U.O.V.S. BIBLIOTEEK

INVOLVEMENT OF ACTIVE OXYGEN SPECIES AND PHENYLALANINE AMMONIA-LYASE IN THE RESISTANCE RESPONSE OF WHEAT TO THE RUSSIAN WHEAT APHID.

By

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HIERDIE EKSEMPLAAR MAG ONDER GEEN OMSTANDIGHEDE UIT DIE BIBLIOTEEK VERWYDER WORD NIE

Preface

The work presented here is a result of an original study conducted at the Department of Botany and Genetics, 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 have been learned about the defense mechanism of wheat against the Russian wheat aphid, but it is still unclear to what extend active oxygen species are involved during the hypersensitive response in eliciting the defense genes. The induction of phenolic compounds has been found to play an important role in the defense mechanism and in this study I have aimed to identify some of these phenolic compounds involved.

I have not previously submitted the dissertation submitted here to any other university/faculty. I therefore cede its copyright in favour of the University of the Free State.

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Table of Contents

List of abbreviations 1
List of figures 3
List of chemicals 5

Chapter 1
Introduction 7

Chapter 2
Literature review 14

2.1 Defense mechanisms 15
2.1.1 Host recognition by pathogens and non-pathogens 16
2.1.2 Perception of the elicitor stimulus 17
2.1.3 Transduction of elicitor signal 18
2.1.3.1 Oxidative burst 19
2.1.4 Regulation of plant responses 23

2.2 Phenylalanine ammonia-lyase activity 26
2.2.1 Characteristics of PAL 29
2.2.2 PAL linking primary and secondary metabolism 29

2.3 Phenolic acids 30
2.3.1 Physical and chemical properties 31
2.3.2 Synthesis and induction of phenols 32
2.3.3 Defense strategy 34

Chapter 3

Materials and Methods 36

3.1	Materials	37
3.1.1	Chemicals	37
3.1.2	Plant material	37
3.2	Methods	37
3.2.1	Determination of phenylalanine ammonia-lyase (PAL) activity	37
3.2.2	Separation and quantification of phenolic compounds	38
3.2.2.1	Phenolic standards	38
3.2.3	Autofluorescence detection of lignin	39
3.2.4	Determination of superoxide dismutase activity	39
3.2.5	Determination of hydrogen peroxide (H_2O_2) concentration	39
3.2.6	Determination of peroxidase (POD) activity	40
3.2.7	Determination of chitinase activity	40
3.2.8	Inhibition of the hypersensitive response by allopurinol	41
3.2.9	Determination of protein concentration	41

Chapter 4

Results 42

4.1	Phenylalanine ammonia-lyase activity	43
4.2	Phenolic compounds	43
4.3	Lignification	48
4.4	Active oxygen species	51
4.4.1	Hydrogen peroxide (H_2O_2) concentration	51
4.4.2	Superoxide dismutase (SOD) activity	51
4.5	Effect of allopurinol on SOD activity	52
4.6	Effect of allopurinol on peroxidase activity	52
4.7	Effect of allopurinol on chitinase activity	53
4.8	The *in vitro* effect of allopurinol on chitinase activity	53

Chapter 5
Discussion 56

Abstract 67
Keywords 68
Opsomming 69

References 71

LIST OF ABREVIATIONS

AOS	active oxygen species
APX	ascorbate peroxidase
CAD	cinnamyl alcohol dehydrogenase
CAT	catalase
CHS	chalcone synthase
CM-chitin-RBV	carboxymethyl-chitin-remazol brilliant violet 5R
cv	cultivar
DTT	1,4-dithiothreitol
EDTA	ethylene diamine tetra-acetic acid
et al.	*et alia* (and others)
GA	gentisic acid
GDP	gross domestic product
GR	glutathione reductase
h.p.i.	hours after infestation
H_2O_2	hydrogen peroxide
HCl	hydrochloric acid
HPLC	high performance liquid chromatography
HR	hypersensitive response
IR	infested resistant
IS	infested susceptible
JA	jasmonic acid
mol	mole
NBT	nitroblue tetrazolium
O_2	oxygen
O_2^-	superoxide anion
PAL	phenylalanine ammonia-lyase
PMSF	phenylmethylsulfonyl fluoride
POD	peroxidase
PR	pathogen related
PVP	polyvinylpolypyrolidone
R	resistant

Rp	plant receptor proteins
RSA	Republic of South Africa
RWA	Russian wheat aphid
S	susceptible
SA	salicylic acid
SAR	systemic acquired resistance
SOS	superoxide dismutase
USA	United States of America
UV	ultraviolet
v/v	volume to volume

LIST OF FIGURES

Figure 2.1	Signal generation and interaction during responses to insects, pathogens and abiotic stresses.
Figure 2.2	A speculative model showing possible components involved in AOS generation and effects of AOS.
Figure 2.3	Signalling events controlling activation of defense genes.
Figure 2.4	The deamination of *L*-phenylalanine catalyzed by PAL
Figure 2.5	Postulated control of PAL, Cinnamate 4-hydroxylase and the PAL inactivation system.
Figure 2.6	Plant phenolics are biosynthesized in several different ways.
Figure 2.7	Metabolic pathway and enzymes involved in lignin biosynthesis.
Figure 4.1	Effect of RWA infestation on PAL activity.
Figure 4.2 (a-g)	The effect of RWA infestation on individual phenolic acid concentrations.
Figure 4.3 (a-d)	The effect of RWA infestation on the anatomical structure of: a = infested resistant, Tugela DN wheat, b = resistant wheat, Tugela DN, c = susceptible, Tugela wheat, d = infested susceptible, Tugela wheat.
Figure 4.4	The effect of RWA infestation (I) on H_2O_2 levels of susceptible (S), Tugela and resistant (R), Tugela DN wheat plants.
Figure 4.5	The effect of RWA infestation (I) on SOD levels in the susceptible (S), Tugela, and resistant (R), Tugela DN, wheat plants.
Figure 4.6	SOD activity in infested (I) susceptible (S), Tugela and resistant (R), Tugela DN wheat plants.
Figure 4.7	POD activity in infested (I), uninfested susceptible (S), Tugela and resistant (R), Tugela DN wheat and the effect of allopurinol on the induced POD activity.
Figure 4.8	Chitinase activity in infested (I), uninfested susceptible (S), Tugela and resistant (R), Tugela DN wheat and the effect of allopurinol on the induced chitinase activity.

Figure 4.9 The *in vitro* and *in vivo* effect of allopurinol on chitinase activity of infested resistant (IR) (Tugela DN) wheat plants; IR* infested resistant- *in vivo* treatment with allopurinol; IR**, infested resistant wheat- *in vitro* treatment with allopurinol.

Figure 5.1 Possible mechanism of oxygen radical production consequent to the activation of purine catabolism during hypersensitivity expression in the incompatible host-pathogen interaction.

Figure 5.2 Speculative model showing the defense mechanism of resistant wheat against RWA infestation.

LIST OF CHEMICALS

1,4-Dithiothreitol (DTT)

3,4,5-Trihydroxybenzoic acid

3,5-diHydroxybenzoic acid

3,5-Dimethoxybenzoic acid

4-Hydroxycinnamic acid

Acetic acid

Acetonitrile

Allopurinol (4-hydroxypyrazolo(3,4-*d*)pyrimidine

BioRad

Caffeic acid (3,4-dihydroxycinnamic acid)

Carboxymethyl-chitin-remazol brilliant violet 5R (CM-chitin-RBV)

Cinnamic acid

Diethyleter

Dowex 1 (1x4-200) basic anion exchange resin

Ethanol

Ethylene diamine tetra-acetic acid (EDTA)

Ferulic acid (4-hydroxy-3-methoxycinnamic acid)

Gentisic acid (2,5-dihydroxybenzoic acid)

Guaiacol

Hydrochloric acid (HCl)

Hydrogen peroxide

Mercaptoethanol

Methanol

Methionine

Na-acetate

Nitroblue tetrozolium (NBT)

Phenylalanine

Phenylmethylsulfonyl fluoride (PMSF)

Phloroglucin

P-Hydroxybenzoic acid

p-Hydroxyphenylacetic acid

Polyvinylpolypyrolidone (PVP)

Protocatechuic acid (3,5-dihydroxybenzoic acid)

Pyranine (8-hydroxypyrene-1,3,6-trisulfonic acid)

Riboflavin

Salicylic acid

Syringic acid (4-hydroxy-3,5-dimethoxybenzoic acid)

Vanillic acid (4-hydroxy-3-methoxybenzoic acid)

α -3,5-Resorcylic acid

γ -Globulin

CHAPTER 1

INTRODUCTION

Plants and animals have evolved together over many millions of years, which resulted in an intricate interaction and interdependence. One outstanding example of this interaction and interdependence is the pollination of the flowering plants by animals. Darwin was one of the first biologists to be impressed by the close association of plants and their pollinators and the remarkable adaptation in the structure and behaviour of plants and animals that make pollination effective (Edwards and Wratten, 1980).

A totally different kind of relationship between animals and plants occurs between the so-called insectivorous plants and their prey. Species such as the sundews (*Drosera* spp.), the venus flytrap (*Dionaea muscipula*), the pitcher plant (*Nepenthes* spp.) and the bladderworths (*Utricularia* spp.) exhibit a remarkable range of adaptations enabling them to trap and digest animal food (Edwards and Wratten, 1980).

A major kind of plant/animal interaction, herbivory, received little attention until recently. Modern research is unraveling a fascinating study, which demonstrates that the relationships between plant and herbivore are quite complex and highly evolved as those in pollination. It would be surprising if it proves otherwise, since clearly plants must always have been under selective pressure to escape from the damaging effects of grazing, while herbivorous animals must have evolved to be closely adapted to their food supplies (Edwards and Wratten, 1980).

One such example of a plant/animal interaction is that of the Russian wheat aphid and wheat plants. The Russian wheat aphid (RWA), *Diuraphis noxia*, is of Palaearctic origin and is widespread in southern Europe, central Africa, the Middle East, and North Africa. The RWA was observed as a pest of wheat in the Republic of South Africa during 1978 (Du Toit and Walters, 1984) and it has persistently remained a serious pest. It has been a pest of small grains in areas of Russia since 1912. In the Crimea, this species has decreased the crop yield by as much as 75% in some years and in south central Turkey heavy damage was reported in 1962 in wheat and barley crops (Burton and Webster, 1993). Reports of damage are known from countries around the Mediterranean Sea and other areas of Asia. Damage is greatest when crops start to ripen concurrently with peak aphid numbers, which can result in considerable reduction of the crop yields of barley and wheat, the most important hosts. The pest also occurs on oats, rice, corn, sorghum, brome, canary grass, wheat grass and other native grasses.

The RWA became a serious wheat pest in South Africa during 1978. At this point of time very little was known about the relationship between *D. noxia* and the host plant, nor the means to control it (Du Toit and Walters, 1984; Du Toit, 1992). Finding the resistance for the control of *D. noxia,* 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 *D. noxia.* The spread of *D. noxia* to the USA and Mexico during the 1980's intensified the search for resistance to *D. noxia.*

Agriculture plays a very important role in the national economy of the RSA as well as a very distinctive role in expanding the economy and social options of the rural people, and consequently improving their quality of life. Agriculture in South Africa generates almost R43 billion a year, which is more than 10% of the gross domestic product (GDP). Twenty five percent of the employment in South Africa is sustained by agricultural activity. Agriculture contributed 9.4% of the foreign exchange earnings in 1994. Maize and wheat are the most important grain crops and constitute 36% and 21% of the arable land, respectively. The gross value of wheat estimated to the amount of R1,354 million during the 1994/1995 season. Wheat production contributed to 3.59-6.3% to the gross agricultural production. Wheat production amounted to 1.9 million metric tons in 1995/96 (Marasas *et al.*, 1997).

Under experimental field conditions it was found that wheat losses due to RWA mounted to 90%. From 1987 to 1993 the loss in the USA as a result of the RWA exceeded $890 million, with $83 million being spent on control, $349 million in lost production and $460 million in additional lost in economic activity in the local communities (Marasas *et al.*, 1997).

The world's first resistant wheat cultivar (Tugela DN) with effective resistance was bred by the Small Grain Institute at Bethlehem, South Africa (Du Toit, 1988; 1989). In 1996 South Africa had already developed seven different cultivars with RWA resistance. In comparison, only one other Russian wheat aphid resistant cultivar has been released in Colorado, USA (Central Bureau Report, 1996). Recently the Small Grain Institute developed a new RWA resistant wheat cultivar "Elands", with a high yield potential, compared to Tugela DN, and generally a better yield potential than Gariep and Betta DN. Elands also performs well under a wide range of cultivation conditions, and additionally has resistance to leaf rust, is moderately susceptible to yellow rust, and the Russian wheat aphid has no effect on it (Hayes, 1999).

The development of these RWA resistant cultivars does not necessarily solve the RWA problem. Resistance breaking biotypes of aphids can form when constantly exposed to resistant cultivars; in the same way as ticks build up resistance to a certain dip over a number of years (Martin, 1992; Central Bureau Report, 1996; Hayes, 1998). Therefore it is advisable to apply supportive control measurements such as biological or chemical control (Central Bureau Report, 1996).

The problem with chemical control is that it is expensive and can also be harmful to the environment. Natural enemies of the RWA show good potential as a supportive measure to control the RWA, especially because the leaves of the resistant cultivars do not roll close and the aphids are therefore exposed to the parasites and predators (Central Bureau Report, 1996; Hayes, 1998).

The Small Grain Institute developed an integrated control program using resistant cultivars and effective natural enemies. This ensures that the RWA can now be controlled viably cost effectively, to a large extent negating the need for insecticides. The results they achieved were excellent, because they managed to achieve an outstanding yield and reduction in both the percentage of infested tillers and the number of RWA per infested tiller (Hayes, 1998).

When the resistance gene Dn1 is incorporated into different agronomic lines, the expression of the resistance differs. For this reason, the symptoms of the different cultivars differ (Central Bureau Report, 1996).

There is growing concern associated with the system. The first concern being whether or not it is necessary or desirable to apply any form of chemical aphid control on these cultivars. Secondly, there is a demand for "threshold values" for the different cultivars as the levels of resistance differ (Central Bureau Report, 1996). During 1993, trials showed that when both plant resistance and biological control were used in the field, the reduction in wheat aphid numbers was so extreme that no other control measures were necessary. At the same time the chance that a resistance breaking biotype of the Russian wheat aphid could form was reduced (Central Bureau Report, 1996). Furthermore, as the leaves of the resistant cultivars do not roll close, predators such as ladybirds, which were not effective in the past, may also exercise control. The integrated program requires no technical knowledge or equipment in its application and is therefore suitable for both commercial and subsistence farmers (Central Bureau Report, 1996).

Data collected has shown that there is no advantage in spraying resistant wheat with insecticides to control the RWA. The use of chemicals may still be needed to control other sporadic wheat pests. The application of chemicals however could influence the natural enemies of the RWA and should therefore be used with extreme caution (Hayes, 1998).

The development of new cultivars is focused on benefiting the producers' pockets. The use of biotechnology is making a major contribution to the development of more improved cultivars. The Green Revolution dominated the international agriculture in the later half of the twentieth century. Observing the success and achievements of plant biotechnology, the next revolution in the next century can be a Gene Revolution (Van Rooyen, 1999).

New information obtained from studying the biochemical interaction can be used to identify resistant genes, to identify molecular and biochemical markers and for developing alternative environmental friendly combating methods (Chrispeels and Sadawa, 1994) to control the RWA. Successful breeding programs during the last few years led to the development of wheat cultivars with resistance to the RWA, but the biochemical mechanism involved is still poorly understood. If the mechanism of resistance is known, it can help in terms of modern molecular biology to develop suitable resistant cultivars with desired properties, presumably in shorter time than traditional breeding methods (Chrispeels and Sadawa, 1994). Examples exist of improved resistance where genes with known defensive functions, are manipulated into crops, where they are expressed constitutively (Loggemann and Schell, 1994))

Identification of genes and the cloning of them can lead to the development of good DNA-probes. These primers can be used very effectively in DNA-hybridization to develop more effective selection methods, which will in turn accelerate traditional breeding programmes (Loggeman and Schell, 1994). Biochemical markers other than DNA such as, phenolic compounds may be found. It is known that enzyme activities of certain PR-proteins are connected to the level of resistance in wheat to the RWA and that can serve as a quantitive measure of resistance. Some studies have shown that peroxidase, chitinase and β-1,3-glucanase are involved in the defense mechanism of wheat against the RWA (Van der Westhuizen *et al.*, 1998 a and b; Botha *et al.*, 1998).

Resistance to pathogens that is conditioned by a gene-for-gene interaction is an active process. Many defense functions are induced during a resistance response in different plant-pathogen systems. These include the *de novo* induction of mRNA transcripts and proteins

termed defense related proteins. These transcripts and proteins are often induced during a susceptible interaction as well as a resistant interaction, but the timing and abundance of the transcript or protein is either faster or higher, respectively, when resistance occurs (Greenberg, 1997).

Defenses that can be induced during a resistance response are alterations in the plant cell walls. At least one protein is cross-linked in the cell walls possibly by dityrosine bridges in a process that is likely to depend on H_2O_2 (Bradley *et al.*, 1992; Brisson *et al.*, 1994). Phenolic compounds become cross-linked in the cell walls, which become lignified during the resistant response. Modifications of the cell wall protect the cell wall from digestion by pathogens (Brisson *et al.*, 1994). The H_2O_2 that is required for cell wall modifications may be supplied by the activation of membrane-localized NADPH-dependant oxidase in a process termed the oxidative burst (Mehdy, 1994).

The induction of the phenylpropanoid pathway, which leads to phytoalexin and lignin biosynthesis, localized synthesis of callose and increased production of the stress hormone ethylene (Grosskopf *et al.*, 1991), is one of the pathways that are induced upon infection. Increases in phenylalanine ammonia-lyase (PAL) activity are considered to be an indicator of resistance since PAL is essential for the synthesis of phenols, compounds associated with resistance (Nicholson and Hammerschmidt, 1992). PAL catalyses the deamination of phenylalanine to cinnamic acid (Jones, 1984), which is a phenylpropane, an important building block of more complex phenolic compounds. In the interaction between wheat and the RWA it has been established beyond reasonable doubt that metabolic changes occur in resistant wheat and that there is an increase in phenolic content that might have a deterrent effect on the RWA (Van der Westhuizen and Pretorius, 1995). The sequence of events in a defense response can be thought to include host cell death and necrosis, accumulation of toxic phenols, modification of cell walls by phenolic substituents or physical barriers (Jones, 1984).

Most cells have the ability to produce and detoxify active oxygen species (AOS). Under normal conditions AOS appear in cells as inevitable by-products formed as a result of successive one-electron reduction of molecular oxygen (Alvarez *et al.*, 1998). The antioxidant defence system consists of low molecular weight antioxidants such as ascorbate, glutathione, α-tocopherol and cartenoids as well as several enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), glutathione reductase (GR) and

ascorbate peroxidase (APX). SOD converts superoxide into hydrogen peroxide (H_2O_2) and O_2. The antioxidants participate in both enzymatic and nonenzymatic H_2O_2 degradation. CAT dismutates H_2O_2 into water and O_2, whereas POD decomposes H_2O_2. It has been found that AOS being produced in the oxidative burst could not only serve as protectants against pathogens, but could also be signals activating further defense reactions, including the hypersensitive response (HR) of infected cells. AOS in the form of H_2O_2 or O_2^- are the key mediators of pathogen-induced programmed cell death (Levine *et al.*, 1994 and 1996; Hammond-Kosack and Jones, 1996; Mehdy, 1994), and may function as part of a signal transduction pathway leading to the induction of defense mechanisms and cell death (Hammond-Kosack and Jones, 1996).

