Nitric Oxide is an Upstream Signal Involved in the Multisignalling Network during the Russian Wheat Aphid Resistance Response and its Application Enhances Resistance

M.J. Moloi*, A.J. van der Westhuizen and A. Jankielsohn

University of the Free State, Bloemfontein, Free State, South Africa

The Russian wheat aphid (RWA) is serious pest of wheat in South Africa since its discovery in 1978. Nitric oxide (NO) plays an essential role in the RWA resistance response of wheat. This study was conducted to establish whether NO acts upstream or downstream of salicylic acid (SA) during the RWA defence response and also to investigate the effect of NO application on RWA control. In addition, the involvement of peroxynitrite in the RWA resistance response of wheat was studied. Resistant and/or susceptible plants grown under controlled conditions (25 ± 2°C) were used. Using a NO donor, sodium nitroprusside (SNP), and a NO production inhibitor, sodium tungstate (NaWO4), it was proved that NO acts upstream of SA during the RWA resistance response of wheat. Furthermore, a significant decrease in RWA intrinsic increase rate (rm) and disease symptom development after SNP application emphasized the role of NO in the RWA resistance responses. High levels of peroxynitrite (by-product of NO) content in the RWA infested resistant plants and inhibition of secondary defence enzymes (β-1,3-glucanase and peroxidase) after urate (inhibitor of peroxynitrite production) application proposed the involvement of this molecule in the signalling events of the RWA resistance.

Keywords: nitric oxide, peroxynitrite, resistance, Russian wheat aphid, salicylic acid

Introduction

The Russian wheat aphid (*Diuraphis noxia,* RWA) has been an important pest of wheat since 1978 when it was first reported in South Africa (Walters et al. 1980). 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 wheat include white, yellow, purple to reddish-purple longitudinal chlorotic streaks and severe rolling on the leaves, which result in lower grain yield/poor quality and even death in the case of extreme infestation (Walters et al. 1980).

* Corresponding author; E-mail: 1996688105@ufs4life.ac.za

Extensive research on the RWA resistance responses of wheat has been conducted in our lab. One of the events during the RWA resistance response is the extra production of nitric oxide (NO) to high levels. Nitric oxide plays an essential role in the early signalling events leading to induction of the secondary defence responses of RWA resistance such as the increased intercellular β-1,3-glucanase and peroxidase activities (Moloi and van der Westhuizen 2014), which are usually used as biochemical markers of systemic acquired resistance (SAR) in plants (Fritig et al. 1998). It was previously discovered that nitrate reductase (NR) is a key enzyme responsible for NO production during the RWA resistance responses. This finding was confirmed with NR inhibition studies, which showed that NR inhibition consequently leads to a significant reduction in NO production (Moloi and van der Westhuizen 2014). Preliminary studies using nitric oxide synthase (NOS) inhibitor further substantiated that NR is the main source of NO production during RWA resistance response (unpublished data).

Salicylic acid (SA) is required for the establishment of SAR (Hayat et al. 2010). A co-operative action between NO and SA has been suggested (Klessig et al. 2000). Although contradictory, some studies suggest NO to act upstream SA during resistance (Song and Goodman 2001) and others suggest the opposite (Zottini et al. 2007; Gaupels et al. 2008). Involvement of SA in the RWA resistance response network has been established. A time-dependant study showed that the RWA infested resistant plants produce higher SA levels than susceptible plants. This selective induction was only observed from 48 hours post infestation (h.p.i) and coincided with high peroxidase activity (Mohase and van der Westhuizen 2002).

In addition, Moloi and van der Westhuizen (2006) indicated that the reactive oxygen species (ROS) and ROS generating enzymes are involved in the early events of the RWA resistance response. The ROS and NO may rapidly react in the absence of enzyme catalysis to form a powerful oxidizing agent, peroxynitrite (Blough and Zafiriou 1985; Saito et al. 2006).

On the basis of the above described work, this study was conducted to explore whether NO acts upstream or downstream of SA and also to investigate involvement of peroxynitrite during the RWA resistance response. Since previous studies clarified the importance of NO in the RWA resistance, the effect of sodium nitroprusside (SNP, a NO donor) application was investigated as a possible tool for RWA control. Parameters used in this case were the intrinsic increase rate (r_m) of RWA populations and the RWA feeding symptom development on the leaves.

Materials and Methods

Plant material

Resistant wheat (*Triticum aestivum* L.) (cv. *Tugela DN*), containing the *Dn1* (PI 137739) resistance gene (Du Toit 1989) and/or near-isogenic susceptible wheat (cv. *Tugela*) were grown under greenhouse conditions (25 ± 2ºC).

