# INVOLVEMENT OF REACTIVE NITROGEN SPECIES IN THE RUSSIAN WHEAT APHID RESISTANCE RESPONSE OF WHEAT

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By

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# DECLARATION

I declare that the Thesis hereby handed in for the qualification ***Philosophiae Doctor*** at the University of the Free State, is my own independent work and that I have not previously submitted the same work for a qualification at/ in another University/ Faculty.

Furthermore, I cede copyright of the Thesis in favour of the University of the Free State.

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**M J Moloi** **Date**

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# CHAPTER 1

## 1. INTRODUCTION

Wheat is one of the leading cereal grain crops produced, consumed and traded in the world. It provides over 20 % of the calories for the world population, and is a staple food for 35 % of the world population (FAO, 1998). In South Africa, wheat is next to maize the most important grain crop produced. The largest wheat producing areas in South Africa since 1994 are the Free State (35 %), Western Cape (34 %) and Northern Cape (15 %). Most of the wheat produced in South Africa is mainly for human consumption with the remainder used for animal feed and seed (Department of Agriculture, Land Reform and Rural Development, 2009). The cultivated wheat belongs to two main classes: common or bread wheat (*Triticum aestivum* L.), which accounts for 95 % and durum wheat (*Triticum durum*), which accounts for 5 % of the world wheat production. Common wheat is used to make bread and biscuits, whereas durum wheat is used to make pasta (Kiplagat, 2005).

Statistics indicates that since 2003/04, wheat production has decreased dramatically. Since then to 2007/08, South Africa could only produce about 60-70 % of the wheat consumed (Department of Agriculture, Land Reform and Rural Development, 2009). This decrease can be attributed to unusual climatic conditions and diseases. Wheat is a host for one of the most destructive insect pests in the world called the Russian wheat aphid (RWA), *Diuraphis noxia* (Mordvilko), particularly in the dry areas. The RWA is

believed to have its origin in the Caucasus region. However, it has been reported in several countries including the USA, Chile, Iran, Canada, Ethiopia, China and most countries bordering the Mediterranean (El Bouhssini and Nachit, 2000). This aphid was first reported by Mordvilko and Grossheim around 1900 in the Mediterranean Sea region and southern Russia. From here, it is believed that the aphid spread from west Asia to the USA and Canada via South Africa and Mexico (Saidi and Quick, 1996). Since 1978 when it was first observed in South Africa, it has also become a major pest of wheat in South Africa (Walters et al., 1980; Du Toit and Walters, 1984). Significant yield and quality losses attributed to RWA infestation of wheat and barley have been documented. In South Africa alone, yield losses of between 35-60 % were recorded (Du Toit and Walters, 1984) and still, great economic losses are being incurred (Basky, 2003).

Plant selection mechanisms used by phloem-feeding insects vary. Upon landing, adults evaluate the tactile and chemical cues of the plant surface to determine the suitability of a plant as a shelter or as a feeding and/or oviposition host. On a good host, the next generation will thrive and on a poor host, insect populations will decline (Walling, 2008). During the initial encounters with a plant, aphids often use their stylets to tap on and make shallow probes on the leaf surface. They secrete a salivary sheath that lines the stylet path. The saliva may contain numerous enzymes such as oxidases, pectinases, and cellulases (Miles, 1999; Walling, 2008). Afterwards, they ingest the phloem sap from their host through their stylets. The nature of cell punctures and the nature of

salivary effectors will determine the defense-signaling pathways that are activated, as well as metabolites and proteins that accumulate in the infested plant (Walling, 2008).

Aphids can have a dramatic negative impact on their host plant, partly due to their capacity for extremely rapid population growth (Goggin, 2007). The RWA feeding symptoms on susceptible small grains include: longitudinal chlorotic streaks (white, yellow, or purple) on the leaves and stems, reduced tillering and root development, spike deformation (trapped), leaf rolling and stunting in the host plant, which results in lower grain yield/ poor quality and even death in the case of extreme infestation (Walters et al., 1980; Fouche et al., 1984; Peairs, 1990; Burd et al., 1998). Extensive chlorosis leads to the death of plants, while leaf rolling retards plant development. Rolling of the flag leaf causes delayed ear emergence, leading to decreased fertility of the florets (Kazemi et al., 2001). It is believed that these insects inject a phytotoxin into their hosts' phloem as part of their pierce-and-suck feeding process, and that this compound is responsible for the symptoms observed in plant (Belefant-Miller et al., 1994). Lately, Saheed *et al* (2007) reported that leaf streaking, curling and necrosis is probably due to tapping of the xylem for water. This will lead to a salivary ejection that decreases offloading of water to the vascular parenchyma and phloem, thereby increasing water, nutrient and photosynthetic stress. Macedo *et al* (2009) reported that RWA infestation negatively affects the net photosynthesis rate of *Tugela* wheat cultivar, where it causes a greater photosynthetic rate reduction.

## 1.1 RWA management

Various RWA management approaches have been employed to control this pest. However, each one has its own advantages and disadvantages.

### 1.1.1 *Insecticides*

Initial efforts to control the RWA were made through the use of insecticides. Since RWA feeding causes rolling of the leaves (RWA colonies are found within the tubes formed by these tightly curled leaves), it is difficult to administer and achieve good insecticide coverage (Baker and English, 1988; Peairs, 1990). In South Africa, large-scale aphicide applications were made annually to protect crops. This was achieved by application of expensive mixtures of systemic and contact insecticides, supplemented by the eradication of volunteer wheat, which served as a host between the seasons (Du Toit and Walters, 1984). Another disadvantage of pesticides is that they pose a threat to human health and to the environment, causing among other, undesirable effects such as phytotoxicity, pollution, development of insecticide resistance, or negative effects on non-target organisms (Hatchett et al., 1994; Isman, 1999).

### 1.1.2 *Biological control*

Biological control on the other hand, is a naturally occurring phenomenon. Natural enemies have been used successfully in green houses to control aphid populations (Van Lenteren and Woets, 1988). Not only do they increase aphid mortality, but also trigger avoidance behaviors that reduce feeding and reproduction (Nelson et al., 2004). However, in open agricultural ecosystems, farmers have relied almost exclusively on

insecticides (Jones, 2001), because natural enemies mostly maintain aphid populations below the economic injury level (Hatchett et al., 1994).

### 1.1.3 *Agronomic practice manipulation*

Manipulating agronomic practices such as irrigation could be another alternative for RWA control. Archer *et al* (1995) discovered that water stress is more important for RWA increase than the amount of fertilizer available to a crop. Their work suggests supplemental irrigation during periods of low precipitation as an alternative management option to reduce RWA increase rate.

### 1.1.4 *Host plant resistance*

Internationally, host plant resistance is an important avenue of pest management, and is one of the favored control tactics for the cereal aphids. Advanced wheat breeding lines that exhibit resistance to the cereal aphids have been developed (Quick et al., 1996; Souza, 1998). For many crops, breeders have identified quantitative loci (QTLs) or single dominance resistance genes (*R* genes) that reduce aphid performance on certain cultivars (Moharramipour et al., 1997).

Ten *Diuraphis noxia (Dn)* resistance genes from wheat and closely related cereals have been identified and described. Included are *Dn*1 in common wheat accession PI137739, *Dn*2 in PI262660, *Dn*3 in goat grass, *Dn*4 in PI372129, *Dn*5 in PI294994, *Dn*6 in PI243781, *Dn*7 derived from rye, *Dn*8 and *Dn*9 in PI294994, and *Dnx* in PI220127 (Liu

et al., 2005). In South Africa *Dn*1, *Dn*2 and *Dn*5 are used in RWA resistance breeding (Prinsloo, 2000).

Despite the availability of resistance genes, eruption of new RWA biotypes is the biggest problem, because new biotypes are virulent to most resistant varieties. During the year 2005, Eastern Free State (South Africa) wheat producers reported unusual RWA damage in resistant cultivars. Greenhouse experiments conducted at the Agricultural Research Council- Small Grain Institute (ARC-SGI), Bethlehem, confirmed the possibility of a new resistance-breaking RWA biotype (Jankielsohn and Lindeque, 2006). Additional evidence on the existence of this biotype in South Africa was further provided by Tolmay *et al* (2007). This resistance breaking phenomenon prompted renewed research to increase knowledge on the biochemical mechanisms of resistance to the RWA.

### 1.2 Plant defense mechanisms

Plants defend themselves from pathogen invasion or insect attack via an arsenal of defense mechanisms, both passive (pre-existing) and active (induced). The pre-existing defense mechanisms include structural barriers such as thick cuticle and cell wall reinforcement (to prevent pathogen invasion) or strategically positioned reservoirs of antimicrobial compounds which prevent colonization of the tissue (Zhao et al., 2005). Once the structural barriers of the host are breached, plants induce other defense reactions such as the hypersensitive response (HR), production of phytoalexins and pathogenesis related (PR) proteins, ion fluxes across the plasma membrane, oxidative

burst, lignifications, and the reinforcement of the cell wall (Hammond-Kosack and Jones, 1996; Repka, 2001). The efficacy of these defense responses often determines whether plants are susceptible or resistant to pathogenic infection. In many plants, resistance to diseases or to avirulence (*Avr*) determinants is known to be genetically controlled by plant resistance genes which confer resistance to pathogens with a matching avirulent gene by specific recognition events (Zhao et al., 2005). However, triggering resistance is not always due to *Avr* products, which activate defense responses in cultivars possessing the matching resistance genes but, instead, proceeds from the action of general elicitors able to activate defenses in different cultivars of one or many species (García-Brugger et al., 2006). Elicitors are the molecules that are able to induce physiological or biochemical responses with the expression of resistance. They can be secreted by the microbes (exogenous elicitors) or generated as a result of physical and/ chemical cleavage of the plant cell wall (Kogel et al., 1988; Somssich and Hahlbrock, 1998).

An accumulating body of evidence indicates that during the HR, one of the early events is the rapid accumulation of the reactive oxygen species (ROS) and the reactive nitrogen species (RNS) (Levine et al., 1994; Baker and Orlandi, 1995; Lamb and Dixon, 1997; Wendehenne et al., 2004; Zago et al., 2006; Zaninotto et al., 2006; Arasimowicz and Floryszak-Wierczorek, 2007; Hong et al., 2008). Ample evidence point to the involvement of ROS in early signal events leading to induction of defense reactions during plant-pathogen interactions (Levine et al., 1994; Alvarez et al., 1998; Orozco-Cárdenas et al., 2001).

In plants, there is increasing evidence for a role of nitric oxide (NO) as an endogenous plant growth regulator as well as a signal molecule in the transduction pathways leading to the induction of local and systemic defense responses against pathogens, and in damage initiating cell death (del Río et al., 2006). During incompatible plant-pathogen interactions, NO may work in conjunction with ROS for the induction of HR or may act independently of ROS for the induction of various defense genes, including PR proteins and enzymes of the phenylpropanoid metabolism (Delledonne et al., 1998; Delledonne et al., 2001).

Information pointing to a correlation between NO and salicylic acid (SA) during plant defense responses is accumulating (Song and Goodman, 2001; Zottini et al., 2007; Gaupels et al., 2008). During the defense responses, SA and NO may work synergistically to transduce the defense signal or, SA may antagonize the NO signaling pathway (Klessig et al., 2000).

NO can freely react with other free radicals such as $O_2^-$ without requiring enzymatic catalysis to form peroxynitrite ($ONOO^-$), a very powerful oxidant (Padmaja and Huie, 1993; Tuteja et al., 2004; Kozak et al., 2005), which may cause a variety of toxic effects in animals and plants (Stamler et al., 1994; Hooper et al., 1998; Bolwell, 1999; Durner and Klessig, 1999). $ONOO^-$ may also be involved in the induction of secondary defense related reactions (Alamilo and García-Olmendo, 2001). In addition, $ONOO^-$ may have an antioxidative role during the defense responses (Wink et al., 1995).

The discovery that ROS play a vital role in the RWA resistance response of wheat (Moloi and van der Westhuizen, 2006) and the knowledge that the resistance response of wheat to the RWA is a typical HR (Belefant-Miller et al., 1994) prompted us to get more insight information on the involvement of the RNS (particularly NO) in the RWA defense response of wheat.

# CHAPTER 2

## 2. LITERATURE REVIEW

Plants are exploited as sources of food and shelter by a wide range of parasites, including viruses, bacteria, fungi, nematodes, insects and even other plants. They have evolved mechanisms of antimicrobial defense which are either constitutive (pre-existing) or inducible (Scheel, 1998).

Upon pathogen attack, plants defend themselves by activating a multi-component defense response. In host defense, pathogen invasion is recognized by proteins encoded by plant disease resistance (*R*) genes that bind specific pathogen-derived avirulence (*Avr*) proteins. In non-host resistance, specific pathogen or plant cell wall derived exogenous or endogenous elicitors are recognized (Odjakova and Hadjiivanova, 2001). Defenses that are shared by all genotypes of a plant species and that prevent species from being a host for a particular pest constitute non-host resistance. Traits that deter herbivory/infestation (antixenosis) or reduce herbivore survival and reproduction (antibiosis) on a host species, are the sources of host plant resistance (Moharramipour et al., 1997; Smith and Boyko, 2007). Other defenses may include basal defenses. Plant traits that have been implicated in these defenses include cell wall modifications, proteins or secondary metabolites that have antixenotic or antibiotic properties, and plant volatiles that repel or attract their natural enemies (Smith and Boyko, 2007).

During incompatible plant-pathogen interactions, recognition of a potential pathogen often results in a hypersensitive response (HR). HR is characterized by localized cell and tissue death at the site of infection (Van Loon, 1997). As a result, the pathogen remains confined to necrotic lesions near the site of infection. A ring of cells surrounding necrotic lesions become fully refractory to subsequent infection, known as localized acquired resistance (Hammond-Kosack and Jones, 1996; Baker et al., 1997; Fritig et al., 1998). These local responses often trigger nonspecific resistance throughout the plant, known as systemic acquired resistance (SAR), providing durable protection against a broad range of pathogens (Sticher et al., 1997; Van Loon, 1997; Fritig et al., 1998).

Just before or concomitant with the appearance of HR is the increased synthesis of several families of the pathogenesis related (PR) proteins (Klessing et al., 2000). Most PR proteins have a damaging action on the structures of the parasite, e.g. PR-1 and PR-5 interact with the plasma membrane, whereas β-1,3-glucanase (PR-2) and chitinase (PR-3, PR-4, PR-8 and PR-11) attack β-1,3-glucans and chitin, which are components of the cell walls in most higher fungi. Increased PR gene expression is frequently used as a marker for SAR in plants (Fritig et al., 1998; Klessing et al., 2000).

Salicylic acid (SA) is required for the activation of defense responses that are mediated by resistance genes and for the establishment of SAR (Vernooij et al., 1994; Chen et al., 1995; Rao et al., 1997; Sticher et al., 1997; Chen et al., 1999; Hayat et al., 2009). Transgenic tobacco plants expressing bacterial salicylate hydroxylase (which

metabolizes SA) were unable to express SAR and even showed enhanced susceptibility to pathogens (Gaffney et al., 1993). SA is not a pre-requisite for HR to take place. Plants that cannot accumulate SA due to the presence of a transgene that encodes SA-degradating enzyme, developed HR after challenge by avirulent pathogens, but did not exhibit systemic expression of defense genes and did not develop resistance to subsequent pathogen attack (Glazebrook, 1999). This further shows that SA is crucial for the establishment of SAR.

Similar to pathogens, aphids induce transcripts associated with plant hormones known to modulate disease resistance such as jasmonic acid (JA), SA, ethylene and abscisic acid (ABA) (Smith and Boyko, 2007). The role of SA and JA in plant aphid-interactions may vary among plant species. Botha *et al* (2005) reported that RWA feeding elicits both SA and JA/ethylene-dependent signaling pathways by mimicking aspects of both pathogen and herbivorous insect attacks.

It is believed that the coordinated activation of HR and other defense mechanisms at the site of infection requires a tight control of the reactive oxygen species (ROS), such as superoxide anion ($O_2^-$) and hydrogen peroxide ($H_2O_2$) (Klessing et al., 2000). Whether or not SA accumulation is preceded by production of the ROS such as $H_2O_2$ during the defense response of plants is not clear. During the cotton hypersensitive response to *Xanthomonas campestris* pv. *malvacearum*, $H_2O_2$ production was found to be a prerequisite for local and systemic accumulation of SA (Martinez et al., 2000). Further discoveries showed that the conversion of benzoic acid (a precursor of SA) to

SA by benzoic acid-2-hydroxylase depended heavily on $H_2O_2$ production (Dempsey and Klessing, 1994; Leon et al., 1995). During the defense response of tobacco infected with tobacco mosaic virus, SA led to induction of $H_2O_2$ production. The mechanism behind this increase involved the binding of SA to a soluble SA-binding protein (SABP)/receptor, characterized as catalase. This binding resulted to inhibition of catalase's ability to convert $H_2O_2$ to $O_2^-$ and water. The resulting elevated levels of $H_2O_2$ led to induction of PR-1 gene expression (Chen et al., 1995). In agreement, the presence of a SA-inhibitable catalase was also observed in wheat (Mohase and van der Westhuizen, 2002). Activity of this protein was however inhibited in both the infested resistant (IR) and the infested susceptible plants (IS), indicating that catalase is not involved in the SA-mediated RWA resistance response (Mohase and van der Westhuizen, 2002), rather another protein/mechanism is involved.

Alternatively during the SA-mediated defense responses, SA may bind with SABP2 (characterized as lipase), generating a lipid-derived signal leading to induction of PR-1 gene expression and SAR development (Kumar and Klessig, 2003). Another protein which may interact with SA was identified as SABP3 (characterized as carbonic anhydrase) in tobacco chloroplasts (Slaymaker et al., 2002). In addition, ascorbate peroxidase (APX) (Durner and Klessig, 1995) and aconitase (Rüffer et al., 1995) have also been associated with SA.

## 2.1 Involvement of nitric oxide in the defense response

Different signal molecules are required for the activation of plant defense responses. In animals, ROS may cooperate with the reactive nitrogen species (RNS) such as nitric oxide (NO) in some pathological conditions, e.g. inflammation, acute phase responses, and programmed cell death (Stamler et al., 1994). NO is a gaseous free radical with a relatively long (in comparison with other free radicals) half-life of 3-5 seconds in biological systems. It is one of the smallest diatomic molecules exhibiting hydrophobic properties, as a result, may not easily migrate in the hydrophilic regions of the cell such as the cytoplasm, but freely diffuse through the lipid phase of membranes (Arasimowicz and Floryszak-Wieczorek, 2007).

NO has attracted a great deal of attention due to its diverse physiological functions and ubiquity, and is now recognized to be an intra- and intercellular mediator of cell functions (Huang et al., 2002). The biological significance of NO was recognized by Science in 1992, which named NO the free radical 'Molecule of the year'. In 1998 the Nobel Prize in Physiology and Medicine was awarded for works that led to the discovery of NO as a biological mediator produced by mammalian cells (del Río et al., 2004).

Due to high diffusivity of NO ($4.8 \times 10^{-5}$ $cm^2s^{-1}$), it can diffuse within a cell from a specific site of generation (e.g. in the mitochondria) to other regions of the cell where it might induce an effect by interacting with specific target proteins. It can also diffuse out of the cell across the plasma membrane (because it is hydrophobic) into adjacent cells, thereby creating a small region of cells responding to it. However, whether or not NO

does diffuse within and between cells, and if it does how far it moves remains unknown (Neill et al., 2008).

The involvement of NO in defense is not only confined to the animal kingdom. NO has proven to be one of the most important signaling molecules involved in the regulation of many physiological and biochemical processes in plants (An et al., 2005). Initial investigations into NO's functioning suggested that plants use it as a signal molecule via pathways remarkably similar to those found in mammals. For example, Durner *et al* (1998) concluded that several critical players of animal NO signaling are also operative in plants. In animals, cGMP and cADP-ribose serve as second messengers for NO signaling. Likewise in tobacco, cGMP was also found to be a second messenger for NO during signaling responses. Inhibitors of guanylate cyclase were found to block NO-induced activation of phenylalanine ammonia lyase (PAL) expression and PAL enzyme activity (Durner et al., 1998), which further validates the involvement of cGMP as a second messenger of NO for induction of the secondary defense responses in plants. Moreover, these inhibitors were found to block NO-mediated root development in cucumber (Pagnussat et al., 2003).

Another suggestion of NO's involvement in physiological processes was the finding that a decrease in NO levels is associated with fruit maturation and flower senescence (Beligni and Lamattina, 2001). It was also found that NO may be a natural senescence-delaying plant growth regulating agent acting primarily, but not solely, by down regulating ethylene emission (Stöhr and Ullrich, 2002). NO seems to also play an

inducing role during seed germination, de-etiolation and hypocotyls elongation. In maize, an increase in tissue expansion was observed after treatment of root segments with low concentrations of NO releasing compounds (Stöhr and Ullrich, 2002). On the other hand, exposure of carrot suspension cells to NO, inhibited respiration rate (Zottini et al., 2002).

NO has other functions to improve the response of plants under diverse abiotic and biotic stress conditions. Garcia-Mata and Lamattina (2001) found that treatment of plants with exogenous NO leads to induction of stomatal closure and enhances drought tolerance of wheat seedlings. Likewise, Neill *et al* (2002) demonstrated that endo- as well as exogenous NO contribute to the ABA-dependent stomatal closure. However, other studies indicated that water stress tolerance is better achieved through a synergistic action of ROS and NO (Zhao et al., 2001; Bright et al., 2006). In agreement, Gachomo and Kotchoni (2008) established that drought stress was better managed through elevated levels of ROS such as $H_2O_2$ and NO.

NO was also found to be involved in resistance against mineral deficiency. Sun *et al* (2006) discovered that application of NO partially reversed iron deficiency-induced retardation of growth, as well as chlorosis in maize leaves.

Several studies confirmed the participation of NO in the plant's response to ultra violet (UV)-B radiation. Neill *et al* (2003) showed that NO treatment of potato tubers prior to UV-B radiation resulted in the development of almost 50 % more healthy leaves in

comparison to non-treated plants. Further investigations by Shi *et al* (2005) showed that NO protected plants from UV-B radiation through increased activity of antioxidative enzymes.

Evidence for the involvement of NO in protection of plants against salinity stress has been documented (Valderrama et al., 2007). In calluses of reed plants, NO was found to induce salt tolerance by increasing the $K^+$ to $Na^+$ ratio (Zhao et al., 2004).

Other studies point to the involvement of NO in wound healing responses of plants (Huang et al., 2002; Huang et al., 2004). Moreover, París *et al* (2007) established that an increase in NO due to wounding of potato plants leads to the induction of callose deposition, and also to an increase in extensin and PAL transcript levels. Their results suggested that NO might potentiate the healing responses of plants leading to rapid restoration of the damaged tissue. Contrary, Orozco-Cárdenas and Ryan (2002) reported that NO can also act as a negative regulator of some other defense responses such as the expression of the proteinase inhibitor (PI) I in tomato.

A plethora of evidence indicates that NO plays a significant role in plant resistance against pathogens. Infection of resistant wheat plants with yellow rust (*Puccinia Striiformis*) produced two NO peaks, of which the earliest was associated with resistance (Guo et al., 2004). The involvement of NO as one of the earliest defense responses was studied in barley epidermal cells infected with *Blumeria graminis*. It was suggested that NO may be important in the initiation and development of effective

papillae (Prats et al., 2005). It was also found that exogenous application of NO significantly conferred higher disease protection against downy mildew in pear millet plants in comparison with the control (Manjunatha et al., 2008). Correa-Aragunde *et al* (2008) established that a low NO concentration in plants can play a significant role in resistance by stimulating cellulose synthesis.

NO also plays an important signaling role during plant defense responses against pathogens by stimulating an increase in production of PAL (Huang et al., 2002; Wang and Wu, 2004; Zeier et al., 2004). Similar results were obtained under UV-light stress where PAL activity was also inhibited due to NO insufficiency (Zeier et al., 2004). It was discovered that treatment of potato tubers with NO stimulated the accumulation of rishitin (another phytoalexin). Additionally, application of a NO scavenger led to synthesis inhibition of this product (Noritake et al., 1996). Biosynthesis of specific phytoalexins was also observed after treatment of soybean cotyledons with NO (Modolo et al., 2002).

### 2.2 Correlation between NO and SA

Ample evidence point to a correlation between NO and SA during plant defense responses. Song and Goodman (2001) discovered that treatment of tobacco mosaic virus (TMV) - infected tobacco plants with NO releasing compounds induced resistance against TMV. NO remarkably reduced the lesion size in both treated and non-treated distant leaves, indicating that NO could induce systemic resistance against TMV infection in tobacco. Investigations in this study led to a conclusion that NO-mediated

disease resistance requires the action of SA and that NO functions upstream of SA in the SAR signaling pathway, although fully dependent on the function of SA. Furthermore, it was found that NO deficiency during UV- stress leads to a decrease in SA and delayed PR-1 gene expression (Zeier et al., 2004). In contrast, Gaupels *et al* (2008) discovered that abundant NO generation in companion cells of V*icia faba* was relying on SA. Zottini *et al* (2007) also found that NO acts downstream of SA in *Arabidopsis thaliana*.

An *et al* (2005) reported that NO is an important signal molecule for the induction of exo- and endo- β-glucanase activity in leaf cell wall. In addition, other studies showed that NO can regulate the glucanase activity, and thereby increase the extensibility of the cell wall framework, i.e. change its composition (Darley et al., 2001; Zhang et al., 2003).

### 2.3 Cross-talk between ROS and NO

ROS alone is not always sufficient to mediate a strong disease resistance in plants, but it can act synergistically with NO to activate a stronger response (Wang and Wu, 2004). Mackerness *et al* (2001) also identified NO and $H_2O_2$ as important early signaling components. NO has been implicated as a potential second messenger during the HR, exerting effects that are both complementary and antagonistic to those of $H_2O_2$ (van Camp et al., 1998; Desikan et al., 2003).

A significant overlap in gene targets for NO and $H_2O_2$ has been established. Catalase deficient tobacco plants were found to have a small number of genes specifically

regulated by either NO or $H_2O_2$ (Zago et al., 2006). Application of NO (SNP) was found to mediate $H_2O_2$–dependent callose deposition along the cell walls adjacent to an appresorium during the *Colletotrichum coccodes*-tomato interaction, which eventually leads to higher resistance, because of the cell wall protein cross-linking (Wang and Higgins, 2006). It was also found that the fungal elicitor from *Fusarium oxysporum* induced a rapid NO production in a *Taxus* cell culture with 12 h of elicitor treatment, which as a result leads to the induction of $H_2O_2$. To further show the relationship between these two molecules, inhibition of NO production further suppressed the elicitor induced $H_2O_2$ production (Wang and Wu, 2004).

Similarly, a genetic approach by Zeier *et al* (2004) showed that reduction of NO levels consequently leads to inhibition of $H_2O_2$ production. In addition, treatment of *Arabidopsis thaliana* or tobacco plants with a high dose of NO for a short period (1 minute) was found to induce many genes that are known to be activated during oxidative stress such as the superoxide dismutase (SOD) (Durner et al., 1998; Huang et al., 2002). In some cases however, NO can act independently from ROS in the induction of specific genes responsible for the synthesis of defense metabolites (Noritake et al., 1996; Delledonne et al., 1998).

When produced simultaneously in large quantities during the defense responses, NO may rapidly react with superoxide anion ($O_2^-$) ($k$ = $6.7 \times 10^9$ liter $mol^{-1}s^{-1}$) generated (Tuteja et al., 2004; Kozak et al., 2005) to form peroxynitrite ($ONOO^-$) without requiring enzymatic catalysis, and hypothetically resulting in the mobility of NO and redox activity

of $O_2^-$ (Padmaja and Huie, 1993). $ONOO^-$ falls in the category of the RNS. The term RNS was coined to designate other related molecules such as dinitrogen trioxide ($N_2O_3$), S-nitrosoglutathione (GSNO), nitrogen dioxide ($NO_2^{\cdot}$), nitrosyl cation ($NO^+$), etc. (Valderrama et al., 2007).

