Salicylic Acid-induced Nitric Oxide and ROS Generation Stimulate Ginsenoside Accumulation in *Panax ginseng* Roots

Rajesh Kumar Tewari · Kee-Yoeup Paek

Abstract We evaluated the involvement of nitric oxide (NO) in salicylic acid (SA)-induced accumulation of ginsenoside in adventitious roots of *Panax ginseng* and its mediation by reactive oxygen species (ROS). Related effects of SA on components of the antioxidant system were also sought. Adventitious roots of *P. ginseng* were grown in suspension culture for 3 weeks in MS medium and treated over 5 days with SA (100 μM) alone, SA in combination with the NO scavenger 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO), or PTIO alone. Nitric oxide, the superoxide anion ($O_2^{.-}$), H_2O_2, nitrite, nonprotein thiol, and ascorbate were monitored together with ginsenoside, NADPH oxidase activity, and several antioxidant enzymes. Salicylic acid did not inhibit root growth but induced accumulation of ginsenoside, lipid peroxidation, and generation of NO and $O_2^{.-}$. It also enhanced activities of NADPH oxidase, superoxide dismutase, catalase, and peroxidase, including ascorbate peroxidase. These effects were suppressed by PTIO. Salicylic acid also decreased glutathione reductase activity. Inclusion of PTIO with SA decreased the activity of glutathione reductase further. Treatment with SA plus PTIO also decreased nonprotein thiol and ascorbate contents but caused nitrite to overaccumulate. Salicylic acid applied to adventitious roots in culture induced accumulation of ginsenoside in an NO-dependent manner that was mediated by the associated increases in $O_2^{.-}$, which gave other antioxidant responses that were dependent on NO.

Keywords NADPH oxidase · Nitric oxide · *Panax ginseng* · ROS · Salicylic acid

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Introduction

Salicylic acid (SA) is a phenolic hormone involved in signaling. It affects numerous physiological processes, including thermogenesis, ethylene synthesis, and fruit ripening (Rhoads and McIntosh 1992). SA accumulation is also associated with a plethora of biotic and abiotic responses because of its signaling effect on the antioxidant defense system (Rao and Davis 1999; Senaratna and others 2000). SA is involved in regulating plant responses to heavy-metal-induced toxicity by enhancing antioxidant defense (Pál and others 2005; Zhou and others 2009). SA interacts with the reactive oxygen species (ROS) signaling pathway. ROS and nitric oxide (NO) have also been shown to regulate SA biosynthesis (Durner and others 1998). NO is a small, diffusible, ubiquitous gas that participates in various pathophysiological and developmental processes of various living organisms (Lamattina and others 2003; Neill and others 2003). In the pathogen-activated hypersensitive response by plants, both NO and ROS are generated and act as signaling molecules (Delledonne 2005). Several models suggest that redox signaling through NO and ROS is enhanced by SA in a self-amplifying process (Klessig and others 2000). Moreover, NO is involved in NADPH oxidase-dependent ROS generation, adventitious root

development (Tewari and others 2008), and accumulation of saponin in *Panax ginseng* (Tewari and others 2007) and phenolics and flavonoids in adventitious roots of *Echinacea purpuria* (Wu and others 2007). NO may also stimulate an increase in phenols in chamomile roots (Kováčik and others 2009a). NADPH oxidase is a mediator of O_2^- generation, and there is reason to believe it is involved in ginsenoside production because of the close relationship between NADPH oxidase activity and saponin accumulation (Tewari and others 2007). It has been reported before that SA induced accumulation of ginsenoside in *Panax ginseng* (Ali and others 2006). SA can affect secondary metabolites (Ali and others 2006) and the accumulation of NO (Zottini and others 2007), whereas NO can affect SA levels (Durner and others 1998). Therefore, we studied SA-induced generation of NO, ROS, and antioxidant responses in the adventitious root of *Panax ginseng* in relation to the accumulation of the commercially important ginsenoside (a steroid glycoside and triterpene saponin believed to explain the medical efficacy of "ginseng"). We also discuss the consequence of SA-induced NO generation in the activation of NADPH oxidase, antioxidant enzymes and metabolites (nonprotein thiol), lipid peroxidation, and ROS formation.

