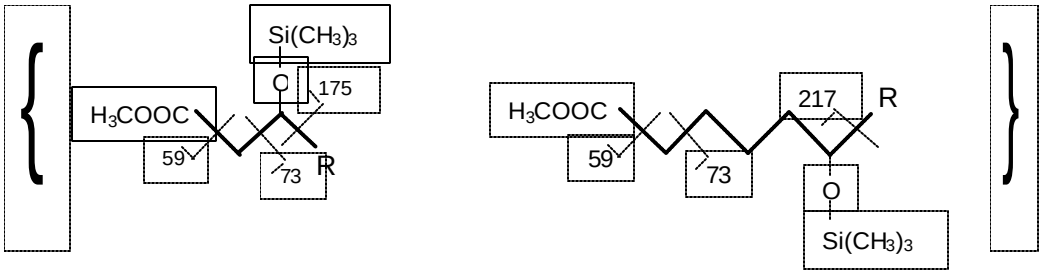


Figure 4. 17 A fatty acid fragment indicating the core structure of hydroxyl fatty acids. m/z = 59, 73, 175 for the hydroxyl group on carbon number three and m/z = 59, 73, 217 for the hydroxyl group on the sixth carbon. Hydroxyl fatty acids do not contain additional keto groups.

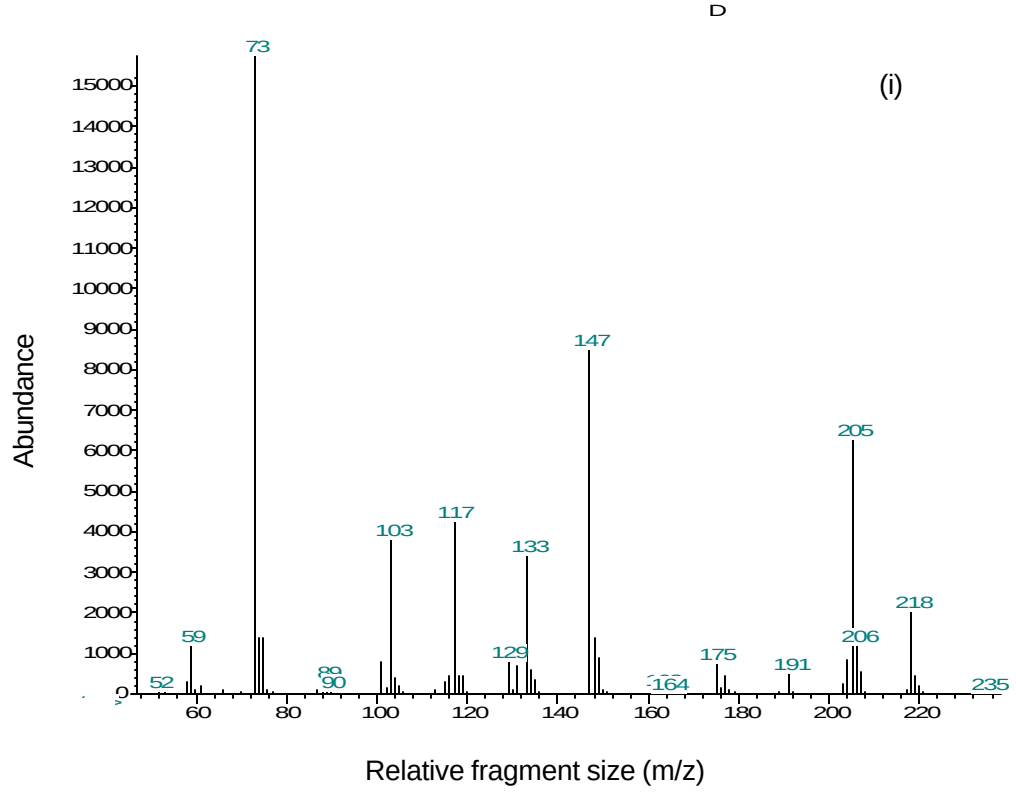


Figure 4.18 A Mass spectrum (i) and expression level (ii) of a hydroxy fatty acid (Rt = 8.95) in the infested (I) susceptible (S), Tugela and resistant (R), Tugela DN wheat 1, 48 and 96 hours post infestation (h.p.i.). Spike numbers on the mass spectrum refer to the m/z values of the fragments.

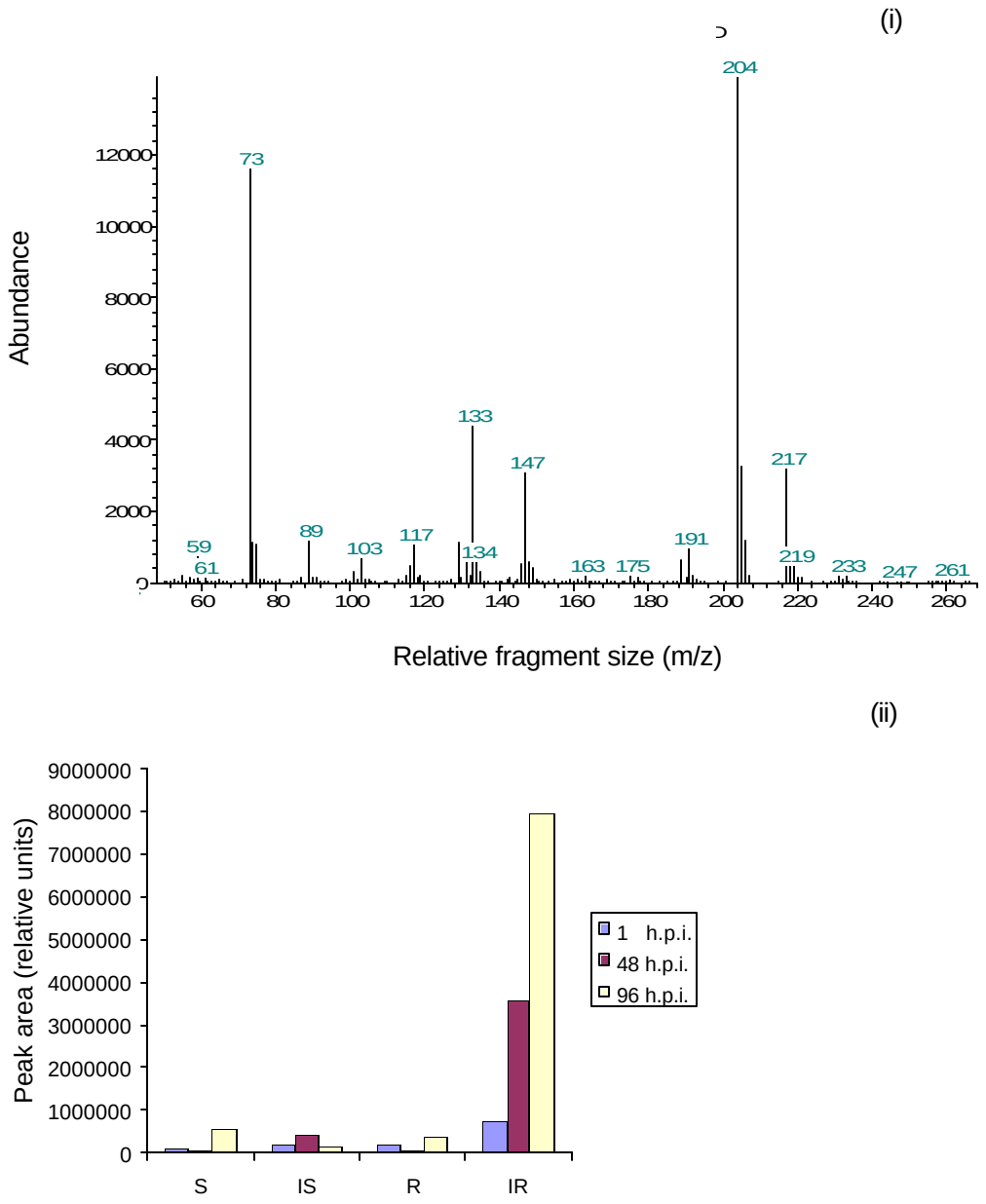


Figure 4.18 B Mass spectrum (i) and expression level (ii) of a hydroxy fatty acid (Rt = 21.68) in the infested (I) susceptible (S), Tugela and resistant (R), Tugela DN wheat 1, 48 and 96 hours post infestation (h.p.i.). Spike numbers on the mass spectrum refer to the m/z values of the fragments.

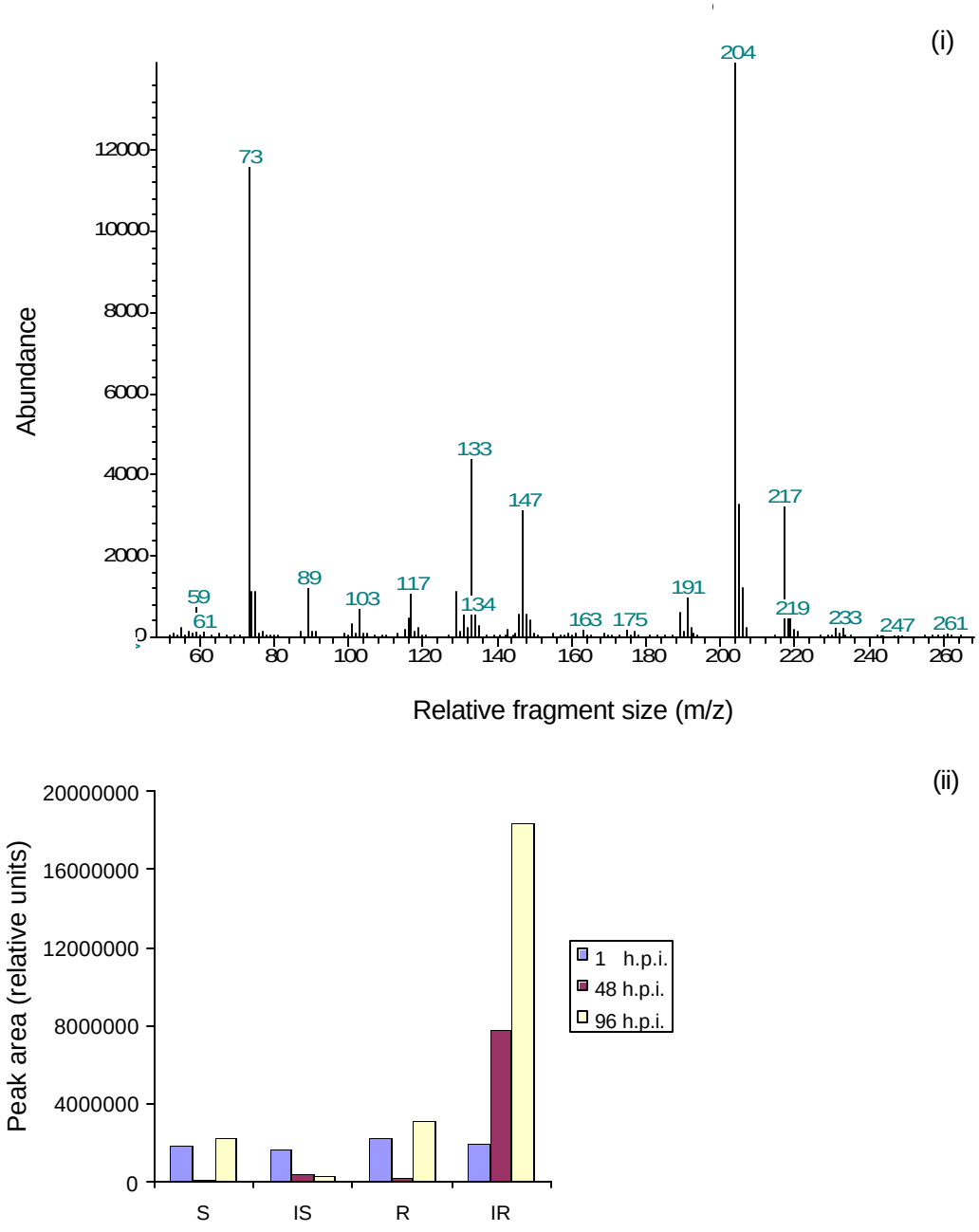


Figure 4.18 C Mass spectrum (i) and expression level (ii) of a hydroxy fatty acid (Rt = 24.69) in the infested (I) susceptible (S), Tugela and resistant (R), Tugela DN wheat 1, 48 and 96 hours post infestation (h.p.i.). Spike numbers on the mass spectrum refer to the m/z values of the fragments.

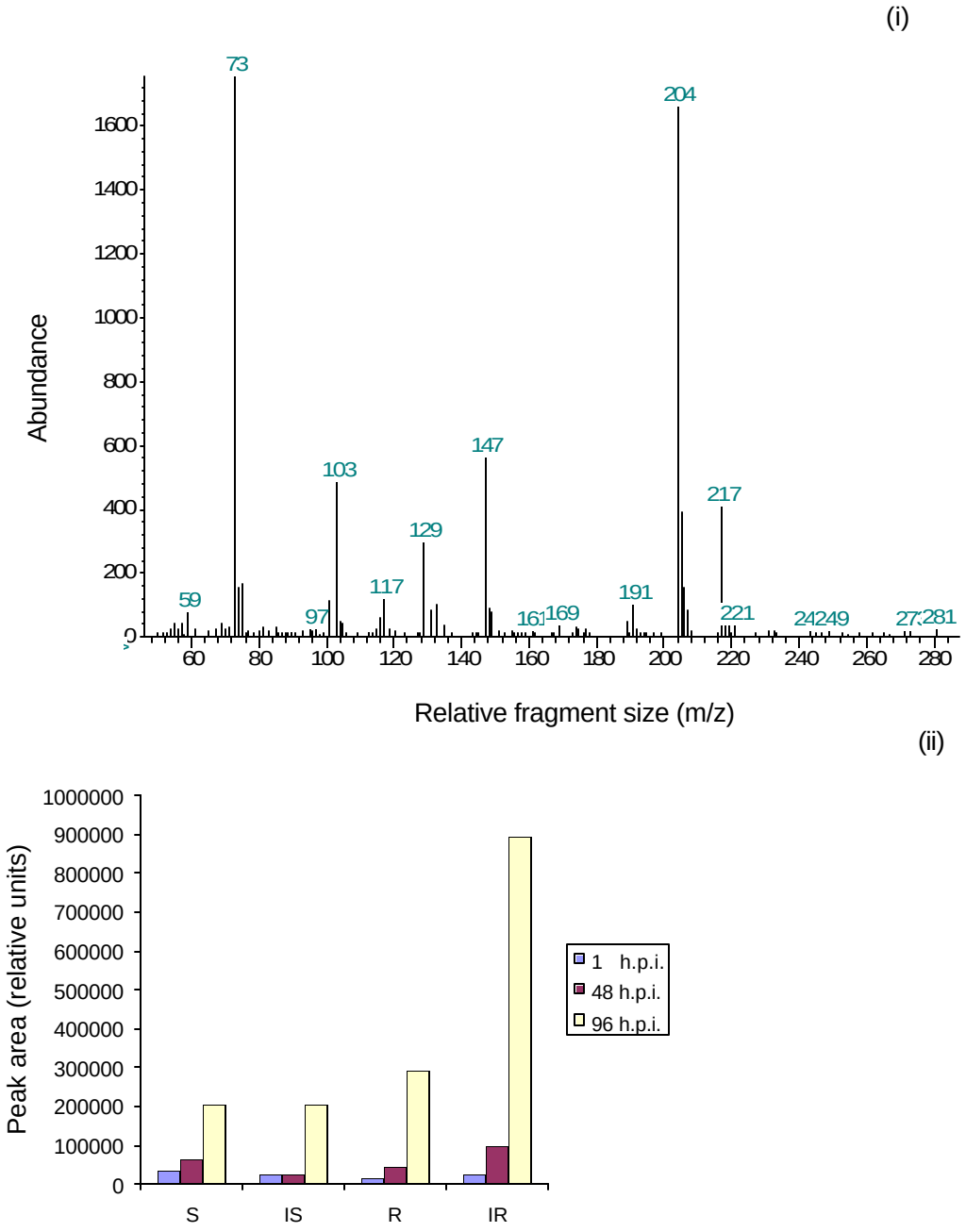


Figure 4.18 D Mass spectrum (i) and expression level (ii) of a hydroxy fatty acid (Rt = 28.68) in the infested (I) susceptible (S), Tugela and resistant (R), Tugela DN wheat 1, 48 and 96 hours post infestation (h.p.i.). Spike numbers on the mass spectrum refer to the m/z values of the fragments.

