THE INVOLVEMENT OF REACTIVE OXYGEN SPECIES IN THE RESISTANCE RESPONSE OF WHEAT TO THE RUSSIAN WHEAT APHID

By

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Submitted in accordance with the requirements for the

Magister Scientiae

in the Faculty of Natural and Agricultural Sciences

Department of Plant Sciences

at the University of the Free State

Bloemfontein

December 2002

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Dedicated to Bonolo Mbokeleng (junior)

ACKNOWLEDGEMENTS

I sincerely wish to thank Prof. AJ van der Westhuizen for his excellent supervision and his constructive comments that contributed towards the success of this study.

I would like to acknowledge the financial support from the National Research Foundation and the University of the Free State towards this study.

I am very grateful to my sister, Mamokete, for the support she gave me throughout the study.

I am also grateful to my parents for giving me an opportunity to study.

I would like to thank Sikhumbuzo for his support and encouragement.

I would also like to thank my colleagues and friends for their assistance.

Finally, I thank Jehovah for giving me life and strength to complete this study.

PREFACE

I declare that the dissertation hereby submitted by me for the Master of Science degree at the University of the Free State is my own independent work and has not previously been submitted by me at another university/faculty.

I furthermore cede copyright of the dissertation in favour of the University of the Free State

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LIST OF ABBREVIATIONS

APX	Ascorbate peroxidase
ARC-SGI	Agricultural Research Council-Small Grain Institute
BMV	Brome mosaic virus
DPI	Diphenylene iodonium
EDTA	Ethylenedinitrilo tetraacetic acid, disodium salt dihydrate
GR	Glutathione reductase
GSSH	Oxidized glutathione
HR	Hypersensitive response
IWF	Intercellular washing fluid
kDa	Kilo Dalton
MAPK	Mitogen activated protein kinase
MAPKK	Mitogen activated protein kinase kinase
MAPKKK	Mitogen activated protein kinase kinase kinase
NBT	Nitroblue tetrazolium
O_2^-	Superoxide anion
PR	Pathogenesis related
PVP	Polyvinylpyrolidone
ROS	Reactive oxygen species
RSA	Republic of South Africa
RWA	Russian wheat aphid
SA	Salicylic acid
SAR	Systemic acquired resistance

SOD	Superoxide dismutase
TMV	Tobacco mosaic virus
Tris	Trishydroxymethyl aminomethane
UV	Ultraviolet
XOD	Xanthine oxidase

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CHAPTER ONE

INTRODUCTION

The agricultural industry plays an important role in the economic growth and development of the Republic of South Africa (RSA) by producing important crops such as maize, wheat, barley, oats, etc. This industry also plays a distinctive role in broadening the economic and social options of rural people, and consequently improving the quality of life (Marasas *et al.*, 1997). The wheat industry and its related secondary industries provide considerable employment on farms and in agribusiness (Howcroft, 1991).

Wheat belongs to the genus *Triticum*, in the grass family Poaceae and the tribe Hordeae in which one to several spikelets are sessile and alternate on opposite sides of the rachis, forming a true spike. Among several cultivated wheat plants, *T. aestivum* L. is by far the most important species. *T. aestivum* is a hexaploid, in other words it has six times seven chromosomes or three genomes. Reproductive cells each contain three sets of seven chromosomes. Varieties in this species fall under the general heading of common wheat of which the flour is best suited for bread. Wheat cultivars that are cultivated in the RSA either have a spring or winter type of growth habit. Winter cultivars require vernalization and must be planted in areas with cold winters. During the winter season, they grow slowly and remain in the vegetative state until early spring when reproductive growth rapidly overtakes and inhibits vegetative growth. Spring cultivars on the other hand do not require vernalization and can be grown in areas with a mild winter (Scott, 1990).

Wheat has been grown the RSA since in the middle of the 16th century. One of the first undertakings of Jan van Riebeeck after his arrival at the Cape in 1652, was to sow wheat on the site of the present–day Cape Town. During the 18th century wheat

production expanded gradually as the early pioneers opened up the country. The crops, however, were irregular due to variation in the climate, unadapted varieties, and later to the incidence of rust (Sim, 1965). Today wheat is successfully produced in the winter rainfall areas of the Western Cape and the summer rainfall areas of the Free State Province, Northern Cape, North West and Northern Province. The Free State Province is the largest wheat-producing region in the RSA, contributing around 40–50 % of the total production (Marasas *et al.*, 1998). The RSA plays a major role in the wheat industry of Southern Africa. It contributes up to 91 % of the South African Development Community's wheat production (Marasas *et al.*, 1997).

Wheat provides more nourishment for the people of the world than any other food source, and enters into the international trade more than any other food. The grain is nutritious and has many natural advantages as food and feed. Because of its small size, it can be easily processed to produce high refined foods. One of the most unique characteristics of wheat grain is the elasticity of the gluten. Unlike any other grain (or any other plant product), wheat gluten enables leavened dough to rise through formation of minute gas cells that retain carbon dioxide formed during fermentation. This unique property forms the basis of bread production, which has been a basic food for man throughout recorded history, and it is still the principal food made from wheat. Wheat too often is thought of as merely a starch food. In addition to easily digested starch, it contains protein, minerals, vitamins and fats (lipids). When wheat or wheat products are used as a main part of the diet and are complemented by small quantities of protein from animal sources, it can be considered a highly nutritious food. In one respect, it offers an advantage over heavily meat-based Western-world diets, in that a wheat diet is significantly lower in fat. Although wheat is considered primarily a food crop, it has extensive feed and industrial use. Wheat grain is a good livestock feed when used as part of the ration. Most wheat milling by-products, especially bran, are utilized in preparations of commercial livestock feeds (Kriel, 1984). Therefore, if no wheat was produced in the RSA, it would be necessary to import it with foreign exchange complications (Howcroft, 1991).

The RSA is a country of extremes in terms of its climate and topography. The highly variable rainfall, temperature, and soil types, undoubtedly have a major effect on wheat yields. However, numerous pathogenic fungi and pests also have a significant effect on the yield (Marasas *et al.*, 1997).

Generally, many insect species that feed on agricultural crops are not serious pests in their native distribution region. In such cases the populations are regulated by both biotic and abiotic factors at a level below that of the economic threshold. If these insects are introduced or find their way into other countries or regions where the biotic and abiotic factors are more favorable, the numbers can increase rapidly and attain pest proportions. Absence of the most successful parasites, predators and pathogens is frequently cited as a prime reason for the population explosion in the country or region of adoption. The noxious wheat aphid, *Diuraphis noxia*, is an example of such an insect (Kriel, 1984; Kovalev *et al.*, 1991).

Diuraphis noxia (Fig. 1.1) originated from the southern parts of Russia, Iran, Afghanistan and countries lining the Mediterranean (Potgieter *et al.*, 1991). This aphid was recorded for the first time in the RSA in 1978, near Bethlehem (in the Eastern Free State), where it was referred to as the Russian wheat aphid (RWA). In September 1979, it had spread over to the greater part of the Free State Province and Lesotho, with isolated foci of infestation in the Western Free State, Northern Cape and Mpumalanga (Du Toit and Walters, 1984). The damage inflicted by the RWA on wheat results in the typical symptoms of susceptibility. Crops damaged by this pest include wheat (*Triticum aestivum* L.), barley (*Hordium vulgare* L.), oat (*Avena sativa* L.), rye (*Secale cereale* L.) and triticale (*Tribicosecale wittmack*), but wheat is the most affected crop (Walters *et al.,* 1980).

In June and July, *D. noxia* move in small numbers from *Bromus* grass and volunteer wheat that are planted for pastures during late summer and autumn into the commercial wheat fields. When the temperature rises in September, *D. noxia* populations increase dramatically. The critical period for the control of this pest often

occurs in late September/early October before the first dependable spring rains. It is common to find that the stored soil moisture is depleted during this period, and that the wheat plants are experiencing moisture stress (Marasas *et al.*, 1997). These conditions are conducive to RWA outbreaks as this aphid thrives on plants under moisture stress (Burd and Burton, 1992).

Figure 1.1: The Russian wheat aphid.

RWA infestation of susceptible wheat cultivars is a major cause of heavy yield losses in the RSA (Du Toit and Walters, 1984; Smith *et al.*, 1991). Due to the severity of the damage caused by the RWA, the fastest and most effective solution to control this pest was the use of chemical control with insecticides. In 1993, the seed dressing called Gaucho became available in the RSA and RWA was controlled by applications of either pre-plant or post-emergence insecticides. This method, though usually effective, is expensive and harmful to the environment. Most commercial farmers have used aerial applications of systemic organophosphates (LD_{50}, 50 milligrams per kilogram) costing approximately R70 per hectare (Marasas *et al.*, 1997).

In view of the several disadvantages of the chemical control program, the Agricultural Research Council-Small Grain Institute (ARC-SGI) started to develop alternative

control methods for this aphid. Natural enemies such as ladybirds and wasp parasites were alternatives for the control of this aphid. This work was started in 1989 with the introduction of a parasitoid *Aphidius matricariae*, which however proved not to be highly effective (Marasas *et al.*, 1997).

The availability of resistant cultivars offers a positive alternative for RWA control. Breeding for resistance was started in 1985 when the genetic resistance of RWA was identified in bread-wheat lines. The first commercial RWA resistant cultivar (*Tugela DN*) in the RSA was released in 1992 by the ARC-SGI. In 1993, the RSA was the first country worldwide to release a RWA resistant wheat cultivar for commercial use, and to date different cultivars have been released. Unlike susceptible wheat cultivars, resistant cultivars are colonized by the RWAs in low numbers, but do not show any reduction in plant height or streaking and rolling damage symptoms caused by this aphid. They also tend to stay green for a longer period, and to be less stressed during the critical period between September and October (Marasas *et al.*, 1997).

Although resistant cultivars have been released, the development of new RWA biotypes may overcome the resistance. This necessitates more rapid development of new cultivars. Any information on the resistance mechanism eventually may contribute to more effective and less time-consuming selection procedures. Furthermore, to transform plants by modern molecular techniques, the availability of resistance genes is essential. In searching for resistance genes or molecular markers, a comprehensive knowledge of the mechanism of resistance would be helpful (van der Westhuizen and Pretorious, 1995).

Plants respond to harmful influences of biotic or abiotic origin by altering their cellular mechanism and invoking various defence mechanisms. The biotic influences include plant pathogens (such as fungi, bacteria, viruses etc.) and other pests (such as insects, nematodes and herbivores). The abiotic influences include the abnormal temperatures (excessively high or low), drought, waterlogging, nutrient deficiencies and air pollutants. Survival under these stressful conditions depends on the plant's

ability to perceive the stimulus, generate and transmit signals and instigate biochemical changes that adjust the metabolism accordingly (Chesin and Zipf, 1990; Enyedi *et al.*, 1992; Mehdy, 1994).

In the case of pathogenesis, the earliest components of cellular response include directed movement of organelles and nucleus towards the site of pathogen attack, generation of the reactive oxygen species (ROS), formation of cell wall apositions mostly consisting of callose at the site of attempted penetration, often followed by cellular collapse which is one type of programmed cell death called the hypersensitive response (HR). These processes are frequently accompanied by the release of phenolics from disintegrating cellular compartments, which upon contact with cytosolic enzymes are chemically modified or polymerized. Accumulation of the defence gene transcripts follows these initial events sometimes in the attacked cells, but mostly in the surrounding tissue. These genes encode pathogenesis related (PR) proteins and the enzymes involved in the biosynthesis of phytoalexins and others, often phenylpropanoid or fatty acid-derived secondary metabolites. Some of these products act directly as defence factors, for example, some PR proteins and phytoalexins, whereas others apparently represent signaling elements such as jasmonate and salicylate, some of which participate in the induction of systemic acquired resistance (Scheel, 1998).

For a long time, the ROS have been considered mainly as dangerous molecules, whose levels need to be kept as low as possible. This opinion has however changed. It has been found that the ROS are the earliest components that play very important roles in the plant's defence system against pathogens (Alvarez and Lamb, 1997; Doke, 1997; Bolwell *et al.*, 2002). The ROS are linked with the key events such as signal transduction, antimicrobial effects, membrane lipoxidation, cell wall modification, induction of cellular protectant and defence genes, and the hypersensitive cell death (Baker and Orlandi, 1995; Lamb and Dixon, 1997; Blumwald *et al.*, 1998; van Breusegem *et al.*, 2001; Neill *et al.*, 2002).

Previous studies have confirmed that RWA infestation of the resistant wheat (cv., *Tugela DN*) leads to induction of the secondary defence related enzymes such as the intercellular β-1,3-glucanases (van der Westhuizen and Pretorious, 1996; van der Westhuizen *et al.*, 1998b and 2002), peroxidases and chitinases (van der Westhuizen *et al.*, 1998a), which highly resemble the defence responses during pathogenesis and forms part of the general defence responses like the HR (van der Westhuizen *et al.*, 1998b).

The role of ROS in resistance against the RWA in wheat was studied to shed more light on the early events that lead to the induction of secondary defence responses. To achieve this, the objectives were:

1. To establish whether ROS production, specifically H_2O_2, is part of the RWA resistance response in wheat.
2. To determine the involvement of the ROS generating enzymes, NADPH oxidase and superoxide dismutase (SOD) in the RWA resistance response and hence ROS production.
3. To establish whether NADPH oxidase inhibition would lead to inhibition of secondary defence reactions.
4. To establish whether the *in vivo* application of H_2O_2 would lead to the induction of secondary defence reactions.

5. To determine whether detoxifying mechanisms such as ROS scavenging enzymes, glutathione reductase and ascorbate peroxidase, were active to prevent subsequent oxidative damage.

CHAPTER TWO

2. LITERATURE REVIEW

2.1 THE RUSSIAN WHEAT APHID

2.1.1 Description

The RWA, *Diuraphis noxia*, is one of the most serious pests of small grains throughout the world (Nkongolo *et al.*, 1992). It is a relatively small (less than 2mm long) pale yellow-green to grey-green aphid with an elongated, spindle shaped body. It can easily be distinguished from other aphids infesting wheat in southern Africa by its extremely short antennae, a characteristic projection above the cauda (or tail), i.e. "double tail", and, to the naked eye, the absence of the prominent siphunculi, which are so typical of other aphids (Walters *et al.*, 1980).

2.1.2 Forms of RWA

Two forms of the RWA exist in the RSA, namely: the winged (alate) and the wingless (apterous) females. The males are not found in the RSA, therefore reproduction takes place without fertilization (parthenogenetically). The winged females develop when the growth stage of a plant is such that it no longer provides a favorable habitat for this pest. They serve to distribute this pest to the nearby fields, or even to other areas where the host plants are in more favorable growth stage or are growing under more favorable conditions. This distribution is achieved, because the winged females can travel long distances on prevailing winds and air convection currents (Walters *et al.*, 1980; Schotzko and Smith, 1991).

2.1.3 RWA-feeding

The RWA use their stylets to feed in the phloem of the host leaf vascular bundle. Before they penetrate the phloem, they probe intercellularly. Sometime penetration is achieved after several probing attempts. During probing, aphids secrete a sheath to protect their stylets. The sheaths may become branched, which is an indication that redirection of the stylet path has occurred. The vascular bundle may be reached from different angles, which will vary with the position of the aphid on the leaf. However, the ultimate destination of the stylets remains the phloem (Fouché *et al.*, 1984).

2.1.4 Symptoms of RWA infestation

The symptoms of RWA infestation in susceptible wheat are very distinct. Infestations are accompanied by white, yellow and purple to reddish longitudinal streaks on the wheat leaves, and the inward curling of the leaf edges (Fig. 2.1). The aphids are mainly found on the adaxial surface of the newest growth of the wheat plants, in the axils of the leaves, or within the curled-up leaves. Heavy infestations in young plants cause the tillers to become prostrate, while in later growth stages, the aphids often infest the flag leaf. The ears often become bent, trapped in the rolled leaf, and turn white (Walters *et al.*, 1980).

2.1.5 Transmission of diseases by RWA

The RWA (like common wheat aphids) are capable of transmitting certain virus diseases (Walters *et al.*, 1980). They were found to be vectors of barley yellow dwarf virus, brome mosaic virus, and barley stripe mosaic virus (Von-Wechmar, 1984). However in the RSA, they are not the effective vectors of brome mosaic virus, with only 20 percent successful transmission under controlled conditions (Cronjé, 1990). The RWA infestation also leads to a drastic reduction in the chlorophyll content,

which when combined with the characteristic of rolling that occurs, causes a considerable loss of photosynthetic effective leaf area on susceptible plants, clearly indicating that the yield will be affected (Walters *et al.*, 1980). Burd and Burton (1992) showed that RWA infestation results in water imbalances in the host plant expressed as a loss of turgor and reduced growth. They also found substantial reduction in plant biomass.

2.1.6 Survival of RWA

Temperature is one of the important factors that controls the RWA survival. The low winter temperatures restrict the aphid population growth, while high temperatures and high rainfall lead to high mortality and reduction in aphid numbers. Explosive increases of RWA populations can occur because of their high reproductive rate and short maturation time. The aphids have a life span of about 25-30 days, and 20-30 generations per year. They produce about 4 nymphs per day. Survival of these nymphs greatly depends on finding an acceptable food source within a few hours of birth and they mature after 7 days (Walters *et al.*, 1980).

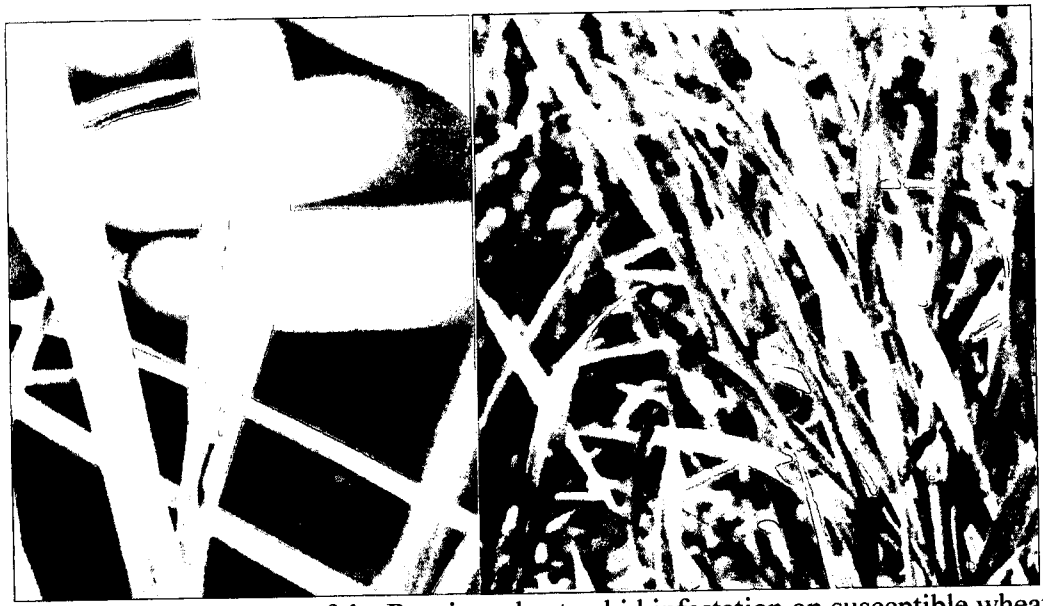

Figure 2.1: Symptoms of the Russian wheat aphid infestation on susceptible wheat.