Substantial research has been done in our laboratory on the defense mechanism of wheat against the RWA, but it is still unclear whether the HR is an absolute requirement for resistance and what role AOS may perform in the establishment of resistance. It is believed that hypersensitive cell death is correlated with cellular lignification (Moerschbacher *et al.*, 1988; Tiburzy and Reisener, 1990) during incompatible interactions between pathogen and host. Xanthine oxidase is thought to be responsible for the generation of oxygen radicals during the HR and superoxide dismutase converts these oxygen radicals to H_2O_2 (Montalbini, 1992 a and b). It is also thought that H_2O_2 is required for the activation of the down stream defense response (Alvarez *et al.*, 1998; Levine *et al.*, 1994; Yahrus *et al.*, 1995) such as PAL, chitinase and β -1,3-glucanase activities. Changes in PAL activity lead to changes in lignin and phenolic compounds when challenged with a pathogen (Cahill and McComb, 1992) and PAL is required for the synthesis of salicylic acid (SA) and precursors for lignification (Mauch-Mani and Slusarenko, 1996). SA is an important signalling molecule, required for the induction of downstream defense responses in wheat infested with the RWA (Mohase, 1998).

In this study we aimed to learn more about the resistance mechanisms of wheat, challenged with the RWA. This includes the role of the HR and AOS, in particular H_2O_2, in establishing the down stream defense response and the involvement of SOD in this regard. Furthermore, the fact that SA and total phenolics in previous studies in our laboratories have been implicated in the resistance response to the RWA, urged us to investigate the effect of RWA infestation on PAL activity and phenolic acid composition and contents in an effort to more closely relate these events to resistance.

CHAPTER 2

LITERATURE REVIEW

2.1 DEFENSE MECHANISMS

In the plant kingdom, like in humans and animals, diseases are rather the exception than the rule. Plants have developed their own defense mechanisms against pathogens and insects, but there are a few pathogens and insects that have managed to overcome plants' natural defense mechanisms and thus cause diseases. All pathogens have a limited host range, e.g. *Pyricularia oryzae*, a pathogen of rice, cannot infect tomato, while *Alternaria solani*, a pathogen of tomato cannot infect rice (Vidhyasekaran, 1988).

Responses of resistant wheat plants to stress induced by RWA infestation in many respects resemble known resistance responses to other biotic as well as abiotic stresses (Van der Westhuizen and Pretorius, 1995). Figure 2.1 illustrates the signal generation and interaction during defense responses to insects, pathogens and abiotic stresses. Castro *et al.* (1999) have screened 26 wheat cultivars for resistance against greenbug (*Schizaphis graminum* Rond) and RWA, and found genetic resistance against both aphid species in several of these cultivars. Castro concluded that the plants have independent defense mechanisms to both pests. Antibiosis against greenbug or RWA appears to be deterrent by two different sets of genes, one affecting development time and the other reducing fecundity and longetivity (Castro *et al.*, 1999). The primary paths of signal/phytochrome induction by "classical" damaging agents (e.g. chewing insects, necrotrophic pathogens) are indicated by solid arrows in Fig. 2.1; secondary paths (arrows with dotted lines) show that some agents do not fit these classical injury modes. The connection with the question mark illustrates the potential for positive and negative interactions (e.g. SA interference with jasmonate synthesis and response; ABA interference with resistance to pathogens, but enhancement of jasmonate-regulated responses). These signals trigger gene expression and metabolism that may or may not contribute to defense against a particular pest, and may actually comprise resistance to some (Bostock, 1999).

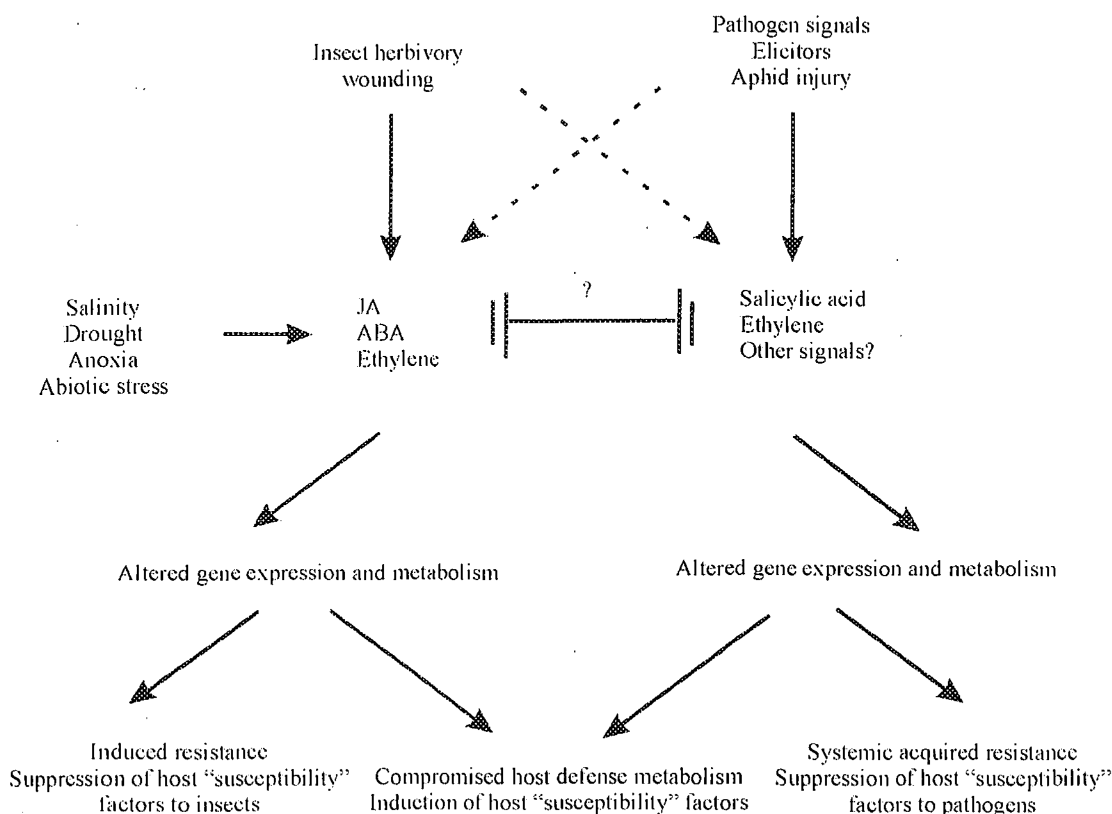


Figure 2.1 Signal generation and interaction during responses to insects, pathogens and abiotic stresses (Bostock, 1999).

2.1.1 Host Recognition by Pathogens and Non-pathogens

The term "elicitor" refers to compounds that stimulate phytoalexin synthesis in plants, the synthesis of cell wall-associated phenylpropanoid compounds, the deposition of callose (1,3-β -glucan), the accumulation of hydroxyproline-rich glycoproteins, and the synthesis of certain hydrolytic enzymes (i.e., β -glucanases and chitinases) (Ebel and Mithöfer, 1998). Recognition of pathogens takes place through elicitors, these elicitors can be released from invading fungal or bacterial pathogens prior to or during ingress (Dixon *et al.*, 1994). Some elicitors are able to stimulate more than one defense mechanism while others only interact synergistically (Scheel, 1998). Non-chemical elicitors would include ultraviolet irradiation, freezing injury, and in some cases merely wounding. It is unclear whether wounding *per se* or the surface-contaminant micro-organisms carried into the wound elicit the low levels of phytoalexin accumulation by wounding.

Several attempts have been made to identify so-called "specific elicitors" that reflect the specificity of certain pathogen races towards a number of host cultivars in interactions

exhibiting gene-for-gene relationships. There are also non-specific elicitors which are complimented by additional factors, that mediate the race/cultivar specificity. Other elicitors include general elicitors – involved in general resistance, biotic elicitors – from fungal and plant cell walls, lipids, microbial enzymes, and polypeptides or glycoproteins (Scheel, 1998).

2.1.2 PERCEPTION OF THE ELICITOR STIMULUS.

It is important to have highly sensitive and specific recognition systems for microbial pathogens for the development of resistance in plants (Nürnberger, 1999). To trigger appropriate protective measures against invading pathogens plants need to distinguish between 'self' and 'non-self'. In contrast to antigen recognition and defense activation by the immune system of vertebrates, which is essentially based on the circulation and interaction of highly specialized cells throughout the whole organism, each plant cell is autonomously capable of sensing the presence of potential phytopathogens as well as mounting defense responses (Nürnberger, 1999). Receptors enable plants to perceive typical fungal chemicals, such as glycopeptides, chitin and ergosterol. Recently it has been discovered that plants can perceive the bacterial motor protein flagellin (Felix *et al.*, 1999). It is possible that most biological elicitors have some receptor in the plant cell wall or on the plasmalemma. A necessary step in the characterization of elicitor receptors is knowledge of structure/activity relationships for the cognate elicitors. The activity of the heptaglucoside of *Pseudomonas megasperma* f.sp. *glycinea* is drastically reduced when the nonreducing terminal 1→6 or 1→3 linked glucose residues are modified, or when the spacing between the two 1→3 residues is shortened. The nature of the reducing end is not critical. The glucopeptide elicitors released from yeast invertase require glycan linked to asparagines as well as the adjacent arginine for activity, whereas the glycan itself suppresses elicitation. The elicitor activity of chitosan depends primarily on the degree of polymerization, suggesting that its interaction with the plant plasma membrane does not require a specific receptor (Dixon *et al.*, 1994).

Lectins are receptors for carbohydrate elicitors, which are either proteins or glycoproteins, which bind to specific saccharides. Glycoproteins are major components of cell membranes, which traverse the lipid bilayer, and the hydrophilic sugar residues protrude from both surfaces of the plasma membrane. Lectins can attach to membrane glycoproteins via projecting sugar residues and cause cells to agglutinate. Lectins are highly specific and can discriminate different types of cells that have only minor variations in the type of membrane glycoproteins (Vidhyasekaran, 1988). Lectins may act as receptors of phytoalexins present in

the cell of an incompatible pathogen, When the elicitors bind with the receptors (lectins), disease resistance is induced. Lectins inhibit the chitin formation in hyphal tips resulting in the inhibition of incompatible pathogens (Vidhyasekaran, 1988).

Certain plant cultivars have the ability to recognize strains or races of pathogen species and, consequently, mount an efficient resistance response. Race-specific pathogen recognition is determined by the action of complementary pairs of (semi) dominant (R) genes in the host plant and (semi) dominant avirulence (*avr*) genes in the pathogen. The lack of or non-functional products of either gene would result in colonization of the plant (Nürnberger, 1999).

Plant defense mechanisms include processes that result from transcriptional activation of defense-related genes, such as the production of lytic enzymes, phytoalexin biosynthesis and systemic acquired resistance (Hammond-Kosack and Jones, 1996). Other plant responses associated with pathogen defense results from allosteric enzyme activation initiating cell wall reinforcement by oxidative cross-linking of cell wall components, apposition of callose and lignins and the production of AOS (Lamb and Dixon, 1997; Dangl *et al.*, 1996). The activation of plant defenses in incompatible plant-microbe interactions results from recognition by the plant of either cell surface constituents of the pathogen or factors that are produced and secreted by the pathogen upon contact with the host plant. Plant-derived elicitors released from the plant by fungal hydrolytic enzymes are thought to act in a way similar to pathogen-derived elicitors. Receptors for pathogen-derived signals do function either on the plant cell surface or intercellular, mediating the conversion of an extracellular signal (Nürnberger, 1999).

2.1.3 Transduction of Elicitor Signal.

Plant defense gene activation requires the transduction of elicitor signals from the site of primary perception at the cell surface to the nucleus where transcription of specific genes is initiated. Secondary messengers in animals include cAMP, cGMP, inositol 1,4,5,-triphosphate, Ca^{2+}, and diacylglycerol. In plants evidence exists for the role of Ca^{2+} as a secondary messenger as well as indirect evidence for inositol-1,4,5,-triphosphate (Ebel and Mithöfer, 1998).

The reduction of extracellular Ca^{2+} concentrations in cell cultures of soybean, carrot, and parsley lowered the levels of phytoalexin accumulated in response to elicitor treatment. In cultured potato cells, the elicitor stimulated increases in the activities of PAL and tyrosine decarboxylase. Omission of Ca^{2+} from the medium of parsley protoplasts resulted in a corresponding reduction in run-off transcription rates of elicitor-responsive genes, but did not affect transcription of constitutively expressed or UV-inducible genes (Scheel, 1998).

In inoculated cells, increased levels of phytoalexins are generally observed only after several hours post-inoculation, whereas increased transcription of phytoalexin biosynthetic genes can be measured within 5 min (Dixon *et al*, 1994). Signal transduction associated with initiation of elicitation should occur prior to the onset of increased transcription. Very early responses to elicitation include changes in a number of parameters associated with signalling in mammalian cells. The involvement of these has been primarily addressed by pharmacological experiments with signal molecules, agonists, and antagonists (Dixon *et al.*, 1994).

2.1.3.3 Oxidative burst.

Plants mount a broad range of responses in response to attempted invasion by a pathogen including the generation of active oxygen species (AOS) (Bolwell and Wojtaszek, 1997). These production of these AOS is one of the earliest events during the hypersensitive response and because AOS are very hazardous to plants they need to be detoxified. Higher plants have developed complex enzymatic and non-enzymatic mechanisms capable of detoxifying these radicals. O_2^- is scavenged through the catalytic activity of SOD, while H_2O_2 is scavenged through the catalytic action of ascorbate peroxidase (APX) and catalase. Maintaining a pool of reducing equivalents in the form of NADPH, ascorbic acid, and glutathione is also important for detoxifying AOS (Mittler *et al.*, 1999)

Hipelli *et al.*, (1999) described the following functions that AOS perform in animals and plants:

- Transmembrane signalling and induction of information transfer, respiratory burst, local defense system and systemic resistance.
- Cell-, tissue-, and organ-damage due to reductive oxygen activation and fenton chemistry in almost all cellular components.
- Defense reactions in the phogosome (animals) or in the apoplast (plants) via formation of AOS and possibly hypohalides.

- Photodynamic damage and phototherapy in humans and animals and light dependent damage and senescence in plants, animals and humans (virus infections, toxins, herbicide action, cancer treatment and sunburn).
- Release of NO and interaction with superoxide producing peroxynitrite.
- Formation of hormone-like messengers from unsaturated membranous fatty acids such as prostaglandins (animals) or jasmonic acid (plants).

The transient induction of hydrogen peroxide at the cell surface initiates 2-3min after addition of elicitor to soybean cell-suspension cultures (Apostol *et al.*, 1989). The burst of hydrogen peroxide production at the surface of elicited bean and soybean cells drives the oxidative cross-linking of repetitive proline rich cell wall structural proteins that are also rich in tyrosine (Bradley *et al*, 1992). This cross-linking is initiated within 5 min of introduction of the stimulus, and the response is completed within 10 – 20 min, depending on the nature of the elicitor.

Addition of catalase or ascorbic acid to soybean cells blocks the induction and accumulation of the phytoalexin, glyceollin, by an elicitor preparation from *Verticillium dahliae*, suggesting that hydrogen peroxide might also function as a signal for the induction of defense genes (Dixon *et al*, 1994). Exogenous application of hydrogen peroxide induces the accumulation of phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS) transcripts in bean leaves and cell cultures (Dixon *et al*, 1994).

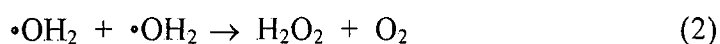
AOS may function in the generation of bioactive fatty acid derivatives analogous to the prostaglandin pathway in mammalian inflammation responses, e.g. H_2O_2 as a substrate for lipoxygenase-mediated production of jasmonic acid precursors from linolenic acid in the plasma membrane (Dixon *et al*, 1994).

The first reaction during the pathogen-induced oxidative burst is believed to be the one-electron reduction of molecular oxygen to form superoxide anion (O_2^-) (Mehdy, 1994). Dioxygen in its ground state is relative unreactive, partial reduction gives rise to AOS, including the O_2^-, H_2O_2 and the hydroxyl radical. O_2^- is a byproduct of mitochondrial electron transport, photosynthesis, and flavin dehydrogenase reactions, and can then be converted to other oxygen species, of which OH^- is the most reactive (Lamb and Dixon, 1997). In contrast

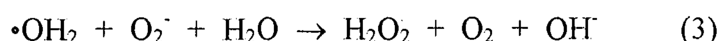
to O_2 AOS species are capable of unrestricted oxidation of various cellular components (Mittler *et al.*, 1999).

The first reaction in the partial reduction of dioxygen is the addition of a single electron to form O_2^-. This can be protonated at a low pH (pKa = 4.8) to yield perhydroxyl radical ($\bullet OH_2$) (equation 1), and $\bullet OH_2$ undergo spontaneous dismutation to produce H_2O_2 (equation 2 and 3) (Lamb and Dixon, 1997).

$$H^+ + O_2^- \leftrightarrow \bullet HO_2 \quad (1)$$



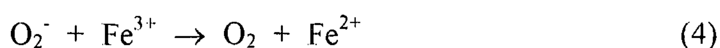
$$\bullet OH_2 + \bullet OH_2 \rightarrow H_2O_2 + O_2 \quad (2)$$



$$\bullet OH_2 + O_2^- + H_2O \rightarrow H_2O_2 + O_2 + OH^- \quad (3)$$

Equation 3 represents the major route for O_2^- decay at cellular pH. Because of the equilibrium in equation 1, spontaneous radical dismutation will decrease as cellular pH increases. Superoxide dismutase catalyzes a highly efficient conversion of $O_2^-/\bullet OH_2$ to H_2O_2 (Lamb and Dixon, 1997).

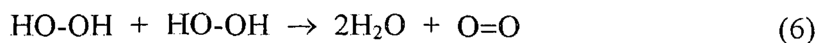
H_2O_2 is stable and less reactive than O_2^-. However, in the presence of reduced transition metals such as Fe^{2+}, which may be free or complexed to chelating agents or proteins, H_2O_2-dependant formation of $\bullet OH$ can occur, and O_2^- can act as the initial reducing agent for the metal (equation 4 and 5).



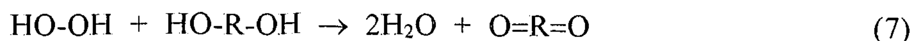
$$O_2^- + Fe^{3+} \rightarrow O_2 + Fe^{2+} \quad (4)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \bullet OH \quad (5)$$

$\bullet OH$ is a very strong oxidant and can initiate radical chain reactions with a range of organic molecules. This can lead to lipid peroxidation, enzyme inactivation, and nucleic acid degradation (Lamb and Dixon, 1997). H_2O_2 is removed by catalase or various peroxidases including ascorbate and glutathione peroxidases. The general equation for catalase and peroxidase reactions (equation 6 and 7) are similar, because the catalase reaction can be viewed as a peroxidative reaction with H_2O_2 as both substrate and acceptor, in equation 7, the R group is often aromatic, in which case a diphenol is converted to a diquinone, a reaction important in lignin polymerization.



$$HO\text{-}OH + HO\text{-}OH \rightarrow 2H_2O + O{=}O \qquad (6)$$



$$HO\text{-}OH + HO\text{-}R\text{-}OH \rightarrow 2H_2O + O{=}R{=}O \qquad (7)$$

Peroxidase can also catalyze the formation of both O_2^- and H_2O_2 by a complex reaction in which NADH is oxidized using trace amounts of H_2O_2 first produced by nonenzymatic breakdown of NADH. The NAD• radical formed then reduces O_2 to O_2^-, some of which dismutates to H_2O_2 and O_2. This reaction is stimulated by monophenols and Mn^{2+} and may be a mechanism for generation of extra cellular H_2O_2 for lignin polymerization (Lamb and Dixon, 1997).