After 1 hour of infestation a hydroxyl fatty acid (Rt = 8.95) was selectively induced in the infested resistant wheat (Fig. 4.18 A). Maximum induction was 13 fold higher 48 h.p.i. whereafter the level of the fatty acid decreased. This fatty acid was detected at low levels in all the samples. Little induction was evident in susceptible plants as infestation proceeded. In spite of the decrease, resistant plants contained more of this fatty acid than susceptible plants.

A hydroxyl fatty acid (Rt = 21.68) was selectively induced in the infested resistant wheat 1 h.p.i. onwards (Fig 4.18 B). The fatty acid level steeply increased as infestation progressed and reached a maximum (16 fold induction) 96 h.p.i., which is a relative high value compared to the other samples.

According to Fig 4.18 C a hydroxyl fatty acid (Rt = 24.69) was differentially induced to much higher levels in infested resistant plants than in infested susceptible plants as infestation proceeded. The peak level which represents an 5 fold induction reached a peak 96 h.p.i. The fatty acid was detected in all of the samples, but only the infested resistant wheat showed differential induction.

A hydroxyl fatty acid (Rt = 28.68) was substantially induced (3 fold higher) in the infested resistant wheat 96 h.p.i. (Fig. 4.18 D). No induction was observed in susceptible plants after infestation. The fatty acid was present in the control plants at higher levels at the 96 h.p.i. time interval than in the earlier time intervals.

4.6.3 OTHER OXYGEN CONTAINING FATTY ACIDS

A group of fatty acids, of which fragmentation patterns did not correspond to that of keto fatty acids or hydroxyl fatty acids, was identified with Rt values of 7.26, 16.81 and 18.65. The fragmentation patterns were compared to that of other known fatty acids, but no match could be found. These fatty acids showed a pattern of gaps of 16 amu followed by 15 amu. The fragmentation pattern showed similarities to that of 3,7,11,15,19-pentaoxa-2,20-disilaheneicosane, 3,7,11,14,18-pentaoxa-2,19-disilaeicosane, 2,2,19,19-tetramethyl and 1-methoxy-7-trimethylsilyloxyheptane. These compounds all had series oxygen molecules between the carbon. Common fragments were identified as m/z: 117, 133, 147 and 207 (Fig.4.19).

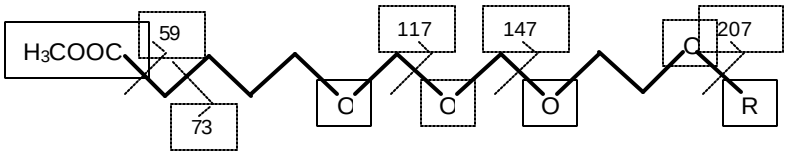


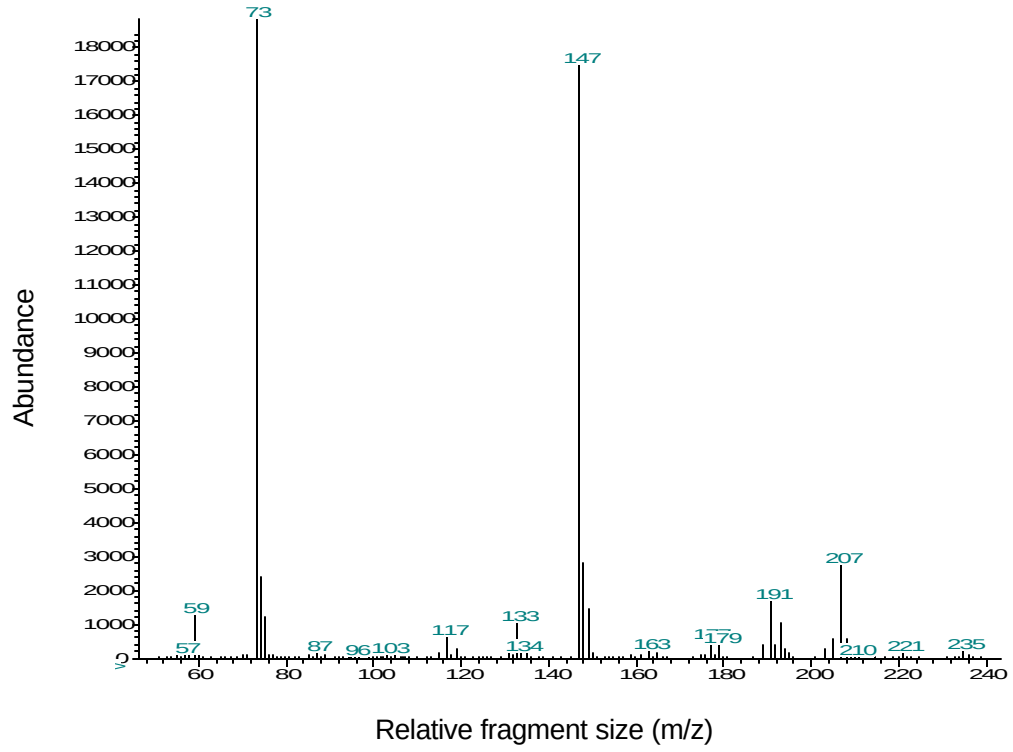
Figure 4. 19 A fatty acid fragment indicating the core structure of a oxygenated fatty acid. Instead of the oxygen connected to a carbon atom by a double bond the oxygen is situated between the carbon molecules; m/z = 59, 73, 117, 147, 207

An oxygen containing fatty acid (Rt = 7.26), other than oxylipin, were found to be selectively induced (25 fold) in the infested resistant wheat (Fig. 4.20 A). An increase in infested resistant wheat was observed at 48 h.p.i. The level, however, declined 96 hours after infestation. In comparison to the induced amount relatively small amounts occurred in the control plants. No induction was observed in susceptible plants after infestation.

A second oxygen containing fatty acid (Rt = 16.81) was detected to be differentially induced in the infested resistant wheat (Fig. 4.20 B). An early increase was found 1 hour after infestation, but there was a significant increase (44 fold) after 48 h.p.i. At 96 h.p.i. a decline in the level of the fatty acid was detected. Induction in infested susceptible plants was negligible compared to that in resistant plants 48 h.p.i. The fatty acids also occurred in the uninfested control plants.

A gradual increase in the content of a third oxygen containing fatty acid (Rt = 18.65) was found in the infested resistant wheat (Fig.4.20 C). The highest level of induction (96 h.p.i.) represents a 5 fold increase. In the infested susceptible and the uninfested controls a gradual increase at a much lower level could also be detected. Interestingly, compared to the previous two oxygen containing fatty acids, the third one continued to increase 96 h.p.i. whereas the former two tended to decrease in content at this time interval.

(i)



(ii)

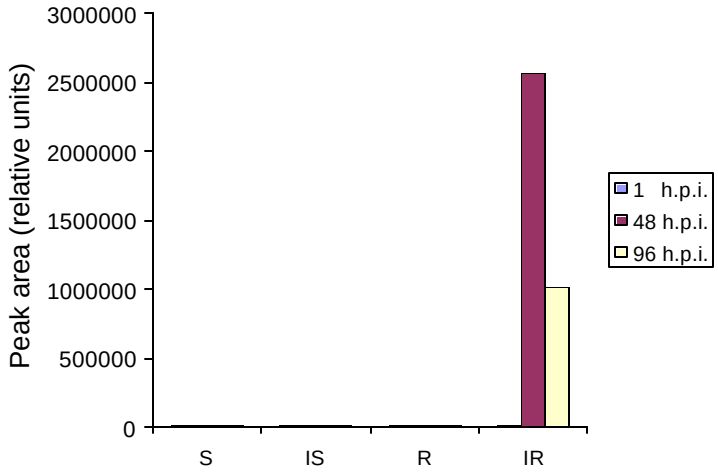


Figure 4.20 A Mass spectrum (i) and expression level (ii) of an oxygen containing fatty acid (Rt = 7.27) in the infested (I) susceptible (S), Tugela and resistant (R), Tugela DN wheat 1, 48 and 96 hours post infestation (h.p.i.). Spike numbers on the mass spectrum refer to the m/z values of the fragments.

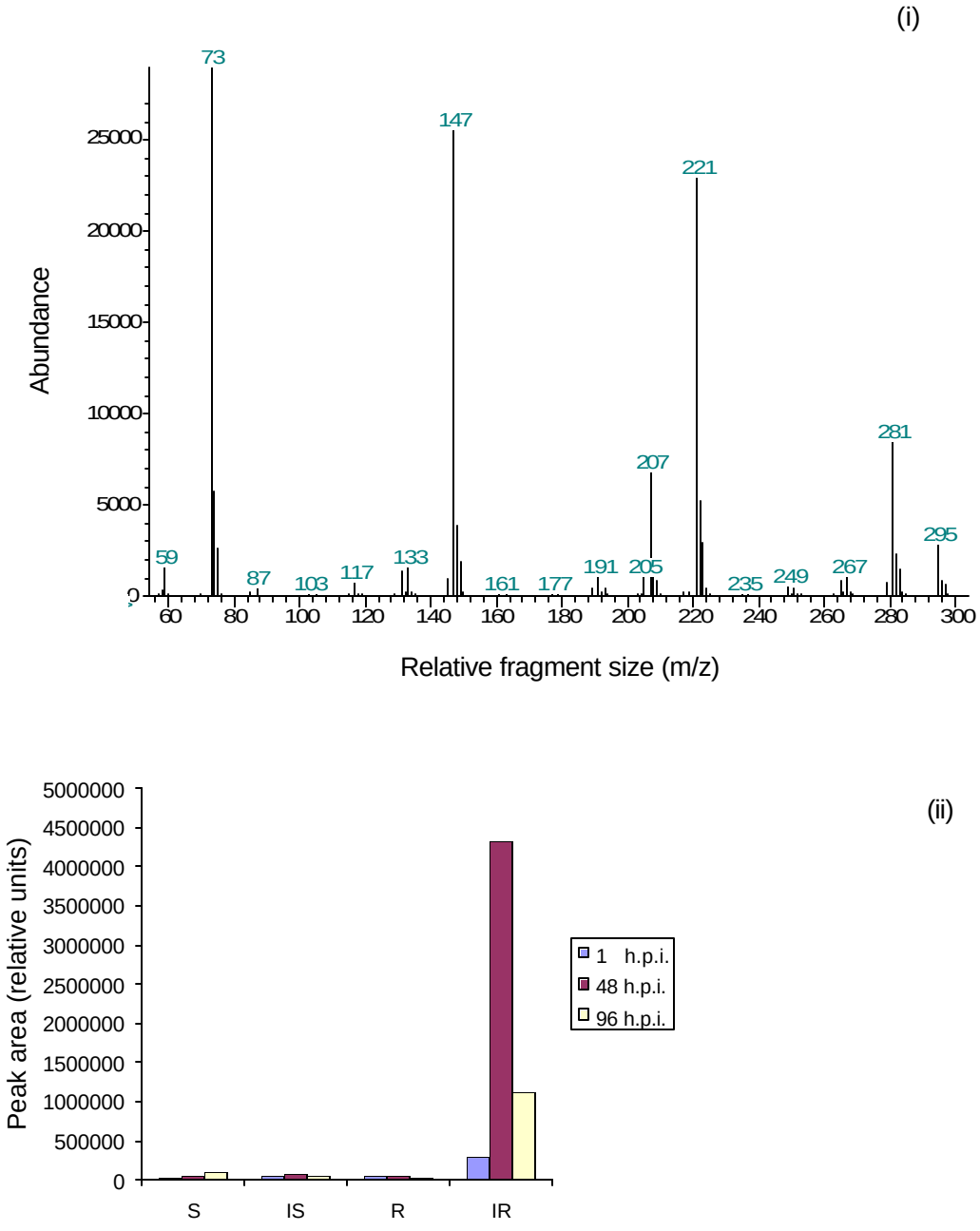
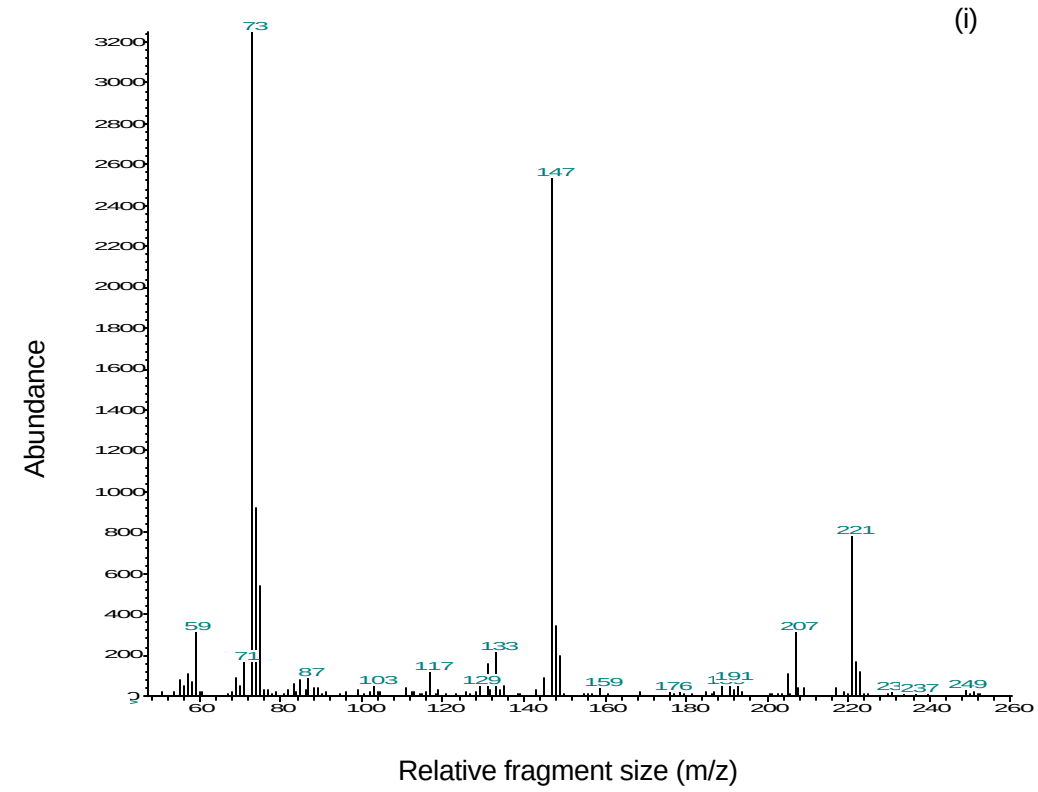


Figure 4.20 B Mass spectrum (i) and expression level (ii) of an oxygen containing fatty acid (Rt = 16.81) in the infested (I) susceptible (S), Tugela and resistant (R), Tugela DN wheat 1, 48 and 96 hours post infestation (h.p.i.). Spike numbers on the mass spectrum refer to the m/z values of the fragments.