2.2 DEFENCE MECHANISMS IN PLANTS

Plants are constantly threatened by a variety of pests and pathogens. Provided that the nutritional requirements are satisfying and the abiotic environment is conducive for growth, these are the major factors limiting crop production and their control is an essential component of modern agriculture. Because of sedentary nature of plants and their potential as a rich source of carbon and nitrogen, plants seem to be an easy and excellent target for parasitic organisms (Kollattukudy, 1985; Ride, 1992; Mehdy, 1994).

2.2.1 Constitutive structural defence

Like other living organisms, plants use many defence mechanisms for the rejection of potential pathogens. These mechanisms can be divided into two categories. The first line of defence, a passive one, is due to the presence of preformed or constitutive factors. The cell wall and cuticle in plants represent the physical barriers that keep most organisms from developing an intimate contact with plants. One important and significant outcome of this barrier is that plants, unlike mammals, rarely develop bacterial diseases (Kollattukudy, 1985; Ride, 1992; Mehdy, 1994).

The constitutive structural defences against insects and herbivores include general tissue toughness, deposition of silica, calcium carbonate or lignin around the vascular bundles or throughout the tissues (Norris and Kogan, 1980). Stem toughness is another resistance mechanism in wheat against insects, e.g. stem sawfy (Wallace *et al.*, 1973). Trichomes and leaf hairs also form part of the structural defences against insects. Most of the plant species (e.g. tomato, potato, cotton, etc.) produce large numbers of glandular trichomes. These trichomes rupture upon contact with insects and produce a rapidly oxidized phenolic mixture that darkens and hardens when exposed to air, immobilizing even moderate sized insects (Berenbaum *et al.*, 1986).

2.2.2 Constitutive chemical defence

The constitutive chemical defence against pathogens often includes the presence of high concentrations of phenolics and alkaloids, although in seeds the major antifungal agents are proteins (Lamb *et al.*, 1992).

The constitutive chemical defenses in most plants have several effects on insects/herbivores. One of the most important effects is that they may be anti-feedant, which provides a plant with the greatest potential protection, because damage may be prevented almost before it begins. They may also be toxic, which has the potential to stop damage quickly (Hammerschmidt and Schutz, 1996).

2.2.3 Induced defence responses

The second line of defence is an active one, it is also called into play (induced) in response to invasion or threat by a potential invader. Like the passive defence, it involves both physical and chemical responses (Johal *et al.*, 1995).

2.2.3.1 Elicitors

The rapid recognition of a potential invader is a prerequisite for the initiation of an effective defence response by the plant. This is achieved through the recognition of specific signal molecules also known as elicitors. Elicitors are the molecules that are able to induce physiological or biochemical responses with the expression of resistance (Kogel *et al.*, 1988). They can be secreted by the microbe (exogenous elicitors) or generated as a result of physical and/or chemical cleavage of the plant cell wall (endogenous elicitors) (Somssich and Hahlbrock, 1998). Some known

elicitors are the oligosaccharides, glycoproteins, peptides, phospholipases, polygalacturonides, β-glucans, chitosan (Dixon *et al.*, 1994; Benhamou, 1996).

Elicitor active structures differ with plant species studied, presumably implying that the plant cell has different receptors which when bound by a ligand, trigger the activation of defence-related genes in the nucleus. Recognition of elicitors by the host cells appears to be strictly dependant on their structure, and the defence responses are stimulated by very low concentrations of these compounds (Darvill and Albersheim, 1984). The ability of pathogens and insects to inhibit or delay induced defence responses is presumed to be mediated by suppressor molecules counteracting elicitor activity (Moerschbacher *et al.*, 1990; Scheel and Parker, 1990; Knogge, 1991; Ryan and Farmer, 1991). Symptoms similar to those described for the highly incompatible interactions like stimulation of phenylalanine-ammonia-lyase (PAL) and other enzymes in the hypersensitive cell death, including lignification, are triggered by application of a *Puccinia graminis f.sp. tritici*-elicitor isolated from *Puccinia graminis f.sp. tritici* germ tubes (Moerschbacher *et al.*, 1986 and 1988; Tiburzy and Reisener, 1990). In genetically susceptible rice cultivars, application of an elicitor mediates induced resistance mechanisms similar to those active in genetically resistant cultivars (Scheinpflung *et al.*, 1995).

It has been found previously that RWA infestation induces the release of an elicitor-active molecule, identified as a lectin-binding glycoprotein. This elicitor accumulates in the apoplast of the resistant wheat plants. It is capable of inducing both local and systemic defence responses (Mohase and van der Westhuizen, 2002b).

2.2.3.2 Wounding

Plants respond to wounding from insect attack by activating a variety of defence mechanisms, including the initiation of processes leading to wound healing. Possible defence genes such as the proteinase inhibitors are induced. Other defence related plant reactions such as the hypersensitive response (HR) and development of pheromones may be induced (Siedow, 1991).

Jasmonic acid acts as a signal for wound healing. Accumulation of proteinase inhibitor I and II, mediated by jasmonic acid, is systemically induced by insect chewing, mechanical wounding and oligonuronide treatment (Dixon *et al.*, 1994).

In the RWA-wheat interactions, however, salicylic acid (SA) was induced differentially in the resistant wheat, indicating a possible involvement of SA in the resistance mechanism, therefore, SA may probably act as a signal molecule mediating the downstream defence responses (Mohase and van der Westhuizen, 2002a). In contrast to wounding, the damage caused on susceptible wheat after RWA infestation is probably caused by a phytotoxin secreted during probing, which results in an early chloroplast breakdown (Fouché *et al.*, 1984; Burd and Burton, 1992). Furthermore, wounding of wheat leaves produced a different expression of chitinase isoforms from RWA infestation (Botha *et al.*, 1998).

2.2.3.3 Pathogens

In some cases of pathogenesis, resistance involves a specific recognition of the invading pathogen by a dominant or semi-dominant plant resistance gene product (*R*-gene). This type of interaction is termed gene-for-gene, where for each gene that confers resistance in the host, there is a corresponding gene in the pathogen that

confers its virulence (Flor, 1956). Dangl (1995) proposed a model suggesting that the direct or indirect interaction of the *Avr* and *R* polypeptides triggers resistance. Jones *et al* (1994) hypothesized that the *R* gene products encode receptors capable of binding the *Avr* products as ligands. Expression of these gene products in susceptible plants resulted in a specific resistance, demonstrating that even susceptible plants possess the underlying biochemical machinery required for defence. Thus, the difference between resistance and susceptibility in this scenario appears to lie on the proper recognition of the *Avr* products (Jones *et al.*, 1994).

There is a resemblance between the RWA-wheat and plant-pathogen interactions. In the case of pathogenesis, as in RWA interactions, SA mediates the expression of both local and systemic resistance (Felton *et al.*, 1999). Van der Westhuizen *et al* (1998b) mentioned that the RWA defence responses they have studied closely resemble certain defence responses during pathogenesis.

Plant defence responses have a number of components, some of which appear to be induced sequentially and others simultaneously. The fastest response is the cross-linking of cell wall proteins, stimulated by the hydrogen peroxide that may have formed during an oxidative burst on attempted invasion. This cell wall modification is often associated with the formation of papilla or aposition at the reaction site (Ride, 1992; Wolter *et al.*, 1993; Bestwick *et al.*, 1997). Both cell wall cross-linking and cell wall appositions represent physical (structural) defence responses, and they occur before the integrity of host cell is threatened (Johal *et al.*, 1995). If the invader is able to breach the structural barriers, the next strategy called upon is the HR.

2.2.4 Hypersensitive response (HR)

Several studies have indicated that there are resemblances between the resistance mechanism against herbivores and resistance mechanism against pathogens

(Hammerschmidt and Schultz, 1996; Botha *et al.*, 1998). Previous studies on the RWA-wheat interaction showed that the resistance response was associated with the HR (Belafant-Miller *et al.*, 1994; van der Westhuizen *et al.*, 1998a, b).

The HR is one of the most efficient natural mechanisms of defence that is induced by infection or infestation itself. It has two main characteristics: Necrosis at and around each point at which the leaf tissue was infected, and localization of the invader to the region of attack. The cells surrounding the necrotic area undergo marked metabolic changes, which are believed to cause, or at least to contribute to the observed resistance (Fritig *et al.*, 1990). Functionally, the HR is sufficient to restrict growth of obligate or biotrophic fungal pathogens, which require living cells for growth in their hosts. However, to contain nectrotrophic pathogens that grow in or on dead tissue, the HR has to be supported by other defence mechanisms (Johal and Rache, 1990).

2.2.4.1 Phytoalexins

One mechanism that often accompanies the HR, is the localized synthesis and accumulation of low molecular weight, broad-spectrum antimicrobial compounds called phytoalexins. Phytoalexins are not produced during biotrophic infections. They are produced by healthy cells adjacent to localized damaged and necrotic cells in response to materials diffusing from the damaged cells. They accumulate around both resistant and susceptible necrotic tissues. There is also an elaboration of structural barriers in host cell walls neighboring the site of infection. Those include, lignification, callose or silicon deposition, suberization, and the production of hydroxyproline-rich proteins, which, singly or synergistically, reinforce the cell wall in the vicinity of infection (Johal *et al.*, 1995).

2.2.4.2 Pathogenesis related (PR) proteins

Several PR proteins are induced during the HR (Durner *et al.*, 1997) and are implicated in the defence and resistance responses of plants. Most of them are monomers with low molecular weights (8-50 kDa), and are very stable at low pH and soluble. They are relatively resistant to both endogenous and exogenous proteolytic enzymes, and are generally localized in the apoplast. The apoplast is known to play a central role in the plant's defence mechanism (Bowles, 1990).

Studies have indicated that during the HR in the RWA-wheat interaction, induction of chitinases, peroxidases and β-1,3-glucanases occur. These downstream products were selectively induced in the resistant cultivars to much higher levels than in susceptible cultivars, which indicated their involvement in the resistance mechanisms (van der Westhuizen *et al.*, 1998a, b).

Some of these PR proteins are lytic enzymes, such as chitinases (Neuhaus, 1999) and glucanases (Leubner-Metzger and Meins, 1999; Zemanek *et al.*, 2002), which probably function by degrading the cell walls of various fungal and bacterial pathogens. The antifungal nature of some other PR proteins appears to be due to their thionine-like (Bohlmann, 1999), or proteinase-inhibitor-like (Heitz *et al.*, 1999) properties, and there are still more whose antipathogen mechanisms are unknown. These PR proteins are induced both locally, around the infection sites and systemically, away from the original infection sites where they contribute to long-lasting and broad spectrum resistance to pathogens that would otherwise cause diseases. This long-lasting resistance throughout the entire plant is called systemic acquired resistance (SAR). SAR enhances resistance of a plant against the same or unrelated pathogens (Ryals *et al.*, 1994; Durner *et al.*, 1997).

2.2.5 Signal transduction

The HR is usually preceded by rapid and transient responses occurring mainly at the cell surface, and based predominantly on activation of the pre-existing components. These include: the ion fluxes, protein phosphorylation and dephosphorylation events, changes in exocellular pH and membrane potential, generation of the reactive oxygen species (ROS) "the oxidative burst" and oxidative cross-linking of the plant cell wall proteins (Wojtaszek, 1997a).

An elicitor binds to a receptor at the plasma membrane. The G-proteins at the receptor activate transfer of the elicitor signals from the receptor to the ion channels, which in turn activate the downstream reactions. The ion channels (i.e. the Ca^{2+} and H^+ influx, and K^+ and Cl^- efflux) open, leading to the induction of the oxidative burst i.e. the reactive oxygen species such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\cdot}OH$), through the action of the plasma membrane associated NADPH oxidase (Fig. 2.2) (Somssich and Hahlbrock, 1998).

2.2.5.1 G-proteins

G-proteins act as molecular signal transducers whose active or inactive states depend on the binding of GTP or GDP respectively. The G-proteins include the two major subfamilies, the heterodimeric G-proteins and the small G-proteins. The heterodimeric G-proteins contain α, β and γ subunits. The small G-proteins appear to be similar to free α subunits, operating without the $\beta\gamma$ heterodimer. Generally, it is the α subunit of the heterodimeric G-protein that has the receptor-binding region and possesses a guanosine nucleotide binding site and GTPase activity (Gilman, 1987). Both classes of G-proteins use the GTP/GDP cycle as a molecular switch for signal transduction. Interaction of the G-protein with an activated receptor promotes the exchange of GDP, bound to the α subunit, for GTP and the subsequent dissociation

of the α-GTP complex from the βγ heterodimer (Blumwald *et al.*, 1998). Aharon (1998) suggested that activation of the defence responses could be G-protein mediated through plasma membrane-delimited pathways. In cultured soybean cells, mastoparan, a G-protein-activating peptide, was found to stimulate calcium influx, increases in cytosolic calcium levels and production of the reactive oxygen species in the absence of an elicitor (Chandra and Low, 1997; Aharon, 1998).

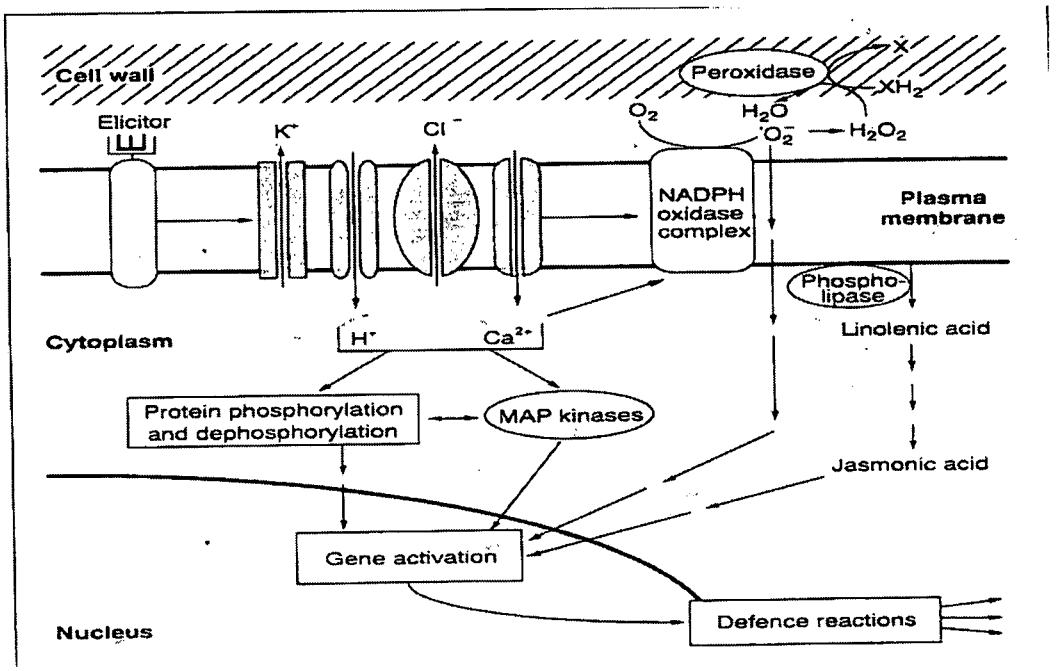

Figure 2.2: Major components of the signal transduction chain in plants (Somssich and Hahlbrock, 1998).

2.2.5.2 Calcium homeostasis

Many cellular processes, including plant responses to pathogens, are regulated by changes in the cytosolic Ca^{2+} concentrations, where free Ca^{2+} can serve to transduce a particular stimulus to target proteins that guide the cellular response. Many of the biochemical responses associated with the defence mechanisms directly correlate with an increase in cytosolic free Ca^{2+} concentration. Measurements of external Ca^{2+} with ion-selective electrodes and of Ca^{2+} fluxes using radiometric techniques have

revealed a large and transient influx with concomitant acidification of the extracellular medium. This suggests a correlation between fungal elicitor activities, hyperpolarization of the host cell plasma membrane, and Ca^{2+} influx (Blumwald *et al.*, 1998).

Able *et al* (2001) discovered that exogenous application of calcium does not have any effect on neither ROS nor HR in infected tobacco suspension cells, but depriving the cell's endogenous calcium significantly suppresses the ROS production and the HR. Blocking inward Ca^{2+} channels at the time of infection completely abolishes ROS production and HR. These results confirm that both ROS production and HR are potentiated by movement of endogenous calcium across the plasmalemma.

The enzyme NADPH oxidase, one of the potential sources of H_2O_2 in plants, also has calcium binding domains (Desikan *et al.*, 1998; Keller *et al.*, 1998; Torres *et al.*, 1998). Moreover, a calcium binding protein, calmodulin, links calcium and H_2O_2 e.g. tobacco cells expressing a constitutively active calmodium showed enhanced HR cell death in response to an incompatible pathogen (Hardig *et al.*, 1997). Calmodulin regulates NAD kinase activity, which generates NADPH for NADPH oxidase activity. Thus cross talk between H_2O_2 and calcium could regulate specificity and/or cross tolerance towards various stresses (Bowler and Fluhr, 2000).

Calcium influx and efflux within a plant cell must be balanced or a host cell might face two disadvantages, namely:

1. inability to sustain the high cytosolic Ca^{2+} levels that are responsible for subsequent biochemical changes,

2. inefficient utilization of energy for maintaining the function of the plasma membrane-bound Ca^{2+}-ATPase (Blumwald *et al.*, 1998).

2.2.5.3 Protein kinases

The phosphorylation cascade, which is probably initiated a receptor, is thought to be involved in signaling at many different levels. Reversible protein phosphorylation is thought to be a key event in regulating the oxidative burst in response to pathogen challenge (Schwacke and Hager, 1992; Baker *et al.*, 1993; Levine *et al.*, 1994; Chandra and Low, 1995; Desikan *et al.*, 1996). It is also involved in the downstream signaling following the H_2O_2 generation and/or perception (Levine *et al.*, 1994; Rajasekhar e*t al.*, 1999; Grant *et al.*, 2000a). Given the large number of protein kinases and phosphatases in plant genomes, and the complexity of signal transduction, it is likely that an interconnecting network of protein kinases and phosphatases (and other signaling components) will eventually be characterized. Moreover, it also likely that the intercellular location of these components will be of critical importance in determining specific outcomes of the signaling pathways that are activated by specific stimuli. As cytosolic calcium elevation is a common and an early response to H_2O_2, it is likely that activation of calcium dependent protein kinases and phosphatases will be an early step, with some enzymes potentially mediating downstream signaling components such as other protein kinases/phosphatases and other effector proteins. To date, though, no calcium dependent protein kinases have shown to be regulated by H_2O_2, although H_2O_2-regulated genes encoding protein kinases and phosphatases have been discovered. However, it is of course possible that constitutively calcium dependent protein kinases are involved in the H_2O_2 signaling (Neill *et al.*, 2002).

A protein phosphorylation cascade that has been shown to be activated by H_2O_2 is a mitogen activated protein kinase (MAPK) cascade. MAPK cascades are evolutionarly conserved in all eukaryotes. Perception of an extracellular signal activates a MAP kinase kinase kinase (MAPKKK). This kinase then phosphorylates a MAPKK, which in turn activates a MAPK by dual phosphorylation on both threonine and tyrosine residues in a conserved T-X-Y motif (Fig. 2.3). Activation of

the MAPK can facilitate its translocation to the nucleus where it can phosphorylate and activate transcription factors, thereby modulating gene expression. In plants, MAPKs can be activated in response to extracellular signals such as drought, cold, phytohormones, pathogen challenge and osmotic stress, that lead to the activation of signal transduction pathways resulting in nuclear gene expression (Hirt, 1997). The *Pto* gene of tomato, which confers resistance to a bacterial speck disease, encodes a cytosolic serine/threonine kinase that interacts with other proteins. Probably by phosphorylating them, some of these target proteins are putative transcription factors thought to activate the PR protein encoding genes (Zhou *et al.*, 1997). It was shown that H_2O_2 induces the activation of MAPK in *Arabidopsis* suspension cultures (Desikan *et al.*, 1999) and H_2O_2 has been shown to activate two MAPKs in *Arabidopsis* plants, at least one of which is activated independently of salicylic acid, jasmonate and ethylene signaling pathways (Grant *et al.*, 2000a).