$ONOO^-$ may have a significant role in amplification of the signal during the incompatible interactions (Marla et al., 1997). In the physiological pH range, $ONOO^-$ is unstable; however, due to its relatively long half-life of approximately 1 second, it may diffuse at considerable distances in the cell. $ONOO^-$ may cause a variety of toxic effects such as lipid peroxidation and cell death in animals, because it is a very powerful oxidant (Stamler et al, 1994; Hooper et al., 1998). Although excessive production of $ONOO^-$ can damage normal tissue, the reactive chemistry of $ONOO^-$ can be considered beneficial when the entire organism is considered (Bonfoco et al., 1995). In plants, this molecule performs a similar function (Bolwell, 1999; Durner and Klessig, 1999). It may react with DNA, thiol groups of proteins and polyunsaturated radicals of fatty acid lipids of membranes, causing serious damage to cell structures and cytotoxicity (Wendehenne et al., 2001; Radi, 2004; Szabó et al., 2007).

Another function of $ONOO^-$ in the defense responses involves induction of the secondary defense related responses such as peroxidase and PAL (Alamilo and García-Olmendo, 2001). $ONOO^-$ may also have a protective role against oxidative stress due to the fact that it can prevent the Fenton reaction [$H_2O_2 + Fe^{2+}$ ($Cu^+$) → $Fe^{3+}$

($Cu^{2+}$) + $^{\cdot}OH$ + $OH^-$ (Wojtaszek, 1997)] by scavenging iron, and thus avoiding the formation of one of the most deleterious ROS, the hydroxyl radical (Wink et al., 1995).

The involvement of $ONOO^-$ in the HR is vague. Alamilo and García-Olmendo (2001) revealed that $ONOO^-$ may be an important component for cell HR to take place. In disagreement, Delledonne *et al* (2002) reported that cell death in plants is activated only when the $NO/H_2O_2$ (not $NO/O_2^-$) ratio is within a limited range, and not when the levels of either NO or ROS are increased independently.

Above literature pinpoints the importance of NO in the defense response of animals and plants. Therefore, a clear understanding of the NO-generating mechanisms/ systems would be beneficial. Different enzymatic and non-enzymatic reactions are involved in NO synthesis.

### 2.4 NO synthesis in animals/mammals

The synthesis of NO in animals is primarily accomplished by three different isoforms of nitric oxide synthase (NOS) (Alderton et al., 2001). Of these, two are constitutive (cNOS) and one is inducible (iNOS) by cytokines and endotoxins. The two sub-types of cNOS are endothelial NOS (eNOS) (which was initially detected in the vascular endothelium) and neuronal NOS (nNOS) (which is present in the central and peripheral nervous system) (Tuteja et al., 2004; Crawford, 2006). These enzymes vary from 130-160 kDa in size, form dimers and are about 50-60 % identical in mammals. The primary differences among these enzymes are in their regulation and in their output rates of NO.

iNOS produces large quantities of NO. nNOS and eNOS produce much lower levels of NO than iNOS and are involved in signaling. The overall reaction for these enzymes in animals is the same and occurs as follows:

L-arginine + NADPH + $O_2$ → $N^{\omega}$-hydroxy-L-arginine (NOHA) + $NADP^+$ + $H_2O$

NOHA + ½ NADPH + $O_2$ → L-citrulline + NO + ½ $NADP^+$ + $H_2O$ (Crawford, 2006).

The physiological functions of NOS are not only limited to NO production. In bacteria, the primary role of NOS may not be producing NO, but rather synthesizing specific molecules, e.g. in *Streptomyces turgidiscabies,* NOS is needed to synthesize the phytotoxic-thaxtomine A (a nitrated dipeptide required for plant pathogenicity). Nitration of lipopeptide arylomyans by *Streptomyces* sp. Tü6075 is associated with increased antimicrobial activity, which may play a significant role during bacterium-bacterium interaction in the soil (Arasimowicz and Floryszak-Wieczorek, 2007).

**2.5 NO synthesis in plants**

The presence of NOS activity in higher plants was for the first time shown using the method of conversion of radiolabeled arginine into radiolabeled citrulline (Cueto et al., 1996; Ninnemann and Maier, 1996). In 1999, the occurrence of this enzyme activity was demonstrated in peroxisomes from pea plants (Barroso et al., 1999). Western blot analysis using antibodies raised against mammalian NOS have enabled the detection of immunoreactive proteins in plant extracts. However, in a proteomic study in extracts from maize, embryonic axis with polyclonal rabbit antibodies against human nNOS and

mouse iNOS, found that many NOS unrelated proteins were recognized by the antibodies (Butt et al., 2003). Such results raised doubts upon the results of NOS presence in plants obtained using immunological techniques with mammalian NOS antibodies (Wendehenne et al., 2003; del Río et al., 2004).

Chandock *et al* (2003) identified iNOS as the first known pathogen-inducible NOS enzyme in plants. They revealed that this protein resembles the mammalian iNOS in that it uses the same co-factors, has comparable kinetic properties, and is induced by pathogens. In the same year, Guo *et al* identified another NOS-like enzyme in plants that is hormone-activated from *Arabidopsis thaliana* (AtNOS1). This AtNOS1 was shown to have the same biochemical properties of mammalian NOS in that it also reduced arginine to citrulline when assayed with a commercial kit. This gene was also shown to encode a novel NOS enzyme that behaves most like the constitutive class of mammalian NOS enzymes (eNOS and nNOS). However, confirmation of NOS enzyme involvement in plant NO production is still a puzzle. It has been found that AtNOS1 may not be a NOS at all, because this gene was found to have no complete sequence similarity to the animal NOS proteins. Also, it had no consensus binding sites for NADPH, FAD or arginine (Crawford, 2006; Neill et al., 2008).

Despite this lack of similarity, AtNOS1 activity was found to induce the defense genes associated with local and systemic responses to interaction of *Arabidopsis thaliana* with *Pseudomonas syringae* (Zeidler et al., 2004). Currently, the view is that although AtNOS1 may not be a NOS *per se*, it is nonetheless an important factor in NO

synthesis/ accumulation. Reflecting this, it has been suggested that the name of the protein be changed to *Arabidopsis thaliana* NO-associated 1 (AtNOA1) (Crawford et al., 2006; Wilson et al., 2008).

Although no plant NOS gene has been identified to date, substantial pharmacological and biochemical data resulting from the use of NOS inhibitors to inhibit NO production indicate that there are enzymes in plants that are affected (Neill et al., 2008). Assays have shown that plants can have arginine-dependent NOS activity, which can be inhibited by classic NOS inhibitors (arginine analogues). These inhibitors are known to block NO production and some NO-mediated responses, showing that plants have orthologues to animal NOS enzymes (Crawford, 2006). Wang *et al* (2006) also performed experiments with NOS inhibitors and their results suggested that NO originated from NOS during UV-B stress. In agreement with this, He *et al* (2007) found that during UV stress, a NOS-dependent NO production was inhibited by $N^{\omega}$-nitrilo-L-arginine-methyl ester (L-NAME), an inhibitor of NOS. In *Arabidopsis thaliana*, it was discovered that NO production was inhibited by another NOS inhibitor named $N^{\omega}$-nitro-L-arginine (L-NNA). This decrease in NO was associated with the fact that NOS may play an important role in NO production (Zhao et al., 2007a and b).

In contrast to the above information, Rockel *et al* (2002) found that NO production by intact leaves or leaf extracts was unaffected by NOS inhibitors. Furthermore, Crawford (2006) reported that mutations in this gene reduced NO accumulation *in vivo* but not

completely. This information indicates that NO production in plants is not only limited to NOS (other mechanisms or genes may be involved).

Nitrate assimilation is a major pathway for nitrogen supply in many plants and microorganisms. Nitrate reductase (NR) has been considered a key enzyme for assimilatory nitrogen metabolism. This enzyme is known to be highly regulated by complex transcriptional and post-translational mechanisms. The distribution of NR was found to be regulated by cell age (with higher NR activity in younger leaves) (Datta and Sharma, 1999; Yamasaki and Sakihama, 2000). The production of NO by the molybdenum cofactor containing enzyme NR is known since the beginning of the 80's. Studies have shown that there are different types of NR in plants, namely, the constitutive NR (EC 1.6.6.2) and the inducible NR (EC 1.6.6.1). This NO-producing constitutive NR was originally unique to the *Leguminosae*. Later on, it was reported that other plant species including sunflower, sugarcane, corn, rape, spruce, spinach and tobacco, emit NO gas under certain conditions (Yamasaki and Sakihama, 2000).

NR (located in the cytoplasm) can generate NO from nitrite ($NO_2^-$) with NADH as an electron donor and catalysis probably involves a molybdenum co-factor. NO production capacity of NR at saturating NADH and $NO_2^-$ concentrations is about 1 % of its $NO_2^-$ reduction capacity. However, *in vivo*, NO production depends on the total NR activity, the enzyme activation state and the intracellular accumulation of $NO_2^-$ and nitrate ($NO_3^-$) (Mahboobi et al., 2002; Rockel et al., 2002; del Río et al., 2004). Quite a number of studies have revealed that post translational modification of NR and $NO_2^-$ may be a

rate-limiting factor/step of NO production by NR (Xu and Zhao, 2003; Yamamoto-Katou et al., 2006).

In most plant systems, it has been discovered that both NR and nitrite reductase (NiR) are inducible by $NO_3^-$ (Pécsváradi and Zsoldos, 1996). Initially, Yamasaki and Sakihama (2000) found that $NO_3^-$ is a substrate for NO production, but with a time-lag. Later on, their work led to a conclusion that the actual substrate for NR-dependent NO production was $NO_2^-$ not $NO_3^-$. In contrast, work by Leleu and Vuylsteker (2004) led to a finding that $NH_3^+$ (not $NO_3^-$) is important for NR activity in *Brasica napus* seedlings. They found that there was a difference of NR activity in roots and shoots when either $NH_3^+$ or $NO_3^-$ was supplied. In roots, it was found that NR activity increased as a function of $NO_3^-$ and decreased when $NH_3^+$ was the only source of nitrogen. However in shoots, NR activity was independent of $NO_3^-$ but dependent on $NH_3^+$. Moreover, the NR mRNA under $NH_3^+$ nutrition was even higher.

Reports by Modolo *et al* (2005 and 2006) further showed that NR is not essential for NO synthesis, but is an important source of $NO_2^-$ for subsequent NO production in *Arabidopsis thaliana* leaf homogenates. Xu and Zhao (2003) found that NO production in non-leguminous plants (wheat, orchid and aloe) was due to an enzyme action rather than a chemical action. They found a strong correlation between NR activity and NO content in wheat. They also found that NR is the main pathway for NO production in wheat seedlings.

Yamamoto-Katou *et al* (2006) reported that NR is involved in INF1 (a major elicitin from *P. infestans*)-induced NO production. It has been found that NO production is more pronounced in leaf homogenates of plants inoculated with an avirulent strain *P. syringae* pv. *maculicola (Psm)* than in non-inoculated plants. In this study, an NR-deficient double mutant (*nia1nia2*) of *A. thaliana* that is deficient in endogenous $NO_2^-$ was used to analyze the response against an avirulent strain of *Psm*. The inoculation of *Psm* in *nia1nia2 A. thaliana* caused leaf chlorosis whereas the HR was induced in wild-type plants. Following inoculation with *Psm*, NO production *in situ* was substantially increased in wild-type plants but not in *nia1nia2* leaves. However, NO production was triggered in *nia1nia2* after infiltration with L-arginine or $NO_2^-$. Furthermore, co-infiltration of $NO_2^-$ and *Psm* restored the HR in the leaves of *nia1nia2* plants. Their findings show that HR is affected in NR-deficient plants, because these plants lack L-arginine and $NO_2^-$, further showing that NR is not responsible for NO, but $NO_2^-$ production.

There are still questions on the reduction of $NO_2^-$. After conversion of $NO_3^-$ to $NO_2^-$ by NR, NiR (localized in the plastids) can reduce $NO_2^-$ to ammonia. Thus ammonia can be delivered either from nitrate reduction, uptake of ammonia, or photorespiration (Mahboobi et al., 2002). Xu and Zhao (2003) found that the reduction of $NO_2^-$ to NO is enzyme dependent; however both NR and NiR do not catalyze this process. In maize leaves, it has been found that there is a biphasic induction of NR and NiR in relation to the varying concentration of $NO_2^-$ . In this study, it was revealed that induction of NR and NiR is strictly regulated by development on a temporal scale. It was also found that although NR and NiR are not dependent on the same substrate for induction; their

developmental programs are not strictly linked. Evidence for this was found from the observation that the maximal activity of NR and NiR were observed on different days. They also found that NiR distribution is regulated by plastid maturity (Datta and Sharma, 1999).

An interesting revelation is that both NR and NOS can work collaboratively. Modolo *et al* (2005) found that in *A. thaliana* infected with *P. syringae*, NO production arise from the co-operation of NOS, NR and the mitochondrial-dependent nitrite-reducing activity. In agreement with this, other researchers discovered that water stress induced NO production was blocked by pre-treatment with inhibitors of both NOS and NR in leaves of maize plants. More importantly, they also found that there is a correlation between NR, NOS and the anti-oxidative enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX) and glutathione reductase (GR). They discovered that treatment of plants with inhibitors of both enzymes led to inhibition of these anti-oxidative enzymes (Sang et al., 2008).

Interestingly, previous studies have shown that these anti-oxidative enzymes (all three) are somehow involved in the Russian wheat aphid (RWA) resistance responses of wheat plants, because they were significantly induced to higher levels in the infested resistant than infested susceptible and control plants (Moloi and van der Westhuizen, 2008). This information is very important, because it shows that there might be an interaction between the ROS, anti-oxidative enzymes and NO; and this relationship

needs to be explored. In agreement with this suggestion, Gould *et al* (2003) proposed that NO emission in plants can be a generalized stress response similar to ROS.

Other systems have been found to generate NO in plants. A plasma-membrane-bound, root specific enzyme, nitrite-NO oxidoreductase (Ni-NOR), may also function as a further source of NO. This enzyme was identified biochemically via its NO-generating activity. However, unlike NR, it does not use NADH as a cofactor, but uses cytochrome *c* as an electron donor *in vitro*. However, neither its physiological role nor its genetic identity is yet known (Stöhr and Stremlau, 2006).

Xanthine oxidoreductase is another enzyme capable of producing NO (in preference to $H_2O_2$) in animals under hypoxic conditions (Millar et al., 1998). It was later on shown that this enzyme is probably not relevant to NO signaling in plants (Planchet and Kaizer 2006). Reports indicated that organelles such as chloroplasts and mitochondria are also capable of producing NO in plants (Bethke et al., 2004; Planchet et al., 2005; Jasid et al., 2006;).

Belefant-Miller *et al* (1994) first recorded that the resistance response of wheat to the RWA is a typical HR, commonly found during pathogenesis. Similar to the reactions that take place during pathogenesis, the intercellular β-1,3-glucanase, chitinase and peroxidase, were found to be involved at a secondary defense level during the RWA resistance response of wheat (van der Westhuizen et al., 1998 a, b). In addition, it was discovered that the ROS, particularly $H_2O_2$, are somehow involved in the RWA

resistance response of wheat by acting as one of the earliest signal molecules for the induction of secondary defense related enzymes (Moloi and van der Westhuizen, 2006).

Literature revealed that during the defense against pathogens, ROS and NO may act synergistically (Wang and Wu, 2004) or independently (Noritake et al., 1996; Delledonne et al., 1998). Therefore, a discovery that $H_2O_2$ is somehow involved in the RWA resistance response of wheat (Moloi and van der Westhuizen, 2006) suggests a need for the establishment of a relationship between NO and $H_2O_2$ during the RWA resistance response of wheat. Moreover, it was discovered that NADPH oxidase, which is a $O_2^-$ generating enzyme, is one of the earliest enzymes stimulated during the RWA resistance response (Moloi and van der Westhuizen, 2006). Therefore it is crucial to explore if a relationship exists between NO and $O_2^-$ during the RWA resistance response, because a reaction between these two can lead to the formation of a very toxic oxidant, peroxynitrite.

It has been discovered that SA plays a very important role as signal molecule for induction of the peroxidase enzyme activity during the RWA resistance response of wheat (Mohase and van der Westhuizen, 2002). This study revealed that the mechanism of action of SA during the RWA resistance does not involve a SA-inhibitable catalase. This revelation denotes that the resistance response of wheat to RWA does not only involve $H_2O_2$, but other molecules such as NO may be involved.

The correlation between NO and SA during pathogenic defense responses (Song and Goodman, 2001; Zottini et al., 2007; Gaupels et al., 2008) stimulates an interest to investigate the relation between NO and SA production during the RWA resistance response of wheat.

## 2.6 Objectives

To date, there are no reports on the involvement of NO in the resistance response of wheat against the RWA. Common resistance responses shared between pathogenesis and the RWA resistance response evoked our interest in elucidating the involvement of RNS in the RWA resistance response of wheat. Specific objectives of this study were to investigate:

1. whether NO is produced during the RWA resistance response;
2. which enzyme (s) is/ are mainly responsible for NO production in the RWA resistance response;
3. the involvement of NO in the secondary RWA defense response;
4. the use of NO in secondary applications such as reduction of symptom development and RWA aphid population;
5. whether ROS and NO can act in conjunction to produce $ONOO^-$ during the RWA resistance;
6. the involvement of $ONOO^-$ in the RWA resistance response of wheat.

# CHAPTER 3

## 3. MATERIALS AND METHODS

### 3.1 Plant material and infestation procedure

Resistant wheat (*Triticum aestivum*) cv. *Tugela DN*, containing the *Dn1* (PI 137739) resistance gene (Du Toit, 1989) and near-isogenic susceptible wheat cv. *Tugela* were grown under greenhouse conditions in trays, at temperatures of 24 $^{o}$C (± 2 $^{o}$C). Culture conditions and infestation procedures were as described by Du Toit (1988). Plants were infested at the early three-leaf stage by scattering Russian wheat aphids (RWAs), *Diuraphis noxia* (Mordvilko), biotype *RWASA1* [originally supplied by Agricultural Research Council- Small Grain Institute (ARC-SGI), Bethlehem, RSA] *,* onto the leaves, at approximately 20 RWAs per plant. Another set of plants (resistant and susceptible) was left uninfested as control. Second and third leaves of plants were harvested after specific time periods (0, 3, 6, 9, 12, 24, 48 and 72 hours post infestation, h.p.i) and frozen immediately in liquid nitrogen.

### 3.2 Treatment of plants with urate

The resistant plants were treated with 1 mM urate [dissolved in Hoagland solution (Hoagland and Arnon, 1950)] through the roots 2 hours before RWA infestation. Leaves were harvested 12 h.p.i (for peroxynitrite determination) and 48 h.p.i (for the measurement of peroxidase and β-1.3-glucanase activities). For the *in vitro* effect on

peroxidase and β-1,3-glucanase activities, urate (at a final concentration of 1 mM in a reaction mixture) was added directly to the reaction mixture.

### 3.3 Sodium nitroprussside (SNP) application

Resistant and/ or susceptible plants (in the early three leaf stage) were supplied with Hoagland solution containing different concentrations of SNP (0.15 mM or 0.5 mM) for the duration of the experiment through the roots (vermiculite was used as a supporting material). Leaves were then harvested at particular time intervals (24, 48, or 72) hours post treatment and/ or infestation.

In the case where SNP was applied as a seed dressing, susceptible and resistant seeds were soaked in different concentrations of SNP (0.15 mM or 0.5 mM) for 1 hour before planting. Control seeds were only soaked in distilled water for 1 hour before planting.

### 3.4 Inhibition studies

#### *3.4.1 Nitrate reductase (NR)*

For the *in vivo* inhibition effect, resistant plants (at early three leaf stage) were infested with RWA and then supplied with a Hoagland solution containing 4.1 mM sodium tungstate ($Na_2WO_4$) for the duration of the experiment. Leaves were then harvested 9 h.p.i (for NR activity). Control plants were only infested with the RWAs. For the *in vitro* effect, $Na_2WO_4$ (at a final concentration of 4.1 mM in a 0.5 mL reaction mixture) was added directly to the reaction mixture.

*3.4.2 Nitric oxide synthase (NOS)*

The RWA infested resistant and susceptible plants were treated with Hoagland solution containing an inhibitor of all the three isoforms of NOS, $N^{\omega}$-nitrilo-L-arginine-methyl ester (L-NAME) (50 mM), through the roots for the duration of the experiment. Control plants were grown in normal Hoagland solution. Nitric oxide (NO) content was afterwards measured after specific time periods of infestation in these plants, to see if NOS has any effect on NO production during the RWA resistance responses.

*3.4.3 β-1,3-glucanase and peroxidase*

For the *in vivo* inhibition effect, resistant plants (at early three leaf stage) were infested with the RWA and then supplied with a Hoagland solution containing 4.1 mM sodium tungstate ($Na_2WO_4$, an inhibitor of NR) or 50 mM $N^{\omega}$-nitrilo-L-arginine-methyl ester (L-NAME, an inhibitor of NOS) through the roots, using vermiculite as a supporting material for the duration of the experiment. Leaves were then harvested 48 h.p.i (for the intercellular pexoxidase and β-1,3-glucanase activities). Control plants were only infested with the RWAs. For the *in vitro* effect, $Na_2WO_4$ (at a final concentration of 4.1 mM in a 0.5 mL reaction mixture) was added directly to the reaction mixture.

**3.5 Involvement of NR and nitrite reductase (NiR) in NO production**

A pathway of NO production was investigated by modifying a method described by Xu and Zhao (2003). Resistant wheat plants were grown in a modified Hoagland solution containing five times higher $Cu^{2+}$ (which is known to inhibit both NR and NiR)

concentration than that in the original Hoagland solution for 14 days, subsequently infested with RWA and then supplied with 1 mM $NaNO_2$ as a substrate except for the control. NO content, NR and NiR activities were then measured 9 hours after treatment.

### 3.6 Collection of the intercellular washing fluids (IWF)

Leaves from both the resistant and susceptible plants were cut in 10 cm long pieces, thoroughly rinsed in distilled water, and then vacuum infiltrated with 50 mM Tris buffer (pH 7.8) for 5 minutes. The leaves were dried on a blotting paper, inserted vertically in a centrifuge tube with a perforated disc at the bottom, and centrifuged (5000 x *g*) at – 4 $^{o}$C for 5 minutes. After centrifugation, the IWF was collected from the bottom of the centrifuge tube, and the procedure was repeated using the same leaves. The combined IWF was frozen in liquid nitrogen and stored at – 20 $^{o}$C for the assay of the intercellular β-1,3-glucanase and peroxidase activities.

### 3.7 Extraction procedure

The extract (for NR, NOS, peroxynitrite and NO assays) was prepared according to the method described by Xu and Zhao (2003). Each 0.5 g frozen leaf tissue was ground in 1 mL of 50 mM potassium phosphate buffer (pH 8.8) consisting of 1 mM EDTA, 25 mM cysteine, and 3 % (m/v) Bovine serum albumin (BSA) (Sigma-Aldrich). The homogenate was centrifuged at 12 000 x *g* for 20 minutes (4 $^{o}$C). The supernatant was used as the enzyme extract.

### 3.8 Protein concentration

The protein content of the enzyme extracts was determined according to a modified method of Bradford (1976). The assay mixture consisted of 160 µL distilled water, 40 µL Biorad (Bio-Rad laboratories GmbH), and 10 µL enzyme extract or standard (0.5 mg $mL^{-1}$). The absorbance was measured at 595 nm using the Biorad microplate reader. BSA was used as a standard.

### 3.9 Nitric oxide (NO) production

NO was determined according to a spectrophotometric method described by Murphy and Noack (1994). This method was proven to be the best suited for the quantification of tissue NO (Ederli et al., 2009). The reaction was initiated by incubating a mixture of 40 µL plant extract (see paragraph 3.7 for extraction procedure), 100 units catalase (Roche Diagnostics GmbH), 100 units superoxide dismutase (Sigma-Aldrich), and 934 µL 50 mM potassium phosphate buffer (pH 7.4) for 5 minutes. Thereafter, 10 µL oxyhemoglobin (1 mM) was added to the reaction mixture and further incubated for 7 minutes. NO production was measured by spectrophotometric measurement (at 401 and 421 nm) of the conversion of oxyhemoglobin to methemoglobin. This technique is based on the direct reaction between NO and the oxygenated, ferrous form of hemoglobin ($HbO_2$), which yields the ferric form, methemoglobin, and nitrate. The reference mixture contained 10 µL phosphate buffer in place of the plant extract. The amount of NO produced was calculated from $A_{401}$-$A_{421}$ ($\Delta\varepsilon$ = 77 $mM^{-1}cm^{-1}$) and expressed as mM NO $min^{-1}$ $g^{-1}$ fresh mass.

Oxyhemoglobin was prepared as follows: 25 mg of hemoglobin (Hemoglobin from rabbit, Sigma-Aldrich) was dissolved in 1 mL of phosphate buffer. Sodium dithionite (2 mg) was dissolved in 1 mL hemoglobin solution to form methemoglobin solution (dark red/maroon), which was then swirled gently under normal air until it turned bright red (oxyhemoglobin). Oxyhemoglobin was desalted by passing through Sephadex G-25 column (2 $cm^2$ area and 15 cm long).

### 3.10 Nitrate reductase activity

Nitrate reductase (NR) activity was assayed according to the modified spectrophotometric stop rate determination method described by Xu and Zhao (2003). The assay mixture of 0.5 mL contained 50 µL 50 mM $KNO_3$, 50 µL 0.5 mM NADH, 100 µL enzyme extract and 300 µL phosphate buffer (50 mM, pH 7.0). For the NR *in vitro* effect, $Na_2WO_4$ (or $H_2O$ for the control) was added at a final concentration of 4.1 mM in a 0.5 mL reaction mixture. The mixture was incubated at 25 $^oC$ for 30 minutes, boiled for 1 minute and then cooled at room temperature. The amount of $NO_2^-$ produced was estimated by adding 0.125 mL 14mM 1-naphthanylamine, 0.125 mL 58 mM sulfanilamide (dissolved in 3N HCl) to the mixture. The final mixture was kept at 25 $^oC$ for 15 minutes and the absorbance was read at 540 nm. The amount of $NO_2^-$ produced was read from a $NaNO_2$ standard curve. NR activity was expressed as mM $NO_2^-$ $mg^{-1}$ prot. $min.^{-1}$

### 3.11 Nitrite reductase (NiR) activity

Nitrite reductase activity was determined according to the spectrophotometric stop rate determination method described by Datta and Sharma (1999). The assay mixture consisted of 1.4 mL of 100 mM potassium phosphate buffer (pH 7.5), 100 µL 9 X diluted enzyme extract, 100 µL 5 mM $NaNO_2$, 100 µL 2 mg $mL^{-1}$ methyl viologen. The volume was made up to 1.8 mL with distilled water. To start the assay, 200 µL (25 mg $mL^{-1}$ in 290 mM $NaHCO_3$) $Na_2S_2O_4$ was added and incubated for 30 minutes at 30 $^oC$. At the end of the incubation period, 100 µL of the assay mixture was added to 1,9 mL of water and vortexed immediately to oxidize the dithionite. The reference reaction contained everything except the enzyme extract, and the blank contained everything except $NaNO_2$ and enzyme extract. The amount of $NO_2^-$ converted by NiR was estimated by adding 1 mL sulfanilamide (1 % w/v in 3N HCl) and 1 mL 0.05 % (w/v) N-(1-naphthyl) ethylene diamine dichloride (NED) solution. The solution was incubated at 30 $^oC$ for 30 minutes and the absorbance was read at 540 nm. NiR activity was expressed as % $NO_2^-$ reduction $mg^{-1}$ prot.

### 3.12 Salicylic acid (SA) content

Total SA (free and conjugated forms) was extracted from SNP or $NaWO_4$ treated resistant wheat plants using a modified method of Tuula *et al* (1994). Leaf tissue (0.5 g) was ground to a fine powder in liquid nitrogen. Thereafter 1 mL 80% (v/v) ethanol was added to the powder, vortexed and centrifuged (20,400 x *g*, 20 min, 4 $^{\circ}C$). Supernatant was collected and the procedure was repeated. The combined supernatant was kept at – 20°C for 1 hour, and then centrifuged again (20,400 x *g*, 10min, 4 °C). The

supernatant was concentrated under reduced pressure in a rotavapor to a tenth of the original volume and adjusted to 4mL with 2% (w/v) metaphosphoric acid in double distilled water. This extract was then partitioned three times with ethyl acetate (1: 1). The combined ethyl acetate extract was evaporated to dryness under reduced pressure (45 °C). The remaining aqueous phase was adjusted to 1mol $L^{-1}$ HCl and heated (80 °C) in a sealed test tube for 1 hour, then cooled and partitioned three times with ethyl acetate (1:1). The combined ethyl acetate extract was dried under reduced pressure (45 °C). Extracts before and after acid hydrolysis were each re-dissolved in 3mL 70% (v/v) methanol, combined and then passed through a C18 Sep Pak cartridge (Waters) that had been pre-equilibrated with 5mL 70% (v/v) methanol. SA was eluted with 6mL 70% (v/v) methanol. The eluate was dried under reduced pressure (45 °C) and re-dissolved in 400µL of the HPLC mobile phase. The mobile phase consisted of water, acetonitrile and 10% (v/v) phosphoric acid (74.5:24.5:1). SA was analyzed by HPLC using a C18 reverse phase column at a flow rate of 0.6 mL $min^{-1}$ at ambient temperature. Detection was by means of an UV-detector at 240 nm. The amount of SA present was determined from SA standard curve which was prepared by using different concentrations (in a range of 0.01 to 1 mM) of SA (Merck) in 70 % (v/v) methanol subjected to similar experimental conditions as the plant extracts. SA content was expressed as mM SA $g^{-1}$ fresh mass.