The RWA stock colonies

The RWA, *Diuraphis noxia* (Kurdjumov), biotype *RWASA1* stock colonies were maintained at the Small Grain Institute of the Agricultural research council (ARC-SGI). These aphids were screened to confirm biotype status and were maintained in gauze cages (gauze73 size: 315 micron) on different wheat cultivars, under greenhouse conditions (25 ± 2ºC).

Infestation procedure for peroxynitrite and SA content

Plants (approximately 20) were infested at the early three-leaf stage by scattering approximately 20 aphids per plant. Leaves were harvested after either time periods (0, 3, 6, 9, 12, 24, 48 h), frozen in liquid nitrogen and stored at –20ºC until used. Only 0.5 g of frozen leaf tissue was used for peroxynitrite and SA extraction.

Sodium nitroprusside (SNP), sodium tungstate and urate application

To investigate the relationship between SA and NO, uninfested resistant plants were supplied with Hoagland solution (Hoagland and Arnon 1950) containing 0.15 mM SNP for the duration of the experiment (48 h) through the roots. If applied to the biological systems, NO donors produce NO and are able to either mimic an endogenous NO-related response or substitute for an endogenous NO deficiency (Floryszak-Wieczorek et al. 2006). Leaves were then harvested for the determination of SA content. Vermiculite was used as a supporting material and this applies to all treatments. The control plants were supplied with Hoagland solution without SNP. The uninfested resistant plants were used here because of their low NO and SA levels as previously discovered (Mohase and van der Westhuizen 2002; Moloi and van der Westhuizen 2014). Therefore, this approach would make it possible to investigate the effect of increased endogenous NO on SA production.

Based on the discovery that NO production mainly depends on the activity of NR, infested resistant plants (which were found to have elevated NR activity and NO production; Moloi and van der Westhuizen 2014) were supplied (through the roots) with a Hoagland solution containing 4.1 mM sodium tungstate (Na_2WO_4, NR inhibitor) 2 hours prior infestation and was continued for the duration of the experiment (48 h). The control plants were not treated with tungstate in order to investigate the effect of NO inhibition on SA production. Leaves were then harvested for SA content determination.

Other sets of plants (susceptible and resistant) were supplied with Hoagland solution that was supplemented with different SNP concentrations (0.1, 0.15 mM and 0.5 mM) through the roots (1 hour prior to RWA infestation). These were used for the RWA intrinsic increase rate (r_m) and disease symptom analysis. Control plants were supplied with Hoagland solution only. In the case where SNP was applied as a seed dressing, seeds were soaked in SNP solutions of different concentrations (0.1, 0.15 and 0.5 mM) for 1 hour before planting. Control seeds were soaked only in distilled water for 1 hour before planting.

The RWA infested resistant plants were treated with 1 mM urate (peroxynitrite inhibitor) in Hoagland solution through the roots (2 hours before RWA infestation). Leaves

were harvested for peroxynitrite content (12 h.p.i), peroxidase and β-1,3-glucanase (48 h.p.i) activities assays.

Intrinsic increase rate and symptom development

First instar aphids were obtained from reproducing adult aphids and transferred to separate wheat plants until they reached adult reproductive stage. Plants were then infested at the early three leaf stage with two reproductive adult aphids per plant. 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:

$$r_m = \text{Ln}(R_o)/T \text{ where } R_o = \Sigma\, l_x m_x \text{ and } T = l_x m_x x$$

(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) (Birch 1948). Each treatment consisted of six replications.

Symptom development on the leaves was recorded 7 and 14 days after RWA infestation according to a scoring system adopted by Tolmay (1995), whereby plants with a score of $1 \geq 3.5$ represented highly resistant plants (no leaf rolling), $3.6 \geq 6.5$ represented medium resistant plants (chlorosis, but no leaf rolling), and $6.6 \geq 10$ represented susceptible plants (rolled leaves and severe chlorosis). Each treatment consisted of 4 replications.

Extract preparation

The extract for the peroxynitrite assay was prepared according to the method described by Datta and Sharma (1999) with no modifications.

Collection of intercellular washing fluids (IWF)

Procedure described by Moloi and van der Westhuizen (2006) was used.

Salicylic acid (SA) content

Total SA (free and conjugated forms) extraction and assay was following a method described by Mohase and van der Westhuizen (2002).