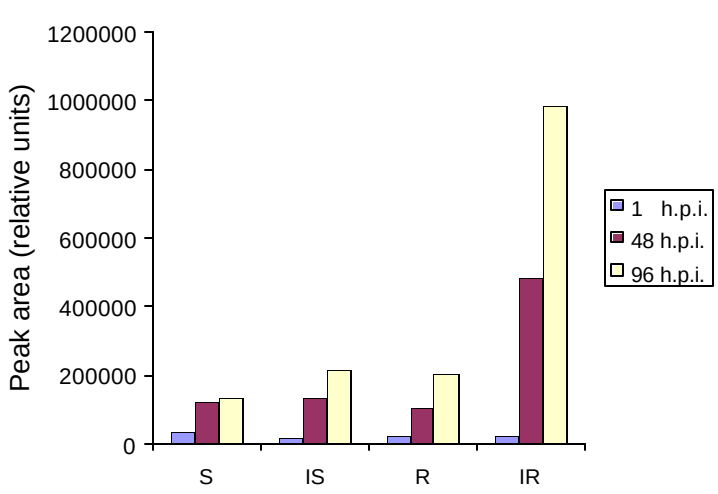


Figure 4.20 C Mass spectrum (i) and expression level (ii) of an oxygen containing fatty acid (Rt = 18.65) in the infested (I) susceptible (S), Tugela and resistant (R), Tugela DN wheat 1, 48 and 96 hours post infestation (h.p.i.). Spike numbers on the mass spectrum refer to the m/z values of the fragments.

CHAPTER 5

Discussion

Many resistance mechanisms in plants have been correlated with increases in LOX activity and lipid peroxidation. Lipid-based signalling pathways, which originate as a result of LOX activity and consequent lipid peroxidation, control many of the cellular processes in eukaryotic cells. In animal cells, lipoxygenase-mediated oxygenation of arachidonic acid and related polyunsaturated fatty acids leads to the formation of leukotrines and a variety of lipid-based products with potent physiological activities, influencing cellular responses (Samuelsson *et* al., 1987). In higher plants known fatty acid-derived metabolites, which affect responses through transcriptional activation of genes are divided into several categories (Farmer *et al*., 1992; Hamberg & Gardner, 1992). These categories include the jasmonate family (octadecanoid-derived cyclopentanones and cyclopentanols), the traumatin family and related alkenals and other classes such as highly oxygenated fatty acid derivatives (oxylipins) (Farmer, 1994). Within these groups of fatty acids, there are volatile compounds, glycosides, conjugates and fatty acids that are potentially unstable and reactive (especially the alkenals) (Farmer, 1994). These metabolites originated from the LOX pathway and are present in most if not all plant species (Parthier, 1991; Staswick, 1992).

Lipoxygenase activity is induced in several incompatible interactions in plants and it seems always to be after a lag phase of several hours (Peever & Higgins, 1989; Slusarenko, 1996; Hornung *et al*., 1999). In this study it was found that LOX activities increased in all infested resistant cultivars 24 hours after infestation (Fig's. 4.1, 4.2 & 4.4). During this 24 hour lag phase other defence reactions are in operation, such as signalling pathways, which are important for the selective induction of LOX activity in resistant plants. The gradual increase of LOX activity over time indicates that this enzyme could play a key role during the resistance response. Young tobacco leaves, with feeding aphids, also showed an increase in LOX activity (Voelckel *et al*., 2004). Research on potato plants, resistant to pathogens, provided evidence of increases in potato LOX mRNA after pathogen infection. Susceptible potato plants did not demonstrate any increases or delayed increases in LOX mRNA (Kolomiets *et al.*, 2000). In transgenic tobacco, expressing antisense LOX, the defence mechanism was suppressed in the tobacco plants when infected with pathogens (Rancé *et al.*, 1998). Transgenic potato plants, devoid of the 13-LOX isoform through antisensing decreased the accumulation of proteinase inhibitors (Royo J *et al.*, 1999). The inhibition and activation of the LOX pathway has been found to inhibit and activate cell death respectively (Rustérucci *et al*., 1999). No increases in LOX activity were found in susceptible wheat plants infested with the

RWA and this is an indication for the possible involvement for LOX during the defence response (Fig.'s 4.1, 4.2 & 4.3). This induction of LOX activity is most probably one of the key steps during the defence response.

The level of LOX activity was found to differ between the different resistant cultivars. The different levels of induction of LOX activity may be attributed to the genetic background in which the *Dn-1* resistance gene was bred into. The highest increase in LOX activity was observed in 'Tugela DN' followed by 'Betta DN' and 'Gariep'. From our results, it is evident that the background, in which the resistance gene is bred into, makes a difference in the expression of LOX activity (Fig.'s 4.1, 4.2 & 4.3). In previous biochemical studies involving these wheat cultivars 'Tugela DN' demonstrated to have the better biochemical defence response (Van der Westhuizen *et al.*, 1998 a & b). The level of LOX expression would therefore seem to be linked to the efficiency of resistance of wheat plants. Higher LOX expression seems to lead to a more effective defence mechanism than a lower expression of LOX. For this reason 'Tugela DN' was further used to study the biochemical resistance mechanism with the focus on the involvement of lipid-based products of wheat against the RWA.

The differential induction of LOX activity in the infested resistant cultivars was confirmed by Western blot analyses (Fig.4.4). A 78 kDa LOX protein was selectively induced by RWA infestation to greater quantities in the resistant wheat. Numerous reports confirmed the differential induction of a LOX protein during incompatible biotic and abiotic stresses (Bohland *et al.*, 1997; Rustérucci *et al*., 1999 & Göbel *et al*.,, 2001) The 78 kDa protein distinctively increased quantitatively as infestation proceeded in the infested resistant wheat. The LOX protein was only expressed after infestation, indicating that the selective induction of LOX is genetically controlled and a signal is required for its induction. The inadequate expression of LOX in susceptible plants can be the result of a very weak expression of LOX gene as a result of the poor induction of the resistance mechanism. The expression of the different isoforms of LOX differs with the type of infestation and the background in which the LOX protein is expressed. Pea roots infested with a nematode selectively expressed a 96 kDa and a 78 kDa LOX protein in resistant plants, but were not selectively induced in the susceptible plants (Leone *et al.*, 2001). The type of LOX isoform induced, will determine the kind of downstream products being formed as a result of LOX activity. In the tomato plant at least 5 different LOX isoenzymes were found to be responsible for the synthesis of various forms of fatty acids (Chen *et* al., 2004). During pathogenesis in wheat it was found that a 92 kDa LOX protein was

predominantly formed, which is believed to be responsible for the resistance response of wheat against pathogens. The LOX-92 predominantly catalyzed the formation of 9-hydroperoxy fatty acids (Bohland *et al*., 1997).

A smaller LOX protein (76 kDa) was expressed in the uninfested susceptible line. This isoform was not expressed in the uninfested resistant or the infested susceptible and resistant lines (Fig. 4.4). The existence of this LOX protein in the uninfested susceptible wheat can be of importance to many plant breeders. This will enable plant breeders to screen for susceptible wheat lines. Further investigation is, however, needed to prove that this 76 kDa protein is indeed universal for uninfested susceptible wheat lines.

The function of LOX during a defence reaction may very well coincide with membrane lipid peroxidation and the irreversible membrane damage during the hypersensitive cell death or the production of antimicrobial compounds (Slusarenko, 1996 & Buonaurio & Sevili, 1999). Membrane damage through lipid peroxidation is initiated by ROS (reactive oxygen species), lipid radicals, or enzymatically by the action of LOX (Rosahl, 1996). The enzymatic formation of lipid peroxides by LOXs during the induction of the HR might be of dual advantage to plants. LOX-derived lipid peroxides are less toxic substances for the plant cell, because they are rapidly metabolized by enzymes of the LOX pathway. Several of these metabolites act as specific antimicrobial compounds. Being non-toxic for the plant, they might enhance the defence capacity of the plant in addition to the initial hypersensitive cell death (Göbel *et al.,* 2003)

Lipoxygenase also plays a role in plant defence against herbivorous insects by affecting insect growth and development in direct and indirect ways. The products of LOX may be repellent to insects and operate as an antixenosis basis of resistance (Felton *et al*., 1994, Rayapuram & Baldwin, 2006). Dietary proteins treated with linoleic acid and LOX declined in nutritional value which in turn resulted in a decline in larval growth (Felton *et al*., 1994). Foliar LOX and lipid peroxidation increases when corn earthworm feeds on soybean plants (Felton *et al*., 1994). Larvae and mites feeding on soybean foliage also resulted in increased lipid peroxidation products in these plants (Hildebrand *et al*., 1986). The success of these defence strategies, lies in the ability of the plant to produce oxylipins via LOX (Rayapuram & Baldwin, 2006). The initial products of LOX, the hydroperoxides, are chemically and enzymatically degraded to reactive aldehydes, ?-ketols and epoxides, which are toxic

to insects (Gardner, 1991). Lipid hydroperoxides also cause chemical changes in proteins such as lipid-proteins, amino acid damage, protein scission and protein-protein cross-links (Gardner, 1979), thus lowering the nutritional value of the plant.

The hypersensitive response (HR) induced in plants by pathogens is characterized by the rapid death of cells at the site of pathogen infection, which restricts the further spread of the pathogen. Lipoxygenase participates in membrane destruction at the site of infection by the generation of highly reactive molecules such as free radicals and thus contributing to the HR (Croft, 1993 & Slusarenko, 1996). It seems that the main contribution of LOX is the formation of hydroperoxides from fatty acids that will be degraded to physiological important products through several enzymatic systems. These hydroperoxides are highly reactive and are converted very easily to their various products (Blée, 1998). Linoleic- and linolenic acid, components of plant lipids, represent potential substrates for LOX and these general free fatty acids have been regarded as the natural LOX substrates (Bohland *et al.*, 1997).

Wounding of a plant often results in the emission of volatile compounds such as 3(*Z*)-hexenal, 2(*E*)-hexenal and the alcohol and ester metabolites of these aldehydes. These are all well-known products of leaf wounding initiated by oxygen dependent LOX reactions (Gardner, 1991). These semiochemicals emitted from a diverse group of plants and insects mediate key processes in the behaviour of specific insects. Volatile phytochemicals can serve as airborne semiochemicals, promoting or deterring interactions between plants and insect herbivores. Wheat seedlings without herbivore damage attract aphids, whereas odors released from wheat seedlings with a high density of aphids repel other aphids (Quiroz *et al.,* 1997).

Pepper fruits inoculated with *Xanthomonas campestris* pv. *vesicatoria* produced volatiles, which were derived from 13-hydroperoxylinoleic and 13-hydroxylenolenic acid. *Trans*-2-hexenal and *cis*-3-hexenol were the two most abundant volatiles being formed, both exhibiting antimicrobial activity, followed by *trans*, *trans*-2,4-hexadienal and 1-hexanol. Other volatiles that are selectively induced upon infestation was 2,4-hexadienal, 2,4-eptcedienal, 3-hexan-1-ol and α- and β-ionone (Buonauria & Servili., 1999). Croft *et al.* (1993) found a substantial increase in *trans*-2-hexenal and *cis*-3-hexenol when *Phaseolus vulgaris* leaves were inoculated with *Pseudomonas syringae*. An increase in 13-hydroxy lyase activity was

found during the hypersensitive response (HR) and also an increase in the C6-alcohols and a decrease in their corresponding C6-aldehydes (Buonauria *et al.*, 1999). The enzymatic breakdown of linolenic acid produces a range of products and intermediates of which many contribute to characteristic odours of cut or damaged plant tissue (Hatanaka *et al.*, 1987; Siedow, 1991). These volatile compounds serve as semiochemicals to attract parasitic wasps, can repel herbivores and can also function as long distance signal compounds to induce a defence response in a nearby plant (Croft *et al.*, 1993; Mattiaci *et al*., 1995; Paré & Tumlinson, 1998; Mercke *et al*., 2004).

Lipid-based compounds not only originate from the LOX-pathway, but it seems that a cyclooxygenase pathway also exists generating lipid-based compounds. A pathogen-induced oxygenase was found to be selectively induced in tobacco leaves after treatment with a pathogen elicitor. This oxygenase has shown great homology to animal cyclo-oxygenase (COX) or prostaglandin endoperoxide synthase (Sanz *et* al., 1998). Cyclo-oxygenase catalyzes the first committed step in the formation of prostanoids, which are lipid-derived signal molecules that mediate many cellular processes (Sanz *et* al., 1998). In vertebrates COX is a key enzyme in the production of lipid-like signal molecules that regulate diverse cellular processes (O'Banion *et* al., 1992 & Serhan *et al.*, 1996). Cyclo-oxygenase catalyses the conversion of polyunsaturated fatty acids, such as arachidonic acid (20:4), to prostaglandin and other eicosanoids. In vertebrates two isoforms of COX are expressed: COX-1 is expressed constitutively in most tissues and is present under normal conditions at very low levels in the brain, COX-2 is acutely expressed after head injuries, in cerebral ischemia and in Altzheimer's disease (Straus & Glass, 2001). Prostanoids, from the COX-2 pathway, induce antioxidative enzymes during the above-mentioned injuries (Zhuang *et al*., 2003). The oxygenase in tobacco plants has a structural core similar to that of that of mammalian COX. Studies on the protein's functionality demonstrated that the tobacco oxygenase protein possesses at least one of the two-enzymatic activities of prostaglandin endoperoxide synthase (Sanz *et* al., 1998). These compounds regulate the immune response and inflammation reaction in response to pathogen infection in mammalian cells. In addition, salicylic acid, jasmonic acid, wounding and oxygen radicals can activate the oxygenase gene in plants (*Sanz et* al., 1998; Hermsmeier *et* al., 2001; Kim *et al.,* 2002). Oxygenases seem therefore to play an important role in various forms of biotic stresses. An oxygenase protein was also induced by the RWA in resistant wheat (Fig. 4.5). The inability of the susceptible wheat to express the oxygenase

protein to higher levels indicates that the combination of the LOX-pathway and the cyclooxygenase pathway forms part of the defence strategy of wheat to the RWA.