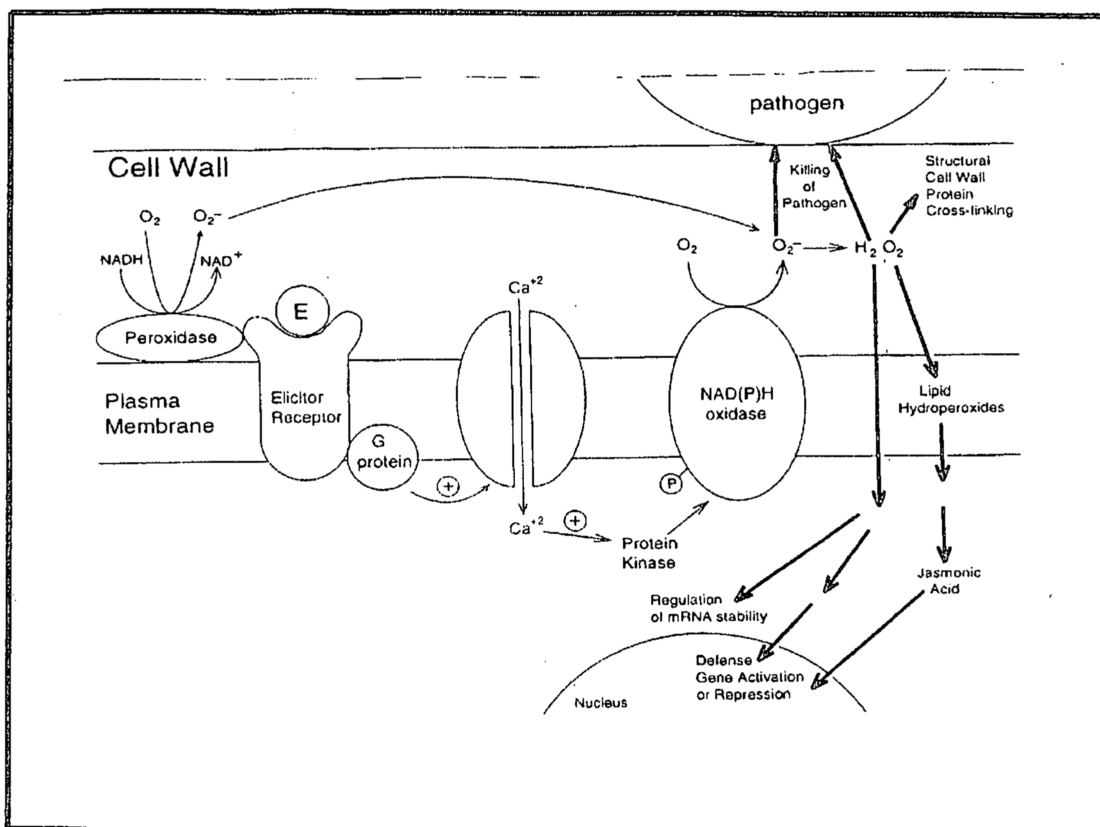


Figure 2.2 A speculative model showing possible components involved in AOS generation and effects of AOS. Assignment of actual components and sequence of components requires additional data. Elicitor receptors may be coupled to AOS synthesis via G proteins, increased intracellular Ca due to Ca channel opening, activation of a protein kinase that activates a membrane-bound NAD(P)H oxidase by phosphorylation. Alternatively, occupation of elicitor receptors may stimulate a membrane-associated peroxidase by unknown mechanisms, which results in O_2^- synthesis. O_2^- spontaneously dismutates to H_2O_2, which is membrane permeable. O_2^- and H_2O_2 contribute to killing the pathogen, whereas H_2O_2 also participates in the oxidative cross-linking of cell wall proteins and regulation of host gene expression (Mehdy, 1994)

Figure 2.2 presents a working model that depicts possible signalling pathways leading to the production of extracellular AOS during the HR. The actual identities of the components and their location in the signal-transduction pathway remain to be clarified (Mehdy, 1994). Several plant receptors that bind plant and fungal cell wall-derived elicitors have been localized in the plasma membrane (Mehdy, 1994). In soybean, plant cell wall-derived polygalacturonic acid and fungal cell wall-derived carbohydrate/(glyco) protein preparations are known to stimulate the oxidative burst, and their receptors were found to be on the plasma membrane (Horn *et al.*, 1989). It is likely that other fungal or plant cell wall carbohydrates or (glyco) protein elicitors that promote the oxidative burst also bind to plasma membrane-bound receptors. Agents known to interact with heteromeric G proteins were shown to promote AOS generation in the presence or absence of elicitor (Mehdy, 1994).

G-proteins are coupled to transmembrane receptors, where they link ligand reception at the cell surface to intracellular signal release. Ligand binding to the receptor results in binding of GTP to the α -subunit of an intracellular heterotrimeric G-protein complex, which activates the α -subunit and releases it from the $\beta\ \gamma$ -subunit comple.g. The released α -subunit then activates an effector such as adenylate cyclase or phospholipase C, and the GTP is hydrolyzed to GDP (Mehdy, 1994; Dixon *et al*, 1994).

AOS generation in several species appears to depend on increased intracellular Ca^{2+} and protein kinase activation. Depletion of Ca^{2+} in the medium reduced active oxygen formation, whereas a Ca^{2+} ionophore induced active oxygen formation (Mehdy, 1994). One reasonable pathway is that the receptor coupled to a G protein leads to Ca^{2+} influx, which then activates a Ca^{2+} -dependant protein kinase and ultimately the O_2^- -generating oxidase (Mehdy, 1994) (Fig. 2.2).

It is likely that the oxidase resides in the plasma membrane or is associated with its external surface, because the release of O_2^- is extracellular (O_2^- is poorly diffusible across membranes). The NADH-dependant oxidase associated with the oxidative burst in plants may be structurally related to the multisubunit NADPH oxidase in mammalian phagocytes responsible for O_2^- generation (Mehdy, 1994).

2.1.4 Regulation of Plant Responses

Phytoalexin accumulation is stimulated by the specific activation of genes encoding the appropriate biosynthetic enzymes (Dixon and Lamb, 1990). Rapid increase in transcription rates of these genes are generally accompanied by the activation of an entire set of additional plant defense genes (Dixon and Lamb, 1990). Plant gene activation is a transient process, which involves receptor mediated transmembrane signalling. Efficient regulation requires the removal of the elicitor from its primary target site in the plasma membrane (Scheel, 1998).

The increased production of AOS during the HR leads to the induction of the genes encoding for the cytosolic isozymes of SOD and APX in tobacco plants infested with TMV (Mittler *et al.*, 1996). The regulation of cAPX expression during the HR is controlled at mRNA and protein levels (Mittler *et al.*, 1998). Transcripts encoding cAPX are induced during the HR and the levels of cAPX protein are suppressed. Increases in mRNA levels occur as part of the antioxidative response of plants to elevate AOS production, while the suppression of cAPX protein, is thought to be unique to the HR, and reflects the need for increased production of AOS (Mittler *et al.*, 1998). This mode of regulation may result from the dual role that AOS play in the life of plants. They are toxic compounds that are produced during stress and need to be scavenged. AOS accumulation is also required for the defense response of plants against invading pathogens (Mittler *et al.*, 1999).

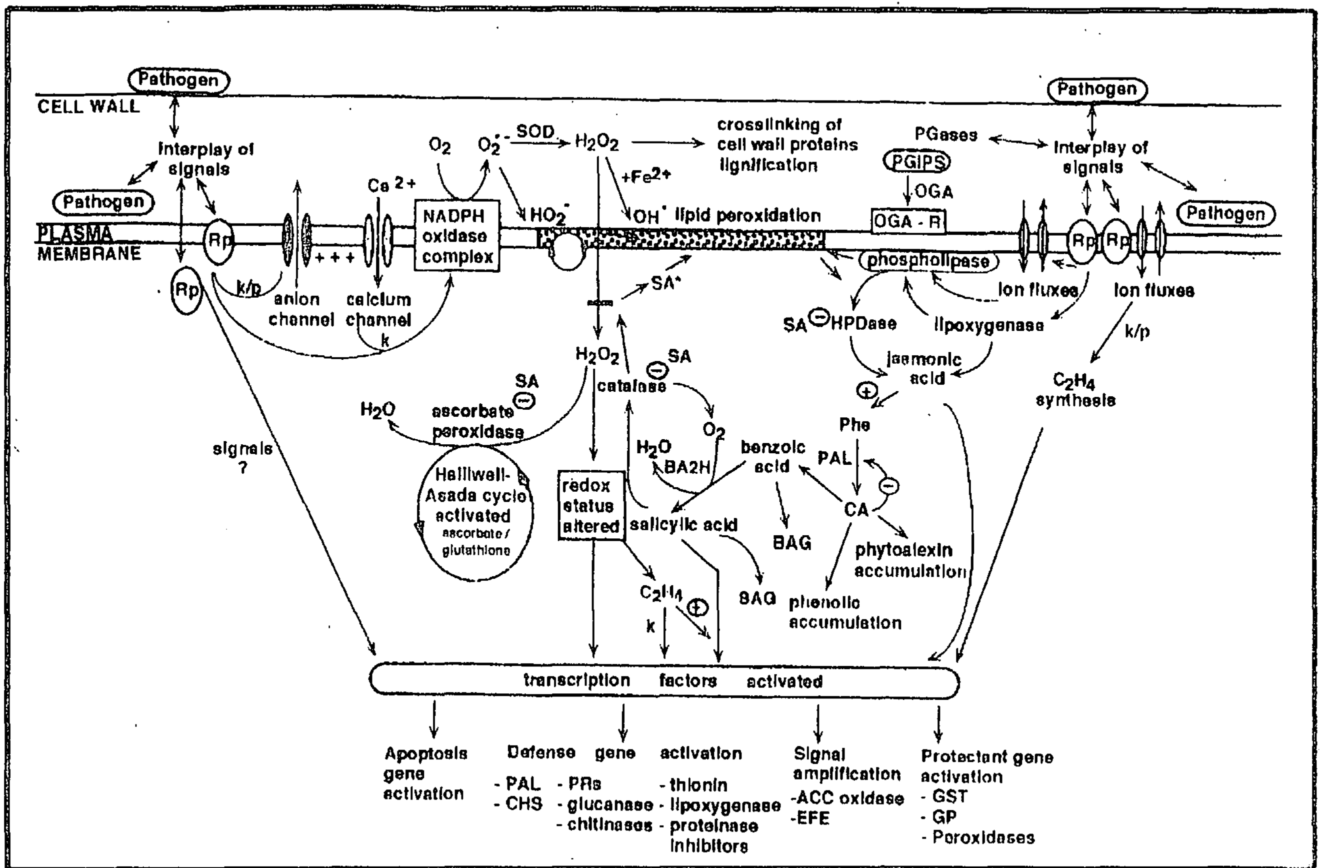


Figure 2.3 Signaling events controlling activation of defense genes (Hammond-Kosack and Jones, 1996). [ACC oxidase, 1-aminocyclopropane-1-carboxylate oxidase; BAG, benzoic acid glucoside; BA2H, benzoic acid-2 hydroxylase; CA, cinnamic acid; CHS, chalcone synthase; EFE, ethylene-forming enzyme; HO_2, hydroperoxyl radical; HPDase, hydroxyperoxide dehydrase; GP, glutathione peroxidase; GST, glutathione S-transferase; k, kinase; $O_2^{\cdot-}$, superoxide anion; hydroxy radical; OGA and OGA-R, oligalacturonide fragments and receptor; p, phosphatase; PAL, phenylalanine ammonia-lyase; Pgases, polygalacturonases; PGIPS, plant polygalacturonic acid inhibitor proteins; Phe, phenylalanine; PR, pathogenesis related; Rp, plant receptor protein; SA, salicylic acid; SAG, salicylic acid glucoside; SA*, SA radical; SOD, superoxide dismutase; (+) indicates positive and (-) indicates negative interactions] (Hammond-Kosack and Jones, 1996)

Plant receptor proteins (Rp) intercept pathogen-derived or interaction-dependent signals. These signals include the direct or indirect products of *Avr* genes, physical contact, and common components in an organism, such as chitin, enzymes, and plant cell wall fragments. Plant receptor proteins may or may not be the products of *R* genes. The immediate downstream signalling events are not known, but involve kinases, phosphatases, G proteins, and ion fluxes (Fig. 2.3). Several distinc and rapidly activated outcomes are recognized, including the production of AOS, direct induction of defense gene transcription, or possibly apoptosis genes, jasmonic acid (JA) biosynthesis, and/or ethylene biosynthesis. Amplification of the initial defense response occurs through the generation of additional signal molecules, that is, other AOS, lipid peroxides, benzoic acid (BA), and SA. These, in turn, induce other defense-related genes and modify defense proteins and enzymes (Sticher *et al.*, 1997).

Concomitant alterations to cellular redox status and/or cellular damage will activate preformed cell protection mechanisms (that is, the Halliwell-Asada cycle, plastid-localized SODs, and catalase) and induce genes encoding various cell protectants (Fig. 2.3). Defense related stress might also induce cell death. Cross-talk between the various induced pathways will coordinate the responses (Hammond-Kosack and Jones, 1996).

2.2 PHENYLALANINE AMMONIA-LYASE ACTIVITY

Plants react to invasion by potentially pathogenic micro-organisms with an array of inducible biochemical defenses, including induction of the phenylpropanoid pathway, which leads to phytoalexin and lignin biosynthesis (Kuhn *et al.*, 1984; Cahill and McComb, 1992), and localized synthesis of callose (Grosskopf, *et al.*, 1991). PAL is a key enzyme linking primary and secondary metabolism and plays an important role in the regulation of these biosynthetic routes leading to the production of phenolic compounds and lignin biosynthesis (Cahill and McComb, 1992; Tena and Valbuena, 1982).

In the initial establishment phase of a pathogen within host tissue, PAL activity often increases and in several host-pathogen interactions increased levels have been shown to be correlated with incompatibility (Hughes and Dickerson, 1989; Miklas *et al.*, 1993). In diseases caused by *Phytophthora spp.* there is good evidence that PAL plays a key role in the development of resistance (Cahill and McComb, 1992). The regulation of the production of the phytoalexin, glyceollin, in soybeans in incompatible interactions with *P. megasperma* f.sp. *glycinea* is closely associated with increased PAL activity and it has been demonstrated that there is increased transcription of PAL mRNA (Coquoz *et al*, 1998). Changes in PAL activity and transcription generally do not occur in compatible interactions. The switching-on of PAL genes is thus an important early step in the development of incompatibility in such systems (Cahill and McComb, 1992). Observations made by Mauch-Mani and Slusarenko (1996) showed that the PAL promoter in *Arabidopsis* was suppressed by specific inhibition of PAL activity in pathogen-treated tissue. That indicated that a product of the phenylpropanoid pathway is involved in a feedback stimulation of the PAL gene. SA has been reported to potentiate the expression of PAL and other defense-related genes, allowing higher levels of expression in response to elicitors (Shirasu *et al.*, 1997).

PAL catalyzes the elimination of NH_3 from *L*-phenylalanine (Fig 2.4) to form *trans*-cinnamic acid (Hanson and Havir, 1981). This reaction is the first step in the phenylpropanoid pathway

and its product, *trans*-cinnamic acid, provides phenyl propane sceletons which can serve as building blocks for lignin or to be utilized in the synthesis of flavonoids and other phenolic derivatives.

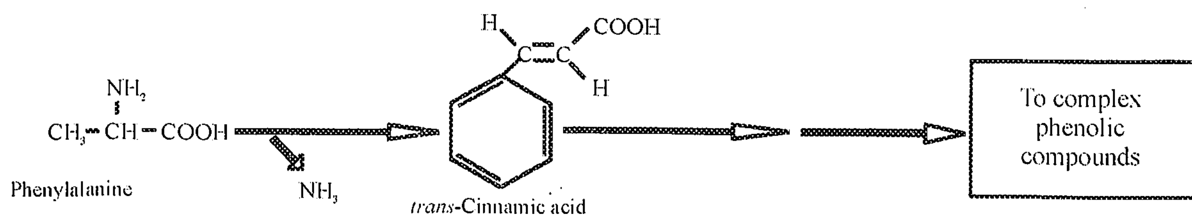


Fig. 2.4 The deamination of L-phenylalanine catalyzed by PAL. *Trans*-cinnamic acid a phenylpropane, is an important building block of more complex phenolic compounds. (Taiz & Zeiger, 1991)

Different mechanisms of PAL activity regulation, including induction of its synthesis by environmental factors, allosteric effects, and product and macro molecular inhibition have been documented (Shirasu *et al.*, 1997).

Although PAL, where isolated, is a relatively stable enzyme, its activity can be lost in tissues and crude extracts (Hanson and Havir, 1981). This loss in activity can be contributed to enzymes hereafter referred to PAL-IS (IS – inactivating system) Figure 2.5 gives an explanation for the regulation of PAL activity.

1. The stimulus initiates transcription and translation of the PAL gene. PAL-IS is already present at a steady state level, so that the first order of decay takes place as soon as it is formed. A steady state plateau of activity is reached when PAL synthesis is maintained.
2. PAL-IS is unstable or subject to inactivation. There is coinduction of both PAL and PAL-IS, and production of mRNA for both decreases.
3. Cinnamate and other metabolite levels may directly affect the rate of translation on cytoplasmic ribosomes of mRNA for PAL and, perhaps PAL-IS.

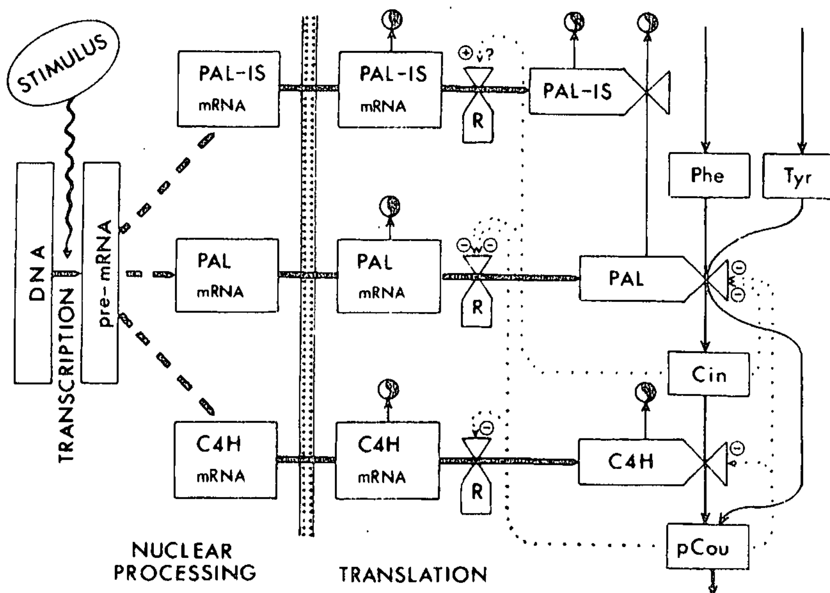


Figure 2.5 Postulated control of PAL, Cinnamate 4-hydroxylase, and the PAL inactivation system. No distinction is made in this scheme between different regions of the cytoplasm, although differences between chloroplast and extrachloroplast PAL have been reported. Dotted lines indicate positive or negative modulation of ribosome or enzyme activity (Hanson and Havir, 1981).