Peroxynitrite (ONOO⁻) content

Peroxynitrite content was measured spectrophotometrically according to Yamasaki and Sakihama (2000). Forty µL plant extract was used.

Intercellular peroxidase activity (EC 1.11.1.7)

A method of Zieslin and Ben-Zaken (1991) was used.

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

Original method of Fink et al. (1988) was used according to modifications described by Moloi and van der Westhuizen (2014).

Protein concentration

The protein content of the enzyme extracts was determined according to Bradford (1976) using bovine γ-globulin as standard.

Statistical analysis

The data were analysed using Sigma Plot version 9.0 of SYSTAT software followed by the *t*-test statements. At least three replications were used.

Results

Exogenous application of NO donor (SNP) on the uninfested resistant plants led to increased production of SA (44%) compared to the control plants (Fig. 1a). Inhibition of NR activity with Na_2WO_4 (NR inhibitor) resulted in a significant reduction (53%, $P = 0.0230$) in total SA content. However, SA content was still high in the infested resistant than uninfested resistant plants (Fig. 1b).
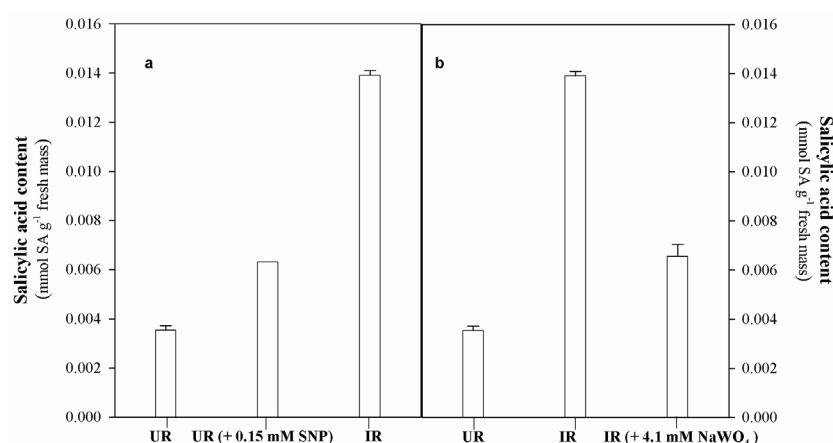

Figure 1. Effect of SNP (a) and Na_2WO_4 (b) application on the total SA content of uninfested resistant and RWA infested resistant wheat cultivar. Values are means ± SD ($n = 3$).
UR: uninfested resistant; IR: infested resistant

The infested susceptible control plants (i.e. with no SNP application) had significantly higher RWA r_m ($P = 0.036$) than the infested resistant controls (Fig. 2a, b). Application of different SNP concentrations through the roots had no significant effect on the r_m of both susceptible and resistant plants (Fig. 2b). In addition, no correlation between SNP and r_m was observed ($r = -0.15$).

In contrast, pre-treatment of seeds with SNP (as a seed dressing) before planting had a substantial impact on r_m of susceptible plants. The 0.15 and 0.5 mM SNP application had significant effects in reducing the RWA r_m by 35% ($P = 0.023$) and 21% ($P = 0.015$), re-

spectively. Correlation between the RWA r_m and treatment of susceptible plants with different SNP concentrations was –0.6. Contrary to root applications, the RWA population was decreased by 20% and 25% when 0.15 and 0.5 mM SNP was applied as a seed dressing on resistant plants (Fig. 2a).
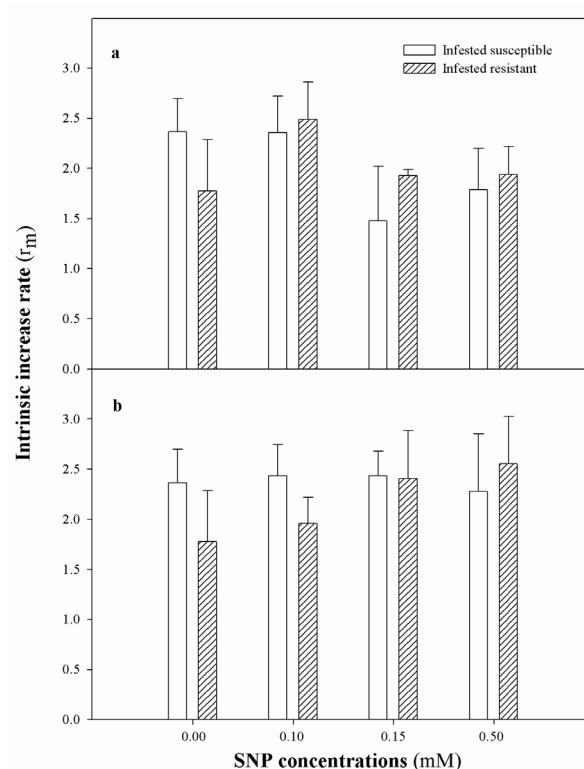