Alpha-oxidation in mammals is of critical importance for the degradation of phytanic and ß-methyl branched fatty acids (Verhoeven *et al.*, 1998). The function of the corresponding pathway in plants is not yet fully understood. The fact that PIOX, established as a fatty acid a-dioxygenase involved in a-oxidation, is pathogen and also aphid inducible, suggests that the importance of the a-oxidation pathway in plants may be related to the successful establishment of a resistance mechanism towards pathogens and aphids. The 2-hydroperoxides that are generated as a result of a-oxidation can act as signalling molecules for the induction of defence related genes (Hamberg, 1998). A direct toxic effect of the hydroperoxides or its degradation products on invading pathogens and insects is also conceivable. The function of fatty acids originating as a result of a-oxidation is still unclear in plants.

Additionally, the initiation of lipid peroxidation in plants forms an important feature of hypersensitive cell death (Montillet, 2005). This is a typical defence response during incompatible interactions of plants with pathogens (Heath, 2000; Göbel *et al.*, 2003). Membrane damage by peroxidation of polyunsaturated fatty acids can be initiated by reactive oxygen species and lipid radicals. In plants, LOX and COX introduces molecular oxygen into polyunsaturated fatty acids, leading to the formation of the corresponding hydroperoxides. The production of hydroperoxides and lipid-like products are not only the result of increases in LOX activity, but also due to increases in COX activity. Salicylic acid, JA and hydrogen peroxide are regarded as cellular signals for the activation of plant defence responses including lipid peroxidation (Hammond-Kosack & Jones, 1996; Reymond & Farmer, 1998).

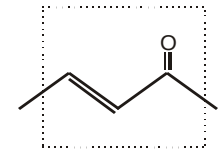
On the other had, one of the best-known lipid oxidation products is malonaldehyde (MDA), which is commonly used to determine lipid peroxidation. Little is known about the activity of MDA on the regulation of gene expression. A few MDA responsive genes have been identified in vertebrate cells (Garcia-Ruiz *et* al., 2002; Chojkier *et al*., 1989). Low levels of MDA upregulates many genes implicated in abiotic stresses. However, genes involved in biotic stress, pathogenesis-related genes, do not respond to MDA treatment (Weber *et al*., 2004). The increase in MDA levels in resistant wheat after infestation (Fig.4.6), most probably serve as building blocks for the synthesis of more complex defence compounds. The increase in lipid

peroxidation and the generation of MDA fragments is an important step in the defence mechanism.

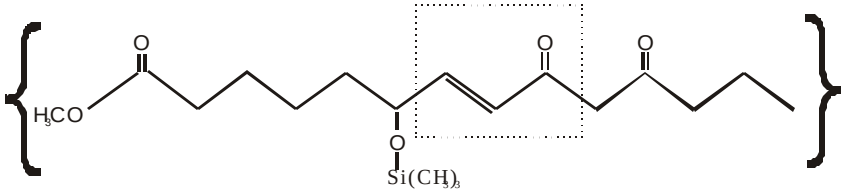
The HR reaction is a common response of plants to incompatible interactions (Mehdy, 1994). It initiates a variety of metabolic changes in the host cell. Amongst these metabolic changes is the generation of reactive oxygen species (ROS). In many incompatible interactions the generation of ROS is crucial for defence (Mehdy, 1994). In the interaction between Tugela DN and the RWA the generation of ROS is of great importance to initiate a successful defence response (Berner, 2000). The generation of superoxide anions can include lipid peroxidation, leading to the loss of membrane integrity and finally to tissue necrosis development (Adam *et al.*, 1989). The generation of ROS in infested Tugela DN is one of the earliest reactions detected to take place (Berner, 2000). Lipid peroxidation is initiated 24 hours after infestation (Fig. 4.6) and it could be that the generation of ROS is required for lipid peroxidation. Increases in lipid peroxidation of the infested resistant wheat (Figure 4.6) corresponded to the increase of LOX activity. Infested susceptible plants did not show any selective increase in lipid peroxidation. As with LOX activity, lipid peroxidation increased 24 hours after infestation and this increase was sustained over time. Tomato plants treated with a fungal elicitor induced both LOX and lipid peroxidation at about the same time (Peever & Higgins, 1989). Lipid peroxidation has also been detected in several other incompatible interactions (Buonaurio & Servili, 1999; Enoki *et al*., 1999) proving to be a very important step during the defence response. Lipoxygenase can participate in lipid peroxidation of the membranes and/or use the fragments generated during lipid peroxidation to produce lipid-like products such as oxylipins. Not only in biotic stress, but also in abiotic stresses lipid peroxidation has been linked to resistance (Gong *et al*., 1998; Kurganova *et al.*, 1999; Borsani *et al.*, 2001 & Juszczuk *et al.*, 2001).

The incompatible interaction between resistant wheat and the RWA is also characterized by the induction of several new lipid-like products and also increases in the production of other already existing lipid products. Many of these lipid-like products were identified as hydroxyl and keto fatty acids. A third group of fatty acids, containing an oxygen molecule between two carbons (C-O-C), were also found to be selectively induced. Though these compounds have been shown to exist, no record of these fatty acids could be found to be involved in defence responses of plants or animals. Various functions of hydroxyl and keto fatty acids have been reported in incompatible interactions. These fatty acids containing oxygen molecules have all

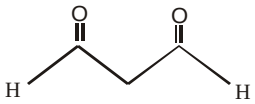
collectively been termed oxylipins. The structures of the keto fatty acids found to be selectively induced show similarities to that of MDA (Fig. 5.1).



Carbonyl group



Keto fatty acid



MDA

Figure 5.1 Structures of a carbonyl, MDA and a keto fatty acid (Vollenweider *et al.,* 2000; Weber *et al*., 2004*)*

Hydroperoxy, hydroxyl and keto fatty acids are produced in considerable amounts during pathogenesis and especially during the hypersensitive response via LOX (Weber, 2002). In this study it was reported that the defence response to sucking insects also led to the accumulation of several keto and hydroxyl fatty acids (Fig. 4.16 & 4.18). In tobacco plants it is has been shown that LOX is required for the formation of fatty acid hydroperoxides leading to the development of hypersensitive cell death (Rusterucci *et al*., 1999). The formation of keto fatty acids can also be a LOX catalyzed reaction or the hydroperoxides can be further converted to hydroxyl fatty acids, which can then be dehydrogegenated to keto fatty acids. Hydroperoxide,

hydroxyl and keto fatty acids are all involved in activating different sets of genes during resistance, with the keto fatty acids being the most reactive (Weber, 2002).

Plants can synthesize a large variety of oxylipins (enzymatically oxygenated fatty acids) and many of them display biological activity (Gerwick *et al*., 1991). Studying the 'oxylipin signature' of plants during stress provides a tool by which to search for oxylipins with regulatory properties (Weber, 2002). The enzymatic and non-enzymatic oxygenation of fatty acids result in the generation of a wide variety of compounds, including fatty acid hydroperoxides (Rusterucci *et al*, 1999), fatty acid ketodienes (Kuhn *et al*., 1990), 4-hydroxynonenal (Esterbauer *et al*., 1991) as well as smaller aldehydes such as MDA and acrolein (Esterbauer *et al*., 1991; Uchida *et al.*, 1998) and many other oxygenated fatty acids. Wounded and infected *Arabidopsis* plants accumulated 9-keto-octadecadienoic acid (9-KODE) and 13-keto-octadecadienoic acid (13-KODE) (Vollenweider *et al*., 2000). Products from keto-octadecanoic fatty acid induce glutathione-*S*-transferase and causes cell death (Vollenweider *et al*., 2000). The formation of fatty acid hydroperoxides via the LOX-pathway is needed for the development of HR (Rusterucci *et al*, 1999). Hydroperoxy, hydroxy and keto acids accumulate during pathogenesis and especially during the HR (Rusterucci *et al*, 1999; Vollenweider *et al*., 2000). Keto fatty acids were shown to powerfully activate *GST1* (involved in photomorphogenesis) gene expression (Vollenweider *et al*., 2000).

Stress-related lipid oxygenation produces a diversity of compounds covering a wide range of molecular masses. Features common to these compounds are the presence of carbonyl groups (Alméras *et al*., 2003). The presence of an unadulterated double bond increases the electrophyllic properties of the carbonyl groups, enhancing their chemical reactivity. The molecules are prone to several chemical reactions including Michael addition whereby nucleophillic atoms (S, N, etc.) react with the ß-carbon in the double bond. Michael addition is the general term for a reaction in which a carbanion (usually as an enolate) adds to the carbon-carbon double bond of an alpha, beta-unsaturated carbonyl compound, or to a double bond activated by conjugation with a strongly electronegative group such as cyanide. The accumulation of these sorts of molecules, including MDA, hexenals, nonenals and keto fatty acids, in diseased and wounded plant tissue is well known (Vollenweider *et al*., 2000; Alméras *et al*., 2003).

When plants are attacked by insects, volatile chemical signals can be released, not only from the damaged parts, but also systemically from other parts of the plant and this continues after cessation of feeding by the insect (Picket *et al*., 2001). Plant volatiles are lipophilic molecules with a high vapour pressure. The biosynthesis of plant volatiles are believed to all originate from a single substrate and with the action of various biosynthetic enzymes produce multiple products (Pichersky *et* al., 2006). Among the chemical signals involved in the induction of defence reactions are cyclic oxylipins derived from C_{18}- or C_{16}- unsaturated fatty acids, the octadecanoids and the hexadecanoids (Weiler *et al.*, 1999). Certain plants have the ability to release stress signals even when undamaged, and that these can cause defence responses in intact plants (Baldwin *et al*., 2006; von Dahl *et al.*, 2006). These discoveries provide the basis for new crop protection strategies that are either delivered by genetic modification of plants or by conventionally produced plants to which the signal is externally applied. An example of such a volatile signal is (E)-2-hexenal, which are derived from the lipoxygenase or octadecanoid pathway (Picket *et al*., 2001). Monoterpenes such as (*E*)-ocimene, and sesquiterpenes such as (-)-germacrene *D*, can be produced by plants and cause repellency to herbivores (Bruce *et al*., 2005). Jasmonic acid can act internally as a plant hormone associated with a damage/stress response but, when methylated can be released by the plant as a semiochemical affecting intact plants by upregulating defense related and other genes (Birkett *et al.*, 2000). Methylated jasmonic acid (Me-JA) is highly volatile and have the ability to up- or down regulate defence genes, especially genes involved in oxylipin metabolism (Schmidt & Baldwin, 2006). Volatile chemicals also serve as semiochemicals promoting or deterring interactions between plants and insect herbivores. For example, wheat seedlings without herbivore damage attract aphids, whereas odors released from wheat seedlings with a high density of aphids repel other aphids (Quiroz *et al*., 1997).

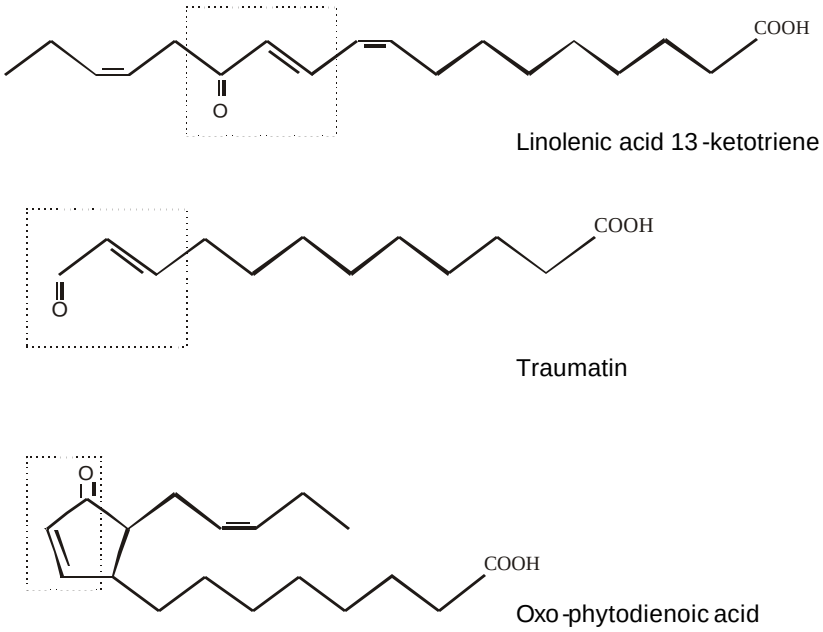


Figure 5.2 Examples of naturally occurring a,ß-unsaturated carbonyl compounds in plants. These oxylipins are strongly implicated in stress responses and pathogenesis. (Vollenweider *et al.*, 2000; Weber *et al.*, 2004)

A large number of fatty acid derivatives often present in minute concentrations were discovered to have profound effects on cellular physiological or pathophysiological reactions. Because of their diversity, these compounds have been classified according to the type of their enzymatic formation. Thus, the term "prostanoids" relates strictly to the products of the cyclooxygenase pathway and includes prostaglandins, prostacyclins, and thromboxanes. The lipoxygenase products include leukotrienes, lipoxins and various peroxy- or hydroxy-fatty acid derivatives. Furthermore, the term "eicosanoids" is used as a collective name for molecules derived from 20-carbon fatty acids. Fatty acids of the linoleic acid family (deriving from 18:2n-6) are the main source of eicosanoids, arachidonic acid (20:4n-6) being the major precursor. Some other 20-carbon acids (20:3n-6 and 20:5n-3) also form important metabolites which have different pharmacological properties. Eicosanoids are synthesized *in vivo* through several routes; some compounds being formed by more than one mechanism. In the plant kingdom, several potent derivatives originating from linolenic acid (octadecanoid-derived compounds) have hormone-like functions (phytohormones) (Schulze *et al*., 2006).

To investigate the possible involvement of prostanoid molecules during the defence response of wheat to the RWA, inhibition studies were performed using indomethacin, an inhibitor of prostanoids (Fig. 4.7 & 4.8). Lipoxygenase and peroxidase (POD) (Van der Westhuizen *et al.*, 1998b) activities were used as parameters of the resistance response, since both enzymes are involved in the defence response of wheat to RWA. Prostanoid inhibition resulted in the inhibition of both LOX and POD activities (Fig. 4.7 & 4.8). This suggests that prostanoid-like compounds are involved in regulating processes in plant defence mechanisms. Prostanoids have been detected in many different plants and certain microorganisms and have various physiological effects in plants. They have been implicated in the control of flowering (Groenewald & Van der Westhuizen, 1997), it has an effect on gibberellic acid controlled responses (Curry & Galsky, 1975), inhibition effect of crown gall tumour on potatoes (Favus *et al.,* 1977) and certain electron-flow reactions in isolated chloroplasts (Barr & Crane, 1977). In maize it was found that prostaglandins have an effect on photosynthesis, nucleic acid metabolism and protein synthesis (Forster *et al*., 1984). There are also reports that prostanoids have a regulatory role in cell membranes (Christov & Vaklinova, 1987). Inhibiting prostanoid synthesis in tobacco plants with indomethacin resulted in the loss of the cyclic monophosphate peak in the S phase of cells and the inhibition of mitotic division (Ehsan *et al.*, 1998).

