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Nitric Oxide Elicitation Induces the Accumulation of Secondary Metabolites and Antioxidant Defense in Adventitious Roots of *Echinacea purpurea*

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Nitric oxide and reactive oxygen species are important signal molecules that play key roles in plant defense responses. We investigated the involvement of nitric oxide elicitation in the synthesis of secondary metabolites within the adventitious roots of *Echinacea purpurea*. When roots were treated with 100 μM sodium nitroprusside (SNP), an exogenous nitric oxide producer, the accumulation of phenolics, flavonoids, and caffeic acid derivatives was enhanced. This level of SNP also induced an antioxidant defense, as indicated by increases in superoxide dismutase, ascorbate peroxidase, and ascorbic acid, along with decreases in hydrogen peroxide, lipid peroxidation, and dehydroascorbate/ascorbic acid. However, a higher concentration (250 μM SNP) acted as a pro-oxidant, thereby raising the levels of hydrogen peroxide, lipid peroxidation, and dehydroascorbate/ascorbic acid while diminishing ascorbic acid, ascorbate peroxidase, and the accumulation of secondary metabolites compared with our observations at 100 μM SNP. Therefore, we conclude that eliciting *E. purpurea* adventitious roots with a concentration of 100 μM SNP is beneficial to their accumulation of secondary metabolites.

Keywords: antioxidant defense, bioreactor, *Echinacea purpurea*, elicitation, nitric oxide, secondary metabolites

The production of secondary metabolites via plant cell/organ cultures is an important area of investigation because of their commercial and medicinal importance. However, these compounds exist at only very low concentrations in plants, which limits their yields. Plant cells respond to various biotic and abiotic elicitors by activating a wide array of reactions [viz., ion fluxes across the plasma membrane, synthesis of reactive oxygen species (ROS), and phosphorylation and dephosphorylation of proteins]. These are all putative components of signal transduction pathways that lead to elicitor-induced defense responses, e.g., the activation of defense genes and hypersensitive cell death (Dietrich et al., 1990; Nürnberger et al., 1994; Baker and Orlandi, 1995). It has been suggested that ROS alone cannot mediate a sufficient disease resistance response in plants, but in combination with nitric oxide (NO) can function synergistically to activate a stronger response (Wang and Wu, 2005). Therefore, NO is a diffusible, bioactive signalling molecule (Beligni and Lamattina, 2000; Neill et al., 2003; Romero-Puertas et al., 2004).

The synthesis of secondary metabolites in plants is believed to be part of their defense response against biotic (pathogenic attack) and abiotic stresses. Applying the elicitors obtained from microorganisms (fungi and bacteria) is an effective strategy for improving the productivity of secondary metabolites in plant cell/organ cultures (Roberts and Shuler, 1997). The generation of NO is a hallmark of such responses to fungal elicitors (Delledonne et al., 1998; Durner et al., 1998). NO has multiple functions, e.g., the stimulation of seed germination (Sarath et al., 2006), formation of adventitious roots (Pagnussat et al., 2002, 2003; Tewari et al., 2007) and root hairs (Lombardo et al., 2006), and the induction of plant defense responses and activation of related genes (Beligni and Lamattina, 2000; Delledonne et al., 2001; Morot-Gaudry-Talarmain et al., 2002). In cell suspension cultures, NO plays a crucial role in the synthesis of secondary metabolites via chemical (e.g., methyl jasmonate; Wang and Wu, 2005), physical, ultrasound (Wang et al., 2006), or microbial elicitors (Wang and Wu, 2004; Wach et al., 2005; Xu et al., 2005).

ROS production is an early event in the elicitation-induced manufacture of secondary metabolites in plant cell cultures through biotic and abiotic means (Yuan et al., 2001; Wu and Lin, 2003). NO also induces DNA fragmentation and cell death in *Taxus* haploid cultures (Pedroso et al., 2000). However, no reports have been published on the relationship among NO, oxidative burst, and secondary metabolite synthesis in the adventitious roots of *Echinacea purpurea* (L.) Moench. Purple coneflower is an important native of North America, and is grown worldwide for its medicinal benefits. Extracts of this plant have antioxidative, antibacterial, antiviral, and antifungal properties, and are used for treating common respiratory and urinary diseases (Barrett, 2003). Here, we examined the effect of an *in vitro* graded supply of sodium nitroprusside (SNP), an NO producer, on *in vivo* NO status. We also investigated the relationship between nitric oxide and other elicitor responses, e.g., H_2O_2 production, the activation of antioxidant defenses, and the production of useful secondary metabolites (phenolics, flavonoids, and caffeic acid derivatives) from *E. purpurea* adventitious root cultures in a bioreactor system.

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Abbreviations: APX, ascorbate peroxidase; AsA, ascorbic acid; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; MDA, malondialdehyde; POD, peroxidase; ROS, reactive oxygen species; SNP, sodium nitroprusside; SOD, superoxide dismutase; TCA, trichloroacetic acid.

