ELICITING AND SIGNAL TRANSDUCTION EVENTS OF THE RUSSIAN WHEAT APHID RESISTANCE RESPONSE IN WHEAT

By

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Dedicated to
'Mantsilele

Preface

The work presented here is a result from an original study conducted at the Department of Botany and Genetics, University of the Orange Free State, Bloemfontein, under the supervision of Prof. AJ van der Westhuizen.

It is a fact that the Russian wheat aphid/wheat interaction ultimately results in the induction of the downstream defense related responses. However, the full events of the pathway signaling these responses has not yet been accounted for by any previous studies. This report attempts to explain the mechanisms involved in the eliciting and signaling events leading to the activation of these defense related responses.

The report employs simple but strictly scientific methods of investigation, the results of which are reproducible. It is therefore hoped that it will provide valuable information to plant breeders employing biotechnology based techniques to enhance resistance in wheat plants.

The dissertation submitted here has not previously been submitted by me to any other university/faculty. I therefore cede its copyright in favour of the University of the Orange Free State.

Acknowledgements

I would like to thank Prof. AJ van der Westhuizen for his excellent supervision. His enthusiastic and constructive comments highly contributed to the success of the study.

I would like to thank my friends and my colleagues whose names could not be mentioned, for their support and assistance.

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A special word of thanks to Morena for his inspiration, encouragement and support throughout the study.

Glory to the Lord for the strength to complete
this study!

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Abbreviations

3 A-T	3-amino-1,2-4-triazole
AOS	Active oxygen species
APS	Ammonium persulfate
APX	Ascorbate peroxidase
ARC-SGI	Agricultural Research Council-Small Grain Institute
Asp	Asparagine
BA	Benzoic acid
BA2H	Benzoic acid-2-hydroxylase
con A	Concanavalin A
cvv	Cultivars
EDTA	Ethyl diamine tetra-acetic acid
Gal NAc	N-acetylgalactosamine
Glc NAc	N-acetylglucosamine
GO	Glucose oxidase
GST	Glutathione-S-Transferase
GTase	UDP-glucosyltransferase
HPLC	High performance liquid chromatography
HR	Hypersensitive reaction
IWF	Intercellular wash fluid
KD	Kilo Dalton
PAL	Phenylalanine ammonia-lyase
pgt	*Puccinia graminis* f. sp.*tritici*
PMSF	Phenyl methylsulfonyl fluoride
PR	Pathogenesis-related
PVP	Polyvinylpyrrolidone
RSA	Republic of South Africa

RWA	Russian wheat aphid
SA	Salicylic acid
SABP 2	Salicylic acid-binding protein 2
SAG	β-O-D-glucosylsalicylic acid
SAR	Systemic acquired resistance
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SP	Spectra Physics
TBS	Tris-buffered saline
Tris	Tris(hydroxymethyl)-aminomethane
TMV	Tobacco mosaic virus
TNV	Tobacco necrosis virus
USA	United States of America
USSR	Union of Soviet Socialist Republics
UV	Ultraviolet
v/v	volume per volume
w/v	weight per volume

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Chapter 1

Introduction

The world depends to a large extend on cereals such as wheat, barley, rye and oat for human and animal nutrition. Any substantial loss in grain production can have a negative impact on the lives of many people, especially in underdeveloped areas of the world. Pests and pathogens more often cause significant losses in yield throughout the world. Plant resistance mechanisms is an exciting field of research, and knowledge of mechanisms involved in resistance promises to be useful in developing new strategies for crop protection.

The Russian wheat aphid (RWA) (*Diuraphis noxia*) (Mordvilko) is a major pest in numerous wheat producing areas worldwide (Nkongolo *et al.* 1989). *Diuraphis noxia* is endemic to Southern Russia, countries bordering the Mediterranean Sea, Iran and Afghanistan (Hewitt *et al.* 1984). The earliest published reference of *Diuraphis noxia* as a pest was in the Crimea (Grossheim 1914). Sporadic outbreaks of this pest have occurred in the former USSR since then. At present, damage caused by *Diuraphis noxia* is restricted to the steppe zone of the Ukraine and Russian Soviet Socialist Republic (Kovalev *et al.* 1991).

The RWA is also widespread in Africa. Haile (1981) reported the RWA to be the leading pest of cereals in the highlands of Ethiopia. Attia and El-Kaddy (1988) observed the RWA on wheat and barley in the Beni-Suef province of Egypt in 1985. In 1980 RWA was found to be present in Mexico (Gilchrist *et al.* 1984). The RWA was first reported in the USA in 1986 and is now found in 17 western states of this country (Miller *et al.* 1994). In July 1988, the RWA was reported in Canada (Jones *et al.* 1989). The RWA has also been

reported in Chile (Jones *et al.* 1989). To date the RWA has not been reported in Australia. Hughes and Maywald (1990) used the CLIMAX model to forecast the suitability of the Australian environment for this pest. The study showed that the climatic conditions in Australia are suitable for RWA population development, and that severe losses could occur, should the RWA spread to Australia.

The RWA is also widespread in the Republic of South Africa (RSA). It was first reported in Bethlehem in the Eastern Free State in 1978, and by September of 1979, it had spread over the greater part of the Free State province and Lesotho. Isolated loci of infestation were also reported in the Western Free State, Northern Cape and Mpumalanga. Figures 1.1 and 1.2 show the distribution of *D. noxia* in the RSA and Lesotho, and in the Free State province. This pest has had a major impact on the South African wheat industry since the early 1980s. It has caused substantial yield losses annually, and prevented the planting of late intermediate and spring wheat in the Free State (Du Toit and Walters 1984; Du Toit 1992). The Free State province forms the largest wheat growing region in the RSA, contributing 40-50% of the total production of the country in normal years.

Research on the RWA in the RSA began in 1980. Due to the severity of the damage caused, the fastest and the most effective solution was the use of insecticides. At the onset, the insecticides registered for the control of other grain aphids were found to be ineffective against the RWA. However, in the eighties progress was made with combinations of parathion and some systemic insecticides. In 1993, the seed dressing Gaucho became available in the RSA, and *D. noxia* has until now been controlled by applications of either pre-plant or post-emergence insecticides. Damage to the plant can be limited by the chemical control but the cost is becoming prohibitive, and the effects of the chemicals are detrimental to the environment, especially in circumstances where harsh climate in the dry land wheat producing areas (of the Free State province) reduces the efficacy of these insecticides (Du Toit 1988).

In view of the several disadvantages of chemical control, alternative methods were sought and plant resistance work was began in 1985, when genetic resistance to the RWA was identified in bread-wheat lines. Following the finding of sources of resistance to RWA (Du Toit 1988, 1989), the backcrossing technique has been used to introduce some resistance in susceptible wheat lines with more acceptable agronomic characteristics. The first RWA resistant cultivar, Tugela *Dn 1*, was released to the market in 1993 by Agricultural Research Council-Small Grain Institute (ARC-SGI).

The resistant cultivars are colonized by RWA in low numbers, but do not show a reduction in the plant height or the typical streaking and leaf rolling damage symptoms usually caused by this aphid. Resistant cultivars tend to survive longer, and to be less stressed during the critical period between September and October. Under field conditions it appears that the infestation of susceptible Tugela and resistant Tugela *Dn 1* follows the same pattern throughout the season, but the RWA numbers on Tugela *Dn 1* are drastically lower. The aphids are found mainly on the adaxial surface of the newest growth, in the axils of leaves or within rolled leaves. Heavy infestation on the susceptible plants causes white streaks along the leaf and tight rolling of the leaf (Du Toit 1988). In the resistant cultivar, only scattered chlorotic spots are seen on the entire surface of the leaf (Fig. 1.3, van der Westhuizen and Botha 1993).

Another form of RWA control is the use of natural enemies. The work began in 1989 with the introduction of parasitoids from areas where the RWA originates. The incorporation of parasitoids in the RWA control program may however, prove not to be highly effective since parasitoids are highly host specific and were not found on the aphids available during summer (Marasas *et al.* 1997). In addition, some farmers still use chemical spraying on their fields to control other pests, pathogens and weeds and the parasitoids can as well be eradicated. An integrated control program for RWA control in which the resistant cultivar is used as the main control method, supported by natural enemies (parasitoids and predators) and entomopathogenic fungi, only resorting to chemical control when really necessary, can be beneficial to the farmers. The efficacy of this control program can be

amplified through information transfer to farmers and their appreciation of the RWA as a problem together with their cooperation with the standards of control (Marasas *et al.* 1997).

Even though resistant cultivars have been released, the development of new RWA biotypes might overcome this resistance. This necessitates more rapid development of new cultivars, and an understanding of the molecular techniques to modify plants for agronomic and yield characters, requires an understanding of the biochemical events associated with plant resistance. In an incompatible RWA\wheat interaction some of the resistance associated events have already been documented. Wheat plants under infestation are under stress, and indications are that RWA induces the hypersensitive reaction (HR) in resistant plants. This includes increased respiration rate, increased levels of phenolics, formation of new phenolics and accumulation of pathogenesis related (PR) proteins (van der Westhuizen and Botha 1993; van der Westhuizen and Pretorius 1995, 1996; van der Westhuizen *et al.* 1998a,b). The HR is the most efficient plant defense mechanism associated with pathogen attack and other stress conditions. Typical symptoms of the HR are necrotic lesions and accumulation of a variety of defense related products, such as PR proteins (Fritig *et al.* 1990; Chrispeels and Sadava 1994).

In the absence of RWA infestation the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) polypeptide profiles of the intercellular proteins are the same in both susceptible and resistant cultivars. However, RWA infestation induces a dramatic change in the protein profiles of the resistant cultivars. Three groups of RWA infestation related proteins were induced in the resistant cultivar and van der Westhuizen and Pretorius (1995, 1996) confirmed that they were serologically related to β-1,3-glucanases and chitinases belonging to the PR 2 and PR 3 of tobacco, respectively (Stintzi *et al* 1993a,b). Some enzymes such as peroxidases are also induced as part of the HR during RWA infestation (van der Westhuizen *et al.* 1998b). Peroxidases are known to play a crucial role in wound repair, wall strengthening and the formation of phenoxy radicals and lignification (Bowles 1990). The enhanced mechanical strength of resistant wheat leaves

after RWA infestation is an indication that lignification might be induced by RWA infestation (van der Westhuizen[1], personal communication).

The interaction between pathogens and plant cells initiates a number of metabolic changes in the host cell. The initial biochemical signal recognized by the plant cells is contained in elicitor molecules. Recognition of these elicitors by host cells appears to be strictly dependent on their structure, and defense responses are stimulated by very low concentrations of these compounds (Darvill and Albersheim 1984). Signal molecule(s) are then synthesized and the signal is transduced within the host cells, leading to activation of defense responses. These responses are also referred to as secondary events and some of them in RWA infested resistant wheat have been documented (van der Westhuizen and Botha 1993; van der Westhuizen and Pretorius 1995, 1996; van der Westhuizen *et al.* 1998a,b). Little is known about the signal tranduction pathway(s) leading to the activation of these defense responses. This study therefore aimed at elucidating the eliciting events and some of the signal transduction events associated with the RWA induced HR in wheat.

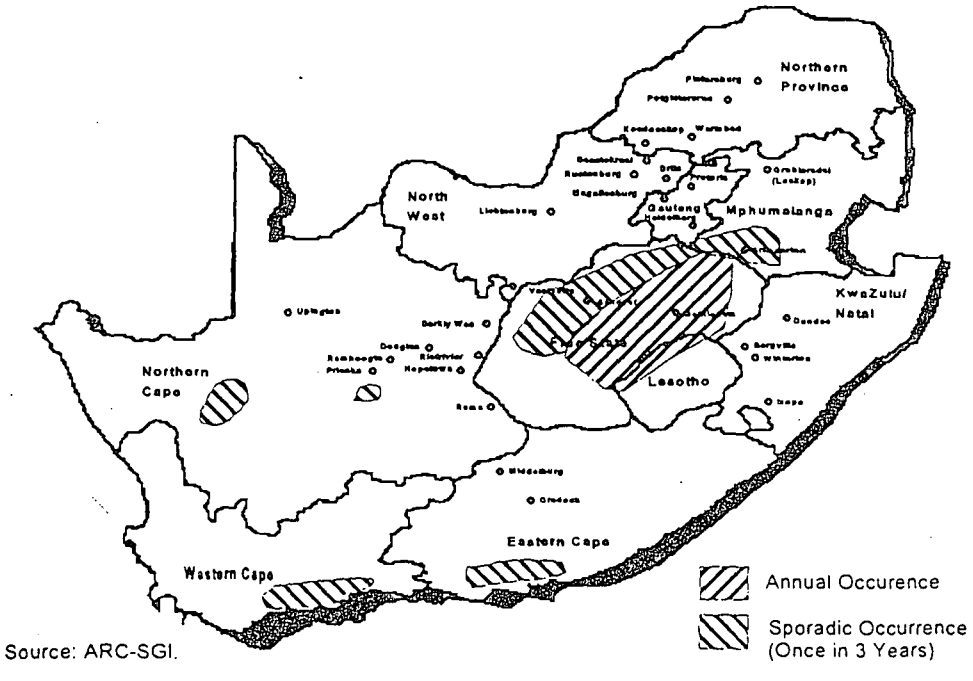
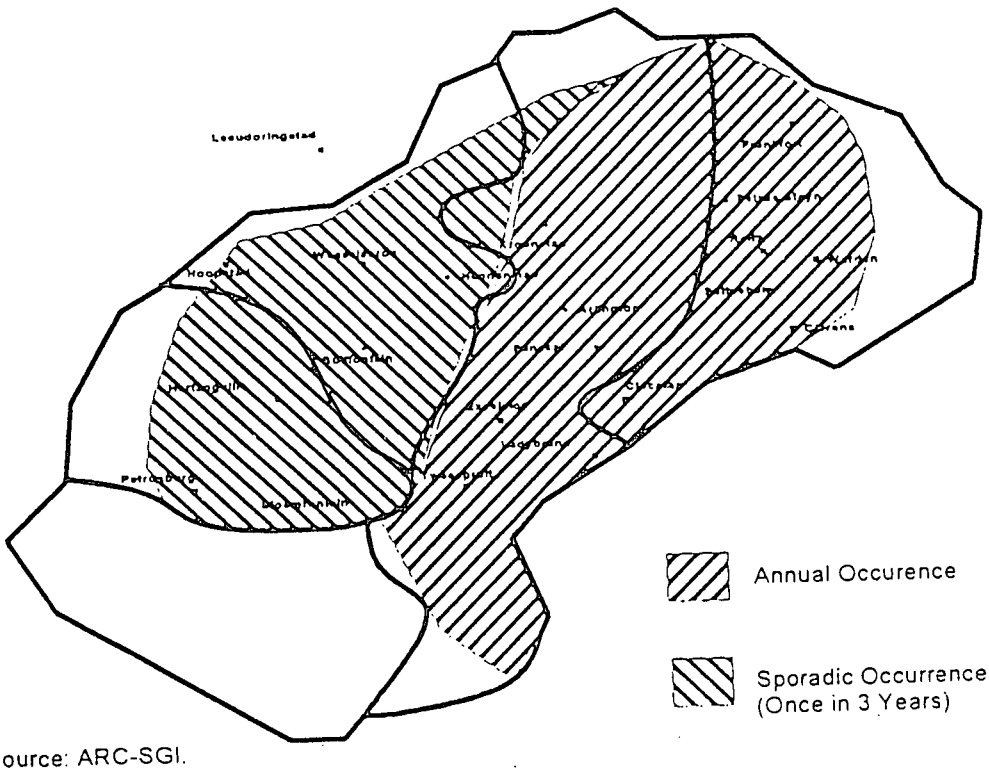


Fig. 1.1 The distribution of RWA in the RSA and Lesotho (Marasas *et al.* 1997).

[1] Van der Westhuizen AJ, Department of Botany and Genetics, UOFS, Bloemfontein.



Source: ARC-SGI.

Fig. 1.2 The distribution of RWA in the Free State province (Marasas *et al.* 1997)

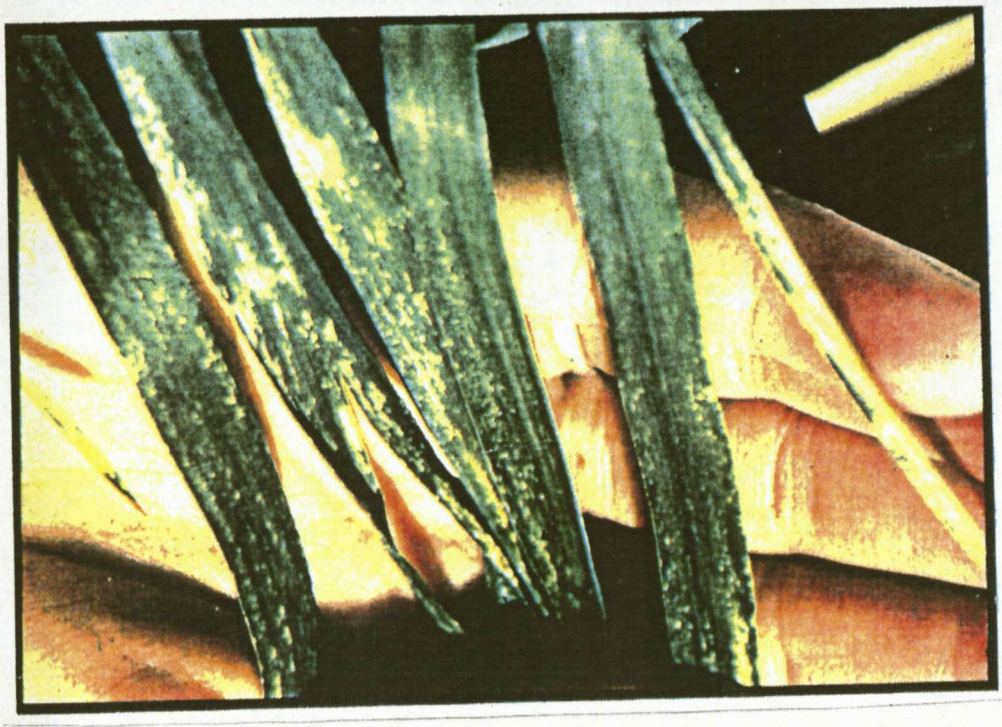


Fig. 1.3 Symptoms of RWA infestation on resistant wheat plants (van der Westhuizen and Botha 1993).

Fig. 1.4 Susceptible (right) and resistant (left) wheat plants under RWA infestation (field conditions).

Chapter 2

Literature Review

2.1 The Russian wheat aphid (RWA) - Features

The RWA is a soft-bodied spindle shaped insect (Fig. 2.1). The body is greenish-yellow measuring about 1.5 to 1.8 mm in length. It is characterized by black specks at the front antennae which are extremely short. The RWA lacks the prominent siphunculi which are pronounced in other aphids. Above its cauda, there is a second protruberance, giving its characteristic " double tail" (Du Toit and Aalbersberg 1980; Walters *et al.* 1980). Several members within a generation comprise parthenogenetic females which are also viviparous. Polymorphism is also a characteristic feature of the RWA. Different morphins can occur within one species (Dixon 1985).

Fig. 2.1 The RWA (*Diuraphis noxia*)

2.2 Origin and distribution of the RWA

The RWA is indigenous to the USSR (Grossheim 1914), countries bordering the Mediterranean sea, Iran and Afghanistan (Hewitt *et al* 1984; Walters *et al.* 1980). It has since spread to South Africa (Walters *et al.* 1980), Mexico (Gilchrist *et al.* 1984), America (Webster *et al.* 1987), Egypt (Attia and El-Kaddy 1988), Ethiopia (Haile 1981), Chile (Jones *et al.* 1989), Canada (Morrison 1988) and South America (Zerene *et al.* 1988). In 1978 the RWA was first reported in South Africa in the Eastern Free State. By September of the following year it was reported in most of the Western Free State and the neighbouring Lesotho. Isolated infestations were reported in Eastern Cape, Northern Cape, Western Cape and Mpumalanga (Walters *et al.* 1980).

The RWA is not only a pest in Southern Africa. In 1980 it was observed for the first time in Mexico (Gilchrist *et al.* 1984). By March 1986 it was reported in Texas (Webster *et al.* 1987) and since then has spread to sixteen states in America and three provinces in Canada (Porter *et al.* 1990). To date the RWA has not been reported in Australia. However a study by Hughes and Maywald (1990) showed that the climatic conditions in Australia are suitable for RWA population development and that severe losses could occur should the RWA spread to Australia.

2.3 Feeding by the RWA

Aphids are phloem feeders with a low frequency of phloem feeding on the resistant lines (Kindler *et al.* 1992). During feeding the aphid moves over the surface of the leaf, on the way obtaining information on the physical properties of the surface and the internal chemistry of the plant. This prior assessment involves little or no probing at all but often enables an aphid to sense the suitability of the host within 60 seconds (Dixon 1985). The stylet is then inserted into the cell wall. The path of the stylet is intercellular until it has pierced the phloem vessels (Fouche *et al.* 1984). This of course induces internal leaf damage as shown by the rapid collapse of the mesophyll and bundle sheath cells of RWA-infested barley leaves along the aphid's stylet pathway (Belafant-Miller *et al.* 1994). The stylet seems to secrete a lipoprotein sheath that surrounds it and may protect it against plant wound reactions (Miles 1990), or the sheath itself may act as an elicitor. As the aphids probe the leaf, they secrete saliva which contains pectinases. This salivary material may contain a phytotoxin and thus act as an elicitor (Brigham 1992). The RWA damages

the plant by destroying the chloroplasts and cellular membranes (Fouche *et al.* 1984), and this damage has been ascribed to a phytotoxin injected into the leaf during feeding (Burd and Burton 1992). This consequentially results in stunted growth of the host, water imbalances and sterility (Burd *et al.* 1989; Kindler *et al.* 1991).

2.4 Plant resistance to aphids - Natural resistance

2.4.1 Physical resistance

Information on the natural resistance to aphids in plants mainly deals with the physical barriers and chemical toxins which deter feeding on the hosts.

a) Trichomes

The glandular trichomes on the leaves of wild potato, *Solanum neocardensii,* adversely affect the feeding behavior of the green peach aphid, *Myzus persicae,* by delaying the time for initiation of feeding (Lapointe and Tingey 1986).

b) Surface wax

The surface of plant leaves is protected against desiccation, insect predation and disease by a layer of surface wax over the epicuticle. The plant waxes are esters formed by the linkage of a long-chain fatty acid and an aliphatic alcohol (Smith 1989). Hybridization experiments between a susceptible and a resistant line showed that resistance was correlated with characteristics of the surface wax of plants. There were qualitative and quantitative differences in wax composition between resistant and susceptible varieties (Corcuera 1993).

c) Tissue thickness

A thick cortex layer in the stems of the wild tomato, *Lycopersicon hirsutum,* deters feeding by the potato aphid, *Macrosiphum euphorbiae* (Thomas) (Quiras *et al.* 1977).

d) Plant pectin

It has been proposed that the degree of methylation and branching of plant pectin (located in middle lamellae and the cell wall), is important in the resistance of sorghum to the green bug, since it may interfere with the activity of the pectinase which is injected into the plant by the aphid (Dreyer and Campbell 1987).