Secondary plant metabolites, those believed not to be primary for sustaining life of an organism, have become an important growing area of research. The areas of interest have focussed on interactions involving chemicals metabolized by plants. Many of these secondary plant metabolites are oxygenated fatty acids originating from different pathways, but mostly from the lipoxygenase pathway. The discovery of a cyclooxygenase pathway has implicated the involvement of prostanoids in plant defence responses.

Lipid fragments that are released as a result of lipid peroxidation are further metabolized by LOX to leukotrines and eicosatetraenoids and by COX to prostaglandins and thromboxanes. The formation of these lipid products from the LOX and COX pathways are most probably involved in the defence response of the resistant, (Tugela DN), wheat to the RWA. Russian wheat aphid infestation resulted in the biosynthesis of especially the keto fatty acids (Fig. 4.16) via the LOX pathway and prostanoids via the COX pathway (Fig. 4.5). Lipoxygenase products have been

shown to play a role in the plant's response to biotic and abiotic stresses both as antimicrobial compounds and as signal molecules that lead to the activation of specific defence genes (Fidantsef *et* al., 1999, Royo *et al*., 1999 & Weber, 2002). Though the involvement of prostanoids in plant defence mechanisms is a new field of study, their discovery will open a new dimension in plant defence mechanisms.

The induction of the LOX pathway is an active process of membrane degradation leading to hypersensitive cell death (Göbel *et al.* 2003). The increase in lipid peroxidation which coincides with the increase in LOX activity was a source for the increase in the production of fatty acid hydroperoxides. Differential expression of LOX activity in compatible and incompatible interactions reinforces the hypothesis of a relationship between LOX and resistance. Analysis of defence responses and resistance patterns in these plants should help us to gain insight into the role of LOX during plant-aphid interactions. The massive production of lipid-like products in this study can be considered as a defence mechanism against aphids. The identification of specific lipid-like molecules and the pathway in which they originate will supply plant breaders with useful knowledge in the breeding of resistant cultivars.

Our knowledge of phyto-oxylipins and their relationships to the defence of plants against insect stress and pathogens is increasing rapidly. Products derived from the LOX-pathway and their role in resistance mechanisms are increasingly considered pivotal in these action mechanisms. Plant oxylipins are no longer non-desired cytotoxic metabolism curiosities. Evidence are accumulating suggesting that oxylipins are potent bioregulators, playing important roles in signalling cascades, plant growth and development, senescence, organogenesis and maintenance of homeostasis (Grechkin, 1998). At the same time, biochemical and especially physiological aspects of the plant LOX pathways remain poorly understood.

The production of AOS (active oxygen species) during the early steps of pathogenesis and during later stages has also been associated with lipid peroxidation in the past (Rustérucci *et al*., 1996). The strong reactivity of oxygen species leads to nonenzymatic membrane lipid peroxidation, which in turn results in electrolyte leakage, disruption of cell membranes and subsequent removal of unsaturated fatty acids from the bilayer (Keppler & Baker, 1989). The increase in extracellular oxygen derivatives gives rise to the release of lipid-like compounds from the membranes and to the synthesis of lipid-derived signal molecules through enzymatic pathways involving LOX and COX (Serhan *et al.*, 1996). The diversity of

signals and signalling pathways are of great advantage to the plants. Such a modular action of different signalling molecules allows the plant to respond to diverse environmental factors in a very specific manner.

The arachidonic acid cascade in mammals is the major source of numerous powerful bioregulators, including prostaglandins, thromboxanes, leukotrienes, lipoxins, hepoxilins and others. In plants, several known pathways of the octadecanoid cascade are the biogenetic origin of many oxylipins (Grechkin, 1998). Comparison of plant and mammalian LOX cascades reveal more differences than similarities. Enzymes of hydroperoxide metabolism abundant in plants, like allene oxide synthase, hydroperoxide lyase and divinyl ether synthase, are not detected in any mammalian tissue. At the same time, mammalian LOXs produce the same hydroperoxy fatty acids from linoleate and linolenate. Evidence is accumulating that these octadecanoids play important regulatory roles both in mammalian and plant defence mechanisms along with the eicosanoids (Grechkin, 1998).

Though the role of many oxylipins derived from C_{18} fatty acids are poorly understood in plants, their role in mammals is very well known. In particular, hydroperoxy and hydroxy derivatives of linoleate and linolenate are involved in the regulation of functions of blood cells and endothelial cells (Brash, 1999). One can therefore propose that that many important physiological functions of plant oxylipins remain to be revealed. The existence of oxylipins with unknown biogenetic origins allows one to speculate that some routes of oxylipin metabolism are bound to be discovered.

The breakdown of resistance to aphids poses serious problems not only for plant breeders, but also to the entire wheat industry. The advances made in this study in understanding the biochemical defence mechanism will open a whole new area for plant breeders and molecular geneticists to select and develop resistant cultivars expressing differential pathways in resistance. Wheat cultivars using different modes of resistance will reduce the risk of aphids to to overcome the resistance. The discovery of an oxygenase pathway in plants might even shed some light on the regulation of other biochemical processes involved in everyday growth and development.

The discovery of new fatty acids is not only of advantage to plant resistance breeding, but also to the food industry. The knowledge gained should further

encourage the development of crops containing new oils with medium-chain triglycerides. The elucidation of pathways for new types of fatty acids such as epoxy, acetylenic and branched chain or cyclic fatty acids could lead to the production of new oils of economic value. However, further progress depends on the elucidation of the regulatory mechanisms and identification of key steps in fatty acid metabolism. The knowledge gained will substantially contribute to our understanding of the synthesis of distinct fatty acids and of the regulation of the lipid synthesis pathway and will allow the movement from empirical technology to predictable oil design.

The importance of knowing and understanding the biochemical defense responses has now become even more important than before, especially with the development of new RWA biotypes, which overcame the existing resistance. Knowledge gained from these biochemical studies will assist in the search for resistance genes, which can lead to better resistance breeding. Resistance genes have been used in breeding programmes with different degrees of success. The greater understanding of the downstream defense response in this study have contributed to better insight in how resistance genes might operate which, in turn, will enhance the use of natural resistance for aphid control. The use of resistance genes has various attractive features to offer to control diseases and also insects. The concerted response of resistance genes can halt the growth and development of the aphids. This will reduce additional inputs from the farmer and there are no adverse environmental effects. It still remains a major problem that aphids will eventually overcome the effects of the resistance genes and it is therefore important to study resistance genes and to find new genes. Single resistance genes are more easily overcome than resistance mechanisms based on multiple resistance genes. These genes are also specific to one insect or pathogen and do not provide a broad spectrum of resistance. Like in the case of the resistant gene to the RWA, which is believed to be a single gene, does not provide broad-spectrum resistance and is also more easily overcome by new biotypes. To include genes which provide broad spectrum resistance will reduce the selective pressure placed on single resistance genes. Broad-spectrum resistance might also be achieved through manipulation of defense signaling components that act downstream during the defense response. Many of the fatty acids that were identified could act as important signal molecules during broad-spectrum resistance. An alternative to the transgenic approach to resistance is to apply natural occurring compounds directly on crops. For example, a synthetic analogue of SA is used in foliar applications to increase systemic acquired resistance in crops (Görlach, 1996).

The advances that were made in this study, especially regarding the involvement of lipid-like products, will initiate a new perspective to plant defense research. Many of these compounds still need to be identified, but the identification and elucidation of their specific roles will introduce a new era in aphid resistance research. The discovery of the involvement of an oxygenase protein and prostanoid compounds not only provide new insight into resistance research but also to general research in plant growth and development. These unidentified compounds might serve as signalling molecules, can act as master switches of resistance and therefore be useful tools for transformation purposes. On the other hand they could be developed into plant activators, which induce resistance after application to plants. Some might even serve as chemical cues or as elicitors thereof to attract natural enemies or act as repelling compounds or they can interfere with the dietary requirements of the aphids leading to reduced colonies. The final outcome might be less pesticide usage and hence healthier environment. For the farmer it might contribute to cheaper and simpler farming practices.

5.1 ABSTRACT

Physical damage and disease are known to cause changes in the lipid composition of plants. The biochemical pathways that lead to changes in the lipid composition were investigated in a comparative study using Russian wheat aphid (RWA) (*Diuraphis* noxia) infested and uninfested resistant ('Tugela DN', 'Gariep' and 'Betta' DN) and near isogenic susceptible ('Tugela', 'Molopo' and 'Betta') wheat cultivars. Lipoxygenase (LOX) activity was determined spectrophotometrically while LOX proteins were analysed by means of Western blots. Lipid peroxidation was determined by measuring malondialdehyde levels spectrophotometrically. To investigate the involvement of prostanoids in the RWA resistance response, prostanoid biosynthesis was inhibited with indomethacin where after the activities of defence related enzymes peroxidase (POD) and LOX were determined spectrophotometrically. Lipid-like products were analysed by means of gas liquid chromatography (GC/MS) and mass spectrometry. Western blots were used to analyse the cyclooxygenase (COX) proteins. LOX, which catalyzes the first step of the lipoxygenase pathway, was selectively induced in the infested resistant wheat. This increase in LOX activity coincided with lipid peroxidation. Several lipid-like compounds were found to be newly induced after infestation and the synthesis of others were enhanced after infestation in the resistant wheat. Many of these lipid products were identified as hydroxyl and keto fatty acids. Some of these fatty acids could be detected as early as 1 h.p.i. (hours post infestation). The levels of some fatty acids increased as infestation proceeded while the levels of others reached a peak 48 h.p.i. and declined towards 96 h.p.i. Hydroxyl and keto fatty acids are important signalling compounds during defence responses. A second pathway, COX pathway, which was believed to exist only in mammals, was also found during the wheat-RWA interaction. The COX protein was selectively induced in resistant wheat after infestation. It has high homology to mammalian COX, which is responsible for prostanoid synthesis. The involvement of prostanoids in the RWA resistance response was confirmed by inhibition studies of prostanoid biosynthesis. Downstream defence reactions e.g. LOX and peroxidase (POD) activities were inhibited upon inhibition of the prostanoid biosynthetic pathway. These results emphasize the importance of two lipid biosynthesis pathways (LOX and COX) that may be essential for the establishment of a successful defence response in wheat to the RWA.

5.2 KEYWORDS

Triticum aestivum, wheat, *Diuraphis noxia*, Russian wheat aphid, lipoxygenase, cyclooxygenase, oxylipins, prostanoids, fatty acids

5.3 OPSOMMING

Fisiese beskadiging en siektes is bekend daarvoor dat dit veranderinge in die lipiedsamestelling van plante teweegbring. Die biochemiese weë wat verantwoordelik is vir hierdie veranderinge is in ‘n vergelykende studie met Russiese koringluis (RKL, (*Diuraphis noxia*) geïnfesteerde en ongeïnfesteerde weerstandbiedende (Tugela DN, Gariep & Betta DN) en naby-isogeniese vatbare (Tugela, Molopo & Betta) koringkultivars bestudeer. Lipoksigenase (LOX)-aktiwiteit is spektofotometries bepaal, terwyl LOX-proteïene deur middel van Westernkladanalise ondersoek is. Lipiedperoksidering is ondersoek deur maloondialdehiedvlakke spektofotometries te bepaal. Om die betrokkenheid van prostanoïede in die RKL-weerstandsrespons te bepaal, is prostanoïedbiosintese met indomatasien gerem waarna die aktiwiteite van vededigingsverwante ensieme, peroksidase (POD) en LOX spektrofotometries bepaal is. Lipiedagtige verbindings is met behulp van gasvloeistof-chromatografie en massaspektrometrie (GC/MS) geanaliseer. Die Westernkladmetode is gebruik om siklo-oksigenase (COX)-proteïene te ondersoek. LOX, wat die eerste stap van die LOX-roete kataliseer, was selektief in geïnfesteerde weerstandbiedende koring geïnduseer. Hierdie toename in LOX-aktiwiteit het met ? toename in lipiedperoksidering gepaard gegaan. Verskeie lipiedagtige verbindings is nuut in die weerstandbiedende koring geïnduseer en baie ander se vlakke is aansienlik verhoog. Baie van die lipiedagtige verbindings is as hidroksiel- en ketovetsure geïdentifiseer. Sommige kon so vroeg as 1 uur na infestering opgespoor word. Die vlakke van sommige vetsure het met verloop van infestering toegeneem terwyl ander piekvlakke 48-uur na infestering bereik het en daarna weer by 96-uur na infestering afgeneem het. Hidroksiel en ketovetsure is belangrike seinverbindings tydens verdedegingsreaksies. ? Tweede roete, die COX-roete, is ook met betrekking tot die koring-RKL-interaksie geïdentifiseer. Hierdie roete is tot dusver nog net in mammalia beskryf. ‘n COK-proteïen is selektief na RKL-infestering in die

weerstandbiedende koring geïnduseer. Dit het hoë homologie met die COX-proteïen wat in mammalia verantwoordelik vir prostanoïedsintese is. Prostanoïedinhibering het gelei tot 'n verlaagde stroomaf-verdedegingsreaksies, gemeet in terme van LOX- en POD-aktiwiteite. Hierdie resultate beklemtoon die belangrikheid van twee lipiedbiosinteseroetes (LOX en COX) wat noodsaaklik is vir suksesvolle verdedeging reaksie in koringplante teen RKL.

5.4 SLEUTELWOORDE

Triticum aestivum, koring , *Diuraphis noxia*, Russiese koringluis, lipoksigenase, siklo-oksigenase, oksilipiene, prostanoïede , vetsure,

REFERENCES

Aalsberg YK, Van Rensburg NJ, & Hewitt PH (1988) Natural enemies and their impact on *Diuraphis noxia* (Mordvilko) (Hemiptera: Aphididae) populations. Bull Entomol Res 78: 111-120

Adam A, Farkas T, Somlyai G, Hevesi M & Kiraly Z (1989) Consequence of O_2 generation during a bacterially induced hypersensitive response in tobacco: deterioration of membrane lipids. Physiol Mol Plant Pathol 34: 13–26

Agrios GN (1988) Plant Pathology, 3rd ed. Academic Press, Inc., San Diego, Calif 1988. In KG Mukerji, and KL Garg (ed.), Biocontrol of plant diseases.

Alméras E, Stolz S, Vollenweider S, Reymond P, Mène-Saffrané L & Farmer EE (2003) Reactive electrophile species activate defence gene expression in *Arabidopsis*. Plant J 34: 205–216.