4. The above also applies to other enzymes associated with phenylpropanoid metabolism such as 4-hydroxylase flavanone synthase, and the transferase leading to chlorogenic acid formation (Fg. 2.5).

Additional possibilities:

5. PAL mRNA may be stored in a protected form for emergency use.
6. A precursor of PAL may accumulate and be converted to PAL at a fixed or variable rate.
7. PAL may be stored bound to a proteinaceous inhibitor. An increase in PAL activity could occur through slow dissociation followed by destruction of the inhibitor. A loss in PAL activity could be the result of synthesis of more inhibitor (Hanson and Havir, 1981).

2.2.1 CHARACTERISTICS OF PAL

PAL has been purified and characterized from a number of plant and fungal sources, but there are no reports of the occurrence of the enzyme being in animals. The enzyme is also present in certain algae, e.g., *Dunaliella marina* (Hanson and Havir, 1981).

PAL not only occurs in the cytoplasm, but also in the plastids, mitochondria and microbodies. The cell or the cell organelles release most of the enzyme activity, but a portion remains associated with miscellaneous membrane fragments (microsomal fractions) and with thylakoid preparations from chloroplasts (Hanson and Havir, 1981).

PAL activity provides precursors for lignin biosynthesis and other phenolics that accumulate in response to infection, e.g. SA. It is shown that SA is essential for systemic acquired resistance (SAR) and for the expression of genetic determined primary resistance (Grosskopf *et al.*, 1991). One function of SA might be to inhibit catalase activity, which, by removing H_2O_2, suppresses the oxidative burst necessary for the HR (Mauch-Mani and Slusarenko, 1996).

Southern blot analysis indicated that, in tobacco, a small family of two or four clustered genes encodes PAL. Northern blot analysis shows that PAL genes are weakly expressed under normal physiological conditions, they are moderately and transiently expressed after wounding, but they are strongly induced during the hypersensitive reaction in response to tobacco mosaic virus or in response to a fungal elicitor. Ribonuclease protection experiments confirmed this evidence and showed the occurrence of two highly homologous PAL messengers originating from a single gene or from two tightly co-regulated genes. .By *in situ* RNA-RNA hybridization PAL transcripts were shown to accumulate in a narrow zone of leaf tissue surrounding necrotic lesions caused by tobacco mosaic virus infection or treatment with the fungal elicitor (Pellegrini *et al.*, 1994)

2.2.2 PAL LINKING PRIMARY AND SECONDARY METABOLISM.

SA is synthesized from cinnamic acid by decarboxylation and side chain shortening to benzoic acid, followed by hydroxylation (Mauch-Mani and Slusarenko, 1996). Radiolabeling proved that SA is synthesized from phenylalanine and that both cinnamic acid and benzoic acid are intermediates in the biosynthetic pathway (Coquoz *et al.*, 1998). SA is required for signal transduction at the local level and that its mode of action may include inhibition of

catalase activity, leading to increased levels of H_2O_2 (Coquoz *et al.*, 1998). The hydroxylated and methoxylated cinnamic acid lignin precursors are synthesized from CoA esters in a two-step process via cinnamoyl-CoA reductase and cinnamyl alcohol dehydrogenase (CAD). CAD activity is regarded as specific for lignin synthesis and was reported to increase rapidly after infection (Mauch-Mani and Slusarenko, 1996).

2.3 PHENOLICS

Plants produce a large variety of secondary products containing a phenol group, a hydroxyl function on an aromatic ring. These substances are classified as phenolic compounds. Phenols play a variety of roles in the plant. Many of them have some role in defense against herbivores and pathogens. Others function in mechanical support, in attracting pollinators and fruit disperses (fragrances), or in reducing the growth of nearby competing plants (Taiz and Zeiger, 1991).

The shikimic acid pathway and the malonic acid pathway are the two basic pathways in which plant phenolics are biosynthesized. The shikimic pathway participates in the biosynthesis of most plant phenolics (Taiz and Zeiger, 1991).

Most classes of secondary phenolic compounds in plants are derived from phenylalanine and tyrosine, and in most plant species the key step in their synthesis is the conversion of phenylalanine to cinnamic acid by the elimination of an ammonia molecule. This reaction is catalyzed by PAL, an important regulatory enzyme of secondary metabolism. In a few plants, particularly grasses, the key reaction in phenolic formation appears to be the analogous conversion of tyrosine to 4-hydroxycinnamic acid.

The activity of PAL in plants is under the control of various external and internal factors, such as hormones, nutrient levels, light, fungal infection, and wounding. Fungal invasion, for example, triggers the transcription of messenger RNA that codes for PAL, thus increasing the synthesis of PAL in the plant and stimulating the synthesis of phenolic compounds (Fig. 2.6) (Taiz and Zeiger, 1991).

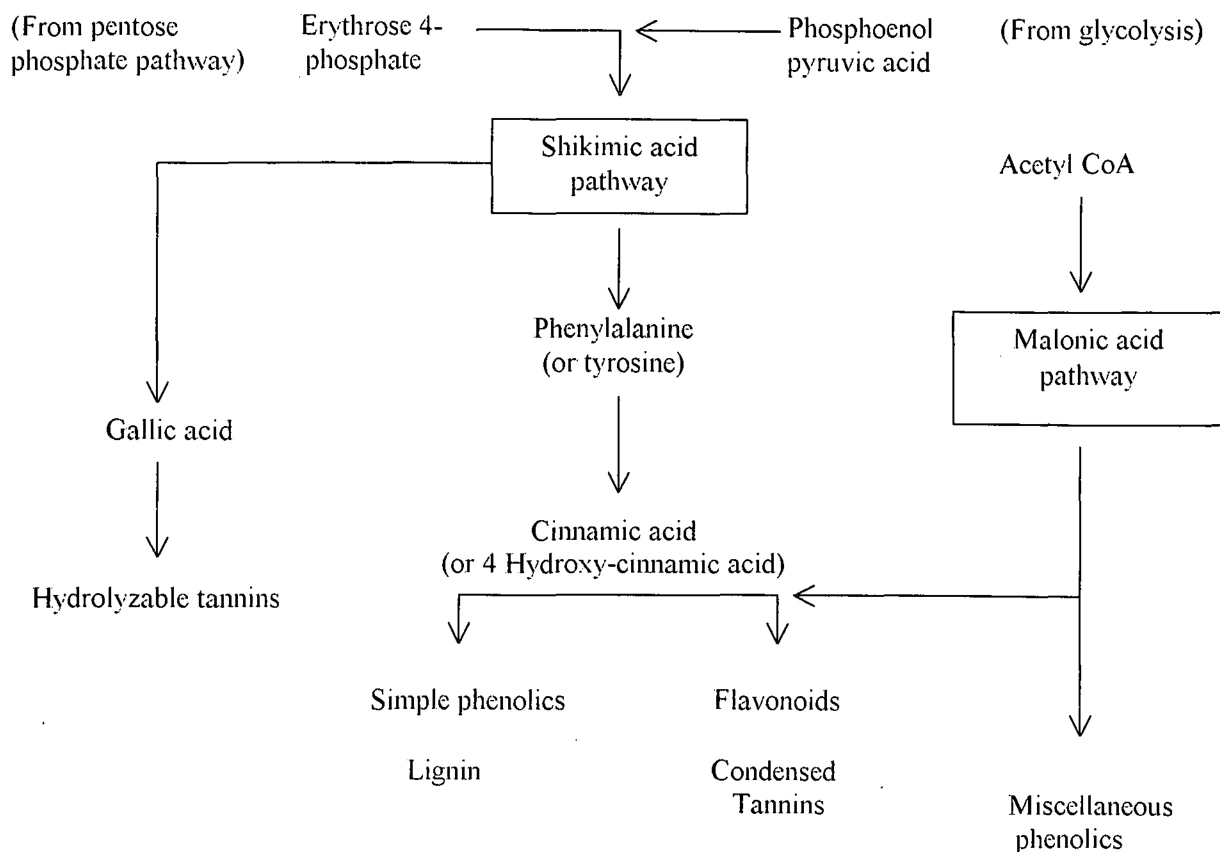


Fig 2.6. Plant phenolics are biosynthesized in several different ways. In higher plants, most secondary phenolics are derived at least in part from phenylalanine, a product of the shikimic acid pathway (Taiz and Zeiger, 1991).

2.3.1 Physical and Chemical properties

Phenols are colourless in the pure form and they tend to be sensitive to oxidation and may turn brownish or dark when exposed to air. These phenolics are normally soluble in polar organic solvents unless they are completely esterified or glycosylated. Water solubility increases with the number of hydroxyl groups present. Phenolic substances are aromatic and therefore have intense absorption in the UV region of the spectrum (Van Sumere, 1989). Phenolics make up a vast class of compounds, comprising anthocyanins, leucoanthocyanins, anthoxanthins, hydroxybenzoic acids, glycosides, sugar esters of quinic and shikimic acids, esters of hydroxycinnamic acids, and coumarin derivatives (Goodman *et al.*, 1967).

Phenolic substances are known to participate in a number of physiological processes, which are essential for growth and development, such as oxidation-reduction reactions, lignification, and stimulation as well as inhibition of auxin activity. Phenols and their oxidation products (quinones) are also potent uncouplers of oxidative phosphorylation, inhibitors of enzymes,

and chelators of metal cofactors (Misaghi, 1982).

2.3.2 Synthesis and induction of phenols

The response of plants to pathogens based on host and non-host interactions are characterized by the early accumulation of phenolic compounds at the infection site and that limited development of the pathogen occurs as a result of rapid (hypersensitive) cell death (Nicholson and Hammerschmidt, 1992).

Rapid accumulation of phenols may result in the effective isolation of the pathogen (or non-pathogen) at the original site of ingress. These responses include the formation of lignin, the accumulation of cell-wall appositions such as papillae, and the early accumulation of phenols within the host cell walls (Sherwood and Vance, 1976). Low molecular weight phenols, such as the benzoic acids and the phenylpropanoids, are formed in the initial response to infection. Evidence strongly suggested that the esterification of phenols to cell wall materials is a common theme in the expression of resistance. The accumulation of polymerized phenols occurs as a rapid response to infection. A common host response is the esterification of ferulic acid to the host cell wall, and it has been suggested that cross linking of such phenylpropanoid esters leads to the formation of lignin like polymers (Nicholson and Hammerschmidt, 1992).

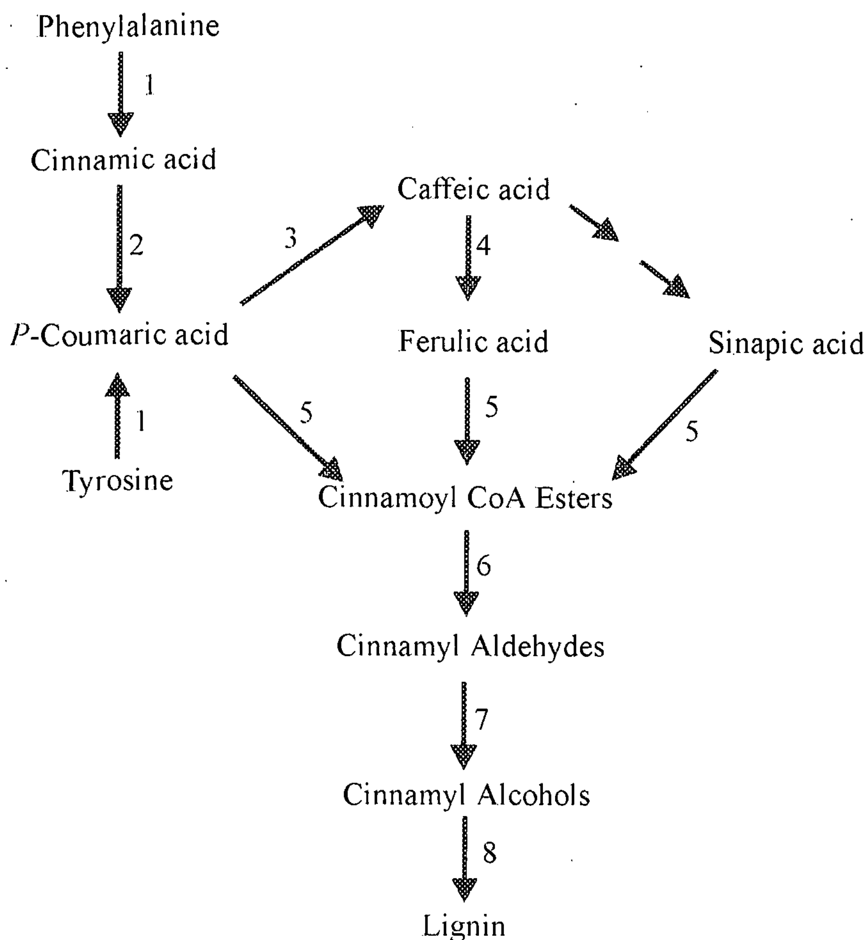


Figure 2.7 Metabolic pathway and enzymes involved in lignin biosynthesis: 1. PAL. 2. cinnamic acid-4-hydroxylase, 3. *p*-coumaric hydroxylase, 4. *o*-methyltransferase, 5. cinnamate acid-CoA-oxidoreductase. 7. cinnamyl alcohol dehydrogenase, 8. peroxidase (Vance *et al.*, 1980)

The pathway of lignin biosynthesis and the enzymes involved are well established (Figure 2.7). PAL catalyzes the conversion of phenylalanine to cinnamic acid. Cinnamic acid is hydrolyzed by cinnamic acid-4-hydroxylase to form *p*-coumaric acid; however *p*-coumaric acid may also be formed by the deamination of tyrosine catalyzed by tyrosine ammonia-lyase. *p*-Coumaric acid is further hydroxylated by *p*-coumaric acid hydroxylase to give caffeic acid. Sinapic acid is formed by hydroxylation and methylation of ferulic acid. Coumaric-, ferulic-, and sinapic acid are converted to their respective CoA esters by cinnamate acid-CoA-ligase. The esters of cinnamic acid derivatives are reduced to their corresponding aldehydes and further reduced to alcohols by cinnamoyl-CoA-oxidoreductase and cinnamyl alcohol dehydrogenase. The final step in biosynthesis is the oxidation of the cinnamyl alcohols to free radicals by peroxidase/H_2O_2 (Vance *et al.*, 1980).

2.3.3 DEFENSE STRATEGY

Resistance in plant-pathogen interactions is accompanied by the rapid employment of a multicomponent defense response. The individual components of this include the hypersensitive reaction (HR), chemical weapons and structural defensive barriers (Dixon *et al.*, 1994). Signals for activation of these various defenses are initiated in response to recognition of elicitors by plant receptors. The defense response may be induced specifically or nonspecifically by a range of biotic and abiotic elicitors (Dixon *et al.*, 1994).

Ferulic and *p*-coumaric acids in corn leaves infected with *Colletotrichum graminicola* were inhibitory to spore germination (Nicholson *et al.*,1989). Several unidentified phenolic compounds were fungitoxic to *Colletotrichum trifolli* in incompatible interactions with sorghum (Nicholson *et al.*,1987) and accumulation of phenolic compounds in clones of *Medicago sativa* were responsible for resistance to certain races of *C. trifolli* (Baker *et al.*, 1989). Resistance of oats to *Erysiphe graminis*, phenolic compounds contributed greatly to the high level of resistance (Carver *et al.*, 1996). Sinapic acid and and an unknown compound 'C' was found to accumulate in wheat challenged with stem rust (Menden *et al.*, 1994). *p*-Coumaric acid and ferulic acid has been found to be the dominant phenolic acids in wheat to cereal aphids (Havlič kova *et al.*, 1996).

Salicylic acid (SA) performs a central role in mediating systemic acquired resistance (SAR) in incompatible plant-pathogen interactions (Ryals *et al.*, 1996; Cao *et al.*, 1997, Malamy *et al.*, 1992, 1996; Rasmussen *et al.*, 1991). This is also the case regarding the interaction between resistant wheat and the RWA (Mohase, 1998). Exogenous applied SA resulted in induced SAR (Mohase, 1998; Malamy *et a.l*, 1996) thus indicating that SA is an important in signalling. Another phenolic acid that has been found to induce systemic acquired resistance is gentisic acid (GA) (Bellés, *et al.*, 1999). SA can be converted to GA in certain species displaying incompatible interactions (Schultz *et al.*, 1993; Bellés, *et al.*, 1999). SA and GA induce different PR-proteins in tomato challenged with tomato mosaic virus. The fact that SA and GA induce different PR-proteins suggest that SA and GA plays a complementary signalling role in the activation of defenses (Bellés, *et al.*, 1999)

The defense mechanism exists in two parts: the first is assumed to involve the rapid accumulation of phenols at the infection site, which functions to slow or even halt the growth of the pathogen and to allow for the activation of "secondary" strategies that would be more

thoroughly to restrict the pathogen. Secondary responses would involve the activation of specific defenses such as the *de novo* synthesis of phytoalexins or other stress related substances (Matern and Grimmig 1994). The initial defense must occur so rapidly that it is unlikely to involve *de novo* transcription and translation of genes, which would be a characteristic of the second level of defense. Thus the sequence of events in a defense response can be thought to include host cell death and necrosis, accumulation of toxic phenols, modification of cell walls by phenolic substituents or physical barriers such as appositions or papillae, and, finally, the synthesis of specific antibiotics (Nicholson and Hammerschmidt, 1992).

Studies have shown that rapid necrosis of mesophyll cells within hours of infection distinguished the incompatible from the compatible response. Histochemical staining of tissue with toluidine blue together with clearing and fluorescence analysis demonstrated that accumulation of phenols at the infection site occurred as early as 3 hours after inoculation, indicating an association of phenols with the initial stages of the response. This included cell death and necrotization at the site of initial penetration. The infection site is not the site of maximum PAL response. Rather, the healthy as yet uninfected cells surrounding the infection site exhibits a marked accumulation of PAL m-RNA. This is a transient response in that by 6 hours after inoculation, the accumulation of PAL messenger fell markedly and was only marginally greater than that of non-inoculated tissue. In a compatible interaction with the same host cultivar, the PAL messenger become progressively elevated as the time after inoculation lengthened, and the pattern of RNA accumulation was diffused throughout the tissue rather than localized within the immediate zone of infection site. The PAL gene activation that occurs in a zone of living cells surrounding the area of cell death at the infection site is presumed to require signal transmission in advance of the fungus intrusion (Nicholson and Hammerschmidt, 1992).

MATERIALS AND METHODS

CHAPTER 3

3.1 MATERIALS

3.1.1 CHEMICALS

All chemicals were of analytical grade or HPLC grade where applicable.

3.1.2 PLANT MATERIAL

Wheat (*Triticum aestivum*) plants, resistant (cv Tugela DN) and susceptible (near isogenic cv. Tugela) to the Russian wheat aphid *Diuraphis noxia* (Mordvilko) were grown in a glasshouse at a night temperature of 21 °C and a day temperature of 25 °C. Leaves were infested with Russian wheat aphids (20-30 aphids per plant), when the plant reached the second leaf stage.

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

3.2 METHODS

Enzyme activities were determined in triplicate (n=3) on the same plant material and the standard deviation of enzyme activity values were calculated using Microsoft excel and the graphs were created using Sigma Plot. In most cases the tendencies of graphs were confirmed in additional experiments.