### 3.13 Intercellular peroxidase activity

A modified method of Zieslin and Ben-Zaken (1991) was used. The assay mixture (1 mL) contained 10 µL IWF, 50 µL 8.2 mM $H_2O_2$, 100 µL 50 mM guaiacol, 340 µL double distilled water and 500 µL 80 mM potassium phosphate buffer (pH 5.5). The absorbance increase was measured at 470 nm for 3 minutes at 30 °C against a blank containing all the reagents except for the IWF, which was replaced by 50 mM Tris buffer (pH 7.8). The increase in absorbance (tetraguaiacol formed) represented the rate of $H_2O_2$ reduction by peroxidase with guaiacol as a hydrogen donor. The enzyme activity was expressed as mM tetraguaiacol $mg^{-1}$ prot. $min^{-1}$ (using guaiacol extinction coefficient of 26.6 $mM^{-1}cm^{-1}$).

### 3.14 Intercellular β-1,3-glucanase activity

A modified method of Fink et al. (1988) was used. The assay mixture contained 10 µL IWF, 250 µL 2 mg $mL^{-1}$ laminarin and 240 µL 50 mM sodium acetate buffer (pH 4.5). After incubation at 37 $^oC$ for 10 minutes, 500 µL of Somogyi reagent [0.2 g $CuSO_4$, 9 g $Na_2SO_4$, 1.2 g $NaCO_3$, 0.8 g $NaHCO_3$, and 0.6 g potassium tartrate in 50 mL double distilled water, (Somogyi, 1952)] was added and incubated at 100 $^oC$ for 10 minutes. After cooling under tap water, 500 µL of Nelson's reagent [2.65 g $(NH_4)_6$ $Mo_7O_{24}$, 2 mL 95-97 % $H_2SO_4$, 0.32 g $Na_2HAsO_4.7H_2O$ in 50 mL double distilled water, (Nelson, 1944)] was added. The absorbance (which represented the amount of glucose formed) was measured at 540 nm. The blank and the glucose standards used to prepare a standard curve were subjected to the same procedure. The quantity of glucose

produced from laminarin was determined from the glucose standard curve (which was subjected to similar experimental conditions) and β-1,3-glucanase activity was expressed as mg glucose $mg^{-1}$ prot. $min.^{-1}$

### 3.15 Peroxynitrite ($ONOO^-$) content

Peroxynitrite content was measured spectrophotometrically according to the method described by Yamasaki and Sakihama (2000). The reaction mixture (1 mL) contained: 20 mM potassium phosphate buffer (pH 7.0), 100 µM 2',7'-dichlorodihydrofluorescein (DCDHF), 2 mM $NaNO_2$, 1 mM NADH and 40 µL plant extract. The mixture was incubated at room temperature for 20 minutes and thereafter absorbance of dichlorofluorescein (DCF) was measured at 500 nm for 10 minutes. DCDHF is supplied as the diacetate ester. Following enzymatic or base-catalyzed cleavage of the diacetate groups by $ONOO^-$, it is readily oxidized to the highly fluorescent product DCF. $ONOO^-$ efficiently mediates this oxidation and neither NO, superoxide, nor hydrogen peroxide alone appears to oxidize DCDHF (Crow, 1997; Kooy et al., 1997). $ONOO^-$ concentration was expressed in $A_{500\ nm}$ $mg^{-1}$prot.

### 3.16 Statistical analysis

For all assays, at least two separate experiments (i.e. planting new sets of resistant and susceptible plants) were conducted and, within each experiment, assays were done in triplicate. The data were analyzed using Sigma Plot version 9.0 of SYSTAT software followed by the *t*-test statements to ensure that mean values of the two data columns

(infested resistant and uninfested resistant plants, or infested resistant and infested susceptible plants, or treated and non-treated plants) are significantly different (i.e. if *P* value is ≤ 0.05).

## 3.17 Reverse transcriptase polymerase chain reaction (RT-PCR)

### *3.17.1 RNA extraction*

A quantity of liquid nitrogen ground leaf material (frozen) was transferred to a 1.5 mL eppendorf tube (up to a 100 µL mark). Trizol reagent (Invitrogen) (500 µL) was added to the powder, vortexed for 10 seconds and then incubated at room temperature for 10 minutes. After this step, 100 µL chloroform was added and the tube was inverted 15 times to precipitate the DNA and proteins, and subsequently incubated at room temperature for 5 minutes and centrifuged (12 000 x *g* for 15 min, 4 $^{o}$C). The clear supernatant (250 µL) was transferred to a new tube containing 250 µL isopropanol, mixed well and then incubated at room temperature for 10 minutes. The tube was again centrifuged (12 000 x *g* for 10 minutes, 4 $^{o}$C) to pellet the RNA. Supernatant was removed by suction (water jet pump) and 500 µL 70 % ethanol was added to the remaining pellet in the tube to dissolve all the salts. The tube was centrifuged at 7 500 x *g* for 10 minutes, 4 $^{o}$C. The supernatant was completely removed (water jet pump) and the sample was left for 5 minutes to dry. DEPC water (200 µL) was added to the sample before incubation on ice for 1 hour. The liquid in the tube was drawn up and down to dissolve the RNA, and then centrifuged for 5 minutes (12 000 x *g* at 4 $^{o}$C) to pellet any undissolved RNA. The RNA (supernatant) was transferred to a new tube.

*3.17.2 RNA concentration*

Extracted RNA was diluted 50X with DEPC water and absorbance for each sample was measured at 260 and 280 nm. These values were used to determine the RNA concentration on the excel template. The RNA (500 ng) was separated on a 1.2 % (w/v) agarose gel to confirm the quality and quantity. RNA was further diluted to final concentration of 5 ng $^{-1}$ µL, and 2 µL was used for each RT-PCR reaction.

*3.17.3 RT-PCR*

The Robust T II-PCR kit (Finnzymes) was used. The reaction mixture (10 µL) contained: 10 ρmol primers (reverse and forward primers), 10 x reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphates (dNTPs), 10 ng RNA, 5 U MuLVRT and 1 U DyNAzyme. Amplification was performed under the following conditions:

48 $^{o}$C at 30 min, 94 $^{o}$C for 2 min, 30 cycles of 94 $^{o}$C for 30 sec, 61 $^{o}$C for 30 sec and 72 $^{o}$C for 1 min. And a final step at 72 $^{o}$C for 5 min.

**3.18 The effect of sodium nitroprussside (SNP) on RWA attraction / repulsion**

SNP treated plants were arranged around a cardboard circle (30 cm in diameter). Forty RWAs were released in the center of each circle. Plants were left for 24 hours in the glasshouse [24 $^{o}$C (± 2 $^{o}$C)] before the aphid count on each plant. Seven plants in total were used per replication; 3 for the seed treatment (0.1 mM; 0.15 mM and 0.5 mM) and

3 for the root treatment (0.1 mM; 0.15 mM and 0.5 mM). The total number of replications was 6.

### 3.19 Intrinsic RWA increase rate

SNP treated plants were infested with two RWA adult apterae (biotype *RWASA1*) each. Age-specific survival ($l_x$) of nymphs and adults and age-specific fecundity ($m_x$) were recorded at 24 hour intervals for a period of 72 hours. Intrinsic rate of increase ($r_m$) was determined using the formula:

$r_m = Ln(R_o)/T$ where $R_o=\sum l_x m_x$ and $T=l_x m_x x$;

x-age at beginning of interval

$l_x$-age specific survivorship

$m_x$-expected daughters

$l_x m_x$-reproductive expectation

T-mean generation time

$R_o$-net reproductive rate

### 3.20 Symptom analysis

Symptom development on plants was analyzed according to a method described by Tolmay (1995), by using a scoring system whereby a plant with a score of 1 ≤ 3.5 represented a highly resistant plant (no leaf curling), 3.6 ≤ 6.5 represented a medium resistant plant, and 6.6 ≤ 10 represented a susceptible plant (curled leaves and severe chlorosis).

# CHAPTER 4

## 4. RESULTS

For all investigations, representative results of an independent experiment are presented here. **See appendix for the results of independent replicate experiments.**

### 4.1 Nitric oxide (NO)

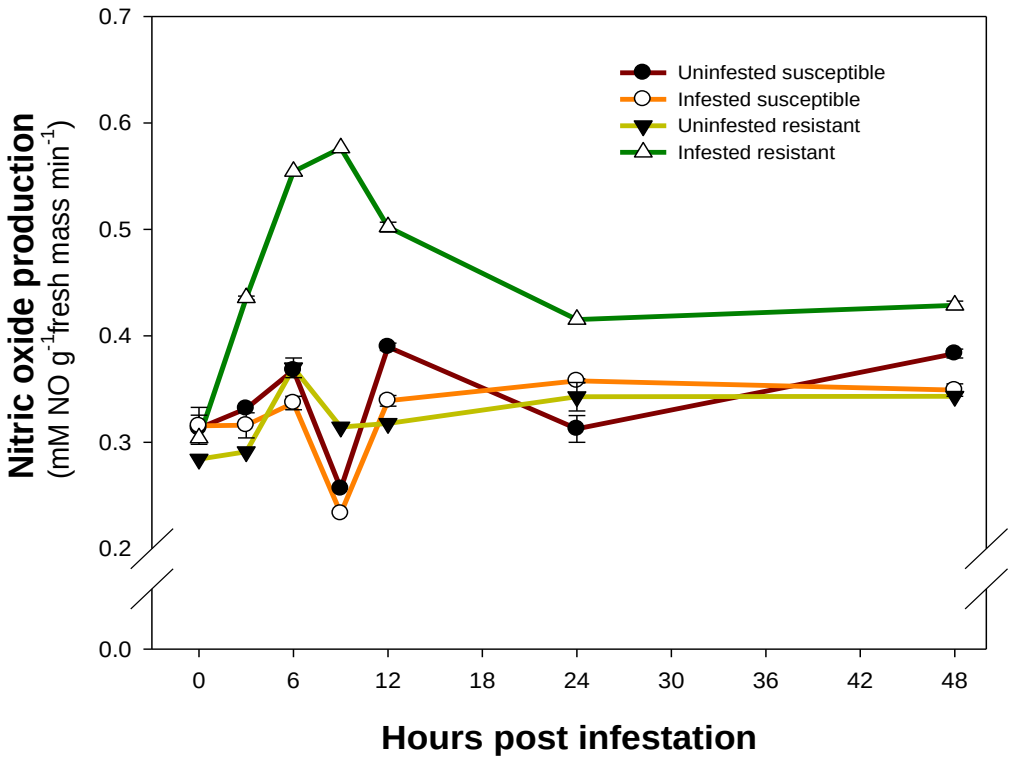


**Figure 4.1.** Effect of RWA infestation on NO production of resistant (*Tugela DN*) and susceptible (*Tugela*) wheat cultivars. Values are means SD ± (n = 3).

An early transient induction of NO production occurred in RWA infested resistant plants. Induction occurred as early as 3 hours post infestation (h.p.i) and peaked 9 h.p.i, representing a 45 % ($P$ = 0.0046) increase in the infested resistant plants. Later on, a continuous drop in NO production occurred (Fig. 4.1).

## 4.2 Potential NO producing enzymes involved in the RWA resistance response of wheat

### *4.2.1 Nitrate reductase (NR)*

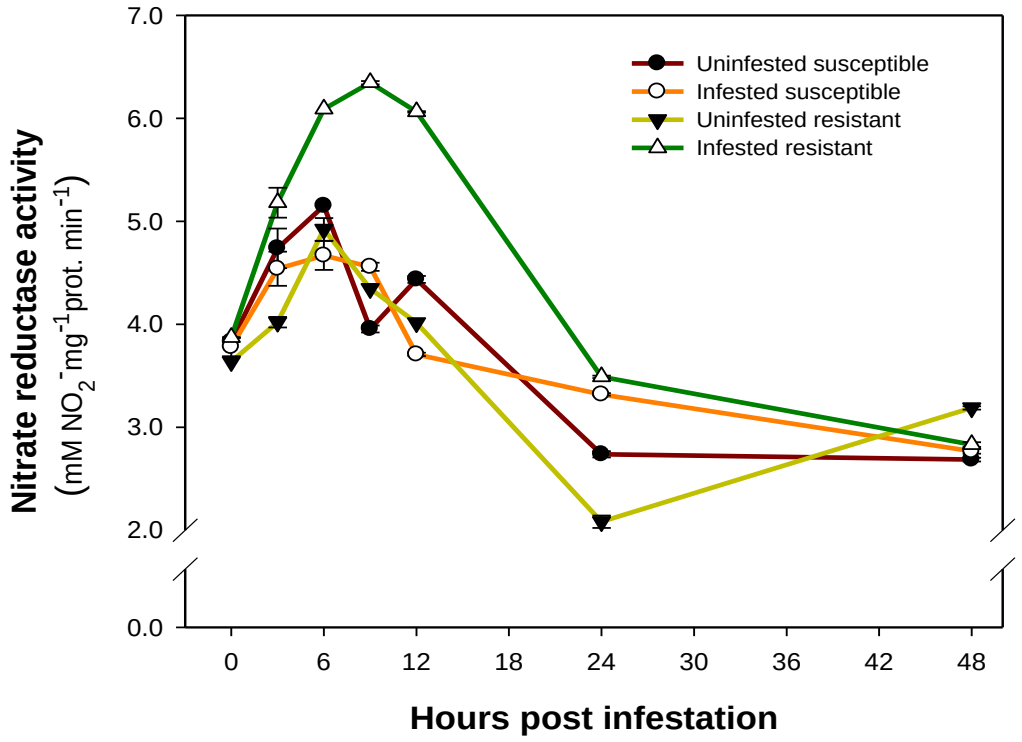


**Figure 4.2.** Effect of RWA infestation on NR activity of susceptible (*Tugela*) and resistant (*Tugela DN*) wheat cultivars. Values are means ± SD (n=3).

RWA infestation caused an early (3 h.p.i) induction of NR activity to higher levels in the resistant than susceptible plants. Peak activity, representing a 39 % increase (*P* = 0.000005), was reached 9 h.p.i, followed by a slow decrease. This early transient increase is also illustrated in Figure 4.3. NR activity in the controls and the infested susceptible plants was only slightly induced for a shorter period (3- 6 h.p.i) (Fig. 4.2).

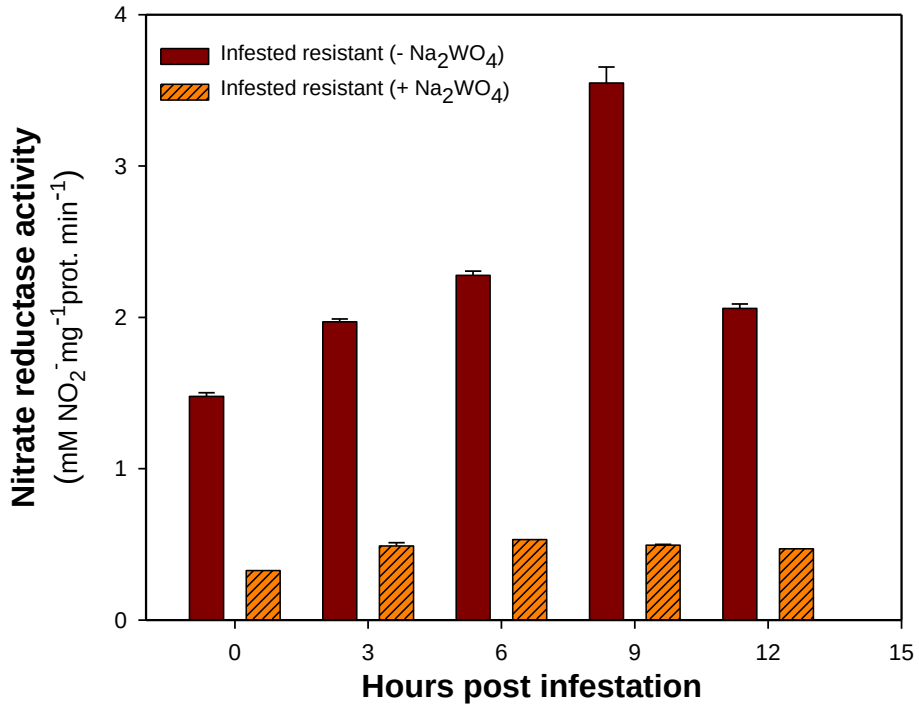


**Figure 4.3.** Effect of sodium tungstate ($Na_2WO_4$) on NR activity (*in vitro*) of a RWA infested resistant (*Tugela DN*) wheat cultivar. Values are means ± SD (n=3).

*In vitro* inhibition of NR activity [by adding sodium tungstate ($Na_2WO_4$) to the reaction mixture] led to significant inhibition of NR activity (> 70 % reduction for all samples tested) (Fig.4.3).

Treatment of the infested resistant plants with $Na_2WO_4$ (*in vivo*) led to a 40 % reduction in NR activity. Consequently, 48 % (*P* = 0.024) alleviation in NO production followed (Fig. 4.4).

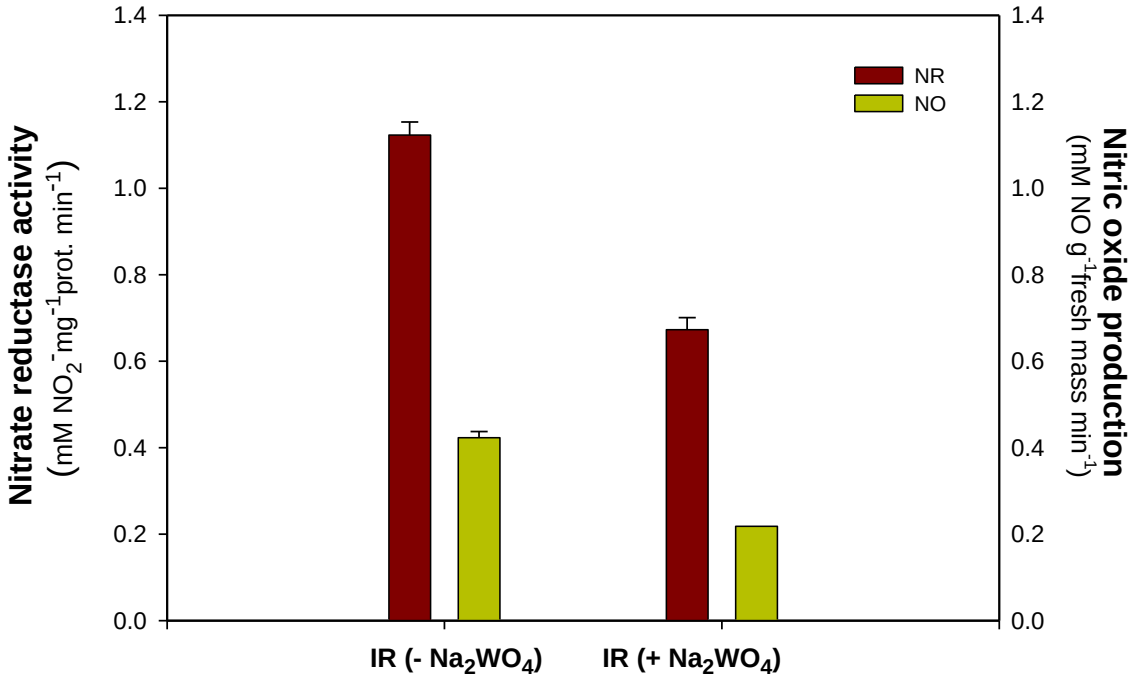


**Figure 4.4**. Effect of sodium tungstate ($Na_2WO_4$) on NR activity and NO production (*in vivo*) of a RWA infested resistant (*Tugela DN*) wheat cultivar (9 h.p.i). Values are means ± SD (n=3).

*4.2.2 Nitrite reductase (NiR)*

In the resistant plants, an increase in NiR activity (10 %) was observed as early as 3 h after RWA infestation. Peak activity, representing a 27 % increase ($P$ = 0.004), occurred 9 h after infestation and thereafter gradually decreased to levels which were still high compared to the control and the infested susceptible plants (Fig. 4.5).

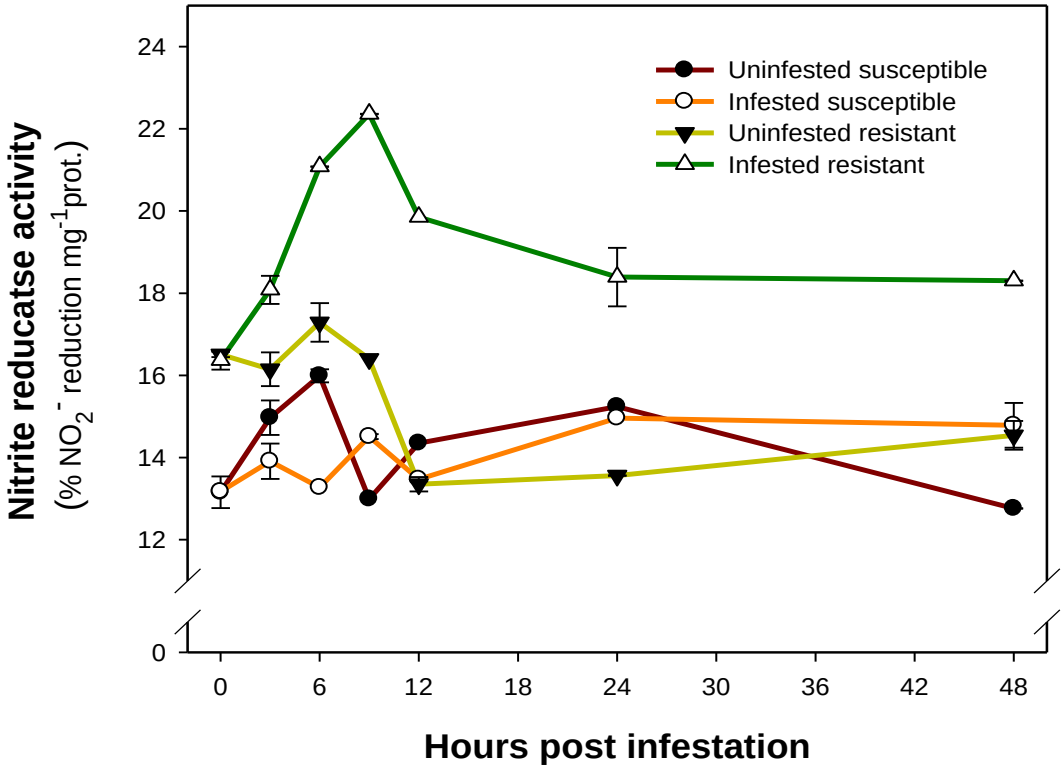


**Figure 4.5.** Effect of RWA infestation on NiR activity of susceptible (*Tugela*) and resistant (*Tugela DN*) wheat cultivars. Values are means ± SD (n=3).

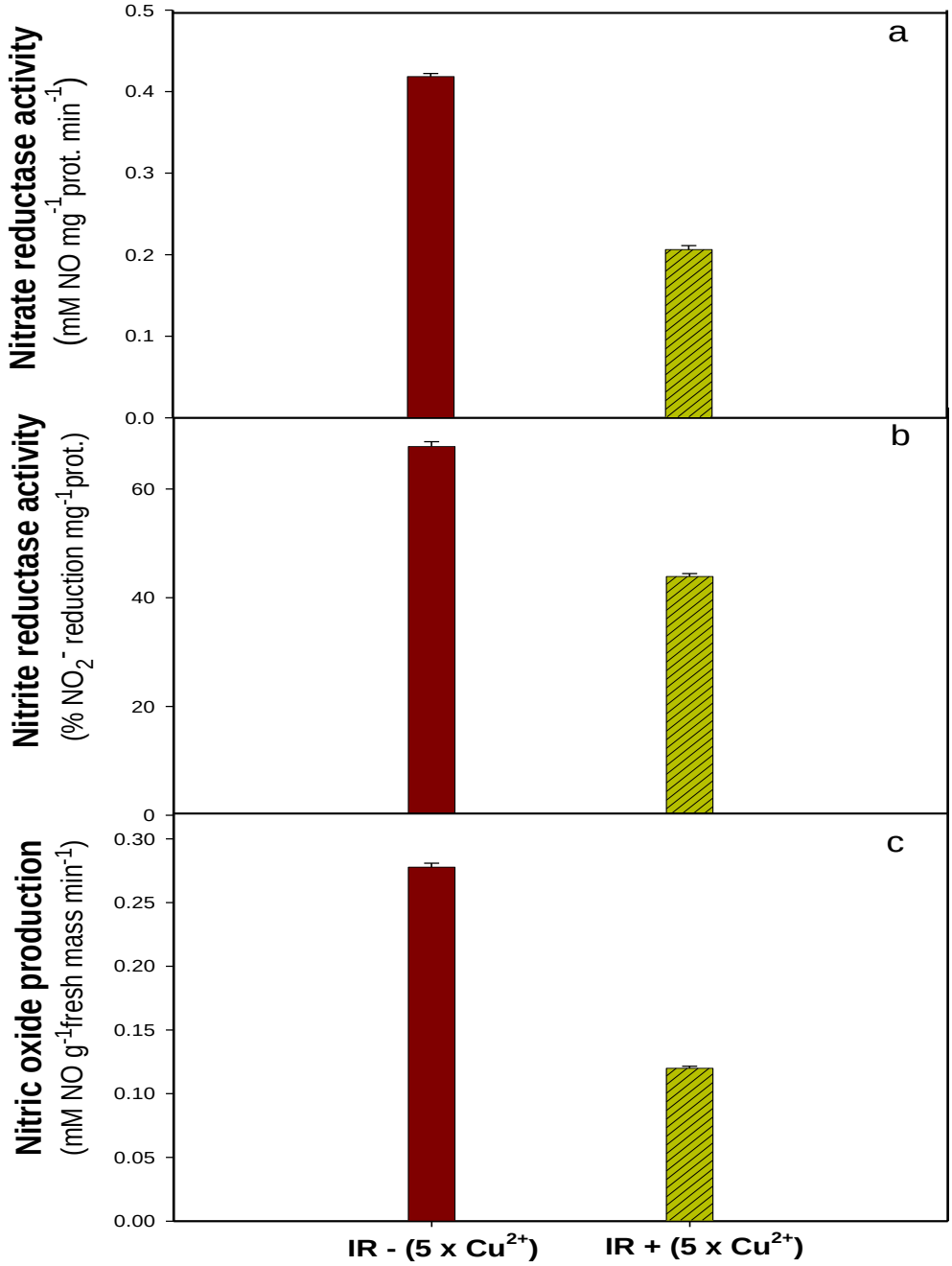


**Figure 4.6**. Effect of excess $Cu^{2+}$ (five times more) in the growth medium (Hoagland and Arnon, 1950) supplemented with 1mM $NaNO_2$ on the activities of NR (a) and NiR (b), as well as on NO production (c) of a RWA infested (9 h.p.i) resistant (IR) wheat cultivar. Values are means ± SD (n=3).

Growing RWA infested resistant plants in Hoagland solution (Hoagland and Arnon, 1950) containing five times more $Cu^{2+}$ [which is known to inhibit the activities of both NR and NiR (Liorens et al., 2000)], substantially inhibited NR and NiR activities (51 %, *P* = 0.0005 and 36%, *P* = 0.013 respectively) 9 h.p.i (Fig. 4.6 a and b). Consequently, NO production was reduced (56 %, *P* = 0.00036) despite nitrite supplementation (Fig. 4.6 c).