2.4.2 Chemical resistance

a) Toxic chemicals

Some constitutive or induced secondary chemicals in plants retard aphid growth, stop aphid feeding or have some other properties which interfere with insect development (Dreyer and Campbell 1987). These compounds are phenolics (van der Westhuizen and Pretorius 1995; Niraz *et al.* 1985), which include gramine and hydroxamic acids (Corcuera 1990; Argandonia *et al.* 1983; Bohidar *et al.* 1986; Barria *et al.* 1992).

b) Nutrition and environmental factors

(i) Sucrose is a feeding stimulant for probing aphids. Substitution of sucrose for other common sugars results in an unacceptable diet (Dreyer and Campbell 1987). Resistant wheat cultivars have a lower sucrose content than susceptible ones (Corcuera 1993). Resistant plants sometimes lack a critical nutritional element, usually nitrogen containing compounds such as amino acids (Dreyer and Campbell 1987), or contain a lower amount of other nutritional components (Niraz *et al.* 1985).

(ii) The population growth rate of the aphid, *Schizaphis graminum* on plants grown in the presence of KNO_3 fertilizers was lower than on plants grown in the absence or low concentration of KNO_3 (Corcuera 1993). The growth rate of the aphid populations also decreased with the accumulation of NaCl in wheat leaves where wheat seedlings were irrigated with saline Hoagland solution (Araya *et al.* 1991).

(iii) High temperature stress increased the gramine content in new leaves of barley, and thereby increased resistance to the green bug (Corcuera 1993).

2.5 RWA control

In the Republic of South Africa, the major wheat growing region is the Free State province. The planting season extends from the end of April to the end of July. In June and July, the RWA moves in small numbers from the *Bromus* grass and volunteer wheat into commercial wheat fields (Kriel *et al.* 1984). When the temperature rises in September the RWA populations increase drastically. The critical period for the control of this pest often occurs in late September to early October before the first spring rains. It is common to find that the stored soil moisture is depleted during this period, and that wheat plants are experiencing moisture stress. These conditions are conducive to the RWA outbreaks as the RWA thrives well on moisture stressed plants (Burd and Burton 1992).

The RWA is mainly found on the adaxial surface of newest growth, in the axils of leaves or within the rolled leaves. Heavy infestation in young plants causes the tillers to become prostrate, while heavy infestations in later growth stages cause the ears to become trapped in the rolled flag leaf (Walters *et al.* 1980). Severe damage is associated with these symptoms. The level of infestation, the growth stage of the host plant, and the duration of the infestation, all influence the severity of the damage caused by the RWA. Du Toit and Walters (1984) concluded that wheat plants were most susceptible to the RWA from the flag leaf stage to flower initiation. Burd and Burton (1992) indicated that the duration of infestation, rather than the level of infestation, may be more important when damage is caused to the host plant.

Several control measures have been implemented to curb the devastating effects of the RWA. These involve chemical, biological and plant resistance measures.

2.5.1 Chemical control

At the onset of the RWA as a problem in the Republic of South Africa (RSA), all the insecticides registered for control of other grain aphids were found to be ineffective against the RWA. This

prompted the wheat producers to turn to combinations of contact and systemic insecticides to attain results which could be tolerated (Walters *et al.* 1980). During the early eighties, considerable progress was made with regard to reducing RWA spread by means of chemical control. The program was heavily reliant on combinations of parathion and some series of systemic insecticides. In 1993, the seed dressing Gaucho was available and the RWA has since then been controlled by applications of either pre-plant or post-emergence insecticides. The tight rolling of leaves protecting aphid colonies within, also makes insecticidal control inefficient. On the other hand chemical control is rather expensive, labour intensive, and requires proper knowledge of pest development. Most commercial farmers have used aerial applications of systemic organophosphates costing approximately R70 per hectare. The financial resources and management skills required to ensure economically viable RWA management are high. In low input agricultural systems where financing, necessary equipment, and knowledge is not readily available, the use of insecticides is very limited and even prohibitive (Marasas *et al.* 1997).

2.5.2 Biological control

The use of natural enemies such as the wasp and ladybird parasites can play an important role in the control of RWA populations (Walters *et al.* 1980). A pathogenic fungus has been observed infecting the RWA during the warm and moist periods, but it is of no practical importance during the normal winter growing season (Walters *et al.* 1980).

A total of four wasp species has been imported from countries where the RWA originated. They were evaluated under South African conditions and amongst them *Aphelinus hordei* showed the best potential for biological control of the RWA (Prinsloo 1995). The female wasp lays an egg within the aphid and the larvae start eating the aphid from inside and within 7 days the aphid dies. One female wasp can lay more than a hundred eggs (Prinsloo 1995). The first wasps were released in 1993 in the Eastern Free State and they parasitized from 48% to 83% aphids. Within a year the wasps had spread across 30 km from the initial site of release. Under laboratory conditions the wasps reduced the aphid population on resistant plants by 50% (Prinsloo 1995). The RWA in the RSA has however, several characteristics which are not favorable for biological control. The rate of reproduction and multiplication of this aphid is much higher than that of

natural enemies present in the RSA. The aphid has a high reproductive potential. The wheat agro-ecosystems are unstable and therefore the natural enemy host is present for only 3-4 months of the year. Chemical controls are used in this agro-ecosystem. The environment is dry and high temperatures normally prevail. Lastly, after wheat, RWA infests the *Bromus* grass and drastic RWA population changes occur between seasons (Kriel *et al.* 1984).

The introduction of biological organisms in the RWA control program can reduce the possibility of the formation of RWA resistance breaking biotypes.

In addition, since 1984 increasing efforts have been made to find sources of resistance to the RWA as an alternative to other methods of control (Du Toit, 1988). *Diuraphis noxia* from various parts of the world differ in their reaction to resistant wheat lines (Puturka *et al.* 1992). All the germ plasm found resistant to RWA should be screened using South African *D. noxia* to find superior sources of resistance. Currently, five sources of resistance are being incorporated into well-known and well adapted South African wheat cultivars by means of backcrossing breeding program. The sources of resistance include PI 137739 (*Dn 1*), PI 262660 (*Dn 2*), PI 294994 (*Dn 5*), CItr 2401 and Aus 22498. The RWA resistant cultivars so far released are namely; Tugela *Dn 1*, Betta *Dn 1*, Gariep, Limpopo, Caledon (all released by ARC-SGI), SST 333, SST 936 (released by Sensako seed company) and PAN 3235 (released by Pannar seed company). The first resistant wheat cultivar to be released worldwide was Tugela *Dn 1* in 1993 (Marasas *et al.* 1997).

Trials on the efficacy of RWA resistance in comparison to susceptible wheat have shown that the resistant cultivars give yields of the same order as susceptible cultivars that have received foliar sprays to control the RWA. This implies that the producer using resistant cultivars can obtain the same yield as with the susceptible ones but with less input costs. Insecticidal seed treatment and foliar spray treatment with a demeton-S-methyl/parathion mixture gave highest yield in both the susceptible and resistant cultivars. So far no negative quality characteristics have been associated with the inclusion of RWA resistance genes (Marasas *et al.* 1997).

2.6 Elicitation of the HR

2.6.1 Elicitors

The term 'elicitor' has been used to describe molecules which are able to induce physiological or biochemical responses associated with the expression of resistance (Kogel *et al.* 1988). Elicitors may be classified into exogenous and endogenous groups. The exogenous elicitors induce the hydrolysis of pathogen components by the plant enzymes, whereas the endogenous elicitors act by inducing the hydrolysis of plant components by the pathogen enzymes. Some elicitors are race-specific, that is, they mimic the gene-for-gene response in being able to induce a response only in the host cultivar on which that race of pathogen is avirulent. Nevertheless most elicitors are race non-specific (Smith 1996).

Many elicitors are extracellular microbial products, or breakdown products from entities in the microbial or plant cell wall. Fungal cell wall elicitors include β-linked glucans (Anderson 1978; Sharp *et al.* 1984), chitosan (Friestensky *et al.* 1985) and the unsaturated lipids, arachidonic and eicosopentanoic acids. In addition a number of metabolites of pathogenic origin also act as elicitors, including; polysaccharides (Hadwiger and Beckman 1980; Sharp *et al.* 1984), galactose and mannose-rich glycoproteins (Darvill and Albersheim 1984; Dixon 1986; Hamdan and Dixon 1987), chitosan (De Wit *et al.* 1985; Mayama *et al.* 1986), fatty acids (Bostock *et al.* 1981), and hydrolytic enzymes (Collmer and Keen 1986). The criteria used to assess elicitor activity are based on the visual estimation of cellular necrosis (Albersheim and Anderson-Prouty 1975), the measurement of electrolyte leakage (Dow and Callow 1979), the determination of extractable activities of induced enzymes (Dixon *et al.* 1981; Hahlbrock *et al* 1981) and the accumulation of phytoalexins (De Wit and Roseboom 1980), or hydroxyproline-rich cell wall glycoproteins (Esquerre-Tugaye *et al.* 1979). Elicitor activity has also been correlated with pectic fragments that arise from partial degradation of the plant cell wall. Degradation products from hydrolases and lyases are active, with maximum activity associated with oligomers of 7-15 units (Collmer and

Keen 1986). This detection of elicitor activity may suggest the involvement of pectic degrading enzymes from pathogens in the elicitation of the HR.

There is a possibility that a single pathogen can produce a number of elicitor-active components *in planta*. Glucans, glycoproteins and mannose-containing polysaccharides have been reported to rise from *Colletotricum lindemuthianum* (Hamdan and Dixon 1987; Tepper and Anderson 1986). Glucans, glycoproteins, and arachidonic acid have been documented as elicitors from *Phytophthora infestans* (Bloch *et al.* 1984; Doke 1985). Interactions occur among different elicitor molecules to modify the elicitor potential. In potato, glucans and arachidonic acid act synergistically as elicitors. Synergism also occurs between fungal cell wall elicitors and pectic fragments in legumes (Davis *et al.* 1986). There is also synergism between a glucan elicitor and the agrichemical probenazole in rice (Dixon and Lamb 1990). These interactions may constitute a highly sensitive detection scheme for the plant.

From a biochemical perspective, elicitors may serve as useful tools in the studies of plant metabolic pathways typically activated during the expression of resistance, and in studies concerned with primary recognition phenomenon between plant and pathogens or pests. The prerequisite for both kinds of studies is the provision of well defined homogenous elicitor molecules (Kogel *et al.* 1988).

2.6.2 Elicitor bioactivity

The diversity of elicitor active structures presumably implies that the plant cell has different receptors which when bound by ligand, trigger activation of the defense-related genes in the nucleus. Evidence suggests that fungal elicitors including the *Puccinia graminis* f. sp. *tritici* (pgt)-elicitor bind to the high affinity receptors in the plasma membrane (Kogel *et al.* 1991; Cosio *et al.* 1992). The activation may be relayed through common or distinct pathways. Recognition of elicitors by the host cells appears to be strictly dependent on their structure, and defense responses are stimulated by very low concentrations of these compounds (Darvill and Albersheim 1984).

The ability of a pathogen to inhibit or delay induced defense responses has been presumed to be mediated by suppressor molecules counteracting elicitor activity (Ouchi and Oku 1981; Callow 1984; Lamb *et al.* 1989; Moerschbacher *et al.* 1990; Scheel and Parker 1990; Knogge 1991; Ryan and Farmer 1991). In wheat it has been found that the *Puccinia graminis* f. sp. *tritici* (pgt)-elicitor activity can be suppressed by simultaneous application of a fraction derived from intercellular washing fluid of susceptible stem rust infected leaves (Beißmann and Kogel 1992). In the incompatible interactions the defense reactions are triggered by elicitor active materials derived from the penetrating stem rust (Arz and Grambow 1995). Symptoms similar to those described for the highly incompatible interactions like stimulation of phenylalanine ammonia-lyase (PAL) and other enzymes in the HR, 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, 1988; Tiburzy and Reisener 1990). In genetically susceptible rice cultivars, application of an elicitor mediates induced resistance mechanisms similar to those active in the genetically resistant cultivars (Scheinpflug *et al.* 1995). However, not much is known about signal transduction pathways leading to the activation of defense reactions in response to the perception of an elicitor signal.

2.6.3 Glycoproteins

Attempts to fractionate elicitors have resulted in the identification of some active macromolecules. These were both structural proteins as well as enzymes. They included glycoproteins, polysaccharides and lipids (Smith 1996).

Glycoproteins are widespread in both plants and animals. They are defined as proteins that are covalently bound through O- or N-glycosidic linkages. They occur in cells, both in soluble or membrane-bound forms, as well as in extracellular fluids. They are important in complex recognitions such as cell-molecule, cell-virus or cell-cell interactions. Some O-linked glycans appear to function in intercellular targeting.

O-linked glycoproteins

The O-glycosidic linkage is found between N-acetylgalactosamine (Gal NAc) and the hydroxyl group of the amino acid residue, serine or threonine. The carbohydrate composition may vary from 1% to over 85% of the total molecular weight. The carbohydrates may include the oligosaccharides and polysaccharides.

N-linked glycoproteins

The bond is formed between the N-actylglucosamine (Glc NAc) and the amino acid residue, asparagine (Asp). This class is often referred to as asparagine-linked.

These glycoproteins occur in three forms; the high mannose (Glc NAc + mannose), the complex (fucose, galactose or sialic acid + Glc NAc + mannose), and the hybrid which has features of both the high mannose and complex types.

2.6.3.1 Glycoproteins as elicitors

In glycoproteins acting as elicitors, the activity may be bestowed either in the carbohydrate or in the polypeptide moiety. In various elicitor preparations, activity has been associated with fractions rich in carbohydrate, containing galactosyl, glucosyl or mannosyl residues as common constituents. The significance of carbohydrate moiety to elicitor activity has been indicated by the reduction in activity caused by periodate treatment of elicitors from *C. lindemuthianum,* while the activity had been unaffected by pronase K treatment (Hamdan and Dixon, 1987). The elicitor activity retained following treatment with pronase indicated that the integrity of the protein is not necessary for biological activity.

On the other hand, in a glycopeptide isolated from *P. megasperma* f. sp. *glycinea* the polypeptide moiety has been shown to be responsible for eliciting activity (Scheel *et al.* 1991; Scheel and Parker 1990; Parker *et al.* 1991; Renelt *et al.* 1993; Sacks *et al.* 1993; Nurnberger *et al.* 1994). The glycoprotein with a Mr 42KD elicited the accumulation of coumarin phytoalexins in *Petroselinum crispum* tissues. It contained oligosaccharide residues of high mannose-type. The elicitor activity was retained following deglycosylation, but treatment with pronase or trypsin

completely abolished it (Parker *et al.* 1991). This was an indication that the protein moiety was responsible for its biological activity. However, few protein elicitors have been identified in which only a polypeptide residue is present. Since most peptide-containing elicitors are glycoproteins, it is difficult in such cases to ascertain whether the polypeptide or the glycosyl moieties are the determinants of activity (Smith 1996).

2.7 The hypersensitive response

Plants respond to infection in a variety of ways, ranging from mounting a strong defense mechanism to no defense mechanism at all. The defense mechanisms are elaborated both locally and systemically. In many cases local resistance is manifested as a hypersensitive response (HR). This is characterized by the development of necrotic lesions around the point of entry and localization of the pathogen within that area. Associated with the HR is the induction of a diverse group of defense related genes. The products of many of these genes play a vital role in restricting pathogen growth either indirectly by strengthening the host cell walls or directly by providing antimicrobial enzymes and metabolites. These products include cell wall polymers, such as lignin and suberin, as well as phenylpropanoids and phytoalexins. Several pathogenesis-related (PR) proteins are also induced during the HR (Durner *et al.* 1997).

Most of the PR proteins are monomers with low molecular weights (8-50 KD). They 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 cell wall, the apoplast and the vacuole (Stintzi *et al.* 1993a,b). Some of these proteins are hydrolytic enzymes [ß-1,3-glucanase (PR 2) and chitinase (PR 3)], but the functions of other PR proteins have not yet been determined. Most of the PR proteins have been shown to enhance disease resistance when overexpressed in transgenic plants (Ryals *et al.* 1996; Wobbe and Klessig 1996).

The rapid increase in the active oxygen species, termed the oxidative burst, may be another facet of the HR. This precedes and then accompanies the lesion-associated host cell death. The response has been observed in diverse monocotyledonous and dicotyledonous species including rice, tobacco, soybean and spruce following pathogen or elicitor treatment (Mehdy 1994). Within

hours to days after the primary infection, the systemic acquired resistance (SAR) builds up throughout the plant. It manifests itself as enhanced and long-lasting resistance to subsequent challenge by the same or other unrelated pathogens (Durner *et al.* 1997).

2.8 Peroxidases

These enzymes use hydrogen peroxide in a range of oxidations. The peroxidases have been intensively studied in higher plants and their activities can be correlated with growth, development and defense responses in plants (Bowles, 1990).

2.8.1 Induction of peroxidases

The activity of peroxidase in plants can be induced by a variety of factors. Infection by pathogens (Svalheim and Robertsen 1990; Reimers *et al.* 1992; Candela *et al.* 1994), wounding (Svalheim and Robertsen 1990), ethylene application (Ingemarsson 1995), low concentration of CO_2 (Takeda *et al* 1993), abscisic acid (Roberts and Kolattukudy 1989; Chaloupkova and Smart 1994), selenite (Takeda *et al.* 1993), indoleacetic acid (Birecka and Miller 1974), sodium chloride (Mittal and Dubey 1991), and elicitors from fungal cell walls (Moerschbacher *et al.* 1986; Gotthardt and Grambow 1992), have all been reported to induce peroxidase activity.

The induction of peroxidase by feeding insects has also been noted. The spotted alfalfa aphid (Jiang and Miles 1993; Bronner *et al.* 1991) and root-knot nematodes (Huang *et al.* 1971; Ganguly and Dasgupta 1979; Zacheo *et al.* 1982; Bajaj *et al.* 1985, Molinari 1991; Mateille, 1994), have enhanced the peroxidase activity in their host plants.

2.8.2 Roles of peroxidases in plants

In this context peroxidases will be discussed in relation to the four main events that occur in the extracellular matrix, namely oxidative burst, lignin synthesis, suberin synthesis, and the construction of intermolecular linkages.

2.8.2.1 The oxidative burst

Several rapid processes characteristic of the HR appear to involve primarily, the activation of preexisting components rather than changes in gene expression. One of these rapid events is the striking release of the active oxygen species (AOS) known as the oxidative burst. This response to pathogens or elicitors has been observed in diverse monocotyledonous and dicotyledonous species including rice, tobacco, soybean and spruce (Mehdy 1994). The AOS are toxic intermediates that result from successive one-electron steps in the reduction of molecular oxygen. The predominant species detected in plant-pathogen interactions are superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($OH^·$).

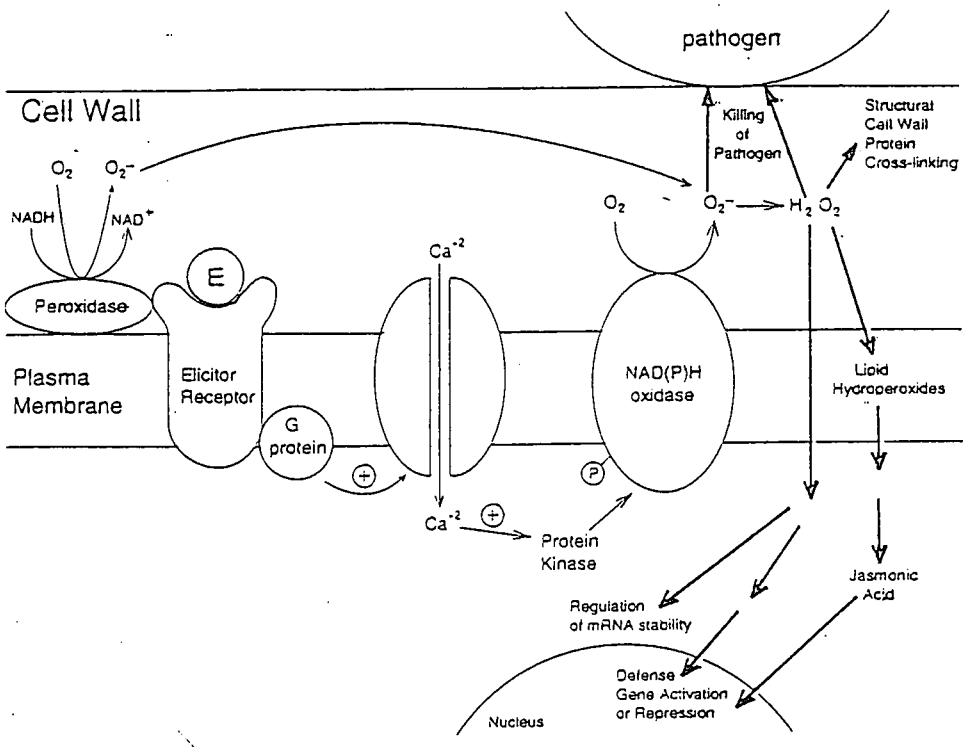


Fig. 2.2 Speculative model showing possible components involved in AOS generation and effects of AOS (Mehdy 1994).

The first reaction during the pathogen-induced oxidative burst is the one-electron reduction of molecular oxygen (O_2) to form superoxide anion ($O_2^{\cdot}$). This reaction is catalyzed by an NADH-dependent peroxidase which is associated with the external surface of the plasma membrane (Sutherland 1991; Vera-Estrella *et al.* 1992). In aqueous solutions, the superoxide anion undergoes spontaneous dismutation catalyzed by superoxide dismutase to H_2O_2 and O_2. The superoxide anion can also act as a reducing agent for transition metals such as Fe^{3+} and Cu^{2+}. An important consequence of metal reduction is that it can lead to the H_2O_2-dependent formation of hydroxyl radicals ($OH^{\cdot}$). The hydroxyl radicals initiate radical chain reactions including lipid peroxidation, enzyme inactivation, and degradation of nucleic acids (Mehdy, 1994). The possible components involved in the AOS are shown in Fig. 2.2.

2.8.2.2 Roles of AOS in plant defense

The current evidence indicates that AOS, particularly H_2O_2 directly reduce growth and viability of the pathogen (Peng and Kuc 1992; Kiraly *et al.* 1993; Wu *et al.* 1995). Spore germination for a number of fungal pathogens has been shown to be inhibited by micromolar concentrations of H_2O_2 (Peng and Kuc 1992). The toxicity of AOS or AOS-derived compounds may contribute to host cell death during the HR due to lipid peroxidation and the generation of lipid free radicals following pathogen or elicitor treatment (Adam *et al.* 1989; Keppler and Baker 1989). In different bean cultivars, lower levels of antioxidant enzymes were correlated with greater resistance to pathogen infection (Buonaurio *et al.* 1987). The AOS, in addition, play a novel role in strengthening the cell wall. The elicitor of bean or soybean cells was shown to result in H_2O_2-mediated oxidative cross-linking of specific structural proteins (Bradley *et al.* 1992). This response was rapid, appeared to depend on *de novo* synthesized H_2O_2, and increased wall resistance to the action of fungal wall degrading enzymes. H_2O_2 has also been implicated to play a role in limiting the spread of cell death by the induction of cell wall protectant genes such as glutathione-s-transferase (GST) in the surrounding cells (Levine *et al.* 1994; Tenhaken *et al.* 1995).