3.2.1 DETERMINATION OF PHENYLALANINE AMMONIA-LYASE (PAL) ACTIVITY.

PAL activity was determined spectrophotometrically as described by Smith-Becker *et al.* (1998). Leaf tissue of the entire plant was ground by means of a mortar and pestle and acid washed sand in 0.1M Na-borate buffer (pH 8.8) containing 1mM ethylene diamine tetra-acetic acid (EDTA), 1mM 1,4-dithiothreitol, 60 mg insoluble polyvinylpolypyrolidone (PVP) and 60 mg Dowex 1 (1x4-200) basic anion exchange resin. Plant material: buffer ratio (1:12). The homogenate was centrifuged at 25 000g for 20 min at 4 °C and the resulting supernatant was used for PAL activity and protein determination.

The reaction mixture consisted of 0.1M (600µ l) Na-borate buffer (pH 8.8), 60mM, (100µ l) L-phenylalanine and 300µ l sample. The change in absorbance was measured

for 20 min at 290 nm (Hitachi U-200 double beam spectrophotometer) at 40 °C. Before starting the measurement the sample and 0.1M Na-borate buffer was incubated for 1 min at 40 °C. The activity is expressed as nmol cinnamic acid.h^{-1}.mg^{-1} protein.

A cinnamic acid calibration curve was composed using cinnamic acid concentrations ranging from 0.0001g.ml^{-1} to 0.001g.ml^{-1}. The absorbance of the different calibration samples was read at 290 nm at 40 °C (1A = 108 nmol cinnamic acid.ml^{-1}).

3.2.2 SEPARATION AND QUANTIFICATION OF PHENOLIC COMPOUNDS.

Leaf material was frozen in liquid N_2 and ground to a fine powder. The grounded material was hydrolysed in 2N HCl for 30min at 85 °C after which the solution was cooled down and filtered through a sintered glass filter. The filtrate was extracted 3x with 70ml diethylether whereafter the extract was dried under vacuum in a Buchi rotavapor. The residue was dissolved 95% (v/v) ethanol.

The phenolic compounds were separated on a HPLC (Spectra-Physics) using a UV detector (270nm) (Spectra-Physics) and a Luna 5µ C-18 reverse phase column (Phenomenex); 250 x 4.60mm. The column was developed at 1ml.min^{-1} with a mixture of acetonitrile (A), methanol (C) and 0.57% acetic acid (C). The following gradient was applied: starting conditions; 6% A, 88% B, 6% C and after 60 min ended with 6% A, 48% B, 46% C (Baiocchi *et al.*, 1993).

3.2.2.1 PHENOLIC STANDARDS.

The following phenolic standards were used: 3,5-dimethoxy-4-hydroxybenzoic acid, 4-hydroxy-3-methoxybenzoic acid (vanillic acid), 2,5-dihydroxybenzoic acid (gentisic acid), 3,4-dihydroxybenzoic acid (protocatechuic acid), 4-hydroxy-3,5-dimethoxybenzoic acid (syringic acid), 3,4-dihydroxycinnamic acid (caffeic acid), 4-hydroxy-3-methoxycinnamic acid (ferulic acid), α-3,5-resorcylic acid, p-hydroxyphenylacetic acid, 4-hydroxy-3,5-dimethoxybenzoic acid (syringic acid) , 3,5-dihydroxybenzoic acid, p-hydroxyphenylacetic acid, p-hydroxybenzoic acid, 4-hydroxycinnamic acid, salicylic acid.

3.2.3 AUTOFLUORESCENCE DETECTION OF LIGNIN.

Fresh leaves were boiled in 70% (v/v) ethanol to extract the chlorophyll from the leaves. The etiolated leaves were put in phloroglucin (2% phloroglucin in 95% ethanol) and after four hours the leaves were placed on a microscope slide and heated while 33.3% HCl was dripped on the leaves until the leaves displayed a red colour. The leaves were observed under a Nikon microphot-FXA fluorescence microscope. Lignin stains red with the phloroglucin/HCl (Moerschbacher, 1988).

3.2.4 DETERMINATION OF SUPEROXIDE DISMUTASE (SOD) ACTIVITY

The extraction procedure was done according to Rao *et al.* (1997). Frozen leaf tissue were ground in100mM potassium phosphate buffer (pH 7.5) containing EDTA and 1% PVP (plant material: buffer ratio is 1:12). The resultant extract was centrifuged (25000g) for 20 min. at 4°C. SOD activity was determined by measuring the inhibition of nitroblue tetrazolium (NBT) reduction according to the method of Keppler and Novacky (1987). The reaction mixture consisted of 50mM phosphate buffer (pH 7.8), 13mM methionine, 75μM NBT, 2μM riboflavin and 50μl sample. The reaction mixture was irradiated by placing it 30 cm below two fluorescent lamps (2x40W) for 10 min. A non-irradiated duplicate was used as a control (reference cuvette). After irradiation the absorbance of the reaction mixture was measured at 560 nm (Hitachi U-2000 double-beam spectrophotometer). In addition, a sample without crude extract was irradiated and used to measure the maximum attainable absorbance at 560 nm. The SOD activity is expressed as log [A_{560} (with crude extract) mg^{-1} / A_{560} (without crude extract)]. One unit of SOD is defined as the amount of enzyme that will inhibit the rate of ferricytochrome c reduction by 50% (Keppler and Novacky, 1987).

3.2.5 DETERMINATION OF HYDROGEN PEROXIDE (H_2O_2) CONCENTRATION.

H_2O_2 production is determined by monitoring the oxidative quenching of the fluorescent peroxide substrate, pyranine (8-hydroxypyrene-1,3,6-trisulfonic acid), according to Legendre *et al.*, (1993). Pyranine was protected from light and stored frozen. The sample (1.5 ml) is treated with 7μl pyranine (0.2 mg. ml^{-1}). The sample preparation was the same as for SOD. The H_2O_2 levels were determined after 10 min by measuring the decrease in pyranine fluorescence (due to H_2O_2-mediated oxidation

of the dye by plant peroxide). An excitation wavelength of 405 nm was used to measure the change in absorbance.

3.2.6 Determination of Peroxidase (POD) Activity.

Frozen leaf tissue was ground in liquid N_2 and proteins were subsequently extracted in 3 ml extraction buffer [100mM Na-acetate (pH 5.5), 10mM mercaptoethanol, 2mM EDTA, 2mM phenylmethylsulfonyl fluoride (PMSF)]. The homogenate was centrifuged (12 000g, 20 min) at 2 °C and the supernatant was used as crude enzyme.

The peroxidase assay mixture consisted of 10-30µ l crude enzyme, 40mM (500µ l) potassiumphosphate buffer (pH5.5), 5mM (100µ l) guaiacol and 8.2mM (340µ l) H_2O_2. The change in absorbance was measured at 470nm for 180 seconds at 30 °C (Hitachi U-2000 double-beam spectrophotometer) (Zieslin and Ben-Zaken, 1991).

3.2.7 Determination of Chitinase Activity.

Chitinase activity was measured according to the method of Wirth and Wolf (1990) with slight modifications. The method is based on the precipitability of CM-Chitin-RBV (Carboxymethyl-chitin-remazol brilliant violet 5R; Loewe Biochemica GmbH) in buffered solutions with HCl.

The reaction mixture for determining chitinase activity consisted of 66.6mM (280µ l) Na-acetate buffer (pH 5.0) and 100µl CM-chitin-RBV (2mg. ml^{-1}) and 20µ l sample. The reaction mixture was incubated at 37 °C and after 30 min the reaction was stopped by the addition of 100µl of 2M HCl. The sample was put on ice for 10 min to allow complete precipitation and subsequently centrifuged at 12 000g for 7 min. Before measuring the absorbancy at 550 nm, the sample was diluted 4x with distilled water. (Wirth and Wolf, 1990)

[The absorbancy values should be in the range of OD_{550nm} = 0.1 – 0.25 when measured against distilled water.]

3.2.8 Inhibition of the hypersensitive response by allopurinol.

The inhibitory effect of allopurinol (4-hydroxypyrazolo(3,4-*d*)pyrimidine) on the HR was done according to the method described by Montalbini (1992b). Allopurinol was applied to plants as a soil drench (0.4mM solution daily; 300ml in 0.016m^3 pots). Application was started 7 days after the wheat was planted and was continued until the last sampling date. Allopurinol is a purine analogue, a competitive inhibitor of xanthine oxidase, since it binds tightly to the reduced molybdenum component of the enzyme. Allopurinol inhibits xanthine oxidase activity and thus the production of oxygen radicals cannot take place (See fig. 5.2).

3.2.9 Determination Protein Concentration.

The protein concentration was determined using a Biorad microplate reader model 3550 (595 nm) as described by Rybutt and Parish (1982). The assay mixture consisted of 160μl distilled water, 40μl BioRAD and 10μl standard (0.5μg. μl^{-1} γ -globulin) (Bradford, 1976).

CHAPTER 4

RESULTS

4.1 Phenylalanine ammonia-lyase activity

The activity of PAL was doubled in infested resistant wheat; peaking 24 hours post infestation (h.p.i.) A less pronounced early induction occurred already 6h after infestation in the resistant wheat plants. Little change in PAL activity occurred in the uninfested resistant (R), infested susceptible and susceptible wheat plants (Fig. 4.1).

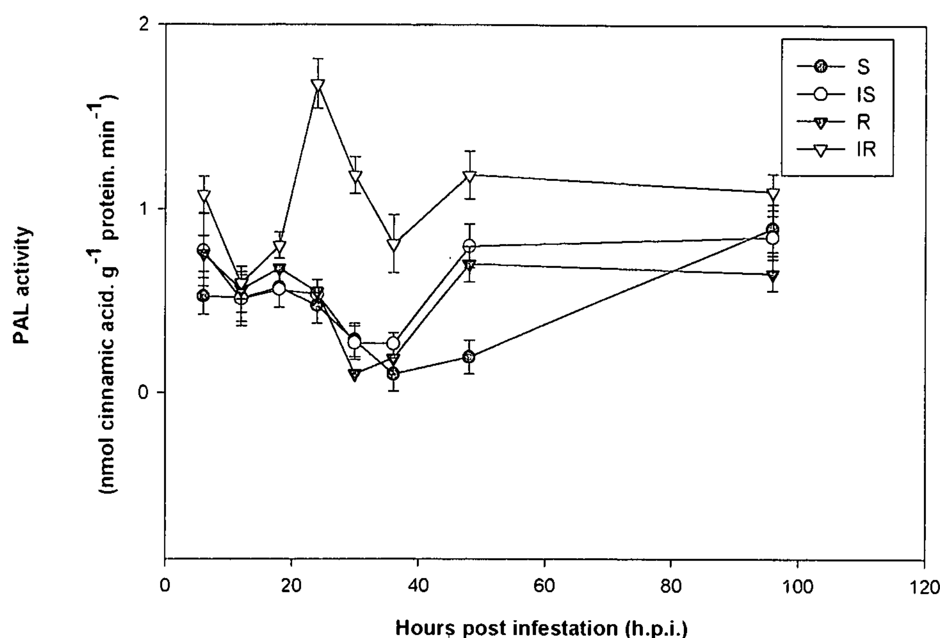


Figure 4.1 Effect of RWA infestation on PAL activity (I) of the susceptible (S), Tugela and resistant (R), Tugela DN wheat plants. Error bars indicate standard deviation. (n=3).

4.2 Phenolic compounds

Several of the phenolic acids tested, were differential induced in the infested resistant wheat. These phenolic acids include *p*-hydroxybenzoic acid,$_{\circ}$syringic acid, resorcylic acid, gentisic acid, caffeic acid, *p*-hydroxyphenylacetic acid and ferulic acid (Fig. 4.2). RWA infestation resulted in a 5-fold increase of gentisic acid concentration in infested resistant wheat, peaking at 264 h.p.i. Very little change occurred in the infested susceptible and uninfested resistant and susceptible wheat plants (Fig. 4.2 a). The *p*-hydroxybenzoic acid concentration was very early selectively induced to much

higher levels than those found in the corresponding controls (Fig. 4.2 b). A 9 fold increase in the concentration of *p*-hydroxyphenylacetic acid occurred as early as 12 h.p.i. in the infested resistant wheat, whereafter there was a 8 fold increase in concentration. Very little change occurred in the infested susceptible and the uninfested resistant and susceptible wheat controls (Fig. 4.2 c). The concentration of syringic acid in infested resistant wheat was selectively induced to a much higher (6 fold) level especially after 24 h.p.i. than in the infested susceptible and the unninfested susceptible and resistant wheat (Fig. 4.2d). RWA infestation resulted in a clear differential induction of caffeic acid (Fig. 4.2 e), ferulic acid (Fig. 4.2 f) and resorcylic acid (Fig 4.2 g) production in infested resistant wheat. The caffeic acid concentration was higher in the infested resistant wheat from 6 h.p.i. than in the controls and it reached a peak 24 h.p.i., which was 24 fold higher than the controls. The concentrations of caffeic, ferulic and resorcylic acid remained low throughout the investigation period in the infested susceptible and the uninfested susceptible, and resistant wheat. The ferulic acid concentration reached a peak (25 fold higher) 30 h.p.i. in the infested resistant wheat (Fig 4.2 f). The resorcylic acid concentration increased from 6 h.p.i. to much higher concentrations throughout the investigation period in the infested resistant wheat. (Fig. 4.2 g).