*4.2.3 Nitric oxide synthase (NOS)*

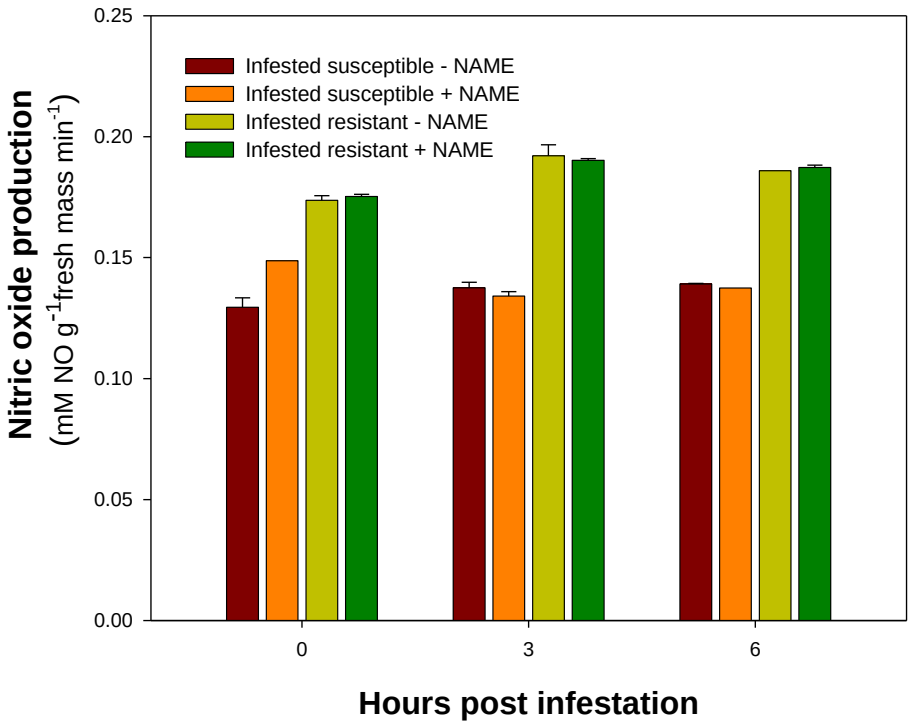


**Figure 4.7.** Effect of L-NAME (NOS inhibitor) on NO production of RWA infested susceptible (*Tugela*) and resistant (*Tugela DN*) wheat cultivars. Values are means ± SD (n=3).

$N^{\omega}$-nitrilo-L-arginine-methyl ester (L-NAME) treatment (*in vivo*) did not have any significant inhibitory effect on NO release of both the RWA infested susceptible and resistant plants. NO production was higher in the infested resistant than infested susceptible plants (Fig. 4.7).

### 4.3 Involvement of NO generating enzymes in the downstream defense response

According to Figure 4.8 (a), inhibition of NO production in the infested resistant plants by a nitrate reductase inhibitor ($NaWO_4$) (for reference, see Fig. 4.3 and Fig. 4.4), consequently leads to a decrease in activities of the secondary defense related enzymes (intercellular β-1,3-glucanase and peroxidase). β-1,3-glucanase and peroxidase activities were reduced by 16 % ($P$ = 0.021) and 70 % ($P$ = 0.009) respectively.

Incorporation of $NaWO_4$ in the reaction mixture (i.e *in vitro*) did not have any effect on activities of the intercellular β-1,3-glucanase and peroxidase (Fig. 4.8 b).

Inhibition studies using L-NAME (NOS inhibitor, Fig. 4.7) have indicated that NOS is not significantly involved in NO production during the RWA resistance response of wheat. Treatment of resistant plants (infested with RWA) with L-NAME for duration of the experiment (48 hours) did not have any significant effect on activities of the intercellular peroxidase (a) and β-1,3-glucanase (b) (Fig.4.9).

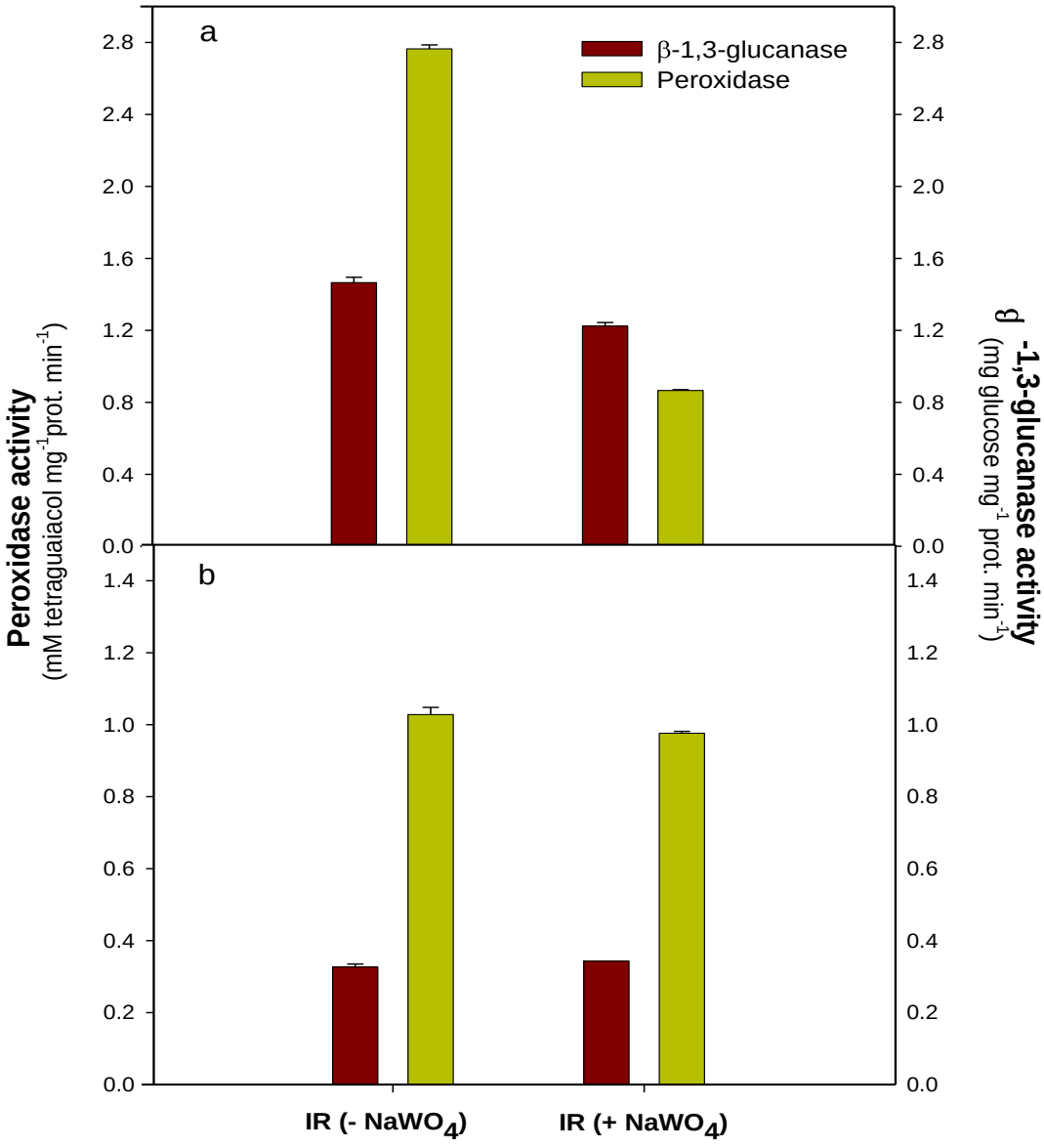


**Figure 4.8.** *In vivo* (a) and *in vitro* (b) effect of sodium tungstate ($NaWO_4$) (nitrate reductase inhibitor) on the intercellular β-1,3-glucanase and peroxidase activities of a RWA infested resistant (IR) (*Tugela DN*) wheat cultivar (48 h.p.i). Values are means ± SD (n = 3).

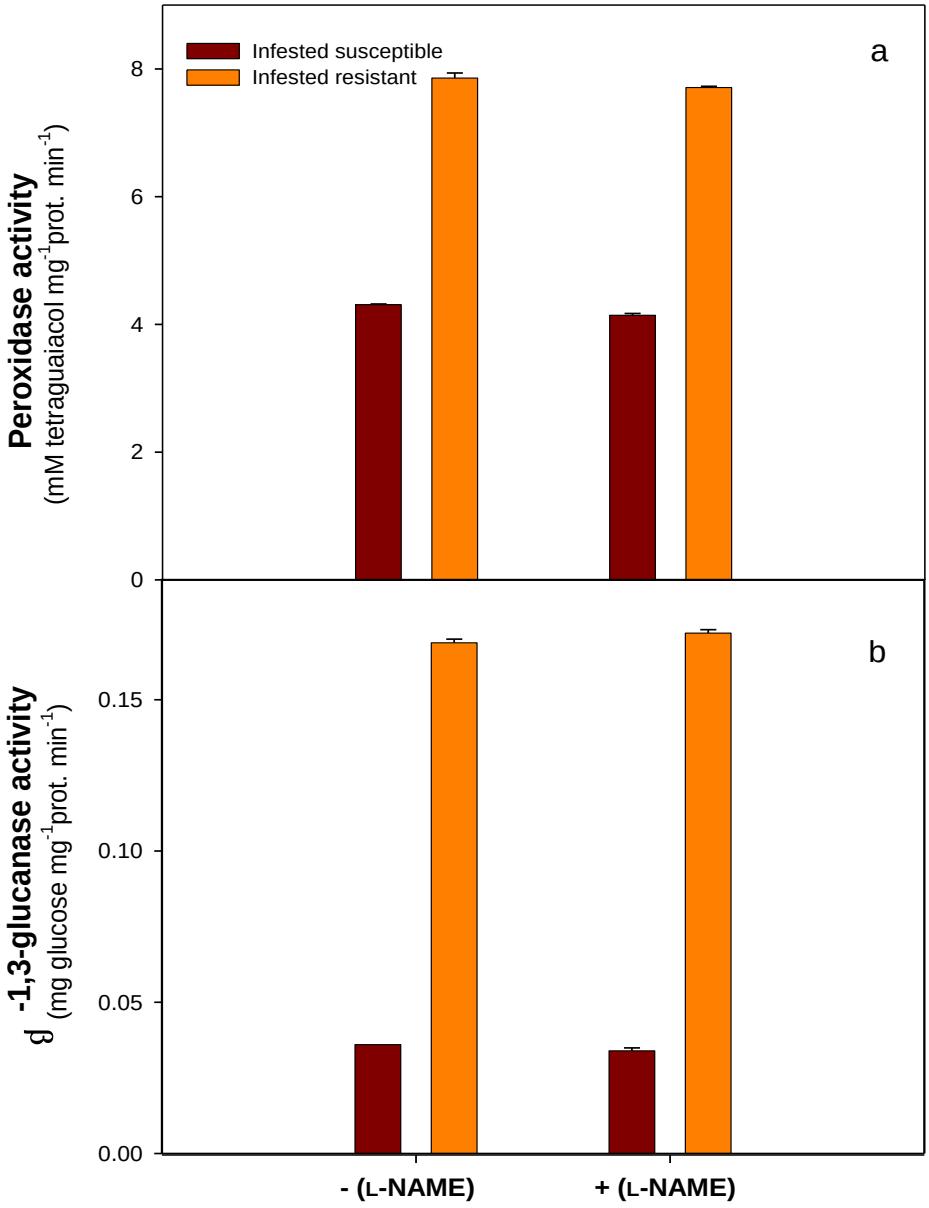


**Figure 4.9.** *In vivo* effect of L-NAME (NOS inhibitor) on the intercellular peroxidase (a) and β-1,3-glucanase (b) activities of RWA infested resistant (*Tugela DN*) and susceptible (*Tugela)* wheat cultivars (48 h.p.i). Values are means ± SD (n =3).

## 4.4 The effect of exogenous NO on the secondary defense response of wheat during the RWA infestation

### *4.4.1 Nitric oxide production, the intercellular β-1,3-glucanase and peroxidase activities*

NO production of RWA infested susceptible plants treated with 0.15 mM and 0.5 mM sodium nitroprusside (SNP), a NO donor, increased with 30 % and 59 % respectively. This amounts to a 0.8-fold higher increase in 0.5 mM SNP than 0.15 mM SNP treated plants (Fig. 4.10 a). Concomitantly, activities of the intercellular β-1,3-glucanase (Fig.4.10 b) and peroxidase (Fig.4.10 c) were abundantly induced (by 5-fold and 2.5-fold respectively) in the 0.5 mM SNP treated plants. No induction of β-1,3-glucanase and peroxidase activities occurred with the 0.15 mM SNP treatment.

Treatment of uninfested resistant plants with 0.15 mM SNP, stimulated significant increase in activities of the secondary defense related enzymes, i.e. 0.7-fold and 0.8-fold increase in the intercellular β-1,3-glucanase activity 24 and 48 hours after treatment respectively (Fig. 4.11 a), and a massive increase (75 %) in intercellular peroxidase activity 24 hours after SNP treatment (Fig. 4.11 b).

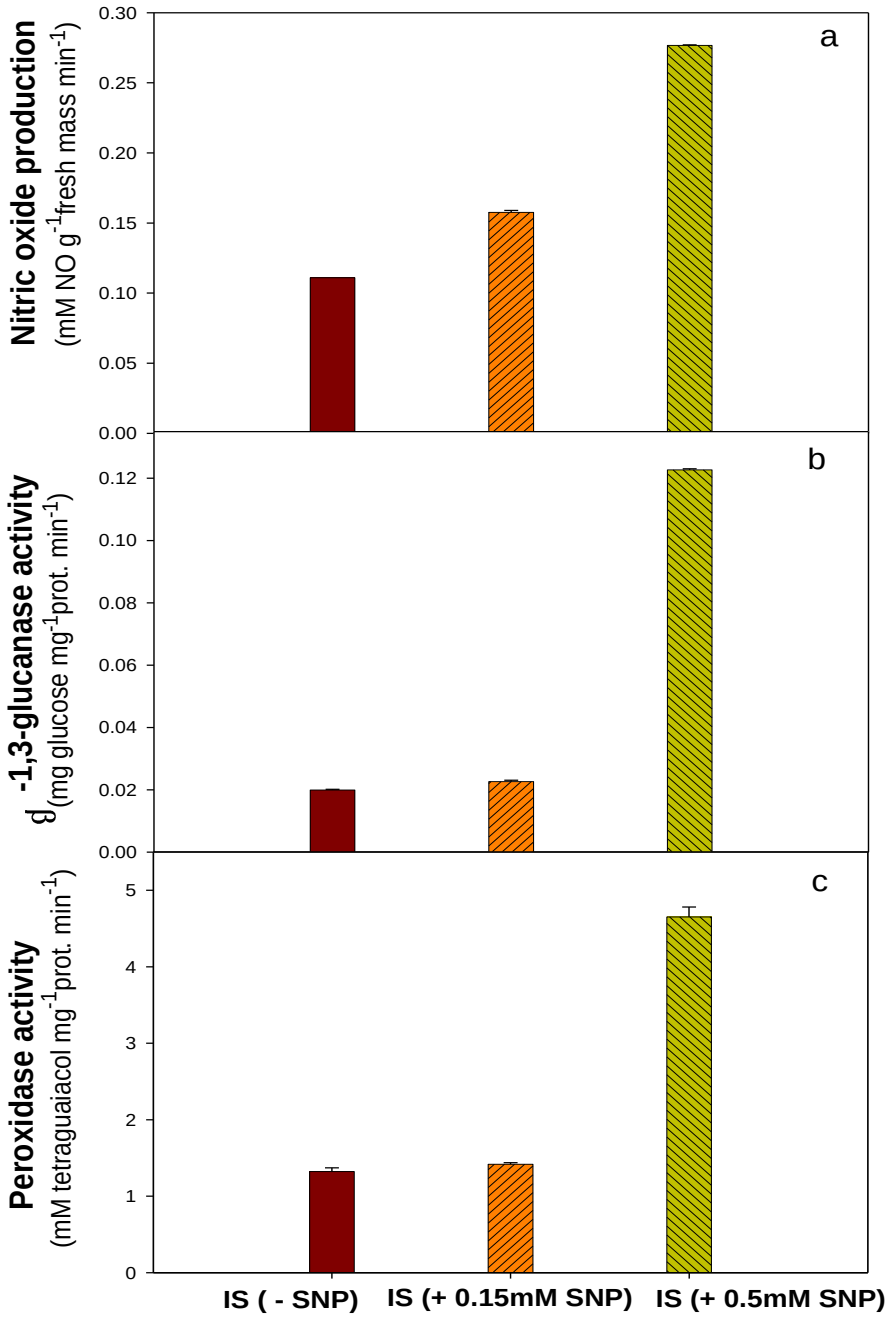


**Figure 4.10.** Effect of sodium nitroprusside (SNP) (root application) on NO production (a), the intercellular β-1,3-glucanase (b) and peroxidase (c) activities of a RWA infested susceptible (IS) (*Tugela*) wheat cultivar (48 h.p.i). Values are means ± SD (n = 3).

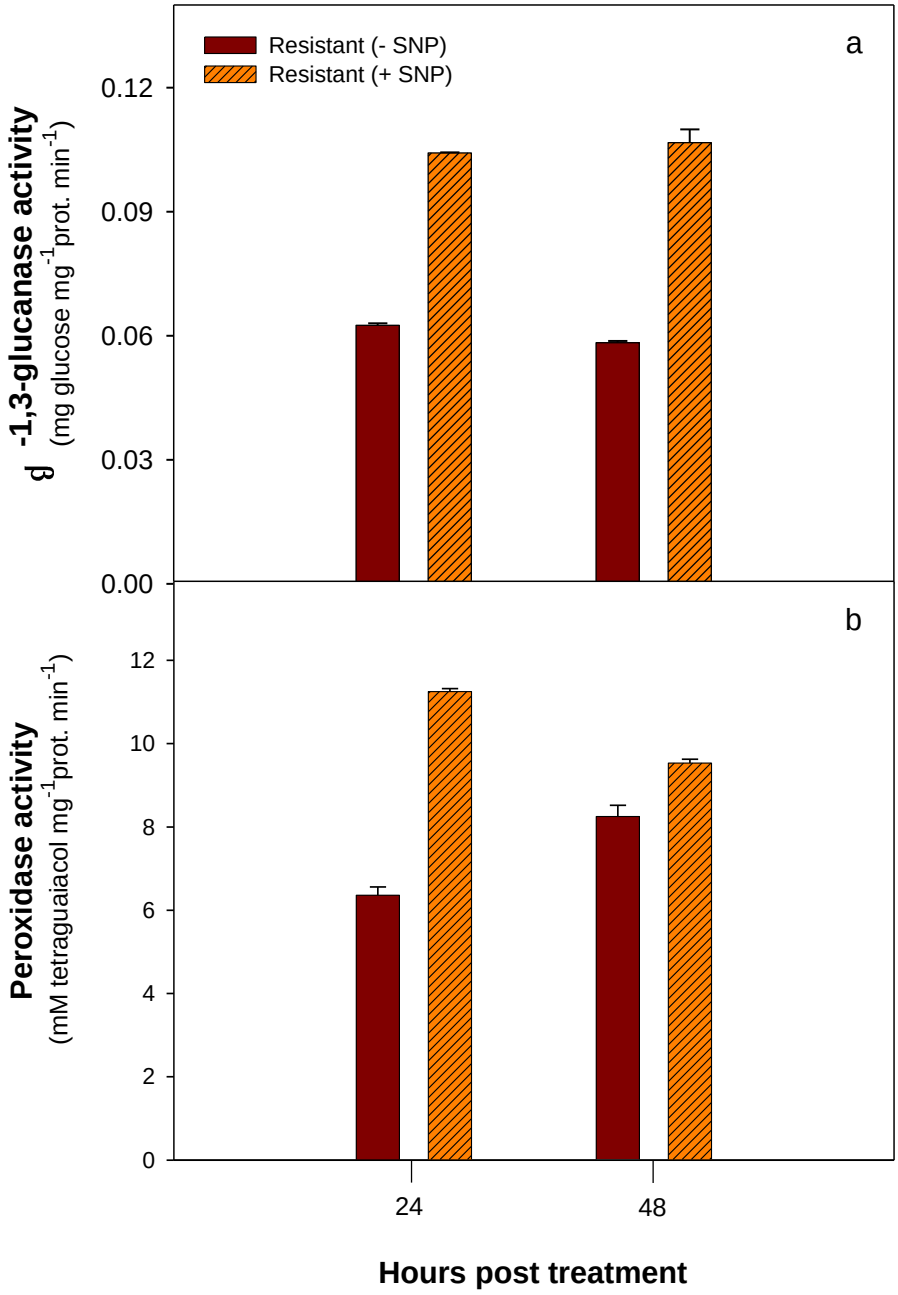


**Figure 4.11.** Effect of 0.15 mM SNP (root application) on the intercellular β-1,3-glucanase (a) and peroxidase (b) activities of a resistant (*Tugela DN*) wheat cultivar. Values are means ± SD (n = 3).

*4.4.2 Pathogenesis related (PR)- gene expression*

Treatment of the uninfested resistant plants with 0.15 mM SNP stimulated an induced expression of the PR-2 gene (β-1,3-glucanase) (24-72 hours post treatment, h.p.t). This gene was not expressed in the control resistant plants, but highly expressed in the infested resistant plants. In infested susceptible plants, PR-2 gene was expressed at much lower levels than in infested resistant plants (Fig. 4.12).

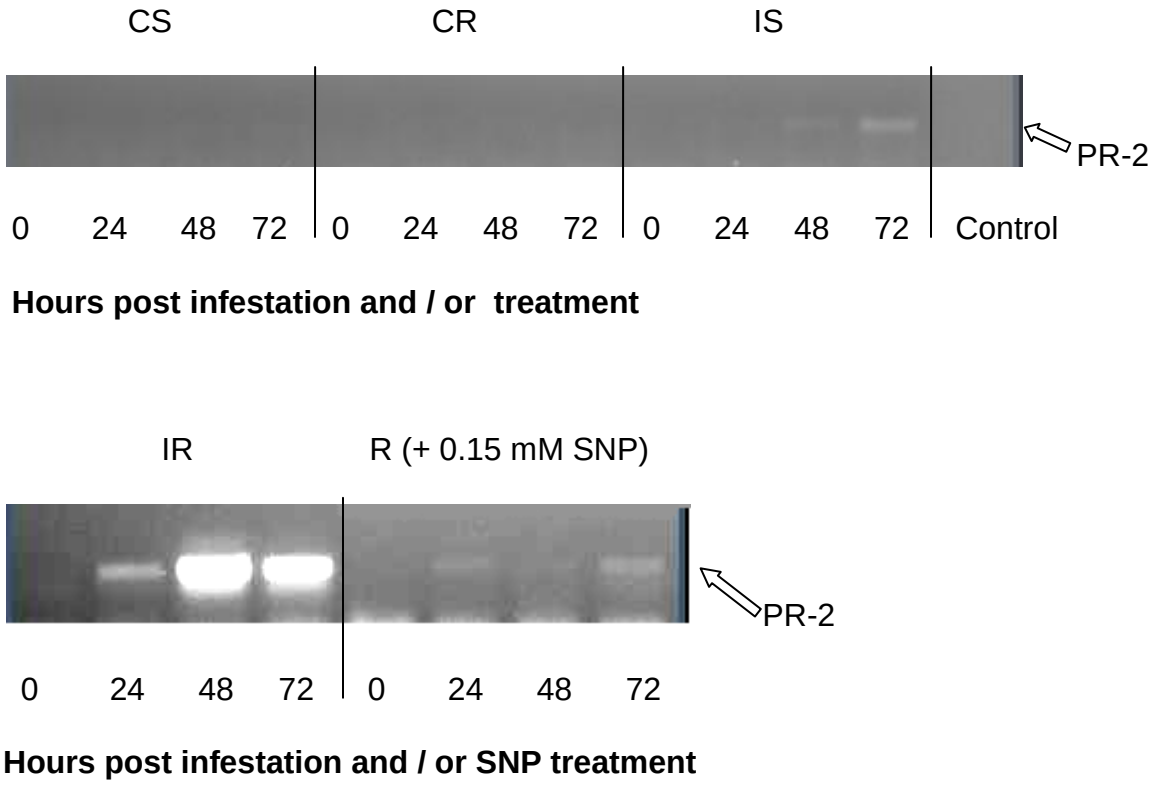


**Figure 4.12.** Effect of SNP (root application) on the β-1,3-glucanase gene expression of resistant (*Tugela DN)* and susceptible (*Tugela)* wheat cultivars. Values are means ± SD (n = 3). CS: control susceptible; CR: control resistant; IS: infested susceptible; IR: infested resistant.

*4.4.3 Salicylic acid content*

The total salicylic acid (SA) content increased substantially in resistant plants following 0.15 mM SNP treatment. A tangible increase of 44 % was obtained 48 hours post SNP treatment (Fig. 4.13).

Inhibition of NO production by $NaWO_4$ resulted in a significant reduction in SA concentration (53 % decrease, $P = 0.0230$) in infested resistant plants (Fig. 4.14).

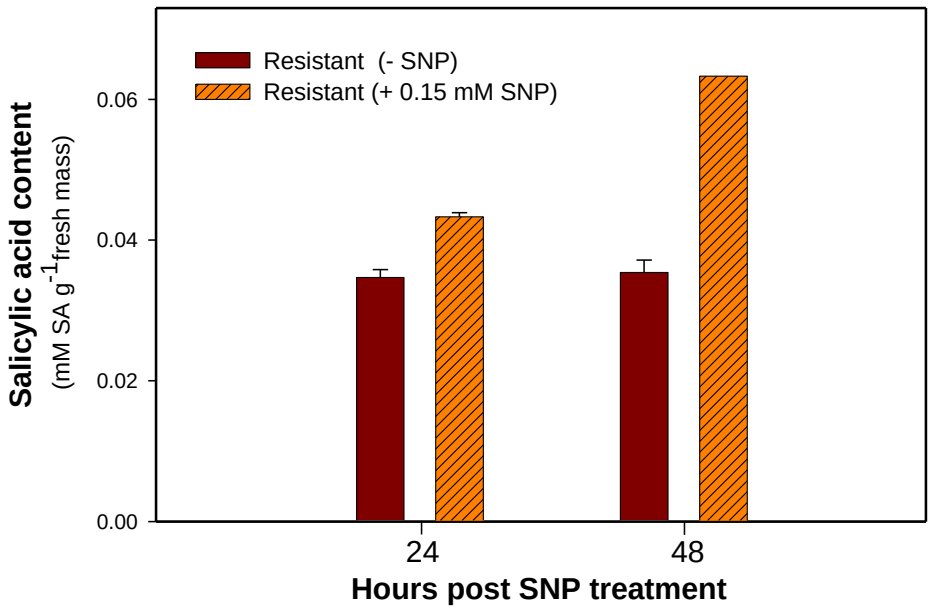


**Figure 4.13.** Effect of SNP (root application) on the total salicylic acid content of a resistant (*Tugela DN*) wheat cultivar. Values are means ± SD (n = 3).

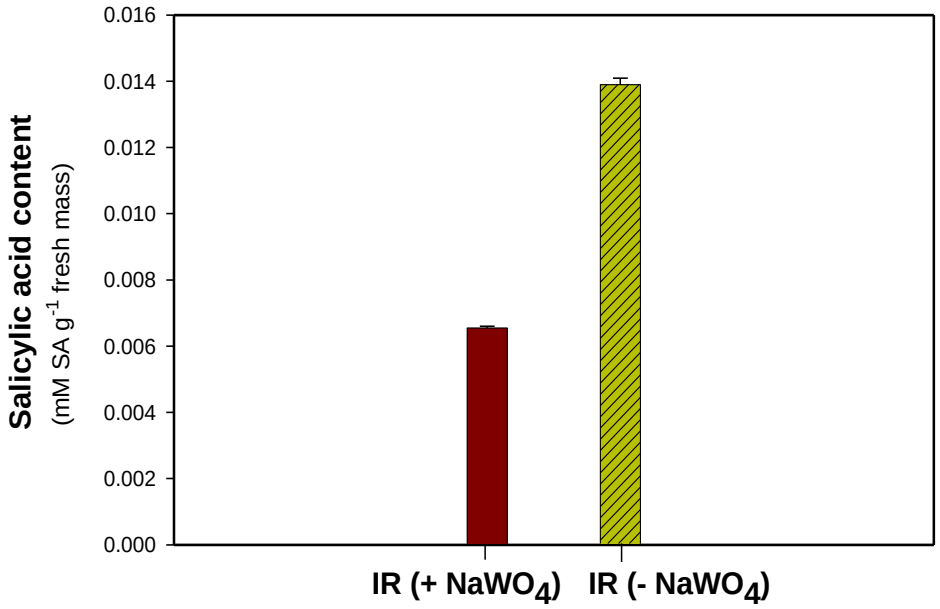


**Figure 4.14.** Effect of NO production inhibition ($NaWO_4$, nitrate reductase inhibitor) on the total SA content of a RWA infested (9 h.p.i) resistant (*Tugela DN*) wheat cultivar. Values are means ± SD (n = 3).