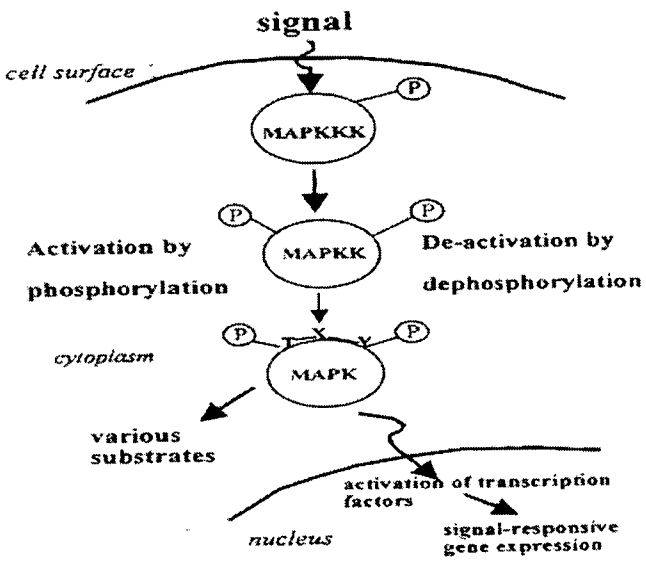

Figure 2.3: Mitogen-activated protein kinase (MAPK) signaling cascade. Schematic representation of a MAPK cascade, which involves activation of a MAPK kinase kinase (MAPKKK) by an extracellular stimulus leading to the sequential phosphorylation of a MAPK kinase (MAPKK) and a MAPK, the latter being dually phosphorylated on conserved threonine (T) and tyrosine (Y) residues (Neill *et al.*, 2002).

2.2.5.4 Plasma membrane H^+-ATPase

Proton extrusion at the plasma membrane provides the electrochemical gradient across the plasma membrane that drives the different H^+-coupled (antiport and symport) and membrane potential-coupled (uniport) transport mechanisms for the uptake and extrusion of solutes. In addition, the membrane potential regulates a number of plasma membrane-bound ion channels, and acidification of the extracellular medium regulates the physical and biochemical properties of the cell wall. Changes in the host plasma membrane H^+-ATPase activity (with the associated changes in ion fluxes across the plasma membrane) are among the earliest events associated with elicitation. In some cases, treatment with elicitors results in inhibition of H^+-ATPase activity and a concomitant depolarization of the plasma membrane potential (Vera-Estrella, 1994). In other cases, treatment with other elicitors results in activation of the plasma membrane H^+-ATPase, with a consequent acidification of the extracellular medium and hyperpolarization of the membrane potential. It has been proposed that the differential effect of elicitors on the plasma membrane H^+-ATPase and the resultant acidification or alkalinization of the extracellular medium is in response to the difference between specific and non-specific elicitors (De Wit, 1995).

2.2.6 Reactive oxygen species "Oxidative burst"

The oxidative burst is an integral component of plant resistance to pathogen and insect attack. It is generally defined as a rapid production of high levels of the reactive oxygen species (ROS) in response to external stimuli (Wojtaszek, 1997b). In plant-insect interactions, one of the defence functions of oxidative burst is direct injury to herbivorous insects. It is also linked with the indirect injury through the oxidative damage of the insects' dietary lipids, proteins, vitamins, antioxidants or through acting as feeding repellents (Felton *et al*, 1994).

Previous studies have shown that the major ROS contributing towards the oxidative burst is hydrogen peroxide (H_2O_2), with possible participation of superoxide anion (O_2^-) (Levine *et al.*, 1994; Alvarez *et al.*, 1998). The occurrence of a transient increase in ROS production is usually very rapid, but vary depending on the plant systems studied, and the challenging factor used. Oxidative burst is a two phase phenomenon. Phase I is an immediate and very transient ROS production, non-specifically stimulated by compatible, incompatible plant pathogen interactions and even saprophytic bacteria. In contrast, phase II is a delayed (1-3 hours after addition of bacteria) and prolonged ROS production that is specifically stimulated by incompatible plant-pathogen interactions. Infection of tobacco (*Nicotiana tabaccum*: incompatible interaction) with necrogenic bacterium, *Erwinia amylovora*, induced a sustained production of the superoxide anion, lipid peroxidation, electrolyte leakage, and concomitant increases of several anti-oxidative enzymes in contrast to the compatible pathogen, *Pseudomonas syringae* pv. *tabaci*, which did not cause any induction of such reactions. The incompatible pear-*P. syringae* pv. *tabaci* interaction also enhanced the superoxide accumulation, lipid peroxidation, electrolyte leakage and antioxidative enzymes (Venissé *et al.*, 2001). Different mechanisms (controlled or non-controlled; enzymatic or non-enzymatic) are involved in the generation of the ROS.

2.2.6.1 Sources of ROS

2.2.6.1.1 Respiration

The mitochondrion is a major source of ROS formation, and it is possible that this organelle could participate in the oxidative burst in plants (Tiwari *et al.*, 2002). Exposure of *Arabidopsis* cells to a mild constant oxidative stress increased respiratory electron transport and oxygen uptake in isolated mitochondria, leading to

increased production of H_2O_2, effectively amplifying the oxidative stress (Braidot *et al.*, 1999).

Plants, as other aerobic organisms, require oxygen (O_2) for the efficient production of energy. During the reduction of O_2 to H_2O, reactive oxygen species such as O_2^-, H_2O_2, and hydroxyl radical ($^{\cdot}OH$) are generated (Fig. 2.4). Initially, the reaction chain requires an input of energy, whereas subsequent steps are exothermic and can occur spontaneously, either catalyzed or not (Vranová *et al.*, 2002).

Acceptance of excess energy by O_2 can additionally lead to the formation of a singlet oxygen ($^1O_2$), a highly reactive molecule when compared to O_2. Singlet oxygen can last for nearly 4μs in water and 100μs in a non-polar environment (Foyer and Harbison, 1994). It can either transfer its excitation energy to other biological molecules or react with them, thus forming endoperoxides or hydroperoxides (Vranová *et al.*, 2002).

Superoxide anion (O_2^-) is a moderately reactive, short-lived ROS with a half-life of approximately 2-4μs. Therefore, O_2^- cannot cross biological membranes and is dismutated readily to H_2O_2. Alternatively, O_2^- reduces quinones and transition metal complexes of Fe^{3+} and Cu^{2+}, thus affecting the activity of metal-containing enzymes (Vranová *et al.*, 2002).

Hydroperoxyl radicals ($HO^{\cdot}_2$) that are formed from O_2^- by protonation in aqueous solutions can cross biological membranes and remove the hydrogen atoms from polyunsaturated fatty acids and lipid hydroperoxides, thus initiating lipid auto-oxidation. H_2O_2 is a moderately reactive, and is a relatively long-lived molecule (half-life of 1μs) that can diffuse some distances from its production site. It may inactivate enzymes by oxidizing their thiol groups (Vranová *et al.*, 2002).

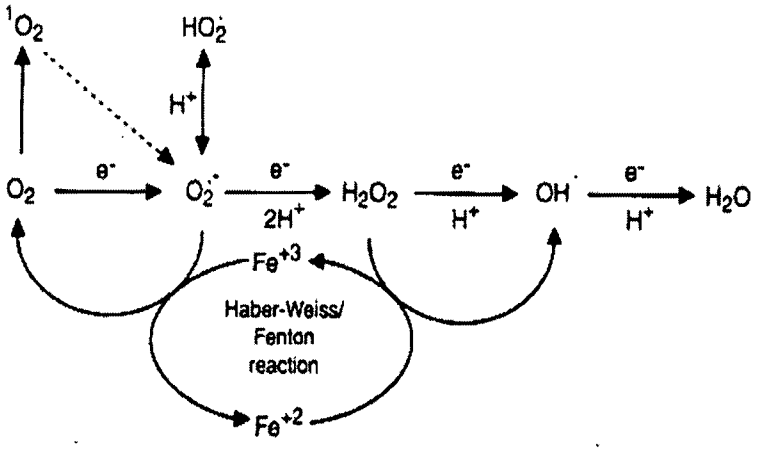

Figure 2.4: Interconversion of the ROS derived from O_2. Ground state molecular oxygen can be activated by excess energy, reversing the spin of one of the unpaired electrons to form $^1O_2$. Alternatively, one electron reduction leads to the formation of $O_2^{\cdot-}$, which exists in equilibrium with its conjugate acid, $HO_2^{\cdot}$. Subsequent reduction steps then form H_2O_2, $OH^{\cdot}$, and H_2O. Metals that are mainly present in cells in the oxidized form are reduced in the presence of $O_2^{\cdot-}$ and , consequently, may catalyze the conversion of H_2O_2 to $OH^{\cdot}$ by the Fenton or Haber-Weiss reactions (Vranova *et al.*, 2002).

The most reactive of all ROS is the hydroxyl radical ($OH^{\cdot}$). It is formed from H_2O_2 through the so called Haber-Weiss or Fenton reaction, using metal as catalysts (Wojtaszek, 1997b):

Haber-Weiss reaction: $H_2O_2 + O_2^{\cdot-} \rightarrow {}^{\cdot}OH + {}^{\cdot}OH^- + O_2$

Fenton reaction: $H_2O_2 + Fe^{2+}(Cu^+) \rightarrow Fe^{3+}(Cu^{2+}) + {}^{\cdot}OH + OH^-$

$O_2^{\cdot-} + Fe^{3+}(Cu^{2+}) \rightarrow Fe^{2+}(Cu^+) + O_2$

The hydroxyl radical can potentially react with all biological molecules, and because cells have no enzymatic mechanism to eliminate this highly reactive ROS, its excess production leads to ultimate death (Vranová *et al.*, 2002).

2.2.6.1.2 Photosynthesis

During photosynthesis under a high light flux, especially in the saturation range of the photosynthetic light curve, more light is absorbed by the photosynthetic apparatus that can be used for the biochemical dark reactions, for example, carbon dioxide fixation. The most important sources of ROS during photosynthetic electron transport are the reduced electron acceptors of photosystem I, especially ferredoxin, which transfer individual electrons to oxygen if the redox chain leading to $NADP^+$ is almost reduced due to accumulation of electrons (photo-reduction of oxygen) (Elstener, 1991; Asada, 1999).

2.2.6.1.3 Biotic and abiotic stress

The ROS generation is also induced in plants following exposure to a wide variety of abiotic and biotic stimuli. These include extreme temperatures, UV irradiation, excess excitation energy, ozone exposure, phytohormone such as abscisic acid, dehydration, wounding, and elicitor and pathogen challenge (Prasad *et al.*, 1994; Lamb and Dixon, 1997; Karpinski *et al.*, 1999; Orozco-Cárdenas and Ryan, 1999; Guan *et al.*, 2000; Langebartels *et al.*, 2000; Pei *et al.*, 2000; A-H-Marckerness *et al.*, 2001). Given that H_2O_2 is produced in response to such a variety of stimuli, it is likely that H_2O_2 mediates cross-talk between signaling pathways and is, an attractive signaling molecule contributing to the phenomenon of "cross-tolerance", in which exposure of plants to one stress offers protection towards the another (Bowler and Fluhr, 2000). For example, exposure to sublethal doses of ozone or UV conferred tolerance to infection by a virulent pathogen (Sharma *et al.*, 1996), and exposure to

heat stress induced tolerance towards subsequent pathogen attack (Vallelian-Bindschender *et al.*, 1998). In addition, exposure to low levels of one stress (e.g. cold) can induce tolerance towards subsequent higher levels of exposure to the same stress, a phenomenon termed acclimation tolerance (Prasad *et al.*, 1994).

2.2.6.2 The ROS generating enzymes

2.2.6.2.1 NADPH oxidases

There is accumulating evidence that the production of ROS is catalyzed by an enzyme with similarities to the phagocytic NADPH oxidase (Amacucci *et al.*, 1998). In several model systems studied in plants, the oxidative burst and the accumulation of H_2O_2 appear to be mediated by the activation of a membrane-bound NADPH oxidase complex (Lamb and Dixon, 1997; del Río *et al.*, 1998a; Potikha *et al.*, 1999; Pei *et al.*, 2000). In animal cells, this enzymatic complex consists of two membrane-associated polypeptides (gp91-phox and gp22-phox) that become active when at least three proteins from the cytosol (p47-phox, p67-phox, and rac) bind to the membrane components (Jones, 1994; Henderson and Chappel, 1996). The NADPH oxidase is thought to consist of at least two plasma membrane redox components, a flavoprotein and a *b*-type Cyt. The mechanism of superoxide anion generation by this enzyme consists of a two-electron transfer from cytosolic NADPH to the flavoprotein components using gp91-phox and gp22-phox as its subunits, one electron transfer to the *b*-type Cyt component, and one electron reduction of O_2 in the following reaction:

$$NADPH + 2O_2 \rightarrow NADP^+ + 2O_2^- + H^+$$

In plants, this enzyme generates ROS at the plasma membrane or extracellularly in the apoplast. Lignifying xylem tissues were able to accumulate H_2O_2 and sustain the H_2O_2 production. The H_2O_2 production in the xylem of *Zinnia elegans* was sensitive to diphenylene iodonium (DPI). Diphenylene iodonium is a suicide inhibitor of

mammalian neutrophil NADPH oxidase. It inhibits NADPH oxidase activity by binding irreversibly to the flavonoid group of the membrane associated gp91-phox subunit of the NADPH oxidase complex (O'Donnel *et al.*, 1993). Further support for the participation of NADPH oxidase-like activity in H_2O_2 production in lignifying xylem was obtained from the observation that areas of H_2O_2 production were superimposed on areas producing superoxide anion, the suspected product of NADPH oxidase, although attempts to demonstrate the existence of superoxide dismutase activity in intercellular washing fluid from *Z. elegans* were unsuccessful. Even so, the levels of NADPH oxidase-like activity in microsomal fractions, and of peroxidase in intercellular washing fluid, were consistent with a role of NADPH oxidase in the delivery of H_2O_2 which may be further used by the xylem peroxidases for the synthesis of lignins (Barcelo, 1998).

The accumulation of H_2O_2 in wounded or systemin-treated tomato leaves was inhibited by DPI (Orozco-Cárdenas and Ryan, 1999). The NADPH dependent production of O_2^- by the plasma membrane and elicitor treated rose cells was also inhibited by DPI. The results obtained in these studies suggest that the enzyme responsible for the synthesis of O_2^- might be similar to the mammalian neutrophil NADPH oxidase, and they are inconsistent with the hypothesis that the synthesis of O_2^- is catalyzed by the extracellular peroxidase (Auh and Murphy, 1995).

2.2.6.2.2 Peroxidases

The production of H_2O_2 by pH-dependent cell wall peroxidases has been proposed as an alternative way of ROS production during biotic stress (Bolwell and Wojtaszek, 1997; Wojtaszek, 1997a, b). This hypothesis was proved when the H_2O_2 accumulation was sensitive to the inhibitors of peroxidases (KCN and NaN_3). Probably, the best characterized model system with respect to the role of peroxidases is the responses of suspension cultured French bean cells to elicitor derived from the

cell walls of *Colletotrichum lindemuthianum.* These cells showed a rapid increase in oxygen uptake, which was followed shortly by the appearance of a burst of ROS, which was probably accounted for by H_2O_2. An essential factor in this production appeared to be a transient alkalinization of the apoplast where the pH rises to 7.0-7.2. Dissipation of this pH change with a number of treatments, including ionophores and strong buffers, substantially inhibited the oxidative burst (Wojtaszek, 1997b).

Overall evidence obtained for a peroxidase dependent oxidative burst in *Arabidopsis* supports a role for French bean peroxidase (FBP) 1-like peroxidases in the oxidative burst. However, this would have to be reconciled with the emerging reverse genetics data for NADPH oxidases in *Arabidopsis.* In this context, the *avr*-mediated oxidative burst in *Arabidopsis* is DPI sensitive (Grant *et al*, 2000b) as in harpin-induced ROS production in *Arabidopsis* cell cultures (Desikan *et al.*, 1996). Treatment of *Arabidopsis* cell cultures with *Fusarium*-derived elicitors showed an oxidative burst which is even more sensitive to DPI than elicitation of French bean cells (Bolwell *et al.*, 2002). Based on these results, Bolwell *et al* (2002) suggested that NADPH oxidase would function in highly specific *R* gene-*avr* gene interactions while the apoplastic peroxidases system would be placed in the realm of responses to elicitor molecules thought by some to represent general defence response. This discrimination between the two types of bursts has been proposed for quite some time (Baker and Orlandi, 1995).

Peroxidase-generated H_2O_2 may function as an antifungal agent in disease resistance. Reduced NAD and NADP in the presence of peroxidase and O_2 may generate antimicrobial quantities of H_2O_2. Hydrogen peroxide inhibits pathogens directly, and or may generate other reactive free radicals that are antimicrobial. The rapid oxidation of reduced NAD and NADP can activate the pentose pathway, which requires oxidized NADP to produce erythrose-4-phosphate and phosphoenolpyruvate. Both of the latter compounds are precursors to cinnamic acid-related phenols via the shikimate pathway. The cinnamic acid-related phenols may

function as phytoalexins or phytoalexin precursors, and may also be polymerized to lignin in a series of reactions that include H_2O_2 and peroxidases. Lignin could further restrict a pathogen within penetrated tissue that contains phytoalexins, H_2O_2, and other antimicrobial compounds. These peroxidases could not only participate in the biosynthesis of antimicrobial compounds and lignin, but also serve as regulators for the entire metabolic process (Peng and Kuc, 1992). Moreover, the peroxidase mediated H_2O_2 generation inhibited the germination of sporangiophores of *P. tabacina in vitro* and disease development of blue mold on tobacco leaf discs (Wojtaszek, 1997b).

2.2.6.2.3 Superoxide dismutases

Superoxide dismutases (SODs) are a family of metalloenzymes that catalyze the disproportionation of O_2^- into H_2O_2 and H_2O (del Río *et al.*, 2002). The superoxide anion is produced at any location where an electron transport chain is present, and hence O_2 activation may occur in different compartments of the cell (Elstener, 1991), including mitochondria, chloroplasts, glyoxysomes, peroxisomes, apoplast, and the cytosol. This being the case, it is not surprising to find that SODs are present in all these subcellular locations (Fig. 2.5) (Alsher *et al.*, 2002). Based on the metal cofactor used by this enzyme, SODs are classified into three groups:

1. the iron SODs are found in both prokaryotes and eukaryotes. In all of the plant species examined to date, it is inferred that they are located in the chloroplasts. They are however absent in animals (Alsher *et al.*, 2002),

2. the manganese SODs are also found in both eukaryotes and prokaryotes. They are located in the peroxisomes and mitochondria (Fridovich, 1986),

3. the copper-zinc SODs have been found mostly in eukaryotes, some are also present in prokaryotes. They are located in the chloroplasts, peroxisomes, and the cytosol (Bordo *et al.*, 1994).

Comparison of deduced amino acid sequences from these three different types of SODs suggest that the manganese and iron SODs are the more ancient types of SODs, and these enzymes most probably have arisen from the same ancestral enzyme, whereas copper-zinc SODs have no sequence similarities to the Mn and Fe SODs and probably have evolved separately (Kanematsu and Asada, 1990; Smith and Doolittle, 1992). The evolutionary reason for separation of SODs with different metal requirements is probably related to the different availability of soluble transition metal compounds in the biosphere in relation to the O_2 content of the atmosphere in different geological areas (Bannister *et al.*, 1991).