Hydrogen peroxide has also been postulated to be involved in mediating some of the defense responses. In transgenic potato, *Solunum tuberosum*, which expresses a foreign gene encoding

glucose oxidase (GO), the constitutively elevated levels of H_2O_2 induced accumulation of total SA in leaf tissues during *Verticilium dahliae* infection. The mRNAs of defense-related genes encoding the anionic peroxidase and chitinases were also induced. Increased accumulation of several isoforms of extracellular peroxidase were also observed. The above responses were also accompanied by a significant increase in the lignin content of stem and root tissues of these transgenic plants (Wu *et al.* 1997).

2.8.2.3 Lignin synthesis

Lignin is an aromatic polymer composed mainly of cinnamyl alcohols such as O-coumaryl, coniferyl and sinapyl alcohols (Lewis and Yamamoto 1990). Lignin biosynthesis involves the hydrogenative polymerization of cinnamyl alcohols to yield phenoxy radicals. These radicals polymerize spontaneously to give a complex net of cross-links among monolignols, proteins and polysaccharides in the cell wall. The formation of phenoxy radicals is catalyzed by peroxidases (Grisebach 1981; Higuchi 1982). Peroxidases have been implicated in these cross-linking reactions for two reasons, firstly they have been localized in cell walls of lignifying tissues, and secondly, they catalyze the production of lignin-like products *in vitro* (Lewis and Yamamoto 1990). Lignification is induced in plant pathogen and plant-insect interactions, and has been correlated both with local (Matern and Kneusel 1988; Vance *et al.* 1980) and induced systemic resistance (Kuc 1983), and the enhanced peroxidase activity (Polle *et al.* 1994; Padu 1995).

2.8.2.4 Construction of intermolecular linkages

The properties of the cell wall, including rigidity can be affected to a large extend by the cross-linking of the polymers. Peroxidases are known to play a crucial role in the formation of these cross-linkages. Two principle polymer-bound phenolic groups that act as substrates for the formation of these cross-linkages include; the side-chain of tyrosine and the products derived from O-coumaric acid. These phenolics are attached to wall polysaccharides. The consequence of peroxidase action on these wall phenolics consists of two parts; the glycoproteins become cross-linked via isotyrosines, leading to an extremely stable, insoluble network, and the gelling of polysaccharides can be substantially increased by diferuloyl bridges (Rombouts and Thibault 1985). In principle, regulation of this cross-linking during a defense response could arise from

changes in levels of the relevant peroxidases, the temporal /spatial availability of H_2O_2 in the wall micro environment, and/or from the degree of feruloylation of the polysaccharides that are secreted (Bowles 1990).

2.8.2.5 Suberin synthesis

Suberin is a polymer composed of aliphatic and aromatic domains (Kolattukudy 1981, 1984). The aromatic polymer is thought to be similar to lignin. It has been proposed that the polymerization of the aromatic monomers of suberin involves an isoperoxidase (Kolattukudy 1981) in a manner similar to lignin biosynthesis (Gross 1977; Grisebach 1981). Suberin is a cell wall component and mainly enhances cell wall rigidity. Its formation is developmentally regulated, but can be induced at a specific site during a defense response, especially when a diffusion barrier has to be constructed to delimit supply of nutrients from the cells. Suberin is however of minor importance in plant defense.

2.9 Salicylic acid

2.9.1 General properties

Salicylic acid (SA) is a phenolic acid with an aromatic ring bearing a hydroxyl group or its derivative (Fig. 2.3). Free SA exists as a crystalline powder with a melting point of 157-159°C. It is moderately soluble in water and very soluble in polar organic solvents. A saturated aqueous solution of SA has a pH value of 2.4.

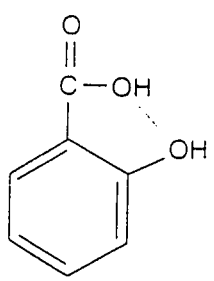


Fig. 2.3 Structure of SA

According to a mathematical model (Kleier 1988; Hsu and Kleier 1990), the physical properties of SA [Pka = 2.98, log K_{ow} (octanol, water partitioning coefficient) = 2.26] are nearly ideal for long distance transport in the phloem. Unless free SA is actively transported, metabolized or conjugated it should move rapidly from the point of initial application or synthesis to distal tissues.

The presence of SA in plants has been suggested a long time ago (Procter 1843; Griffiths 1958) and has lately been confirmed by investigators using modern analytical techniques (Mendez and Brown 1971; Cleland and Ajami 1974; Baardseth and Russwurm 1978). A survey of SA in the leaves and reproductive structures of thirty-four agronomically important species confirmed the ubiquitous distribution of this compound in plants (Raskin *et al.* 1990). Rice, crabgrass, green foxtail, barley and soybean had SA levels in excess of 1μg g^{-1} fresh weight. The highest levels of SA were recorded in inflorescences of thermogenic plants and in plants infected with necrotizing pathogens (Raskin 1995).

Plants are one of the world's richest sources of natural medicines. The use of plants and their extracts for healing dates back to earliest recorded history. The use of the willow tree (*Salix*) to relieve pain is believed to be as old as the 4th century BC. The active principle of willow remained a mystery until the 19th century when the salicylates, including salicylic acid, methyl salicylate, saligenin (the alcohol of SA) and their glycosides, were isolated from different plants including willow. Soon thereafter, SA was chemically synthesized and subsequently replaced by the synthetic derivative acetylsalicylic acid (aspirin) which has similar medicinal properties and produces less irritation. Despite the long history of SA, its mode of action is not fully understood.

The findings that it plays a role in disease resistance in plants raises the possibility of some parallels between SA action in plants and animals.

2.9.2 SA as an endogenous signal

The first conclusive evidence implicating endogenous SA as a regulatory molecule resulted from studies of the thermogenic voodoo lilies, *Sauromatum gutatum* (Raskin *et al.* 1987, 1989). The

spadix of the voodoo lily exhibits dramatic increases in temperature during flowering. There are two periods of temperature increases and a large transient rise in endogenous SA levels was found to precede both periods. In addition, thermogenesis and the production of aromatic compounds associated with thermogenesis could be induced by treatment of spadix explants with SA, acetyl-salicylic acid, or 2,6-dihydroxybenzoic acid but not with 31 structurally similar compounds (Klessig and Malamy 1994).

2.9.3 Biosynthesis of SA

The potential to manipulate the levels of SA in plants depends on the understanding of the biosynthetic pathway (Fig. 2.4).

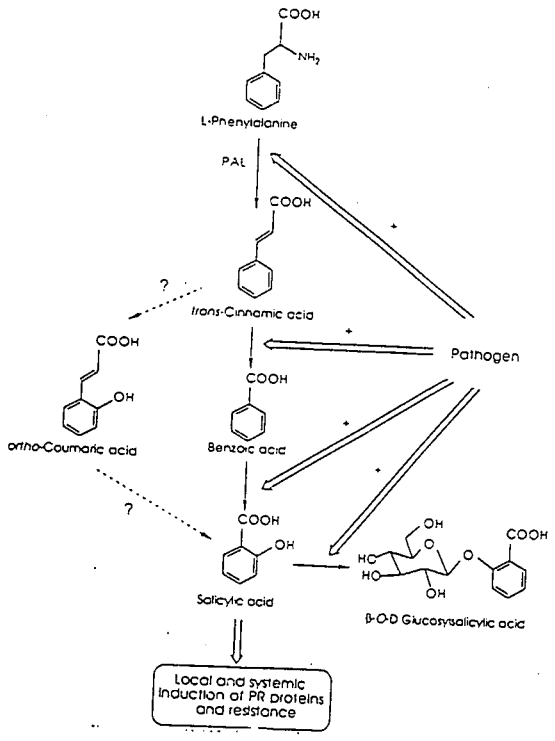


Fig. 2.4 SA biosynthesis (Raskin 1995).

The biochemical logic and some published reports (Leon *et al.* 1993; Metraux *et al.* 1995) suggest that in plants, SA is likely synthesized from t-cinnamic acid, an intermediate of the phenylpropanoid pathway which yields a variety of phenolics with structural and defense-related

functions. Feeding both healthy and TMV-inoculated tobacco leaves with putative precursors of SA showed that only benzoic acid was capable of increasing tissue levels of SA (Raskin 1995). When (^{14}C) - labeled cinnamic acid was fed into the leaves, labeled benzoic acid and SA were formed (Bradley *et al.* 1992). No radioactive O-coumaric acid was formed from cinnamic acid. Feeding leaf tissue with labeled benzoic acid resulted in the formation of SA with specific radioactivity almost equal to that initially supplied as benzoic acid. This suggested that most of the SA in tobacco is formed from benzoic acid. The formation of SA from t-cinnamic acid may occur by a chain-shortening reaction followed by 2-hydroxylation or *vice versa*. In other plant species SA may be formed through ortho-hydroxylation followed by β-oxidation of the O-coumaric acid (Yalpani *et al.* 1993).

2.9.4 Metabolism of SA

Most phenolic acids in plants exist in the form of sugar conjugates. SA in the same manner undergoes conjugation with glucose forming β-O-D-glucosylsalicylic acid (SAG). The production of a glucose ester has also been reported (Edwards 1994). Leaves of xanthi-nc tobacco rapidly metabolized exogenously applied or endogenously produced SA to β-O-D-glucosylsalicylic acid. Endogenously produced SA following TMV-treatment in tobacco was also rapidly conjugated to SAG, in fact most of the SA present in TMV-inoculated leaves of tobacco is in the conjugated form (Enyedi *et al.* 1992). Large amounts of the SA conjugate were found only in leaves that exhibited HR and around the necrotic lesions. Phloem sap and pathogen-free leaves of TMV-inoculated tobacco did not contain significant levels of β-O-D-glucosylsalicylic acid (Enyedi *et al.* 1992) indicating that only free SA can move in the plant.

The enzyme UDP-glucosyltransferase (GTase) occurs constitutively in plants and is also SA-inducible. It catalyzes the glucosylation of SA to β-O-D-glucosylsalicylic acid (SAG). It has been partially characterized from cell suspension cultures of *Mallotus japonicus* (Tanaka *et al.* 1990, Yalpani *et al.* 1992) and tobacco (Enyedi and Raskin 1993). In all these species the enzyme is SA inducible. In tobacco the enzyme activity is enhanced about 7-fold above basal levels between two and three days after TMV-infection, consistent with the rise in SA levels. Available data also indicate that GTase is one of the many proteins induced during the HR (Raskin 1995).

SAG accumulation in systemic parts of the plant is unclear. Enyedi *et al.* (1992) detected free SA, but no SAG in lower halves of tobacco leaves inoculated with TMV at their tips. They also found only free SA in the phloem exudates of inoculated leaves and in upper uninoculated leaves of TMV-infected plants. This suggested that SAG is neither transported to, nor synthesized in the uninoculated sites. The authors speculated that these tissues may lack sufficient GTase activity to convert SA to SAG. In contrast, it has also been found that both SA and SAG are present in the inoculated and uninoculated halves of TMV-infected leaves (Klessig and Malamy 1994). Similarly, conjugated SA was found in uninoculated leaves of *P. lachrymans*-infected cucumber (Meuwly *et al.* 1994).

The existence of SAG suggests an additional complexity in the modulation of the SA signal. To test its bio-activity in the absence of SA, it was synthesized and injected into the extracellular spaces of tobacco leaves and PR-1 gene induction was monitored (Hennig *et al.* 1993). SAG proved to be as active as SA in inducing PR-1 genes. Isolation of extracellular fluid from SAG-injected leaves showed that SAG was hydrolyzed to release SA in the extracellular spaces. Apparently the released SA entered the surrounding cells and was reconjugated to form SAG.

2.9.5 Role of SA in disease resistance

Signaling pathways involved in the initiation and maintenance of the HR and systemic acquired resistance (SAR) are still poorly understood. However, resistance to pathogens and the production of at least some PR proteins in plants can be induced by SA or acetylsalicylic acid even in the absence of pathogenic organisms. The discovery of the protective function of SA was made in 1979 (White 1979) in xanthi-nc tobacco, *Nicotiana tabacum*. It contains the "N" gene originating from *N. glutinosa* and confers HR to TMV (Holmes 1938). Injection of leaves with 0.01% SA solution and 0.02% aspirin solution, as well as watering of tobacco plants with aspirin prior to TMV inoculation caused a dramatic reduction in lesion number (White 1979). Exogenously applied SA also reduced lesion size. The reduction in lesion size has been considered to be more a reproducible measure of increased resistance than reduction in lesion number. Salicylate treatments also resulted in induction of PR proteins in treated leaves. Even when

watered on to the soil, SA reduced the size of tobacco necrosis virus (TNV) lesions of tobacco. Salicylate reduced the symptoms of TNV in asparagus bean by 90% and induced PR proteins and resistance to alfalfa mosaic virus in cowpea protoplasts by up to 90% — depending on the mode of application. Recently SA has emerged as a key signaling component involved in the activation of certain plant defense responses. In the early 1990s it became apparent that SA is an endogenous compound that operates in the signaling pathway for plant defense (Raskin 1992).

In tobacco infected with TMV, SA accumulates to high levels (more than 50-fold) (Malamy *et al.* 1990) at the site of infection with subsequent, but much smaller rise in the uninfected systemic tissues. This increase paralleled the transcriptional activation of PR genes in both inoculated and uninoculated leaves. Strikingly, exogenously applied SA induced the same set of nine genes that were activated systemically upon TMV infection (Durner *et al.* 1997). Increase in SA accumulation in the phloem of cucumber plants infected with either TNV or *Colletotrichum lagenarium* was also shown to precede the development of necrotic lesions (Ryals *et al.* 1996; Wobbe and Klessig 1996).

The TMV inoculation of tobacco cultivars (NN genotype), resistant to TMV led to the elicitation of the HR restriction of the spread of the virus (Rasmussen *et al.* 1991). A minimum 50-fold increase in the endogenous SA level in inoculated leaves was observed 72 h post inoculation (Mauch-Mani and Slusarenko 1996). Salicylic acid was also observed in the phloem exudate of excised leaves following TMV inoculation (Pieterse *et al.* 1996). Concurrent with the appearance of SA in the phloem, there was an increase in the level of SA in the uninoculated leaves above the TMV- inoculated lower leaf (Mauch-Mani and Slusarenko 1996, Pieterse *et al.* 1996). In the same leaves several PR proteins were induced as the SA levels increased. The induction of PR-1 genes paralleled the rise in endogenous SA levels in both the inoculated and uninoculated leaves. It was shown that the increase in endogenous SA was sufficient for the induction of PR-1 genes (Pieterse *et al.* 1996).

Recently, the participation of SA in plant defense responses has been demonstrated through analysis of transgenic tobacco and *Arabidopsis* expressing the nahG gene from *Pseudomonas*

putida. This encodes the enzyme salicylate hydroxylase (Gaffney *et al.* 1993; Vernooij *et al.* 1994). These plants accumulate little, if any SA, and as a consequence show reduced or no PR gene expression, fail to establish SAR and therefore become unable to prevent pathogen growth and spread from the primary site of infection. Salicylate hydroxylase converts SA to catechol which is inactive in inducing disease resistance (Gaffney *et al.* 1993, Delaney *et al.* 1994). The importance of SA in activation of resistance is further underscored by the demonstration that *Arabidopsis* plants become susceptible to avirulent fungal pathogens when PAL is specifically inhibited (Mauch-Mani and Slusarenko 1996). PAL catalyzes the first step in SA biosynthesis, and resistance can be restored in PAL-inhibited plants by treatment with exogenous SA application. Increased susceptibility is presumably caused by a block in SA synthesis.

The disease resistance genes induced by SA can be grouped into two broad classes. The first class consists of genes whose expression is insensitive to protein synthesis inhibitors such as glutathione-transferase genes, 35S promoter of cauliflower mosaic virus, nopaline and octopine synthase genes of *Agrobacterium*. Promoters of this class contain copies of as-1- like cis elements, which mediate SA - induced expression. On the other hand the second class has the acidic PR (class II) genes whose induction by SA is sensitive to inhibitors of protein synthesis (Durner *et al.* 1997).

2.9.6 Salicylic acid and systemic acquired resistance

Systemic acquired resistance (SAR) refers to a distinct signal transduction pathway that plays a major role in the ability of plants to defend themselves against pests and pathogens. It is usually activated following the HR or any disease symptoms and results in a broad spectrum systemic resistance (Hunt and Ryals 1996; Neuenschwander *et al.* 1996). The SAR primes the unaffected parts of the plant to subsequent invasion by the pathogen or pest. It can be detected several days following initial infection and can last for several weeks. It is usually effective against a broad range of pathogens which may sometimes be unrelated to the inducing pathogen. It has often been referred to as plant immunization (Raskin 1995).

Disease resistance mechanisms in plants are expressed at two levels; local resistance at the site of infection and systemic resistance at the distal uninfected parts of the plant (Klessig and Malamy 1992; Metraux 1994; Ryals *et al.* 1994). This separation in time and space of the primary infection and SAR may imply the presence of an endogenous signal which actually moves from the site of infection to distal uninfected tissues. A lot of evidence is accumulating suggesting that SA plays a key role in both disease resistance and SAR signaling. The level of endogenous SA has been found to increase by several hundred-fold in tobacco or cucumber following pathogen infection and the increase correlated with SAR (Malamy *et al.* 1990; Metraux *et al.* 1990; Rasmussen *et al.* 1991). In other plant species the same pattern follows (Dempsey *et al.* 1993; Uknes *et al.* 1993; Yalpani *et al.* 1993; Cameron *et al.* 1994). The accumulated data, coupled with the finding that exogenously applied SA can induce SAR gene expression (Ward *et al.* 1991; Vernooij *et al.* 1995) led to the suggestion that SA was involved in SAR signaling. The compelling evidence has also been brought forward by the analysis of tobacco transgenic plants which produce salicylate hydroxylase. These plants do not accumulate free SA and are unable to mount SAR response against fungal, bacterial or viral infections. On the other hand the isogenic wild type (NahG) plants inoculated with avirulent strains of *P. Parasitica* or *P. Syringae* DC 300 showed no pathogen growth (Gaffney *et al.* 1993; Bi *et al.* 1995; Friedrich *et al.* 1995; Lawton *et al.* 1995).

The biochemical markers for SAR have recently been identified as SAR genes. Their induction is tightly correlated with the onset of SAR in uninfected tissues (Metraux *et al.* 1989; Ward *et al.* 1991; Uknes *et al.* 1992). The genes are so classified if their presence or activity correlates tightly with maintenance of the resistant state (Neuenschwander *et al.* 1996). The analysis shows that many belong to the PR proteins. In tobacco, the set of SAR markers consists of at least 9 families made up of acidic form of PR-1 (PR-1a, b, c); β-1,3-glucanase (PR-2a, b, c); class III chitinase (PR-3a, b; Q); hevein-like protein (PR-4a, b); thaumatin like protein (PR-5a, b); acidic and basic isoforms of class III chitinase; extracelullar β-1,3- glucanase (PR-Q`); and basic isoform of PR-1 (Ward *et al.* 1991).

It has been described that SA coordinately induces the full spectrum of SAR genes (Ward *et al.* 1991), including all the well-characterized PR proteins. The relative amounts of individual PR-mRNAs or proteins differ greatly from those seen during the HR. In N gene-containing tobacco SA induces PR-1a, -1b, -1c, -2a and -2b to the same extend as TMV, but PR-2c, -3a, -3b, -4 and -5 remained at low levels and only reached high levels in the presence of other signaling molecules such as ethylene (Van Loon 1977) or jasmonic acid (Xu *et al.* 1994).

2.9.7 Mode of action of SA

Regardless of whether SA is a mobile signal of SAR or not, it appears to be required for the establishment and maintenance of the SAR. However the mechanism by which SA induces SAR is still unclear. Previous studies have demonstrated that SA binds and inhibits catalase activity in tobacco both *in vitro* and *in vivo* (Chen *et al.* 1993; Conrath *et al.* 1995). Thus, one possible function of SA is to inhibit the hydrogen peroxide-degrading activity of catalase leading to an increase in the endogenous level of H_2O_2, which is generated by photorespiration, photosynthesis, oxidative phosphorylation and the hypersensitive response-associated oxidative burst. The H_2O_2 and other reactive oxygen species derived from it, could then serve as second messengers to activate the expression of plant defense related genes such as the PR-1 genes. When endogenous H_2O_2 levels were raised by injecting leaves with H_2O_2 or 3-amino-1,2,4-triazole (3AT), PR-1 gene expression was induced. It was also induced by compounds that promote H_2O_2 generation *in vivo* such as glycolate and paraquat (Klessig and Malamy 1994). Treatment of plants with 3AT or paraquat enhanced resistance to TMV-infection in tobacco as has been demonstrated for SA (Klessig and Malamy 1994). PR genes were activated considerably less effective with H_2O_2 treatment than with SA treatment. Antioxidants suppressed the SA-mediated expression of PR-1 genes (Conrath *et al.* 1995).

In contrast to the hypothesis, several studies questioned the role of H_2O_2 and the SA-mediated inhibition of catalase and ascorbate peroxidase (APX) during the activation of defense responses. No detectable increases in H_2O_2 levels were found during the establishment of SAR (Neuenschwander *et al.* 1995) and significant reductions in catalase activity were not observed in tobacco infected with *Pseudomonas syringae* or in leaf discs that were pretreated with SA (Bi *et*

al. 1995). In addition H_2O_2 and H_2O_2-inducing chemicals were unable to induce PR-1 gene expression in Nah G plants (Bi *et al.* 1995; Neuenschwander *et al.* 1995). Moreover, high concentrations of H_2O_2 (150-1000 mM) were shown to induce SA accumulation (Leon *et al.* 1995; Neuenschwander *et al.* 1995; Summermatter *et al.* 1995). Finally, catalase antisense or co-suppressed sense transgenic tobacco with reduced catalase activity did not exhibit constitutive PR-1 gene expression unless there was a concurrent development of necrosis (Chamnongpol *et al.* 1996; Takahashi *et al.* 1997). Looking at these results it seems that H_2O_2 acts upstream of SA in the signal transduction cascade rather than or in addition to, acting downstream of SA. The results may also suggest that the activation of defense responses is mediated through the interaction of SA with other cellular factors such as salicylic acid binding protein 2 (SABP 2) rather than or in addition to catalase and APX (Du and Klessig 1997).