Materials and Methods

Plant Material and Growth Conditions

Adventitious root cultures of mountain ginseng (*Panax ginseng* 'CBN-1') were obtained from an established root line collection generated at Chungbuk National University (South Korea) from 4-year-old mountain ginseng through callus culture (Yu and others 2000). Selected roots were proliferated further in a 5-L airlift balloon-type bioreactor under sterile conditions in 4 L of 3/4-strength MS (Murashige and Skoog 1962) liquid medium supplemented with 5 mg L^{-1} indole butyric acid, 0.1 mg L^{-1} kinetin, and 5% sucrose for 4 weeks. Explants were grown for an additional 3 weeks in 500-ml Erlenmeyer flasks containing 250 ml of 3/4-strength MS medium supplemented with 5% (w/v) sucrose and 5 ppm of indole butyric acid. After 3 weeks, the roots were treated (elicited) with either sterile water (control), 100 μM salicylic acid (SA), SA in combination with 100 μM 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO, an NO scavenger), or 100 μM PTIO alone. Roots were sampled and analyzed 5 days later.

Dry Mass Measurement

After 5 days, treated roots were washed with distilled water and blotted dry with a paper towel, dried in an oven at 60°C for 48 h, and weighed.

Extraction and Determination of Ginsenoside Content

Extraction and determination of saponin (ginsenoside) were modified from previous methods (Fuquay and Yoshikawa 1987; William and others 1996). The ginsenoside fraction was analyzed by high-pressure liquid chromatography (HPLC) (Waters 2690 separation module; Waters 996 photodiode array detector; Waters Millennium 2010 chromatography manager) on a Nova-pak Platinum C18 column (4 μm, 3.9 mm × 150 mm), with water and acetonitrile as the mobile phase. The ratio of water and acetonitrile was gradually changed from 84:16 to 10:90 during 101 min and then reverted back to the initial ratio of 84:16 by 110 min of total separation time. The flow rate of the mobile phase was 0.7 ml min^{-1} and the ginsenoside was detected at 203 nm. Total ginsenoside content was calculated as the sum of ginsenoside fractions by area under the peak as described by Yu and others (2000).

In situ Localization of NO

Endogenous NO was analyzed using the NO-sensitive dye 4,5-diaminofluorescein diacetate (DAF-2DA, Sigma-Aldrich) according to Corpas and others (2004). The roots were stained with 10 μM of DAF-2 DA dissolved in loading buffer (10 mM Tris-HCl, pH 7.4) for 1 h at 25°C in the dark, followed by 15 min of washing with loading buffer. NO was detected as green fluorescence of DAF-2 (excitation = 495 nm and emission = 515 nm) under a laser confocal scanning microscope (Leica TCS SP2, Leica DM IRE2, GmbH, Germany).

Quantification of Superoxide Anion

The superoxide anion was quantified after Able and others (1998) by monitoring the reduction of XTT [3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide sodium salt] by O_2^-. Root tissue (1.0 g) was homogenized with 1.0 ml of 50 mM Tris-HCl buffer (pH 7.5) and centrifuged at 14,000 ×g for 20 min. The reaction mixture contained 50 mM Tris-HCl buffer (pH 7.5), 50 μl of extract, and 0.5 mM XTT. The reduction of XTT was monitored at 450 nm for 4 min. Superoxide anion production was calculated using an extinction coefficient of 2.16×10^4 M^{-1} cm^{-1}.

Hydrogen Peroxide and Lipid Peroxidation

Hydrogen peroxide (H_2O_2) concentration was determined as the H_2O_2-titanium complex formed by reaction of tissue-H_2O_2 with titanium tetrachloride (Brennan and Frenkel 1977). The extent of lipid peroxidation was determined by the method of Heath and Packer (1968) in terms of

malondialdehyde (MDA) content using the thiobarbituric acid (TBA) reaction. The amount of TBA reactive substance (TBARS) was calculated from the difference in absorbance at 532 nm and at 600 nm using an extinction coefficient of 155 mM^{-1} cm^{-1}.