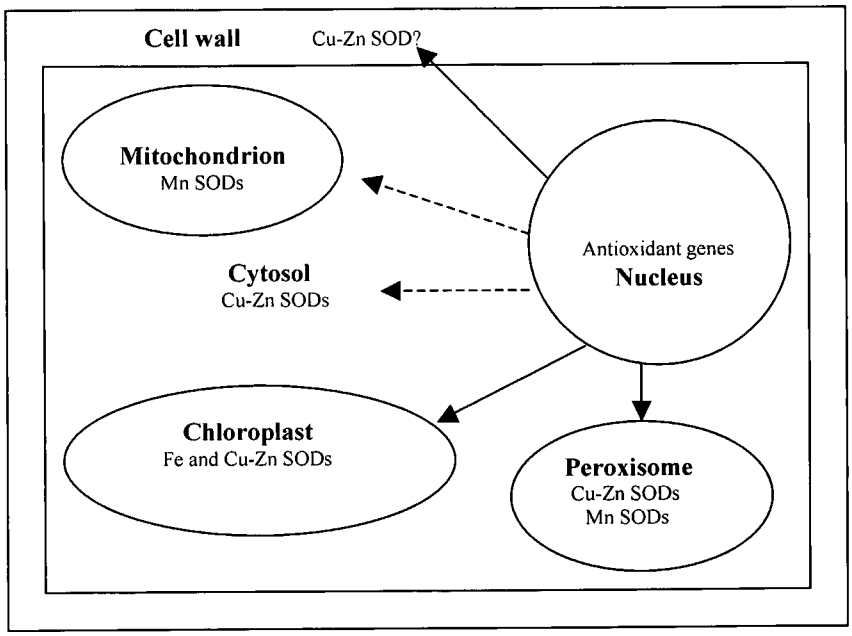

Figure 2.5: Locations of superoxide dismutases throughout the plant cell (redrawn from Alscher *et al.*, 2002).

2.2.6.2.4 Xanthine oxidases (Xanthine: oxygen oxidoreductase)

Xanthine oxidase is a complex of metalloflavoproteins containing one molybdenum, one FAD, and two iron-sulphur centres of the ferredoxine type in each of its two independent subunits. It catalyses the oxidation of xanthine and hypoxanthine to uric

acid in the peroxisomes (Fig. 2.6), and is a well-known producer of superoxide radicals. The presence of xanthine and uric acid, substrate and product, respectively, of the XOD reaction, as well as allantoin, the product of the uricase reaction, was detected in leaf peroxisomes by HPLC analysis (Corpas *et al.*, 1993). The occurrence of xanthine, uric acid, and allantoin in leaf peroxisomes indicate a role of these organelles in the catabolism of xanthine produced as a result of the turnover of nucleotides, RNA and DNA in leaf cells (Corpas *et al.*, 1993; del Río *et al.*, 1998b).

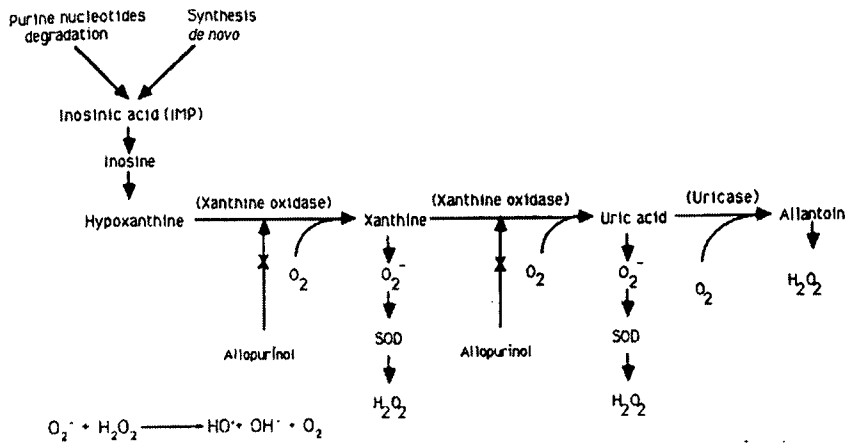

FIGURE 2.6: Possible involvement of xanthine oxidase in the production of the reactive oxygen species (Montalbini, 1992).

In the incompatible bean rust response, toxic effects are reached as a consequence of strong activation of both XOD and uricase of the host, presumably associated with unscavenged toxic oxygen species production and primarily responsible for HR (Montalbini, 1992b). Montalbini (1992a, b) also suggested that XOD could be a source of ROS during tobacco mosaic virus (TMV) infection and various rust-induced HR's in tobacco, bean, and wheat plants. Allupurinol treatment of bean leaves suppressed the development of HR symptoms as well as the electrolyte leakage caused during incompatible rust infection. Allupurinol (4-hydroxypyrazolo (3,4-d) pyrimidine) is a purine analogue, a competitive inhibitor of XOD, effective *in*

vitro and *in vivo* since it binds tightly to the reduced molybdenum component of the enzyme (Montalbini, 1992b).

In contrast to this hypothesis, Ádám *et al* (2000) suggested that XOD is not the main ROS generator in wheat during the HR to leaf rust. In their investigations, they found that allupurinol treatment does not affect the incompatible interaction between *Triticum aestivum* and *Puccinia recondita* f.s.p. *tritici,* even at the highest concentration studied. Moreover, HR did not change after treatment with allupurinol.

2.2.6.2.5 Oxalate oxidases

The oxalate oxidases are located in the extracellular matrix of plants, and they were also found to be involved in plant defence. They catalyze the conversion of oxalate to carbon dioxide and H_2O_2 according to the reaction:

$HOOC\text{-}COOH + O_2 \rightarrow 2CO_2 + H_2O_2$

According to Peng and Kuc (1992), ROS generated by oxalate oxidases could be directly toxic to microorganisms. Oxalate oxidase activity is suggested to be a marker of general defence responses rather than a cultivar resistance marker (Hurkman and Tanaka, 1996).

2.2.6.2.6 Amine oxidases

The copper containing amine oxidases catalyze the oxidation of a wide variety of biogenic amines, including mono-, di-, and polyamines to the corresponding aldehydes with the release of NH_3 and H_2O_2 according to the reaction:

$RCH_2NH_2 + O_2 + H_2O \rightarrow RCHO + NH_3 + H_2O_2$

The plant amine oxidases are predominantly localized in the extracellular matrix. Hydrogen peroxide formed from the oxidation of amines may be directly utilized by the cell wall-bound peroxidases in lignification and cell wall strengthening, both during normal growth and in response to external stimuli such as wounding and pathogenesis (Allan and Fluhr, 1997; Bolwell and Wojtaszek, 1997).

2.2.6.2.7 Urate oxidases (uricases)

Urate oxidase has been found to be strongly induced in the incompatible response between *P. vulgaris* and *Uromyces phaseoli*. Therefore it may be supposed that the superoxide derived from XOD activation, and H_2O_2 derived from uricase activation and superoxide disproportionation, may react in the "Haber-Weiss reaction or in the presence of chelates or in Fenton-like reaction" to form a highly reactive hydroxyl radical, which in turn may alter the integrity of the membrane, probably via lipid peroxidation and the associated free-radical chain reaction (Montalbini, 1992a).

2.2.6.3 Involvement of ROS in the HR

Hydrogen peroxide generated during the oxidative burst may perhaps be sufficient as a local trigger for the programmed cell death of challenged cells (Levine *et al.*, 1994). Treatment of non-photosynthetic *Arabidopsis* cells with glucose oxidase-glucose (H_2O_2-generating mixture) resulted in a high induction of H_2O_2 and caused cell death in 66.9 % of cells. To test whether the continuous oxidative stress activated an active signalling mechanism, cell cultures were pre-incubated with protease inhibitors that blocked the H_2O_2 dependent cell death in soybean and *Arabidopsis* cultures. Cell death was reduced significantly, indicating that oxidative stress induced an active HR-like programmed cell death pathway (Levine *et al.*, 1996; Solomon *et al.*, 1999).

This hypothesis was further proved in DPI treated tomato inoculated with *P. syringae*, where DPI blocked H_2O_2 accumulation and also reduced the induction of programmed cell death (Alvarez *et al.*, 1998). Moreover, tobacco cells that undergo HR upon infiltration with fungal elicitors showed a decrease in catalase activity level which was paralleled by a strong H_2O_2 accumulation (Dorey *et al.*, 1998). The ability of H_2O_2 to induce cell death was also demonstrated *in vivo*. In transgenic plants with lower H_2O_2 scavenging or in others that overproduce H_2O_2 generating enzymes, cell death appeared spontaneously or could be easily induced by stress (Chamnongpol *et al.*, 1998; Kazan *et al.*, 1998).

Transgenic catalase/peroxidases-deficient tobacco plants (i.e. in which endogenous H_2O_2 will not be readily catabolized) were hyperresponsive to pathogen challenge, thus providing direct evidence for a role of H_2O_2 in cell death (Mittler *et al.*, 1999). Recently, cell death triggered in barley aleurone by phytohormone gibberrellin was also found to be mediated by H_2O_2 (Bethke and Jones, 2001; Fath *et al.*, 2002), implying a role for H_2O_2 in developmental programmed cell death in addition to that induced by pathogen challenge.

2.2.6.4 Signaling role of ROS in plant defence

The H_2O_2 can act as a diffusible signal for the induction of cellular protectant genes in surrounding cells. Although microbial elicitors, insect or pathogen attack induce a massive switch in host gene expression, H_2O_2 from the oxidative burst functions as a selective signal for the induction of only a subset of defence genes. It is a small diffusible molecule, and demonstration of catalase-sensitive signal transmission across dialysis membranes from infected cells to adjacent uninfected cells, indicate that H_2O_2 functions as a mobile intercellular signal for selective activation of cellular protectant genes (Levine *et al.*, 1994).

Hydrogen peroxide generated in the vascular bundles of tomato leaves in response to wounding can also act as a diffusible signal for the expression of defence genes or "late genes" (such as the proteinase inhibitors) in the mesophyll cells, but not for signaling pathway genes "early genes" in the vascular bundle cells (Orozo-Cárdenas *et al.*, 2001). Transgenic plants with elevated levels of H_2O_2 due to the constitutive overproduction of glucose oxidase or repression of peroxisomal catalase were more resistant to pathogens, accumulated salicylic acid, and expressed pathogenesis related genes and proteins (Wu *et al.*, 1997; Chamnongopol *et al.*, 1998).

Alvarez *et al* (1998) investigated the involvement of ROS generated at the site of the immunizing inoculation with an avirulent pathogen in the establishment of systemic acquired resistance. They discovered that localized generation of H_2O_2 *in situ* by glucose oxidase-glucose mixture infiltrated into small regions of two primary leaves caused systemic induction of *PR-2* transcripts which serve as molecular markers for the SAR, accompanied by the development of resistance in secondary leaves to subsequent challenge with a virulent strain of *P. syringae* pv. m*aculicola.*

Under stress conditions, one of the strategies that plants have adopted is to slow down growth. The ability to reduce cell division under unfavorable conditions may not only allow conservation of energy for defence purposes, but also may limit the risk of heritable damage (May *et al.*, 1998). The ROS, as ubiquitous messenger of stress responses, probably play a signaling role in these adaptive processes. Exogenous application of micro molar concentrations of reduced glutathione (GSH) raises the number of meristematic cells undergoing mitosis, whereas depletion of GSH has the opposite effect (Sánchez-Fernández *et al.*, 1997). While the cell cycle progression is under negative control of ROS, H_2O_2 stimulates somatic embryogenesis (Cui *et al.*, 1999) and is essential for root gravitropism (Joo *et al.*, 2001).

2.2.6.5 Oxidative stress and antioxidative mechanisms

Oxidative stress arises from an imbalance in the generation and metabolism of ROS, with more ROS (such as H_2O_2) being produced than are metabolized. Well-established deleterious effects of ROS include damage of DNA and proteins, and lipid peroxidation. Hydrogen peroxide also inhibits chloroplast sulfhydryl-containing enzymes by readily oxidizing their sulfhydryl groups (Loggini *et al.*, 1999). However, plants possess a battery of antioxidant mechanisms, both enzymatic and non-enzymatic, by which ROS are removed from the cell (Noctor and Foyer, 1998). Thus, critical balance between the production and metabolism of ROS determines the fate of the cell (Neill *et al.*, 2002). In non-stressing conditions, the antioxidative defence system of cells provides adequate protection against ROS by the concerted action of both enzymatic and non-enzymatic antioxidants (Halliwell and Gutteridge, 1989; Asada, 1999).

2.2.6.5.1 Carotenoids

Chloroplasts contain low molecular weight lipophylic antioxidants such as the carotenoids and tocopherols, which protect membranes from oxidative damage (Fryer, 1992; Havaux, 1998; Asada, 1999). Apart from their light harvesting function and structural role, carotenoids prevent the chlorophyll-photosynthesized formation of highly destructive singlet oxygen by scavenging any additional singlet oxygen present. An even more important function of carotenoids is their ability to accept directly electronic excitation energy from triplet chlorophyll. In particular, β-carotene is in close contact with the chlorophyll and can therefore be excited at the triplet level by radiationless energy transfer. The energy is then released in the form of heat. In the absence of carotenoids, chlorophyll is not only destroyed by light (visible as bleaching), but also all structures in the chloroplast are destroyed (Siefermann-Harms, 1987; Havaux *et al.*, 2000).

2.2.6.5.2 α-tocopherols

On the other hand, α-tocopherol (vitamin E) is also able to scavenge singlet oxygen and lipid peroxyradicals, to form quinone derivatives and chromanoxyl radicals, respectively. Chromanoxyl radicals may be recycled back to α-tocopherol by ascorbic acid and reduced glutathione, whereas the scavenging of singlet oxygen by α-tocopherol appears not to be reversible. Both α-tocopherol and carotenoids are bound to the lipid matrix of thylakoid and chloroplastic membranes (Fryer, 1992; Havaux *et al.*, 2000).

2.2.6.5.3 The ascorbate-glutathione cycle

The ascorbate-glutathione cycle, also called the Foyer Halliwell-Asada cycle (Fig. 2.7), is an efficient way for plant cells to dispose H_2O_2 in certain cellular compartments where this metabolite is produced and no catalase is present (Halliwell and Gutteridge, 2000). This cycle makes use of the non enzymatic antioxidants, ascorbate and glutathione, in a series of reactions catalyzed by four antioxidative enzymes, and has been demonstrated in chloroplasts, cytosol, root nodule mitochondria and peroxisomes (Foyer and Mullineaux, 1994).

The characteristics of ascorbate peroxid were well studied in *Euglena* (Shigeoka *et al.*, 1980a, b). Ascorbate peroxidase utilizes ascorbate as its specific electron donor to reduce H_2O_2 to water with the concomitant generation of monodehydroascorbate (MDA) (Shigeoka *et al*, 2002). Monodehydroascorbate is spontaneously disproportionated to ascorbate and dehydroascorbate (DHA). It is also directly reduced to ascorbate by the action of NAD(P)H-dependant MDA reductase. Dehydroascorbate reductase utilizes glutathione (GSH) to reduce DHA and thereby

regenerate ascorbate. The oxidized glutathione (GSSH) is then regenerated by glutathione reductase, utilizing reducing equivalents from NAD(P)H. Thus, APX in combination with the effective ascorbate-glutathione cycle functions to prevent the accumulation of toxic levels of H_2O_2 in photosynthetic organisms (Asada, 1992 and 1997).

The APX isoenzymes are critical components that prevent oxidative stress in photosynthetic organisms. Additionally, recent studies on the response of APX expression to some stress conditions and pathogen attack indicate the importance of APX activity in controlling the H_2O_2 concentration in intracellular signaling (Shigeoka *et al.*, 2002). They are distributed in at least four distinct cellular compartments. The stromal APX (sAPX) and thylakoid membrane-bound APX (tAPX) in chloroplasts, microbody (including glyoxysome and peroxisome), membrane bound APX (mAPX), and cytosolic (cAPX) (Chen and Asada, 1989; Miyake *et al.*, 1993; Yamaguchi *et al.*, 1995a, b; Bunkelmann and Trelease, 1996; Ishikawa *et al.*, 1996a, c and 1998). A fifth APX isoenzyme (mitAPX) occurs in a mitochondrial membrane-bound form (Jimenéz *et al.*, 1997; Leonardis *et al.*, 2000).

2.2.6.5.3.1 Properties of the APX isoenzymes

The APX isoenzymes have high specificity for ascorbate as the electron donor, especially in the case of chloroplastic APX (chlAPX) and (mitAPX) isoenzymes (Yoshimura *et al.*, 1998; Asada, 1999; Leonardis *et al.*, 2000). In addition to oxidizing ascorbate, cAPX and mAPX of higher plants and algal APX's can also oxidize artificial electron donors such as pyrogallol or guaiacol at appreciable rates (Chen and Asada, 1989; Ishikawa *et al.*, 1995 and 1996b; Yoshimura *et al.*, 1998; Asada, 1999). Plant APX isoenzymes cannot reduce lipid hydroperoxides. APX, a haem-containing enzyme with a protoporphyrin prosthetic group is inhibited by cyanide. It is also inhibited by thiol modifying reagents such as *p*-

chloromercuribenzoate and suicide inhibitors such as the hydroxylamine, *p*-aminophenol, and hydroxyurea (Chen and Asada, 1989 and 1990). One of the most characteristic properties APX is its instability in the presence of ascorbate. The instability of APX seems to be a reason that APX was not found for a long time in photosynthetic organisms. ChlAPX isoenzymes exist in a monomeric form, but cAPX is a dimer consisting of identical subunits with a molecular mass of 28 kDa (Mittler and Zilinkas, 1991; Miyake *et al.*, 1993). As for chlAPX isoenzymes, the molecular mass of tAPX and sAPX is about 4.5kDa larger than that of sAPX (33.2 kDa), the difference between tAPX and sAPX is related to the requirement of membrane binding (Chen and Asada, 1989; Miyake *et al.*, 1993; Ishikawa *et al.*, 1996a). The molecular masses (31 kDa) of mAPX and mitAPX are similar (Yamaguchi *et al.*, 1995a; Ishikawa *et al.*, 1998; Leonardis *et al.*, 2000).

2.2.6.5.4 Catalases

Catalase converts H_2O_2 to H_2O and O_2 in the following reaction:

$HO\text{-}OH + HO\text{-}OH \rightarrow 2H_2O + O{=}O$

Plants, unlike animals, have multiple isoforms of catalase, which are present in the peroxisomes, chloroplasts, and mitochondria (Baker and Orlandi, 1995; Shigeoka *et al.*, 2002). The plant catalases can be divided into three classes:

1. class I catalases are most prominent in photosynthetic tissues and are involved in the removal of H_2O_2 produced during photorespiration,
2. class II are abundantly produced in the vascular tissues and may play a role in lignification, but their exact biological role remains unknown,
3. class III are highly abundant in seeds and young plants (van Breusegem *et al.*, 2001).

Due to its high Km, catalase is very inefficient at scavenging low levels of H_2O_2 produced in cells. The scavenging systems described here are believed to play a more

important role than catalase in reducing levels of H_2O_2 during early plant-pathogen interactions. An exception might be if the ascorbate-glutathione system becomes overloaded in a stress situation and NADPH levels are depleted. Unlike this system, catalase does not require NADPH and so might be a more important antioxidant under these conditions (Baker and Orlandi, 1995).