If the predominant mechanism by which SA induces defense responses is not through increased H_2O_2 levels caused by inhibition of catalase and ascorbate peroxidase, then one possibility could be through the generation of SA free radicals (Fig. 2.5). This is a likely product of the interaction of SA with catalase and peroxidases (Durner and Klessig 1996).

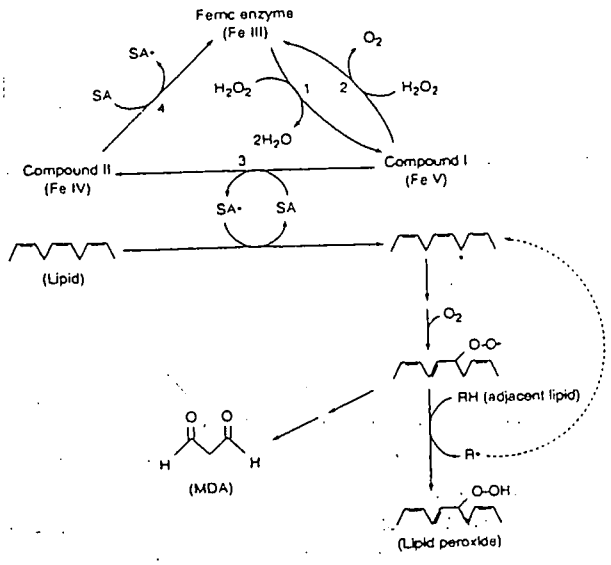


Fig. 2.5 Proposed interaction between catalase and SA (MDH; Malate dehydrogenase, Anderson *et al.* 1998)

Free radicals derived from compounds can induce lipid peroxidation, and the products of this reaction, such as lipid peroxides are potent signaling molecules in animals and possibly in plants as well (Durner and Klessig 1996; Anderson *et al.* 1998).

SA inhibition of catalase is thought to occur via shunting of an oxidized catalase intermediate (compound I) from the rapid cycling catalase reaction, which restores enzyme function into the much slower peroxidative reaction cycle, which results in the trapping of catalase in an inactive and partially reduced state (compound II) (Durner and Klessig 1996). As SA donates an electron to compound I catalase, it is converted to the oxidised form, SA. This SA free radical could initiate lipid peroxidation and may also modify other macromolecules (Savenkova *et al.* 1994). However the SA free radical does not inhibit peroxidases involved in lignin biosynthesis (Hammond-Kosack and Jones 1996).

Catalase is inactivated by its own substrate H_2O_2 (Kirkman *et al.* 1987; DeLuca *et al.* 1995). The rate and amount of inactivation rises with increasing levels of H_2O_2 (Durner and Klessig 1996). At relatively low concentrations of H_2O_2 the addition of SA inhibited catalase activity. When the rate of H_2O_2 generation was increased 10-fold, SA accelerated the inactivation of catalase by H_2O_2. However, as the reaction time increased SA protected the enzyme against further inactivation. Due to different kinetics of catalase inactivation by H_2O_2 and SA different results were obtained with respect to the effects of SA on catalase activity depending on the concentration of H_2O_2 present (Kirkman *et al.* 1987).

Even though SA and SA signal transduction pathways are involved in resistance against many pathogens, in some cases PR gene expression and resistance can be activated in a SA-independent manner. For instance, activation of systemic induced resistance in *Arabidopsis* by root inoculation with biocontrol bacterium *P. fluorescens* is not associated with increases in endogenous SA or PR gene expression (Pieterse 1996). In addition, systemic resistance induced by *P. fluorescens* is manifested equally well in transgenic *Arabidopsis* expressing nahG. In the same way the presence of nahG gene

does not compromise either Cf-2 or Cf-9 gene-mediated resistance to *Cladosporium fulvum* in tomato (Hammond-Kosack and Jones 1996). The role of SA in defense is complex and may also differ from species to species. In rice, no increases in endogenous SA levels occur following pathogen infection, but constitutive SA levels are relatively high (30μg g^{-1} fresh weight) and correlate well with general disease resistance (Silverman *et al.* 1995).

Chapter 3

Materials and Methods

3.1 Materials

3.1.1 Chemicals

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

3.1.2 Plant material

Wheat (*Triticum aestivum* L. cv Tugela, Tugela *Dn 1* [PI137739 (= SA 1684)/5* Tugela], Tugela *Dn 2* [PI262660], and Tugela *Dn 5* [PI294994], Du Toit 1989, 1992) plants were grown in trays under green house conditions and prevailing day\night periods. The temperature was maintained at 22±4°C. When the plants were at the second-leaf growth stage, they were infested with RWA according to Du Toit (1989). The plants were watered daily.

3.2 Methods

3.2.1 Extraction of intercellular wash fluid (IWF)

After different RWA-infestation periods, the leaves were harvested and IWF collected. The leaves were cut into 8 cm pieces, thoroughly rinsed in distilled water and immersed in distilled water, then vacuum infiltrated (3 min). The water-soaked pieces were thoroughly dried on blotting paper and placed vertically in the centrifuge tubes with tips facing

downwards. They were then centrifuged (2000xg) for 15 min at 2°C. The procedure of IWF collection was repeated with the same leaves and the combined extract was stored at -20°C.

3.2.2 Treatment of plants with intercellular wash fluid (IWF)

The second leaves of plants at the beginning of the third-leaf growth stage were injected intercellularly with the IWF (200 µl) of infested susceptible (Tugela) and resistant (Tugela *Dn 1*) plants. The control plants were intercellularly injected with double distilled water (200 µl). The apparatus shown in Fig. 3.1 was used to intercellularly inject the plants. A leaf to be injected was placed across a petri dish (5 cm diameter), and then injected in the middle and the syringe pressed until the fluid had moved the entire distance across the petri dish.

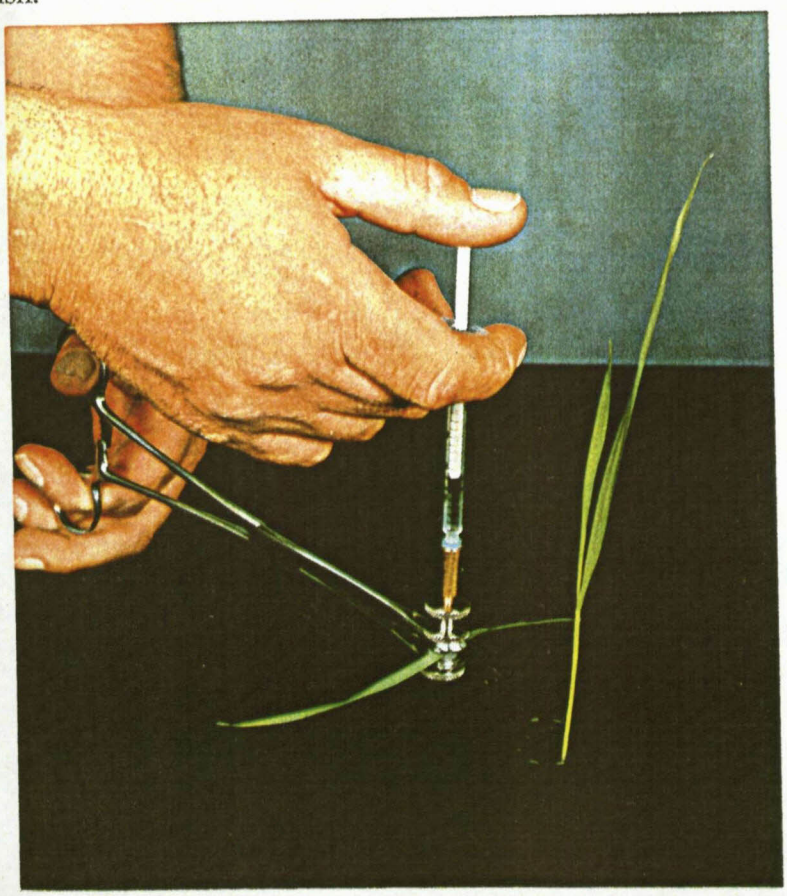


Fig. 3.1 Apparatus used for intercellular injection of plants.

3.2.3 Determination of protein content

The amount of protein in the samples was determined according to the dye-binding assay technique (Bradford 1976). Bovine γ-globulin was used as a standard.

3.2.4 Determination of elicitor activity of IWF

IWF collected from RWA infested susceptible and resistant plants was intercellularly injected into the second leaves of plants at the beginning of the third-leaf growth stage. Double distilled water was injected into the control plants. The injected leaves were harvested 24 and 48 h after injection.

Peroxidase and β-1,3-glucanase activities were determined as described in section 3.2.5.1 and 3.2.5.2.

3.2.5 Determination of enzyme activities

A weighed amount of leaf tissue was ground in liquid nitrogen and homogenized in 100 mM sodium acetate buffer (pH 5.5) containing 10 mM mercaptoethanol, 2 mM ethylene diamine tetra-acetic acid (EDTA) and 2 mM phenylmethylsulfonyl fluoride (PMSF). The ratio of plant material to buffer was 1:5. The extract was centrifuged (12000xg) for 20 min at 2°C and the supernatant was stored at -20°C.

3.2.5.1 Peroxidase activity

The peroxidase activity was determined using a modified method of Zieslin and Ben-Zaken (1991). The assay mixture contained 500 µl of 80 mM potassium phosphate buffer (pH 5.5), containing 2 mM EDTA, 340 µl water, 100 µl 5 mM guaiacol, 50 µl 8.2 mM H_2O_2 and 10 µl enzyme extract. The change in absorption was measured at 470 nm for 180 sec at 30°C.

3.2.5.2 β-1,3-glucanase activity

β-1,3-glucanase activity was assayed using a modified method of Fink *et al* (1988). The assay mixture contained 10 µl enzyme extract, 250 µl laminarin (2 mg ml^{-1} water) and 240 µl 50 mM sodium acetate buffer (pH 4.5). After incubation at 37°C for 10 min, 500 µl of Somogyi reagent [0.4 g $CuSO_4$, 18 g Na_2SO_4, 2.4g Na_2CO_3, 1.6 g $NaHCO_3$ and 1.2 g potassium tartrate in 100 ml distilled water, (Somogyi 1952)] was added. After cooling, 500 µl Nelson's reagent [5.3 g $(NH_4)_6Mo_7O_{24}$, 4.4 ml H_2SO_4 (100%), 0.64 g $Na_2AsO_4.7H_2O$ in 100 ml distilled water] was added and the absorbance measured at 540 nm.

3.2.6 Isolation of elicitor active material from IWF

IWF (50 ml) collected from RWA infested susceptible and resistant plants was freeze-dried and the residue dissolved in 5 ml 70 % (v/v) methanol. An aliquot of this solution was fractionated by reverse phase chromatography on a pre-equilibrated [5 ml 70% (v/v) methanol] C-18 Sep Pak cartridge (Separations Waters). The column was eluted with 10 ml 70% (v/v) methanol and all the eluent collected as 1 ml fractions. The fractions were individually dried *in vacuo* at ambient temperatures and redissolved in 2 ml double distilled water.

Eluent fractions were individually injected intercellularly into the second leaves of both susceptible and resistant plants which were at the beginning of the third-leaf growth stage. After 48 h the injected leaves were harvested and peroxidase activity was determined as described in section 3.2.5.1.

3.2.7 Isolation of glycoproteins

The glycoproteins were isolated from peroxidase inducing fractions recovered during reverse phase chromatography. The procedure was carried out as described by Kogel *et al.* (1992) with slight modifications.

After the concanavalin A (con A) sepharose column (Pharmacia) was equilibrated with 20 ml of 20 mM Tris-HCl buffer (pH 7.4), containing 0.5 M NaCl, 1.0 mM $MgCl_2$, 1.0 mM $MnCl_2$ and 1.0 mM $CaCl_2$, 1 ml of the sample was applied. The eluent collected during application was tested for glycoproteins. The column was firstly eluted with 4 ml of the equilibration buffer and subsequently with 40 ml of 0.1 M glucoside (Sigma) and 0.5 M α-D-methylmannoside (Sigma). Eluents from the glucosides were collected together and the volume reduced to 7.5 ml using a freeze-dryer. The eluents were desalted on PD 10 columns (Pharmacia), equilibrated and eluted with water according to instructions of the manufacturer. All the buffers contained 0.1 % n-butanol (v/v) to prevent microbial growth.

The isolated glycoproteins were injected intercellularly into the second leaves of both the susceptible and the resistant plants at the beginning of the third-leaf growth stage. The injected leaves and the third leaves were harvested after 48 h and peroxidase activity was determined on both the second and the third leaves as described in section 3.2.5.1.

3.2.8 Spot dot assay for concanavalin A (con A) binding glycoproteins

The assay was carried out using a combined method of Hawkes (1982) and Hawkes *et al.* (1982) with some modifications.

An 8x8 mm square grid was drawn on the nitrocellulose membrane. The piece of nitrocellulose membrane with two small squares on it was washed for 5 min in distilled

water by gentle agitation and left at room temperature to dry. A small sample containing 1.5 µg protein was applied on the membrane and an equal volume of double distilled water on the other square as a control. The membrane was incubated at 4°C for 10 min, the individual squares were cut out and washed for 5 min in Tris buffered saline (TBS) [50 mM Tris-HCl (pH 7.4) and 200 mM NaCl]. The squares were then incubated in 3 % (w/v) milk powder in TBS for 15 min. The blocking solution was replaced with freshly prepared con A (50 µg ml) in blocking solution and the membranes incubated for 30 min with agitation, then washed for three consecutive 5 min periods in TBS. A secondary antibody, horseradish peroxidase [Mannheim-Boehringer (GmbH)] (50 µg ml^{-1}) in blocking solution was used to incubate the membrane for another 30 min. The membrane was washed for three consecutive 3 min periods in TBS. Freshly prepared solution of 0.06% (w/v) 4-chloro-1-naphthol and 0.01% (v/v) hydrogen peroxide in TBS was added to stain the membranes. The stained membrane was washed in distilled water, air-dried and stored in the dark.

3.2.9 SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) was carried out according to Laemmli (1970). A 12% separating gel with 6% stacking gel (acrylamide:N,N-methylene-*bis*-acrylamide, 100:1) was used. The polypeptides bands were analyzed using the Coomassie staining procedure. After electrophoresis the gels were placed in staining solution (2-3 h) containing 0.1% (v/v) Comassie blue R-250 in 40 % methanol and 10 % acetic acid (Zehr *et al.* 1989) and destained in 40 % methanol and 10 % acetic acid in water.

3.2.10 Extraction of salicylic acid (SA)

Different wheat cultivars, Tugela, Tugela *Dn 1*, Tugela *Dn 2* and Tugela *Dn 5* were infested with RWA at the second-leaf growth stage and harvested after 4, 8, 12, 24, 48,

72, 96, and 120 h. SA was extracted using the procedure adapted from Tuula *et al.* (1994).

Leaf tissue (0.5 g) was ground in liquid nitrogen and homogenized in 1 ml pre-cooled 80 % (v/v) ethanol. After centrifugation (13 000xg) for 20 min, the pellet was resuspended in 80 % (v/v) ethanol and centrifuged again. The combined supernatant was incubated at -20°C for 60 min, centrifuged (13 000xg, 10 min) and dried under reduced pressure to one tenth of the original volume, then, metaphosphoric acid was added to give a final concentration of 2 % (w/v) in 4 ml water. This extract was then partitioned 3 times with ethyl acetate (v/v) and the top phases pooled and evaporated to dryness in a rotorvapor at 45°C. The remaining bottom phase was adjusted to 1 M with HCl and heated at 80°C in a sealed tube for 60 min. The extract was then partitioned three times with ethyl acetate (v/v). The top phases were pooled and evaporated to dryness in a rotorvapor at 45°C. Extracts from both cycles were combined and redissolved in 3 ml 70 % (v/v) methanol and passed though a C-18 Sep Pak cartridge equilibrated with 5 ml 70 % (v/v) methanol. SA was eluted with 10 ml 70 % (v/v) methanol. This was dried under reduced pressure at 45°C and redissolved in 400 µl of the HPLC mobile phase [water: acetonitrile: phosphoric acid (10 % v/v); 74.5: 24.5: 1].

3.2.11 HPLC analysis of salicylic acid

The extracts were separated on a C-18 reverse phase column (Phenomenex, Separations) with a high performance liquid chromatograph (HPLC) (Spectra Physics (SP)-400) equipped with a UV detector (SP 1000). The mobile phase consisted of water, acetonitrile and 10 % (v/v) phosphoric acid in the ratio, 74.5: 24.5: 1. Salicylic acid was detected at 240 nm. To account for SA recovery, a known concentration of SA (BDH) was run through the column and its peak area determined. The same SA standard was run through C-18 Sep Pak cartridges as described in section 3.2.10 and then run through the C-18 reverse phase column (Phenomenex, Separations) and its peak area determined. The

percentage loss due to experimental procedure was calculated and accounted for in the final calculation of the salicylic acid content in the susceptible and resistant plants.

3.2.12 Catalase activity

Frozen leaf tissue (1 g) was ground in liquid nitrogen and homogenized in 3 ml extraction buffer [50 mM phosphate buffer, pH 7.0, containing 0.1 % (v/v) Triton X-100, 1.0 % (w/v) polyvinylpyrrolidone (PVP) and 0.04 % (w/v) sodium metabisulphite]. The homogenate was centrifuged (15 000xg, 15 min) at 4°C. The supernatant (2.5 ml) was passed through a PD 10 column (Pharmacia) and eluted with 3.5 ml 50 mM phosphate buffer (pH 7.0). Glycerol was added to the eluate to produce a final concentration of 12.5 % (v/v) and stored at -80°C.

The activity of catalase was determined using an oxygen electrode on a Gilson polarograph, model NI (Hansatech) equipped with a recorder (Gilson). All the reactions were carried out at 25°C. The reaction buffer was 50 mM potassium phosphate buffer (pH 7.0) containing 30 % (v/v) H_2O_2. The reaction was began by adding 20 µl enzyme extract to 4 ml reaction buffer in a reaction chamber. The amount of liberated oxygen was recorded on the chart recorder. The activity of catalase was calculated from the slope of the graph.

3.2.13 Effect of applied salicylic acid on peroxidase activity

Sodium salicylate [(univAR) 0.5 mM] was injected intercellularly (using apparatus in Fig. 3.1) into the second leaves of susceptible and resistant plants at the beginning of the third-leaf growth stage. Double distilled water was injected into the control plants. The entire second leaves were harvested after 24, 48, 72 and 96 h and frozen separately. Peroxidase activity was determined in the second leaves as described in section 3.2.5.1.

3.2.14 Effect of applied salicylic acid on catalase activity

Salicylic acid [(0.5 mM), BDH] was injected intercellularly (using apparatus in Fig. 3.1) into the second leaves of susceptible and resistant plants at the beginning of the third-leaf growth stage. Double distilled water was injected into the control plants. The entire second leaves and third leaves were harvested after 3, 12, and 24 h and frozen separately. Catalase activity was determined in both the second and the third leaves as described in section 3.2.12.

3.2.15 Effect of applied hydrogen peroxide on salicylic acid content

Hydrogen peroxide [(300 mM), Leon *et al.* 1995] was injected intercellularly (using apparatus in Fig. 3.1) into the second leaves of susceptible and resistant plants at the beginning of the third-leaf growth stage. Double distilled water was injected into the control plants. The entire second leaves and third leaves were harvested after 4, 8, and 12 h and the amount of SA was determined as in section 3.2.10 and 3.2.11.

Chapter 4

Results

4.1 Eliciting effect of the IWF of RWA infested susceptible (Tugela) and resistant (Tugela *Dn 1*)

The RWA feeds by inserting its stylet into the intercellular spaces until it reaches the phloem. During feeding it injects salivary material which may contain a phytotoxin and it digests the cell walls, the products of which may act as elicitors (Hewitt *et al* 1984). All these products accumulate in the apoplast, hence we investigated the presence of the eliciting potential of the intercellular wash fluids of uninfested and infested susceptible and resistant plants. Peroxidase and β-1,3-glucanase enzyme activities were used as parameters to quantify the eliciting activity. These enzymes were found to be involved in the resistance response against the RWA (van der Westhuizen and Pretorius 1995, 1996; van der Westhuizen *et al.* 1998a,b).

4.1.1 Peroxidase activity

The effect of IWF of infested susceptible and resistant plants on peroxidase activity of the susceptible (Tugela) and resistant (Tugela *Dn 1*) plants also determined. The IWF of infested susceptible plants induced a non-significant increase (1.002-fold) in peroxidase activity of susceptible plants and a higher 50-fold increase in the peroxidase activity of resistant plants. The IWF of infested resistant plants on the other hand differentially induced a higher 42.5-fold increase in peroxidase activity of the susceptible plants and a

massive 87-fold increase in peroxidase activity of the resistant plants (Fig. 4.1). This was observed after 24 h.

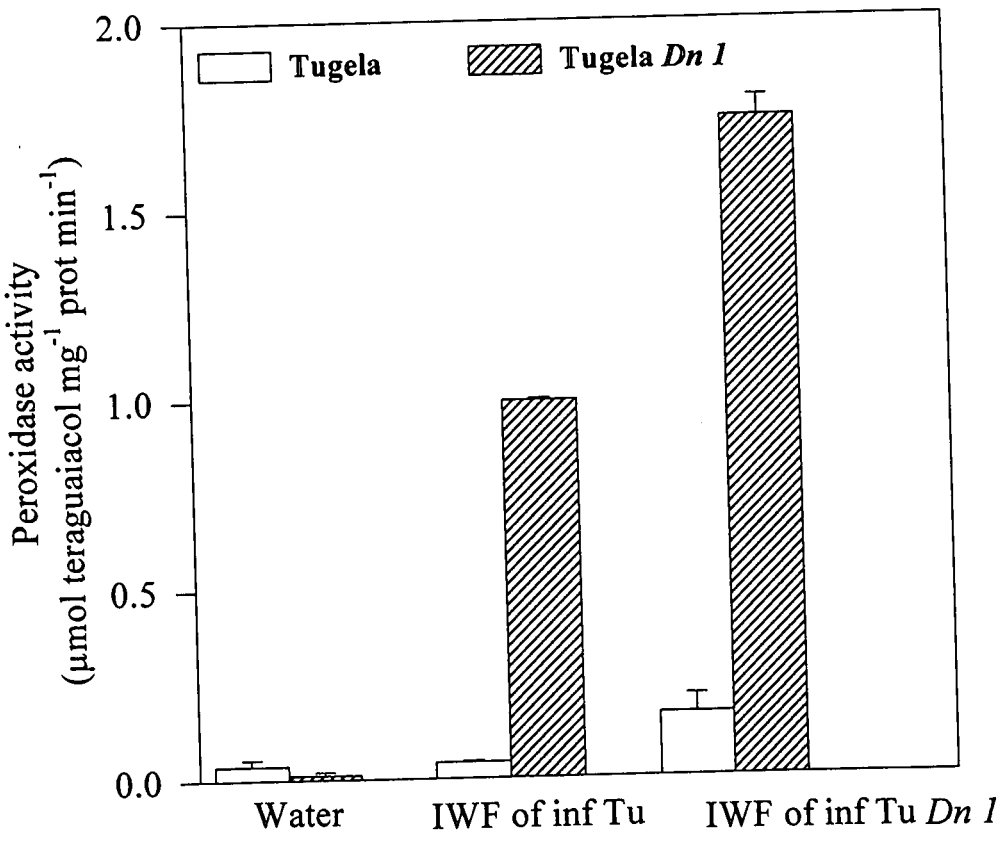


Fig. 4.1 Effect of intercellularly applied IWF of RWA infested susceptible (Tugela, Tu) and resistant (Tugela *Dn 1*, Tu *Dn 1*) plants on the peroxidase activity of susceptible and resistant plants after 24 h of injection. Values are means ± SD (n=3).