Ascorbate, Nonprotein Thiol, and Nitrite Contents

Root tissue (250 mg) was homogenized in 2 ml of 5% (w/v) trichloroacetic acid (TCA) and centrifuged for 5 min at 10,000 $\times g$. Total ascorbate (after reducing dehydroascorbate [DHA] to ascorbic acid [AsA] by dithiothreitol) and AsA in the supernatant were measured as the Fe^{3+}-bipyridyl complex (absorbance max = 525 nm) formed from the reduction of $FeCl_3$ by AsA (Law and others 1983). DHA content was calculated from the difference between total ascorbate and AsA. For nonprotein thiol, root tissue (1 g) was homogenized in 10 ml 5% (v/v) TCA and centrifuged at 10,000 $\times g$ for 5 min. Aliquots of 0.5 ml of the homogenates were mixed with Ellman reagent (0.5 ml of 0.01 M DTNB solubilized in 1 M potassium phosphate buffer, pH 7.8). The contents were mixed and absorbance was read within 5 min at 412 nm against a reagent blank. Total sulfhydryl groups were calculated from the extinction coefficient of 13.1 mM^{-1} cm^{-1}. Nitrite content was determined according to Zhou and others (2005). Roots (0.5 g) were ground in a mortar and pestle in 2 ml of 50 mM cool acetic acid buffer (pH 3.6, containing 4% zinc diacetate). The homogenate was centrifuged at 10,000 $\times g$ for 15 min at 4°C and the supernatant was collected. The pellet was washed twice by 0.5 ml of extraction buffer and centrifuged as before. The two supernatants were combined and 0.05 g of activated charcoal added. After vortexing and filtration, the filtrate was leached and collected. The mixture of 0.5 ml of filtrate and 0.5 ml of the Greiss reagent (Fluka) was incubated at room temperature for 30 min. Absorbance was determined at 540 nm. Nitrite content was calculated by comparison to a standard curve of $NaNO_2$.

Enzyme Extraction

Fresh root tissue (1 g) was powdered in liquid nitrogen in a chilled pestle and mortar and homogenized in 4 ml of chilled 50 mM potassium phosphate buffer (pH 7) containing 1% (w/v) insoluble polyvinylpolypyrrolidone and 1 mM phenylmethylsulfonylfluoride, 1 mM EDTA, 1 mM dithiothreitol, and 0.2% (v/v) Triton X-100. The homogenate was centrifuged at 10,000 $\times g$ for 10 min at 2°C. The supernatant was stored at 2°C and used for enzyme assays within 4 h. For the assay of ascorbate peroxidase (APX) activity, 5 mM ascorbic acid (AsA) was also included in the extraction medium.

Assays of Enzymes

NADPH oxidase (NOX, EC 1.6.3.1) activity was determined after Sagi and Fluhr (2001). NOX was assayed by a modified assay based on reduction of XTT by $O_2^{\cdot-}$ radicals. The assay reaction medium contained 50 μl of enzyme extract, 0.3 mM 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide sodium salt (XTT), 0.2 mM NADPH, 0.1 mM $MgCl_2$ and 1.0 mM $CaCl_2$ in a 1 ml of 50 mM Tris-HCl buffer (pH 7.4). The reaction was initiated with the addition of NADPH. XTT reduction was determined at 470 nm in the presence and absence of 50 U of CuZn-SOD. XTT reduction by $O_2^{\cdot-}$ is shown corrected for reduction in the presence SOD (50 U ml^{-1}).

Superoxide dismutase (SOD, EC 1.15.1.1) activity was assayed by its ability to inhibit the photochemical reduction of NBT at 560 nm. The reaction mixture (5 ml) contained 25 mM phosphate buffer (pH 7.8), 65 μM nitro blue tetrazolium (NBT), 2 μM riboflavin, enzyme extract (equivalent to 10 mg fresh weight [FW]), and 15 μl of N,N, N',N'-tetramethylethylenediamine (modified after Beauchamp and Fridovich 1971). The reaction mixture was exposed to light (350 μmol m^{-2} s^{-1}) for 15 min. The activity is expressed as U min^{-1} mg^{-1} protein. Enzyme extract corresponding to 50% inhibition of reaction was considered as one enzyme unit.

Catalase (CAT, EC 1.11.1.6) activity was estimated in a reaction mixture containing 500 μmol H_2O_2 in 10 ml of 100 mM phosphate buffer (pH 7) and tissue extract (equivalent to 20 mg FW). The amount of H_2O_2 decomposed after a 5-min reaction was assayed by reading absorbance at 240 nm of both blanks and samples (modified after Bisht and others 1989). Corresponding blanks were maintained by adding 1 ml of 2 N H_2SO_4 prior to addition of enzyme extract. CAT activity is expressed as U mg^{-1} protein.

Peroxidase (POD, EC 1.11.1.7) activity was estimated after Bisht and others (1989) in a reaction mixture containing 5 ml of 100 mM phosphate buffer (pH 6.5), 1 ml of 0.5% p-phenylenediamine, 1 ml of 0.01% H_2O_2, and tissue extract (equivalent to 5 mg FW); change in absorbance after 5 min was measured at 485 nm. Enzyme activity is expressed as U per mg protein basis.