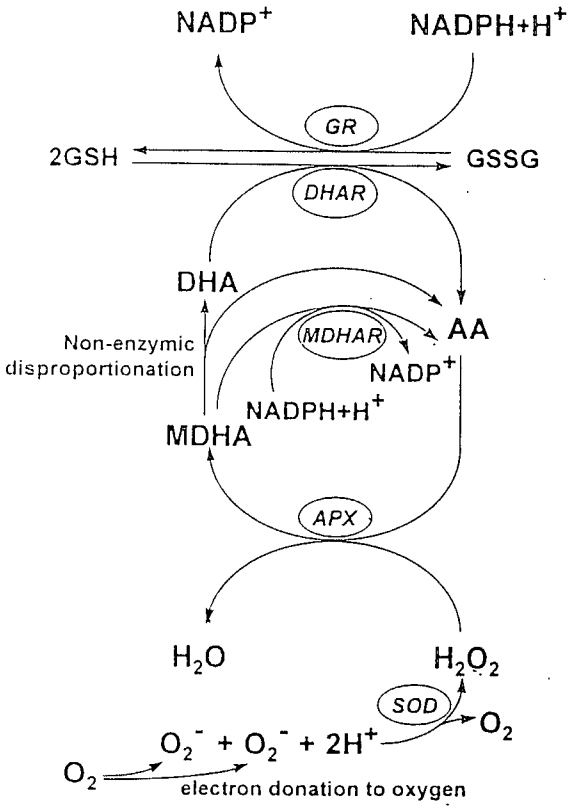

Figure 2.7: The ascorbate-glutathione cycle (Noctor and Foyer, 1998).

CHAPTER THREE

3. MATERIAL AND METHODS

3.1 Plant material

The Russian wheat aphid (RWA) (*Diuraphis noxia*, Mordvilko) resistant [cv., *Tugela DN*, containing the *Dn 1* (PI 137739) resistance gene (Du Toit, 1989)] and the near isogenic susceptible (cv., *Tugela*) wheat (*Triticum aestivum* L.) plants were grown under greenhouse conditions in trays, at temperatures of 24°C ± 2°C. Plants in the early three leaf stage were infested by scattering the aphids (approximately 20 RWAs per plant) on the leaves. Another set of plants (resistant and susceptible) was left uninfested as control. All leaves from each plant were excised at the base of a stem after 3, 6, 9, 12, 24, and 48h of infestation for analysis. They were frozen in liquid nitrogen and stored at –20°C.

3.2 Methods

For all assays performed in this study, two separate experiments (i.e. planting new sets of resistant and susceptible plants) were conducted. Assays were done in triplicate.

3.2.1 Treatment of plants with diphenylene iodonium, *in vivo*

Resistant plants were treated with diphenylene iodonium (DPI), an inhibitor of NADPH oxidase, according to the method of Orozco-Cárdenas *et al* (2001) to determine if NADPH oxidase is involved in the production of H_2O_2 in the RWA-wheat interaction. Plants were excised at the base of the stem with a razor blade. The cut stem was placed in 10mM potassium phosphate buffer (pH 6.0) alone

(control) for 2h or in the same buffer containing 100μM DPI for 2h or for the duration of the experiment, under the same growth conditions described in paragraph 3.1. After these treatments, excised plants were infested, and the control and 2h DPI treated plants were transferred to distilled water. After 6h of infestation, the leaves were used for the determination of NADPH oxidase activity and H_2O_2 content. The intercellular washing fluid (IWF) was collected from the leaves after 48h of infestation.

3.2.2 Treatment of plants with an H_2O_2-generating mixture of glucose and glucose oxidase

Resistant plants were treated with glucose plus glucose oxidase according to the method described by Orozco-Cárdenas *et al* (2001), to establish the signaling role of H_2O_2 in induction of the secondary defence reactions. Plants were excised at the base of the stem with a razor blade. The excised stems were placed in 10mM potassium phosphate buffer (pH 6.0) alone as control or in the same buffer containing (50mM) glucose plus glucose oxidase (2.5 units/mL) for 2h. Thereafter, plants were incubated in distilled water. After 6h, the leaves were used for H_2O_2 assay. These treatments were performed in the green house under the same conditions described in 3.1. The IWF was collected from the leaves after 48h of incubation for the intercellular β-1,3-glucanase and peroxidase activity.

3.2.3 Collection of the intercellular washing fluid (IWF)

Leaves from the resistant plants were cut in 10cm long pieces, thoroughly rinsed in distilled water, and then vacuum infiltrated in 50mM Tris buffer (pH 7.8) for 5 minutes. The leaves were dried on blotting paper, inserted vertically in a centrifuge tube with a disc at the bottom, and centrifuged (500x g) at –4°C for 5 minutes. After centrifugation, the IWF was collected from the bottom of the centrifuge tube, and the procedure was repeated using the same leaves. The combined IWF was frozen in

liquid nitrogen and stored at –20°C for the assay of the intercellular β-1,3-glucanase and peroxidase activity.

3.2.4 Hydrogen peroxide concentration

H_2O_2 is a major ROS contributing to the oxidative burst (Wojtaszek, 1997b) and apparently plays a role in the induction of the defence responses (Alvarez *et al.*, 1998). The hydrogen peroxide content was assayed using a modified method of Ferguson *et al* (1983). Frozen leaf material (1g) was homogenized in 5mL cold acetone whereafter the extract was centrifuged (5930x g) at –4°C for 15 minutes. The supernatant was collected and 0.5mL titanium tetrachloride ($TiCl_4$) reagent (20 % in concentrated HCl) was added to it. After dropwise addition of 3.5mL NH_4OH (25 %), the solution was mixed. The sample was centrifuged (5930x g) at –4°C for 5 minutes. The supernatant was discarded and precipitate was washed repeatedly with 5mL volumes cold acetone until the supernatant was colorless. The washed precipitate was dissolved in 20mL 2N H_2SO_4, and filtered prior to measurement of absorbance at 415nm against a blank, which had been subjected to the same procedure. Standards in the range of 0.75 to 5.35mM H_2O_2 were also treated with $TiCl_4$ and subjected to the same procedure.

3.2.5 Protein concentration

Protein content of the enzyme extracts was determined according to the method of Bradford (1976) as modified by Rybutt and Parish (1982). The assay mixture consisted of 160μL distilled water, 40μL Biorad (Bio-Rad laboratories GmbH), and 10μL enzyme extract or standard. The absorbance was measured at 595nm using the Biorad microplate reader. Bovine γ-globulin (0.5μg μL^{-1}) was used as a standard.

3.2.6 The ROS generatig enzymes

3.2.6.1 NADPH oxidase activity

NADPH oxidase, a key enzyme that catalyzes the formation of the ROS, O_2^-, from molecular oxygen at the plasma membrane of plants (Somssich and Hahlbrock, 1998), was extracted using a modified method of Mulosevic and Slusarenko (1996). Frozen leaf material (1g) was ground to a fine powder in liquid nitrogen using a pre-cooled mortar and pestle. To the powder, 4mL of the extraction buffer (50mM potassium phosphate buffer, pH 7.0, containing 0.1 % Triton X-100, 1 % polyvinylpyrolidone (PVP), 0.04 % $Na_2S_2O_5$, and 10mM EDTA) was added. The homogenate was centrifuged (17400x g) at 4°C for 15 minutes, and the supernatant obtained served as the enzyme extract. All steps in the preparation of this enzyme extract were carried out on ice.

NADPH oxidase activity was measured spectrophotometrically according to the method of Askerlund *et al* (1987) as modified by Rao *et al* (1996), to establish whether NADPH oxidase is involved in ROS production that will eventually lead to induction of the defence responses against RWA infestation in wheat. The assay mixture (1mL) contained 540µL 50mM potassium phosphate buffer (pH 7.0), 300µL 150µM NADPH, 100µL 100µM KCN, and 60µL enzyme extract. The reaction was initiated by addition of enzyme extract and the decrease in absorbance at 340nm was followed for 3 minutes at 25°C.

In the experiments where NADPH oxidase activity was inhibited with DPI *in vitro* to establish whether DPI has a direct effect on NADPH oxidase activity, a 1mL reaction mixture contained 200µL 1mM DPI, 100µL 100µM KCN, 340µL 50mM potassium phosphate buffer (pH 7.0), 300µL 150µM NADPH, and 60µL enzyme extract.

3.2.6.2 Superoxide dismutase (SOD, EC 1.15.1.1) activity

SOD catalyzes the dismutation of O_2^- to form H_2O_2 and H_2O (del Río *et al.*, 2002). Extraction of SOD was done according to a modified method of Mulosevic and Slusarenko (1996) as described in 3.2.6. SOD activity was measured spectrophotometrically as described by Keppler and Novacky (1987) to establish its contribution to ROS production during the RWA resistance response. A 1mL reaction mixture contained 30µL enzyme extract and a 970µL solution of: 2µM riboflavin, 0.1mM EDTA, 75µM 4-nitro blue tetrazolium chloride (NBT), and 13 mM methionine. Irradiation was performed by placing the sample 30cm below two fluorescent lamps (2x40 W) for 30 minutes in an aluminum foil covered box. A non-irradiated duplicate was used as a control (reference cuvette) during the measurement of absorbance at 560nm. Also, a sample without enzyme extract was irradiated and used to measure the maximum attainable absorbance at 560nm. The SOD activity was expressed as log [A_{560nm} (with enzyme extract) mg^{-1}protein divided by A_{560nm} (without enzyme extract)].

3.2.7 The ROS scavenging enzymes

The activities of the ROS scavenging enzymes, glutathione reductase and ascorbate peroxidase, were determined to establish their contributions to the ROS balance during the resistance response.

3.2.7.1 Glutathione reductase (GR, EC 1.6.4.2) activity

The extraction procedure (Mulosevic and Slusarenko., 1996) described in 3.2.6 was used for GR. The activity of GR was determined by monitoring the GSSG-dependent oxidation of NADPH at 340nm and 25°C for 3 minutes according to a modified method of Foyer and Halliwell (1976). The reaction mixture (1mL)

contained 230µL 0.5mM GSSG (oxidized glutathione), 30µL 2mM EDTA, 230µL 0.2mM NADPH, 470µL 100mM potassium phosphate buffer (pH 7.8), and 40µL enzyme extract. The reaction was started by the addition of NADPH.

3.2.7.2 Ascorbate peroxidase (APX, EC 1.11.1.11) activity

APX was extracted using the method described by Mishra *et al* (1995). Frozen leaf material was ground to a fine powder in liquid nitrogen, and 5mL of 50mM potassium phosphate buffer, pH 7.0, (containing 0.25 % Triton X-100, 1 % PVP) was added to it. To prevent the inactivation of APX during the extraction procedure, the buffer was supplemented with 0.5mM sodium ascorbate. The homogenate was centrifuged (17400x g) at –4°C for 15 minutes. The supernatant obtained, was used as enzyme extract for the APX assay.

The APX activity was determined by following the oxidation of ascorbate as a decrease in absorbance at 290nm as described by Mishra *et al* (1993). For calculations, an extinction coefficient of $2.8mM^{-1}cm^{-1}$ was used. The reaction mixture (1mL) contained 575µL 50mM potassium phosphate buffer (pH 7.0), 150µL 0.5mM sodium ascorbate, 50µL 0.1mM EDTA, 200µL H_2O_2, and 25µL enzyme extract. The reaction was initiated by adding the enzyme extract.

3.2.8 Intercellular peroxidase (EC 1.11.1.7) activity

The intercellular peroxidase activity served as one of the parameters of secondary defence reactions. A modified method of Zieslin and Ben-Zaken (1991) was used for the assay of the intercellular peroxidase activity to establish the role of H_2O_2 in resistance against RWA.The assay mixture (1mL) contained 10µL IWF, 50µL 8.2mM H_2O_2, 100µL 50mM guaiacol, 340µL double distilled water, and 500µL 80mM potassium phosphate buffer (pH 5.5). The increase in absorbance was measured at 470nm for 3 minutes at 30°C.

3.2.9 Intercellular β-1,3-glucanase (EC 3.2.1.39) activity

In addition to the intercellular peroxidase activity, the intercellular β-1,3-glucanase activity served as a parameter of secondary defence reactions. A modified method of Fink *et al* (1988) was used for the determination of β-1,3-glucanase activity to establish whether induction of H_2O_2 by RWA infestation will eventually lead to the induction of β-1,3-glucanase activity. The assay mixture contained 10μL IWF, 250μL 2mg mL^{-1} laminarin, and 240μL 50mM sodium acetate buffer (pH 4.5). After incubation at 37°C for 10 minutes, 500μL of Somogyi reagent [0.2g $CuSO_4$, 9g Na_2SO_4, 1.2g $NaCO_3$, 0.8g $NaHCO_3$, and 0.6g potassium tartrate in 50mL double distilled water, (Somogyi, 1952)] was added and incubated at 100°C for 10 minutes. After cooling under tap water, 500μL of Nelson's reagent [2.65g $(NH_4)_6$ Mo_7O_{24}, 2mL 95-97% H_2SO_4, 0.32g $Na_2Has.7H_2O$ in 50mL double distilled water, (Nelson, 1944)] was added. The absorbance was measured at 540nm. The blank and the glucose standards were subjected to the same procedure.

CHAPTER FOUR

4. RESULTS

4.1 The effect of RWA infestation on the hydrogen peroxide content of resistant and susceptible wheat

RWA infestation selectively induced a 1.2-fold increase in the H_2O_2 content of resistant plants 3h after infestation. From 6 to 9h after infestation, a slight decrease in H_2O_2 content was observed. Inspite of this decrease, the level of the H_2O_2 content was still high in infested resistant plants compared to non-infested control plants. The highest induction of 1.4-fold was obtained 9h after infestation compared to the controls. In contrast, the infested susceptible plants showed an insignificant induction of the H_2O_2 content throughout the entire 48h study period (Fig. 4.1).

4.2 The effect of RWA infestation on the activities of the ROS generating and scavenging enzymes of resistant and susceptible wheat

4.2.1 NADPH oxidase activity

NADPH oxidase activity was determined in infested and uninfested resistant and susceptible plants. An induction representing a 1.3-fold increase in NADPH oxidase activity was observed as early as 6h after infestation in resistant plants. Peak activity (1.4-fold increase) was observed 9h after infestation, and 48h after infestation it had dropped to the original level. In contrast, the activity in the infested susceptible plants was only slightly induced (Fig. 4.2).

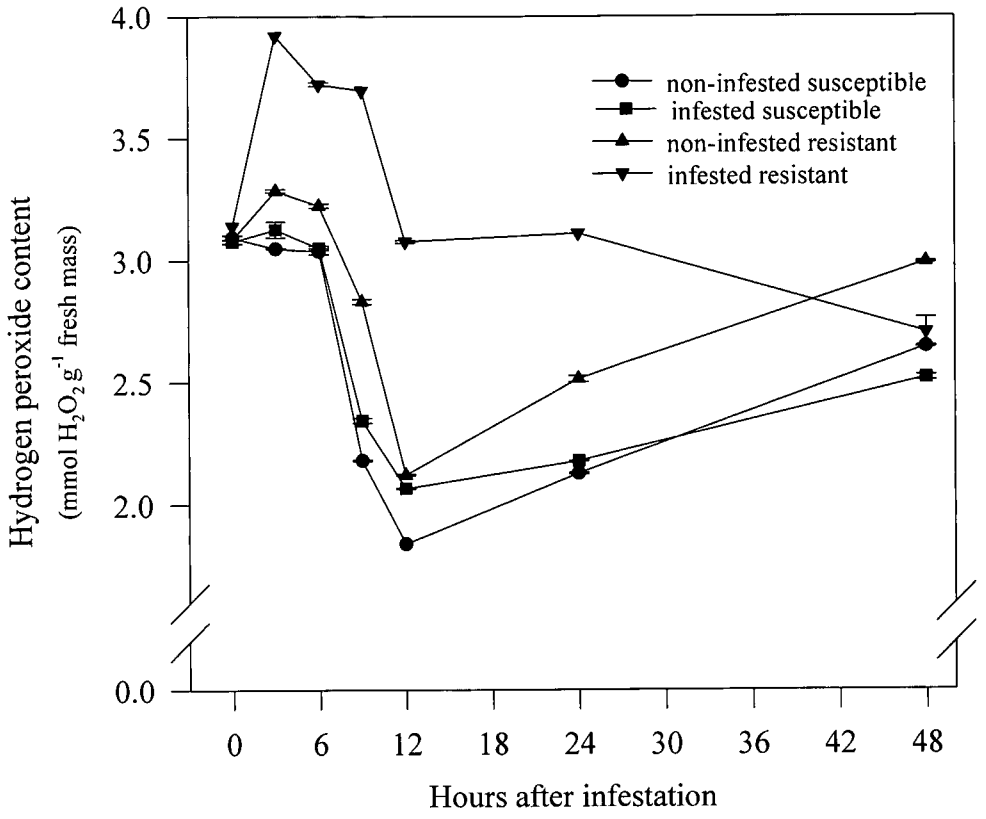

Figure 4.1: Effect of RWA infestation on the hydrogen peroxide content in resistant (*Tugela DN*) and the near isogenic susceptible (*Tugela*) wheat. Error bars indicate standard deviation (n=3).

4.2.2 Superoxide dismutase (SOD) activity

SOD activity was induced to significant higher levels in resistant than in susceptible plants by RWA infestation. An early, sharp induction (1.2-fold increase) in SOD activity was observed already 6h after infestation in resistant plants and declined afterwards. However, the level of activity was still high compared to infested susceptible plants, where very little induction in SOD activity occurred (Fig. 4.3).

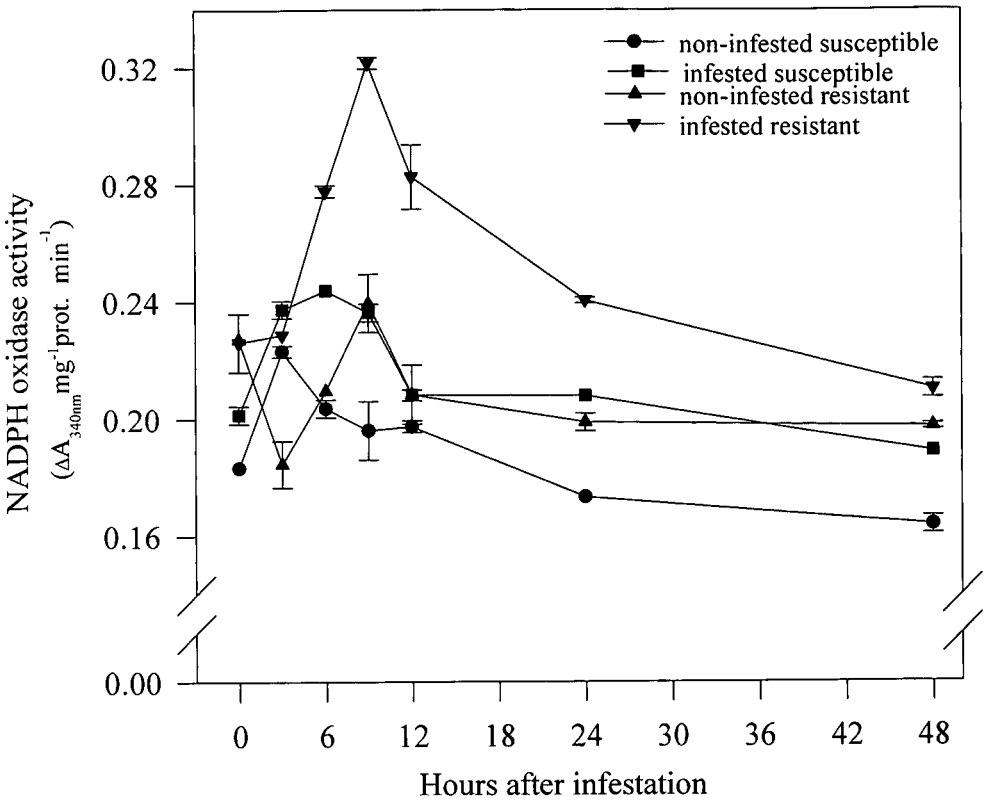

Figure 4.2: Effect of RWA infestation on NADPH oxidase activity in resistant (*Tugela DN*) and the near isogenic susceptible (*Tugela*) wheat. Error bars indicate standard deviation (n=3).