The effect of IWF on peroxidase activity was further followed up to 48 h after application. The IWF of infested susceptible plants induced a 10-fold increase in peroxidase activity of

susceptible plants and a 1.5-fold in the resistant plants. The IWF of infested resistant plants induced a 13-fold increase in the susceptible and a 7-fold increase in peroxidase activity in the resistant plants (Fig. 4.2).

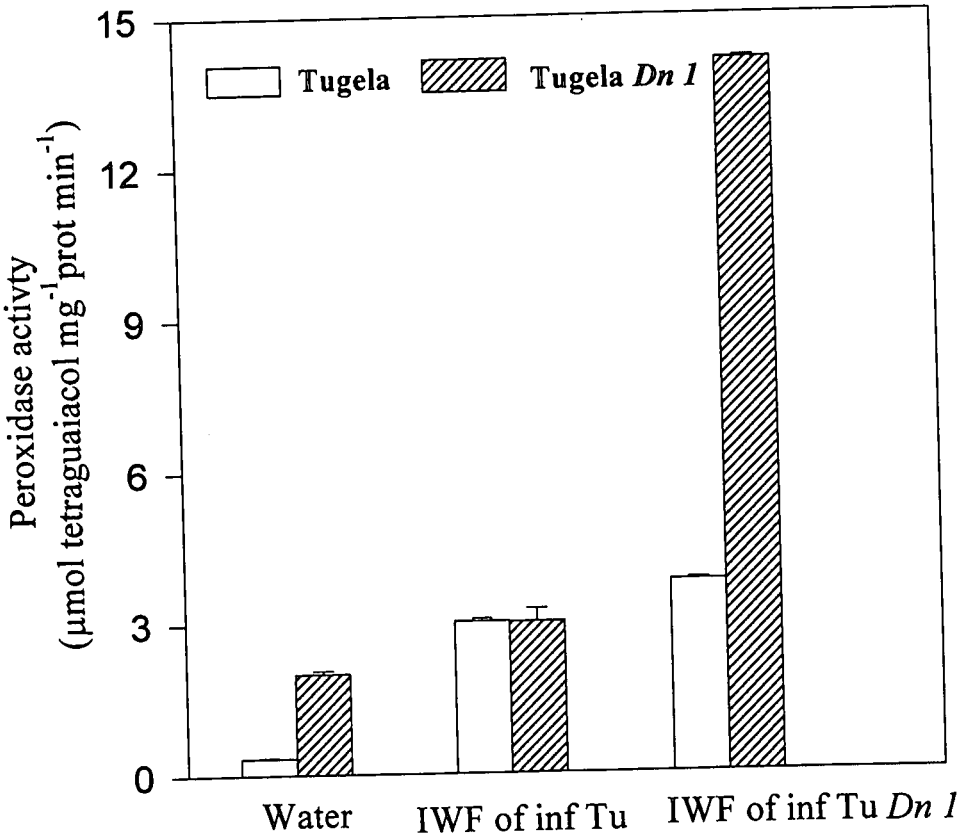


Fig. 4.2 Effect of intercellularly applied IWF of RWA infested susceptible (Tugela, Tu) and resistant (Tugela *Dn 1*, Tu *Dn 1*) plants on the peroxidase activity of susceptible and resistant plants after 48 h of injection. Values are means ± SD (n=3).

4.1.2 β-1,3-glucanase activity

The IWF of infested susceptible (Tugela) plants differentially induced β-1,3-glucanase activity in both the susceptible and the resistant (Tugela *Dn 1*) plants. The IWF of infested susceptible plants induced a 1.6-fold increase in β-1,3-glucanase activity of the susceptible plants and a 1.4-fold increase in the resistant plants. The IWF of infested resistant plants on the other hand, induced a higher 2.4-fold increase in β-1,3-glucanase activity of susceptible plants and a 2.3-fold increase in the resistant plants (Fig. 4.3).

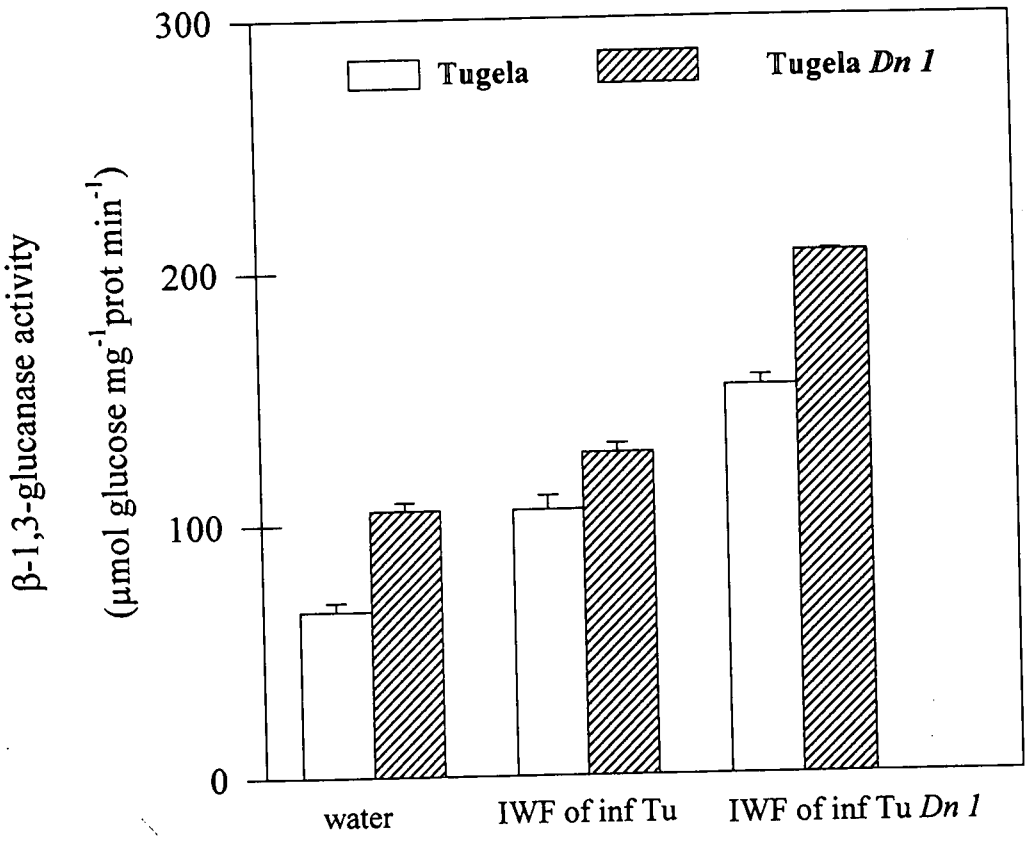


Fig.4.3 The effect of intercellularly applied IWF of RWA infested susceptible (Tugela, Tu) and resistant (Tugela *Dn 1*, Tu *Dn 1*) plants on β-1,3-glucanase activity of susceptible and resistant plants after 24 h of injection. Values are means ± SD (n=3).

The effect of IWF from RWA infested susceptible and resistant plants was also determined after 48 h of application (Fig. 4.4). The IWF of infested susceptible plants induced no significant increase in β-1,3-glucanase activity in susceptible plants and a 1.2-fold increase in resistant plants. The IWF of infested resistant plants on the other hand, showed a higher eliciting effect, inducing a 1.5-fold increase in β-1,3-glucanase activity in the susceptible plants and a 1.7-fold increase in the resistant plants. The induced β-1,3-glucanase was however, quantitatively higher in the resistant plants than in the susceptible ones.

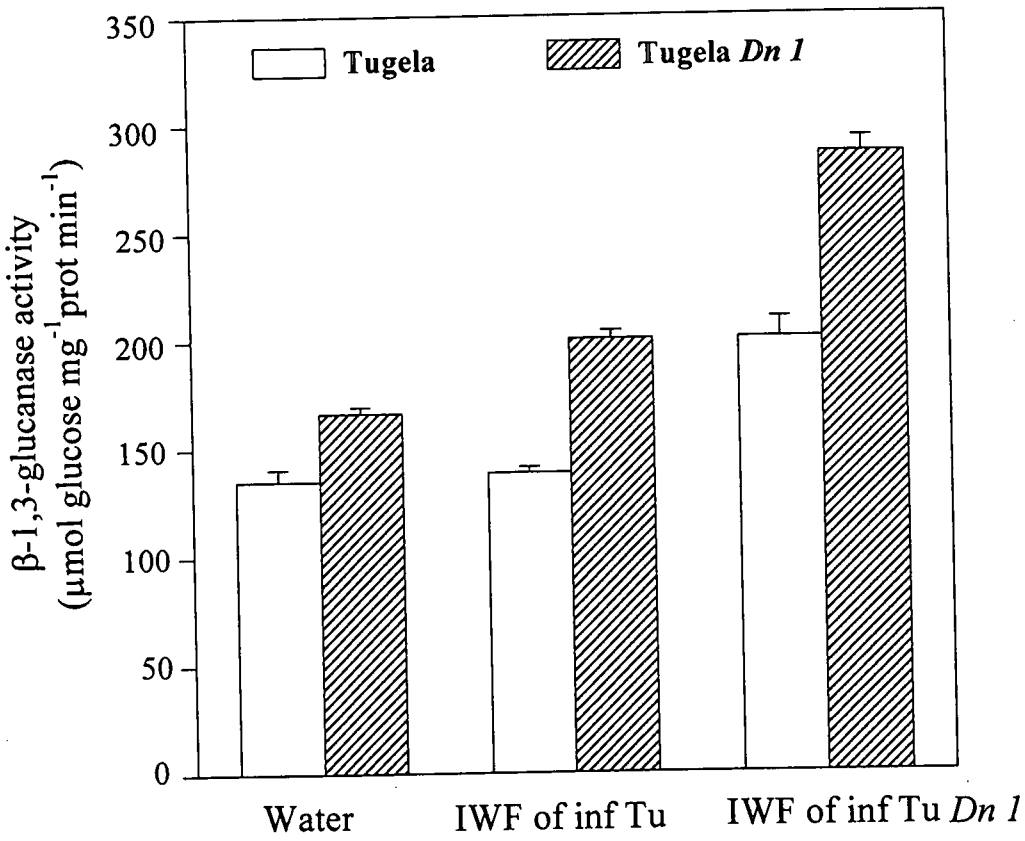


Fig 4.4 The effect of intercellularly applied IWF of RWA infested susceptible (Tugela, Tu) and resistant (Tugela *Dn 1*, Tu *Dn 1*) plants on β-1,3-glucanase activity of susceptible and resistant plants after 48 h of injection. Values are means ± SD (n=3).

4.1.3 Fractionation of the IWF of RWA infested resistant plants

The IWF of infested resistant plants showed a more pronounced eliciting activity on susceptible and resistant plants than IWF of susceptible plants. To isolate the potential elicitor, the IWF of infested resistant plants was fractionated using C-18 reverse-phase chromatography. All the fractions obtained were intercellularly injected into susceptible and resistant plants and peroxidase activity of the plants determined. The first fraction of the C-18 reverse-phase chromatography selectively induced the highest peroxidase activity in the resistant plants (Fig. 4.5).

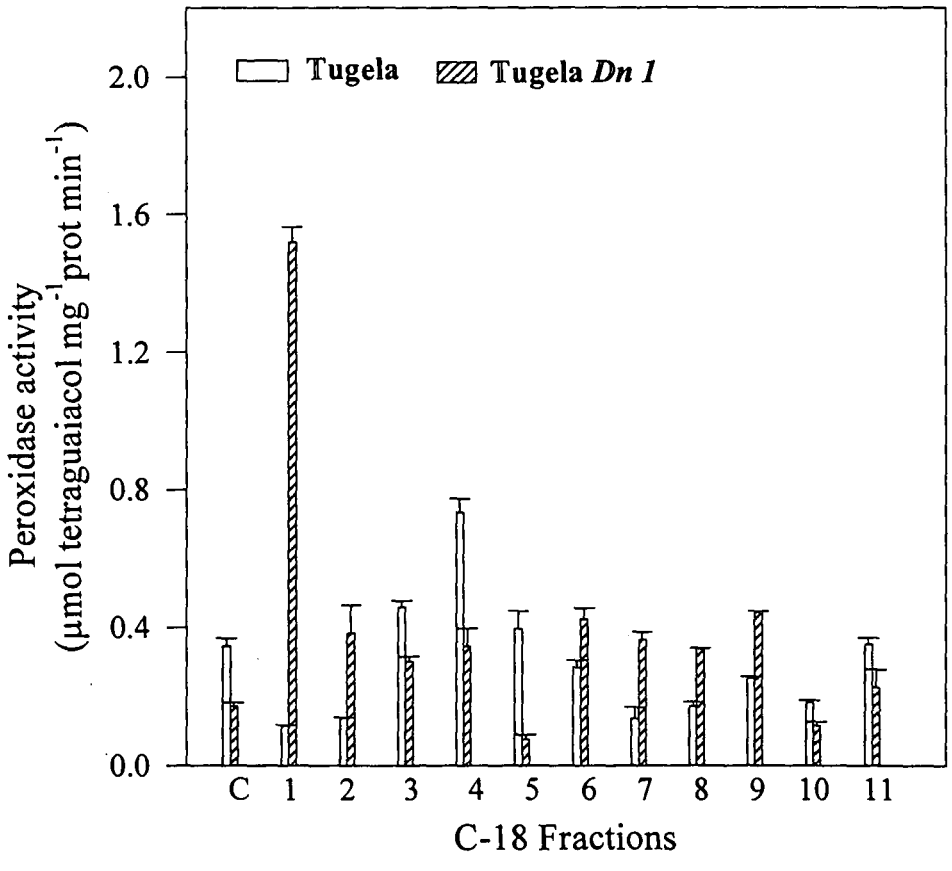


Fig. 4.5 The effect of eluent fractions after C-18 reverse-phase chromatography on the peroxidase activity of the susceptible (Tugela) and resistant (Tugela *Dn 1*) wheat plants. Peroxidase activity was determined 48 h after the fractions were intercellularly injected into the plants. Values are means ± SD (n=3).

4.1.4 Isolation of glycoproteins

The identity of the eliciting fraction (fraction-1) was investigated by a spot-dot assay for glycoproteins. The fraction tested positive for glycoproteins as indicated by the blue dot as opposed to the control which contained only distilled water (solvent) (Fig. 4.6).

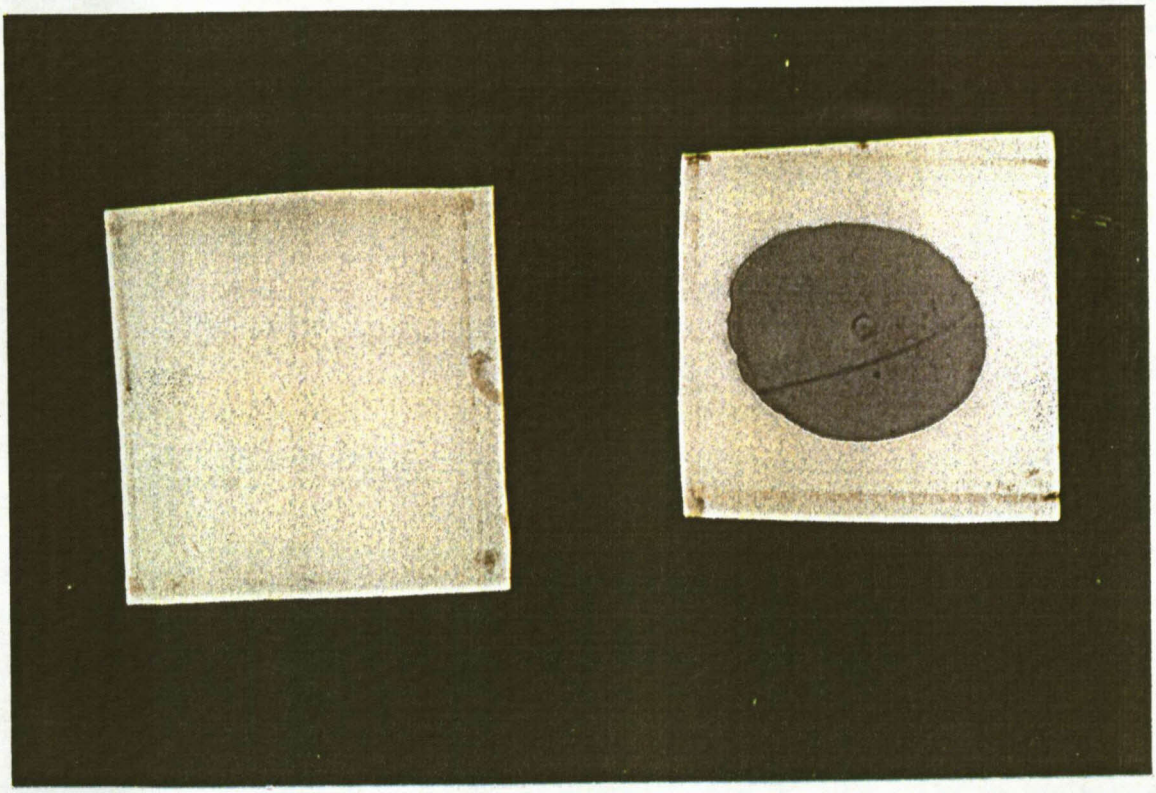


Fig. 4.6 Spot dot assay for glycoproteins after C-18 reverse-phase chromatography of the IWF of infested resistant plants. The control (left) contained solvent (distilled water) and sample (right) contained fraction-1 after C-18 reverse-phase chromatography.

The glycoproteins were isolated from fraction-1 (Figs. 4.5 and 4.6) using concanavalin A (con A) binding chromatography. The glycoproteins were displaced by mannopyranosides and mannoglucosides and again tested positive for glycoproteins (Fig. 4.7).

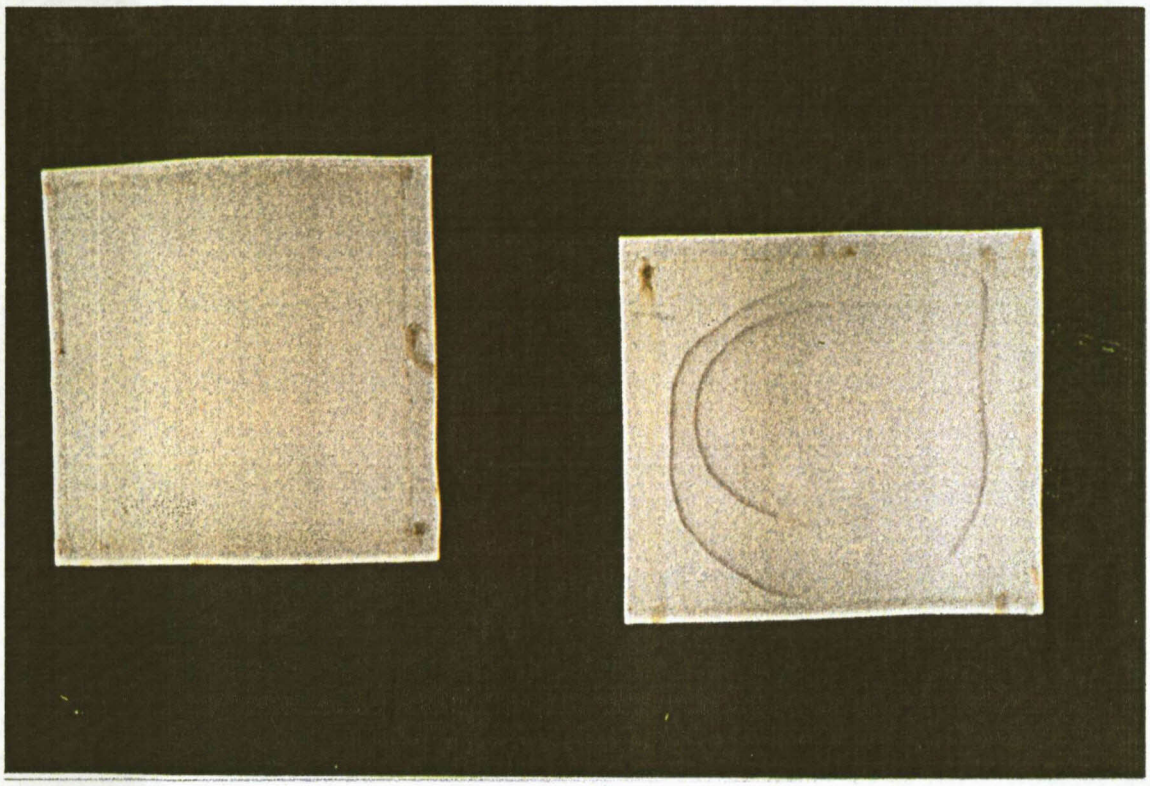


Fig. 4.7 Spot dot assay for glycoproteins after con A binding chromatography. The control (left) contained solvent (distilled water) and the sample (right) isolated glycoproteins.

Polypeptide profiles of the glycoproteins separated by con A binding chromatography revealed three bands. The polypeptide bands were corresponding to molecular weights: 46, 23 and 17 KD (Fig. 4.8).

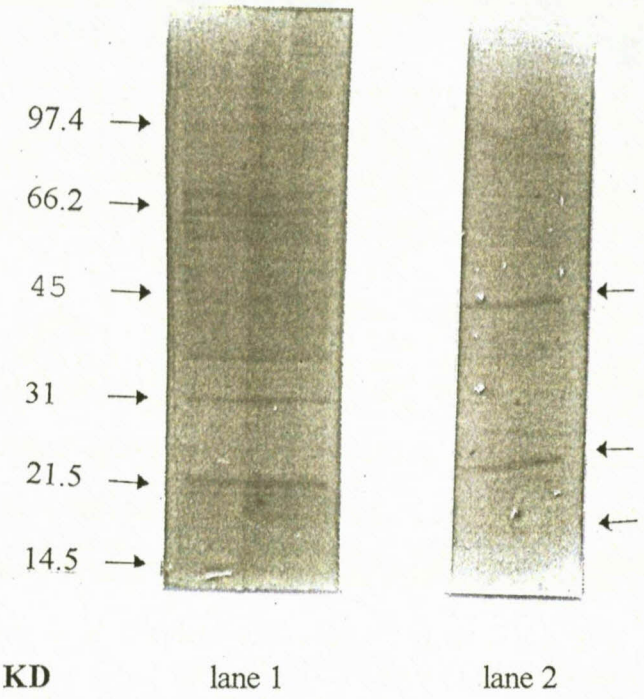


Fig. 4.8 Polypeptide profile of glycoproteins separated by con A binding chromatography, after SDS-PAGE. The same amount of protein (30 µg) was loaded in each lane. Lane 1 contains the standard proteins and lane 2 the isolated glycoproteins.