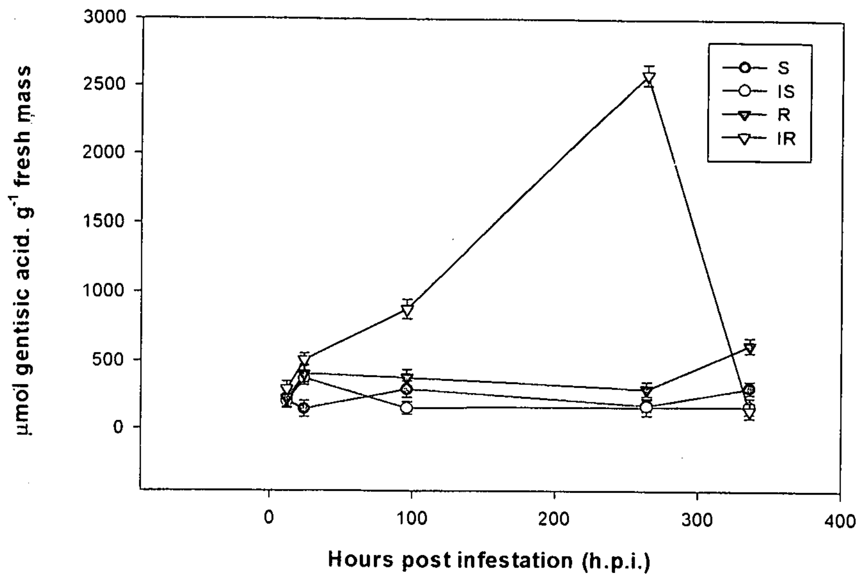


Figure 4.2 a

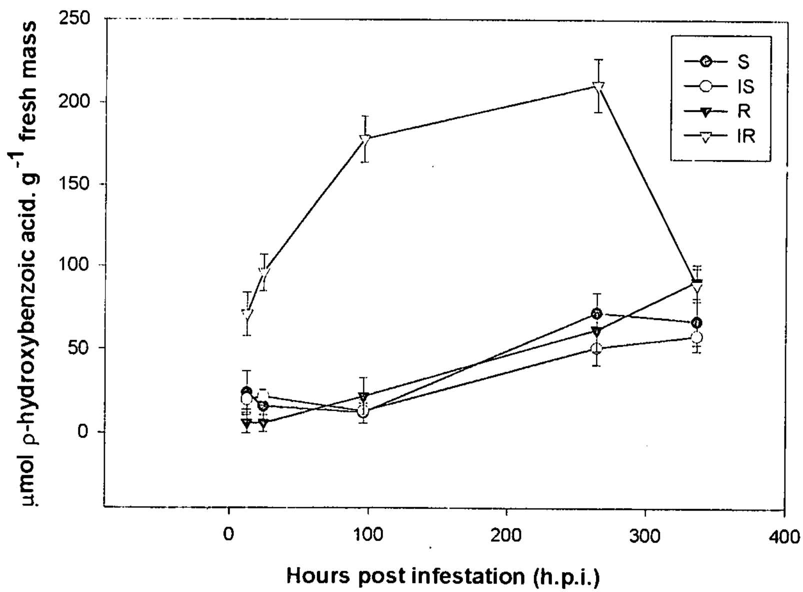


Figure 4.2 b

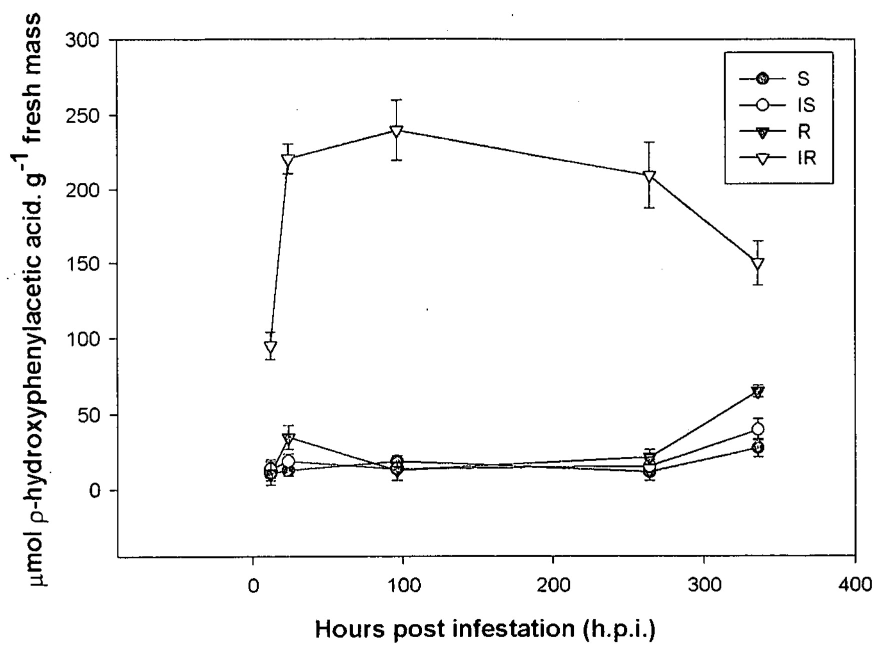


Figure 4.2 c

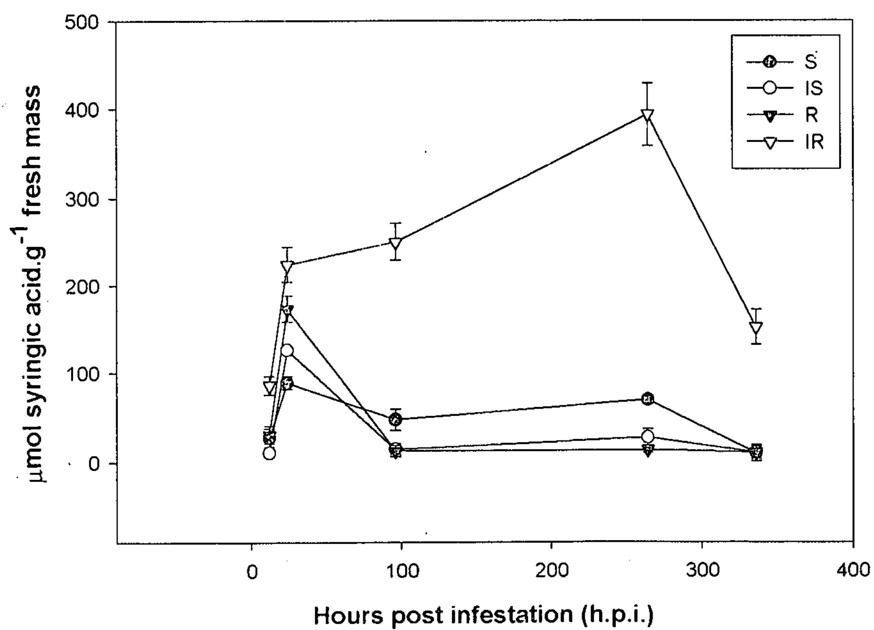


Figure 4.2 d

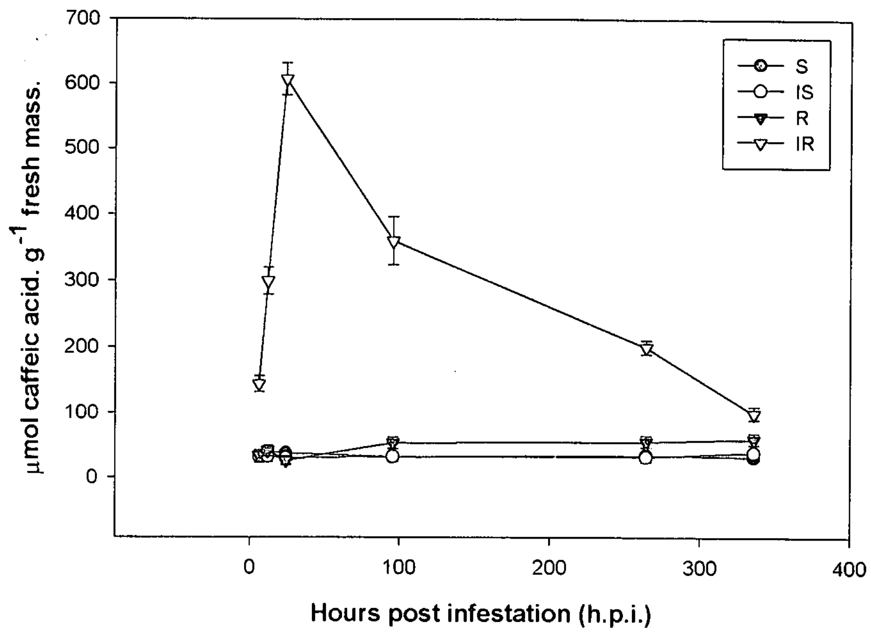


Figure 4.2 e

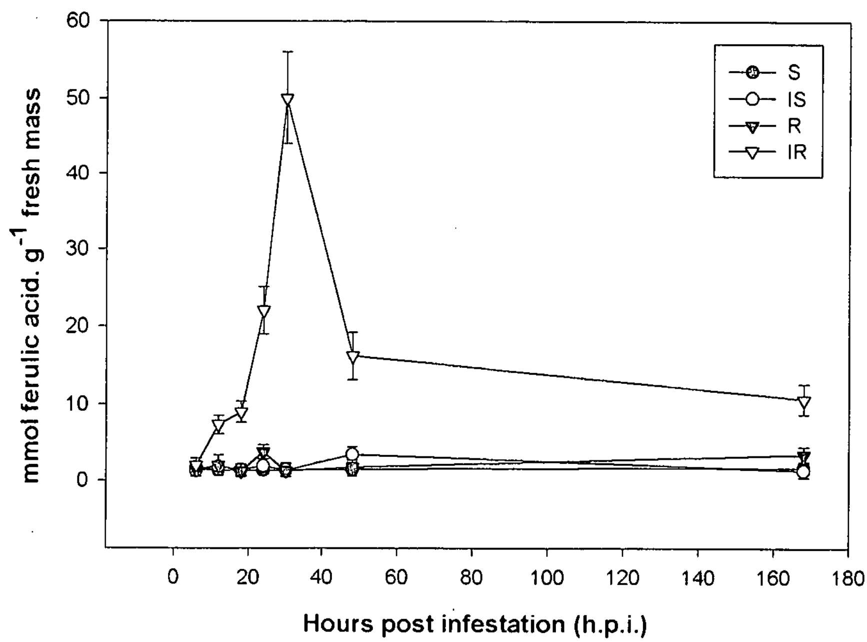


Figure 4.2 f

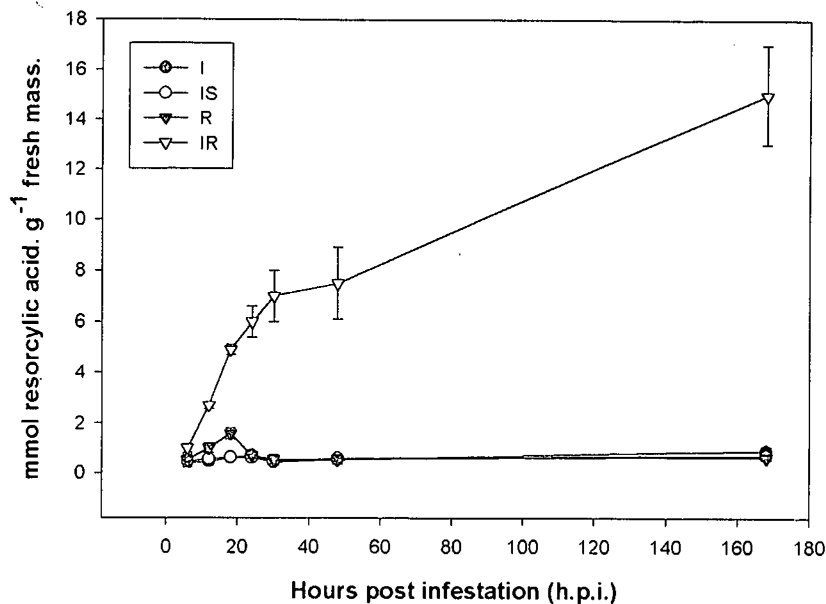
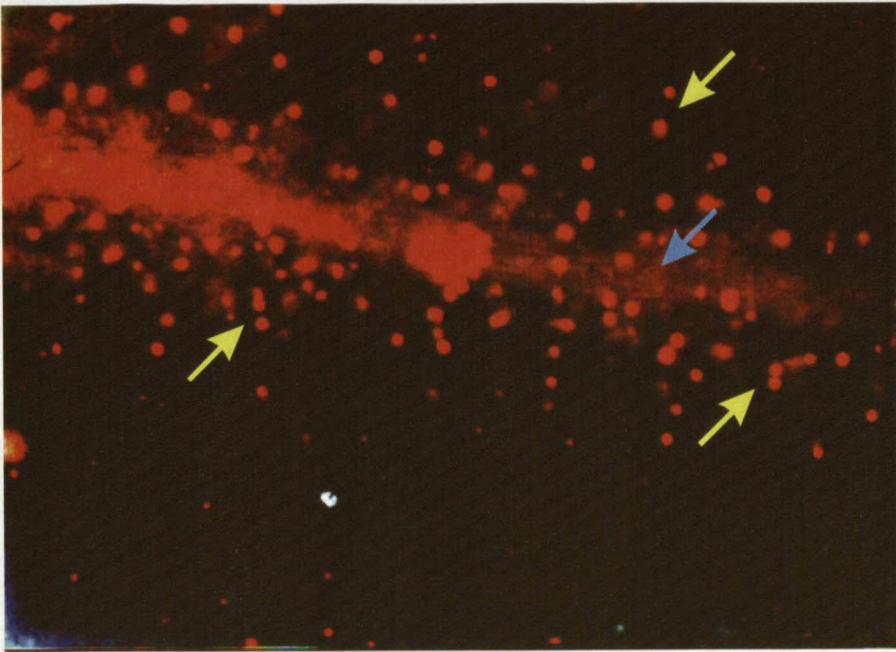


Figure 4.2 g

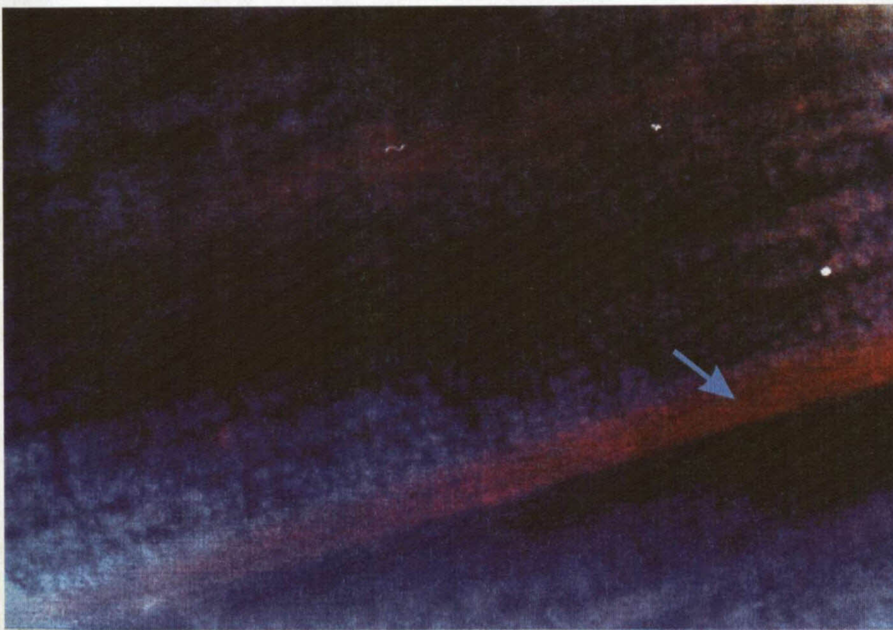
Figure 4.2 (a-g) The effect of RWA infestation (I) on the individual phenolic acid concentrations in the resistant (R), Tugela DN and susceptible (S), Tugela wheat plants. Error bars indicate standard deviation (n=2).

4.3 LIGNIFICATION

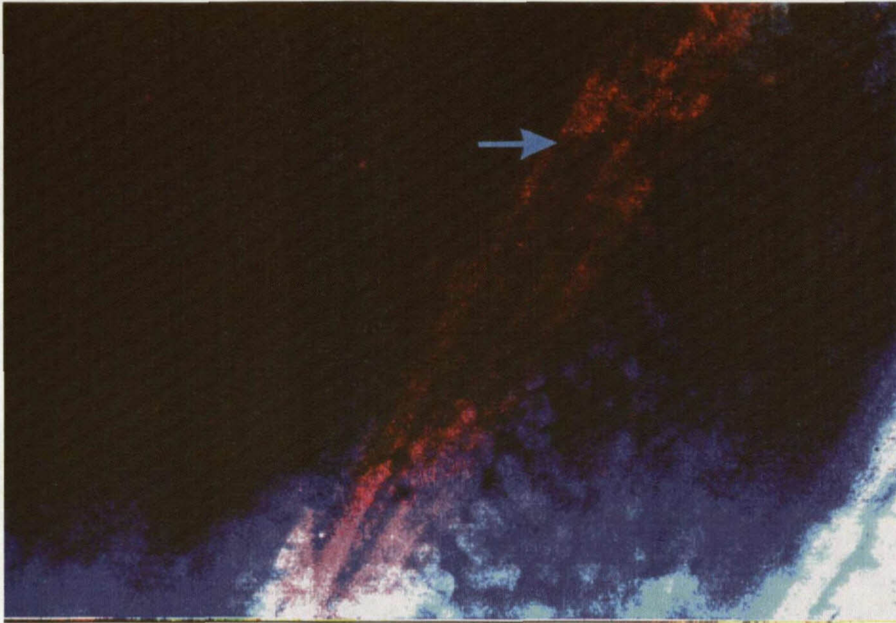
Phloroglucinol /HCl staining is specific for lignin which appears red, when viewed under a fluorescence microscope (Moerschbacher *et al.*, 1990; Sherwood and Vance, 1976). In Figure 4.3a lignified cells of infested resistant wheat leaves showed up as red dots, the yellow arrows indicate some of them. The lignified cells are concentrated around the vascular tissue of the leaf. These lignified cells were only observed in infested resistant wheat and no lignified cells formed in infested susceptible and the uninfested susceptible and resistant wheat plants (fig. 4.3 b-d). The red 'lines' that are seen in Figure 4.3 a-d, indicated by blue arrows, represent the vascular tissue of the wheat leaf which naturally contains lignin and thus coloured red.



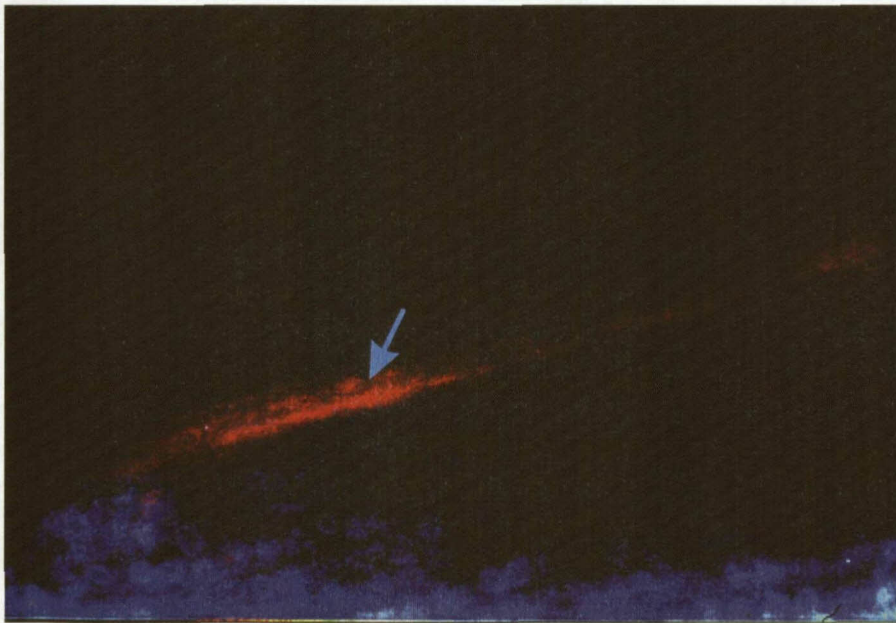
a.



b.



c.



d.

Figure 4.3 (a-d) The effect of RWA infestation on the anatomical structure of: a = infested resistant, Tugela DN wheat, b = uninfested resistant, Tugela DN wheat, c = uninfested susceptible, Tugela wheat, d = infested susceptible, Tugela wheat.

4.4 ACTIVE OXYGEN SPECIES

4.4.1 HYDROGEN PEROXIDE (H_2O_2) CONCENTRATION

H_2O_2 accumulated differentially in resistant wheat in response to RWA infestation. An early peak value was reached 8 h.p.i. The induction of H_2O_2 did not occur in the corresponding controls

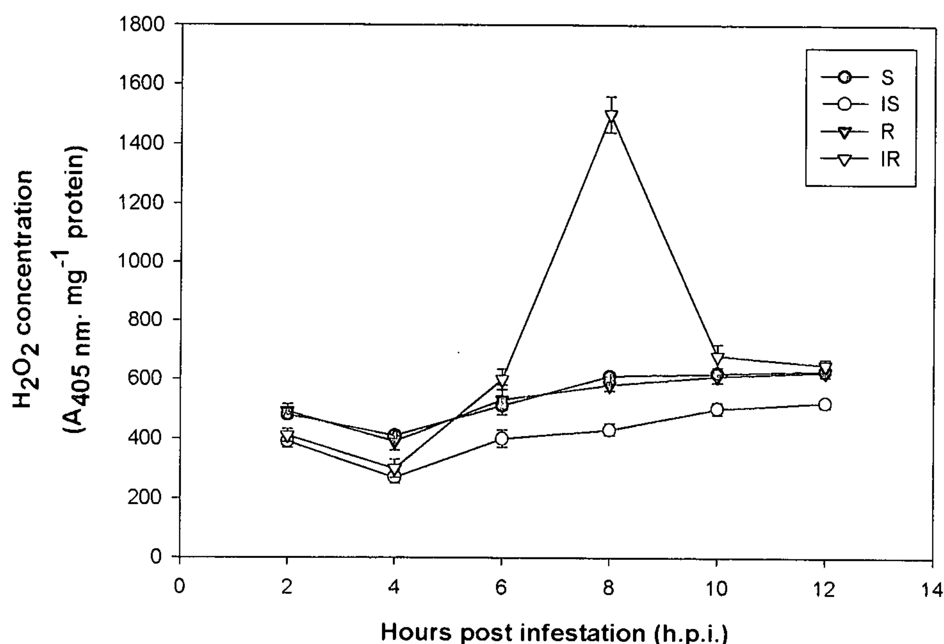


Figure 4.4 The effect of RWA infestation (I) on H_2O_2 concentrations of susceptible (S), Tugela and resistant (R), Tugela DN wheat plants. Error bars indicate standard deviation, (n=3).

4.4.2 SUPEROXIDE DISMUTASE ACTIVITY

RWA infestation led to a selective induction of SOD activity in the resistant wheat (Fig. 4.5) and the SOD activity remained constantly low during the investigation period in the infested susceptible and the uninfested susceptible and resistant wheat. The activity reached a peak 8 h.p.i. in the infested resistant wheat representing a 15 fold increase in activity relative to the controls.

150 067 85

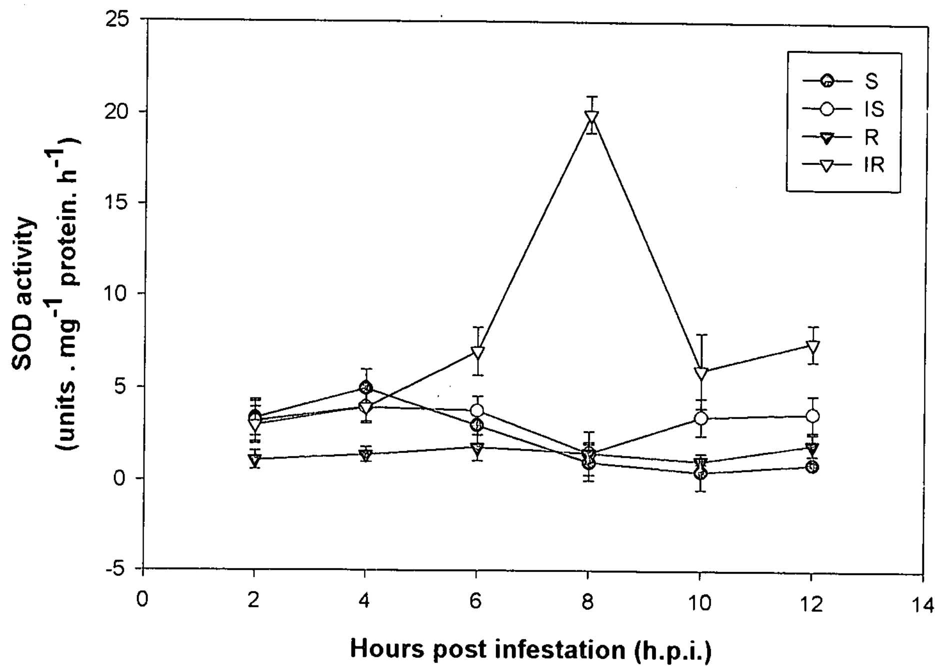


Figure 4.5 The effect of RWA infestation (I) on SOD activity in the susceptible(S), Tugela and resistant (R), Tugela DN wheat plants. Error bars indicate standard deviation, (n=3).

4.5 EFFECT OF ALLOPURINOL ON SOD ACTIVITY

Allopurinol inhibited the induced SOD activity by 85% (Fig. 4.6) in the infested resistant wheat. Figure 4.6 clearly illustrates again that RWA infestation selectively induced SOD activity to much higher levels in resistant wheat than in the controls.