4.2.3 Glutathione reductase (GR) activity

RWA infestation induced GR activity to higher levels in resistant than in susceptible plants. Unlike the NADPH oxidase and SOD activities, where the induction occurred during the early hours of infestation in resistant plants, induction of the GR activity was delayed. Peak activity (1.5-fold increase) occurred 12h after infestation in the resistant plants. In spite of a slight drop in activity after 12h, the level of GR activity remained relatively high compared to the uninfested controls and infested susceptible plants (Fig. 4.4).

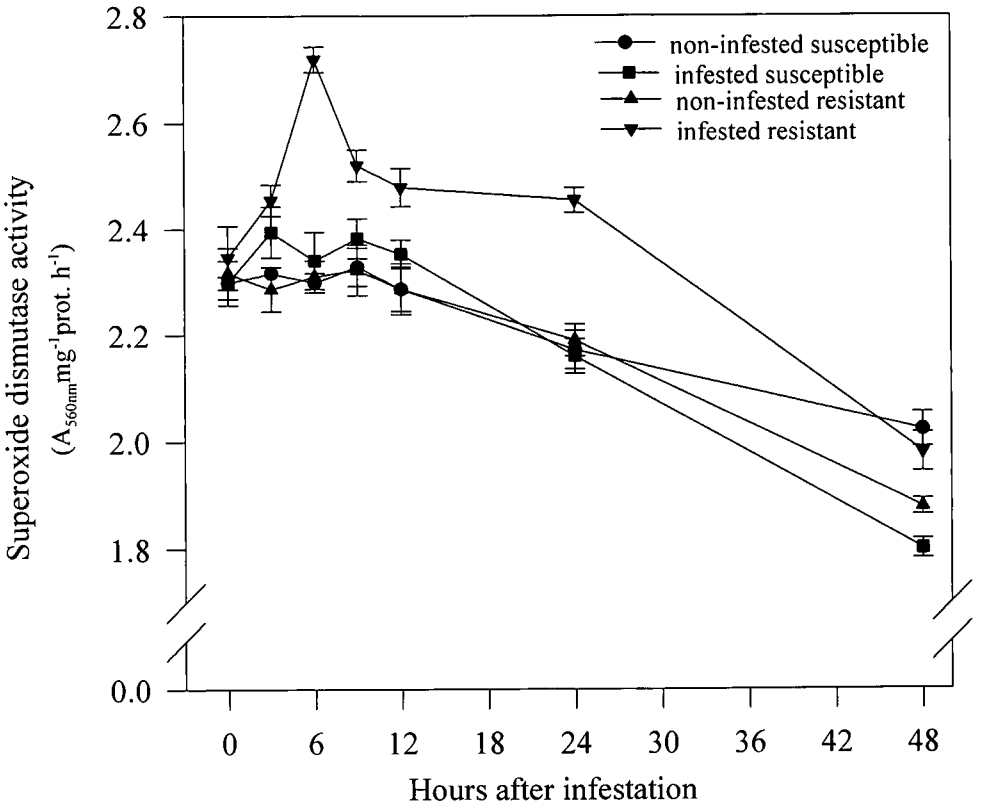

Figure 4.3: Effect of RWA infestation on superoxide dismutase activity in resistant (*Tugela DN*) and the near isogenic susceptible (*Tugela*) wheat. Error bars indicate standard deviation (n=3).

4.2.4 Ascorbate peroxidase (APX) activity

APX activity was induced to much higher levels in resistant than in susceptible plants by RWA infestation. A maximum induction representing a 1.4-fold increase was reached 12h after infestation, whereafter the activity dropped to a level that was still higher than in uninfested control or in infested susceptible plants. In contrast, APX activity in infested susceptible plants was only slightly induced during the early hours of infestation (Fig. 4.5).

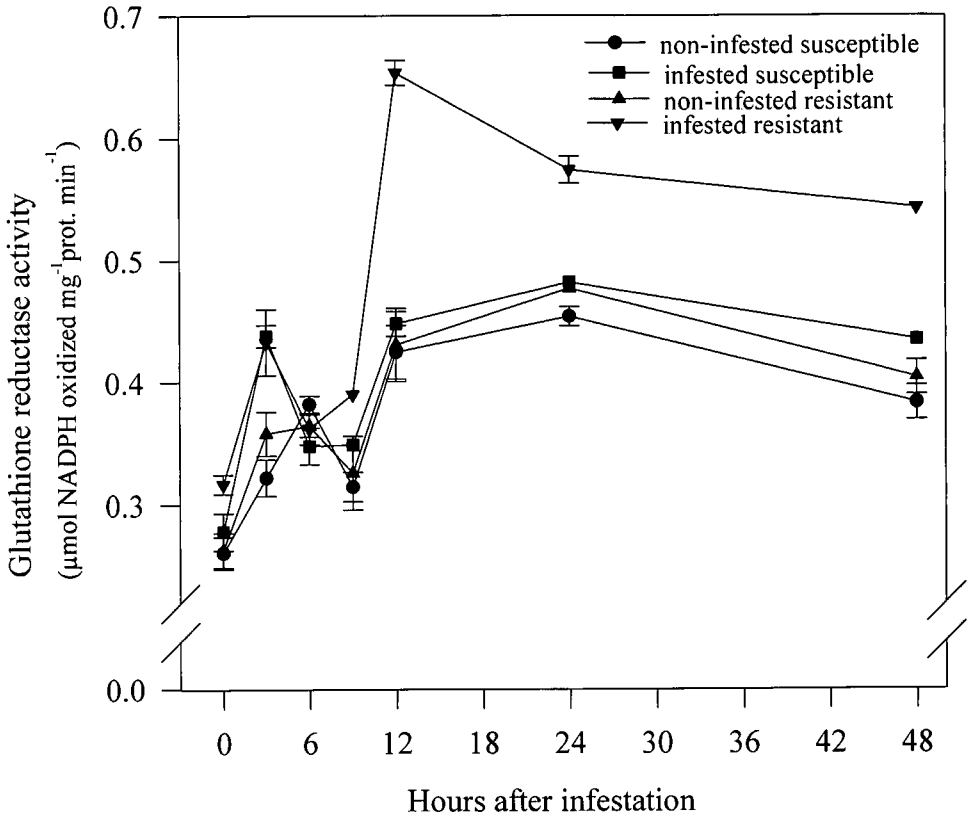

Figure 4.4: Effect of RWA on the glutathione reductase activity in resistant (*Tugela DN*) and the near isogenic susceptible (*Tugela*) wheat. Error bars indicate standard deviation (n=3).

4.3 The effect of diphenylene iodonium (DPI), *in vitro,* on the activities of NADPH oxidase, intercellular β-1,3-glucanase and peroxidase in resistant wheat

Figure 4.6a clearly illustrates the induced NADPH oxidase activity in resistant plants after 6h of infestation. Addition (*in vitro*) of DPI, a suicide inhibitor of the NADPH oxidase, to a reaction mixture containing the enzyme extract from resistant plants infested with RWA, substantially inhibited (70 %) the NADPH oxidase activity compared to a reaction where no DPI was added.

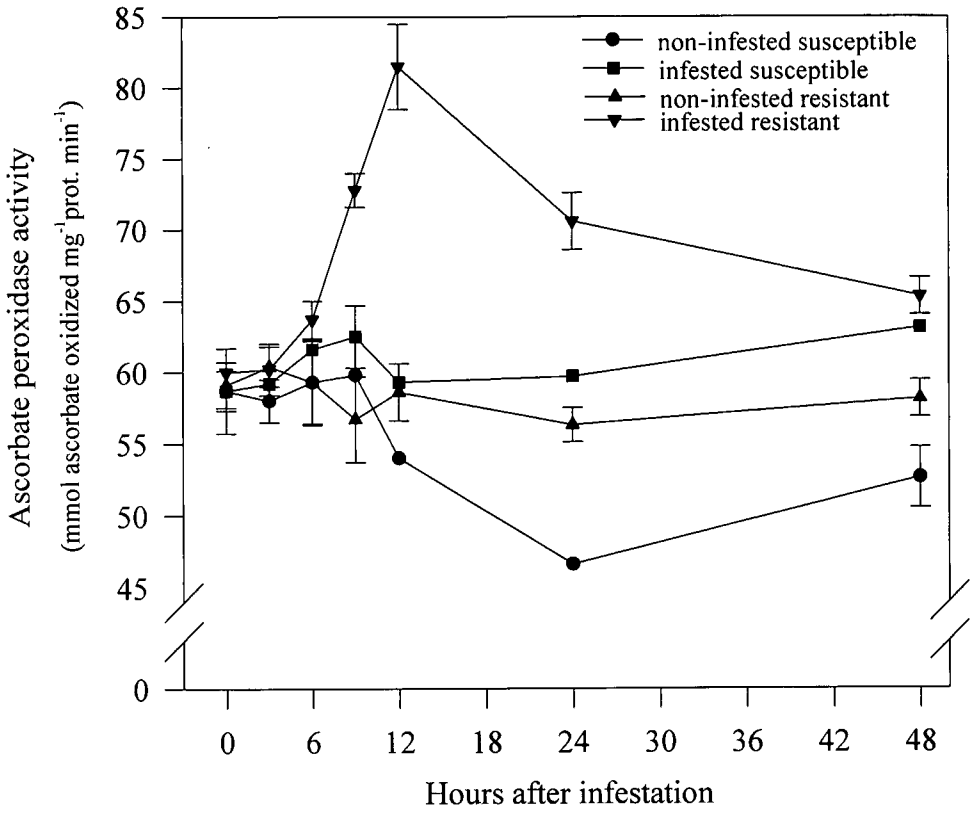

Figure 4.5: Effect of RWA infestation on the ascorbate peroxidase activity in resistant (*Tugela DN*) and the near isogenic susceptible (*Tugela*) wheat. Error bars indicate standard deviation (n=3).

The intercellular β-1,3-glucanase activity was significantly induced (57 %) after 6h of infestation in the resistant plants. Addition (*in vitro*) of DPI to the reaction containing an extract from the infested resistant plants did not inhibit the β-1,3-glucanase activity (0 % inhibition) (Fig. 4.6b).

Figure 4.6c clearly illustrates the induced intercellular peroxidase activity in resistant plants after 6h of infestation. Addition (*in vitro*) of DPI to the reaction mixture containing an extract from infested resistant plants (6h after infestation), did not have any inhibition effect (0 %) on the intercellular peroxidase activity.

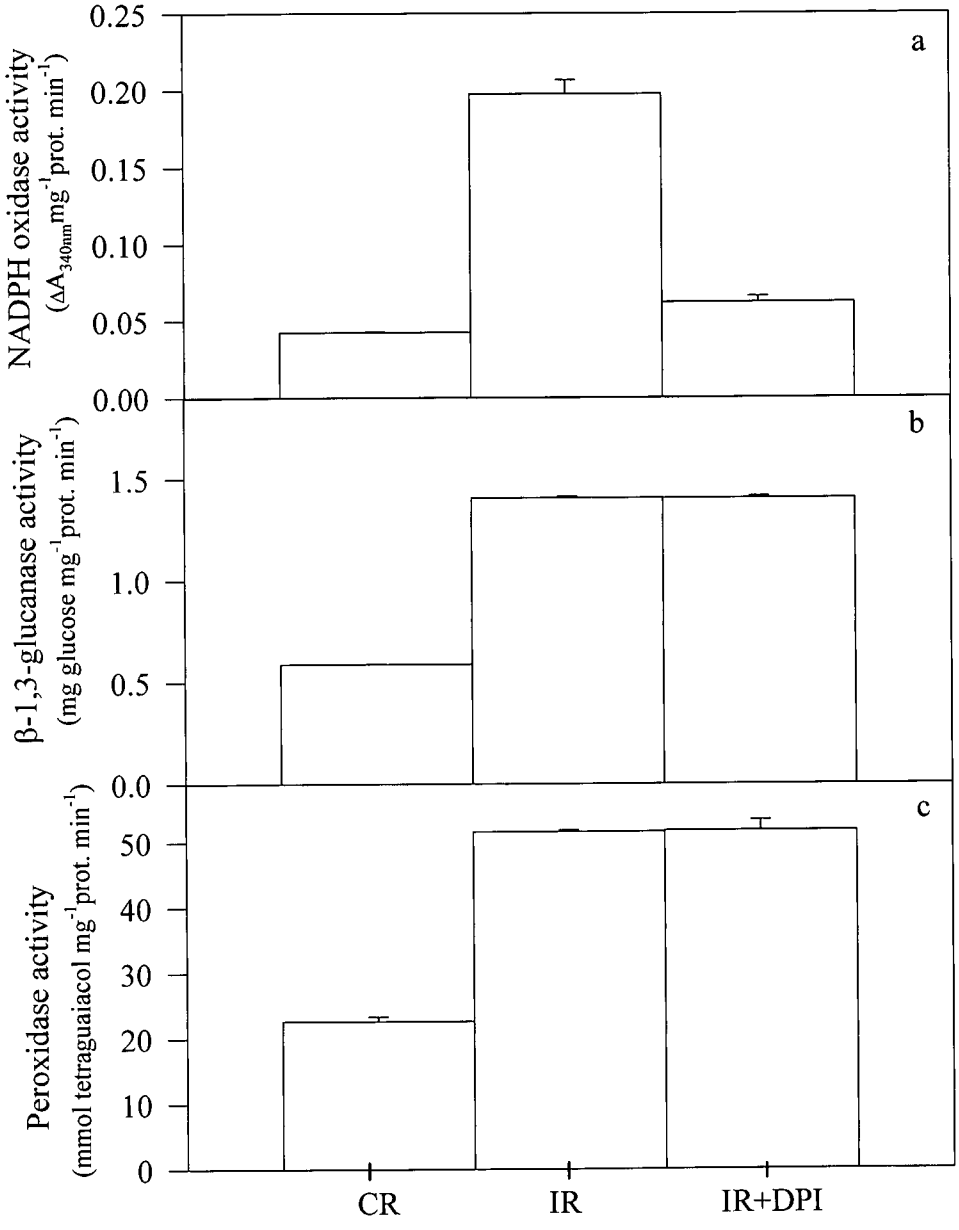

Figure 4.6: Effect of diphenylene iodonium (DPI) (*in vitro*) on the NADPH oxidase (a), intercellular β-1,3-glucanase (b) and peroxidase (c) activities of extracts from infested resistant (*Tugela DN*) wheat. Error bars indicate standard deviation (n=3). CR= uninfested resistant; IR= infested resistant.

4.4 The effect of diphenylene iodonium (DPI) , *in vivo,* on the hydrogen peroxide content and the activities of NADPH oxidase, intercellular β-1,3-glucanase and peroxidase in infested resistant wheat

4.4.1 NADPH oxidase activity

Treatment of RWA infested resistant plants with DPI significantly inhibited NADPH oxidase activity. The NADPH oxidase activity of resistant plants that were treated with DPI for a period of 2h and infested for 6h with RWA, were inhibited by 43 % compared to untreated, infested control resistant plants. Extending the period of DPI treatment to 8h resulted in a 54 % inhibition (Fig. 4.7).

4.4.2 Hydrogen peroxide content

The infested (6h) buffer treated resistant plants had a high H_2O_2 content. DPI treatment (2h) of resistant infested plants, significantly inhibited (42 %) the H_2O_2 production. Extending the period of DPI treatment to 8h, caused a further decrease in the H_2O_2 content representing an inhibition of 45 % (Figure 4.8).

4.4.3 Intercellular β-1,3-glucanase activity

The intercellular β-1,3-glucanase activity of infested (48h) resistant plants that were treated with DPI for 2h was inhibited by 30 %. In the case where resistant plants were continuously (48h) treated with DPI, the β-1,3-glucanase activity was even more inhibited (40 %) (Fig. 4.9).

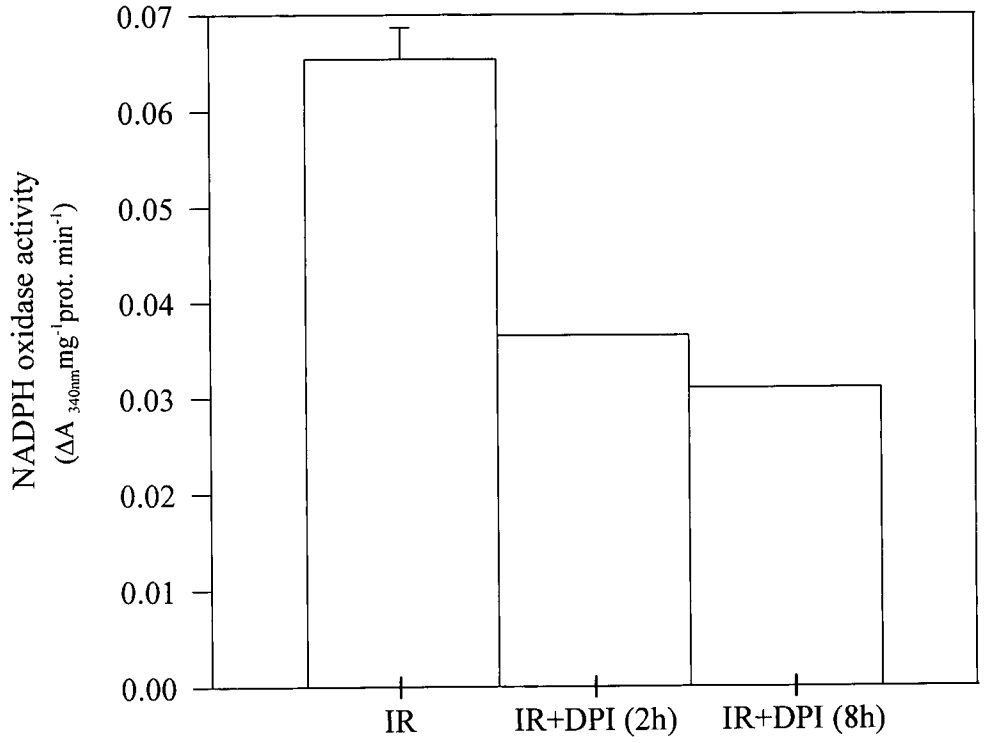

Figure 4.7: Effect of diphenylene iodonium (DPI) (*in vivo*) on NADPH oxidase activity of infested resistant (IR) (*Tugela DN*) wheat. Error bars indicate standard deviation (n=3).