4.1.5 Eliciting activity of glycoproteins

The enzyme peroxidase was used as a parameter to measure the eliciting activity of the glycoproteins separated from the IWF of infested resistant plants by con A binding chromatography. Intercellular injection of the glycoproteins into the leaves of both susceptible and resistant plants differentially induced quantitatively higher peroxidase

activity in the resistant than in the susceptible plants (Fig. 4.9). The degree of induction was however higher in resistant plants (20-fold) than in susceptible plants (9-fold) when peroxidase activity was expressed in terms of protein content.

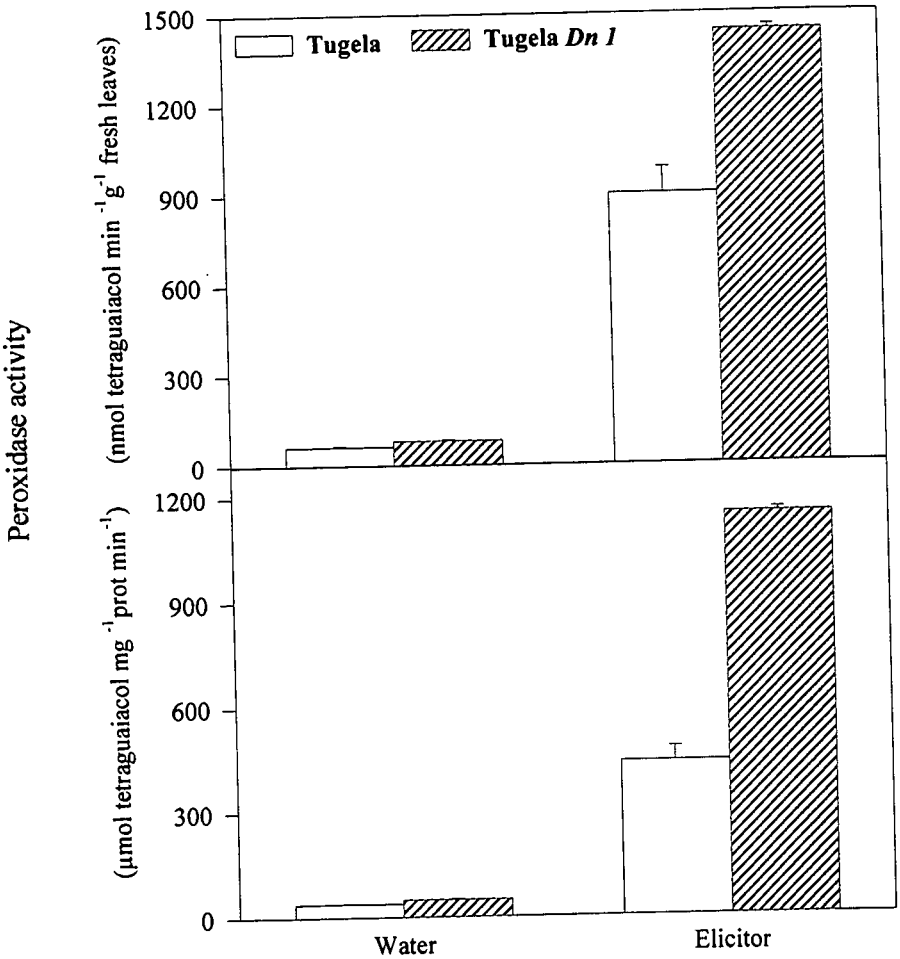


Fig. 4.9 Effect of intercellular glycoproteins (elicitor) isolated by con A binding chromatography on the peroxidase activity of susceptible (Tugela) and resistant (Tugela *Dn 1*) plants. The glycoproteins were intercellularly injected into second leaves and peroxidase activity determined 48 h after injection. Values are means ± SD (n=3).

Peroxidase activity was induced in the third uninjected leaves of wheat plants whose second leaves were injected. There was a 2-fold induction in the resistant plants when peroxidase activity was expressed per fresh mass of leaves, and there was no significant induction in the susceptible plants (Fig. 4.10).

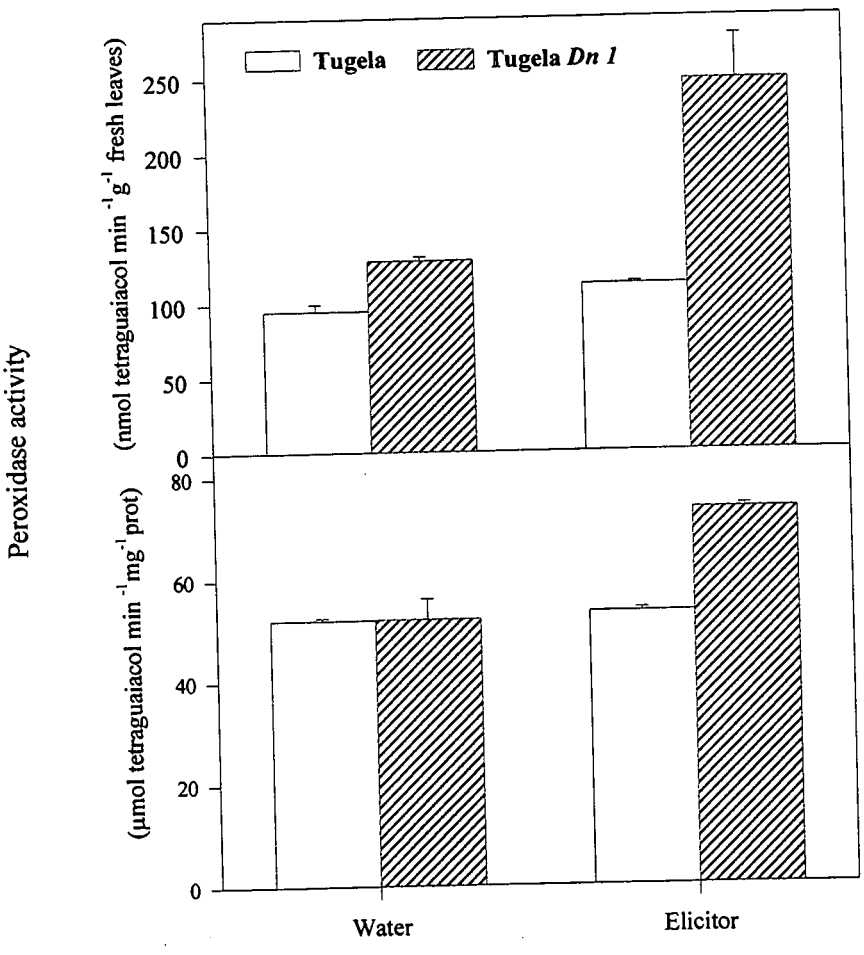


Fig. 4.10 Effect of intercellular glycoproteins (elicitor) isolated by con A binding chromatography on the peroxidase activity of susceptible (Tugela) and resistant (Tugela *Dn 1*) plants. The glycoproteins were intercellularly injected into the second leaves and peroxidase activity was determined on the third uninjected leaves of the same plants 48 h after injection. Values are means ± SD (n=3).

4.1.6 Effect of RWA infestation on salicylic acid (SA) content

RWA infestation differentially induced the accumulation of SA to higher levels in resistant (421-fold) than susceptible (78-fold) plants (Fig. 4.11). The SA content was determined after 7 days of RWA infestation.

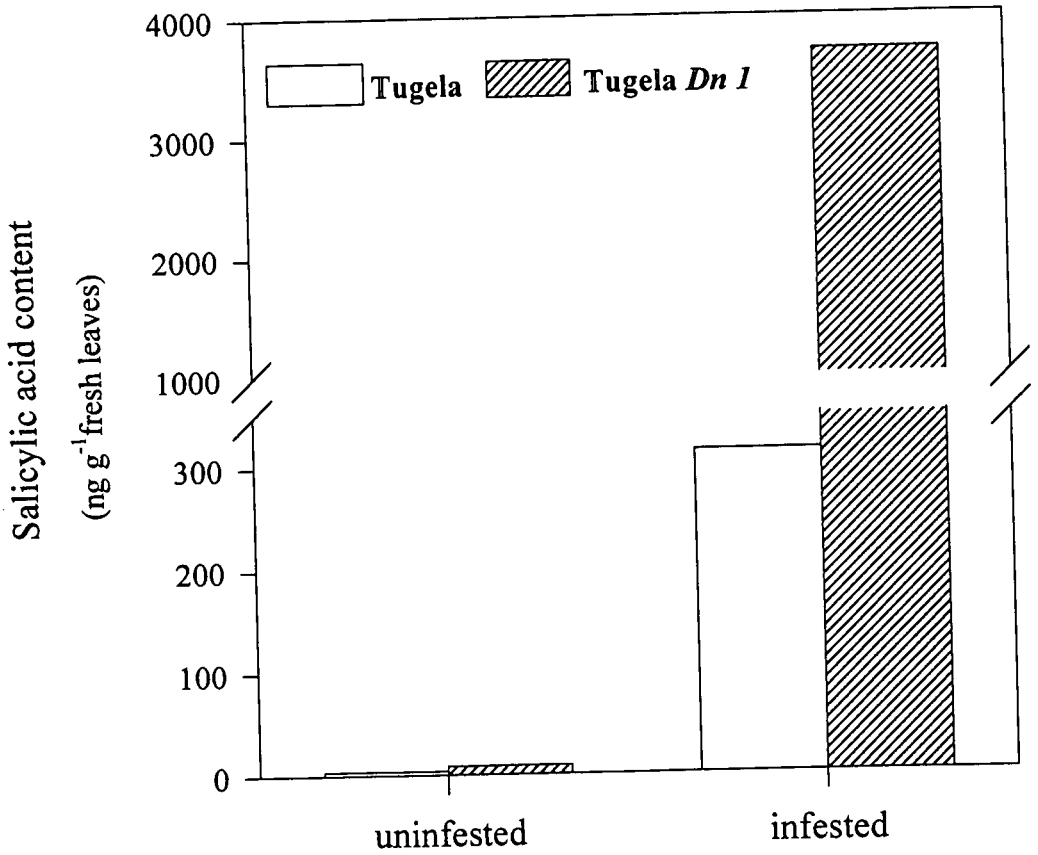


Fig. 4.11 Effect of RWA infestation on the salicylic acid content of susceptible (Tugela) and resistant (Tugela *Dn 1*) plants after 7 days of infestation.

Salicylic acid started to accumulate to higher levels in resistant than susceptible cultivars after 8 h of infestation (Fig. 4.12). The SA level reached a peak after 96 h and thereafter declined. A slight increase was also observed in the susceptible plants .

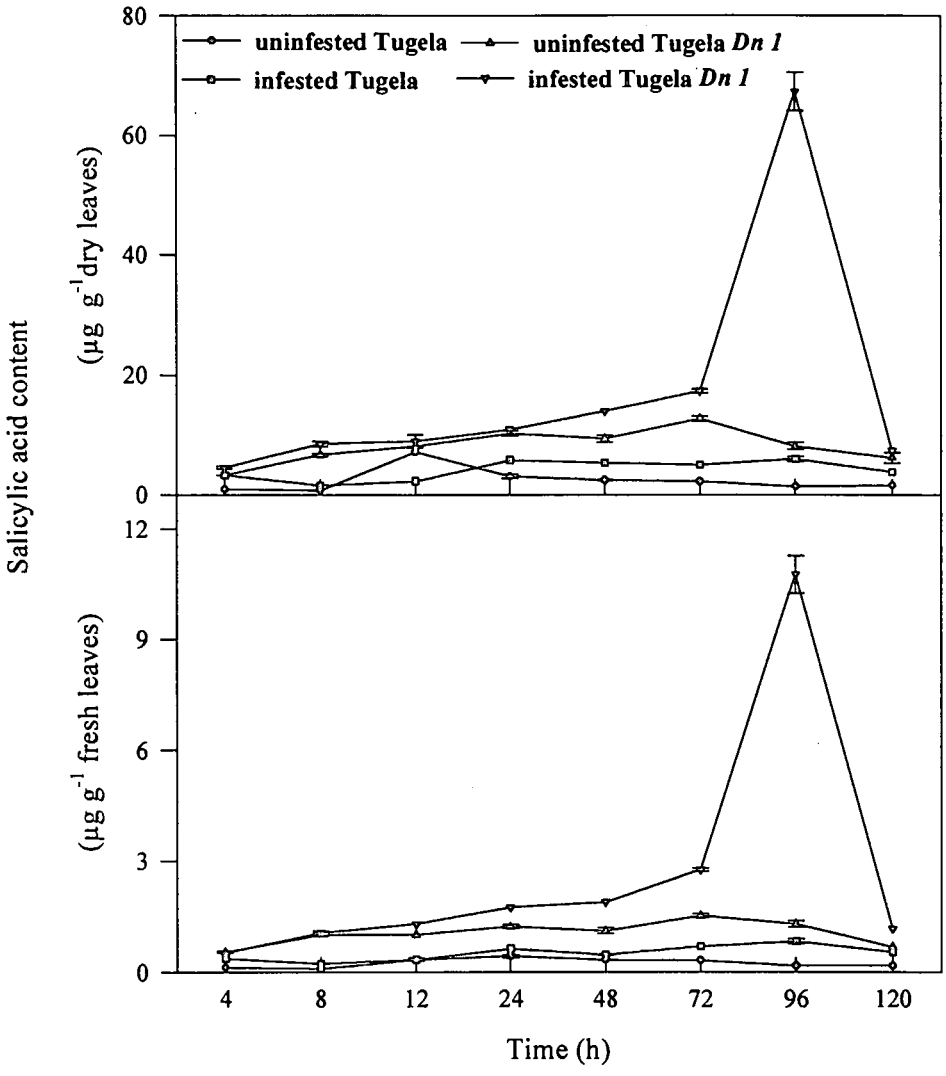


Fig. 4.12 The effect of RWA infestation periods on the SA content of susceptible (Tugela) and resistant (Tugela *Dn 1*) plants. Values are means ± SD (n=3).

RWA infestation selectively induced SA accumulation in the resistant (Tugela *Dn 2*) plants. The selective increase was observed as early as 8 h after infestation. In another resistant wheat cultivar (Tugela *Dn 5*), RWA infestation also induced a selective accumulation of SA content. The selective induction was observed as early as 4 h after infestation and the highest SA content was observed after 72 h (Fig. 4.13), after which

there was a decline in SA content. The RWA induced SA content was much lower in Tugela *Dn 2* than in other resistant wheat lines (Tugela *Dn 1* and Tugela *Dn 5*) (Fig. 4.13).

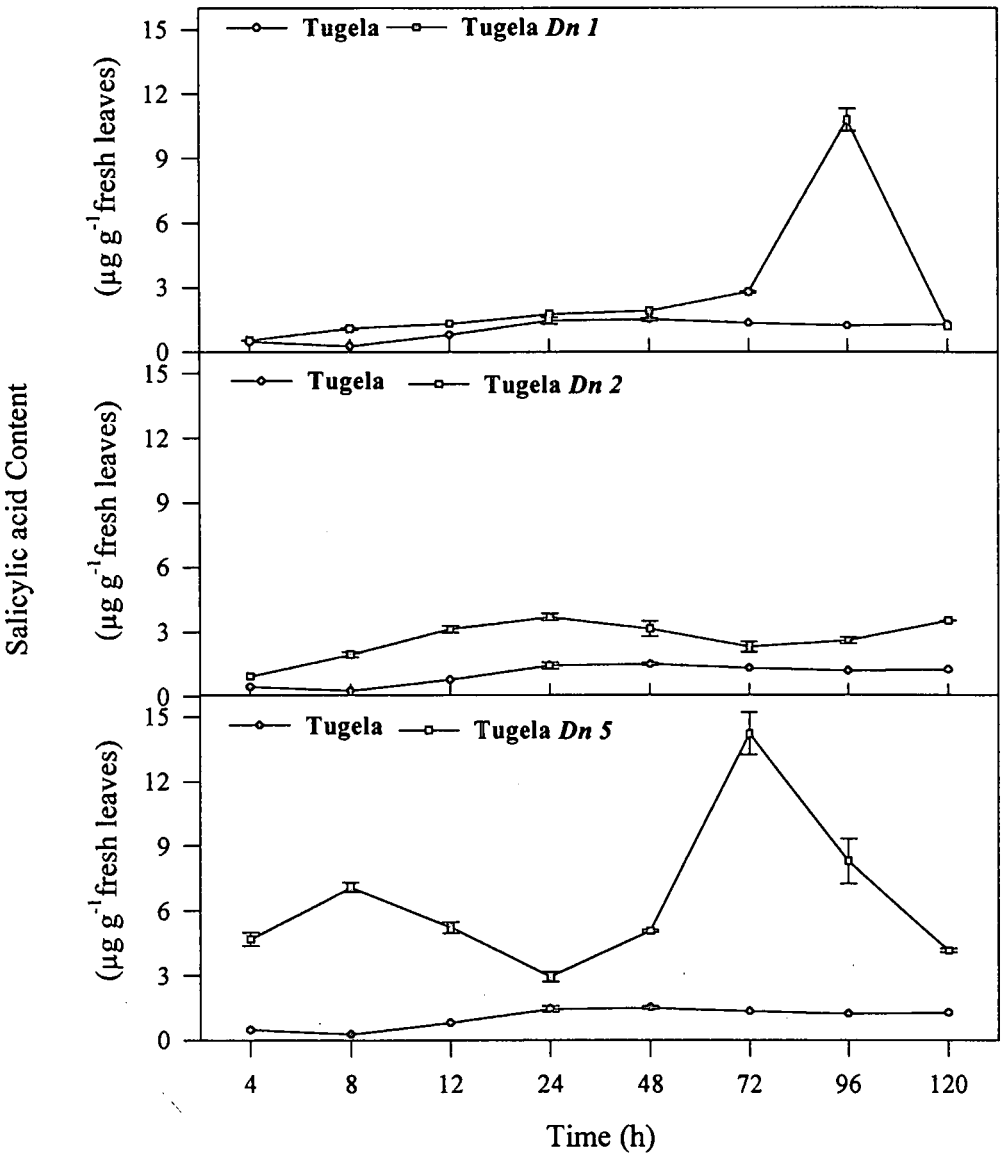


Fig.4.13 Effect of RWA infestation on SA content of resistant wheat cultivars (Tugela *Dn 1*, Tugela *Dn 2* and Tugela *Dn 5*). Values are means ± SD (n=3).

4.1.7 Effect of RWA infestation on peroxidase activity

Peroxidase is one of the enzymes induced as a secondary defense response in RWA/wheat interaction (van der Westhuizen and Pretorius 1996; van der Westhuizen *et al.* 1998b). To ascertain any correlation between SA accumulation and peroxidase activity, SA content during infestation was determined concurrently with peroxidase activity. Our data depicted in Fig 4.14, show an increase in peroxidase activity correlating well with the accumulation of SA. Over the entire 120 h study period RWA infestation differentially induced higher peroxidase activity in the resistant (Tugela *Dn 1*) than susceptible (Tugela) plants. RWA induced SA accumulation as early as 8h and an increase in peroxidase activity was also as early as 8 h (Fig. 4.14).

4.1.8 Effect of RWA infestation on catalase activity

Catalase activity tended to decrease after 48 h in uninfested resistant and susceptible plants, but RWA infestation induced a sharper decrease in catalase activity which was more pronounced in infested resistant plants. After 96 h of infestation catalase activity decreased in infested resistant plants to a level 2.6 times lower than in the infested susceptible plants (Fig. 4. 15).

4.1.9 Effect of exogenously applied SA

a) Catalase activity

The relationship between SA and catalase was investigated by determining the effect of intercellular application of SA. Twelve hours after intercellular injection a significant decrease in catalase activity was observed in the resistant plants. Contrary to this, there was no decrease in catalase activity observed in the susceptible plants (Fig. 4.16).

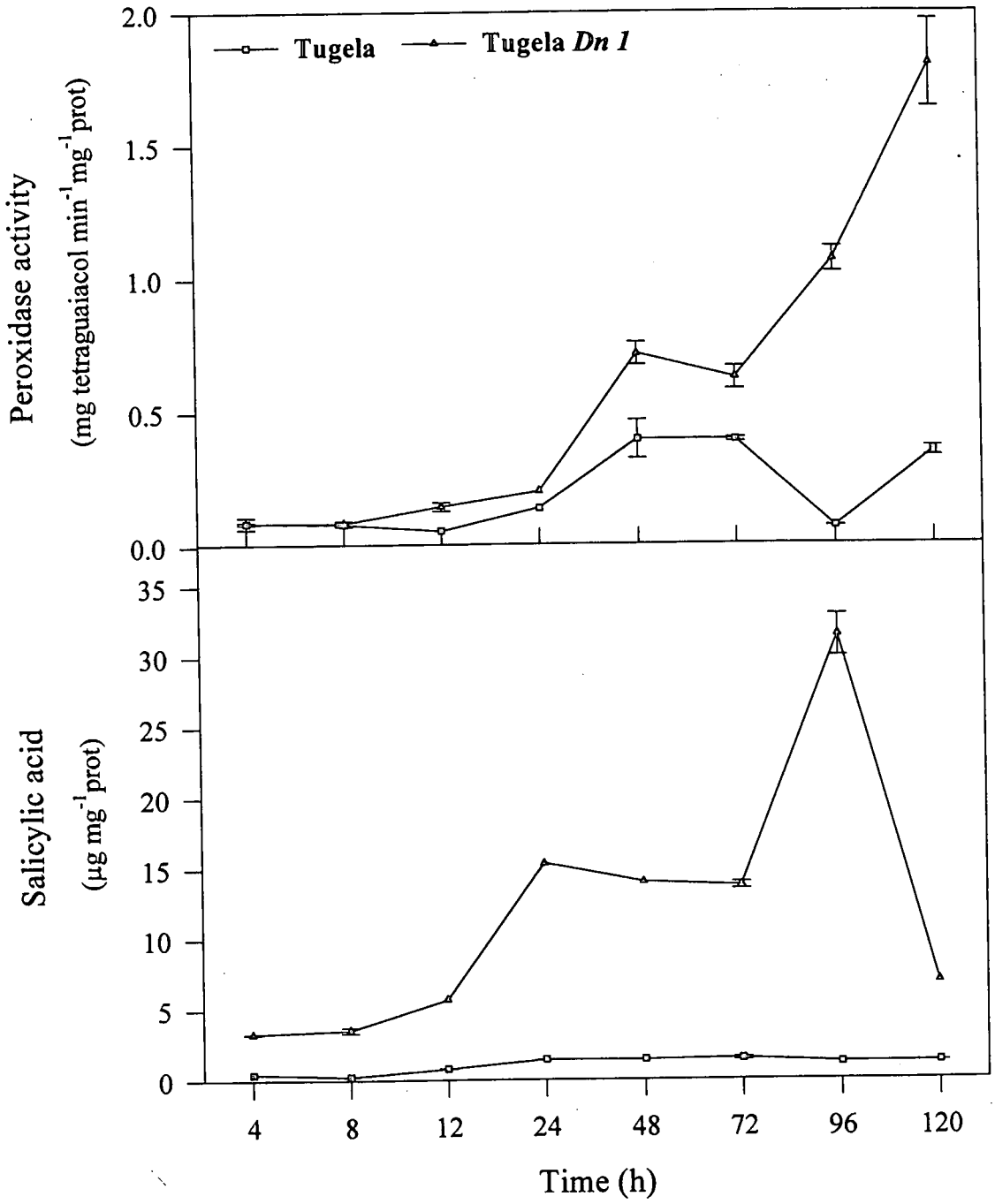


Fig. 4.14 Effect of RWA infestation on peroxidase activity and SA content of susceptible (Tugela) and resistant (Tugela *Dn 1*) plants. Values are means ± SD (n=3).