Ascorbate peroxidase (APX, EC 1.11.1.11) was measured in a 3-ml reaction mixture containing 50 mM phosphate buffer (pH 7), 0.5 mM AsA, 0.1 mM H_2O_2, 0.1 mM EDTA, and enzyme extract (equivalent to 10 mg FW). Both minus tissue extract and minus H_2O_2 blanks were run and the changes in absorbance every 15 s were read at 290 nm (Nakano and Asada 1981). The activity of APX was calculated as U per mg protein basis.

Glutathione reductase (GR, EC 1.6.4.2) was measured in a 1.5-ml reaction mixture of 50 μl of 10 mM GSSG, 200 μl

of 1.0 mM Na$_2$EDTA, 150 μl of 10 mM DTNB, 1 ml of 200 mM phosphate buffer (pH 8), and enzyme extract (equivalent to 20 mg FW) preincubated at 25°C for 5 min. The reaction was initiated by addition of 50 μl of 1 mM NADPH, and the rate of reduction of GSSG was monitored at 412 nm. Enzyme activity has been expressed as units per mg protein (modified from Jablonski and Anderson 1978).

Statistical Analysis

All results are means of three independent experimental replicates ($n = 6$). The data were analyzed by analysis of variance (ANOVA) and tested for significance by DMRT using SigmaStat software.
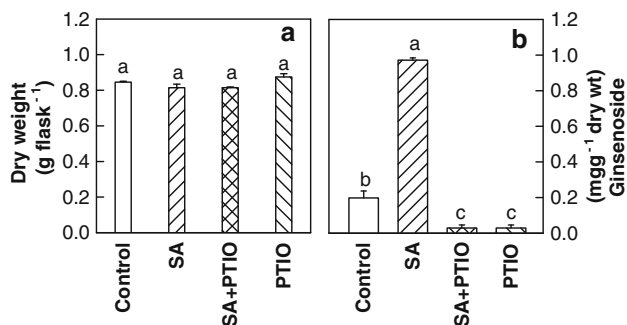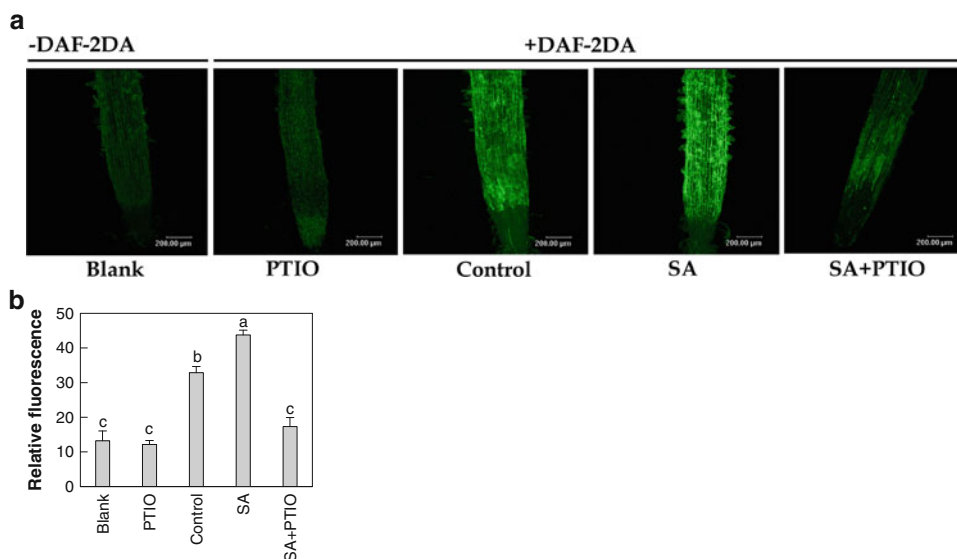

Fig. 1 Effect of supplying salicylic acid (SA), SA plus the NO scavenger 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (SA + PTIO), or PTIO alone on **a** dry mass and **b** ginsenoside accumulation in cultured adventitious roots of *Panax ginseng*. Vertical bars ± SE are the mean of six replicates. Means with different letters are significantly different at $P < 0.05$. Treatments were for 5 days at 100 μM for each substance applied

Results

SA Does not Affect Growth of Adventitious Roots but Enhances the Accumulation of Ginsenoside

SA (100 μM) did not affect root growth over 5 days in terms of dry weight. However, accumulation of ginsenoside increased almost fourfold (Fig. 1a, b). Application of PTIO alone or with SA decreased ginsenoside content almost to zero (Fig. 1b).