### 4.5 The effect of NO application on the symptom development

Application of different concentrations of SNP (root application) to infested susceptible and resistant plants, had a notable impact on the resistance response of susceptible plants. Symptom analysis (168 h.p.i) shows that SNP delayed disease symptom development in the infested susceptible plants. These plants were transiently transformed from being susceptible (S) to the RWA, to being medium resistant (MR) to

RWA by these SNP treatments (particularly 0.15 mM and 0.5 mM). No significant changes were brought by SNP treatments in the infested resistant plants (Fig. 4.15 a).

Later on (336 h.p.i), the infested susceptible plants developed more disease symptoms than earlier on, irrespective of SNP treatments. Again, no difference worth noting was observed in the SNP treated infested resistant plants (Fig. 4.15 b).

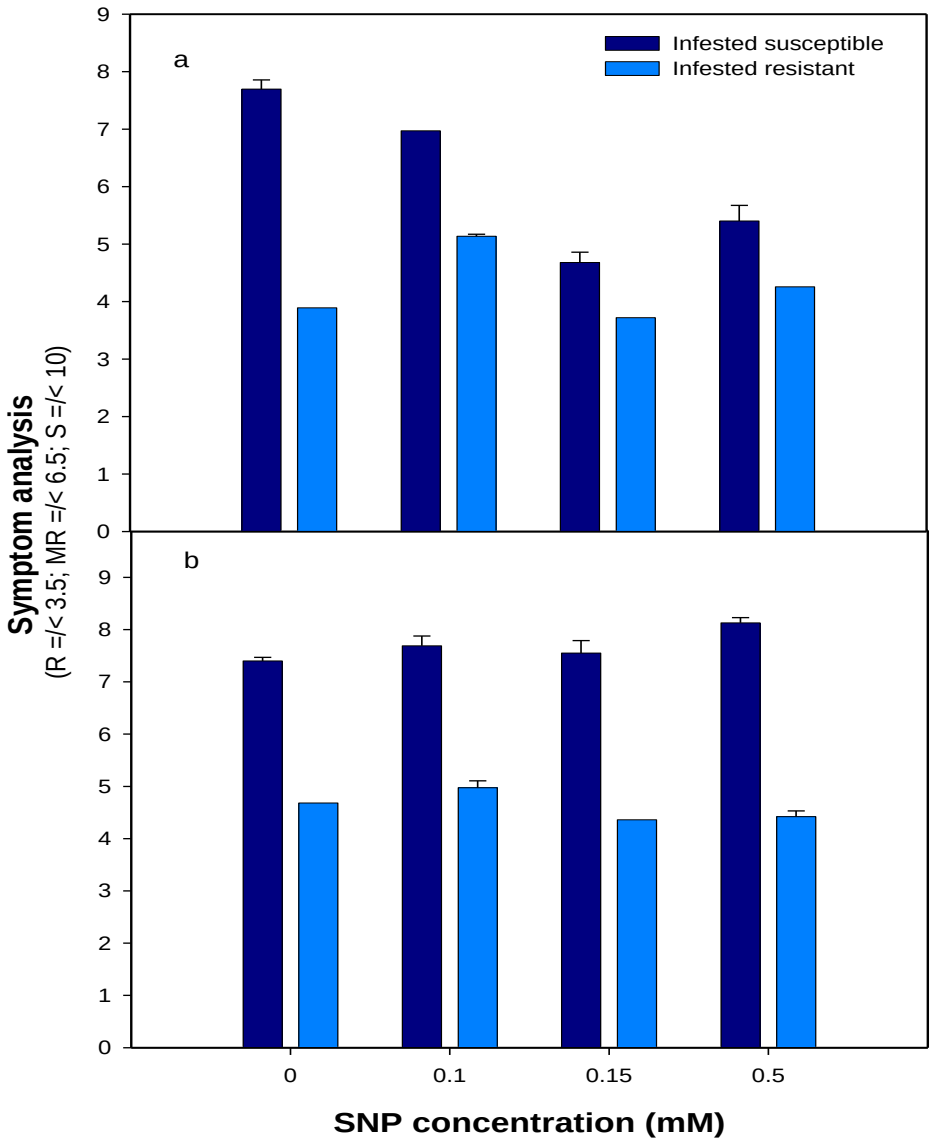


**Figure 4.15.** Effect SNP (root application at different concentrations) on symptom/ disease development of RWA infested resistant (*Tugela DN*) and susceptible (*Tugela*) wheat cultivars 168 (a) and 336 (b) h.p.i. Values are means ± SD (n = 4). A score of 1 ≤ 3.5 = highly resistant (R) (no leaf curling); 3.6 ≤ 6.5 = medium resistant (MR); and 6.6 ≤ 10 = susceptible plant (S) (curled leaves and severe chlorosis).

### 4.6 The effect of NO application on RWA attraction/ repulsion

Treatment of the resistant and susceptible plants with SNP at different concentrations (root application) did not have any reduction impact on the number of aphids (adults and nymphs) attracted to the plants 24 h.p.i. In fact, aphid numbers (especially the nymphs) increased by at least 50 % in all SNP treatments (Fig. 4.16 a).

Results of a trial where different SNP treatments were applied as a seed dressing before planting proved to be different from the above. No aphids were found on the leaves of the resistant and susceptible plants treated with 0.15 and 0.5 mM SNP (24 hours after the release of aphids for settlement on the plants). In comparison with the control, aphid numbers in the 0.1 mM SNP treated plants increased (especially for the infested susceptible plants), with the exception of *Tugela DN* nymphs (Fig. 4.16 b).

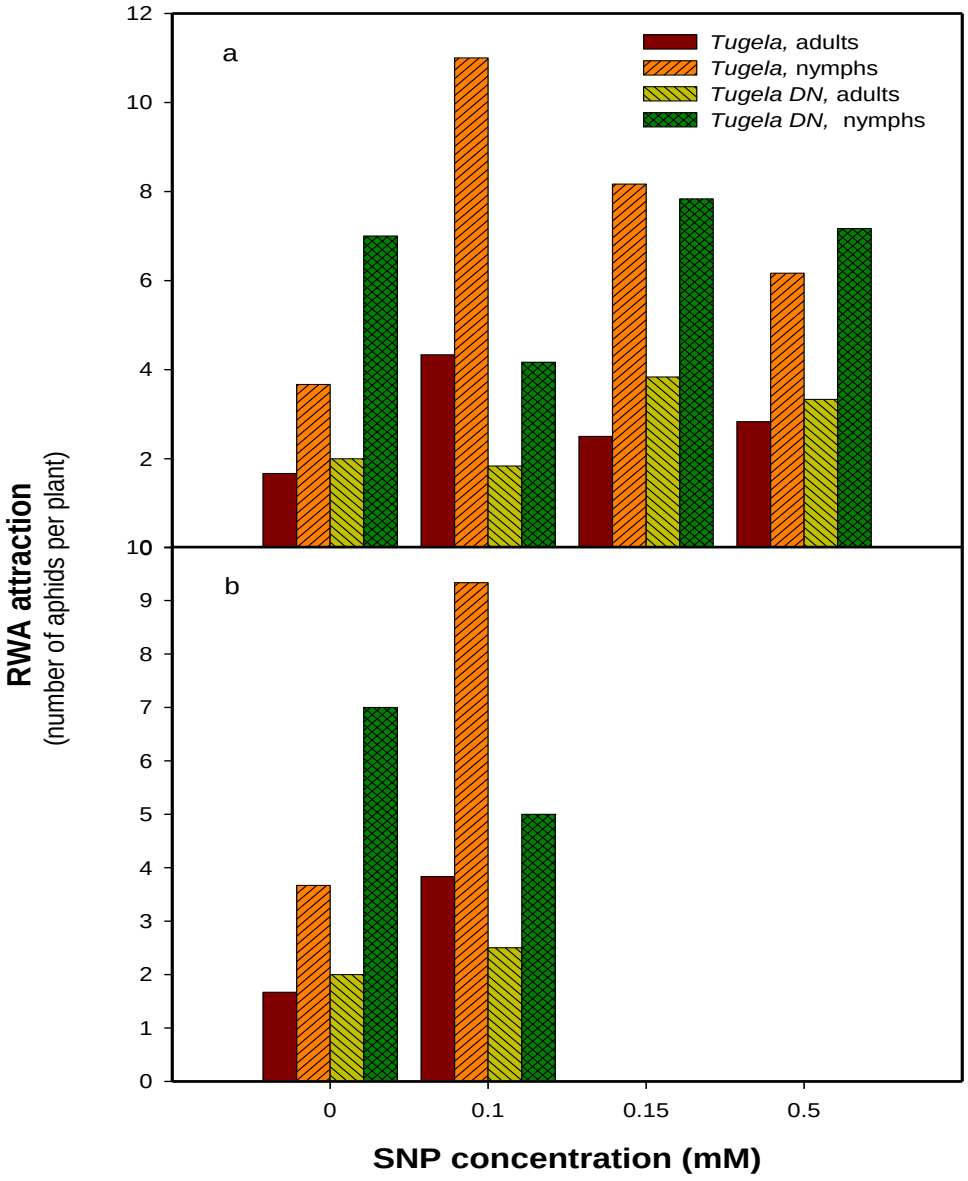


**Figure 4.16.** Attraction of the RWA (nymphs and adults) to the leaves of resistant (*Tugela DN*) and susceptible (*Tugela*) wheat cultivars (24 h.p.i) after SNP treatments at different concentrations (root application; a) (seed dressing; b). Values are means (n = 6).

### 4.7 The effect of NO application on the intrinsic RWA increase rate

SNP (root application at the concentration of 0.15 mM and 0.5 mM) had no RWA reduction (adults + nymphs) effect on the leaves of infested resistant plants (72 h.p.i). Contrary, the 0.1 mM SNP treated infested resistant plants showed a 22 % decrease in the aphid population increase rate. In the infested susceptible plants, a slight decrease of 8 % and 6 % in the aphid population was observed after 0.15 mM and 0.5 mM SNP (root application) respectively (Fig. 4.17 a).

Pretreatment of seeds with SNP led to a significant decrease in the RWA increase rate on the susceptible plants. A 35 % (0.15 mM SNP) and 21 % (0.5 mM SNP) decrease in intrinsic rate of RWA increase was obtained 72 h.p.i for the susceptible plants. In contrast to the root applications, the RWA population was now decreased by 20 % and 25 % on the 0.15 and 0.5 mM SNP seed treated resistant plants respectively (Fig. 4.17 b).

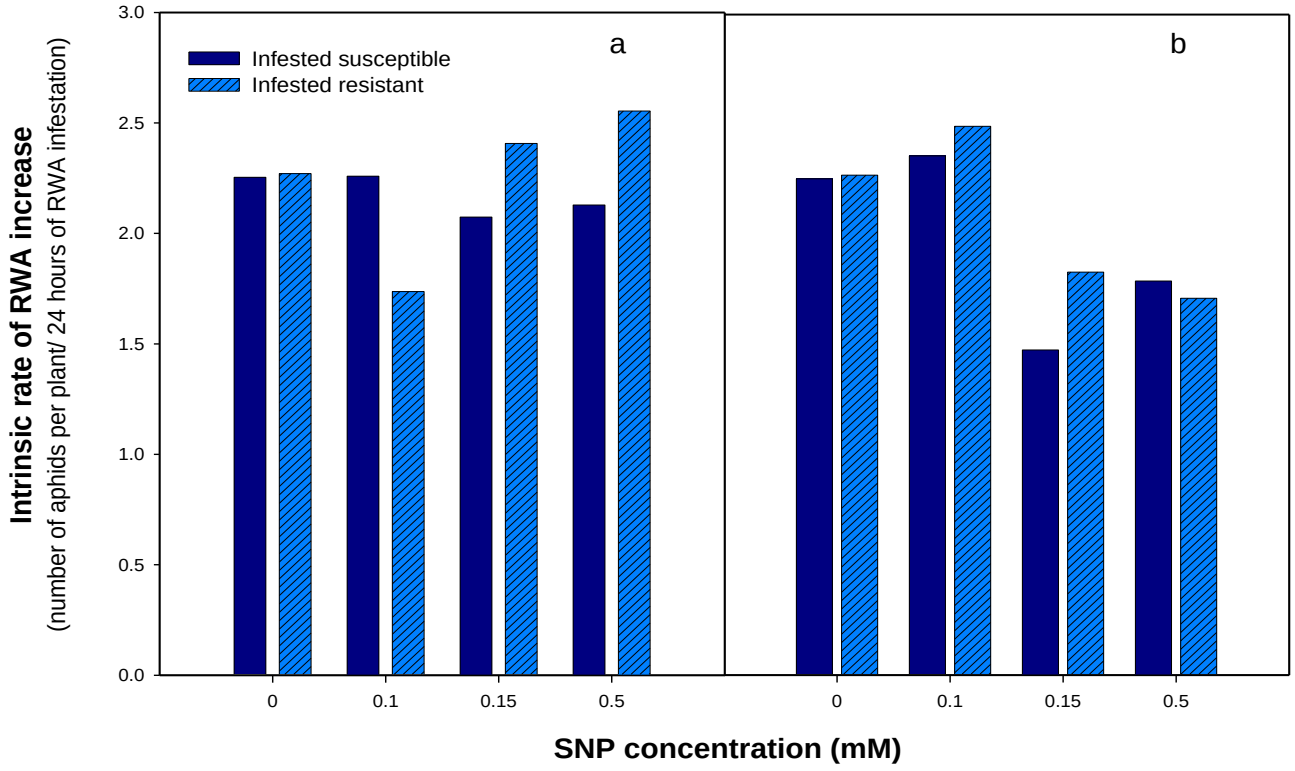


**Figure 4.17.** Intrinsic RWA increase rate (72 h.p.i) on susceptible and resistant wheat cultivars treated with various concentrations of SNP (root application) (a) or seed dressing (b). Values are means (n = 6).

### 4.8 The effect of RWA infestation on the peroxynitrite content

The peroxynitrite ($ONOO^-$) content was selectively induced to higher levels in RWA infested resistant than susceptible plants. A sharp increase in $ONOO^-$ content was observed after 3 h of infestation, which peaked at 12 h.p.i, representing a 45 % increase. Subsequently, the $ONOO^-$ content dropped to the original level (Fig. 4.18).

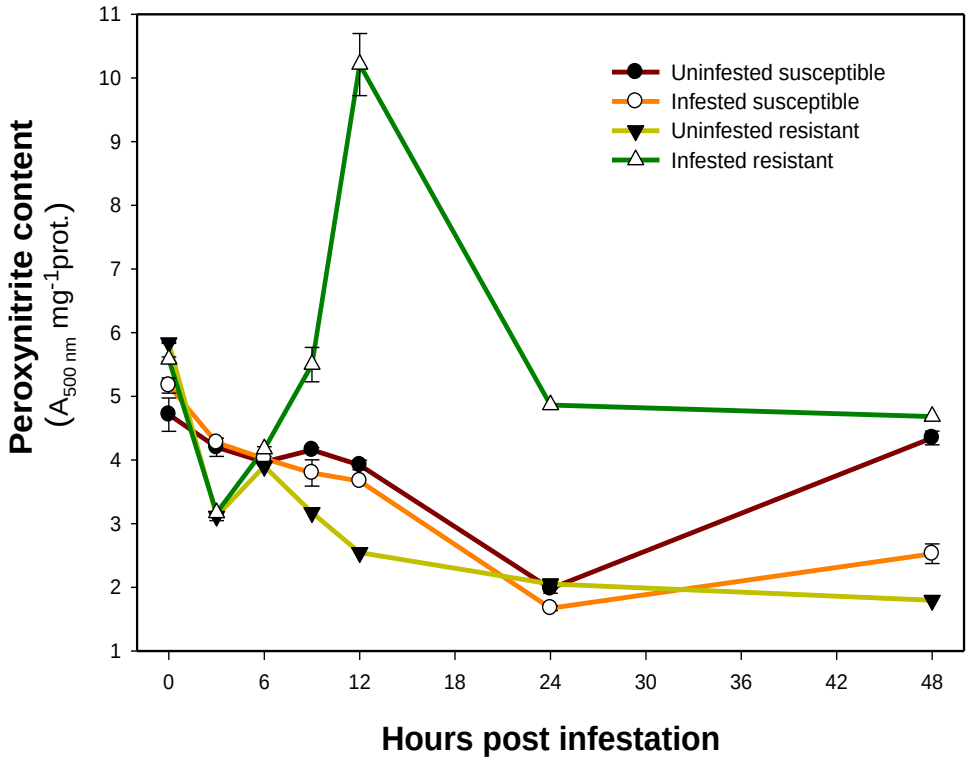


**Figure 4.18.** Effect of RWA infestation on the peroxynitrite content of resistant (*Tugela DN*) and susceptible (*Tugela*) wheat cultivars. Values are means ± SD (n = 3).

### 4.9 The role of peroxynitrite in the RWA resistance response

Compared to the control, treatment of the infested resistant plants with urate (*in vivo*) (a selective inhibitor of $ONOO^-$ production) significantly reduced (47 % decrease) the amount of $ONOO^-$ produced (Fig. 4.19 a).

Treatment of infested resistant plants with urate (*in vivo*) led to a significant reduction in the activities of the intercellular β-1,3-glucanase (51 %, $P$ = 0.0012, Fig. 4.19 b) and peroxidase (53 %, $P$ = 0.00004, Fig. 4.19 c).

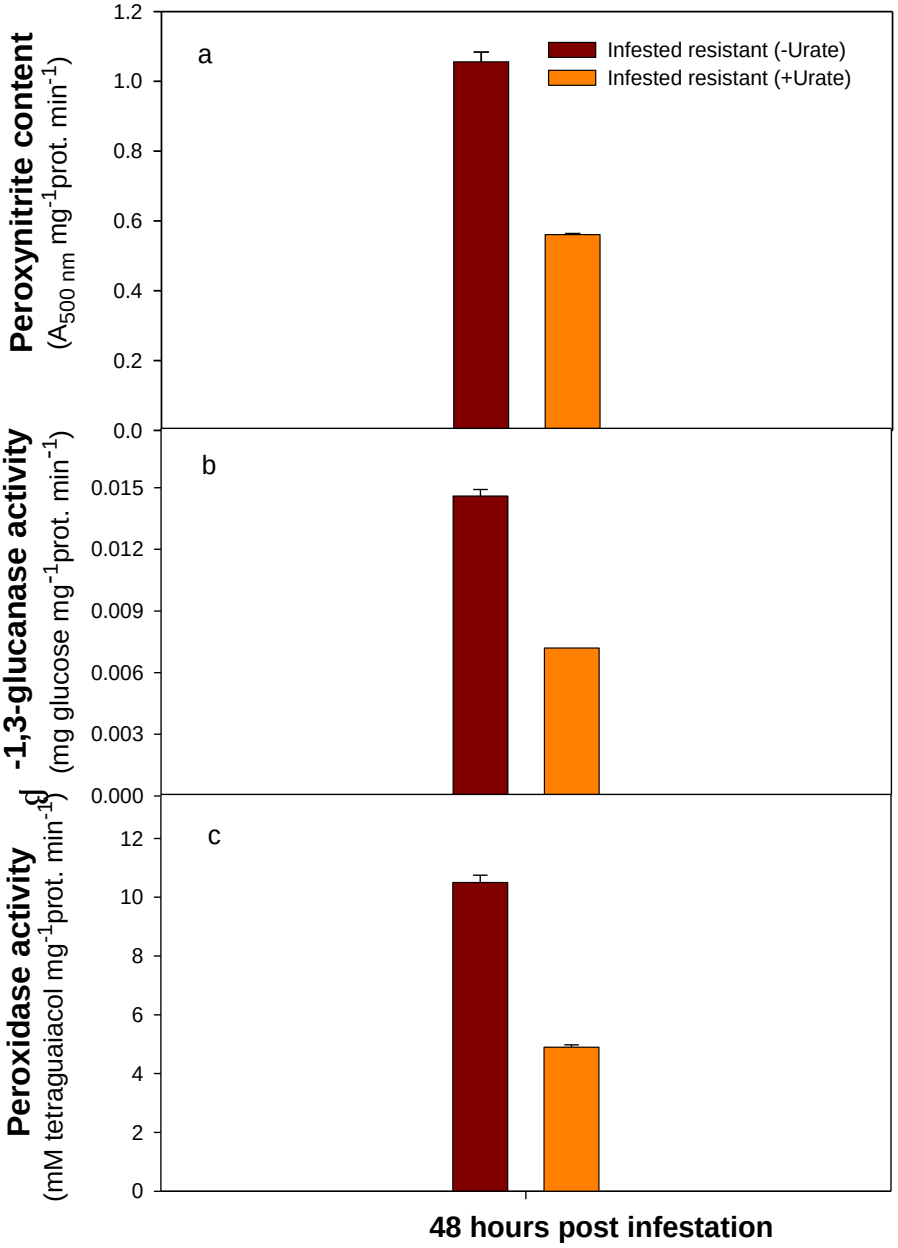


**Figure 4.19.** Effect of urate ($ONOO^-$ formation inhibitor) (*in vivo*) on the peroxynitrite content (12 h.p.i) (a), intercellular β-1,3-glucanase (b) and peroxidase (c) activities (48 h.p.i) of a RWA infested resistant (*Tugela DN*) wheat cultivar. Values are means ± SD (n = 3).

Addition of urate to the reaction mixture (i.e. *in vitro*), did not inhibit the activities of the intercellular β-1,3-glucanase and peroxidase (Fig. 4.20 a and b)

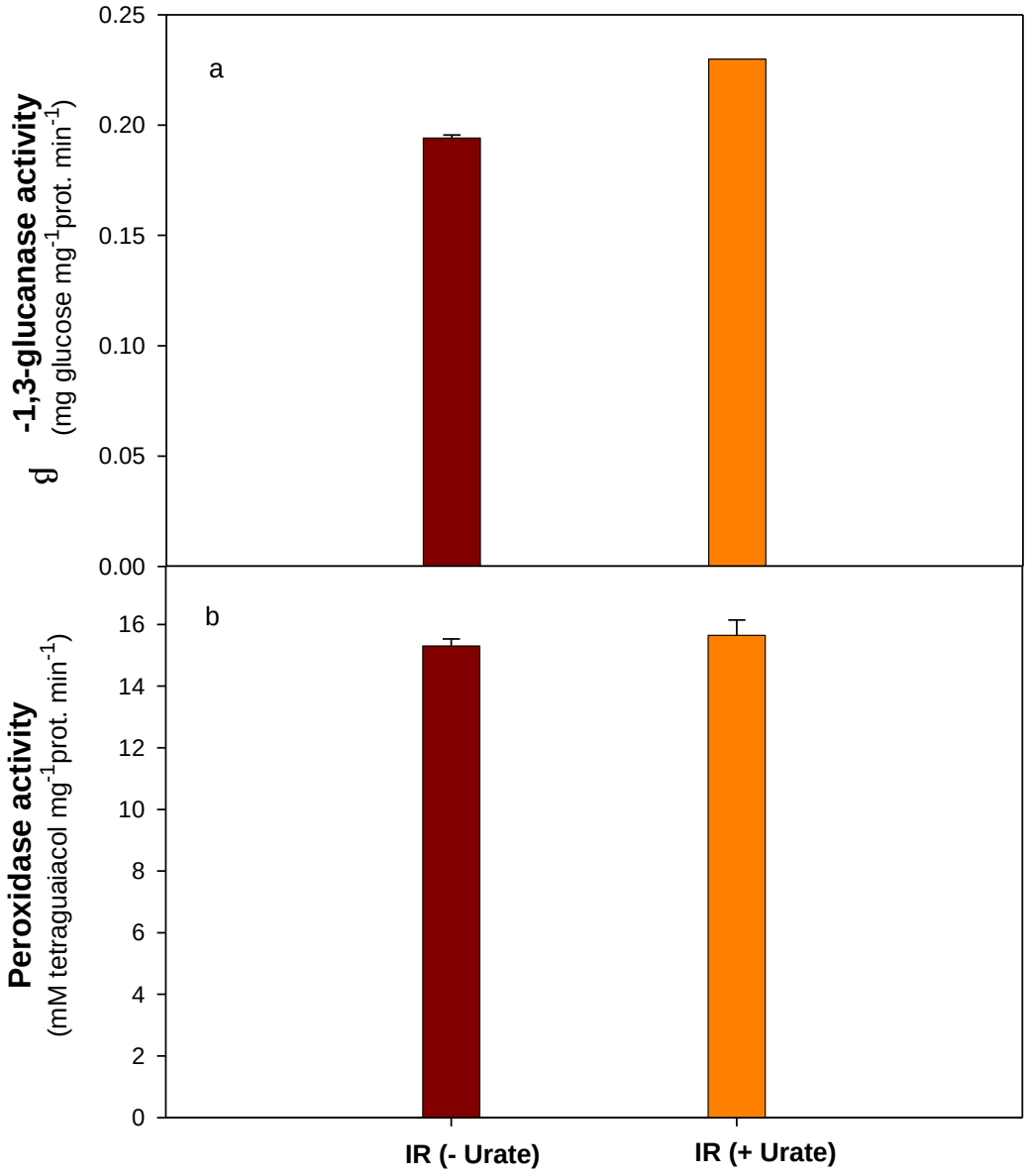


**Figure 4.20.** Effect of urate ($ONOO^-$ formation inhibitor) (*in vitro*) on the intercellular β-1,3-glucanase (a) and peroxidase (b) activities (48 h.p.i) of a RWA infested resistant (*Tugela DN*) wheat cultivar. Values are means ± SD (n = 3).

# CHAPTER 5

## 5. DISCUSSION

Pest and disease control by means of chemicals is expensive, often complicated and in addition can be harmful to the environment. A relative cheap and environmental friendly alternative to the use of chemicals is plant resistance. Resistance can be obtained by breeding or genetic engineering by introducing pest or pathogen resistance genes into crop plants. Progress in biotechnology over the last couple of years has contributed towards new and improved resistance in many instances and might pave the way towards the development of novel safe control methods (McDowell and Woffenden, 2003).

In South Africa, the control of the Russian wheat aphid (RWA), *Diuraphis noxia*, a destructive pest of wheat, has to a great extent been achieved through the use of resistance genes such as *Dn*1, *Dn*2 and *Dn*5 (Prinsloo, 2000). However, already new biotypes that are capable of breaking the current resistance bred into wheat have developed (Haley et al., 2004; Jyoti et al., 2006; Qureshi et al., 2006). Evidence for the existence of a resistance breaking RWA biotype in South Africa was provided by the researchers at the Agricultural Research Council- Small Grain Institute (ARC-SGI) (Jankielsohn and Lindeque, 2006; Tolmay et al., 2007). In order to stay ahead of this problem and also to establish advanced strategies for controlling pests and diseases, a better understanding of the plant resistance mechanisms is necessary.

It was documented that the RWA resistance response is not a wounding response, but a typical hypersensitive response (HR) characteristic of pathogenesis (Belefant-Miller et al., 1994). This was confirmed by van der Westhuizen *et al* (1998 a and b). One of the earliest events during the HR is the production of the reactive oxygen species (ROS) such as hydrogen peroxide ($H_2O_2$) and superoxide anion ($O_2^-$) (Levine et al., 1994; Baker and Orlandi, 1995; Klessing et al., 2000). Although important in plant disease resistance, the ROS alone may not be a sufficient requirement. There is strong evidence that during defense responses against pathogens, ROS production is accompanied by rapid synthesis of nitric oxide (NO) in the infected tissues (Delledonne et al., 2001; Delledonne et al., 2002; Hancock et al., 2002; Wang and Wu, 2004; Wendehenne et al., 2004). It is therefore of great importance to establish the involvement of the reactive nitrogen species (RNS), particularly nitric oxide (NO), during the RWA resistance response in wheat. This information may be useful for future manipulation of RWA resistance.

An accumulating body of evidence strongly suggests the involvement of NO in the defense of plants against various biotic and abiotic stress factors (Garcia-Mata and Lamattina, 2001; Huang et al., 2002; Neill et al., 2002; Guo et al., 2004; Shi et al., 2005; Valderrama et al., 2007; Manjunatha et al., 2008). Currently, there are no reports on the involvement of NO during the RWA resistance response of wheat. The work reported here gives a clear indication on how NO is involved in the RWA (biotype *RWASA1*) resistance response of wheat plants.