Alvarez ME, Pennell RI, Meijer P-J, Ishikawa A, Dixon RA and Lamb C (1998) Reactive oxygen intermediates mediate a systemic network in the establishment of plant immunity. Cell 92: 773-784

Attiga FA, Fernandez PM, Weeraratna AT, Manyak MJ & Patierno SR. 2000. Inhibitors of prostaglandin synthesis inhibit human prostate tumor cell invasiveness and reduce the release of matrix metalloproteinases. Cancer Res 60: 4629-4637

Baker B, Zambryski P & Dinesh-Kumar SP (1997) Signaling in plant-microbe interactions. Science 276: 726-733

Baldwin IT, Halitschke R, Paschold A, von Dahl & Preston CA (2006) Volatile signaling in plant-plant interactions:"Talking trees" in genomics era. Science 311: 812-815

Barr R & Crane FL (1977) The effect of prostaglandins on photosynthesis. Proc Indiana Acad Sci 86: 117-122

Benhamou N (1996) Elicitor induced plant defence pathways. Trends in Plant Sci 1: 233-240

Berner JM (2000) M.Sc. thesis: Involvement of active oxygen species and phenylalanine ammonia-lyase in the resistance response of wheat to the Russian wheat aphid. University of the Free State: 51-55

Birkett MA, Campbell CAM, Chamberlain K, Guerrieri E, Hick AJ, Martin JL, Matthes M, Napier JA, Pettersson J, Pickett JA, Poppy GM, Pow EM, Pye BJ, Smart LE, Wadhams GH, Wadhams LJ and Woodcock CM (2000) New roles for cis-jasmone as an insect semiochemical and in plant defense. Proc Nat Acad Sci 97: 9329-9334.

Blechert S, Brodschelm W, Hölder S, Kammerer L, Kutchan TM, Vlueller MJ, Xia Z-Q & Zenk MH (1995) The octadecanoic pathway: signal molecules for the regulation of secondary pathways. Proc Natl Acad Sci USA 92: 4099-4105

Bleé, E. (1998) Phyto oxylipins and plant defence reactions. Prog. Lipid Res. 37, 33-37

Bohland C, Balkenhohl T, Loers G, Feussner I & Grambow HJ (1997) Differential induction of Lipoxygenase isoforms in wheat upon treatment with Rust fungus elicitor, chitin oligosdaccharides, chitosan and methyl jasmonate. Plant Physiol 114: 679-685

Bohlmann H (1994) The role of thionins in plant protection. Crit Rev Plant Sci 13: 1-16

Boijsen K, Nielsen KK, Gottschalk T & Mikkelsen JD (1993) Genetic transformation *Nicotiana benthamiana* with chitinase and ß-1,3-glucanase genes from *Beta vulgaris* (sugar beet). In: Fritig B, Legrand M (eds) Mechanisms of plant defence responses. Kluwer Academic Publ, Dordtrecht, The Netherlands, 449-510

Bol JF, Linthorst HJM & Cornelissen BJC (1990) Plant pathogenesis-related proteins induced by virus infection. Annu Rev Phytopathol 28: 113-138

Boller T (1995) Chemoperception of microbial signals in plant cells. Ann Rev Plant Physiol Plant Mol Biol 46: 189-214

Bolwell GP, Butt VS, Davies DR & Zimmerlin A (1995) The origin of oxidative burst in plants. Free Rad Res Comm 23: 517-532

Borsani O, Valpuesta V & Botella MA (2001) Evidence for a role of salicylic acid in the oxidative damage generated by NaCl and osmotic stress in *Arabidopsis* seedlings. Plant Physiol 126: 1024–1030.

Bostock RM & Stermer BA (1989) Perspectives on wound healing in resistance to pathogens. Ann Rev of Phytopath 27: 343-371

Bostock RM (1999) Signal conflicts and synergies in induced resistance to multiple attackers. Physiol Mol Plant Pathol 55: 99-109

Botha A-M, Nagel MAC, Van der Westhuizen AJ & Botha FC (1998) Chitinase isoenzymes in near-isogenic wheat lines challenged with Russian wheat aphid, exogenous ethylene, and mechanical wounding. Bot Bull Acad Sin 39: 99-106

Bowles DJ (1990) Defence-related proteins in higher plants. Annu Rev Biochem 59: 873-907

Bradford MM (1976). A rapid and sensitive method for the quantification of micron quantities of protein utilizing the principle of protein dye binding. Anal Biochem 72: 248-254

Bradley DJ, Kjellbom P & Lamb CJ (1992) Elicitor- and wound- induced oxidative cross-linking of a proline-rich plant cell wall protein: A novel, rapid defence response. Cell 70: 21-30

Brash AR (1999) Lipoxygenases: occurrence, function, catalysis and acquisition of substrate. J. Biol. Chem. 274: 23679-23682

Bruce TJ, Wadhams LJ & Woodcock CM (2005) Insect host location: a volatile situation. Trends Plant Sci 10: 269-274

Brunner M, Kocsy G, Rüegsegger A, Schmutz D & Brunold C (1995) Effect of chilling on assimilatory sulfate reduction and glutathione synthesis in maize. J Plant Physiol 46: 743-747

Buonaurio R & Servili M (1999) Involvement of lipoxygenase, lipoxygenase pathway volatiles and lipid peroxidation during the hypersensitive reaction of pepper

leaves to *Xanthomonas campestris* pv. *vesicatoria*. Physiol and Mol Plant Pathol 54: 155-169

Burcham P C & Kuhan Y T (1996) Introduction of carbonyl groups into proteins by the lipid peroxidation product, malondialdehyde. Biochem Biophys Res Commun 220: 996-1001

Burt JD & Burton RL (1992) Characterization of plant damage caused by Russian wheat aphid (Homoptera: Aphididae). J Econ Entmol 85 (5): 2017-2022

Burton R.L. and Webster J.A. (1993). Agricultural Res. Service progress report, Russian wheat aphid, Fith Annual Report. Plant Sciece Res. Lab, Stillwater, OK PSWCL Prog Rep 93-009 Feb

Chen Z, Kloek A, Cuzick A, Moeder W, Tang D, Innes R, Klessig D, McDowell J & Kunkel B (2004) The Pseudomonas syringae type III effector AvrRpt2 functions downstream or independently of SA to promote virulence on Arabidopsis thaliana. The Plant J 37 494-504

Choi D, Bostock RM, Avdiushko S & Hildebrand DF (1994) Lipid derived signals that discriminate wound- and pathogen-responsive isoprenoid pathways in plants: methyl jasmo nate and the fungal elicitor arachidonic acid induce different 3-hydroxy-3-methylglutaryl-coenzyme A reductase genes and antimicrobial isoprenoids in *Solanum tuberosum* L. Proc Natl Acad Sci USA 91: 2329-2333

Chojkier M, Houglum K, Solisherruzo J & Brenner DA (1989) Stimulation of collagen gene-expression by ascorbic acid in cultured human-fibroblasts – a role for lipid-peroxidation. J Biol Chem 264: 16957–16962

Christov AM & Vaklinova SG (1987) Effects of prostaglandins E2 and E2a on the electrofusion of pea mesophyll protoplasts. Plant Physiol 83: 500-504

Coligne DB, Kragh KM, Mikkelson JD, Nielson KK, Rasmussen U & Vad K (1993) Plant chitinases. Plant J 3:31-40

Collins MB, Haley SD, Peairs FB & Rudolph JB (2005) Biotype 2 Russian wheat aphid resistance among wheat germplasm accessions. Crop Sci 45: 1877-1880

Cosgrove DJ, Gilroy S, Kao T-h, Ma H & Schultz JC (2000) Plant signalling 2000. Cross talk among geneticists, physiologists, and ecologists. Plant Physiol 124: 499-505

Croft, K.P.C., Juttner, F. and Slusarenko, A.J. (1993) Volatile products of the lipoxygenase pathway evolved from *Phaseolus vulgaris* (L.) leaves inoculated with *Pseudomonas syringae* pv *phaseolicola*. Plant Physiol. 101, 13-24

Curry SC & Galsky AG (1975) The action of prostaglandins on GA_3-controlled responses. I.Induction of barley endosperm acid phosphatase activity by prostaglandins E_1 and E_2. Plant. Cell Physiol 16: 799-804

De Leon IP, Sanz A, Hamberg M & Castresana C (2002). Involvement of the Arabidopsis alpha-DOX1 fatty acid dioxygenase in protection against oxidative stress and cell death. Plant J 29 (1):61-62

De Moraes CM, Lewis WJ, Paré PW, Alborn HT & Tumlinson JH (1998) Herbivore-infested plants selectively attract parasitoids. Nature 393: 570-572

Delaney TP, Uknes S, Vermoij B, Friedrich L, Weyman K, Negrotto D, Gaffney T, Gut-RellaM, Kessmann H, Ward E & Ryals J (1994) A central role in salicylic acid in plant disease resistance. Science 266: 1247-1250

Desikan R, Clarke A, Atherfold P, Hancock JT and Neil SJ (1990) Harpin induced mitogen activated protein kinases activity during defence responses in *Arabidopsis thaliana* suspension cultures. Planta 210: 97-103

Desikan R, Hancock JT, Coffey MJ & Neill SJ (1996) Generation of active oxygen in elicited cells of *Arabidopsis thaliana* is mediated by NADPH oxidase-like enzyme. FEBS Lett 382: 213-217

Dixon R & Lamb CJ (1990) Molecular communication in interactions between plants and microbial pathogens. Annu. Rev. Plant Physiol. Plant Mol. Biol. 41: 339-367

Dong X (1998) SA, JA, ethylene, and disease resistance in plants. Curr Opin Plant Biol 1: 316-323

Du Toit and Walters (1984). Damage assessment and economic threshold values for the chemical control of the Russian wheat aphid *Diuraphis noxia* (Mordvilko), on winter wheat. In: M.C. Walters (ed). Technical Commun, Department of Agriculture, RSA 191:58-62

Du Toit F (1988). Another source of Russian wheat aphid (*Diuraphis noxia*) resistance in *Triticum aestivum*. Cereal Res. Commun. 16 (1-2):105-106

Duffey SS & Stout MJ (1996) Antinutritive and toxic components of plant defence against insects. Arch Insect Biochem Physiol 32: 3-37

Durner J, Shah J, Klessig DF (1997) Salicylic acid and disease resistance in plants. Trends Plant Sci 2: 266-274

Ehsan H, Reichheld JP, Roef L, Witters E, Lardon F, Vanbockstaele D, Vanmontagu, M Inze D & Vanonckelen H (1998) Effect of indomethacin on cell cycle dependent cyclic AMP fluxes in tobacco BY-2 cells. FEBS Lett 422: 165 169

Enoki H, Izawa T, Kawahara M, Komatsu M, Koh S, Kyozuka J & Shimamoto K (1999) Ac as a tool for the functional genomics of rice. Plant J 19: 605–613

Esterbauer H, Schauer RJ & Zollner H (1991) Activation of Stress Signaling Pathways by the End Product of Lipid Peroxidation Free Radicals Biol Med 11: 81-128

Farmer E.E. (1994) Fatty acid signalling in plants and their associated microorganisms. Plant Mol Biol 26: 1423-1437

Farmer EE & Ryan CA (1990) Interplant communication: Airborne methyl jasmonate induces synthesis of proteinase in plant leaves. Proc Natl Acad Sci USA 87: 7713-7716

Farmer EE & Ryan CA (1992) Octadecanoid precursors of jasmonic acid activate the synthesis of wound-inducible proteinase inhibitors. Plant Cell 4: 129-134

Farmer EE (1994) Fatty acid signalling in plants and their associated microorganisms Plant Mol Biol 26: 1423-1437

Farmer EE, Johnson RR & Ryan CA (1992) Regulation of expression of proteinase inhibitor genes by methyl jasmonate and jasmonic acid. Plant Physiol 98: 995-1002

Farmer EE, Weber H & Vollenweider S (1998) Fatty acid signalling in *Arabidopsis*. Planta 206: 167-174

Favus S, Gonzalez O, Bowman P & Galsky A (1977) Inhibition of crown-gall tumor formation on potato discs by cyclic-AMP and prostaglandins E_1 and E_2. Plant Cell Physiol. 18: 469-472

Felton GW, Bi JL, Summers CB, Mueller AJ & Duffey SS (1994) Potential role of lipoxygenases in defence against insect herbivory. J. Chem. Ecol. 20 (3) 651-665.

Feussner I & Wasternack C (2002) The lipoxygenases pathway. Annu. Rev. Plant Biol. 53: 275-297

Fidantsef AL, Stout MJ, Thaler JS, Duffey SS & Bostock RM (1999) Signal interactions in pathogen and insect attack: expression of lipoxygenases, proteinase inhibitor II, and pathogenesis-related protein P4 in the tomato, *Lycopersicon esculentum.* Physiol Mol Plant Pathol. 54: 97-114

Flach J, Pilet P-E & Jolles P (1992) What's new in chitinase research? Exper 48: 701-716

Forster T,Szarvas T & Tanacs B (1984) Plant physiological effects of PGF_{2a} and prostaglandin synthesis in corn-leaf. Acta Biovhim Biophys Acad Sci Hung 19: 42

Fouché A, Verhoeven RL, Hewitt PH, Walters MC Kriel CF & De Jager J (1984). Russian aphid (*Diuraphis noxia*) feeding damage on wheat, related cereals and a *Bromus* grass species. In: Walters MC (ed) Progress in Russian wheat aphid (*Diuraphis noxia*) Morw. Research in the Republic of South Africa. Tech Commun Dep Agric S Afr No 191: 22-33

Fridovich I (1995) Superoxide radical and superoxide dismutase. Annu Rev Biochem 64: 97-112

Gang OR, Kasahara M, Xia ZQ, Mijnsbrugge KV, Bauw G, Boerjan W, Montagu MV, Davin LB & Lewis NG (1999). Evolution of plant defense mechanisms. Relationships of phenylcoumaran benzylic ether reductases to pinoresinol-lariciresinol and isoflavone reductases. J Biol Chem 274 (11):7516-27.

García Garrido JM & Ocampo JA (2002) Regulation of plant defence response in arbuscular mycorrhizal simbiosis. J Exp Bot 53: 1377-1386.

Garcia-Ruiz I, de la Torre P, Diaz T, Esteban E, Fernandez I, Munoz-Yague T and Solis-Herruzo JA (2002) *Sp1* and *Sp3* transcription factors mediate malondialdehyde-induced collagen alpha 1 (I) gene expression in cultured hepatic stellate cells. J Biol Chem 277, 30551–30558.

Gardner HW (1979) Lipid hydroperoxides reactivity with proteins and amino acids: A review J Agric Food Chem 27: 220-228

Gardner HW (1991) Recent investigations into the lipoxygenase pathway of plants Biochem Biophys Acta 1084: 221-239

Gelli A, Higgins V & Blumwald E (1997) Activation of plant plasma membrane Ca^{++}-permeable channels by race-specific fungal elicitors. Plant Physiol 113: 269-279

Gerwick WH, Moghaddam M & Hamberg M (1991) Oxylipin metabolism in the red alga *Gracilariopsis lemaneiformis*: mechanism of formation of vicinal dihydroxy fatty acids. Arch. Biochem. Biophys. 290: 436-444

Gilchrist LI, Rodrigues R, & Burnett PA (1984) The extent of Freestate streak and *Diuraphis noxia* in Mexico. In: Barley yellow dwarf, a proceedings of the workshop CIMMYT (Mexico) 157-163

Göbel C, Feussner I & Rosahl S (2003) Lipid peroxidation during the hypersensitive response in potato in the absence of 9-lipoxgygenases. J Biol Chem: 278 (52): 52834-52840.