4.6 EFFECT OF ALLOPURINOL ON PEROXIDASE ACTIVITY.

Allopurinol inhibited the induction of POD activity by 80% (Fig. 4.7) in infested resistant wheat. RWA infestation differentially induced POD activity in resistant wheat peaking at 30 h.p.i.

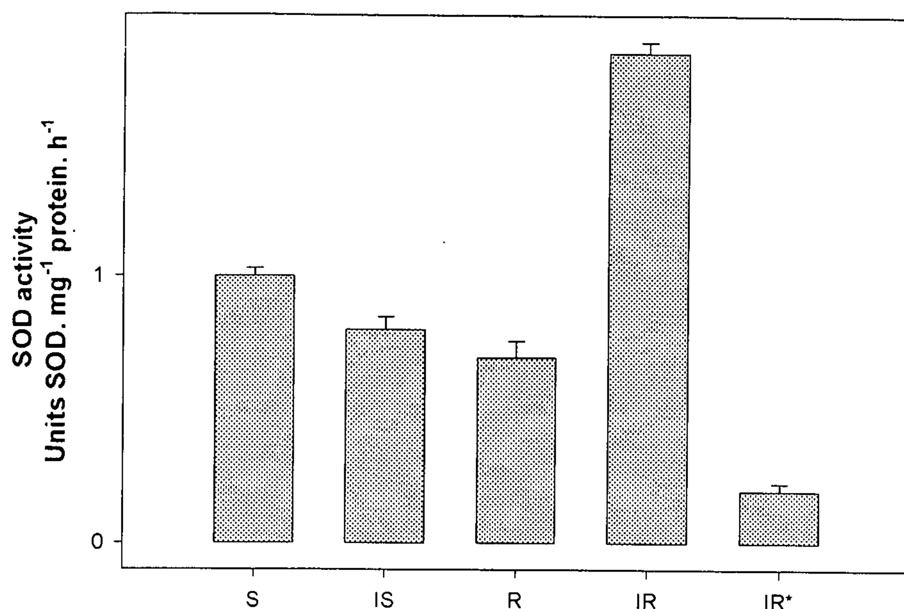


Figure 4.6 SOD activity in infested (I) and uninfested susceptible (S), Tugela and resistant (R), Tugela DN 6 h.p.i. and the effect of allopurinol on the induced SOD activity (*). Error bars indicate standard deviation (n=3).

4.7 Effect of Allopurinol on Chitinase Activity.

The selectively induced chitinase activity was effectively inhibited to levels even lower than the controls. RWA infestation induced chitinase activity differentially in infested resistant wheat (Fig. 4.8).

4.8 The *In Vitro* Effect of Allopurinol on Chitinase Activity.

Allopurinol had virtually no effect on the activity of chitinase when applied *in vitro* in comparison when allopurinol was applied as a soil drench when it resulted in a 97% inhibitory effect on chitinase activity (Fig. 4.9).

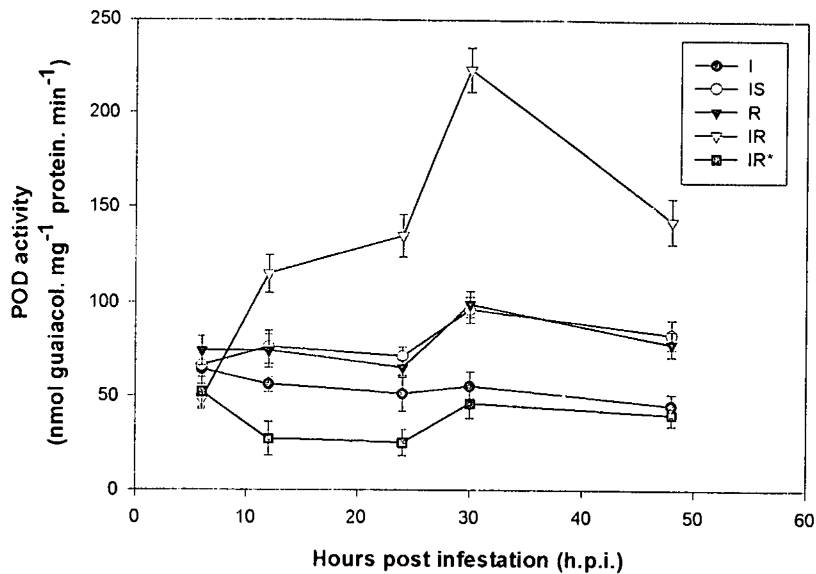


Figure 4.7 POD activity in infested (I) and uninfested susceptible (S), Tugela and resistant (R), Tugela DN wheat and the effect of allopurinol on the induced POD activity (*). Error bars indicate standard deviation, (n=3).

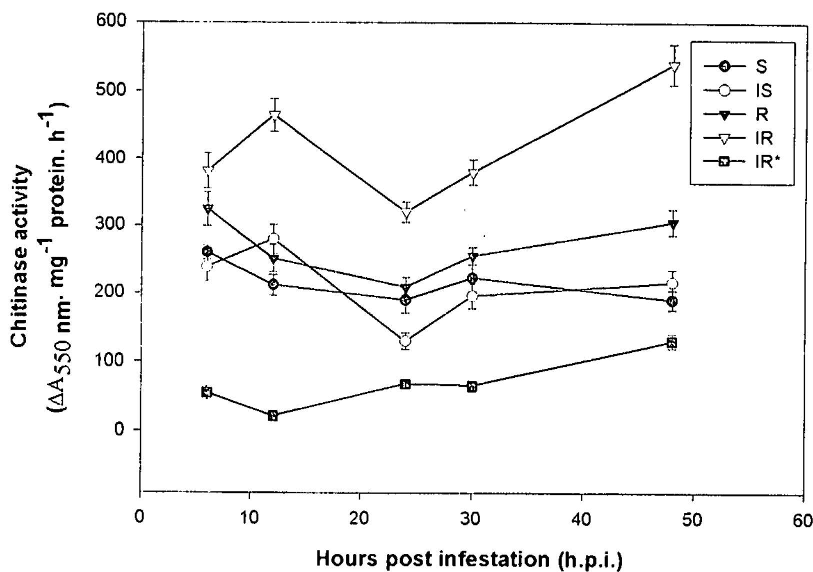


Figure 4.8 Chitinase activity in infested (I) and uninfested susceptible (S), Tugela and resistant (R), Tugela DN, wheat and the effect of allopurinol on induced chitinase activity (*). Error bars indicate standard deviation, (n=3).

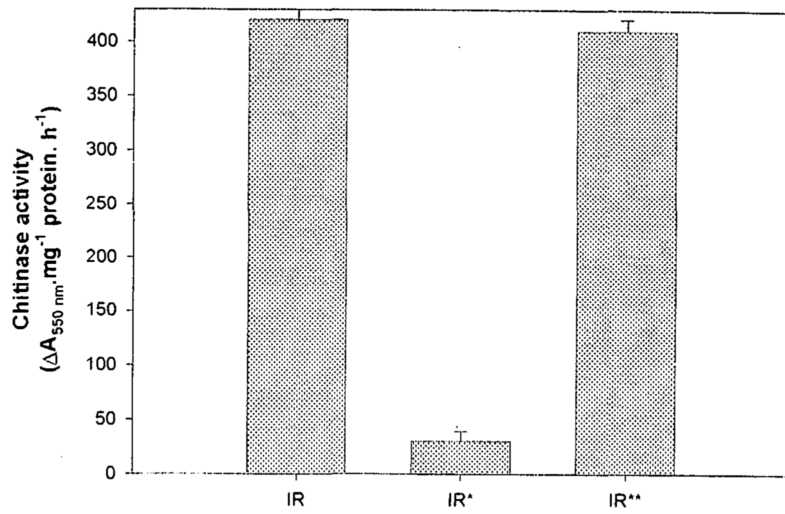


Figure 4.9 The *in vitro* and *in vivo* effect of allopurinol on chitinase activity of infested resistant (IR), Tugela DN wheat plants; IR*, infested resistant wheat – *in vivo* treatment with allopurinol; IR**, infested resistant wheat – *in vitro* treatment with allopurinol.

CHAPTER 5

DISCUSSION

Plant resistance to herbivores is correlated with an enhanced oxidative state of the plant tissues (Felton *et al.*, 1994), which involves the generation of AOS. Possible roles for the oxidative state in antiherbivore defense includes direct oxidative injury to the herbivore, indirect injury to the herbivore through oxidative damage to dietry compounds, signal transduction and elicitating plant defense systems (Felton *et al.*, 1994). Polidoros and Scandalios (1999) have proven that high levels of H_2O_2 induce the expression of the antioxidative defense genes Cat 1, Cat 3 and Gst1 in maize. Low concentrations of H_2O_2 had no effect on these genes. In *Arabidopsis* and tobacco, Leon *et al.* (1995) has demonstrated that H_2O_2 stimulated SA biosynthesis. In the interaction between wheat and the RWA it has been reported that H_2O_2 stimulated salicylic acid (SA) accumulation by 65% (Mohase, 1998). The application of exogenous H_2O_2 to tobacco leaves was responsible for the induction of other phenolic compounds such as benzoic acid, the precursor of SA, as well as the activation of benzoic acid 2-hydroxylase, the key enzyme for SA synthesis (Wu *et al.*, 1997). The AOS produced during the HR in plants after insect attack, are caused by a loss of chemical antioxidants such as cartenoids, ascorbate, glutathione and related thios and / or a decrease in antioxidant generating enzymes such as catalase, glutathione reductase and superoxide dismutase (SOD) activity (Felton *et al.*, 1994).

In the interaction between resistant wheat and the RWA it was found that H_2O_2 was selectively induced (Fig. 4.4) and that the concentrations of H_2O_2 peak values quite soon after infestation (8 h.p.i.). This early development of H_2O_2 can be crucial in the development of resistance, because AOS are known to play numerous functions in defense e.g., driving the synthesis of cell wall structural proteins, the oxidation of cinnamyl alcohols to free radicals by peroxidase/H_2O_2 leading to the formation of lignin (Vance *et al.*, 1980), as local trigger for programmed cell death and as a diffusible signal for the induction of defense genes in neighboring cells (Levine *et al.*, 1994; Milosevic and Slusarenko, 1996)

The production of active oxygen species, which develops during the HR, is believed to be the result of the activity of xanthine oxidase (XO) when hypoxanthine is converted to xanthine and xanthine to uric acid (Fig. 5.1). The conversion of these substrates by XO leads to the generation of active oxygen species (Montalbini, 1992 aandb). Enhanced activity in XO and uricase preceded and accompanied the HR

induced by *Uromyces phaseoli* infection in beans (Montalbini 1989, 1991). The superoxide anion and hydrogen peroxide (Tolbert, 1982) are produced as a consequence of oxygen oxidoreductase activities of XO and uricase. The enhancement of these enzyme activities during the HR, could generate unscavanged toxic oxygen species (Montalbini, 1992b).

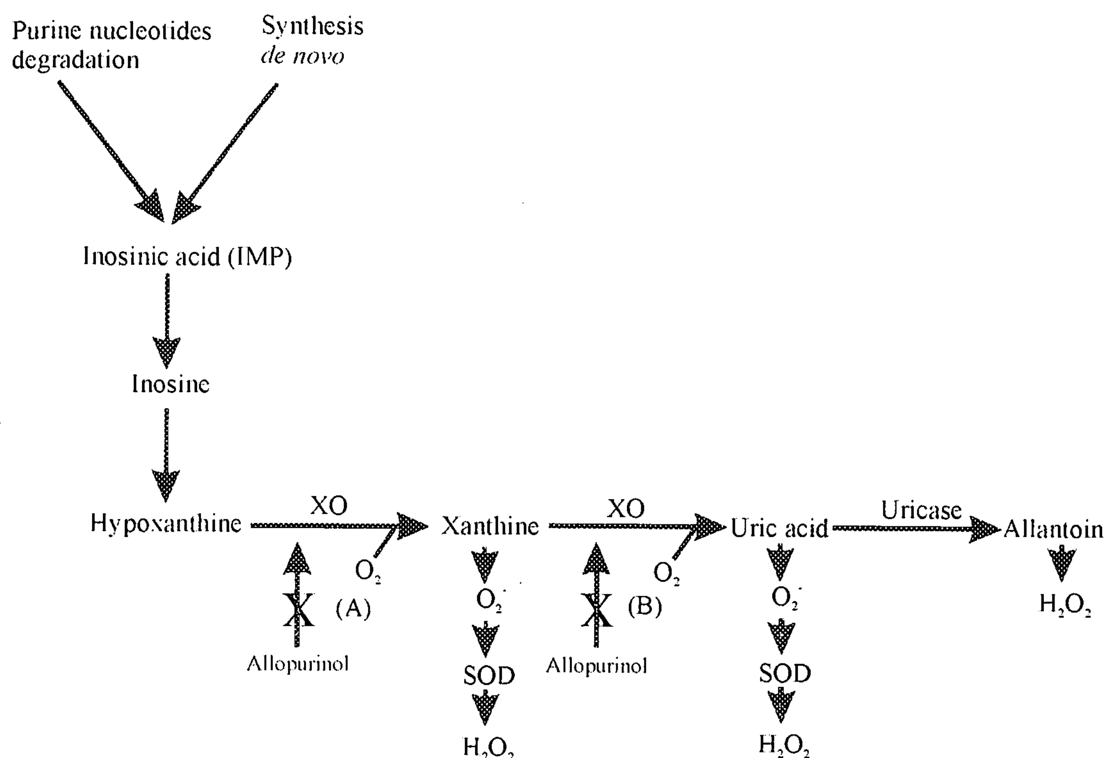


Figure 5.1 Possible mechanism of oxygen radical production consequent to the activation of purine catabolism during hypersensitivity expression in the incompatible host-pathogen interaction. (A) and (B) indicates where allopurinol inhibits the activity of xanthine oxidase. (SOD = Superoxide dismutase; XO = Xanthine oxidase) (Montalbini, 1992b)

The O_2^- that is produced during the HR is converted to H_2O_2 (Fig. 5.1) through the activity of SOD (Montalbini, 1992b). The SOD activity in resistant wheat was selectively induced (Fig. 4.5) when infested with the RWA and the peak activity of SOD was at the same time (8 h.p.i.) as when the H_2O_2 levels peaked in infested resistant wheat (Fig. 4.4). This suggests that SOD might be responsible for the generation of H_2O_2 during the HR.

In this study we wanted to determine whether oxygen radicals and in particular H_2O_2, are responsible for the induction of the down stream defense response. Allopurinol,

which is an inhibitor of XO activity, (Montalbini, 1992b, 1996) leads to the inhibition of the production of O_2^- and H_2O_2 (Fig.5.1). When allopurinol was applied as a soil drench to inhibit the production of AOS, it was found that that the activity of SOD was dramatically reduced (Fig. 4.6) in the infested resistant wheat and since SOD activity corresponded to the H_2O_2 levels, we suggest that little or low levels of H_2O_2 was probably produced. The very low SOD activity is probably the result of low substrate (O_2^-) concentration caused by inhibition of XO by allopurinol. Studies done by Alvarez (1998) on AOS, in particular H_2O_2 and O_2^- have shown that H_2O_2 is responsible for the induction of the defense genes and of cell death in the development of restricted lesions. This observation was also observed by Bolwell *et al.* (1998) and Bolwell and Wojtaszek (1997). Since then it was found that H_2O_2 plays an important role in the defense mechanisms of many plant-pathogen interactions, especially in gene activation. Felton *et al.* (1994 and 1999) found differential increases in H_2O_2 in the incompatible interaction between plants and insects. In the interaction between resistant wheat and the RWA, Mohase (1998) found evidence that H_2O_2 might elicit the accumulation of SA, which is involved in systemic spread of the resistance response against the RWA.

It has been well established that peroxidase (POD) (Van der Westhuizen *et al.*, 1998a, b) and chitinase (Nagel, 1995; Van der Westhuizen *et al*, 1996 and 1998b) activities are involved in the down stream defense response of wheat against the RWA. They were therefore used in this study as indicators whether the down stream defense response are activated or not. In this study it was also found that the POD (Fig. 4.7) and chitinase (Fig. 4.8) activities were differentially induced in infested resistant wheat upon infestation with the RWA. The differentially induced POD activity dropped (Fig. 4.7) as a result to inhibition by allopurinol, which may be the result of insufficient substrate (H_2O_2) and / or insufficient gene induction due to low H_2O_2 levels. For this reason the inhibition of POD activity by allopurinol does not necessarily prove that the H_2O_2 was necessary to induce the down stream defense response. We therefore, additionally, investigated the effect of allopurinol inhibition on the chitinase defense response.

Chitinase activity in infested resistant wheat plants decreased by 97% when treated with allopurinol relative to infested resistant wheat plants where chitinase was

differentially induced (Fig. 4.8). Allopurinol applied *in vitro* had however, no effect on the chitinase activity of infested resistant wheat (Fig. 4.9). Other studies done on the inhibitory effect of allopurinol by Montalbini (1992 a and b; Montalbini and Torr, 1996) have shown that in bean leaves upon infection with rust that cellular permeability and necrosis of host cells, which are characteristic of the HR, were suppressed. Allopurinol treatment further inhibited the hypersensitive necrotic response induced by tobacco necrosis virus infection in bean leaves. It was also very interesting to note that the leaves of resistant wheat displayed signs of rolling when allopurinol was applied (results not shown), a phenomenon only seen in susceptible plants when infested with the RWA.

In many plant tissues it was found that wounding resulted in the selective induction of PAL activity (Hanson and Havir, 1981). Havlíč ková *et al*, (1996) has found that PAL is differentially induced in wheat by cereal aphids. Moerschbacher (1990) found a differential increase in PAL activity in wheat infested with oat crown rust. Differential increase in PAL activity was also found in other incompatible host-pathogen interactions (Moerschbacher *et al.*, 1988; Havlíč ková *et al*, 1996; Cahill and McComb, 1992; Coquoz *et al*, 1998) suggesting that PAL activity plays an important role in the defense mechanism of various incompatible interactions leading to resistance. Not much is known about the role PAL plays in insect defense or whether it is even induced. Cahill and McComb (1992) demonstrated that stimulation of PAL activity in *Eucalyptus calophylla* roots was closely linked to both increased lignification and phenolic contents.

It was shown that PAL is directly involved in the regulation of many physiological processes related to defenses in plants (Jones, 1984). In the initial establishment phase of a pathogen within host tissues, PAL activity often increases and this increased level of PAL activity is correlated with incompatibility (Smith-Becker *et al.*, 1998; Tena and Valbuena, 1982; Cahill and McComb, 1992; Arz and Grambow, 1995). The regulation of the production of glyceollin in soybeans in incompatible interactions with *Phytophthora megasperma* was closely associated with increased PAL activity (Cahill and McComb, 1992) and it has been demonstrated that there was an increase in PAL mRNA (Ward *et al.*, 1989). Changes in PAL activity and transcription generally do not occur in compatible interactions (Esnault *et al.*, 1987). The induction

of PAL genes is thus an important step in the development of incompatibility in such systems.

RWA infestation caused PAL activity to be selectively induced to higher levels in resistant than in susceptible wheat plants (Fig. 4.1). This phenomenon can also be observed in the incompatible interaction between plants and various pathogens as described above. It would therefore not be surprising that PAL also plays an important role in the defense response of wheat against the RWA. As I have described earlier in section 2.2, PAL plays an important role in the regulation and synthesis of phenolic compounds (Cahill and McCamb, 1992; Tena and Valbuena, 1982).