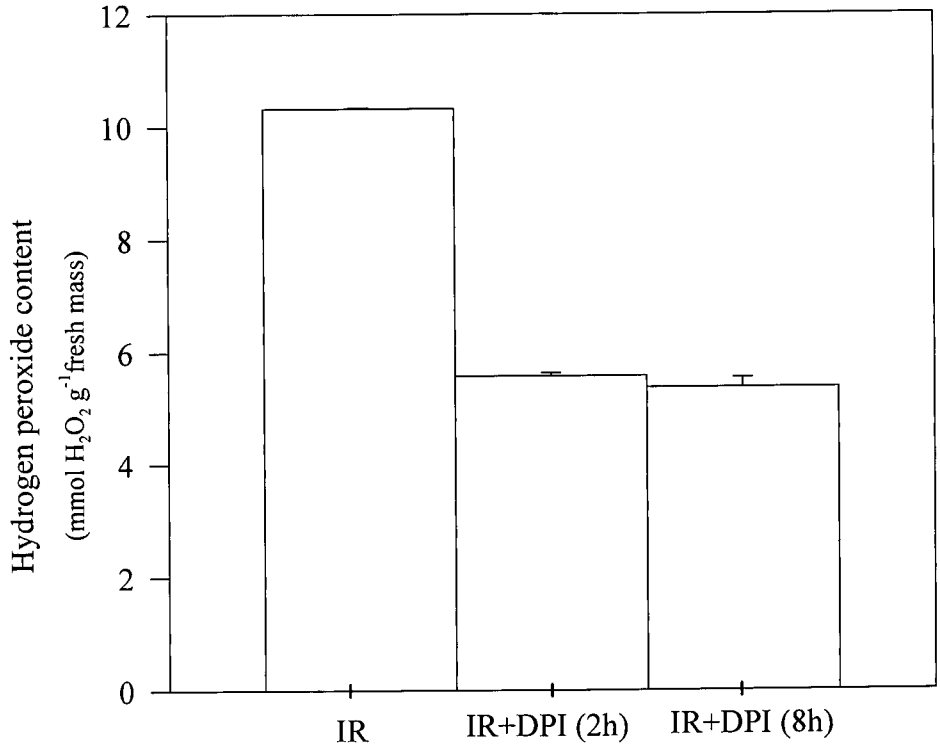

Figure 4.8: Effect of diphenylene iodonium (DPI) (*in vivo*) on the hydrogen peroxide content of infested resistant (IR) (*Tugela DN*) wheat. Error bars indicate standard deviation (n=3).

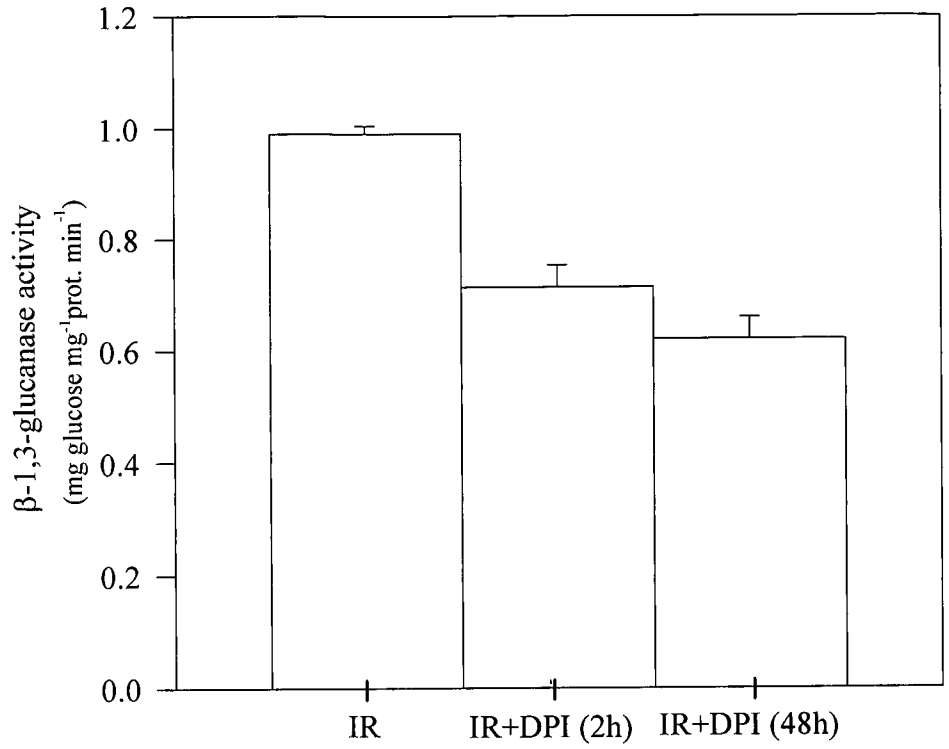

Figure 4.9: Effect of diphenylene iodonium (DPI) (*in vivo*) on the intercellular β-1,3-glucanase activity of infested resistant (IR) (*Tugela DN*) wheat. Error bars indicate standard deviation (n=3).

4.4.4 Intercellular peroxidase activity

The intercellular peroxidase activity was significantly inhibited (51 %) when resistant plants were treated with DPI for 2h, and then infested afterwards for a period of 48h. The activity was also inhibited to about the same extent (53 %) when the infested resistant plants were treated with DPI for 48h (Fig. 4.10).

4.5 The effect of hydrogen peroxide application on the defence related events of resistant wheat

4.5.1 Hydrogen peroxide content

The H_2O_2 accumulation was studied in resistant plants incubated for 2h in a H_2O_2 generating mixture of glucose and glucose oxidase. This treatment resulted in a significant induction (57 %) in the H_2O_2 content (Fig. 4.11).

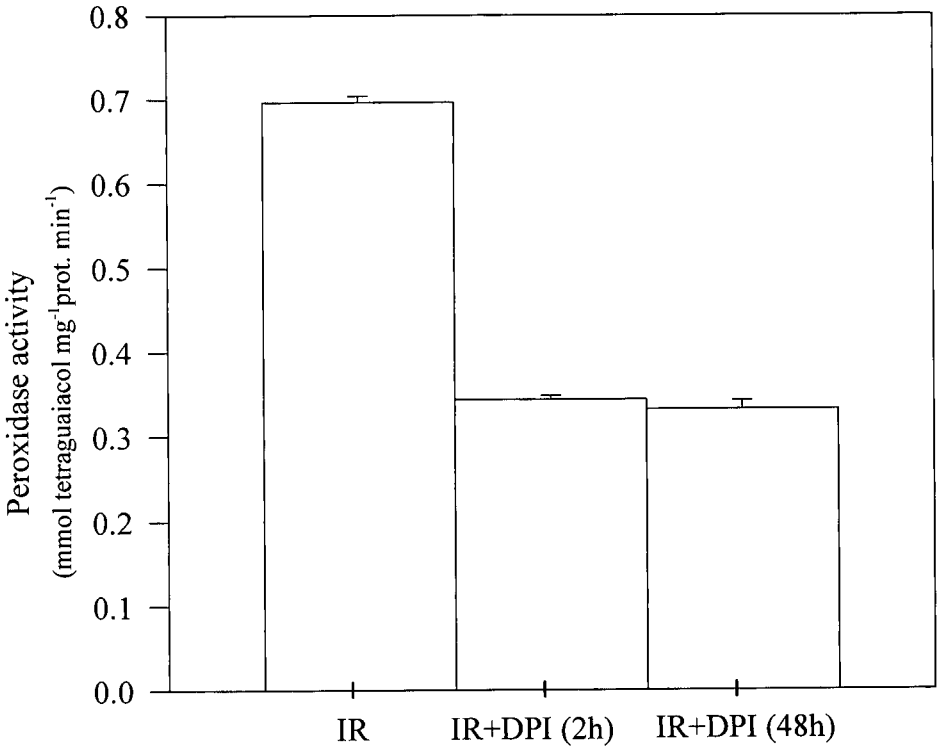

Figure 4.10: Effect of diphenylene iodonium (DPI) (*in vivo*) on the intercellular peroxidase activity of infested resistant (IR) (*Tugela DN*) wheat. Error bars indicate standard deviation (n=3).

4.5.2 Intercellular β-1,3 glucanase and peroxidase activities

Activities of intercellular β-1,3-glucanase and peroxidase were determined after 48h of glucose plus glucose oxidase treatment, to test whether the generated H_2O_2 activated secondary defence responses. In the treated plants, the intercellular β-1,3 glucanase and peroxidase activities were substantially induced (64 and 56 % respectively) compared to buffer treated control plants (Fig. 4.12).

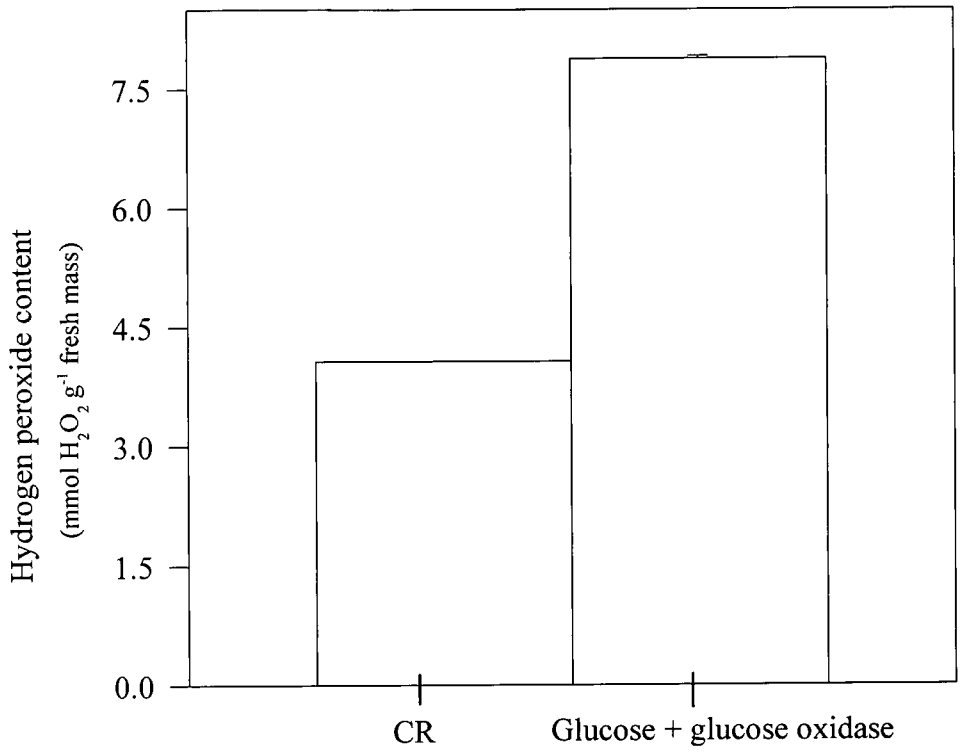

Figure 4.11: Effect of the hydrogen peroxide generating mixture of glucose and glucose oxidase on the hydrogen peroxide content of resistant (*Tugela DN*) wheat. Error bars indicate standard deviation (n=3). CR= non-treated resistant.

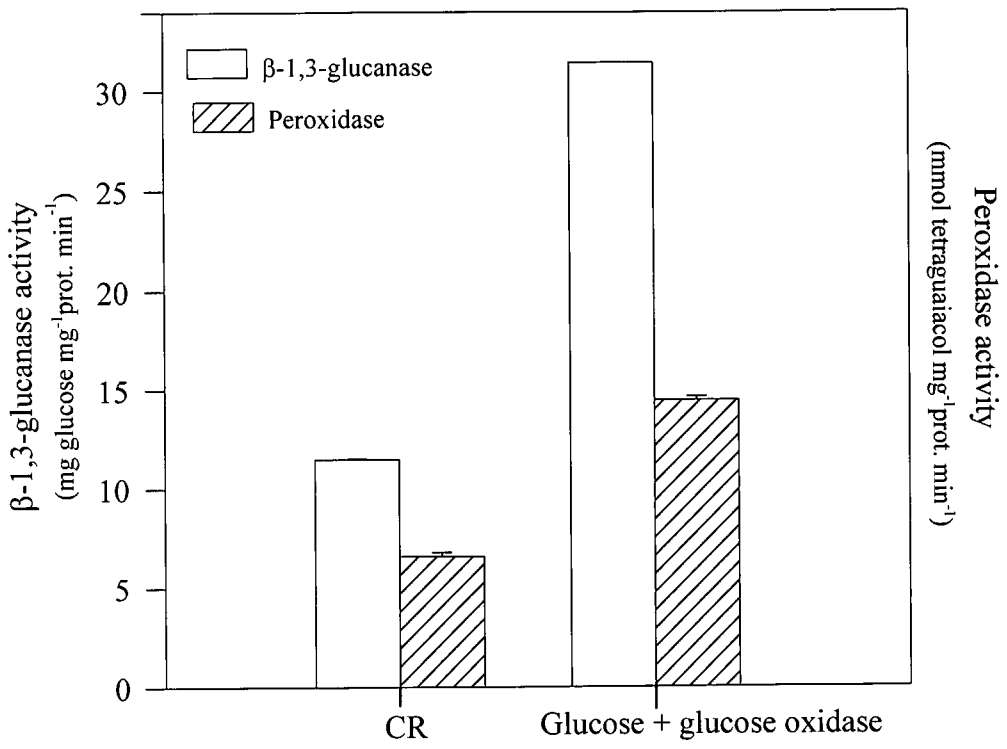

Figure 4.12: Effect of the hydrogen peroxide generating mixture of glucose and glucose oxidase on the intercellular β-1,3-glucanase and peroxidase activities in resistant (*Tugela DN*) wheat. Error bars indicate standard deviation (n=3).

CHAPTER FIVE

DISCUSSION

Plants, unlike animals, are confined to the habitat where they grow. Therefore, they have developed a broad range of defence responses to cope with biotic stress such as herbivore and pathogen attack. The oxidative burst, a rapid production of relative large quantities of the reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2), is one of the earliest observable events of the plant's defence strategy (Wojtaszek, 1997b).

The downstream defence (secondary defence) responses in the RWA-wheat interaction have been studied quite extensively. It has previously been found that RWA infestation resulted in the selective induction of an array of downstream defence responses in resistant wheat plants (van der Westhuizen and Pretorious, 1996; van der Westhuizen *et al.*, 1998a, b). Little is known about the signaling events leading to the induction of downstream defenses in wheat by RWA. Previous studies on the RWA-wheat interaction, have suggested SA to be a signal molecule mediating the downstream defence responses (Mohase and van der Westhuizen, 2002a). Ample evidence indicates that the ROS act as local signals for the induction of defence genes during plant pathogen interactions (Chen and Klessing, 1991; Chen *et al.*, 1993; Levine *et al.*, 1994; Wu *et al.*, 1995; Alvarez *et al.*, 1998; Chamnongopol *et al.*, 1998; Orozco Cárdenas *et al.*, 2001). In an attempt to unravel more of the RWA resistance mechanism, we investigated the possible signaling role of H_2O_2 in the induction of the downstream defence responses, as well as the involvement of the ROS generating and scavenging enzymes in the H_2O_2 production and metabolism.

The H_2O_2 content was selectively induced during the early hours of infestation (3-9h) in the RWA infested resistant than infested susceptible plants (Fig. 4.1). This was confirmed in a second experiment.

H_2O_2 is produced in plants as a result of a wide variety of biotic and abiotic stresses. A burst of H_2O_2 occurred in soybean cells treated with glucan and pectin elicitors (Levine *et al.*, 1994). Plant-pathogen interactions on the other hand have also been reported to induce early H_2O_2 accumulation. Lettuce cells inoculated with *P. syringae* pv. *phaseolicola* resulted in accumulation of the H_2O_2 content in plant cell walls adjacent to attached bacteria (Bestwick *et al.*, 1997). Furthermore, powdery mildew inoculation induced a higher accumulation of H_2O_2 in resistant than susceptible barley plants (Vanacker *et al.*, 2000). Inoculation of soybean cells with a bacterial pathogen *P. syringae* pv. *glycine* (*Psg*) also resulted in high levels of H_2O_2. Moreover, inoculation of soybean cells with virulent *Psg* (*avrC*) did not induce H_2O_2 accumulation, but in contrast, a massive H_2O_2 accumulation occurred about 3h after inoculation with avirulent *Psg* (*avrA*) (Levine *et al.*, 1994). Drought stress has also been implicated in the induction of a higher H_2O_2 content in resistant compared to susceptible wheat plants (Loggini *et al.*, 1999).

There is little information on induction of the ROS in response to insect attack. Differential induction of the H_2O_2 accumulation was found in the incompatible interactions between plants and insects (Felton *et al.*, 1999). The H_2O_2 burst can cause damage to insect's essential amino acids, carotenoids and other dietary lipids (Felton *et al.*, 1994). These findings support our results of the selective accumulation of H_2O_2 in resistant plants, suggesting the involvement of H_2O_2 in the RWA resistance response.

Since RWA infestation caused a selective accumulation of H_2O_2 in resistant wheat, further investigations were conducted to monitor the activities of the ROS

generating enzymes, NADPH oxidase and superoxide dismutase (SOD), after RWA infestation in resistant and susceptible wheat plants.

A plasma membrane-bound NADPH oxidase has been implicated in the production of ROS during the defence responses against various stress conditions in plants (Doke *et al.*, 1996; Low and Merida, 1996; Lamb and Dixon, 1997; Orozco-Cárdenas and Ryan, 1999). RWA infestation differentially induced the NADPH oxidase activity to considerable higher levels during the early hours (6-9h) of infestation in resistant than susceptible plants (Fig. 4.2). These results were confirmed in a second experiment. These results suggest that increased NADPH oxidase activity is one of the earliest defence-related events in the resistance response of wheat to the RWA. The selective induction of the NADPH oxidase activity supports the simultaneous accumulation of H_2O_2 (Fig. 4.1). This correlation has been found in several model systems studied in plants. It was found that the oxidative burst and the accumulation of H_2O_2 are mediated by a plasma membrane NADPH oxidase complex (Doke *et al.*, 1996; Lamb and Dixon, 1997; Ogawa *et al.*, 1997; del Río *et al.*, 1998a, b; Potikha *et al.*, 1999; Pei *et al.*, 2000).

The selective induction of NADPH oxidase activity in plants was also reported for other stress conditions. Incubation of potato tuber slices with an incompatible race of *Phytophthora infestans* induced NADPH oxidase activity, which was detected in the plasma membrane. Contrary to this, no stimulation of NADPH oxidase activity was detected in the plasma membrane fractions from tissues inoculated with the compatible race or from control tissues (Doke, 1995; Doke and Miura, 1995). Furthermore, Vera-Estrella (1994) reported that increased NADPH oxidase activity was also found in the plasma membranes of suspension-cultured cells of a tomato *Cf5* line treated with incompatible race-specific elicitors from *C. fluvum*. These findings support the involvement of NADPH oxidase in resistance against pathogens.

Abiotic stress such as UV-B light has been reported to induce NADPH oxidase activity in resistant *Arabidopsis thaliana* (Rao *et al.*, 1996). Although NADPH oxidase has not been shown to be induced by insects, it is known to be induced by tissue damage/wounding in both plant and animal tissues (Bochkov *et al.*, 2002).

SODs are involved in the generation of H_2O_2. They catalyze the disproportionation of O_2^- to H_2O_2 and water (del Río *et al.*, 2002). The SOD activity was selectively induced to much higher levels (6h after infestation) in the resistant than susceptible wheat plants (Fig. 4.3). These results were supported by results of a second experiment. Since increased SOD activity (6h) correlates with to increased NADPH oxidase activity (6h), it strongly suggests that SOD activity was stimulated as a result of the O_2^- formed by NADPH oxidase during RWA infestation. It was found that the NADPH oxidases use NADPH as their substrate for the production of O_2^- from O_2. The O_2^- is in turn used as a substrate for the SOD activity (Beuchamp and Fridovich, 1971; Askerlund *et al.*, 1987). The fact that SOD activity was selectively induced to higher levels in resistant than susceptible plants is indicative of its involvement in the resistance response against the RWA.