Göbel C, Feussner I, Schmidt A, Scheel D, Sanchez-Serrano J, Hanberg M, Rosahl S (2001) Oxylipin profiling reveals the preferential stimulation of the 9-lipoxygenase pathway in elicitor-treated potato cells. J Biol Chem 276 (9): 6267-6273

Gong M, Van der Liut AH, Knight MR & Trewavas AJ (1998) Heat-shock-induced changes in intracellular Ca^{2+} level in tobacco seedlings in relation to thermotolerance. Plant Physiol 116: 429–437

Görlach J, Volrath S, Knauff-Beiter G, Hengy G, Beckhove U, Kogel KH, Oostendorp M, Staub T, Kessmann H & Ryals J (1996) Benzothiadiazole, a novel class of inducers of systemic acquired resistance, activates gene expression and disease re sistance in wheat. Plant Cell 8: 629 643.

Grechkin A (1998) Recent developments in biochemistry of the plant lipoxygenase pathway. Prog Lipid Res 37: 317–352.

Groenewald EG & Van der Westhuizen AJ (1997) Prostaglandins and related substances in plants. The Bot Review 63(3): 199-220

Grossmann S & Zakut R (1979) Determination of lipoxygenase (lipoxydase). Methods Biochem Anal 25: 303-329

Haile A (1981) Cereal aphids: Their distribution, Biology and management on highland barley. M.Sc Thesis. School of graduate studies, Addis Ababa University.

Halliwell B & Gutteridge JMC (1990) Role of radicals and catalytic metal ions in human disease. An overview. Methods Enzym 186: 1-85

Hamberg M & Gardner HW (1992) Oxylipin pathway to jasmonates: biochemistry and biological significance. Biochim et Biophys Acta 1165: 1-18

Hamberg M (1988) Biosynthesis of 12-oxo-10,12(z)-phytodienoic acid: identification of an allene oxide cyclase. Biochem Biophys Res Commun 156: 543-550

Hamberg M (1998) A pathway of biosynthesis of divinyl ether fatty acids in green leaves. Lipids 37: 427-433

Hamberg M (2000) New cyclopentenone fatty acids formed from linoleic acid and linolenic acids in potato. Lipids 35: 353-363

Hamberg M, Sanz A, Castresana C (1999). a -Oxidation of fatty acids in higher plants. Journal of Biological Chemistry 274, 24503–24513

Hamberg M, Sanz A, Rodriguez MJ, Calvo AP & Castresana C (2003). Activation of the fatty acid alpha-dioxygenase pathway during bacterial infection of tobacco leaves. Formation of oxylipins protecting against cell death. J Biol Chem 278(51): 51796-51805

Hamberg M. & Samuelson B (1967) On the specificity of the oxygenation of unsaturated fatty acids catalysed by soybean lipoxidase. J Biol Chem 242: 5329-5335

Hammond-Kosack KE & Jones JDG (1996) Resistance gene-dependent plant defence response. Plant Cell 8:1773-1791

Hanne H, Refsgaard F,Tsai L & Stadtman ER (1999) Modifications of proteins by polyunsaturated fatty acid peroxidation products. Proc Nat Acad Sci USA 97 (2): 611-616

Hatanaka A, Kajiwara T & Sekiya J (1987) Biosynthesis pathway for C6-aldehydes formation from linolenic acid in green leaves. Chem Phys Lipids 44, 341–361.

Hattinga JL, Humberb RA, Poprawskic TJ & Millerd RM (1999) A survey of fungal pathogens of aphids from South Africa, with special reference to cereal aphids. Biol Control 16 (1): 1-12

Hayes P (1998) Beating Russian wheat aphid: The farmer's allies. Farmer's Weekly, January 23: 22-26

Hayes P (1999) Elands: a new wheat cultivar. Farmer's Weekly, January 22, 20-21

Heath M (2000) Hypersensitive response-related cell death. Plant Mol Biol 44: 321–334

Hermsmeier D, Schittko U & Baldwin IT (2001) Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata*. Part I. Large-scale changes in the accumulation of growth- and defense-related plant mRNAs. Plant Physiol. 125: 683–700.

Hewitt PH, Van Niekerk GJJ, Walters MC, Kriel CF & Fouchè A (1984) Aspects of the ecology of the Russian wheat aphid, *Diuraphis noxia*, in the Bloemfontein District. I. The colonization and infestation of sown wheat, identification of summer hosts and cause of infestation symptoms. Pages 3-13 in; Progress in Russian wheat aphid (*Diuraphis noxia* Mordw.) research in the republic of South Africa. Proceedings of a meeting of the Russian wheat aphid task team held at the University of the Orange Free State, Bloemfontein, 5-6 May 1982 MC Walters, ed. Technical Communication No 191, Dept of Agriculture, South Africa

Hildebrand DF, Rodriquez JG, Brown GC, Luu KT & Volden CS (1986) Peroxidative responses of leaves in two soybean genotypes injured by two spotted spider mites (Acari: Tetranychidae) J Econ Entomol 79: 1459-1465

Holub EB, Beynon JL & Crute IR (1994) Phenotypic and genotypic characterization of interactions between isolates of *Peronospora parasitica* and accessions of *Arabidopsis thaliana*. Mol Plant Microbe Interact 7: 223-239

Hornung E, Walther M, Kuhn H & Feussner I (1999) Conversion of cucumber linoleate 13-lipoxygenase to a 9-lipoxygenating species by site-directed mutagenesis. Proc Natl Acad Sci USA 96: 4192-4197

Howe GA & Schimiller AL (2002) Oxylipin metabolism in response to stress. Curr. Opin. Plant Biol. 5: 230-236

Jones JW, Byers JR, Butts RA & Harris JL (1989) A new pest in Canada: Russian wheat aphid, *Diuraphis noxia* (Mordvilko) (Homoptera: Aphididae). Can Entomol 121: 623-624

Juszczuk I, Malusa E & Rychter AM (2001) Oxidative stress during phosphate deficiency in roots of bean plants (*Phaseolus vulgaris* L.). J Plant Physiol 158: 1299–1305.

Jyoti JL, **Qureshi JA, Michaud JP & Martin TJ (2006)** Virulence of two Russian wheat aphid biotypes to eight wheat cultivars at two temperatures. Crop Sci 46: 774-780

Karban R & Baldwin IT (1997) Induced responses to herbivory. University of Chicago Press, Chicago, Illinois, USA

Kato H, Kogure K, Araki T, Liu XH, Kato K, Itoyama Y (1995) Immunohistochemical localization of superoxide dismutase in the hippocampus following ischemia in a gerbil model of ischemic tolerance. J Cereb Blood Flow Metab 15:60-70

Kenner & Stenhagen E (1964) Location of double bonds by mass spectrometry. Acta Chem Scand 18: 1551-1552

Keppler LD & Baker CJ (1989) O_2 initiated lipid peroxidation in a bacteria-induced hypersensitive reaction in tobacco cell suspensions. Physiol Biochem 79: 555 562.

Kim Y-C, Yi S-Y, Mang HG, Seo YS, Kim WT & Choi D (2002) Pathogen-induced expression of cyclo-oxygenase homologue in hot pepper (*Capsicum annuum* cv. *Pukang*). J Exp Bot 53: 383–385

Koch T, Krumm T, Jung V, Engelberth J & Boland W (1999) Differential induction of plant volatile biosynthesis in the lima bean by early and late intermediates of the octadecanoid signaling pathway. Plant Physiol 121: 153-162

Kock JLF, Jansen van Vuuren D, Botha A, Coetzee, DJ, van Dyk MS, Coetzee, DJ, Botes PJ, Shaw N, Friend J, Ratledge C, Roberts AD & Nigam S (1997) The production of biologically active 3-hydroxy-5,8,11,14-eicosatetraenoic acid (3-HETE) and linoleic acid metabolites by Dipodascopsis. System Appl Microbiol 20: 39-49

Kolomiets MV, Chen H, Gladon RJ, Braun EJ & Hannapel DJ (2000) A leaf lipoxygenase of potato induced specifically by pathogen infection. Plant Physiol 124: 1121-1130.

Kombrink E & Somssich IE (1997) Pathogenesis-related proteins and plant defence, in *The Mycota* (Part A, Plant Relationships) (Carrol G & Tudzynski P eds.) 107-128, Springer

Kovalev OV, Poprawski TJ, Stekolshchikov AV, Vereshchagina AB & Gandrabur SA (1991) *Diuraphis Aizenberg* (Hom. Aphididae); Key apterous viviparous females, and review of Russian literature on the natural history of *Diuraphis noxia* (Kurdjumov, 1913). J Appl Entom 112: 425-436

Kuhn H, Sprecher H & Brash AR (1990) On singular or dual positional specificity of lipoxygenases. The number of chiral products varies with alignment of methylene groups at the active site of the enzyme. J. Biol. Chem. 265: 16300-16305

Kurganova LN, Veselov AP, Sinitsina YV & Elikova EA (1999) Lipid peroxidation products as possible mediators of heat stress response in plants. Russ J Plant Physiol 46: 181–185

Laemmli U.K. (1970) Cleavage of structure proteins during the assembly of the head bacteriophage T4. Nature 227: 680-685

Lamb C and Dixon R.A. (1997) The oxidative burst in plant disease resistance. Ann. Rev. Plant Physiol. Plant Mol. Biol. 48:251-275

Léon J, Lawton MA & Raskin I (1995) Hydrogen peroxide stimulates salicylic acid biosynthesis in tobacco. Plant Physiol 108: 1673-1678

Leone A, Melillo MT & Bleve-Zacheo T (2001) Lipoxygenase in pea roots subjected to biotic stress. Plant Sci 161 (4): 703-717

Ligterink W, Kroj T, zur Nieden U, Hert U & Scheek D (1997) Receptor-mediated activation of a MAP kinase in pathogen defence of plants. Science 276: 2054-2057

Linthorst HJM (1991) Pathogenesis related proteins in plants. Crit Rev Plant Sci 10: 123-150

Liochev S & Fridovich I (1994) Role of superoxide anion radicals in the production of hydroxy radicals: *in vitro* and *in vivo*. Free Rad Biol Med 16: 19-33

Lucas PW, Bita T, Dorvell BW, Dominy NJ & Yamashita (2000) Mechanical defences to herbivores. Ann Bot 86: 913-920

Malamy J, Carr JP, Klessig DF & Raskin I (1990) Salicylic acid: a likely endogenous signal in the resistance response of tobacco to viral infections. Sci 250: 1001-1004

Marasas C, Anandajayasekeram P, Tolmay V, Martella D, Purchase J and Prinsloo G (1997) Socio –economic impact of the Russian wheat aphid control Res. program. Published by: South African Center for Cooperation in Agricultural and Natural Resources Res. & Training. 1 -103

Marasas CN (1999) Socio-economic impact of the Russian wheat aphid integrated control program. PhD thesis, University of Pretoria

Matsui K, Shibutani M, Hase T & Kajiwara T (1996) Bell pepper fruit fatty acid hydroperoxide lyase is a cytochrome P540 (CYP74B). FEBS lett 394: 21-24

Mattiaci L, Dicke M & Posthumus MA (1995) ß-Glucosidase: An elicitor of herbivore-induced plant odor that attracts host-searching parasitic wasps. Proc Natl Acad Sci USA 92: 2036-2040

Mauch F & Staehelin LA (1989) Functional implications of the subcellular localization of ethylene-induced chitinase and ß-1,3-glucanase in bean leaves. Plant Cell 1: 447-457

Mauch F, Hadwiger LA & Boller T (1988) Antifungal hydrolases in pea tissue. I. Purification and characterization of two chitinases and ß-1,3-glucanases differentially regulated during development and in response to fungal infection. Plant Physiol. 87: 325-333

McDowell JM & Dangl JL (2000) Signal transduction in the plant innate immune response. Trends Biochem Sci 25: 79-82

Mehdy MC (1994) Active oxygen species in plant defence against pathogens. Plant Physiol. 105:467-472

Melchers LS, Apotheker-de Groot M, van der Knaap, Ponstein AS, Sela-Buurlage M, Bol JF, Cornelissen BJC, van den Elzen PJM, & Linthorst HJM (1994) A new class of tobacco chitinase homologous to bacterial exo-chitinase displays antifungal activity. Plant J 5: 469-480

Mercke P, Kappers IF & Verstappen FWA (2004) Combined transcript and metabolite analysis reveals genes involved in spider mite induced volatile formation in cucumber plants. Plant Physiol 135: 2012-2024

Messina FJ & Sorenson SM (2001) Effectiveness of lacewing larvae in reducing Russian wheat aphid populations on susceptible and resistant wheat. Biol Control 21 (1): 19-26

Métraux JP, Singer H, Ryals, Ward E, Wyss-Benz M, Gaudin J, Raschdorf K, Schmid E, Blum W & Inverardi B, (1990) Increase in salicylic acid at the onset of systemic acquired resistance in cucumber. Sci 250: 1004-1006

Michaud JP & Sloderbeck PE (2005) Russian wheat aphid: An introduced pest of small grains in the High Plains. Kansas State University, May (http://www.oznet.ksu.edu, Downloaded January 2006)

Miller H Porter DR, Burd JD, Mornhinweg DW & Burton RL (1994) Physiological effects of Russian Wheat Aphid (*Homoptera: Aphididae*) on resistant and susceptible barley. J Econ Entomol 887 (2): 493-499

Mohase L & Van der Westhuizen AJ (2002) Salicylic acid is involved in resistance responses in the Russian wheat aphid-wheat interaction. J Plant Physiol 159: 585-590

Montalbini P (1992) Changes in xanthine oxidase activity in bean leaves induced by *Uromyces phaseoli* infection. J. Phytopathol. 134: 63-74

Montillet J-L, Chamnongpol S, Rustérucci C, Dat J, Van de Cotte B, Agnel J-P, Battesti C, Inzé D, Van Breusegem F & Triantaphylidès C (2005) Fatty Acid hydroperoxides and H_2O_2 in the execution of hypersensitive cell death in tobacco leaves. Plant Physiol 138: 1516-1526

Mornhinweg DW, Brewer MJ & Porter DR (2005) Effect of Russian wheat aphid on yield and yield components of field grown susceptible and resistant spring barley. Crop Sci 46: 36-42

Ni X & Quisenberry SS (2005) *Diuraphis noxia* and *Rhopalosphum* and *padi* (Hemiptera: Aphididae) Interactions and their injury on resistant and susceptible cereal seedlings. J Econ Entomol 99: 551-558

Nicolaou KC, Ramphal JY, Petasis NA & Serhan MH (1991) Lipoxins and related eicosanoids: Biosynthesis, biological properties, and chemical synthesis. Angew Chem Int Ed Engl 30: 1100-1116

Nürnberger T (1999) Signal perception in plant pathogen defence. Cell Mol Life Sci 55: 167-182

O'Banion MK, Winn VD & Young DA (1992) cDNA cloning and functional activity of a glucocorticoid-regulated inflammatory cyclooxygenase. Proc Natl Acad Sci USA 89: 4888–4892.