SA Induces the Generation of NO

SA elicitation enhanced accumulation of NO as indicated by DAF fluorescence (Fig. 2a, b) whereas the application of PTIO in combination with SA and alone eliminated almost all DAF fluorescence (Fig. 2a, b). However, nitrite content was unaffected by SA; however, when SA was applied in association with PTIO, it enhanced the accumulation of nitrite more than 70-fold (Fig. 3a). This large rise in nitrite was associated with the almost complete disappearance of nonprotein thiol (NPT) in the roots (Fig. 3b). PTIO alone did not cause major changes to nitrite and NPT concentrations (Fig. 3a, b).

SA-induced ROS Generation is NO Dependent

SA induced generation of O_2^- and H_2O_2 in a NO-dependent manner. An enhancement in O_2^- and H_2O_2 (Fig. 4a, b), compounded with an intense signal of DAF fluorescence indicating high tissue NO, was observed when SA was applied. Scavenging for NO with PTIO prevented the SA-induced rise in O_2^- and H_2O_2 and returned them to the levels of control roots (Fig. 4a, b). Lipid peroxidation, as

Fig. 2 In situ localization of nitric oxide (NO) in adventitious roots of *Panax ginseng* supplied for 5 days with PTIO, SA, or SA + PTIO (**a**) and relative fluorescence (**b**). Vertical bars ± SE are the mean of five roots. Unstained roots are labeled as *Blank*. Generation of NO was revealed by green fluorescence in a 4,5-diaminofluorescein diacetate (DAF-2DA) assay. Treatment concentrations were at 100 μM for each substance applied. Treatment abbreviations are defined in Fig. 1
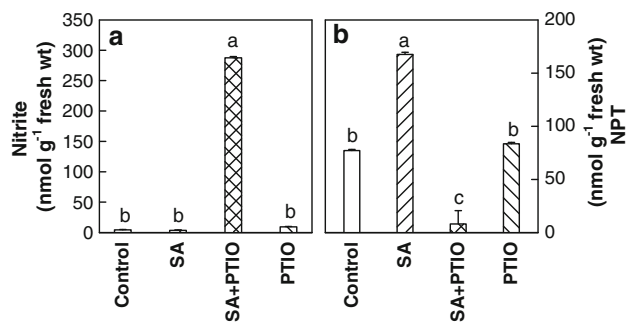
Fig. 3 Effect of SA, SA + PTIO, or PTIO alone on concentrations of **a** nitrite and **b** nonprotein thiol (NPT) in adventitious roots of *Panax ginseng*. Vertical bars ± SE are the mean of six replicates. Means with *different letters* are significantly different at $P < 0.05$. Treatments were for 5 days at 100 μM for each substance applied. Treatment abbreviations are defined in Fig. 1

assessed by malondialdehyde assay, was also enhanced by SA. This effect too was NO-dependent because levels were suppressed when PTIO was combined with SA (Fig. 4c). PTIO alone did not cause any major change in O_2^- and H_2O_2 (Fig. 4).

SA Enhances the Activities of NADPH Oxidase, SOD, CAT, POD, and APX

Activity of NADPH oxidase (NOX), an enzyme involved in O_2^- generation, was enhanced by SA and reverted back to the control levels when PTIO was supplied with SA (Fig. 5). Antioxidant enzymes SOD, CAT, and POD were also enhanced by SA and reverted back to the control levels when PTIO was included (Fig. 5). The activity of APX was also enhanced by SA, but in the presence of PTIO, SA lowered APX activity to less than half that of control roots (Fig. 5). In contrast to NOX, SOD, CAT, and POD, the activity of GR was downregulated by SA and was decreased further if PTIO was applied with SA (Fig. 5). PTIO applied alone did not cause any major change in the activities of NOX, SOD, POD, APX, or GR (Fig. 5).
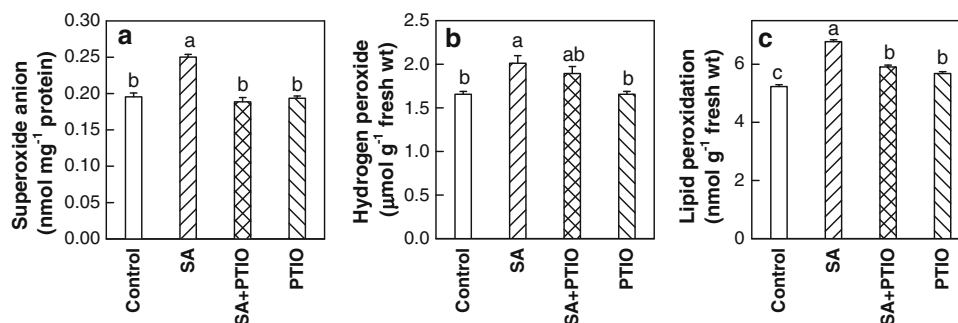