Figure 2. The RWA r_m (72 h.p.i) of susceptible and resistant wheat cultivars treated with various concentrations of SNP (seed dressing) (a) or root application (b). Values are means ± SD (*n* = 6)

Susceptible control plants had more severe RWA infestation symptoms than the resistant control plants 168 (*P* = 0.015) and 336 (*P* = 0.0098) h.p.i. Application of different concentrations of SNP (root application) had a notable impact on the resistance response of susceptible plants. Significant reduction in the RWA infestation symptoms was observed in the susceptible plants (168 h.p.i) after SNP application. These plants were transiently transformed from being susceptible (S, 7.7), to being medium resistant (MR, 4.7 and 5.4) by 0.15 mM and 0.5 mM SNP application. In addition, correlation between treatment and symptom development on susceptible plants was observed (*r* = –0.63). No significant changes were brought by SNP treatments in the infested resistant plants (Fig. 3a).
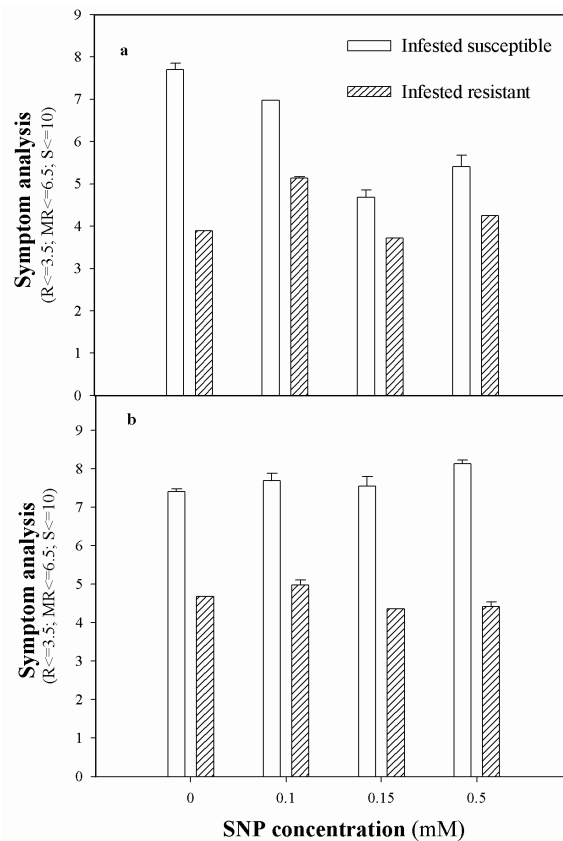
Figure 3. Effect SNP (root application) on symptom/disease development of RWA infested resistant *(Tugela DN)* and susceptible (*Tugela*) wheat cultivars 168 (a) and 336 (b) h.p.i. Values are means ± SD (*n* = 4). S: susceptible, MR: medium resistant, R: highly resistant

Later-on (336 h.p.i), the infested susceptible plants developed more disease symptoms than earlier-on, irrespective of SNP treatments (Fig. 3b).

Selective induction of peroxynitrite to higher levels in the resistant plants was observed. A peak increase representing 45% was reached 12 hours post infestation (h.p.i) and subsequently dropped. The RWA infested susceptible and control plants had no significant induction (Fig. 4).