Higher levels of NO production in infested resistant than infested susceptible plants suggests it may be involved in the RWA resistance response of wheat. Constitutive production of NO in the control plants, suggests that resistance to the RWA may be linked to elevated NO levels (Fig. 4.1). NO could also be constitutively produced, because it is involved in other physiological processes such as fruit maturation and flower senescence (Beligni and Lamattina, 2001), seed germination and plant growth (Stöhr and Ullrich, 2002). Early induction of NO [3 hours post infestation (h.p.i)] in the infested resistant plants, strongly indicates that it is involved in the early events of the RWA resistance response. This increase was however transient, suggesting that NO could just be acting as an early signal for triggering the secondary defense responses of wheat plants (Fig. 4.1). NO was also reported to function as a critical signal for disease resistance in plants (Delledonne et al., 1998; Durner et al., 1998; Durner and Klessig, 1999). Guo *et al* (2004) discovered that resistance to stripe rust disease in wheat was dependent on the first peak of NO produced during the early infection stage.

Induction of nitrate reductase (NR) 3 h.p.i in the resistant plants only, suggests that it is somehow also involved in the early events of the RWA resistance response (Fig. 4.2). This early increase correlates with the activity of some of the ROS generating enzymes (such as NADPH oxidase) that are also found to be linked with the early events of the RWA resistance responses of wheat (Moloi and van der Westhuizen, 2006). In the infested susceptible plants, NR activity was only induced at a relative low level up to 6 h.p.i. These results also suggest that resistance is linked not only to the level of induction, but also to the time/ duration of NR induction. This confirms early work that

critical factors contributing to the defense response of plants include speed and strength, occurrence as well as timing of a resistance reaction, with weaker detection in the susceptible than resistant plants (Fritig et al., 1998; Somssich and Hahlbrock, 1998). Important to note is that the pattern of NR increase in the infested resistant plants correlates with that of NO production (Fig. 4.1), strongly implicating the involvement of this enzyme in NO production. Xu and Zhao (2003) also showed that there is a strong correlation between NR and NO production in wheat seedlings. They also found that NR is the main pathway for NO production. Similarly, it was discovered that NR-dependent NO production does take place during pathogenesis (Yamamoto-Katou et al., 2006). A positive correlation between NO and NR was also recorded in maize seedlings exposed to ultraviolet (UV) -B radiation (Zhang et al., 2008).

It is well known that tungstate can be substituted for molybdenum and inhibit the NR activity by preventing formation of an active molybdenum cofactor, indispensable for the catalytic activity of NR (Xu and Zhao, 2003). Results on the effect of sodium tungstate ($Na_2WO_4$) (*in vitro*) confirm the inhibitory effect of sodium tungstate (Fig. 4.3). Furthermore, it is found that inhibition of NR (*in vivo*) consequently leads to a significant reduction of NO production (Fig. 4.4) in the infested resistant plants 9 h.p.i, strongly suggesting that NR is a key enzyme involved in NO production during the RWA resistance response.

Although results in this study suggested NR to be an enzyme involved in NO production during RWA resistance response, the pathway for NO production from $NO_2^-$ still needs

to be resolved. It has been reported that the reduction of $NO_2^-$ may lead to the production of $NH_3$ instead of NO, and that this reduction is facilitated by the enzyme, nitrite reductase (NiR) (Mahboobi et al., 2002). The same enzyme may also be used by plants to reduce $NO_2^-$ to NO. As a result, NiR activity was measured in the RWA infested resistant and susceptible plants (Fig. 4.5). An early increase (3 h.p.i) in NiR activity suggests that it may also be involved during the early events of the RWA resistance responses. A significant higher peak activity was obtained 9 h.p.i in the infested resistant than susceptible plants. The fact that peak NiR activity was matched by peak NR activity (Fig. 4.2) and maximum NO production (Fig. 4.1), supports the idea that these two enzymes might be mutually involved in NO production during the RWA resistance response. In contrast, studies by Datta and Sharma (1999) indicated that maximal activity of these enzymes was observed on different days in dark or light grown maize seedlings exposed to various nitrate concentrations, and that their developmental programs were not tightly linked.

Since it is obvious that there is a correlation between high NO production and high activities of NR and NiR, infested resistant plants (9 h.p.i) were selected for further elucidation studies to investigate whether reduction of $NO_2^-$ to NO is facilitated by NiR or by another mechanism. The results show that the substantial reduction of NO production (56 %) is a consequence of NR and NiR inhibition by excess $Cu^{2+}$ (Fig. 4.6 a, b and c). This NO inhibition occurred despite of $NO_2^-$ supplementation (in the form of $NaNO_2$). If there was another mechanism (whether enzymatic or not) involved in the reduction of $NO_2^-$ to NO, then NO production would not have been inhibited. These

results indicate that NiR is involved in NO production during the RWA resistance response of wheat (Fig. 4.6 a, b and c), in accordance to Mahboobi *et al* (2002). These results may be summarized as follows:

$$NO_3^- \xrightarrow{NR} NO_2^- \xrightarrow{NiR} NO$$

In contrast, Xu and Zhao (2003) performed an experiment involving inhibition of NR and NiR, as well as heat treatments in higher non-leguminous plants, which led to a discovery that the reduction of $NO_2^-$ to NO is not dependent on NiR, instead another enzyme was responsible for catalyzing this process. Although NR may not be directly responsible for NO production, it is an important source of $NO_2^-$ for subsequent NO production (Modolo et al., 2005 and 2006).

It is also suggested that NR and NOS might act in concert to produce NO in plants. Modolo *et al* (2005) found that in *A. thaliana* infected with *P. syringae*, NO production arise from the co-operation of NOS, NR and the mitochondrial-dependent nitrite-reducing activity. In support, Sang *et al* (2008) reported that water stress induced NO production was blocked by pre-treatment of maize leaves with inhibitors of both NOS and NR.

In addition, a NO producing NOS-like enzyme is not unique to animals only, because it has also been detected in plants (Cueto et al., 1996; Ninnemann and Maier, 1996; Barroso et al., 1999; Chandock et al., 2003; Crawford, 2006). Evidence for the

existence of a NOS-like enzyme in roots and nodules of *Lupinus albus* inoculated with *Bradyrhizhobium* sp. *Lupinus* was provided by Cueto *et al* (1996). These findings provided an incentive to investigate the contribution of NOS towards NO production during the RWA resistance response. The study included the use of a NOS inhibitor, $N^{\omega}$-nitro-L-arginine methyl ester (L-NAME). The results (Fig. 4.7) indicate that NOS has no significant effect on NO production during the RWA resistance response. This is proven by the fact that the NO content in both RWA infested susceptible and resistant plants was neither blocked nor inhibited by L-NAME treatment. In disagreement, treatment of UV-B exposed maize seedlings with a NOS inhibitor, $N^{\omega}$-nitro-L-arginine (L-NNA), resulted to inhibition of both NOS activity and NO production, suggesting a NOS-dependent NO production (Wang et al., 2006). Furthermore, He *et al* (2007) also found that during UV-B stress, a NOS-dependent NO production was inhibited by L-NAME in *Paulownia tomentosa.*

The RWA resistance response is a typical HR (Belefant-Miller et al., 1994). In a previous study, Moloi and van der Westhuizen (2006) discovered that the RWA resistance response of wheat involves early induction of the ROS such as $H_2O_2$, which is a pre-requisite for the activation of HR (Klessig et al., 2000). The HR often triggers non-specific resistance throughout the plant known as systemic acquired resistance (SAR), which provides durable protection against a broad range of pathogens (Sticher et al., 1997; van Loon, 1997; Fritig et al., 1998). According to van der Westhuizen *et al* (1998 a and b), the resistance response of wheat to the RWA involves induction of the pathogenesis related (PR) proteins such as β-1,3-glucanase, chitinase as well as

peroxidase, which are usually used as markers for SAR (Ryals et al., 1996; Fritig et al., 1998). PR proteins are often referred to as defense proteins functioning in limiting the multiplication and spread of pathogens (van Loon, 1997). They are known to be induced in many plants in response to fungal infection and other stress-related factors (Bowles, 1990; Stinzi et al., 1993; Fritig et al., 1998; Xue et al., 1998; Cooper et al., 2004).

Preceding results (Figures 4.1, 4.2 and 4.4) indicated that NR is participating in NO synthesis during the RWA resistance response. This was substantiated by the fact that NR inhibition resulted in the inhibition of NO production. A significant decrease in the activities of the secondary defense related enzymes, such as the PR proteins, β-1,3-glucanase and peroxidase in the infested resistant plants after sodium tungstate (an inhibitor of NR) treatment (Fig. 4.8 a), further suggests the involvement of NO, probably as a signal, in the RWA defense response. The non-inhibitory *in vitro* effect of sodium tungstate on the β-1,3-glucanase and peroxidase activities (Fig. 4.8 b), confirms a possible signaling role for NO. In agreement, An *et al* (2005) reported that NO is an important signal molecule for the induction of exo- and endo- β-glucanase activity in the leaf cell wall of maize seedlings exposed to ultraviolet (UV) B radiation. Other studies demonstrated that NO signaling can regulate the β-1,3-glucanase activity, and thereby increase the extensibility of the cell wall framework, i.e. change its composition (Darley et al., 2001; Zhang et al., 2003).

In an effort to confirm the previous findings that NOS is not involved in NO production (Fig. 4.7) during the RWA resistance response and that NO seems to act as another

signal for downstream defense reactions (Fig. 4.10 and 4.11), an investigation was launched on the effect of NOS inhibition (by using L-NAME, the inhibitor of all the three isoforms of NOS) on downstream (secondary) defense reactions (β-1,3-glucanase and peroxidase activities). According to results in Figure 4.9, NOS inhibition had no effect on the secondary defense related enzyme activities, which supports the absence of NOS in the synthesis pathway of NO, which acted as a signal to induce secondary defense related enzyme activities (Fig. 4.8 a). This suggestion is also confirmed by the results presented in Figure 4.7.

Since results have indicated that NO is a possible signal molecule during the RWA resistance response, this prospect has been investigated further by applying a NO donor, sodium nitroprusside (SNP), to plants. NO donors are compounds which produce NO when applied to biological systems and are able to either mimic an endogenous NO-related response or substitute for an endogenous NO deficiency (Floryszak-Wieczorek et al., 2006). RWA infested susceptible plants were also used in this investigation, since NO levels are generally low in these plants after infestation (Fig. 4.1). Low NO production after RWA infestation could be a contributing factor for susceptibility. The activities of the secondary defense related enzymes, β-1,3-glucanase and peroxidase, after RWA infestation, were also at a low level in the infested susceptible plants (van der Westhuizen et al., 1998 a and b).

The cytoprotective and cytotoxic action of NO on plant metabolism depends to a large extent on its concentration, and is affected by the rate of synthesis, displacement and

efficiency of removal of this reactive nitrogen species (RNS) (Wojtaszek, 2000; Beligni and Lamattina, 2001; Romero-Puertas; 2004). Importantly, it has been found that a too high NO donor concentration has inhibitory effects (instead of stimulating NO production). The commonly applied donor concentrations range from 10-500 µM (Modolo et al., 2002; Floryszak-Wieczorek et al., 2006).

SNP (root application, 0.15 mM) produced a significant amount of NO in the infested susceptible plants (Fig. 4.10 a). However this concentration does not seem to be high enough to stimulate significant intercellular β-1,3-glucanase (Fig. 4.10 b) and peroxidase (Fig. 4.10 c) activities in these plants. A higher SNP concentration (0.5 mM) on the other hand stimulated the highest NO level in the infested susceptible plants (Fig. 4.10 a), and this coincided with significant increases in the activities of intercellular β-1,3-glucanase (Fig. 4.10 b) and peroxidase (Fig. 4.10 c). These findings further suggest that NO could play a significant role in the signaling events leading to induction of the downstream defense enzymes during the RWA resistance response of wheat; and that the level of NO present in the cells may play an important role in resistance.

In agreement, results presented in Figure 4.11 show that NO applied to uninfested resistant plants stimulated a substantial increase in the intercellular β-1,3-glucanase (Fig. 4.11 a) and peroxidase activities (Fig. 4.11 b). In contrast to the results obtained with the SNP treated infested susceptible plants, 0.15 mM SNP was sufficient to trigger the activity of these secondary defense related enzymes in the resistant plants. This indicates further that the level of NO in the resistant and susceptible plants is crucial for

the stimulation of the defense response, and that resistant plants respond better to NO than infested susceptible plants, which require higher concentration of SNP (0.5 mM) to trigger the defense response due to the low NO production in these plants (Fig. 4.1).

According to Figure 4.12, β-1,3-glucanase [pathogenesis related (PR)-2] gene expression was much higher in the infested resistant than infested susceptible plants. This correlates with results of van der Westhuizen *et al* (1998 b) on β-1,3-glucanase activity. They also found that a higher β-1,3-glucanase protein level is linked to the resistance response. Transcript accumulation for PR-2 was higher in the resistant wheat leaves infected with stripe rust than the control (Moldenhauer et al., 2008), indicating that infection triggers higher production of this protein in the resistant plants. PR-2 is believed to act primarily on glucans present in the cell walls of most fungal pathogens to release fragments (oligosaccharides) that may be perceived by plants as elicitors that serve to trigger further defense responses in addition to the direct harmful effect on the pathogen (Mauch and Staehelin, 1989; Wolski et al., 2006). Induction of β-1,3-glucanase gene expression after application of NO (0.15 mM SNP) to the uninfested resistant plants (Fig. 4.12) also implicates NO in the signaling events of the RWA resistance response. SNP (0.5 to 1 mM) treatment triggers high expression of the defense genes such as phenylalanine ammonia lyase (PAL), PR-1 and β-1,3-glucanase (Beligni et al., 1997; Durner et al., 1998, Klessig et al., 2000). Additional evidence for the involvement of NO in the defense response was provided by Polverari *et al* (2003). They observed that NO leads to the activation of β-1,3-glucanase transcript accumulation in SNP treated (1 mM) *Arabidopsis thaliana*. NO was also associated with

the defense response of wounded potato plants by inducing callose deposition, extensin and PAL transcript accumulation (París et al., 2007). Furthermore, biosynthesis of specific phytoalexins was observed after treatment of soybean cotyledons with NO donors (Modolo et al., 2002).

Salicylic acid (SA) is one of the most important signals for the establishment of SAR in plants (Vernooij et al., 1994; Rao et al., 1997; Sticher et al., 1997; Chen et al., 1999; Glazebrook, 1999; Hayat et al., 2009). A high increase in SA content following NO application (Fig. 4.13) suggests that NO may be a signal molecule that stimulates SA production during the RWA resistance response of wheat. This was further substantiated by a significant reduction in SA content after tungstate (inhibitor of NR and thus NO production) treatment (Fig. 4.14). Previously, it was suggested that SA plays a key role in the signal events of the RWA resistance response of wheat, e.g. by inducing peroxidase activity (Mohase and van der Westhuizen, 2002). This information correlates with findings in this study, suggesting that NO acts upstream SA, which consequently induces the intercellular peroxidase and β-1,3-glucanase activities, contributing to resistance in the infested resistant plants. In support of this idea, research conducted by Song and Goodman (2001) revealed that in tobacco, NO-mediated disease resistance requires the action of SA and that NO functions upstream of SA in the SAR signaling pathway. Likewise, Durner *et al* (1998) found that administration of NO donors increased total SA levels in susceptible tobacco infected with tobacco mosaic virus. In contrast, it was reported that NO acts downstream of SA during the defense response of *Arabidopsis thaliana* (Zottini et al., 2007). Similarly,

Gaupels *et al* 2008 also indicated that NO generation in the companion cells of *Vicia faba* was dependent on SA.

It was previously reported that the RWA resistance response includes higher production of hydrogen peroxide ($H_2O_2$) and that $H_2O_2$ could be involved in signaling events (Moloi and van der Westhuizen, 2006). Interestingly, it was discovered that catalase activity is not involved in the SA-mediated RWA resistance response (Mohase and van der Westhuizen, 2002). Other studies revealed that NO production can inhibit the activity of tobacco catalase, leading to subsequent increases in the intracellular $H_2O_2$ concentration. NO may inhibit catalase activity by binding to its prosthetic heme group, resulting in the heme nitrosylation. This in turn prevents the interaction of $H_2O_2$ with the iron centre of catalase, a prerequisite for the reduction of $H_2O_2$ (Clark et al., 2000; Floryszak-Wieczorek et al., 2007).

There is significant overlapping between the NO and $H_2O_2$ signaling pathways in plants (Zeier et al., 2004; Zago et al., 2006). NO may also contribute to an increase in $H_2O_2$ concentration by stimulating the $H_2O_2$ generating systems (Ederli et al., 2009). $H_2O_2$ can, in addition, be produced through the dismutation of superoxide anion by superoxide dismutase (SOD) during the RWA resistance response (Moloi and van der Westhuizen, 2008). Whether or not NO has anything to do with this $H_2O_2$ production, still remains to be discovered. Ederli *et al* (2009) also showed that NO released by SNP causes $H_2O_2$ production in tobacco leaf tissues. Other reports suggest that $H_2O_2$ emerges following synthesis of NO and that it may act synergistically with NO in plant

disease resistance (Delledonne et al., 2001; Hancock et al., 2002; Wendehenne et al., 2004; Urszula and Rozalska, 2005; Fan et al., 2008). In contrast, it was reported that $H_2O_2$ production of wounded tomato plants was not dependent on NO production (Orozco-Cárdenas and Ryan, 2002).

The simultaneous production of $H_2O_2$ and NO (both induced during 3 h.p.i in the RWA infested resistant plants) (Moloi and van der Westhuizen, 2006; Fig. 4.1), suggests an interdependence between these molecules during the RWA resistance response. However, it still remains to be elucidated whether these molecules act independently or not during the signaling events of RWA resistance response.

During defense responses, NO may very rapidly react with $O_2^-$, provided there is sufficient NO to form peroxynitrite ($ONOO^-$), in the absence of enzymatic catalysis (Tuteja et al., 2004; Saito et al., 2006). The chemistry of $ONOO^-$ formation is complex and strongly dependent on pH (Denicola et al., 1998). $ONOO^-$ is a reactive nitrogen species (RNS) with potent oxidizing power (Blough and Zafiriou, 1985). Under circumstances of high NO steady-state concentration (i.e. high nitrite concentration in the chloroplast), generation of $ONOO^-$ may lead to impairment of the photosynthetic machinery (Jasid et al., 2006). Although a sharp increase in $ONOO^-$ production was observed after 3 h.p.i in resistant plants (Fig. 4.18), the level of induction was still low at this stage. This could be attributed to higher SOD activity (converts $O_2^-$ to $H_2O_2$) 3-6 h.p.i in RWA infested resistant than in susceptible plants (Moloi and van der Westhuizen, 2006), preventing the reaction between NO (which was already high) and

$O_2^-$. The increase in $ONOO^-$ formation observed later-on (9 h.p.i), with a peak induction (12 h.p.i) in the infested resistant plants, could be a consequence of high NO production and sufficient $O_2^-$ levels, because at this period the SOD activity was already low, with high NADPH oxidase activity (Moloi and van der Westhuizen, 2006 and 2008), meaning that there was probably little conversion of $O_2^-$ to $H_2O_2$. This increase was only transient probably due to excessive production of $ONOO^-$, which could damage a normal tissue (Delledonne et al., 2002) and also, because NO production had already dropped after 9 hours of infestation (Fig. 4.1). The selective induction of $ONOO^-$ in the RWA infested resistant plants suggests involvement of this molecule in the RWA resistance response (Fig. 4.18).

Treatment of the RWA infested resistant plants with urate (*in vivo*), an inhibitor of $ONOO^-$ production, substantially inhibited $ONOO^-$ production (Fig. 4.19 a). The RWA infested resistant plants (12 h.p.i) were used in this study, because a peak for $ONOO^-$ production was reached at this point, and it would therefore be easier to see the consequences of $ONOO^-$ production inhibition. Studies have showed that urate is a selective inhibitor $ONOO^-$, not NO and/ or $H_2O_2$ (Alamilo and García-Olmendo, 2001).

To measure the involvement of $ONOO^-$ in the RWA resistance response, activities of the secondary defense related enzymes were determined after urate treatments. Figure 4.19 indicates that inhibition of $ONOO^-$ by urate (*in vivo*), consequently leads to significant decreases in activities of the intercellular β-1,3-glucanase (Fig. 4.19 b) and peroxidase (Fig. 4.19 c). Furthermore, activities of these enzymes were not inhibited by

urate addition *in vitro* (Fig. 4.20 a and b), which indicates that observed reduction is not a consequence of direct effect of urate. These results suggest that $ONOO^-$ could also be involved in the RWA resistance responses as one of the signal molecules in addition to NO and $H_2O_2$. However, the interaction between these molecules during the RWA resistance still needs to be elucidated.

Reports indicate that $ONOO^-$ production is involved in the secondary defense responses through induction of PR-1 (Durner and Klessig, 1999), peroxidase and PAL accumulation in *Arabidopsis thaliana* infected with a bacterial pathogen *Pseudomonas syringae.* It was also found to be involved in induction of HR-mediated cell death, necrotic lesion formation and alterations of cell wall (Alamilo and García-Olmendo, 2001). In animals, $ONOO^-$ may cooperate in killing microorganisms such as *Rhodococcus equi* (Darrah et al., 2000). However, it has not been clarified whether it is directly toxic to pathogens or not in plants (Arasimowicz and Floryszak-Wieczorek, 2007).

Another aspect which needs to be explored in order to fully understand what happens during the early events of the RWA resistance response involves NO and the antioxidative enzymes. Studies using tobacco blight-yellow 2 cells treated with SNP, led to a discovery that NO has an antioxidative role, because it co-operates with ascorbate in protection against oxidative processes (high ascorbate was a consequence of increased NO levels) (de Pinto et al., 2002). We have discovered that RWA resistance response also involves induction of the antioxidative enzymes such as ascorbate

peroxidase and glutathione reductase (Moloi and van der Westhuizen, 2008). This brings about questions on whether there is any relationship between increased NO levels and these enzymes during the RWA resistance responses of wheat.

Currently, there are no records on the use of the NO donor, SNP, as a tool for reducing the RWA damage symptoms and the aphid numbers under field or glasshouse conditions. The report below will shed light on whether NO can be used for the RWA damage control.

Application of the NO donor (SNP) through the roots also leads to induced resistance, i.e. reduced damage symptom (chlorosis and leaf rolling) development on the leaves of the RWA infested susceptible plants. However, it seems that NO application through the roots can only confer a short-term resistance to these plants, because its effect diminished later-on (336 h.p.i) (Fig. 4.15 a and b). This finding correlates with results in Figure 4.10, where NO application induced the activities of the secondary defense PR proteins in the infested susceptible plants, suggesting that NO could reduce the damage symptom development through elevated activities of the secondary defense related enzymes.

In contrast to the above, application of NO through the roots had no effect on the number of aphids (adults and nymphs) attracted to the plants (Fig. 4.16 a). It seems that application of NO as a seed dressing was more effective in reducing aphid attraction within a 24 hour period (Fig. 4.16 b), which is an indication that NO application method

plays an important role in inducing the plant's resistance. These results also suggest that the signaling effect of NO may lead to the release of chemicals that have a repellent action on the aphids. To support this, it was discovered that the RWA settling was significantly reduced after exposure of wheat to methyl salicylate, a volatile product (Prinsloo et al., 2007) of the PAL pathway that leads to SA accumulation (Mohase and van der Westhuizen, 2002). Based on these studies, it could be that aphids were not attracted to the plants due to high SA accumulation as a result of NO production in these plants (Fig. 4.13 and 4.14).

A significant reduction in the intrinsic rate of aphid increase (Fig. 4.17 b), where NO was applied as a seed dressing (compared to where NO was applied through the roots; Fig. 4.17 a), further proves that the method of NO application is very important in inducing the defense responses of plants. The seed dressing method would also be very practical and easy to apply. These results also point out the importance of elevated NO in the reduction of aphid population increase rate.

# CHAPTER 6

## 6. OBJECTIVES ACHIEVED

The understanding of the plant's defense mechanisms is still fragmentary. Although disease and pest management in plants has been to a great extend achieved through the use of resistance genes, a major challenge is biotype evolution, whereby pathogens mutate to overcome plant resistance. In South Africa for example, one of the biggest threats to the wheat industry is crop losses caused by the Russian wheat aphid (RWA), *Diuraphis noxia* (Mordvilko), a destructive pest of wheat. This pest has been successfully controlled through the use of resistant cultivars containing resistance genes such as *Dn1*, *Dn2* and *Dn5* (Prinsloo, 2000). However, the advent of a new resistance breaking RWA biotype (*RWASA2*) in South Africa (Jankielsohn and Lindeque, 2006; Tolmay et al., 2007) prompted continued research on the resistance mechanism in wheat against the RWA, for advancement of resistance breeding.

This research was conducted in an attempt to increase our understanding of the early biochemical reactions of the RWA resistance response by investigating the involvement of reactive nitrogen species (RNS), specifically nitric oxide (NO) and peroxynitrite ($ONOO^-$). Results of this research may provide new insights for the understanding of the RWA resistance response, which may be useful in designing new and effective RWA control measures, especially considering the possible scenario of the development of new resistance breaking RWA biotypes. Effective resistance will be beneficiary to the wheat industry as a whole, but more specifically will benefit emerging farmers with no

access to expensive modern pesticides and little knowledge and equipment to apply it. Modern technology is provided in the form of seed, which is simple to use.

New ground was broken with the discovery of the involvement of NO in the RWA resistance response. Various aspects of this discovery include the following:

1. Early production of NO in infested resistant plants indicates that NO is involved in the early events of RWA resistance response. Noteworthy, higher NO levels in resistant than susceptible plants may suggest that resistance is somehow associated with the level of NO production. However, this needs to be explored further.
2. In contrast to the knowledge that nitric oxide synthase (NOS) is involved in NO production, the results proved that nitrate reductase (NR) produces NO during RWA resistance response, however not directly. Consensually, high NR activity corresponded with high NO production. It was indicated that NR is only involved in the reduction of nitrate to nitrite. Results essentially revealed that reduction of nitrite to NO is facilitated by nitrite reductase (NiR). In accordance, production of NO during RWA resistance can be summarized as:

$$NO_3^- \xrightarrow{NR} NO_2^- \xrightarrow{NiR} NO$$

Moreover, resistance was found to be linked with induced NR activity, further suggesting the involvement NO in RWA resistance response of wheat (because NR subsequently leads to the production of NO).

A low constitutive production of NO in both uninfested resistant and susceptible plants may be an indication that NO is not only important for resistance, but plants need this molecule for other physiological processes.

3. Studies using NR inhibitor (sodium tungstate, which subsequently leads to inhibition of NO production) or NO donor (sodium nitroprusside, SNP), revealed that NO is involved in the signal events leading to induction of the secondary (downstream) defence enzymes (intercellular β-1,3-glucanase and peroxidase), which are used as markers of resistance.

   In an attempt to unravel the signal network of RWA resistance response, which involves salicylic acid (SA) (Mohase and van der Westhuizen, 2002), an important discovery that NO acts upstream SA during RWA resistance was made. This is a crucial revelation towards understanding of the RWA signal reactions.

4. During the RWA resistance response, the reactive oxygen species (ROS) such as superoxide anion ($O_2^-$) produced by NADPH oxidase (Moloi and van der Westhuizen, 2006), react with NO to form peroxynitrite ($ONOO^-$). Importantly, results of $ONOO^-$ production inhibition suggested a possible involvement of $ONOO^-$ in the RWA resistance mechanism, probably as another signal molecule for the induction of downstream defense response. This accentuates the possibility of cross-talk between signaling pathways.

5. Application of NO has a potential to alleviate the RWA damage symptoms and also to reduce the aphid population on the susceptible plants. More importantly, the method of NO application as a seed dressing is of great interest, because it is a simple control method that can be used by emerging farmers. This method may also be applicable to traditional farmers.

Although findings of this study may not be applicable under field conditions due to various biotic and abiotic factors, they may be used as a base-line for measuring what may be expected. For the first time, this study has revealed the involvement of the RNS as one of the prerequisites for the RWA resistance response in wheat. The proposed model herein (Fig. 6.1), indicates how this study fits into the existing knowledge of the early signal reactions leading to RWA resistance.