Fig. 5 Effect of SA, SA + PTIO, or PTIO alone on the activities of NADPH oxidase (*NOX*), superoxide dismutase (*SOD*), catalase (*CAT*), peroxidase (*POD*), ascorbate peroxidase (*APX*), and glutathione reductase (*GR*) in the adventitious roots of *Panax ginseng*. Vertical bars ± SE are the mean of six replicates. Means with *different letters* are significantly different at $P < 0.05$. Treatments were for 5 days at 100 μM for each substance applied. Treatment abbreviations are defined in Fig. 1

Effect of SA on Molecular Antioxidants

Salicylic acid had no significant effect on AsA and DHA concentrations compared to control; however, PTIO with SA decreased concentrations of AsA, DHA, total ascorbate (TA) (Fig. 6a), and nonprotein thiol (Fig. 3b). PTIO alone also decreased ascorbate content relative to controls

Fig. 4 Effect of SA, SA + PTIO, or PTIO alone on **a** superoxide (O_2^-), **b** hydrogen peroxide (H_2O_2), and **c** lipid peroxidation in adventitious roots of *Panax ginseng*. Vertical bars ± SE are the mean
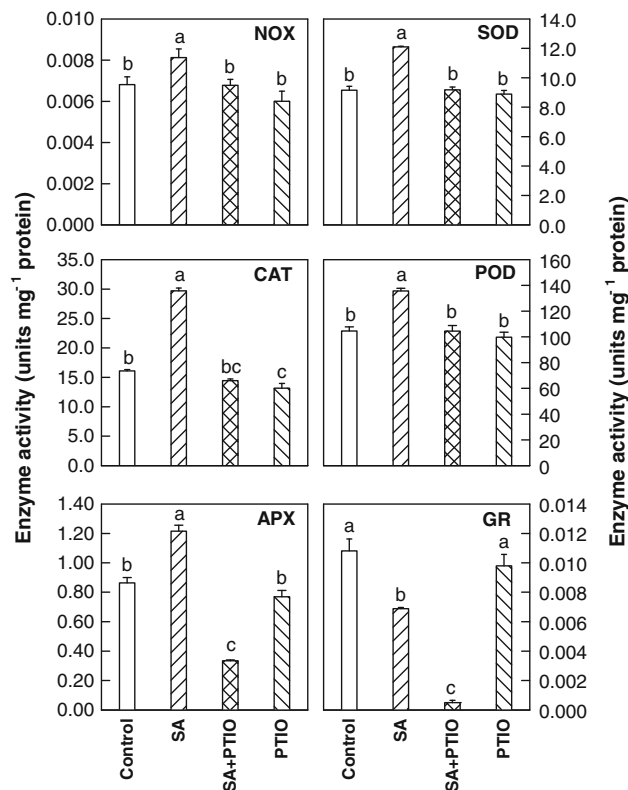
of six replicates. Means with *different letters* are significantly different at $P < 0.05$. Treatments were for 5 days at 100 μM for each substance applied. Treatment abbreviations are defined in Fig. 1
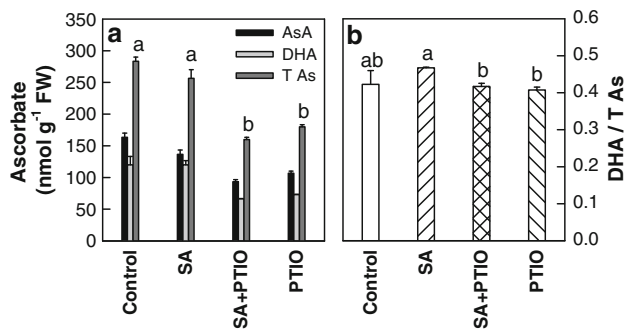
Fig. 6 Effect of SA, SA + PTIO, or PTIO alone on **a** concentrations of ascorbic acid (*AsA*), dehydroascorbic acid (*DHA*), and total ascorbate (*TAs*) content, and **b** the DHA/TAs ratio in the adventitious roots of *Panax ginseng*. Vertical bars ± SE are the mean of six replicates. Means with *different letters* are significantly different at $P < 0.05$. Treatments were for 5 days at 100 μM for each substance applied. Treatment abbreviations are defined in Fig. 1