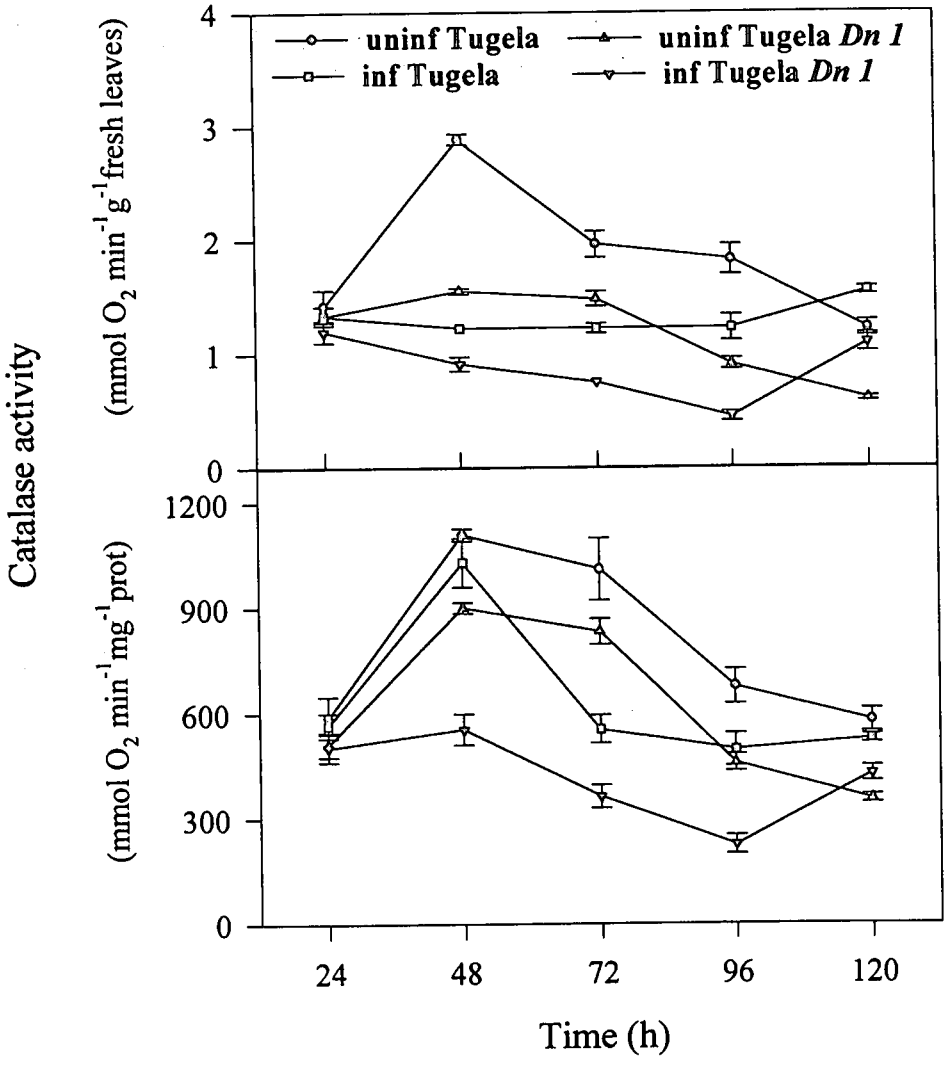


Fig. 4.15 Effect of RWA infestation on catalase activity of susceptible (Tugela) and resistant (Tugela *Dn 1*) plants. Values are means ± SD (n=3).

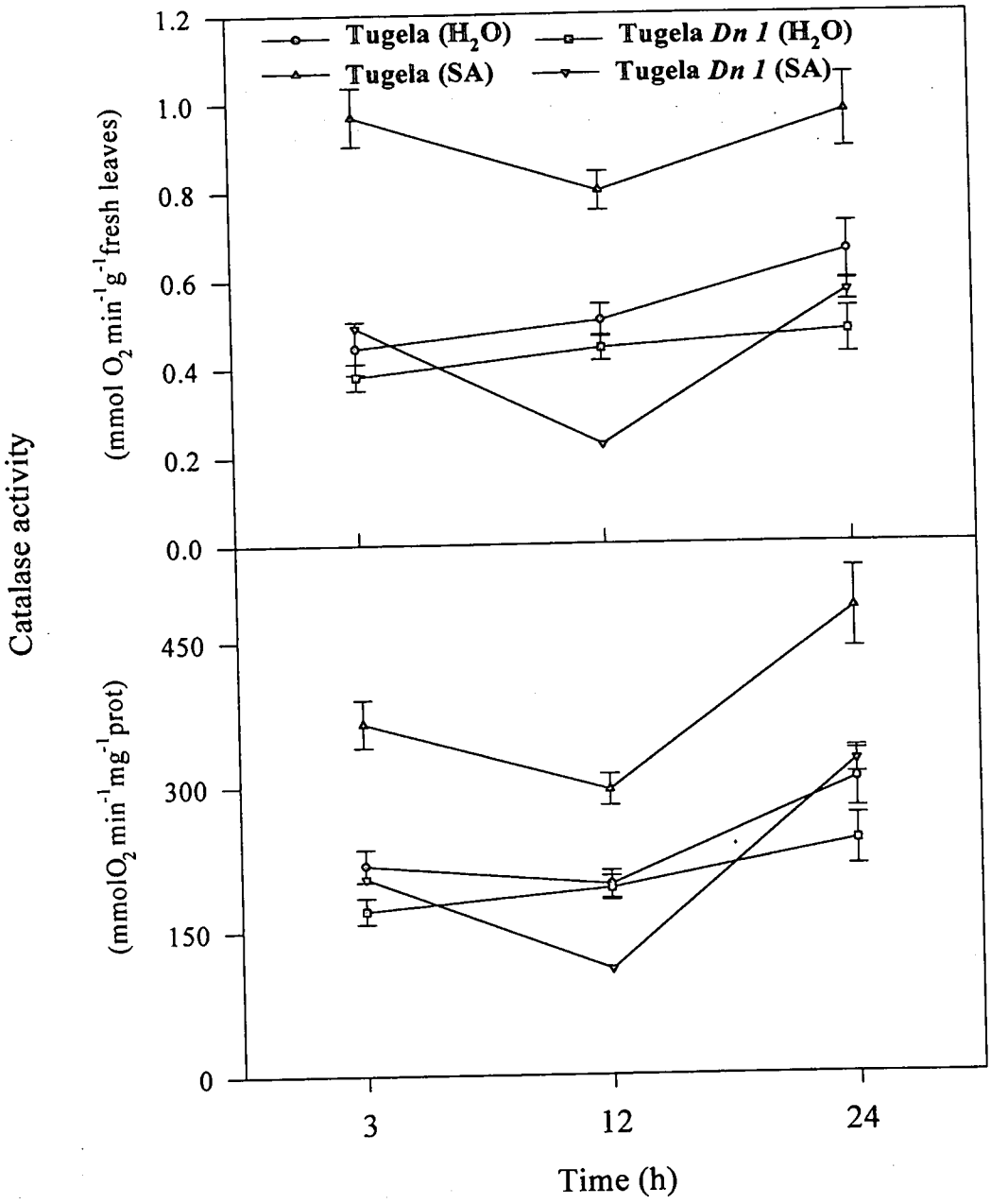


Fig. 4.16 Effect of intercellularly applied SA on catalase activity of susceptible (Tugela) and resistant (Tugela *Dn 1*) plants. Values are means ± SD (n=3).

In the resistant plants about 47% catalase inactivation was observed after 12 h of SA application. Extending the study period to 24 h, a totally different scenario was observed.

Catalase activity was no longer inactivated in both the susceptible and the resistant plants. In the distal third leaves which were not injected, no catalase inactivation was observed throughout the 24 h study period (Fig 4.17).

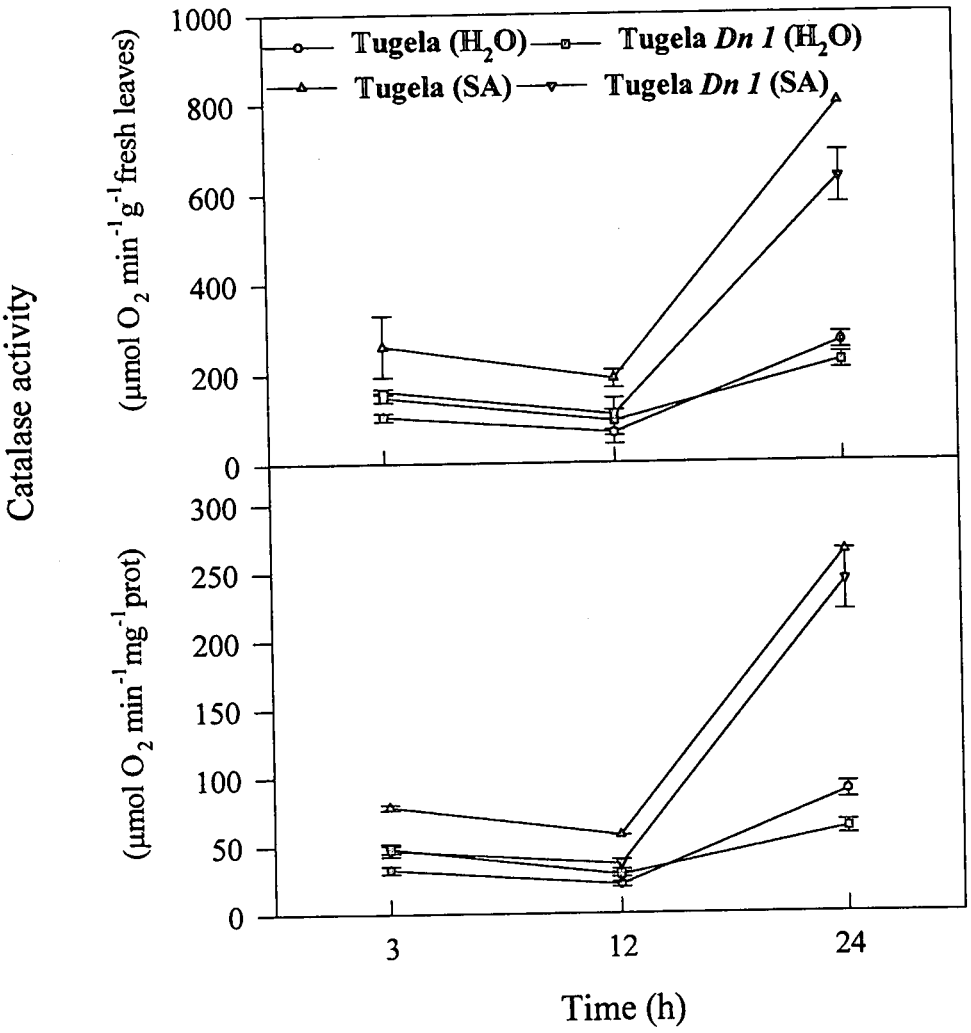


Fig. 4.17 Effect of intercellularly applied SA in the second leaves, on catalase activity of the third (untreated) leaves of susceptible (Tugela) and resistant (Tugela *Dn 1*) plants. Values are means ± SD (n=3).

b) Peroxidase activity

The effect of exogenously applied SA on the peroxidase activity of susceptible plants was observed 72 h after treatment, when a slight induction of peroxidase activity (1.2-fold) was observed. A much higher peroxidase induction was observed in the resistant plants (1.8-fold) 72 h after SA injection (Fig. 4.18). After 96 h of SA application the same degree of peroxidase induction (1.2-fold) was observed in the susceptible plants. The induction in the resistant plants had slightly increased (2.2-fold).

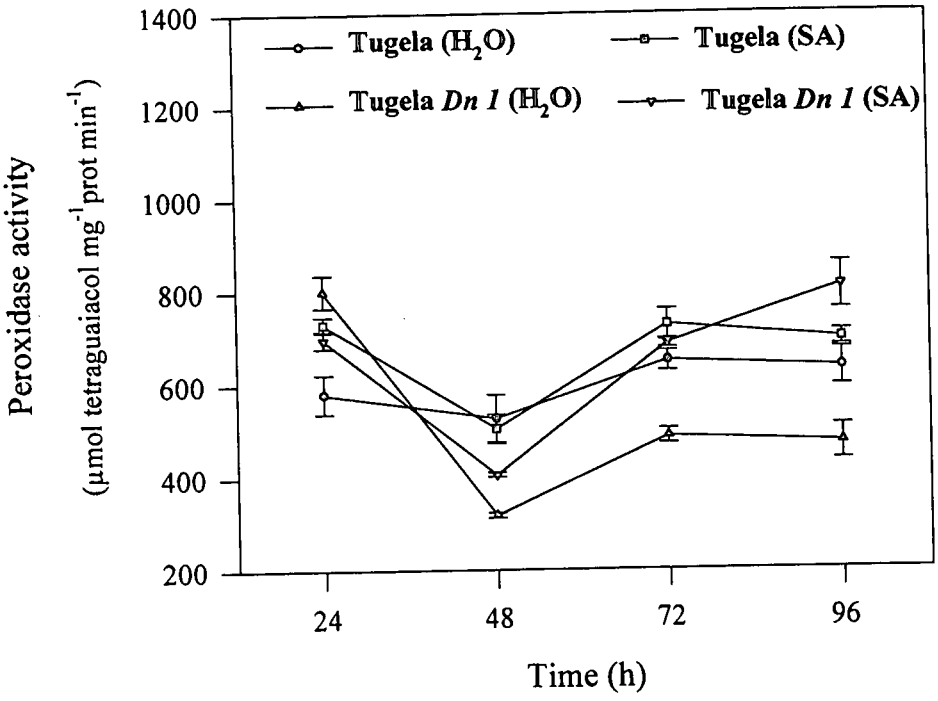


Fig. 4.18 Effect of exogenously applied SA on the second leaves, on peroxidase activity of the second leaves of susceptible (Tugela) and resistant (Tugela *Dn 1*) plants. Values are means ± SD (n=3).

SA, exogenously injected into the second leaves, slightly induced peroxidase activity in the untreated leaves of susceptible plants (1.3-fold) 72 h after SA treatment. A higher induction (2-fold) was observed after 72 h in the resistant plants (Fig.4.19).

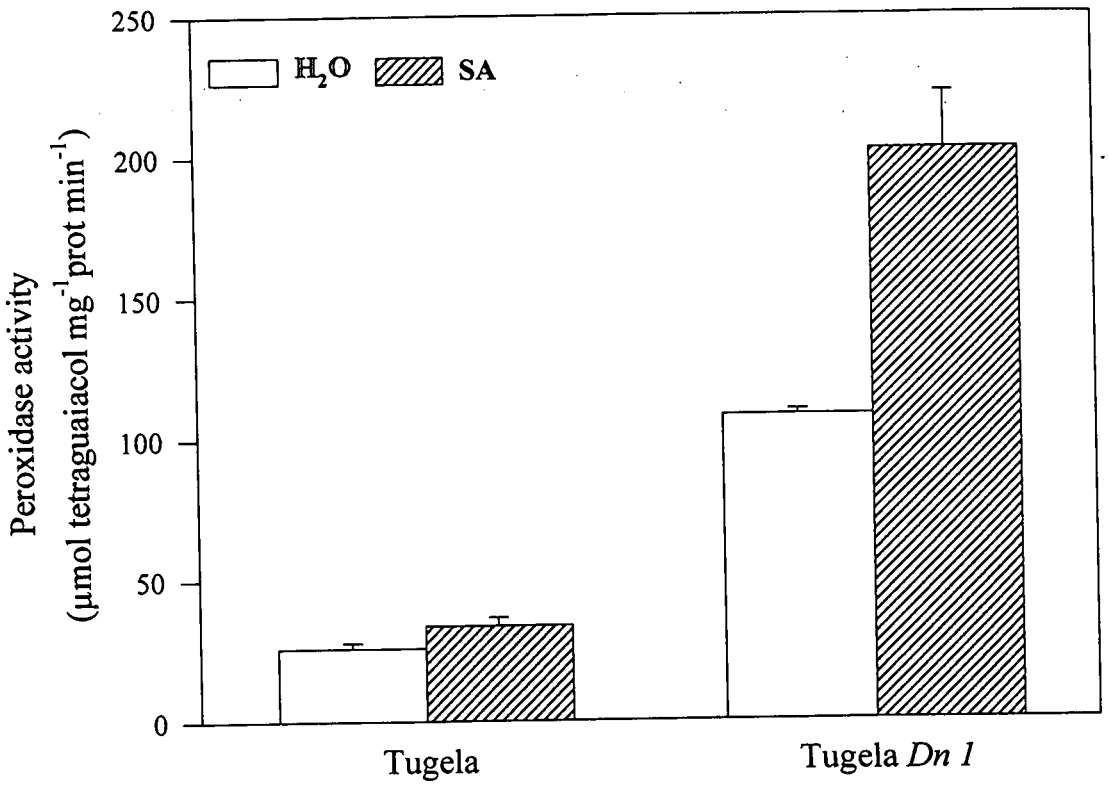


Fig. 4.19 Effect of exogenously applied SA in the second leaves, on the peroxidase activity of the third (uninjected) leaves of susceptible (Tugela) and resistant (Tugela *Dn 1*) plants after 72 h. Values are means ± SD (n=3).

4.1.10 Effect of exogenously applied H_2O_2 on the *in vivo* SA content

Hydrogen peroxide intercellularly injected into the leaves of susceptible and resistant plants, selectively induced SA accumulation in the resistant plants. Within 4 h of injection a significant increase in SA content was observed in the resistant plants (Fig. 4.20). Extending the study period further to 12 h did not result in any increase in SA content, in fact, a decrease in SA content was observed.

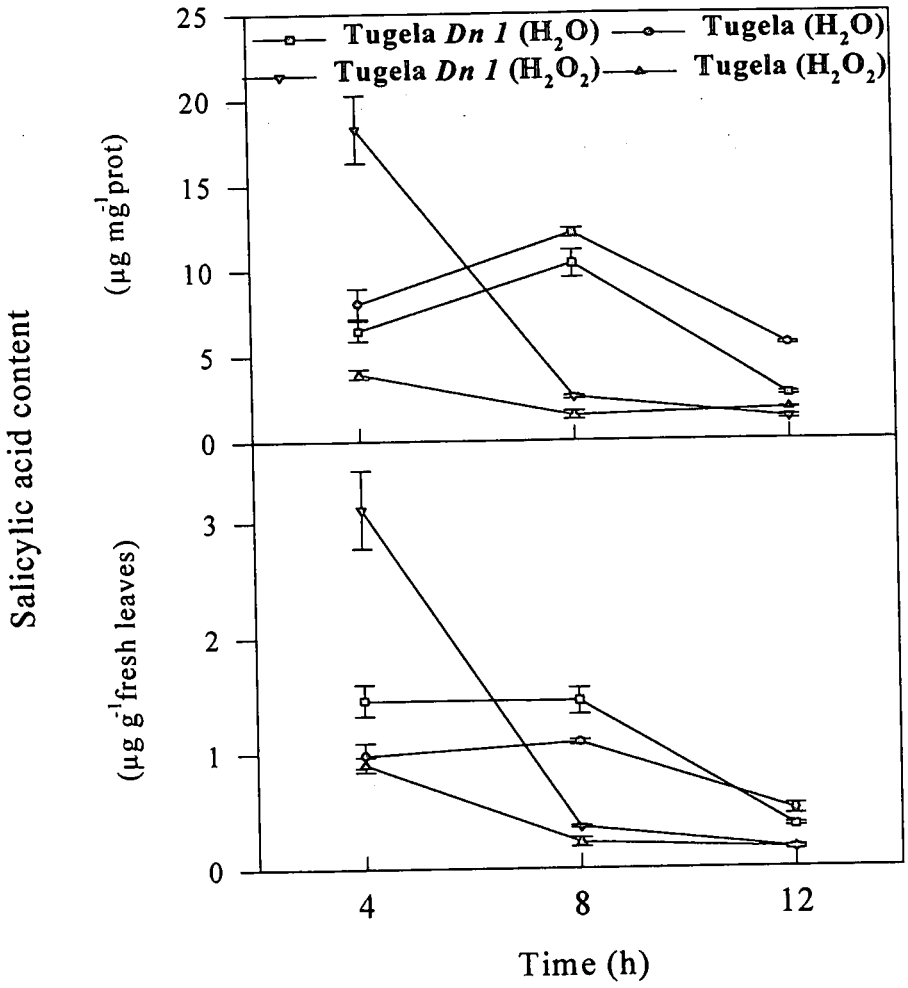


Fig. 4.20 Effect of exogenously applied H_2O_2 on the SA content of susceptible (Tugela) and resistant (Tugela *Dn 1*) plants. Values are means ± SD (n=3).

Chapter 5

Discussion

There are marked differences in the induction of defense responses in the resistant and susceptible plants associated with RWA infestation. There is considerable interest in the eliciting and signaling pathway(s) involved in the induction of these defense responses, alternatively referred to as downstream defense responses. Necessary biotechnological manipulation of plants for crop resistance will likely come from stepwise biochemical analysis of the pathways, either upstream from eliciting and signaling or downstream from the activation of defense related genes.

The RWA feeds by inserting its stylet into the apoplast and probes until it reaches the phloem. The local events in the immediate zone of the RWA stylet entry trigger systemic events. The apoplast is the site from which signals from local events are transferred to the whole plant to elicit defense responses (Ouchi and Oku 1981). In addition, the apoplast is also the site where many defense related products accumulate (Bowles 1990). As the aphids probe intercellularly they may spill out a salivary phytotoxin. This phytotoxin has been reported by Brigham (1992) and identified as a polypeptide. On the other hand, the aphids may digest the cell walls as they probe the intercellular spaces and the cell wall fragments may act as elicitors (Collmer and Keen 1986). These products accumulate in the apoplast and the eliciting activity may occur in the intercellular wash fluids (IWF) of infested susceptible and resistant plants.