Inoculation of primary roots of *Eucalyptus species* with *Phytophora cinnamomi* led to changes in PAL activity, lignin concentrations and the concentration of soluble phenolics in roots (Cahill and McComb, 1992). Induced synthesis of phenolic compounds is a common feature of host-pathogen interactions and specific phenolics have been implicated in host resistance (Ikegawa *et al.*, 1996). Infection of potato by *Phytophthora infestans* resulted in the accumulation of chlorogenic acid in infected tissue (Friend *et al.*, 1973). Ferulic and *p*-coumaric acids in bound form were involved in the resistance of wheat leaves to *Puccinia recondita* f.sp. *tritici* (Southern and Deverall, 1990) and also to cereal aphids (Havlič ková *et al*, 1996). RWA infestation differentially induced total phenolics in resistant wheat (Van der Westhuizen and Pretorius, 1995) indicating that the phenolic contents play an important role in the defense response. Since phenols have long been associated with defense responses of plants, and due to its high toxicity to cereal aphids (Todd *et al.*, 1971) this increased phenolic content in infested resistant plants may be a factor contributing to RWA resistance.

Simple phenolic compounds such as cinnamic acid, coumaric, caffeic, ferulic, protocatechuic, chlorogenic, and quinic acids exhibit antimicrobial activities. Certain coumarins and flavonoids are toxic to micro-organisms and plants. Mono- and ortho-dihydroxyphenols occur generally in the form of sugar esters and glycosides, and many form toxic substances upon hydrolysis (Misaghi, 1982).

The concentrations of caffeic acid (Fig. 4.2 e) and ferulic acid (Fig. 4.2 f) were differentially induced in infested resistant wheat and they peaked at the same time as PAL activity did (Fig. 4.1). This indicates that increases in these phenolic acid concentrations are the result of the increased PAL activity that was found in the infested resistant wheat (Fig. 4.1). Caffeic and ferulic acid are responsible for the process leading to lignification during incompatible interactions (Vance *et al.*, 1980, Bradley *et al.*, 1992).

The production of *p*-hydroxybenzoic acid (Fig. 4.2 b), *p*-hydroxyphenylacetic acid and caffeic acid (Fig. 4.2 e) was very early selectively induced resulting in higher concentrations after infestation compared to the controls. Resorcylic acid (Fig. 4.2 g), gentisic acid (Fig. 4.2 a) and syringic acid reached peak concentrations mostly 96h after infestation. This suggests that phenolic compounds have different functions during resistance. Some of these tasks may be related to signaling and lignification. Certain phenolics may even serve as deterrents to insects and pathogens. Gentisic acid, possibly, plays a role in the activation of certain PR-proteins, as was found to be in the case of tomato infected with tomato mosaic virus (Bellés *et al.*, 1999). It has been found that gentisic acid and salicylic acid acted synergistically, inducing different defense genes (Bellés *et al.*, 1999). The specific function of gentisic or which gene it induces in the resistance response of wheat to RWA is unknown.

In the hypersensitive response of resistant wheat plants to rust fungi, the mechanism of resistance is attributed to cellular lignification (Moerschbacher *et al.*, 1990), which may serve to reduce fungal growth by increasing resistance of the cell wall to fungal penetration and by lignifying the fungal hyphae (Vance *et al.*, 1980). This response is considered an active mechanism of resistance of plants to fungi and involves induction of enzymes in the lignin biosynthetic pathway (Moerschbacher *et al.*, 1990).

Wounded leaves treated with chitosan displayed lignification around the wounded margins, which is characterized by the deposition of lignin that is extremely rich in syringyl residues and is thought to act primarily as a physical barrier to limit further invasion by potential pathogens (Mitchell *et al.*, 1999). Near-isogenic wheat lines carrying the *Sr5* gene for resistance displayed differential induction in the activities of PAL and 4-coumarate:CoA ligase, as well as the enzyme activities of the lignin

biosynthesis pathway, cinnamyl-alcohol dehydrogenase (CAD) and POD (Moerschbacher *et al*, 1988). Fluorescence microscopy revealed that cells of wheat leaves infested with oat crown rust became necrotic and exhibited bright yellow autofluorescence when stained with diethanol and viewed under UV-light. These fluorescent cells stained positively with phloroglucinol/HCl, suggesting that hypersensitive cell death is correlated with lignification (Moerschbacher *et al.*, 1990). In the same experiment Moerschbacher *et al.* (1990) found differential induction of the lignin biosynthetic enzymes; i.e. PAL, 4-coumarate:CoA ligase, CAD, and POD activities. Barley infested with RWA displayed yellow collapsed, autofluorescent cells (CAC) when viewed under blue or UV-light (Belefant-Miller, 1994). The CAC of mesophyll and bundle sheath tissues in response to RWA infestation is visually similar to the hypersensitive response of barley to incompatible fungi. In both cases whole cells collapsed and became yellow fluorescent (Bellefant-Miller *et al.*, 1994).

In Figure 4.3(a) lignified cells can clearly be seen where the aphids penetrated the wheat leaves. It is only in infested resistant wheat where lignified cells are observed. Infested susceptible wheat displayed no lignification of mesophyll cells. These results suggest that hypersensitive cell death and accompanied lignification is correlated with the resistance response in wheat to the RWA. The lignification process in *Eucalyptus* species is closely related to induced PAL activity and phenolic acids (Cahill and McComb, 1992). As in many other plant-pathogen interactions as I have mentioned earlier, lignification played an important role in isolating the pathogen and/or toxins from the surrounding cells and so preventing the spread of the pathogen and/or toxin(s) to other parts of the plant. This might also be the case in the interaction between wheat and the RWA.

According to the present study and previous studies in this laboratory it has been established that RWA infestation of resistant wheat resulted in the differential induction of POD (Fig. 4.7) and chitinase activities (Fig. 4. 8) (Van der Westhuizen *et al.*, 1998b). The differential induction of these enzymes in resistant wheat indicates to us that the defense mechanism is induced, particularly the hypersensitive reaction (HR). Mohase (1998) showed that H_2O_2 was necessary to stimulate the down stream defense response (chitinase activity) since chitinase was induced by exogenous applied H_2O_2 and our results confirm this demonstration (Fig. 4.8). Further it was

found that SOD activity corresponded to the production of H_2O_2 (Fig. 4.4 and 4.5). SOD activity was also inhibited when H_2O_2 production was decreased by allopurinol (Fig. 4.6). From Fig. 4.4, Fig. 4.5 and Fig. 4.6 it seems that there is a relationship between the inhibition of SOD activity and the simultaneous decrease in H_2O_2 concentration. Taking into account the Model of Montalbini (Fig 5.1) our results suggest that SOD catalyzes a reaction that results in H_2O_2 as a product.

The peak PAL activity (Fig. 4.1) corresponded to gallic (Fig. 4.2d), resorcylic (Fig. 4.2g) caffeic (Fig. 4.2e) and ferulic acid (Fig. 4.2f) concentrations in infested resistant wheat. This not only indicates a regulatory role of PAL in the synthesis of phenolics; but also the involvement of phenolics in the resistanse response of wheat to the RWA. The selective induction of PAL activity, caffeic acid and ferulic acid concentrations in resistant wheat may have contributed, to amongst others, the formation of lignified cells (Fig. 4.3a). Previous studies in this laboratory confirmed the possible involvement of phenolics in the resistance response (van der Westhuizen and Pretorius, 1995). It is not known whether one or more of these phenolic compounds have a deterrent effect on the RWA. An increase in PAL activity can also lead to an increase in SA accumulation which is essential for maintaining SAR, (Mohase, 1998) during RWA infestation, since the formation of SA is dependent on PAL activity.

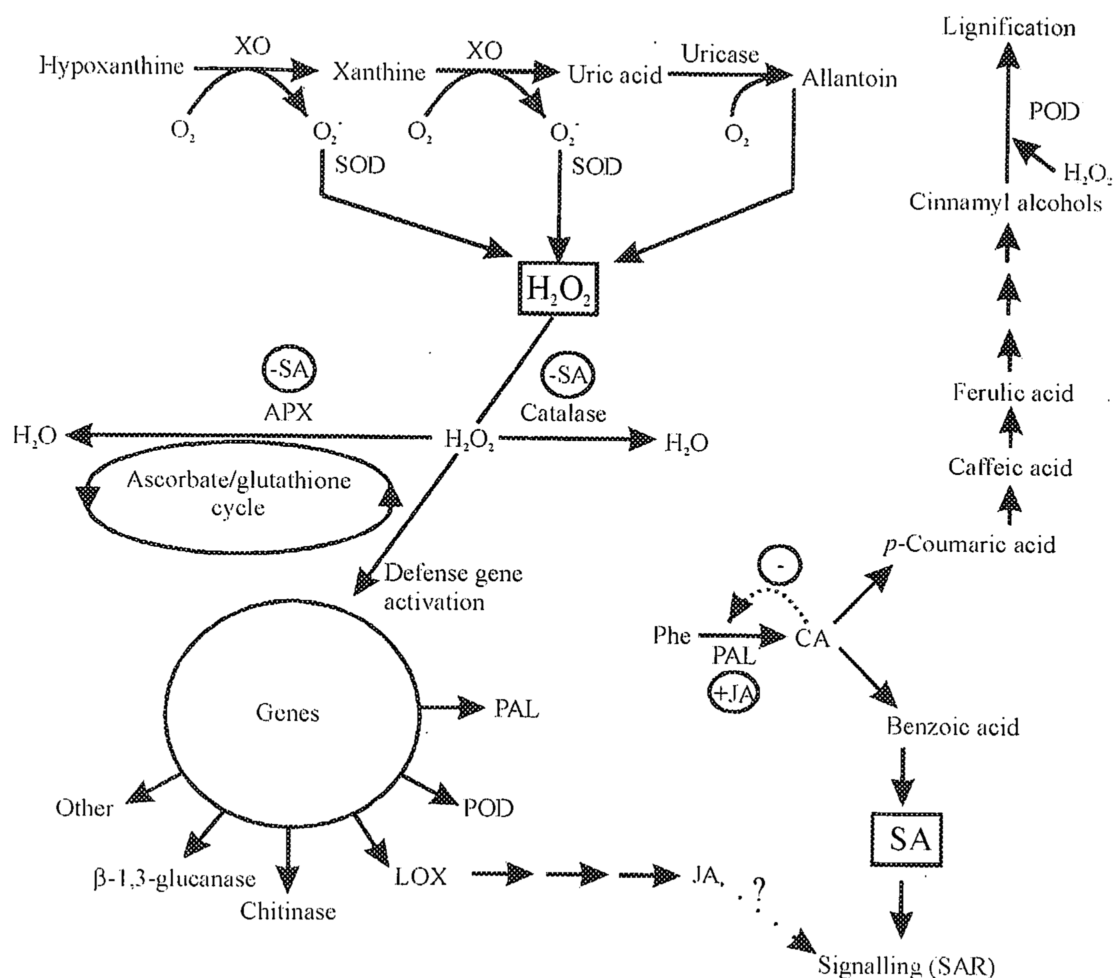


Figure 5.2 Speculative model showing the defense mechanism of resistant wheat against RWA infestation. SA – salicylic acid; JA – jasmonic acid; Phe – phenylalanine; PAL – phenylalanine ammonia-lyase; CA – cinnamic acid; POD – peroxidase; LOX – lipoxygenase; APX – ascorbate peroxidase; XO – xanthine oxidase; SOD – superoxide dismutase; (+) - stimulate; (-) - inhibit

Taking into account previous results of research done in our laboratory on the biochemical defense mechanism of wheat against the RWA and the model of Hammond-Kosack & Jones 1996 (Fig. 2.3) as well as the results of the present study, a speculative scheme has been compiled in Fig. 5.2. Xanthine oxidase catalyzes the production of O_2^-, which is converted to H_2O_2 by SOD. H_2O_2 is involved in the activation of defense genes and can assist in cross-linking of cell wall proteins catalyzed by POD. Catalase catalyzes the conversion of H_2O_2 to H_2O to regulate H_2O_2 levels and prevent it from reaching toxic levels. It was reported that ascorbate peroxidase and the ascorbate/glutathione cycle is involved in the scavenging of H_2O_2 in incompatible host pathogen interactions (Hammond-Kosack and Jones, 1996).

However, its involvement in the defense mechanism of wheat against the RWA needs still to be established. H_2O_2 induces chitinase activity in resistant wheat. It is speculated that H_2O_2 induces PAL, POD, LOX and β -1,3-glucanase activities. PAL plays an important role in the synthesis of phenolic acids, which play a role in lignification, as deterrent agents, and in signalling. The concentrations of ferulic and caffeic acid are directly dependent on the activity of PAL. SA concentrations are also dependent on the activity of PAL. SA inhibits the activities of catalase and ascorbate peroxidase (Hammond-Kosack and Jones, 1996) thereby maintaining high H_2O_2 levels when needed to maintain the SAR (Mohase, 1998). POD together with H_2O_2 contributed to lignification during the HR. LOX activity leads to the synthesis of JA (Swart, 1998), which has a stimulating effect on PAL (Hammond-Kosack and Jones, 1996). The stimulation effect of JA on PAL needs to be proven in RWA/wheat interaction. Swart (1998) has found that JA does not induce LOX, POD or β -1,3-glucanase activity, suggesting another role for JA in the RWA/wheat interaction.

ABSTRACT

The effect of Russian wheat aphid (RWA), *Diuraphis noxia* (Mordvilko) infestation on phenylalanine ammonia-lyase (PAL) and superoxide dismutase (SOD) activities as well as internal hydrogen peroxide (H_2O_2) levels and phenolic acid composition was studied in resistant (Tugela DN1) and susceptible (Tugela) wheat cultivars. In this study we aimed to learn more about the resistance response of wheat against the RWA with special reference to the role of active oxygen species (AOS), in particular H_2O_2, in establishing the down stream defense response and furthermore to investigate the involvement of phenylalanine ammonia-lyase (PAL) and phenolic compounds in the resistance response.

SOD, PAL, POD (peroxidase) and chitinase activities were determined by means of spectrophotometric essay procedures. The phenolic compounds were separated using HPLC and a C-18 reverse phase column. Allopurinol was used to inhibit xanthine oxidase activity and concomitant H_2O_2 generation. The expression of the down stream defense response was measured in terms of POD and chitinase activities.

RWA infestation selectively induced H_2O_2 production to a maximum concentration 8 h.p.i. in resistant wheat. The increase in H_2O_2 concentration corresponded with the increase in SOD activity, which was also differentially induced in infested resistant wheat to a maximum value 8 h.p.i. These results suggest that SOD is involved in the generation of H_2O_2 during the hypersensitive reaction (HR). Inhibitory studies done, have proven that H_2O_2 was needed for the induction of the down stream defense response. Chitinase activity was extremely low when the production of H_2O_2 was inhibited by allopurinol.

PAL activity increased differentially in infested resistant wheat to higher levels (200% increase) than in infested susceptible wheat. Peak activity of PAL was reached between 18-48 h.p.i. The increase in the activity of PAL, a key enzyme in the synthesis of phenolic compounds, was reflected in increased phenolic acid concentrations. Some of the phenolic acids that were selectively induced in resistant wheat upon RWA infestation were, *p*-hydroxybenzoic acid, gallic acid, resorcylic

acid, gentisic acid, caffeic acid, *p*-hydroxyphenylacetic acid and ferulic acid. The *p*-hydroxybenzoic acid, *p*-hydroxyphenylacetic acid, caffeic acid and ferulic acid concentrations corresponded to the peak PAL activity.

Selective increases in PAL activity, caffeic and ferulic acid concentrations probably contributed to the selective lignification of mesophyll cells in infested resistant wheat. This is a manifestation of the HR, which is part of the defense response against the RWA.

Findings in this study were indicative of the involvement of active oxygen species in establishing the down stream defense response and of phenolic compounds in the resistance response of wheat against the RWA.

KEYWORDS

Triticum aestivum, Diuraphis noxia, Russian wheat aphid, wheat, active oxygen species, hydrogen peroxide, hypersensitive response, lignin, phenolics, phenylalanine ammonia-lyase, resistance

Opsomming

Die invloed van Russiese koringluis- (RKL), *Diuraphis noxia* (Mordvilko) infestering op die aktiwiteite van fenielalanineammoniakliase (PAL) en superoksieddismutase (SOD) asook op die interne waterstofperoksied- (H_2O_2) vlakke en fenoliese samestelling is ondersoek in weerstandbiedende (Tugela DN1) en vatbare (Tugela) koringkultivars. In hierdie studie het ons gepoog om meer te wete te kom oor die verdedegingsmeganisme van koring teen die RKL met spesiale verwysing na die rol van reaktiewe suurstofspesies (ROS), in besonder H_2O_2, in die ontwikkeling van die stroomafverdedegingsreaksie. Verder is die betrokkenheid van die fenoliese verbindings en PAL-aktiwiteit in die verdedegingsreaksie ondersoek.

SOD-, PAL-, POD- (peroksidase) en chitinase-aktiwiteite is deur middel van spektrofotometriese bepalingsmetodes ondersoek. Die fenoliese verbindings is met behulp van HPLC en 'n C-18 omgekeerde fase kolom geskei. Allopurinol is gebruik om die xantienoksidase- (XO) aktiwiteit te inhibeer en sodoende die produksie van H_2O_2 te inhibeer. Stroomafverdedeging is in terme van POD- en chitinase-aktiwiteit uitgedruk.

Die H_2O_2-vlakke is selektief deur RKL-infestering geïnduseer en het 'n piekwaarde 8 ure na infestering (u.n.i.) in weerstandbiedende koring bereik. Die toename in H_2O_2 konsentrasie het ooreengestem met die toename van SOD-aktiwiteit 8 u.n.i. Hierdie resultate dui aan dat SOD veranwoordelik was vir die produksie van H_2O_2 gedurende die hipersensitiewe reaksie (HR). Inhiberingstudies het bewys dat H_2O_2 noodsaaklik was vir die indusering van die stroomafverdedegingsreaksies. Allopurinol-inhibering van H_2O_2-produksie het inhibering van chitinase-aktiwitiet (stroomafverdedegingsreaksie) tot gevolg gehad.

RKL-infestering het tot die differensiële toename in PAL-aktiwiteit tot baie hoër vlakke (200% toename in aktiwiteit) in weerstandbiedende koring as in vatbare koring gelei. PAL-aktiwiteit het 'n piekwaarde tussen 18 en 48 u.n.i. bereik. Die differensiële toename in PAL-aktiwiteit, 'n sleutelensiem in die sintese van fenoliesesure is weerspieël in 'n die selektiewe toename in fenoliese suurkonsentrasies. Fenoliese sure

wat selektiewe induksie getoon het in weerstandbiedende koring met RKL infestering is: *p*-hidroksiebensoësuur, 3,5-dihidroksiebensoësuur, *p*-hidroksiefenielasynsuur, resorsielsuur, gentisiensuur, kaffeïensuur en ferulasuur. Die *p*-hidroksiebensoësuur, p-hidroksiefenielasynsuur, kaffeïensuur en ferulansuur se piekkonsentrasies het ooreengestem met die PAL-piek aktiwiteit. Die selektiewe toename in PAL-aktiwiteit, kaffeïensuur- en ferulansuurkonsentrasies het waarskynlik bygedra tot die selektiewe lignifisering van die mesofilselle in die geïnfesteerde weerstandbiedende koring. Hierdie is 'n manifestasie van die HR wat deel is van die verdedegingsreaksie teen die RKL.

Bevindings in hierdie studie het op die betrokkenheid van ROS in die ontwikkeling van die stroomafverdedegingsreaksie gedui asook dat fenoliese sure by die verdedegingreaksie van koring teen die RKL betrokke is.

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