High SOD activity has been linked to the defence strategy of plants against various stress conditions such as the low temperature, high irradiance (Schoner and Krause, 1990), drought conditions (Dhinsa and Matowe, 1981; Burke *et al.*, 1985; Perl-Treves and Galun, 1991), chilling (Clare *et al.*, 1984; van Breusegem *et al.*, 1999a, b), SO_2 fumigation (Tanaka and Sugara, 1980; Rao, 1992), cold treatment (Schoner and Krause, 1990), salt stress (Gómez *et al.*, 1999; Hernádez *et al.*, 2001), UV-B and ozone treatment (van Camp *et al.*, 1994; Willekens *et al.*, 1994), cadmium exposure (Schützendübel *et al.*, 2001).

Based on the results obtained, further investigations were performed to determine whether the induced NADPH oxidase activity was a source of H_2O_2 produced in

RWA infested resistant plants. To achieve this, we first tested if NADPH oxidase could be inhibited *in vitro* by diphenylene iodonium (DPI), a suicide inhibitor of NADPH oxidase. DPI binds irreversibly to the flavonoid group of the membrane associated gp91-phox subunit of the NADPH oxidase complex (O'Donnell *et al.*, 1993). The results obtained from this study were in agreement with this theory, because NADPH oxidase activity was substantially inhibited (70 %) after addition of DPI in a reaction mixture containing an enzyme extract from infested resistant wheat plants (Fig. 4.6a).

Secondly, we investigated whether the inhibition of NADPH oxidase (*in vivo*) by DPI would result in a concomitant inhibition of H_2O_2 production. In this study, the NADPH oxidase activity and the H_2O_2 content were substantially inhibited by DPI treatment (Fig's. 4.7 and 4.8). These results strongly suggest that NADPH oxidase is a major enzyme involved in formation of the H_2O_2 in infested resistant plants during RWA infestation.

Similar results were found by Orozco-Cárdenas *et al* (2001). They found that the wound-inducible accumulation of H_2O_2 was effectively inhibited by treatment of tomato plants with DPI, and they suggested that the production of H_2O_2 was due to the presence of NADPH oxidase. Furthermore, according to Alvarez *et al* (1998), the H_2O_2 accumulation in *Arabidopsis* leaves was also inhibited by DPI, and they concluded that NADPH oxidase was involved in the formation of H_2O_2.

It has been reported that H_2O_2 can act as a local signal for the hypersensitive cell death and also as a diffusible signal for the induction of defensive genes in plants (Alvarez *et al.*, 1998). Transgenic tobacco plants that were engineered to have low levels of antioxidative defenses show higher constitutive or inducible levels of H_2O_2 and PR proteins, together with increased sensitivity and general resistance to pathogen challenge (Chamnongpol *et al.*, 1998; Mittler *et al.*, 1999). Similarly, the

induction of the PR-1 protein in tobacco leaves by UV-B required the accumulation of H_2O_2 (Green and Fluhr, 1995).

Chilling tolerance was induced in maize seedlings that were pre-treated with H_2O_2 or menadione, a superoxide-generating compound (Prasad *et al.*, 1994). Similar results were obtained in plants regenerated from potato nodal explants treated with H_2O_2. The regenerated plants were more thermotolerant than the control plants (Lopez-Delgado *et al.*, 1998). *Arabidopsis* leaves pre-treated with H_2O_2 also became tolerant to excess light (Karpinski *et al.*, 1999). Furthermore, Orozco-Cárdenas *et al* (2001) showed that wounding-dependent H_2O_2 accumulation acts as a second messenger for the induction of a subset of defence genes, including the proteinase inhibitors and polyphenol oxidase.

Since it has been reported that H_2O_2 is involved in induction of the defence responses in plants, additional investigations were performed to study the involvement of H_2O_2 in the secondary defence responses of resistant wheat infested with RWA. Previous studies performed by Van der Westhuizen *et al* (1998a, b) have shown that the activities of the secondary defence related enzymes (intercellular β-1,3-glucanase, chitinase and peroxidase) are induced by RWA infestation in the resistant *Tugela DN* plants. In contrast, they found no significant induction in the susceptible *Tugela* plants. They associated this induction of PR proteins with the resistance response, which is a typical HR against RWA.

In order to investigate the possible involvement of H_2O_2 in the RWA resistance response, the effect of inhibition of H_2O_2 production on the intercellular β-1,3-glucanase and peroxidase activities were studied. These PR proteins accumulate in the apoplast, which is known to play an important role in the plant's defence mechanism (Bowles, 1990).

The relative high intercellular β-1,3-glucanase activity in resistant plants 48h after infestation was inhibited by DPI treatment (Fig. 4.9). These results imply that inactivation of the NADPH oxidase activity by DPI, will lead to the inhibition superoxide O_2^- production. If the production of O_2^- (the substrate for SOD) is inhibited, it will naturally affect the SOD activity and the formation of H_2O_2. The β-1,3-glucanase activity could therefore be inhibited, because H_2O_2 can act as a mobile intercellular signal for the induction of the defence genes such as the proteinase inhibitors and PR proteins (Levine *et al.*, 1994; Alvarez *et al.*, 1998). Van der Westhuizen *et al* (1998b) found that the elevated β-1,3-glucanase activity forms part of a combined defence mechanism such as the HR that culminates in resistance against RWA in wheat.

The intercellular β-1,3-glucanases are well known to be involved in resistance mechanisms against pathogens. They may directly damage the fungal cell walls or play a regulatory role by releasing elicitors from cell walls (Lamb *et al.*, 1989; Krishnaveni *et al.*, 1999). Wheat plants infected with *F. graminearum* have shown a more rapid accumulation of β-1,3-glucanase and other PR proteins in resistant than susceptible plants (Li *et al.*, 2001). Inoculation of soybean plants with the bacterium *Psg* or fungal elicitor from *Phytophthora* spp. has been reported to increase expression of a basic class III isoform of β-1,3-glucanase (Cheong *et al.*, 2000). Similar studies have been conducted on tomato (Joosten and De Wit, 1989; Roulin and Buchala, 1995), tobacco (Wyatt *et al.*, 1991), oat (Daugrois *et al.*, 1990), muskmelon (Fink *et al.*, 1990), bean (Netzer and Kritzman, 1979), chickpea (Volgelsang and Barz, 1993) and potato (Beerhues and Kombrink, 1994).

There is little information on induction of the intercellular β-1,3-glucanase activity in response to insect attack. Bronner *et al* (1991) reported that induced glucanase activity was associated with HR in *Solanum* species invaded by the gall mites. Similarly, glucanase activity was also induced in leaves of potato infested by cyst

nematodes (Rahimi *et al.*, 1996). In addition, the β-1,3-glucanase activity was also induced in roots and stems of chickpea (Cabello *et al.*, 1994), as well as in leaves of barley (Ignatius *et al.*, 1994) and sorghum (Krishnaveni *et al.*, 1999) in response to wounding.

The finding that DPI-inhibition of the NADPH oxidase activity (Fig.4.7) also inhibited the intercellular peroxidase activity (Fig. 4.10), further substantiated the involvement of H_2O_2 (strongly suggested to be generated by NADPH oxidase) in induction of secondary defence responses. These results suggest H_2O_2 to be involved in the resistance response against RWA, by acting as a signal for activation of the defence genes (e.g. peroxidases). Alvarez and Lamb (1997) also found that in plant-pathogen interactions, H_2O_2 induces an orchestrated sequence of reactions involving activation of the peroxidases.

The intercellular peroxidases are involved in the defence related events that occur in the extracellular matrix. These include strengthening of cell walls by lignification, the formation of intermolecular cross-links and suberin formation (Bowles, 1990; Mehdy, 1994). Van der Westhuizen *et al* (1998a) found that RWA infestation leads to induction of cell wall thickening of approximately 12 % in the mesophyll cells of the resistant wheat only. This response might hinder RWA probing. In addition, the resistance response of the wheat cultivar *Prelude-Sr5* against an avirulent race of stem rust fungus *Puccinia graminis* f.sp. *tritici* was correlated with rapid lignification in penetrated host cells (Reisener *et al.*, 1986). Furthermore, in tomato, resistance to the fungal pathogen, *Verticillum albo-atrum*, was correlated with a more rapid deposition of suberin and lignin in a resistant isoline than in a susceptible one (Street *et al.*, 1986; Robb *et al.*, 1987). Similarly, infiltration of rice leaves with suspensions of a bacterial blight pathogen, *Xanthomonas oryzae* pv. *oryzae*, caused a deposition of lignin-like polymers at the site of inoculation during the incompatible interactions (Reimers and Leach, 1991). Little is known about induction of the intercellular peroxidase activity by insects.

Nematode infestation induced an increase in the peroxidase activity in resistant tomato plants (Ganguly and Dasgupta, 1979; Zachero *et al.*, 1982).

Studies were conducted (*in vitro*) to investigate whether inhibition of the intercellular β-1,3-glucanase and peroxidase activity was not a direct effect of DPI. The intercellular β-1,3-glucanase and peroxidase activities were not inhibited (Fig 4.6b, c) by DPI. Therefore, inhibition of the β-1,3-glucanase and peroxidase activities by DPI occurred indirectly. It is evident that inhibition of the H_2O_2 accumulation as a result of NADPH oxidase inhibition by DPI, manifested in reduced activation of defence genes including that of the β-1,3-glucanases and peroxidases.

Additional evidence that ROS has a signaling role in induction of the secondary defence related enzymes was obtained from experiments with the H_2O_2-generating system of glucose and glucose oxidase. When the glucose-glucose oxidase mixture was supplied to excised resistant plants, H_2O_2 accumulated to high levels (Fig. 4.11). In conjunction with this induction, the intercellular peroxidase and β-1,3-glucanase activities were also substantially induced to high levels 48h later (Fig. 4.12), as in the case of infestation (Fig's. 4.6, 4.9and 4.10). These results and results of DPI inhibitory studies were confirmed in a second experiment.

The glucose-glucose oxidase H_2O_2-generating system was previously found to cause the constitutive accumulation of sublethal levels of H_2O_2 in the apoplast of plant tissues and in the medium of cell suspension cultures, with the concomitant activation of a defence response equivalent to systemic acquired resistance (SAR). This treatment caused plants to be more resistant to pathogen attacks (Wu *et al.*, 1995 and 1997; Alvarez *et al.*, 1998). Furthermore, Levine *et al* (1994) also suggested the induction of glutathione-S-tranferases (GST) through the production of H_2O_2 by a glucose-glucose oxidase mixture.

The imposition of biotic and abiotic stress conditions can give rise to excess concentrations of ROS, resulting in oxidative damage at cellular level. H_2O_2 is especially toxic in the chloroplasts, because it inhibits the Calvin cycle enzymes possessing exposed sulfhydryl groups, hence reducing the photosynthetic carbon dioxide assimilation. The antioxidants (such as glutathione and ascorbate) and antioxidative enzymes [such as glutathione reductase (GR) and ascorbate peroxidase (APX)] function to interrupt the cascades of uncontrolled oxidation in each organelle. GR and APX are believed to act in conjunction to metabolize H_2O_2 to H_2O through a metabolic cycle widely known as the ascorbate-glutathione cycle (Fig. 2.7) (Noctor and Foyer, 1998).

Since the activities of ROS generating enzymes as well as the H_2O_2 content were induced to relative high levels during the early hours of infestation in RWA infested resistant plants (Fig's. 4.2, 4.3 and 4.1), further investigations were conducted to study the involvement of GR and APX in conferring protection from oxidative damage indirectly caused by RWA feeding. The results obtained from this study showed that RWA infestation selectively induced the GR activity to significant higher levels in infested resistant than susceptible wheat plants (Fig. 4.4). This ROS scavenging activity was somewhat delayed compared to the ROS generating activities. These results suggest a relationship between the H_2O_2 production and H_2O_2 metabolism. This late induction could mean that H_2O_2 was becoming toxic to the cells after 9h of infestation, therefore the GR activity was stimulated to protect the cells from oxidative damage. In agreement with these results, Argandoña (1994) found the same kind of GR induction in wheat and barley infested with aphids (*Sitobion avenae*) and it was concluded that the GR activity could be stimulated as a defensive mechanism of these plants against the physiological damage indirectly produced by the aphids.

The GR activity was also associated with increased tolerance against various stress conditions. A drought tolerant wheat cultivar, *Ofanto,* showed a balance between

the H_2O_2 production and the GR activity (Loggini *et al.*, 1999). Exposure of resistant and susceptible pea plants to SO_2 inhibited photosynthesis in both plants, with the susceptible being more affected by SO_2 stress than resistant plants. The GR activity was found to be high in the resistant plants (Doulis *et al.*, 1998). Similarly, Rao and Dubey (1992) also suggested that the resistant plants exposed to SO_2 induced H_2O_2 accumulation with no visible symptoms of injury, probably because of their high GR activity. Furthermore, GR activity was also stimulated by UV-B and ozone exposure (Strid, 1993; Willekens *et al.*, 1994; Rao *et al.*, 1996).

The effect of RWA infestation on the APX activity was also investigated in resistant and susceptible wheat plants. The APX activity was selectively enhanced in resistant plants (Fig 4.5). The peak activity was reached after the maximum H_2O_2 content was reached. This could mean that APX activity was stimulated as a result of H_2O_2 toxicity in the cells, implicating APX as an adaptive response of the resistant plants to high amounts of ROS generated during RWA feeding. Results on the GR and APX activities were supported by results of a duplicate experiment.

Various stress conditions stimulate APX activity. In *Arabidopsis thaliana* and tobacco plants, UV-B induced significant changes in APX activity (Willekens *et al.*, 1994; Rao *et al.*, 1996). Mishra *et al* (1995) also reported an induction of APX activity in response to high light within 24h of exposure.

From the investigations performed on the antioxidative enzymes, both the GR and APX activities were induced after 12h of infestation (Fig's. 4.4 and 4.5), suggesting that these enzymes are co-regulated during the RWA resistance response. Pastori and Trippi (1992) also reported this kind of co-regulation in maize: Drought resistant maize showed a greater induction of APX activity than susceptible maize plants, in addition, a significant increase in GR activity was also observed. Furthermore, GR and APX activities were both induced to high levels by water

stress in the resistant genotype. In contrast, the susceptible genotype showed the lowest antioxidative enzyme activities (Sairam *et al.*, 1998).

To conclude: We have established that RWA infestation leads to the selective induction of the ROS, involving the ROS generating enzymes including NADPH oxidase and SOD in resistant plants. It is suggested that induction of NADPH oxidase activity is incidental to activation of the SOD activity. The H_2O_2 may be formed through the dismutation of O_2^- by SOD. However, the rapid and continuous production of H_2O_2 may harm cell metabolism. The selective induction of the GR and APX activities during the resistance response supports their protective role against oxidative damage and to maintain a favourable ROS balance in the cell. Evidence was provided that the H_2O_2 accumulation is involved in the induction of the defence related enzymes β-1,3-glucanases and peroxidases during the RWA resistance response. This information undoubtedly contributes to a better understanding of the resistance mechanisms in wheat against the RWA, especially the early events, and might help in future manipulation of the RWA resistance in wheat.

SUMMARY

The effect of Russian wheat aphid (RWA), (*Diuraphis noxia*, Mordvilko), infestation on the activities of the reactive oxygen species (ROS) generating and scavenging enzymes as well as the hydrogen peroxide content were studied in resistant (cv., *Tugela DN*) and the near isogenic susceptible (cv., *Tugela*) wheat (*Triticum aestivum* L.) cultivars. Infestation resulted in an early induction (6-9h after infestation) of the activities of the ROS generating enzymes, NADPH oxidase and superoxide dismutase (SOD), in resistant plants only. These increased enzyme activities correlated with the increased H_2O_2 content in infested resistant plants. The activities of the ROS scavenging enzymes, glutathione reductase and ascorbate peroxidase were selectively induced somewhat later (12h after infestation) in resistant plants. Results suggest an early involvement of the ROS, and relevant ROS generating and scavenging enzymes in the resistance response of wheat to the RWA.

Inhibitory studies were performed *in vivo* using diphenylene iodonium (DPI), an inhibitor of NADPH oxidase, to shed light on the possible upstream role of ROS in defence against the RWA in infested resistant plants. DPI substantially inhibited the RWA induced NADPH oxidase activity and the concomitant accumulation of H_2O_2. This resulted in the inhibition of activities of the secondary defence related enzymes, β-1,3-glucanase and peroxidase, suggesting a signaling role for H_2O_2 in RWA resistance through activation of defence genes. Accordingly, treatment of plants with the H_2O_2 generating mixture of glucose and glucose oxidase resulted in induction of the H_2O_2 production as well as induction of the β-1,3-glucanase and peroxidase activities in resistant plants.

In vitro inhibition studies confirmed that DPI is a specific inhibitor of NADPH-oxidase and did not have any direct inhibitory effect on the β-1,3-glucanase and peroxidase activities. This finding further substantiated that H_2O_2 generated during

RWA infestation was involved in the signaling events leading to induction of the secondary defence responses in resistant plants.

Key words: Wheat (*Triticum aestivum* L.), Russian wheat aphid (*Diuraphis noxia*), reactive oxygen species, hydrogen peroxide, NADPH oxidase, superoxide dismutase, glutathione reductase, ascorbate peroxidase, peroxidase, β-1,3-glucanase, diphenylene iodonium, resistance.

OPSOMMING

Die invloed van Russiese koringluis-(RKL), (*Diuraphis noxia,* Mordvilko) infestering op reaktiewe suurstofspesies (ROS)-produserende en -opruimende ensiemaktiwiteite asook waterstofperoksiedinhoud van weerstandbiedende (cv., *Tugela DN*) en die na-isogeniese vatbare (cv., *Tugela*) koring-(*Triticum aestivum* L.) kultivars is ondersoek. Infestering het die vroeë induksie (6-9 ure na infestering) van die aktiwiteite van die ROS-produserende ensieme, NADPH-oksidase en superoksieddismutase (SOD) in weerstandbiedende plante tot gevolg gehad. Hierdie verhoogde ensiemaktiwiteite het met die verhoogde H_2O_2-inhoud in geïnfesteerde weerstandbiedende plante gekorrelleer. Die aktiwiteite van die ROS-opruimende ensieme, glutatioonreduktase en askorbaatperoksidase was ietwat later (12 ure na infestering) selektief in weerstandbiedende plante geïnduseer. Die resultate het op die vroeë betrokkenheid van ROS en relevante ROS-produserende en -opruimende ensieme in die weerstandsrespons van koring teen die RKL gedui.

Remmingsstudies is *in vivo* met behulp van difenileenjodonium (DPI), 'n NADPH-oksidaseremstof, uitgevoer om lig op die moontlike stroomoprol van ROS in verdediging teen die RKL in geïnfesteerde weerstandbiedende plante te werp. DPI het die RKL-geïnduseerde NADPH-oksidase-aktiwiteit en gepaardgaande H_2O_2-akkumulering aansienlik gerem. Dit het remming van die aktiwiteite van die sekondêre verdedigingsverwante ensieme, β-1,3-glukanase en peroksidase, tot gevolg gehad, wat op 'n seiningsrol vir H_2O_2 d.m.v. verdedigingsgeenaktivering gedui het. Dienooreenkomstig, het behandeling van plante met die H_2O_2-produserende mengsel, glukose-en glukose-oksidase, tot die induksie van β-1,3-glukanase en peroksidase-aktiwiteite in weerstandbiedende plante gelei.

In vitro remmingsstudies het bevestig dat DPI 'n spesifieke remstof van NADPH-oksidase is en dat dit geen direkte remmingsinvloed op β-1,3-glukanase en peroksidase-aktiwiteite gehad het nie. Hierdie bevinding het bykomstig bevestig dat H_2O_2, geproduseer tydens RKL-infestering, by die seiningsgebeure wat tot die induksie van sekondêre verdedigingsreaksies in weerstandbiedende plante lei, betrokke is.

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