Significant reduction in peroxynitrite content (47% decrease) was obtained after urate (selective inhibitor of peroxynitrite production) application on RWA infested resistant plants 12 h.p.i (Fig. 5a). Consequently, urate application further led to substantial inhibition of β-1,3-glucanase (51%, $P = 0.0012$, Fig. 5b) and peroxidase (53%, $P = 0.0004$, Fig. 5c) activities.
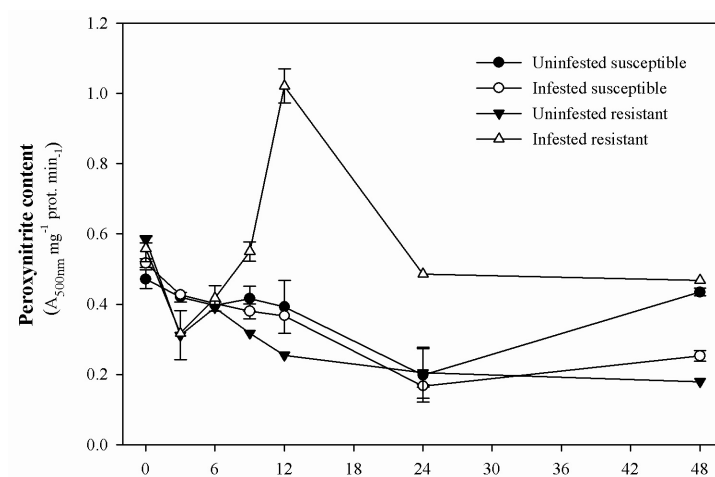
Figure 4. Effect of RWA infestation on the peroxynitrite content of resistant and susceptible wheat cultivars. Values are means ± SD (*n* = 3)
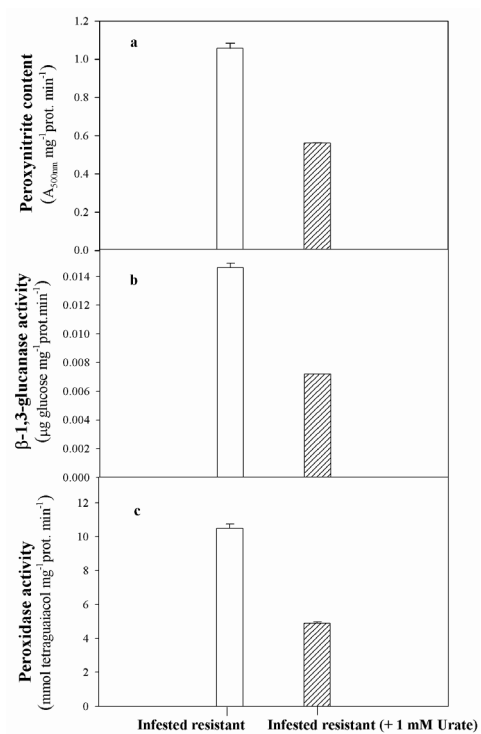

Figure 5. Effect of urate on peroxynitrite content (12 h.p.i; a), intercellular *β*-1,3-glucanase (48 h.p.i; b) and peroxidase (48 h.p.i; c) activities of a RWA infested resistant wheat cultivar. Values are means ± SD (*n* = 3)

Discussion

It was found in previous studies that SA and NO levels increased during the RWA resistance responses. It was also revealed that SA and NO play an important signalling role during the RWA resistance response (Mohase and van der Westhuizen 2002; Moloi and van der Westhuizen 2014). Although these studies are informative, the relationship between the two molecules was not investigated. The current work will provide clarity on this matter. The NO donors are known to mimic an endogenous NO-related response or substitute for an endogenous NO deficiency if applied on biological systems (Floryszak-Wieczorek et al. 2006). A significant increase in SA content of uninfested resistant plants (which were previously found to have very low NO level; Moloi and van der Westhuizen 2014) following SNP application (Fig. 1a), is an indication that NO acts upstream of SA. Strong evidence that NO production depends on the activity of NR during the RWA resistance response of wheat was previously provided (Moloi and van der Westhuizen 2014). Therefore, substantial decrease in SA production after NR inhibition (Fig. 1b) further confirms that SA acts downstream of NO during RWA resistance. In support, 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 signalling pathway. Likewise, Durner et al. (1998) found that administration of NO donors increased total SA levels in susceptible tobacco infected with tobacco mosaic virus. The synergistic effect of SA and NO in decreasing the deleterious effects of biotic and abiotic stress is also suggested (Klessig et al. 2000; Simaei et al. 2011).