Peroxidases are involved in several defense-related events that occur in the extracellular matrix (Bowles 1990). In addition, van der Westhuizen *et al.* (1998a,b) found that RWA infestation induced peroxidase and β-1,3-glucanase activities in resistant plants. The expression of peroxidase activity was therefore mainly used as a measure of the defense response. Since the induction of peroxidase activity could be only one aspect of the

defense responses, β-1,3-glucanase activity was also used to measure the defense response.

The eliciting activity found in the IWF of infested resistant plants differentially induced the downstream defense related peroxidase and β-1,3-glucanase activities to quantitatively higher levels in resistant than susceptible plants (Figs. 4.1, 4.2, 4.3 and 4.4). The detection of the elicitor activity in the IWF of infested resistant plants implied that the source may either be the aphid or the aphid/host interaction. In fact, the higher enzyme activities induced by IWF of infested resistant plants implied that the source of the elicitor is from the interaction of the aphid with the resistant host cultivar.

The resistant (Tugela *Dn 1*) plants contain the *Dn 1* resistance gene whereas the susceptible (Tugela) line does not contain this particular resistance gene. The differential induction of peroxidase and β-1,3-glucanase activities to higher levels in the resistant plants may be due to the presence of the *Dn 1* resistance gene in the resistant plants. In the resistant plants, RWA infestation selectively induced peroxidase and β-1,3-glucanase activities to higher levels in the resistant plants (van der Westhuizen *et al* 1998a,b). According to Gotthardt and Grambow (1992), the pgt-elicitor treatment induced a significant increase in peroxidase activity in the suspension culture of a resistant wheat line while the susceptible culture was not affected. The observed induction of peroxidase and β-1,3-glucanase activities in the susceptible plants, even though to quantitatively lower levels, may be an indication that the susceptible plants lack some component, either a receptor to bind the elicitor or may contain suppressors of the elicitor activity.

However an elicitor without any gene-specificity was reported by Kogel *et al.* (1988). The glycoprotein elicitor isolated from germ tube walls of *Puccinia graminis* f. sp. *tritici* induced a non-cultivar specific induction of PAL activity in wheat. The isolated elicitor from *Pyricularia oryzae* also induced defense reactions in both susceptible and resistant rice cultivars. This shows that it lacked the race specificity which is common for gene-for-gene interactions (Scheinpflug *et al.* 1995).

Furthermore, Deverall and Deakin (1985) found a chemically undefined eliciting activity in intercellular fluids of *P. recondita* infected wheat cv. Chinese Spring. This activity could not be correlated with the presence of the Lr 20 gene for resistance and they concluded that the intercellular fluids were neither rust-strain nor gene-specific in their eliciting activity. In addition, an elicitor isolated from mycelial cell walls of the soybean fungal pathogen *Phytophthora megasperma* f. sp. *glucinea* does not appear to be confined to specific races of the host, and hence may be involved in non-host resistance although race-specificity might reside in the release of the active fragments from mycelial walls or their subsequent breakdown products rather than in the structure of the elicitor intrinsically (Lamb *et al.* 1992). In the RWA/wheat interaction the elicitor may be an inherent product of the interaction between the aphid and the resistant (Tugela *Dn 1*) plants. The non-specific elicitor is usually involved in basic resistance and the binding of an elicitor to a putative receptor in the plasma membrane has been proposed to mediate resistance responses (Albersheim and Anderson-Prouty, 1975; Keen, 1975).

Nevertheless, there are also elicitors which are absolutely gene specific. The race-specific elicitors induce responses only in the cultivars of the host species on which the pathogen race is avirulent. They have been implicated to represent direct or indirect products of avirulence genes (Keen 1990). This has been verified in some instances. However, most elicitors are not race specific (Dangl 1995). The ability of the IWF of the resistant plants to induce the defense responses in both the resistant and the susceptible cultivars made it impossible to categorize it as cultivar specific or non-specific. However, the results indicate that it might be non-cultivar specific in its action

The isolated elicitor also induced defense responses in the leaves which were not treated with the elicitor (Fig. 4.10). This was an indication that the elicitor was also able to induce the defense responses systemically. The elicitor was inducing a similar defense response as the RWA on the uninfested wheat leaves of the infested plant. This was previously shown by the induction of downstream defense related enzymes (β-1,3-glucanase,

chitinase and peroxidase) on the third leaves, when RWA feeding was confined to the second leaves (van der Westhuizen *et al* 1998a,b). Systemic responses have also been implicated during wounding, fungal or bacterial infections and infestation of roots with cysts of nematodes (Bowles 1990).

The susceptibility of the Tugela plants during RWA infestation may be due to failure of the aphid\wheat interaction to produce an eliciting molecule. The ability of the elicitor to induce defense responses, though to a quantitatively lower level in susceptible plants, showed that under appropriate conditions it might be possible to mediate resistance to genetically susceptible host cultivars, for instance by application of an elicitor. This has been reported by Scheinpflug *et al.* (1995) in susceptible rice cultivars. However, the lower response might be due to their inability to recognize the elicitor and as a result a cause of their susceptibility

The presence of the eliciting activity in the IWF of infested resistant plants showed that the elicitor is a water soluble molecule since, the apoplast was infiltrated with water before extraction. The step-wise C-18 reverse phase concanavalin A (con A) binding chromatography also showed the elicitor to be water soluble glycoprotein. The isolated elicitor was not purified to homogeneity since it still contained more than one polypeptide band (Fig. 4.8). Since con A has binding specificity for D-mannopyranose and glucopyranose residues and the elicitor co-eluted with these residues, the lectin-binding glycoprotein may have either mannose or glucose residues as its carbohydrate moiety. The role of lectin-binding glycoproteins as inducers of non-host resistance in cereals was also reported by Kogel *et al.* (1988). Whether the elicitor activity was confined to the carbohydrate or the protein part of the glycoprotein still remains to be investigated. The isolated elicitor also needs further purification steps to homogeneity before it can be labeled as a natural elicitor of defense responses in the RWA/wheat interaction.

The lectin-binding glycoproteins might not be the only elicitors in the IWF of the RWA infested resistant plants. Glucans and mannose-containing polysaccharides, in addition to

glycoproteins, have been reported to arise from *Colletotrichum lagenarium* (Hamdan and Dixon 1987; Tepper and Anderson 1986). Glucans, glycoproteins and arachidonic acid have also been reported as elicitors originating from *Phytophthora infestans* (Bloch *et al.* 1984; Doke 1985). Interactions occur among different elicitors to modify the elicitor potential. Fungal cell wall elicitors and pectic fragments act synergistically in legumes (Davis *et al.* 1986), as well as the glucan elicitor and the agrichemical probenazole in rice (Dixon and Lamb 1990).

The elicitor induced signaling pathway(s) involved in the wheat defense mechanisms in the RWA/wheat interaction is still poorly understood. However, there is increasing evidence that SA plays an important role in plants exhibiting the HR against pathogens (Hammond-Kosack and Jones, 1996) even though very little is known about the role of SA in induced resistance in cereals (Silverman *et al.* 1995).

RWA infestation differentially induced SA accumulation in wheat plants, with the highest induction in the resistant cultivar (Figs. 4.11, 4.12 and 4.13). Previous studies on these cultivars indicate that the downstream defense related enzyme [β-1,3-glucanase (PR 2), chitinase (PR 3) and peroxidase] activities are also induced by RWA infestation (van der Westhuizen and Pretorius 1993, 1995; van der Westhuizen *et al.* 1998a,b). The accumulation of SA following RWA infestation may probably be an important step in the signal transduction pathway leading to the activation of the downstream defense responses. The induction of SA accumulation has also been reported in resistant tobacco (Xanthi-nc) inoculated with tobacco mosaic virus (TMV), with no apparent increase in SA in the susceptible cultivar (Malamy *et al.* 1990). Also in cucumber, a fluorescent metabolite identified as SA, increased dramatically in phloem vessels of plants inoculated with tobacco necrosis virus (TNV) or the fungal pathogen *Colletotrichum lagenarium* (Metraux *et al.* 1990). In tobacco injected with TMV, the accumulation of SA paralleled the induction of PR 1 gene expression in the virus inoculated and systemic leaves (Malamy *et al.* 1990). Parallel studies in cucumber showed that increases in SA levels induced by *Colletotrichum lagenarium* or *Pseudomonas syringae* pv *syringae* preceded both the

appearance of SAR and the induction of peroxidase activity (Singh, 1978; Metraux *et al.* 1990). The importance of SA as a signaling molecule has also been emphasized by the observation made in *Arabidopsis* where depletion of SA caused a breakdown of both SAR and gene-for-gene resistance (Delaney *et al.* 1994).

The RWA induced SA accumulation in wheat cultivars containing different resistance genes showed unique kinetics influenced by the presence of these genes (Fig. 4.13). The resistance genes incorporated into the susceptible (Tugela) wheat plants may play a role in determining the degree of resistance in the different resistant wheat lines. The absence of resistance in the susceptible plants may be explained in terms of the lack of the particular resistance genes and consequently very low levels of induced SA accumulation. This kind of gene influence has also been reported in tomato. The genetically unlinked Cf-9 and Cf-2 genes promoted different levels of SA induction by *Cladosporium fulvum* (Hammond-Kosack *et al.* 1996).

The role of SA as an endogenous signal molecule involved in the activation of defense responses has also been emphasized by exogenously applied SA. The susceptible and resistant wheat cultivars responded to exogenous SA by increasing enzyme activities of defense related β-1,3-glucanase (PR 2), chitinase (PR 3) [Leotlela[1] personal communication] and peroxidase (Fig 4.18). The response of both cultivars to exogenous SA may indicate that a receptor for SA is present in both the cultivars. This response implicated that the biosynthesis of SA may be one of the steps determining susceptibility or resistance in wheat cultivars. Our results were in agreement with the findings of Malamy *et al.* (1990) who reported that while TMV induced PR proteins only in resistant tobacco plants, exogenous application of SA was effective in both susceptible and resistant tobacco plants. In the RWA/wheat interaction, susceptible wheat plants accumulated low levels of SA (Figs. 4.11, 4.12, 4.13) and also expressed low levels of peroxidase activity (Fig. 4.14) as opposed to the resistant plants, but it is more a case that susceptible plants also react to applied SA (Figs. 4.18, 4.19). The response of susceptible

[1] Leotlela P Oxford University, Oxford, UK.

wheat plants to exogenous SA may be a potential step which could be exploited for the enhancement of resistance in susceptible plants.

In an effort to elucidate the signaling role of SA in the defense responses, SA accumulation was related to peroxidase activity during RWA infestation. RWA infestation induced a selective increase in peroxidase activity as early as 12 h, with the highest activity in the resistant plants (Fig. 4.14). The increase in the accumulation of SA in the resistant plants (Fig. 4.14) correlated well with the increase in induced peroxidase activity in the resistant plants. This was an indication that SA was acting as a signaling molecule. In addition, its early accumulation, 4 h after infestation, may also point to its role as a signaling molecule. The signaling role played by SA was similar to findings of Malamy *et al.* (1990) who reported that an increase in an endogenous SA level of resistant tobacco plants infected by TMV occurred concomitant to the accumulation of PR 1 mRNA expression.

In addition, the increase in SA accumulation in the phloem of cucumber plants infected with TNV or *Colletotrichum lagenarium* preceded the development of necrotic lesions associated with the HR (Ryals *et al.* 1996; Wobbe and Klessig 1996). SA accumulation was also observed in the phloem of TMV inoculated and uninoculated leaves and reported to be sufficient for the induction of PR 1 gene expression (Pieterse *et al.* 1996). In these reports SA was signaling the induction of the defense responses.

Chen *et al.* (1993) showed that SA acts by binding to, and inactivating catalase. The resistant (Tugela *Dn 1*) plants showed a marked decrease in catalase activity at a time point when RWA feeding had induced the highest SA accumulation (Figs. 4.14, 4.15). The results are in agreement with the hypothesis that SA inactivates catalase. However this inactivation only occurred at high levels of SA.

The finding that SA inactivates catalase was further shown by exogenous application of SA (Fig. 4.16). Inhibition of catalase in the resistant plants indeed showed that at high

levels SA can inhibit catalase. However, supplied SA failed to inactivate catalase in the distal leaves (Fig. 4.17). This was an indication that SA inhibits catalase activity only at high levels as in the injected leaves, but not in the distal parts of the plant. This was probably related to its translocation and metabolism. Free SA is a more likely translocated form than the conjugated form (Enyedi *et al* 1992). The decline in inactivation of catalase with time could have been due to the rapid metabolism of SA. SA is metabolized to a sugar conjugate β-O-D-glucosylsalicylic acid (SAG) (Enyedi *et al* 1992; Malamy *et al.* 1992).

Whether free SA or the conjugated form is active in defense mechanisms is unclear. Hennig *et al.* (1993) injected SAG into extracellular spaces of tobacco and monitored PR 1 gene expression. In these experiments SAG proved to be as active as free SA. However, isolation of extracellular fluid from SAG-injected leaves demonstrated that SAG was hydrolyzed to release free SA in the extracellular spaces. The transient presence of SA in the injected leaves made it impossible to determine whether free SA or SAG was the active form. However, in studies of both phytohormones and phenolics, the unconjugated forms have been shown to be active, while the glucose-conjugated forms are inactive (Conn, 1984). Therefore the conjugated SA did not serve as catalase inhibitor.

Our results were in agreement with those of Sanchez-Casas and Klessig (1994) who reported that the activity of catalase was inhibitable by SA. The inactivation of catalase probably leads to elevated levels of H_2O_2, but preferably around the injected area and not throughout the entire plant. Recent reports have indicated that at the site of infection SA levels can reach as much as 150μM, which is sufficient to cause substantial inhibition of catalase and ascorbate peroxidase (APX) (Enyedi *et al.* 1992; Gaffney *et al.* 1993; Chen *et al.* 1993; Conrath *et al.* 1995; Durner and Klessig 1995). In the uninfected systemic tissues the SA content is probably too low to increase H_2O_2 levels through inactivation of catalase and APX.

To investigate further the role of SA in defense mechanisms we looked into the effect of reactive oxygen species, particularly H_2O_2 on the content of SA. If high SA levels inhibited catalase, then elevated H_2O_2 levels could potentiate SA accumulation. Leon *et al.* (1995) postulated that O_2 released from the degradation of H_2O_2 can act as a substrate in the synthesis of SA. In tobacco, SA is synthesized from benzoic acid (BA). The enzyme benzoic acid-2-hydroxylase (BA2H) which catalyzes the conversion of BA to SA uses molecular O_2 as one of its substrates (Leon *et al.* 1995).

To investigate whether H_2O_2 acts upstream or downstream of SA, we injected H_2O_2 into the leaves of susceptible and resistant plants. We found that applied H_2O_2 actually induced SA accumulation (Fig. 4.20). The transient induction of SA in the resistant plants occurred as early as 4 h after H_2O_2 treatment. As the incubation time was prolonged no activation of SA synthesis occurred. Hydrogen peroxide is highly oxidative and at high levels phytotoxic. Consequently, it is rapidly degraded (Bi *et al.* 1995). This accounted for the inactivation of SA synthesis with time. This was a clear indication that SA synthesis is potentiated by high levels of H_2O_2 in the signal transduction pathway leading to defense responses. Neuenschwander *et al.* (1995) also found that treating plants with H_2O_2 induced SA accumulation in TMV resistant tobacco leaves. Furthermore, it was reported that high levels of H_2O_2, as well as ozone or ultraviolet treatment, induced SA biosynthesis (Summermatter *et al.* 1995; Neuenschwander *et al.* 1995; Leon *et al.* 1995). Based on these findings, it was suggested that SA synthesis occurs at high levels of H_2O_2.

However, it still remains unresolved whether the elevated levels of H_2O_2 triggering the synthesis of SA result from SA inactivation of catalase. Firstly, SA has to accumulate to high levels before it can induce a significant inactivation of catalase. That is, SA synthesis has to be stimulated by an already existing H_2O_2 pool before it can reach levels capable of inactivating catalase. The H_2O_2 pool may originate from the oxidative burst associated with the HR. Secondly, catalase is inactivated by its own substrate H_2O_2 (Kirkman *et al.* 1987; DeLuca *et al.* 1995). This is in part due to H_2O_2-mediated accumulation of compound II which is formed at low levels of H_2O_2 and conversion of compound II to

compound III at high levels of H_2O_2. The rate of conversion and inactivation rises with increasing levels of H_2O_2 (Kirkman *et al.* 1987; Durner and Klessig 1996). The increase in peroxidase activity during RWA infestation (Fig. 4.14) and the increase in SA content (Fig. 4.14) may be an indirect indication of an increase in H_2O_2 levels, which could have contributed to inactivation of catalase, and finally, SA inactivates catalase only at high levels. When SA was exogenously applied and its effect on catalase determined, inactivation was only evident in the leaves directly injected, but not the in the distal uninjected leaves of the same plant (Figs. 4.16 and 4.17). This was an indication that very high levels of SA are required for the inactivation of catalase which are not necessarily found in distal parts of the plant. We therefore speculated that elevated levels of H_2O_2 triggering the synthesis of SA are not a direct consequence of catalase inactivation by SA.

This being the case, we speculated that SA interaction with catalase results in two potential signals, H_2O_2 and SA free radicals, a likely by-product of the interaction of SA with catalase and peroxidases (Durner and Klessig, 1996). Free radicals from phenolic compounds can induce lipid peroxidation, the products of which are potent signaling molecules in animals and possibly in plants (Anderson *et al.* 1998). SA induced lipid peroxidation in tobacco suspension cells resulted in the formation of lipid peroxides that activated PR 1 genes in these cells (Klessig *et al.* 1996). Furthermore, the shunting of SA into the peroxidative cycle of catalase may be to protect the complete inactivation of catalase. The SA free radicals can donate electrons to compound II converting it to the active ferricatalase form. On the other hand, the inactivation of catalase by high levels of SA leading to elevated levels of H_2O_2, may be to provide sufficient substrate for peroxidase activity. The peroxidase activity was induced during RWA infestation in parallel with the accumulation of SA (Fig. 4.14).

The possibility of SA interacting with catalase through the formation of SA free radicals may also be emphasized by the observed induction of lipoxygenases in resistant (Tugela DN) plants during RWA infestation (Powell[1] personal communication). The lipoxygenases

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are involved in lipid peroxidation and were also found to regulate the downstream defense responses in the RWA defense mechanism. In addition SA induced lipid peroxidation was significantly high in *Arabidopsis* leaves treated with SA (Rao *et al.* 1997) and a significant induction of PR genes was observed (Shah *et al.* 1997).

We therefore conclude that during feeding the RWA induces the release of an elicitor-active molecule which accumulates in the apoplast of resistant plants. This elicitor, identified as a lectin-binding glycoprotein is capable of inducing both local and systemic defense responses. The induction of these defense responses occurred in parallel with the accumulation of SA. The increase in SA content may probably be acting as a signal molecule in mediating these responses. SA is synthesized from high levels of H_2O_2 and it inactivates catalase. It can therefore be suggested that SA acts downstream of H_2O_2 and upstream of catalase in the signal transduction pathway in the defense mechanism of the resistant wheat plants under RWA infestation.

Abstract

Features of the upstream defense response in the interaction between wheat (*Triticum aestivum* L.) and the Russian wheat aphid (RWA) (*Diuraphis noxia*, Mordvilko) were investigated. The study was conducted in an attempt to shed light on the eliciting and signal transduction events of the defense mechanism.

It was found that the intercellular wash fluid (IWF) of infested resistant wheat plants (Tugela *Dn 1*), injected intercellularly into susceptible (Tugela) and resistant (Tugela *Dn 1*) plants had much higher eliciting activity than the IWF of infested susceptible plants. The eliciting activity was measured in terms of the expression of downstream defense related peroxidase and β-1,3-glucanase activities. The defense related enzymes were differentially induced to higher levels in the resistant than susceptible plants. Fractionation of the IWF of infested resistant plants by C-18 reverse-phase and con A binding chromatography revealed the elicitor to be a glycoprotein capable of inducing both local and systemic defense responses.

The signaling role of salicylic acid (SA) in mediating the downstream defense responses was also investigated. RWA infestation induced differential accumulation of SA to higher levels in the resistant (421-fold) than susceptible (78-fold) plants. The increase in the activity of the defense related peroxidase in infested resistant plants correlated well with the increase in SA content. Intercellular application of H_2O_2 induced a 65% increase in SA content only in the resistant plants. As SA was accumulating, catalase activity was declining. In addition, intercellularly applied SA also inhibited catalase activity by 49%.

The results from this study indicated that the intercellular glycoproteins from infested resistant plants can act as elicitors of the downstream defense responses and that SA is most probably a signaling molecule in mediating these responses.

Opsomming

In 'n poging om meer lig te werp op die elisiterings- en seintransduksiemeganismes van die verdedigingsmeganisme van koring, *Triticum aestivum* L., teen die Russiese koringluis (RKL), *Diuraphis noxia* Mordvilko, is aspekte van die stroomop verdedigingsrespons ondersoek.

Deur middel van intersellulêre toediening aan weerstandbiedende (Tugela *Dn 1*) en vatbare (Tugela) koringplante is dit vasgestel dat die intersellulêre wasvloeistof (IWV) van geïnfesteerde weerstandbiedende koring (Tugela *Dn 1*) baie hoër elisiteringsaktiwiteite as die IWV van geïnfesteerde vatbare koring (Tugela) bevat het. Die elisiteringsaktiwiteit is bepaal in terme van die uitdrukking van stroomaf verdedigingsverwante peroksidase- en β-1,3-glukanase-aktiwiteite. Hierdie aktiwiteite is deur die IWV van geïnfesteerde weerstandbiedende plante selektief tot hoër vlakke in weerstandbiedende as in vatbare plante geïnduseer. 'n Glikoproteïen met lokale sowel as sistemiese elisiteringsvermoëns is deur middel van C-18 omgekeerde fase en kon. A-bindingschromatografie uit die IWV van geïnfesteerde weerstandbiedende koringplante geïsoleer.

Die rol van salisielsuur (SA) in die seinmeganisme wat aanleiding tot die stroomaf-verdedigingsresponse gee, is ook ondersoek. RKL-infestering het differensiële akkumulering van SA tot hoër vlakke in weerstandbiedende (421-voud) as in vatbare (78-voud) plante geïnduseer. Die toename in verdedigingsverwante peroksidase-aktiwiteit in geïnfesteerde weerstandbiedende plante het goed met die toename in salisielsuurinhoud gekorreleer. Intersellulêre H_2O_2 -toediening het 'n 65% toename in SA-inhoud slegs in weerstandbiedende plante geïnduseer. Endogene SA-akkumulering het met 'n afname in katalase-aktiwiteit gepaard gegaan. Hierbenewens het intersellulêre toegediende SA, katalase-aktiwiteit met 49% gerem.

Die resultate van hierdie studie het aangetoon dat intersellulêre glikoproteïene van geïnfesteerde weerstandbiedende plante kan as elisitore van die stroomaf verdedigingsrespons optree en dat SA heel moontlik die seinmolekuul is wat hierdie respons uitlok.

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