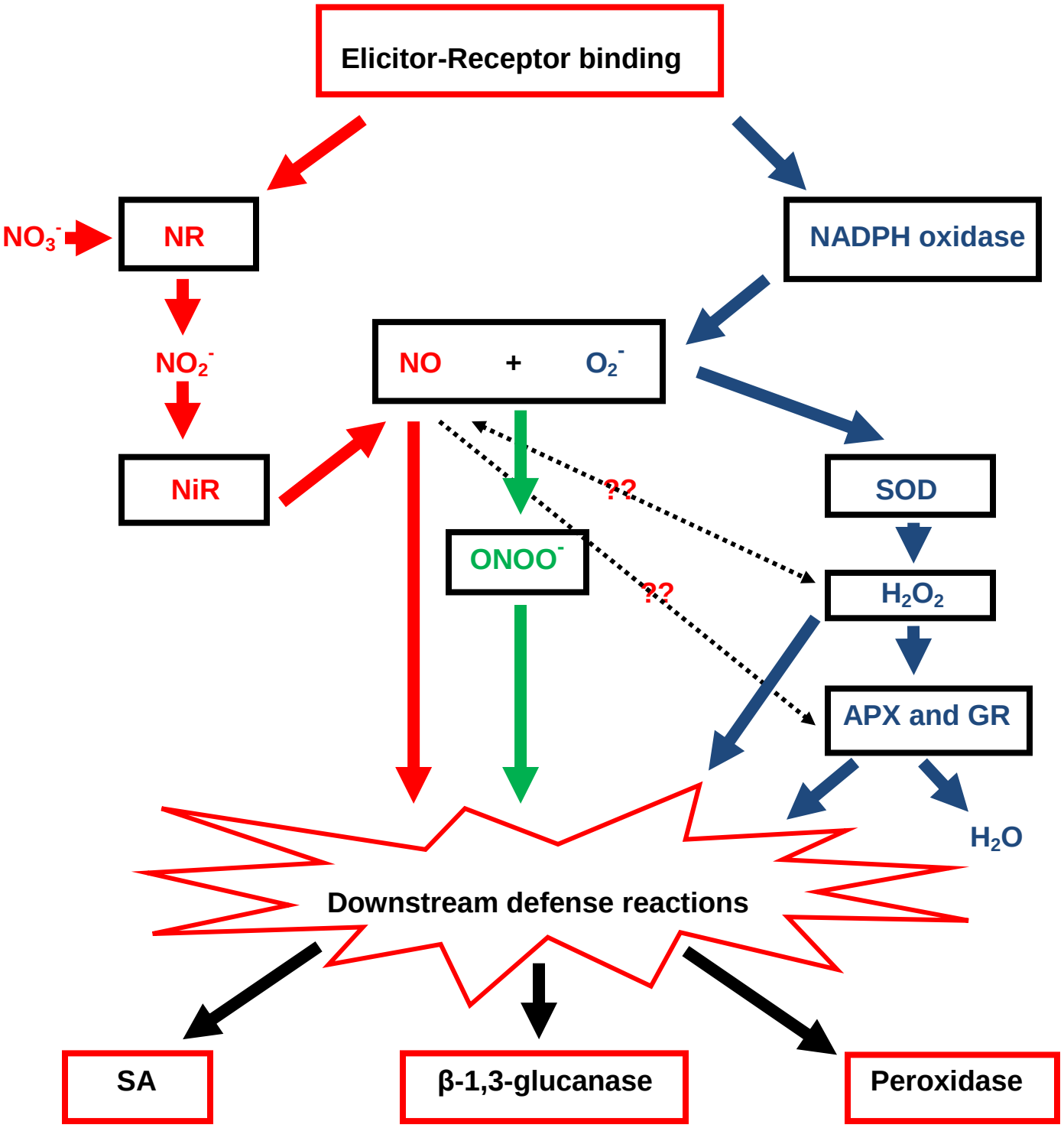


**Figure 6.1.** Proposed model for the involvement of the reactive nitrogen species (RNS) and reactive oxygen species (ROS) in the RWA resistance response of a wheat. After

RWA attack, elicitors bind to receptors at the cell wall. This binding induces early production of nitric oxide (NO) and hydrogen peroxide ($H_2O_2$) (Moloi and van der Westhuizen, 2006), which are involved in the RWA resistance response as early signal molecules. Key enzymes in NO production are nitrate reductase (NR) and nitrite reductase (NiR). Key enzymes in $H_2O_2$ production are NADPH oxidase and superoxide dismutase (SOD) (Moloi and van der Westhuizen, 2006). This model suggests that NO acts upstream of salicylic acid (SA), consequently leading to induction of other downstream defense reactions such as β-1,3-glucanase and peroxidase. The increase in NO production promotes the reaction between NO and $O_2^-$ to yield peroxynitrite ($ONOO^-$), which also plays an important role in activation of the downstream defense reactions. Whether or not NO acts independently of $H_2O_2$ during the RWA resistance response is not clear. Also associated with the secondary RWA defense response is induction of the antioxidative enzymes such as ascorbate peroxidase (APX) and glutathione reductase (GR), which convert $H_2O_2$ to $H_2O$ when it reaches toxic level (Moloi and van der Westhuizen, 2008). The relationship between NO production and these enzymes needs further exploration.

An increase in the production of ROS (Moloi and van der Westhuizen, 2006) and RNS such as NO in resistant plants, which are the prerequisites of hypersensitive response (HR) (Levine et al., 1994; Baker and Orlandi, 1995; Klessing et al., 2000), further confirm initial reports that RWA resistance response is a typical HR characteristic of pathogenesis (Belefant-Miller et al., 1994; van der Westhuizen et al., 1998 a and b).

## CHAPTER 7

# 7. APPENDIX

## *7.1: RESULTS OF INDEPENDENT REPLICATE EXPERIMENTS*

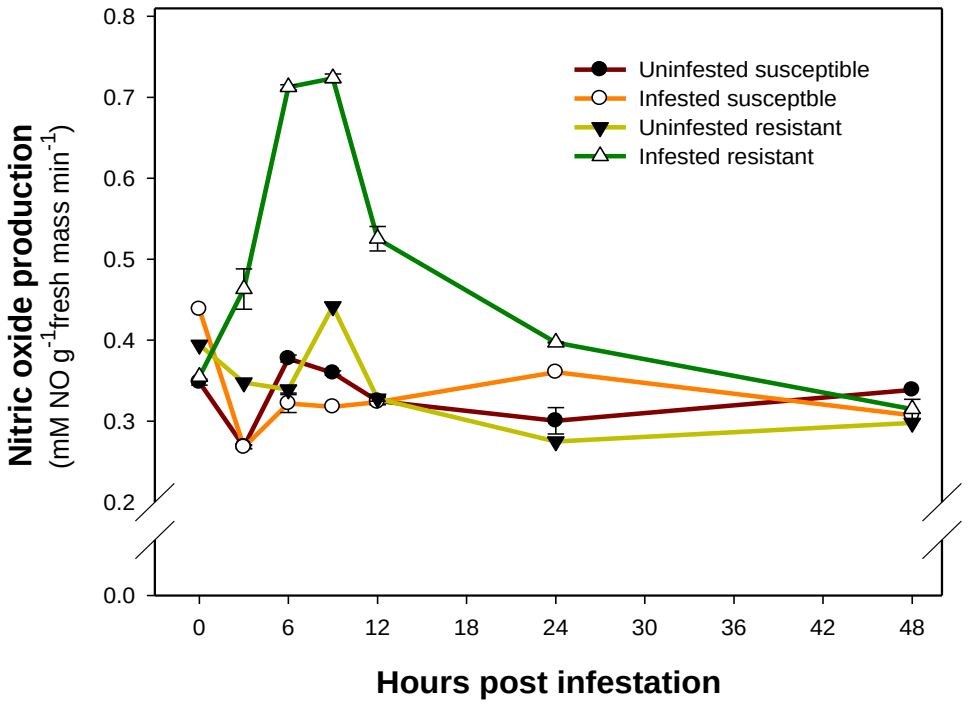


**Figure 7.1.** Effect of RWA infestation on NO production of resistant (*Tugela DN*) and susceptible (*Tugela*) cultivars. Values are means SD ± (n = 3).

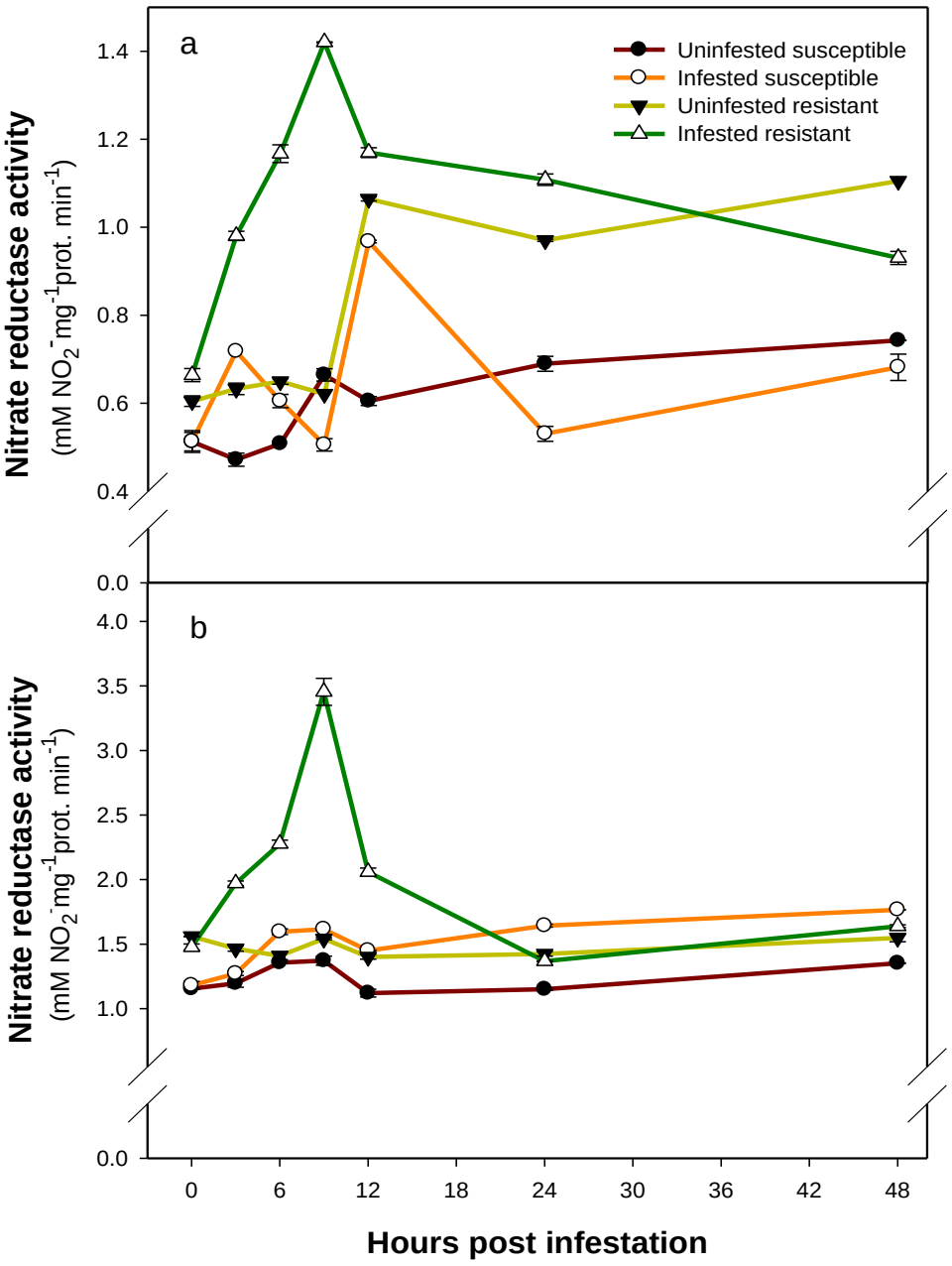


**Figure 7.2.** Effect of RWA infestation on NR activity of susceptible (*Tugela*) and resistant (*Tugela DN*) wheat cultivars. Values are means ± SD (n=3). (a: represents duplicate results; b: represents triplicate results).

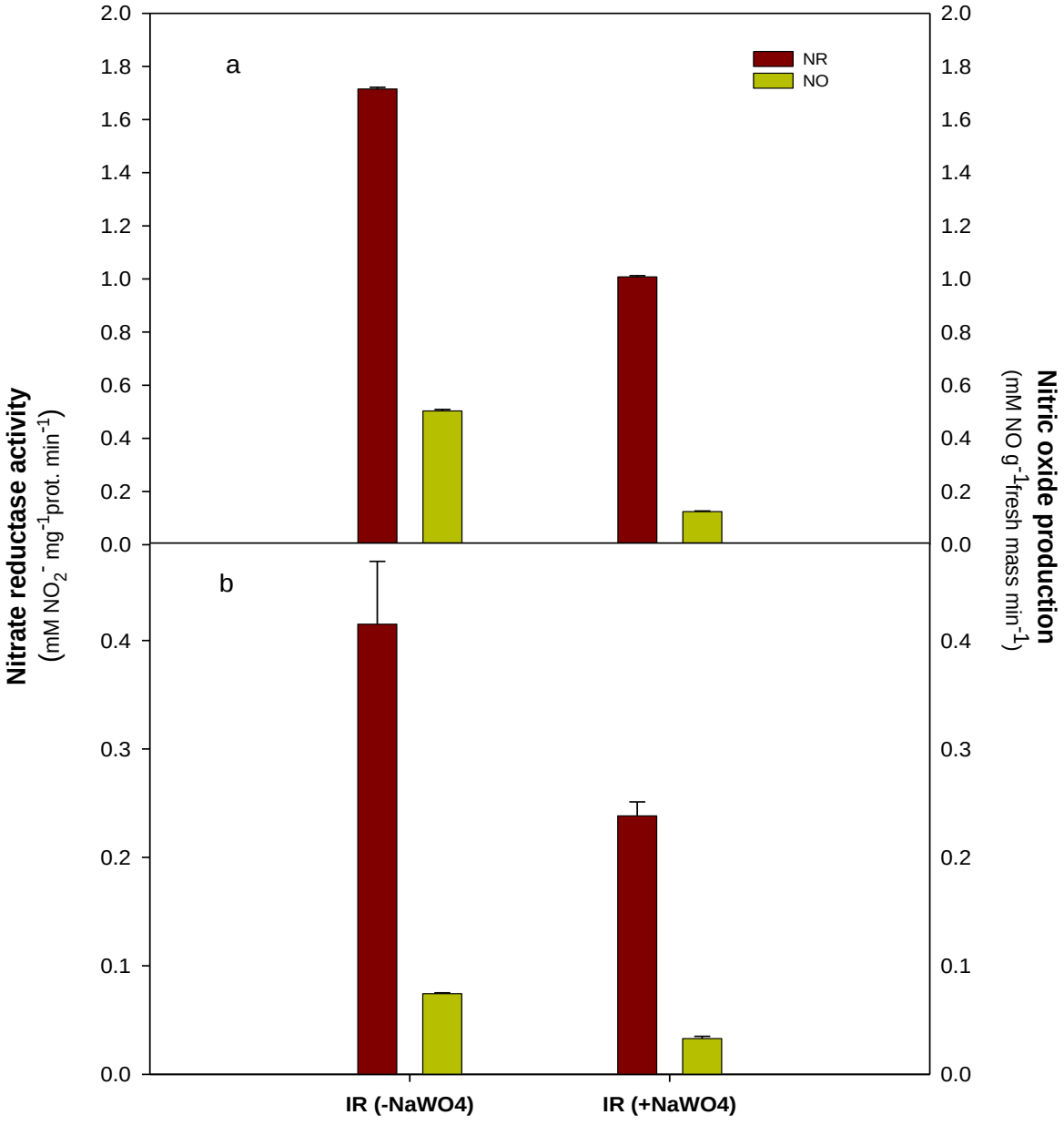


**Figure 7.3.** Effect of sodium tungstate ($Na_2WO_4$) on NR activity and NO production (*in vivo*) of a RWA infested resistant (*Tugela DN*) wheat cultivar (9 h.p.i). Values are means ± SD (n=3). (a: represents duplicate results; b: represents triplicate results).

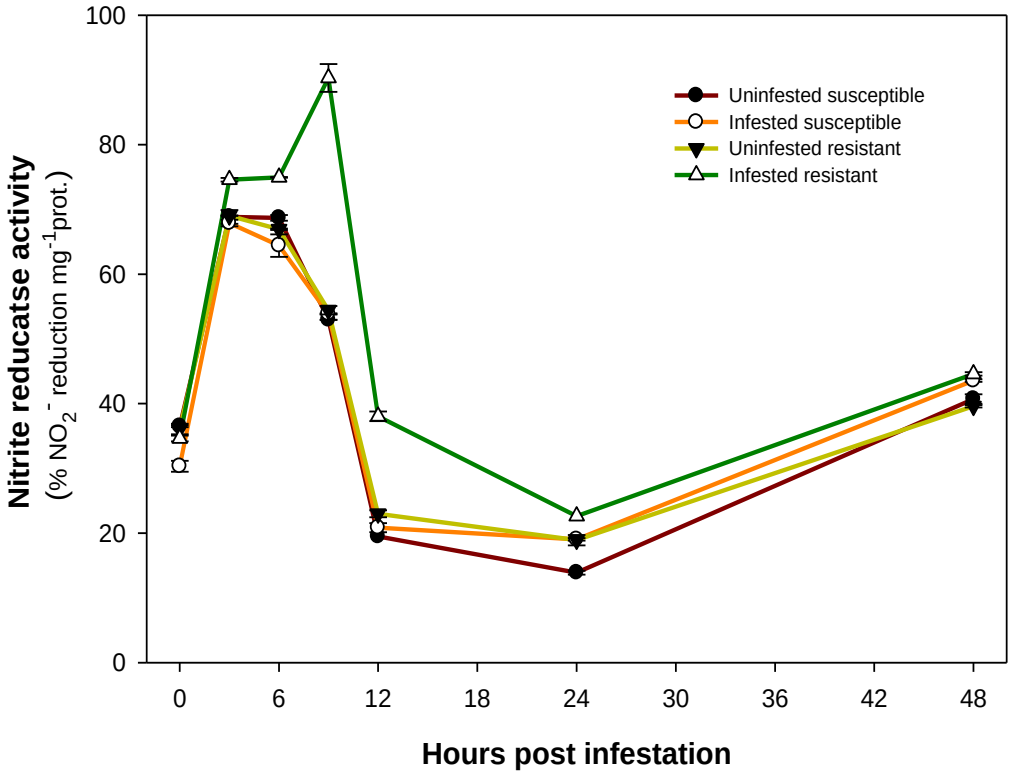


**Figure 7.4.** Effect of RWA infestation on NiR activity of susceptible (*Tugela*) and resistant (*Tugela DN*) wheat cultivars. Values are means ± SD (n=3).

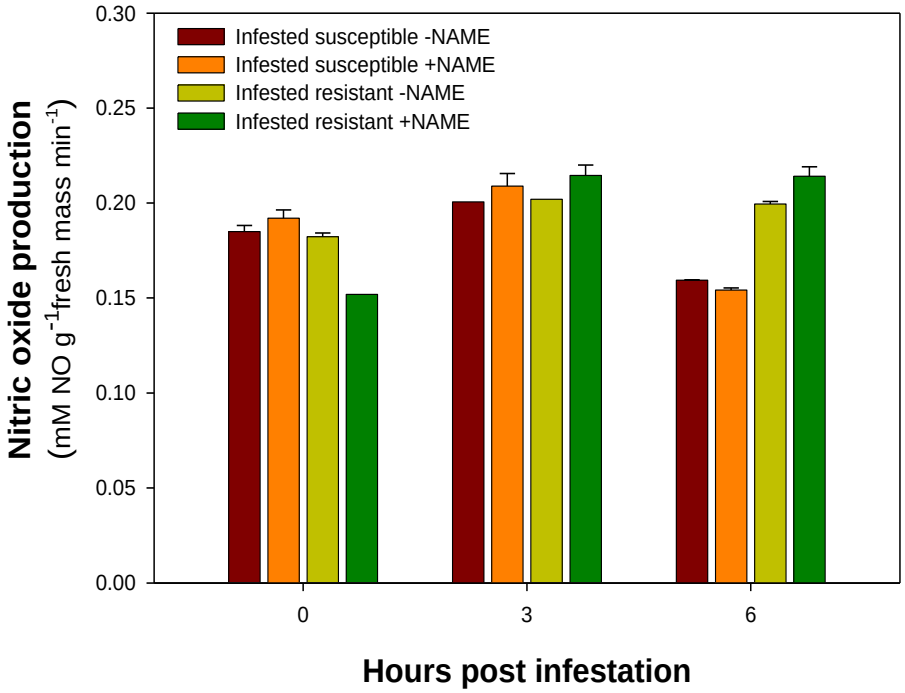


**Figure 7.5.** Effect of L-NAME (NOS inhibitor) on NO production of RWA infested susceptible (*Tugela*) and resistant (*Tugela DN*) wheat cultivars. Values are means ± SD (n=3).

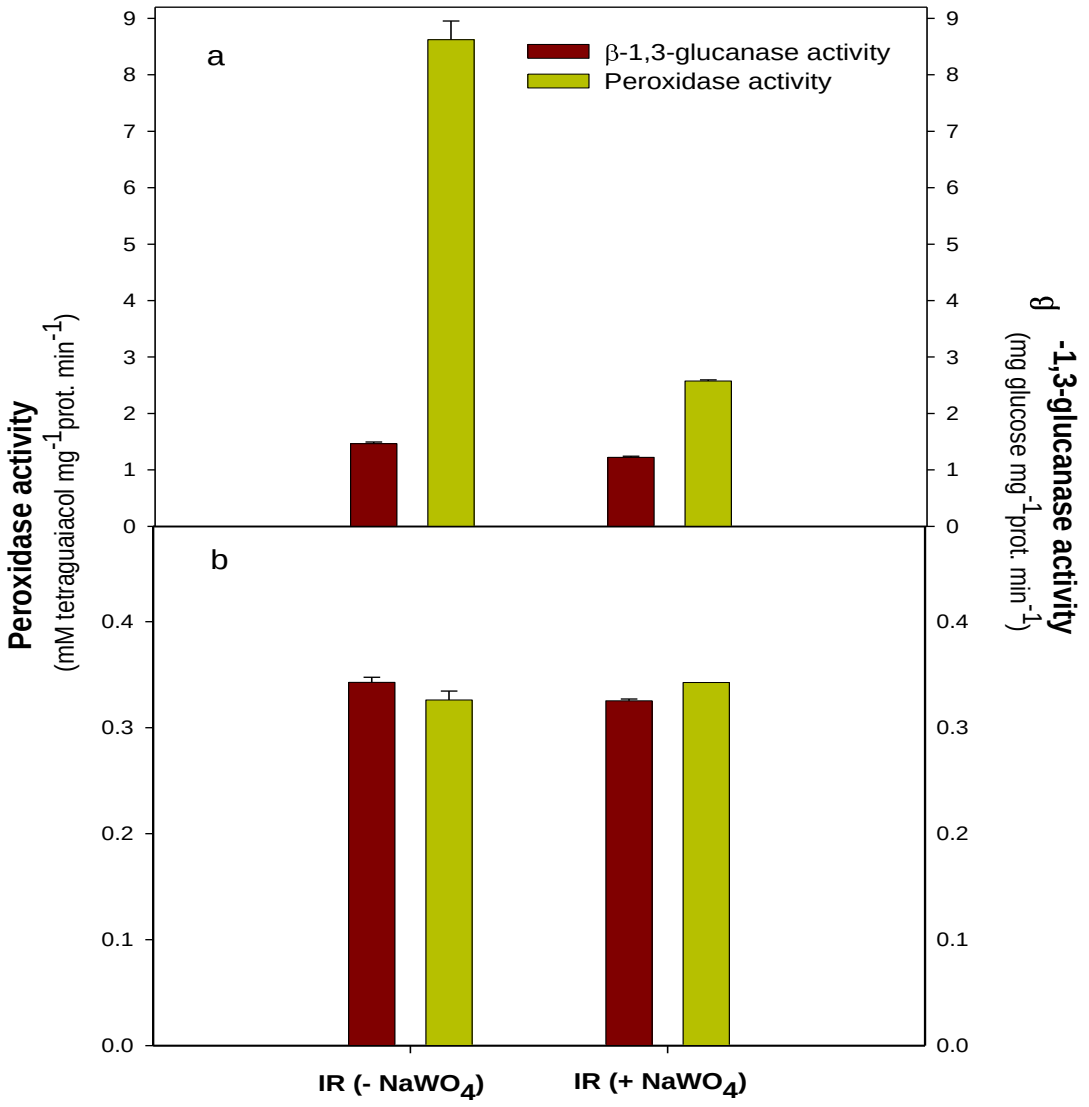


**Figure 7.6.** *In vivo* (a) and *in vitro* (b) effect of sodium tungstate ($NaWO_4$) (nitrate reductase inhibitor) on the intercellular β-1,3-glucanase and peroxidase activities of a RWA infested resistant (*Tugela DN*) wheat cultivar (48 h.p.i). Values are means ± SD (n = 3).

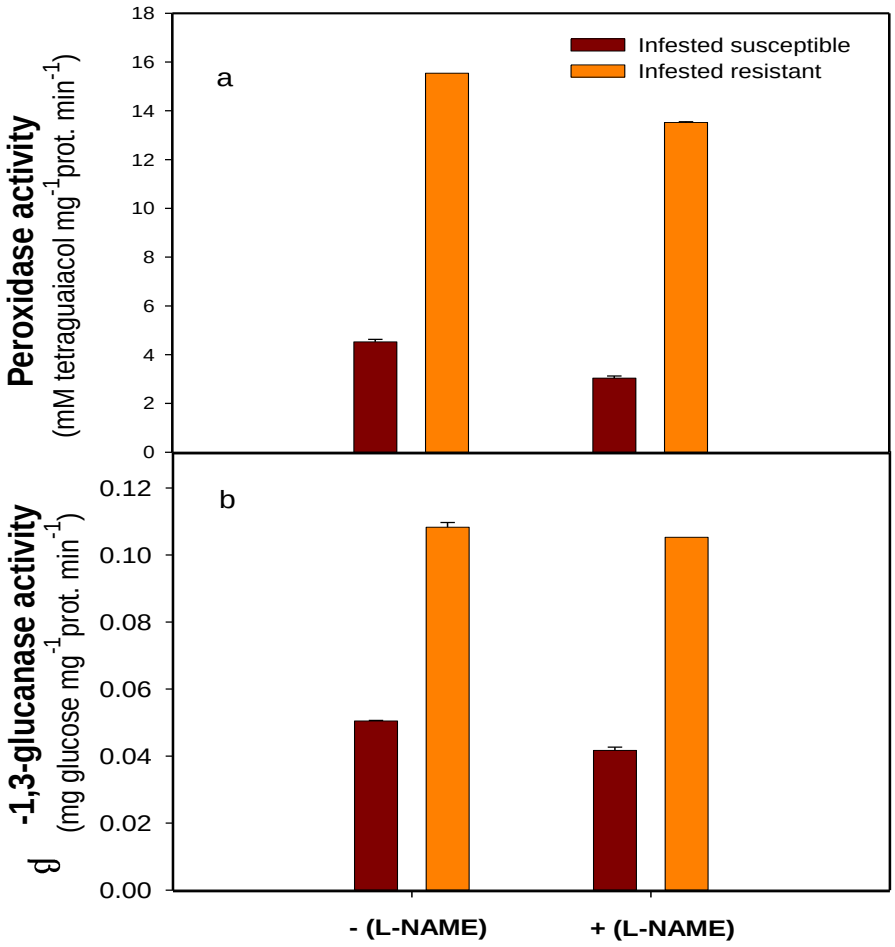


**Figure 7.7.** *In vivo* effect of L-NAME (NOS inhibitor) on the intercellular peroxidase (a) and β-1,3-glucanase (b) activities of RWA infested resistant (*Tugela DN*) and susceptible (*Tugela)* wheat cultivars (48 h.p.i). Values are means ± SD (n =3).