(Fig. 6) but had no effect on nonprotein thiol (Fig. 3b). Dehydroascorbic acid and total ascorbate (TA) ratios were unaffected by these treatments (Fig. 6b).

Discussion

SA induces growth inhibitory effects at lower concentrations (250 μM) and lethal effects at higher concentrations (1 mM, 48 h; 2 mM, 24 h) in chamomile plants (Kováčik and others 2009b). Exogenous application of SA (0.05–0.5 mM) has been considered to induce moderate stress, affecting the oxidative status of the plant (Horváth and others 2007). However, supplying SA at the lower concentration of 100 μM did not decrease root growth, but this low concentration was highly effective in inducing the accumulation of NO, O_2^-, and ginsenoside in the adventitious root of *P. ginseng*. Reversal of these SA effects by combined treatment of PTIO, a NO scavenger, suggests strongly that they are NO-dependent. Kováčik and others (2009c) observed a protective effect of lower concentrations of SA against heavy-metal toxicity. Moreover, SA in combination with metals induces the accumulation of H_2O_2 and O_2^- in roots as a part of the signaling pathway for quantitative changes in phenolic metabolites (Kováčik and others 2009c). In a previous study, Zottini and others (2007) also showed that SA induces NO accumulation in *Arabidopsis thaliana* roots, and NO has been reported previously to participate in ginsenoside accumulation in the adventitious roots of *P. ginseng* (Tewari and others 2007). The superoxide anion may function as a signal for the induction of defense genes (Jabs and others 1997), and, therefore, it could also signal ginsenoside production. Accumulation of ginsenoside coupled with higher NO and O_2^- generation suggests that SA-induced NO and O_2^- are

involved in the activation of the ginsenoside biosynthetic (isoprenoid) pathway.

DAF2-DA is a specific dye for NO detection, fluorescing green under UV light after reacting with NO (Kojima and others 1998). SA produced strong DAF fluorescence in the adventitious roots of *Panax ginseng*. Disappearance of SA-induced DAF fluorescence with PTIO highlights the specificity of the dye and ability of SA to promote the formation of NO. Nitrite has been suggested as an index of NO generation (Stöhr and Stremlau 2006) in plants but does not appear to be reliable because we observed an overaccumulation of nitrite in the SA-treated roots given PTIO to remove NO. A similar accumulation of nitrite in the roots of chamomile plants treated with excess Cu + PTIO has recently been reported (Kováčik and others 2010). The overaccumulation of nitrite by PTIO + SA suggests activation of the nitrogen assimilatory pathway (nitrate reductase) in the adventitious roots of *P. ginseng* by SA, but only in the absence of NO. The nitrite induced by PTIO + SA probably reacted with nonprotein thiols (NPTs) such as GSH and cysteine. This may ultimately cause a severe decline in NPT. Reaction products of active oxygen with NO and NO itself are free radicals (Kumar and others 2010) and may induce nitrosative/oxidative stress in plants (Valderrama and others 2007). Thus, SA supplied enhanced O_2^-, H_2O_2, and lipid peroxidation, and these effects were markedly reversed when NO was removed with PTIO. These observations suggest that SA induced oxidative stress in the adventitious roots of *P. ginseng* by enhancing NOX activity in a NO-dependent manner. Upregulation in NOX-mediated O_2^- generation has also been reported before on addition of SA to tobacco suspension cultures (Kawano and others 1998). As NOX activity was reverted back to control levels by PTIO + SA treatment, NO appears to be instrumental in the activation of NOX activity.

Ascorbate is a major molecular antioxidant and redox buffer of the cellular environment (Noctor and Foyer 1998). Ali and others (2006) have reported an enhanced AsA content in the adventitious roots of *P. ginseng* elicited with 200 μM SA. In the present study, 100 μM SA did not cause a significant change in ascorbate concentration. However, SA in association with PTIO and PTIO alone decreased ascorbate concentration. This decrease is likely to be an individual effect of PTIO unrelated to NO scavenging.