The damage resulting from aphid infestation is partly due to their capacity for extremely rapid population growth on their host plant (Goggin 2007). Since the role of NO in the RWA resistance has already been established (Moloi and van der Westhuizen 2014), it would be interesting to know whether exogenous application of NO (SNP) could have any effect on the RWA r_m. This parameter has often been used as an index for herbivore performance (Calvitti and Remotti 1998; Buitenhuis et al. 2004). In addition, alternative host suitability can be quantified using growth rate parameters, such as the r_m (Pucherelli et al. 2011). For instance, Jyoti and Michaud (2005) compared the performance of two RWA biotypes (*RWA1* and *RWA2*) on three wheat cultivars using r_m. They noted that *RWA1* performed better on the susceptible *Trego* than on either of the two resistant cultivars, *Stanton* and *Halt,* whereas *RWA2* performed well on all three cultivars and formed larger colonies. Furthermore, Michaud et al. (2006) also found a reduced performance of *RWA1* on resistant cultivars with reproductive rate reduced on resistant cultivars by an average of 32 and 41%. Essentially, Hossein (2010) found significant differences in r_m values of RWA on 5 varieties of wheat and concluded that varieties with the highest r_m value were susceptible and those with the lowest r_m values were considered to be resistant. Higher RWA r_m in the susceptible than resistant plants (Fig. 2a, b) further substantiates the role of NO in the signalling events of the RWA resistance responses, because NO production was found to be generally lower in the infested susceptible than resistant plants (Moloi and van der Westhuizen 2014). A significant reduction in the RWA r_m after SNP application as a seed dressing (Fig. 2a) as opposed to root application proves that the

method of NO application is critical in inducing the defence responses of plants. The level of NO production also plays a vital role in resistance because only the plants with 0.15 and 0.5 mM SNP application had significant reduction in RWA population growth (Fig. 2a). Studies indicate that too high NO donor concentrations can have inhibitory effects. The commonly applied donor concentrations range from 10–500 μM (Modolo et al. 2002; Floryszak-Wieczorek et al. 2006). A negative correlation (–0.6) further substantiates the role of NO level in the resistance responses. To add on, our preliminary studies showed that a number of RWAs that settled/that were attracted on the plants were significantly reduced by SNP treatment (unpublished data). As a result, this could be a contributing factor to low RWA r_m.

Nitric oxide application could also enhance resistance by reducing the development of RWA feeding symptoms, which include chlorosis and leaf rolling, on the leaves of susceptible plants (Fig. 3a). A relatively strong correlation of SNP and disease symptoms indicates that NO plays a positive role in resistance. However, it seems that NO application through the roots could only confer a short-term resistance to these plants because its effect diminished later-on (336 h.p.i) (Fig. 3b). These results confirm that application of NO can mimic endogenously produced NO (Floryszak-Wieczorek et al. 2006), further leading to reduction of the RWA r_m and RWA feeding symptom development. This, moreover, supports the role of NO in the RWA resistance response of wheat.

During the defence responses, NO may very rapidly react with O_2^-, provided there is sufficient NO to form peroxynitrite in the absence of enzymatic catalysis (Tuteja et al. 2004; Saito et al. 2006). An increase in peroxynitrite formation (9 h.p.i) in the resistant, with a transient peak induction (12 h.p.i; Fig. 4) could therefore be a consequence of high NO production and sufficient ROS production. The suggestion is supported by our previous work, which showed that at this period the SOD activity (responsible for conversion of O_2^- to H_2O_2) was already low with high NADPH oxidase activity (responsible for O_2^- production) (Moloi and van der Westhuizen 2006; 2008; 2014). Higher induction of peroxynitrite in the infested resistant than susceptible plants strongly suggests involvement of this molecule in the RWA resistance response. Peroxynitrite formation could play an important protective role against oxidative stress by preventing the formation of hydroxyl radical, one of the most deleterious ROS (Wink et al. 1995). In order to further investigate the involvement of peroxynitrite in RWA resistance, plants were treated with urate (a selective inhibitor of peroxynitrite formation) (Alamilo and García-Olmendo 2001). Reduction of peroxynitrite production (Fig. 5a) and consequently inhibition of activities of the secondary defence related enzymes, intercellular β-1,3-glucanase (Fig. 5b) and peroxidase (Fig. 5c), further substantiate the importance of this molecule in RWA resistance. Reports indicate that peroxynitrite production is involved in the secondary defence responses through induction of PR-1, peroxidase and PAL accumulation in *Arabidopsis thaliana* infected with a bacterial pathogen *Pseudomonas syringae* (Durner and Klessig 1999). Activities of these enzymes were not inhibited by urate addition *in vitro* (not shown), indicating that the observed reduction was not a consequence of direct urate effect.

From these studies, it can be concluded that peroxynitrite could be involved in the RWA resistance responses as one of the signal molecules in addition to NO, H_2O_2 and SA (Mohase and van der Westhuizen 2002; Moloi and van der Westhuizen 2006, 2014). During the RWA resistance response of wheat, NO acts upstream of SA. The significant effect of NO application on reduction of RWA population growth and eventually the feeding symptoms further confirm the importance of NO in the RWA resistance.

Acknowledgements

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