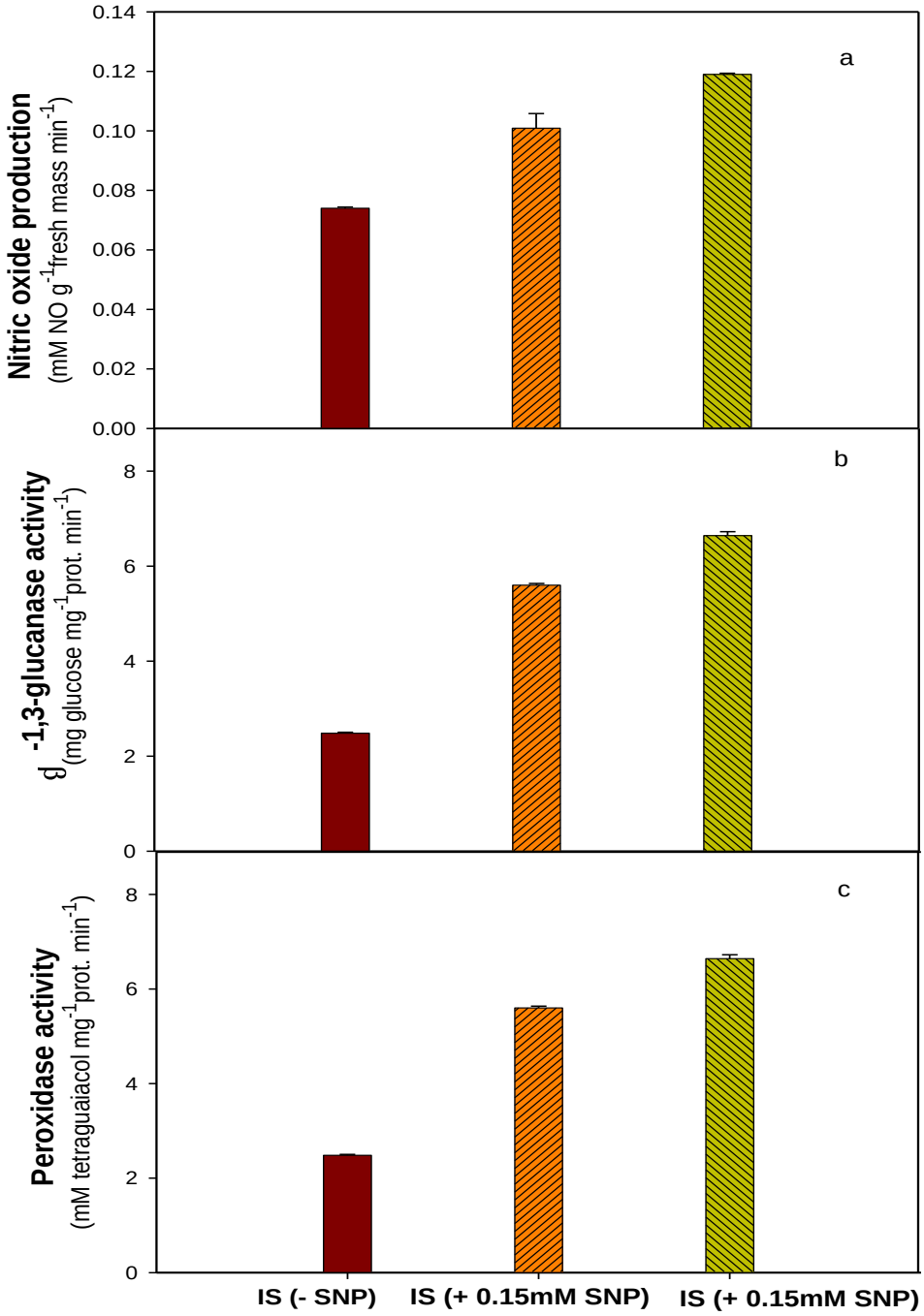


**Figure 7.8.** Effect of sodium nitroprusside (SNP) (root application) on NO production (a), β-1,3-glucanase (b) and peroxidase (c) activities of a RWA infested susceptible (*Tugela*) wheat cultivar (48 h.p.i). Values are means ± SD (n = 3).

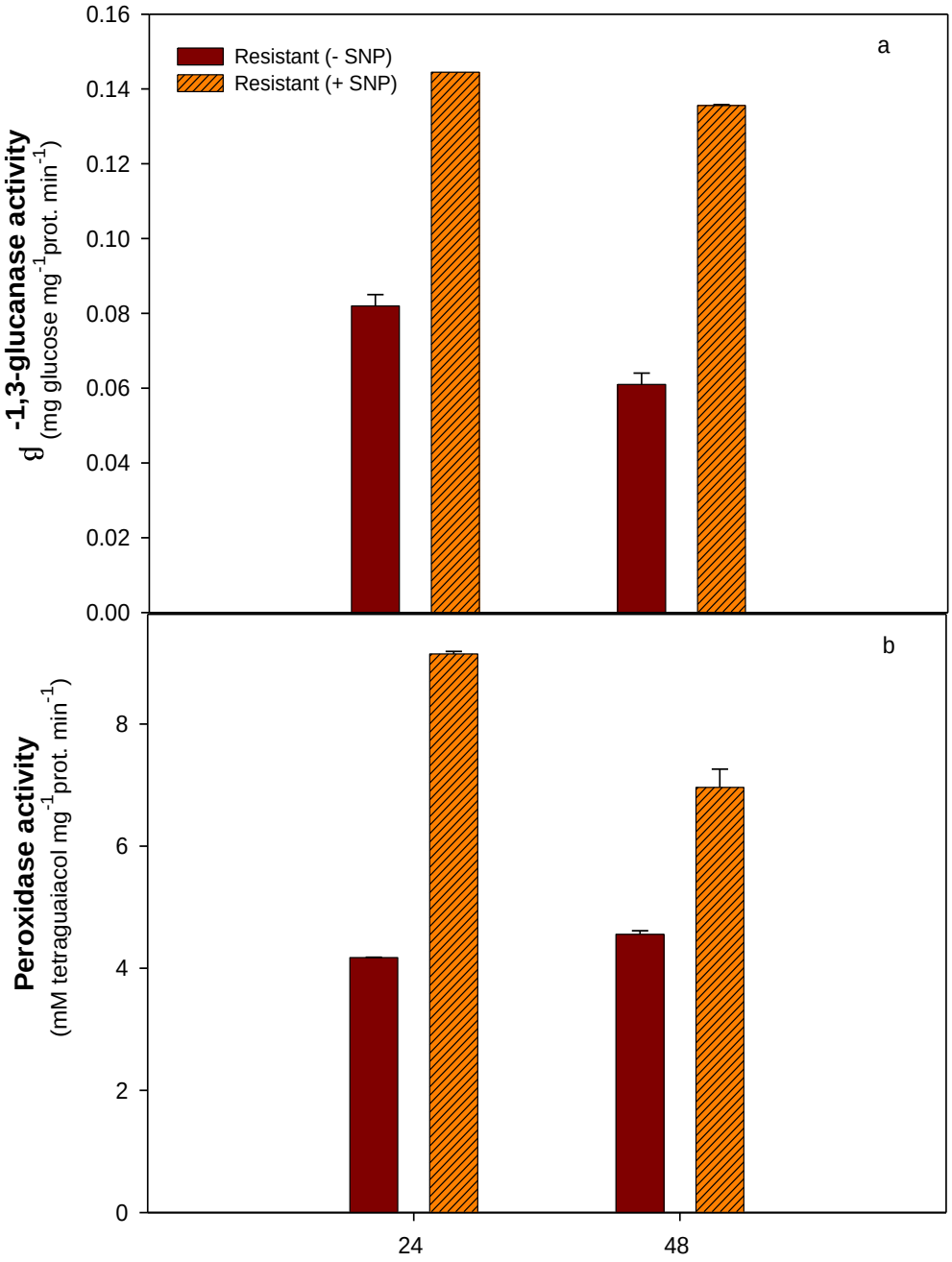


**Figure 7.9.** Effect of 0.15 mM SNP (root application) on the intercellular β-1,3-glucanase (a) and peroxidase (b) activities of a resistant (*Tugela DN*) wheat cultivar. Values are means ± SD (n = 3).

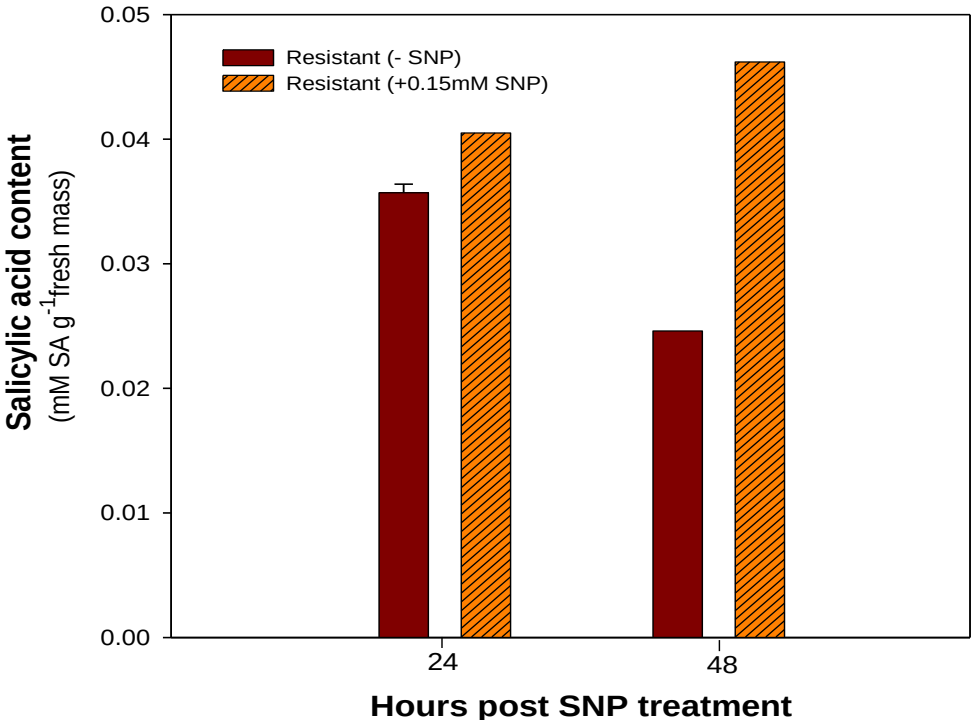


**Figure 7.10.** Effect of SNP (root application) on the total salicylic acid content of a resistant (*Tugela DN*) wheat cultivar. Values are means ± SD (n = 3).

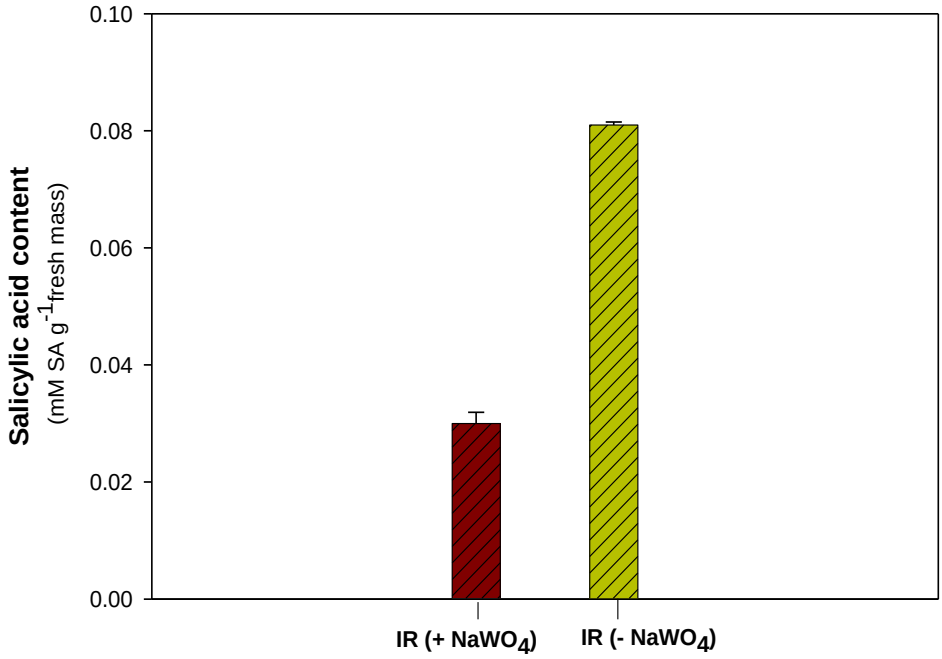


**Figure 7.11.** Effect of NO production inhibition ($NaWO_4$, nitrate reductase inhibitor) on the total SA content of a RWA infested (9 h.p.i) resistant (*Tugela DN*) wheat cultivar. Values are means ± SD (n = 3).

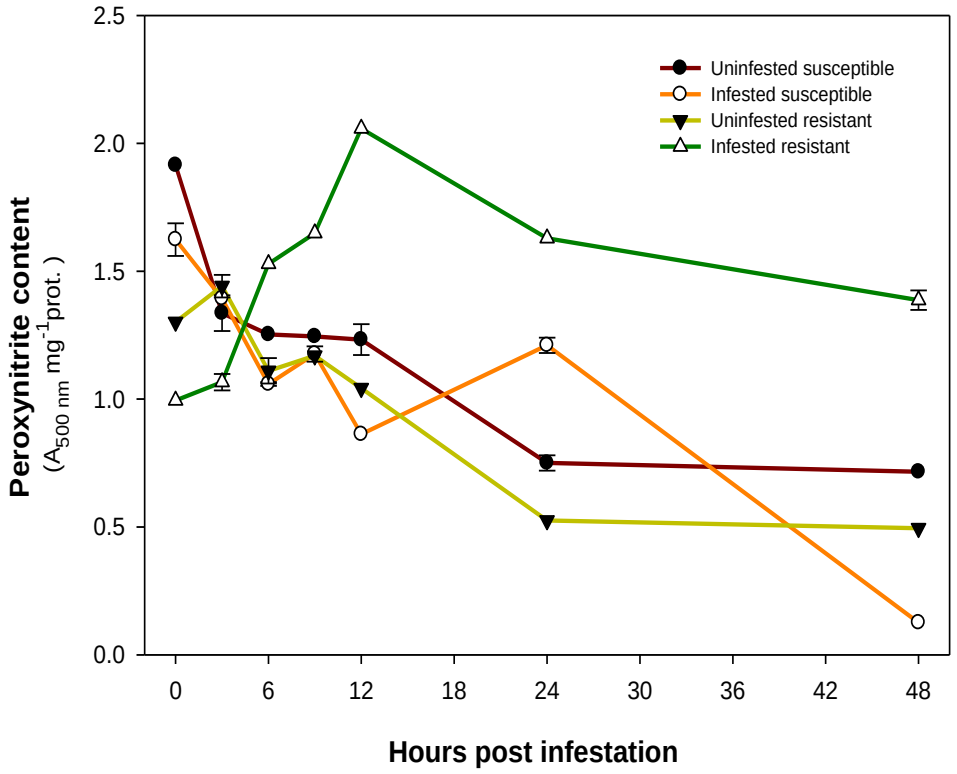


**Figure 7.12.** Effect of RWA infestation on the peroxynitrite content of resistant (*Tugela DN*) and susceptible (*Tugela*) wheat cultivars. Values are means ± SD (n = 3).

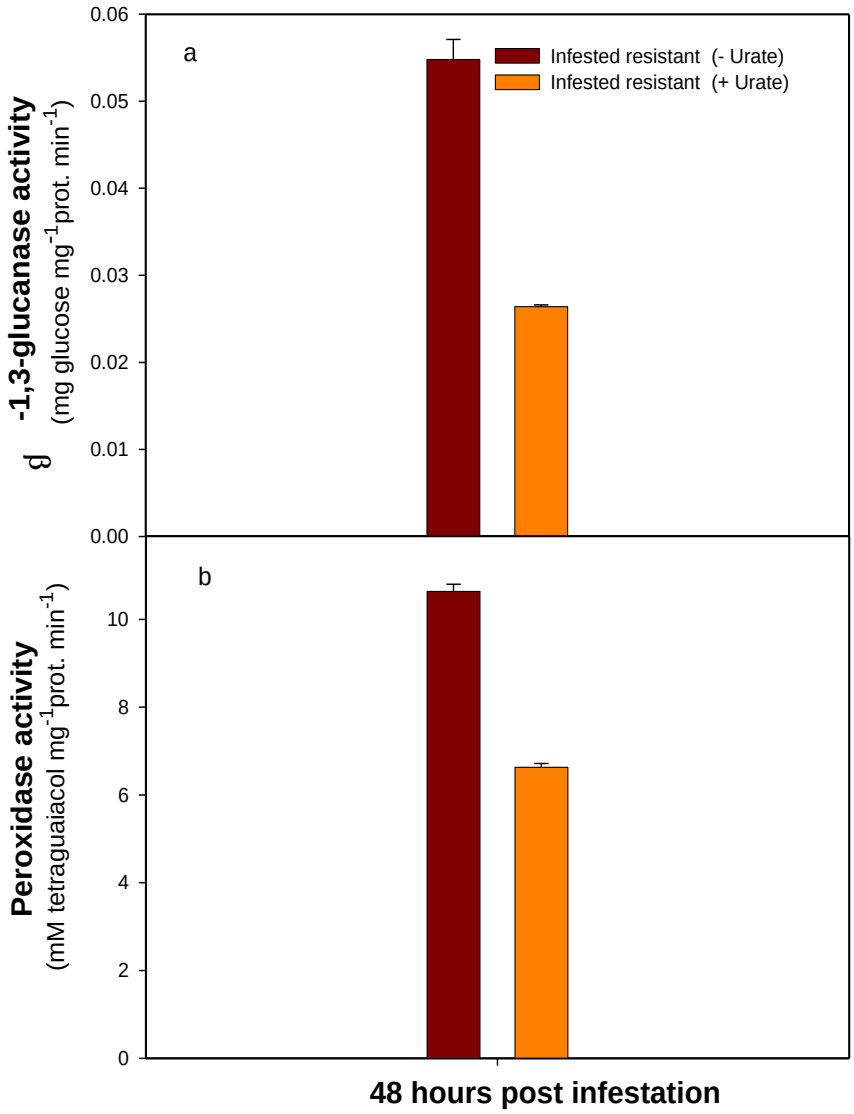


**Figure 7.13.** Effect of urate ($ONOO^-$ formation inhibitor) on the intercellular β-1,3-glucanase (a) and peroxidase (b) activities (48 h.p.i) of a RWA infested resistant (*Tugela DN*) wheat cultivar. Values are means ± SD (n = 3).

## *7.2: LIST OF ABBREVIATIONS*

1. Abscisic acid (ABA).
2. Agricultural Research Council- Small Grain Institute (ARC-SGI).
3. *Arabidopsis thaliana* NO-associated 1 (AtNOA1).
4. *Arabidopsis thaliana* NOS1 (AtNOS1).
5. Ascorbate peroxidase (APX).
6. Avirulence (*Avr).*
7. Bovine serum albumin (BSA).
8. Constitutive NOS (cNOS).
9. Dichlorodihydrofluorescein (DCDHF).
10. Dichlorofluorescein (DCF).
11. *Diuraphis noxia (Dn).*
12. Endothelial NOS (eNOS).
13. Glutathione reductase (GR).
14. Hours post infestation (h.p.i).
15. Hydrogen peroxide ($H_2O_2$).
16. Hypersensitive response (HR).
17. Inducible NOS (iNOS).
18. Infested resistant (IR).
19. Infested susceptible (IS).
20. Intercellular washing fluids (IWF).
21. Jasmonic acid (JA).
22. Neuronal NOS (nNOS).

23. Nitrate reductase (NR).
24. Nitric oxide (NO).
25. Nitric oxide synthase (NOS).
26. Nitrite reductase (NiR).
27. Nitrosyl cation ($NO^+$).
28. $N^{\omega}$-hydroxy-L-arginine (NOHA).
29. $N^{\omega}$-nitrilo-L-arginine-methyl ester (L-NAME).
30. $N^{\omega}$-nitro-L-arginine (L-NNA).
31. Pathogenesis related (PR).
32. Peroxynitrite ($ONOO^-$).
33. Phenylalanine ammonia lyase (PAL).
34. Proteinase inhibitor (PI).
35. Quantitative loci (QTLs) .
36. Reactive nitrogen species (RNS).
37. Reactive oxygen species (ROS).
38. Resistance (R).
39. Reverse transcriptase polymerase chain reaction (RT-PCR)
40. Russian wheat aphid (RWA).
41. SA-binding protein (SABP).
42. Salicylic acid (SA).
43. S-nitrosoglutathione (GSNO).
44. Sodium nitroprusside (SNP).
45. Superoxide anion ($O_2^-$).

46. Superoxide dismutase (SOD).
47. Systemic acquired resistance (SAR).
48. Tobacco mosaic virus (TMV).
49. Ultra violet (UV).

## *7.3: LIST OF FIGURES*

Page

## 8. SUMMARY

A recent accumulating body of evidence points to the importance of nitric oxide (NO) as a signal molecule involved in the regulation of many physiological and biochemical processes in plants. This study was aimed at investigating the involvement of reactive nitrogen species (RNS), in particular NO and peroxynitrite ($ONOO^-$), in the Russian wheat aphid (RWA), *Diuraphis noxia* (Mordvilko), resistance response of wheat (*Triticum aestivum* L.). Resistant (cv. *Tugela DN*) and near isogenic susceptible (cv. *Tugela*) wheat plants were grown under the greenhouse conditions in trays at 24 $^oC$ (± 2 $^oC$) and were infested at an early three leaf stage with approximately 20 RWAs (biotype *RWASA1*) per plant. RWA infestation induced an early accumulation (3-9 hours post infestation) of NO to a higher level in the resistant than susceptible plants. A corresponding increase in the activity of nitrate reductase (NR, EC 1.6.6.1) and inhibition studies using sodium tungstate (NR inhibitor) confirmed its involvement in NO production during the RWA resistance response. In addition, results indicated that the reduction of nitrate ($NO_3^-$) to nitrite ($NO_2^-$) involves NR and subsequently, nitrite reductase (NiR, EC 1.7.7.1) is responsible for the direct production of NO from $NO_2^-$. Inhibition studies using a nitric oxide synthase (NOS, EC 1.14.13.39) inhibitor, $N^{\omega}$-nitrilo-L-arginine-methyl ester (L-NAME), further proved that NOS has no significant effect on NO production during the RWA resistance response.

In an experimental approach using sodium nitroprusside (SNP, NO donor) and / or sodium tungstate (NR inhibitor), it was discovered that NO acts as a signal for the

induction of activities of secondary defense related enzymes such as the intercellular β-1,3-glucanase and peroxidase. Higher β-1,3-glucanase transcript accumulation after SNP treatment in resistant plants further supported the involvement of NO as signal molecule during the RWA defense response. In addition, results revealed that NO acts upstream of salicylic acid (SA) during the signaling events.

High production of $ONOO^-$ [a product of an interaction between superoxide anion ($O_2^-$) and NO] in resistant plants after RWA infestation, suggests its involvement in the RWA resistance response. Inhibition of β-1,3-glucanase and peroxidase activities by urate (a specific inhibitor of $ONOO^-$ production), further suggests the involvement of $ONOO^-$ in the induction of the secondary RWA defense response.

Furthermore, it was found that SNP root application of wheat plants resulted in transient resistance only. However, in contrast to root treatments, application of SNP as a seed dressing was more effective and has indeed a great potential of alleviating RWA damage in the susceptible plants by reducing aphid attraction as well as the intrinsic rate of aphid increase.

## 9. KEYWORDS

Russian wheat aphid; *Diuraphis noxia*; reactive nitrogen species; nitric oxide; nitrate reductase; nitrite reductase; nitric oxide synthase; peroxynitrite; peroxidase; β-1,3-glucanase; RWA resistance.

## 10. OPSOMMING

Die belangrikheid van stikstofoksied (NO) as ‘n seinmolekule by die regulering van fisiologiese en biochemiese prosesse in plante word huidiglik toenemend beklemtoon. Die doel van hierdie studie was om die rol van reaktiewe stikstofspesies (RNS), veral NO en peroksinitriet ($ONOO^-$), in die weerstandsrespons van koring (*Triticum aestivum* L.) teen die Russiese koringluis (RKL), *Diuraphis noxia* (Mordvilko) te bepaal. Weerstandbiedende (cv. *Tugela DN*) en na-isogeniese vatbare (cv. *Tugela*) koringplanteplante is in saadlaaie in ‘n glashuis by 24 °C (± 2 °C) gekweek en op ‘n vroeë drieblaarstadium met nagenoeg 20 RKL (biotipe *RWASA1*) per plant geïnfekteer. RKL-infestering induseer ‘n vroeë akkumulasie (3-9 ure na infestering) van NO, met hoër vlakke in die weerstandbiedende plante as in die vatbare plante. ‘n Ooreenstemmende toename in die aktiwiteit van nitraatreduktase (NR, EC 1.6.6.1) en remmingsstudies met behulp van natriumtungstaat (NR-remstof) het die betrokkenheid van NR by NO-produksie tydens die RKL-weerstandsrespons bevestig. Hierbenewens het resultate aangetoon dat NR by die reduksie van nitraat ($NO_3^-$) na nitriet ($NO_2^-$) betrokke is en dat nitrietreduktase (NiR, EC 1.7.7.1) vir die direkte produksie van NO vanaf $NO_2^-$ verantwoordelik is. Remmingstudies, waar ‘n stikstofoksiedsintase (NOS, EC 1.14.13.39) remstof, $N^{\omega}$-nitrilo-L-arginien-metielester (L-NAME), gebruik is, het verder bewys dat NOS geen betekenisvolle invloed op NO-produksie gedurende die RKL-weerstandsrespons gehad het nie.

‘n Eksperimentele benadering deur van natriumnitroprussied (SNP, NO skenker) en / of natriumtungstaat (NR-remstof) gebruik te maak, het getoon dat NO as ‘n sein vir die induksie van aktiwiteite van sekondêre verdedigingsverwante ensieme soos intersellulêre β-1,3-glukanase and peroksidase dien. Hoër β-1,3-glukanasetranskripakkumulasie na SNP-behandeling van weerstandbiedende plante ondersteun die betrokkenheid van NO as seinmolekule gedurende die RKL-verdedigingsrespons. Resultate het ook getoon dat NO stroomop van salisielsuur (SA) gedurende seintransduksie werksaam is.

‘n Hoë produksie van $ONOO^-$ [‘n produk van die interaksie tussen superoksiedanione ($O_2^-$) en NO] in weerstandbiedende plante na RKL-infestering was aanduidend van die betrokkenheid van $ONOO^-$ by die RKL-weerstandsrespons. Remming van β-1,3-glukanase- en peroksidase-aktiwiteite deur uraat (‘n spesifieke remstof van $ONOO^-$ produksie) toon verder dat $ONOO^-$ by die induksie van die sekondêre RKL-verdedigingsrespons betrokke is.

Voorts is bevind dat SNP, as ‘n worteltoediening, net kortstondige weerstand by koringplante veroorsaak het. Daarenteen was SNP meer doeltreffend wanneer dit as ‘n saadbedekking aangewend was en het dit inderdaad groot potensiaal om RKL-skade in vatbare plante te verlig deur die luisaantrekking te verminder sowel as om die luistoenametempo te verlaag.

## 11. CONFERENCE CONTRIBUTIONS ORIGINATING FROM THIS STUDY:

1. **M.J Moloi and A .J van der Westhuizen** (oral presentation). Involvement of nitric oxide during the Russian wheat aphid resistance. **33rd Annual Conference of the South African Association of Botanists**, 14-18 January 2007, University of Cape Town, Cape Town, South Africa.
2. **M.J Moloi and A.J van der Westhuizen** (oral presentation). Involvement of nitric oxide in the Russian wheat aphid resistance response of wheat. **International Conference on Biotic Plant Interactions,** 27-29 March 2008, Queensland Biosciences Precinct, The University of Queensland, Brisbane, Australia.
3. **M.J Moloi and A.J van der Westhuizen** (oral presentation). Nitric oxide and the Russian wheat aphid resistance response of wheat. **35th Annual Conference of the South African Association of Botanists**, 18-22 January 2009, Stellenbosch University, Cape Town, South Africa.
4. **M. J Moloi and A. J van der Westhuizen** (poster presentation). Involvement of the reactive nitrogen species in the Russian wheat aphid resistance response of wheat. **"Deutsche Phytomedizinische Gesellschaft – Arbeitskreise Mykologie und Wirt-Parasit-Beziehungen" Workshop, Kaiserslautern,** 26-27 March 2009, Germany.
5. **M. J Moloi and A. J van der Westhuizen** (oral presentation). Involvement of nitric oxide in the RWA resistance response of wheat. **The postgraduate symposium,** 27 October 2009, University of Johannesburg, Auckland park campus, South Africa.

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