Ocampo C.A., Moerschbacher B.M. & Grambow H.J. (1986) Increased lipoxygenase activity is involved in the hypersensitive response of wheat leaf cells infected with the avirulent rust fungi or treated with fungal elicitor. Z. Naturforsch. 41c: 559-563

Paré PW & Tumlinson JH (1997) *De novo* biosynthesis of volatiles induced by insect herbivory in cotton plants. Plant Physiol 114: 1161-1167

Paré PW & Tumlinson JH (1998) Cotton volatiles synthesized and released distal to the site of insect damage. Phytochem 47 (4):521-526

Parthier, B (1991) Jasmonates, new regulators of plant growth and development: many facts and few hypothesis on their actions. Bot. Acta 104: 446-454.

Peever TL & Higgins VJ (1989) Electrolyte leakage, lipoxygenase, and lipid peroxidation induced in tomato leaf tissue by specific and nonspecific elicitors from Cladosporium fulvum. Plant Physiol 90: 867-875

Peng M & Kuc J (1992) Peroxidase generated hydrogen peroxide as a source of antifungal activity in vitro and on tobacco leaf disks. Phytopathol 82: 696-699

Pichersky E, Noel JP & Dudareva N (2006) Biosynthesis of plant volatiles: nature's diversity and ingenuity. Sci 311: 808-811

Pickett JA, Rasmussen HB, Woodcock CM, Matthes M & Napier JA (2001) Plant stress signalling: understanding and exploiting plant–plant interactions. Biochem Soc Trans 31: 123-127

Pieterse CMJ & van Loon LC (1999) Salicylic acid-independent plant. defence pathways. Trends Plant Sci 4: 52-58

Prinsloo GJ (1990) Some spects of the Biology of the Aphidophagous parasitoids *Aphelinus asychis* Walker M. Sc. thesis, Univ of the Free State, Bloemfontein, South Africa

Puterka GJ, Burd JD & Burton RL (1992) Biotypic variation in a worldwide collection of Russian wheat aphid (Homoptera: Aphididae). J Econ Entomol 85: 1497-1506

Quiroz A, Pettersson J, Pickett JA, Wadhams LJ and Niemeyer HM (1997) Semiochemicals mediating spacing behavior of bird cherry-oat aphid, *Rhopalosiphum padi* feeding on cereals. J Chem Ecol 23: 2599-2607

Qureshi JA, Michaud JP & Martin TJ (2006) Resistance to biotype 2 Russian wheat aphid (Homoptera: Aphididae) in two wheat lines. J Econ Entomol 99 (2): 554-550

Rancé I, Fourier J & Esquerré-Tugayé M -T (1998) The incompatible interaction between *Phytophthora parasitica* var. *nicotianae* race 0 and tobacco is suppressed in transgenic plants expressing antisense lipoxygenase sequences. Proc Natl Acad Sci USA 95: 6554-6559

Rao MV, Paliyath G, Ormond DG, Murr DP & Watkins CB (1997) Influence of salicylic acid on H_2O_2 production, oxidative stress, and H_2O_2-mediating enzymes. Plant Physiol 115: 137-149

Rasmussen JB, Hammerschmidt R & Zook MN (1991) Systemic induction of salicylic acid accumulation in cucumber after inoculation with *Pseudomonas syringae* pv *syringae*. Plant Physiol 97: 1342-1347

Rayapuram C & Baldwin IT (2006) Using nutritional indices to study LOX3-dependent insect resistance. Plant, Cell and Environ 29: 1585-1594

Reymond P & Farmer EE (1998) Jasmonate and salicylate as global signals foe defence gene expression. Curr Opin in Plant Biol 1: 404-411

Rosahl S (1996) Lipoxygenases in plants—Their role in development and stress response. Z Naturforsch 51:123-138

Royo J, León, ancanneyt G, Albar J-P, Rosahl S, Ortego F, Castañera & Sánchez-Serrano (1999) Antisense-mediated depletion of a potato lipoxygenase reduces wound induction of proteinase inhibitors and increases weight gain of insect pests. Plant Biol 96 (3): 1146-1151

Rustérucci C, Blein J-P, Stallaert V, Ducruet J-M, Pugin A & Ricci P (1996) Relationship between active oxygen species, lipid peroxidation, necrosis, and phytoalexin production by elicitins in Nicotiana. Plant Physiol 111, 885–891.

Rustérucci C, Montillet J-L, Agnel J-P, Battesti C, Alonso B, Knoll A, Bessoules J-J, Etienne P, Suty L, Blein J-P & Triantaphylides C (1999) Involvement of lipoxygenase-dependent production of fatty acid hydroperoxides in the development of the hypersensitive cell death induced by cryptogein on tobacco leaves. J Biol Chem 274 (51): 36446-36455

Ryan CA (1990) Protease inhibitors in plants: genes for improving defence against insects and pathogens. Ann Rev Phytopathol 28: 425-429

Rybutt DB & Parish CR (1982) Protein determination on an automatic spectrophotometer. Anal Biochem 121: 213-214

Ryhage R & Stenhagen E (1960) Mass spectrometry in lipid research. J Lipid Res 1: 361-390

Saffert A, Hartmann-Schreier J, Schon A & Schreier P (2000) A dual function alpha-dioxygenase-peroxidase and NAD(+) oxidoreductase active enzyme from germinating pea rationalizing alpha-oxidation of fatty acids in plants. Plant Physiol 123(4):1545-1552

Samuelsson B, Dahlen SE, Lindgren JA, Rouzer CA, Serhan CN (1987) Leukotrienes and lipoxins: structures, biosynthesis, and biological effects. Sci 237: 1171-1176

Sanz A, Moreno JI & Castresana C (1998) PIOX, a new pathogen-induced oxygenase with homology to animal cyclooxygenase. The Plant Cell 10: 1523-1537

Sayre LM, Arora PK, Iyer RS & Salomon RG (1993) Pyrrole formation from 4-hydroxynonenal and primary amines. Chem Res Toxicol 6 19–22

Schmidt DD & Baldwin IT (2006) Transcriptional responses of *Solanum nigrum* to methyl jasmonate and competition: a glasshouse and field study. Funct Ecol 20:500-508

Schmidt HH & Walter U (1994) NO at work. Cell 78: 919-925

Schulze B, Lauchli R, Mekem Sonwa M, Schmidt A, Boland W (2006) Profiling of structurally labile oxylipins in plants by in situ derivatization with pentafluorobenzyl hydroxylamine Anal Biochem 348(2): 269-283

Serhan CN, Haeggstrom JZ & Leslie CC (1996) Lipid mediator networks in cell signalling: update and impact of cytokines. FASEB Journal 10: 1147-1158

Shimizu T, Radmark O & Samuelsson B (1984) Enzyme with dual lipoxygenases activities catalyses leukotriene A4 synthesis from arachidonic acid. Proc. Natl. Acad. Sci. USA 81:689-693

Shulaev V, Silverman P & Raskin I (1997) Airborne signalling by methyl salicylate in plant pathogen resistance. Nature 385: 718-721

Siedow J (1991) Plant lipoxygenase: structure and function. Annu Rev Plant Physiol Plant Mol Biol 42: 145-188

Slusarenko AJ (1996). Plant lipoxygenase in plant resistance to infection. *In* G. Piazza ed, Lipoxygenase and Lipoxygenase Pathway Enzymes. AOCS Press, Champaign, IL: 176-197

Smith CJ (1996) Accumulation of phytoalexins: defence mechanism and stimulus response system. New Phytol 132: 1-45

Song W-C, Funk CD, Brash AR (1993) Molecular cloning of an allene oxide synthase: cytochrome P540 specialized for the metabolism of fatty acid hydroperoxides. Proc Natl Acad Sci USA 90: 8519-8523

Stakman EC (1915) Relation between *Puccinia graminis* and plants highly resistant to its. attack. J Agric Res IV 193-201

Stamler JS (1994) Redox signaling: Nitrosylation and related target interactions of nitric oxide. Cell 78: 931-936

Staswick PE (1992) Jasmonate, genes, and fragrant signals. Plant Physiol 99: 804–807

Staswick PE, Yuen GY & Lehman CC (1998) asmonate signaling mutants ofArabidopsisare susceptible to the soil fungus *Pythium irregulare*. Plant J 15, 747-754

Stout MJ, Fidantsef AL, Duffy SS & Bostock RM (1999) Signal interactions in pathogen and insect attack: systemic plant-mediated interactions between pathogens and herbivores of the tomato, *Lycopersicom esculentum*. Physiol Mol Plant Pathol 54: 115-130

Straus DS & Glass CK (2001) Cyclopentenone prostaglandins: New insights on biological activities and cellular targets. Med Res Cardiol 18: 458-470

Takabayashi J & Dicke M (1996) Plant carnivore mutualism through herbivore-induced carnivore attractants. Trends Plant Sci 1: 109-113

Taylor CB (1998) Defence responses in plants and animals – more of the same. Plant Cell 10: 873-876

Tjamos EC & Kuc JA (1982) Inhibition of steroid glycoalkaloid accumulation by arachidonic and eicosapentaenoic acids in potato. Science 217: 542-544

Tolmay VL, Prinsloo GJ, Maré R & Hatting JL (1998) Wheat production and the Russian wheat aphid. Pages 30-33 in; Wheat Farmer's Day 1998. ARC-Small Grain Institute, Bethlehem, South Africa

Tumlinson JH, Turlings TCJ & Lewis WJ (1993). Semiochemically mediated foraging behaviour in beneficial parasitic insects. Arch Insect Biochem and Physiol 22: 385-391

Uchida K, Kanematsu M, Motimitsu Y, Osawa T, Noguchi N & Niki E (1998) Acrolein is a product of lipid peroxidation : formation of free acrolein and its conjugate with lysine residues in oxidized low density lipoproteins. J Biol Chem 273:16058–16066.

Van der Westhuizen A.J. and Pretorius Z (1995) Biochemical and physiological responses of resistance and susceptible wheat to Russian wheat aphid infestations. Cer Res Commun 23 (3): 305-313

Van der Westhuizen A.J., Qian X-M and Botha A-M (1998a) ß-1,3-glucanase in wheat and resistance to the Russian wheat aphid. Physiol Plant 103: 125-131

Van der Westhuizen A.J., Qian X-M and Botha A-M (1998b). Differential induction of apoplastic peroxidase and chitinase activities in susceptible and resistant wheat cultivars by Russian wheat aphid infestation. Plant Cell Reports 8: 132-137

Vanacker H, Carver TLW & Foyer CH (1998) Pathogen-induced changes in the antioxidant status of the apoplast in barley leaves. Plant Physiol 117: 1103-1114.

Vancanneyt G, Sanz C, Frarmaki T, Paneque M, Ortego F, Castanera P & Sanchez-Serrano JJ (2001) Hydroperoxide lyase depletion in transgenic potato plants leads to an increase in aphid performance. Proc Natl Acad Sci USA 98: 8139-8144

Verhoeven NM, Wanders RJ, Poll-The BT, Saudubray JM & Jakobs C (1998) The metabolism of phytanic acid and pristanic acid in man: a review. J Inherit Metab Disorders 21: 697–728.

Vick BA & Zimmerman DC (1987) Pathways of fatty acid hydroperoxide metabolism in spinach leaf chloroplasts. Plant Physiol. 85: 1073-1078

Vick BA & Zimmerman DC (1984) Biosynthesis of jasmonic acid in plants. Plant Physiol 75, 458-461.

Vick BA (1993) Oxygenated fatty acids of the lipoxygenase pathway. Moore TS, ed 1993. Lipid metabolism in plants. Boca Raton, FL: CRC Press 167-191

Voelckel C, Weisser W & Baldwin IT (2004) An analysis of plant-aphid interactions by different microarray hybridization strategies. Mol Ecol 13, 3187-3195

Vollenweider S, Weber H, Stolz S, Chételat A & Farmer EE (2000) Fatty acid ketodienes and fatty acid ketotrienes: Michael addition acceptors that accumulate in wounded and diseased *Arabidopsis* leaves. Plant J 24: 467-476

Von Dahl CC, Hävecker M, Schlögl & Baldwin IT (2006) Caterpillar-elicited methanol emission: a new signal in plant herbivore interactions. The Plant J 46: 948-960

Walters MC (1984) Progress in Russian wheat aphid (*Diuraphis noxia* Moerd.) Research in the Republic of South Africa. Proceedings of the meeting of the Russian Aphid task team held at the University of the Orange Free State, Bloemfontein 5-6 May 1982. Ed. MC Walters. Technical communication No 191, Department of Agriculture, Republic of South Africa.

Wasternack C, Ortel B, Miersch O, Kramell R, Beale M, Greulich F, Feussner I, Hause B, Krumm T, Boland W & Parthier B (1998) Diversity in octadecanoid-induced gene expression of tomato. J Plant Physiol 152: 345-352

Weber H (2002) Fatty acid-derived signals in plants. Trends in Plant Sci 7: 217-224

Weber H, Chételat A, Reymond P & Farmer EE (2004) Selective and powerful stress gene expression in *Arabidopsis* in response to malondialdehyde. The Plant J 37: 877-888

Weber H, Vick BA & Farmer EE (1997) Dinor-oxo-phytodienoic acid: a new hexadecanoid signal in the jasmonate family. Proc Natl Acad Sci USA 94: 10473-10478

Weichart H, Stenzel I, Berndt E, Wasternack C & Fuessner I (1999) Metabolic profiling of oxylipins upon salicylate treatment in barley leaves – preferential induction of the reductase pathway by salicylate. FEBS Lett 464: 133-137

Weiler EW, Laudert D, Stelmach BA, Hennig P, Biesgen C & Kubigsteltig I (1999) Octadecanoid and hexadecanoid signalling in plant defence. Novartis Found Symp 223:191-204.

Wojtaszek P (1997) Oxidative burst: an early plant response to pathogen infection. Biochem J 322: 681-692

Yamamoto M, Li M, Mitsuma N, Ito S, Kato M, Takahashi M & Sobue G (2001) Preserved phosphorylation of RET receptor protein in spinal motor neurons of patients with amyotrophic lateral sclerosis: an immunohistochemical study by a phosphorylation-specific antibody at tyrosine 1062. Brain Res 912, 89–99

Zhang Y-Y & Punja ZK (1994) Induction and characterization of chitinase isoforms in cucumber (*Cucumis sativus* L.): effect of elicitors, wounding and pathogen inoculation. Plant Sci 99: 141-150

Zhang Z, Collinge DB & Thordal-Christensen (1995) Germin-like oxalate, a H_2O_2-producing enzyme, accumulates in barley attacked by the powdery mildew fungus. Plant J 8: 139-935

Zhuang H, Sokhon PIN, Xiaoling LI & Sylvain D (2003) Regulation of heme oxygenase expression by cyclopentenone prostaglandins. Soc Exp Biol & Med 499-505

Zieslin N and Ben-Zaken R (1991) Peroxidase, phenylalanine ammonia-lyase and lignification in peduncles of rose flowers. Plant Physiol Biochem 29: 147-151

Zimmerman DC & Coudron CA (1979) Identification of traumatin, a wound hormone, as 12-oxo-trans-dodecenoic acid. Plant Physiol 63: 536-541