Our results indicate that SA induced the generation of O_2^- by enhancing NOX activity. SOD, an O_2^- dismutating enzyme, was also enhanced by SA treatment. Here, O_2^- appears to be signaling the activation of SOD activity. Enhanced activity of SOD would dismutate O_2^- to a relatively less toxic and more stable H_2O_2. Plants have an array of H_2O_2-scavenging enzymes, including CAT and various

peroxidases and molecular antioxidants such as AsA (Cheeseman 2007). Increased activities of CAT, POD, and APX by SA elicitation appear to be antioxidant responses that will scavenge excess H_2O_2 from the cellular environment to protect cells from oxidative damage. Increased activities of CAT, POD, and APX are consistent with previous studies conducted with plant organs treated with SA (Ali and others 2006), exposed to NaCl (Sawada and others 2008) and heavy metals (Shi and Zhu 2008; Zhou and others 2009). Moreover, there are some published results showing that exogenous applications of SA inhibit activities of CAT (Metwally and others 2003; Shi and Zhu 2008) and APX (Shi and Zhu 2008). These differences may be because of differential responses of plants and/or experimental conditions. Reversal of activities of SOD, POD, CAT, and APX under combined treatment of PTIO with SA suggests involvement of NO in modulating the activities of these enzymes. It has been reported previously that NO is involved in the regulation of CAT, POD, and APX in adventitious roots of *P. ginseng* (Tewari and others 2007, 2008), *Phalaenopsis* flower (Tewari and others 2009), and maize leaves (Kumar and others 2010) with respect to root development, flower senescence, and iron homeostasis. Enhanced activities of CAT, POD, and APX coupled with higher NO content in SA-treated roots and

their downregulation in the presence of PTIO + SA clearly suggest a regulatory role for NO in antioxidant defense.

Contrary to the effects on SOD, CAT, POD, and APX, the activity of GR was downregulated by SA and decreased further with inclusion of PTIO. Metwally and others (2003) also reported downregulation of GR transcripts and upregulation in glutathione synthase transcripts in SA-treated roots. The increase in nonprotein thiols despite a decrease in GR activity in SA-treated roots suggests upregulation of the glutathione synthetic pathway by SA.

In conclusion, SA induces accumulation of ginsenoside in adventitious roots of *Panax ginseng* in association with increased NO production. The SA-induced accumulation of ginsenoside was dependent on this NO in association with a SA-induced, NO-dependent rise in superoxide (O_2^-). This suggests that O_2^- is an SA-triggered, NO-dependent signal promoting ginsenoside production (Fig. 7). SA also induces other NO-dependent changes. The induced O_2^- is presumed responsible for an NO-dependent rise in lipid peroxidation that accompanies ginsenoside accumulation. The increase in O_2^- is the likely outcome of activation of NOX by SA because this too is NO-dependent. The chain of action based on NO-dependent SA-induced increases in O_2^- extends to increases in activity of the antioxidant enzymes SOD (generating increased H_2O_2), catalase, and peroxidase. This is evidence of a modulation of oxidative stress and antioxidant responses by SA acting through NO (Fig. 7).
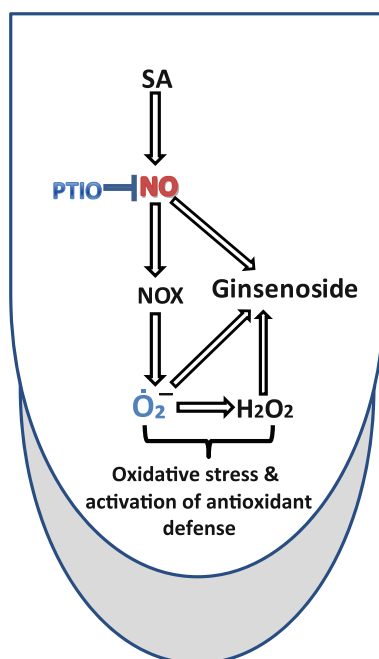
Fig. 7 Schematic representation of the possible mechanism by which SA-induced NO participates in ginsenoside synthesis. NO activates NADPH oxidase activity resulting in the generation of superoxide anion radicals (O_2^-) and hydrogen peroxide (H_2O_2) and activation of antioxidant defense. NO, O_2^-, and H_2O_2, independently or in combination, participate in ginsenoside synthesis

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