MATERIALS AND METHODS

Plant Materials and Induction of Adventitious Roots

Fresh roots of *Echinacea purpurea* collected in Canada were washed with a detergent solution (AIC Co., Korea) for 10 min, then rinsed with running tap water for 10 min. Sterilized root pieces were inoculated on a Murashige and Skoog (1962) medium supplemented with 2.0 mg L^{-1} indole butyric acid (IBA) and 50 g L^{-1} sucrose (pH 6.0). After one month of culturing at 22±1°C in the dark, calli were induced and proliferated on the same medium and under the same culture conditions. To induce adventitious roots, proliferated calli were inoculated on a 1/2-strength MS medium containing 2.0 mg L^{-1} IBA and 50 mg L^{-1} sucrose (pH 6.0). After another month of culturing at 22±1°C in the dark, adventitious roots were induced, which were further proliferated on a 3/4-strength MS medium with 1.0 mg L^{-1} IBA and 50 mg L^{-1} sucrose. Sub-culturing occurred at 4-week intervals.

Treatment Procedure and Root Collection

Four-week-old adventitious roots (1.5 to 2.0 cm long) were cut into small pieces (<0.5 cm); 7 g L^{-1} of this inoculum was added to 5-L bioreactors containing 4 L of 1/2 MS liquid medium, 2.0 mg L^{-1} IBA, and 50 g L^{-1} sucrose (Wu et al. 2006). The air flow rate was adjusted to 0.1 vvm (400 mL min^{-1}), and the air temperature within the bioreactors was controlled at 22±1°C. After 4 weeks of growth, the cultures were elicited with a graded concentration of sodium nitroprusside (0, 50, 100, or 250 μM SNP). Roots were harvested at the end of the 5-week culture period, then washed with deionized water and nanopure water. Samples were blotted dry, weighed to the nearest gram, and stored at –80°C after being fixed in liquid nitrogen.

Root Growth and the Measurement of Phenolics and Flavonoids

Root growth was defined by recording fresh weights (FW) and dry weights (DW), the latter being measured after the blotted tissues were oven-dried at 60±1°C for 48 h. Total phenolic contents were determined as described by Ali et al. (2006). Briefly, 1.0 g of dry roots was homogenized in 10 mL of 95% (v/v) methanol, then frozen for 48 to 72 h before being centrifuged at 19,000×g for 20 min. A 0.1 mL aliquot of the extract solution was mixed with 2.5 mL water and 0.1 mL (2 N) Folin-Ciocalteu reagent (10-times dilution; Sigma Chemical Co., St. Louis, MO). After the tubes were shaken thoroughly for 5 min, a 0.5 mL solution of Na_2CO_3 (20%, w/v) was added and the mixture was allowed to stand for 30 min in the dark. Absorbance was measured at 760 nm. Phenol contents were measured, using gallic acid as our standard.

Flavonoid content was determined by the colorimetric method (Sakanaka et al., 2005). Briefly, 0.25 mL of methanolic plant extract or (+/–) catechin standard solution was mixed with 1.25 mL of distilled water, followed by the addition of 0.75 mL of a 5% (w/v) sodium nitrite solution. After 6 min, 0.15 mL of a 10% (w/v) aluminium chloride solution was added, and the mixture was brought up to 2.5 mL with distilled water and mixed well. Absorbance was measured immediately at 510 nm in a spectrophotometer. The result was expressed as mg of (+/–) catechin equivalent per gram of root DW.

Caffeic Acid Derivatives

Our extraction and analysis of caffeic acid derivatives were done according to the method of Pellati et al. (2004). Caffeic acid fractions were analyzed by HPLC on an XTerraRP 18 column (particle size 3.0 μM, 150 mm 3 mm). The mobile phase was water (A) and acetonitrile (B), and the gradient elution was modified as follows: initial 10% B for 40 min, 25% B for 11 min, 50% B for 1 min, then recycle to initial conditions for 8 min at a flow rate of 0.3 mL min^{-1}. Those caffeic acid derivatives were detected at 330 nm. Standards of caftaric acid, chlorogenic acid, and cichoric acid were obtained from CromaDex (Laguna Hills, CA, USA).

Hydrogen Peroxide and Lipid Peroxidation

H_2O_2 concentration was determined as the H_2O_2-titanium complex formed by the reaction of tissue-H_2O_2 with titanium tetrachloride, per the method of Brennan and Frenkel (1977). Lipid peroxidation was defined in terms of malondialdehyde (MDA) content by a thiobarbituric acid (TBA) reaction (Heath and Packer, 1968). The amount of TBA reactive substance (TBARS) was calculated from the difference in absorbance at 532 nm and 600 nm, using an extinction coefficient of 155 mM^{-1} cm^{-1}.

Nitrite, Ascorbate and Non-protein Thiol

Nitric oxide content was determined via the method described by Zhou et al. (2005). Roots (0.5 g) were ground in a mortar and pestle in 2.0 mL of 50 mM cool acetic acid buffer (pH 3.6) containing 4.0% (w/v) zinc diacetate. The homogenates were centrifuged at 10000×g for 15 min at 4°C. After the supernatant was collected, the pellet was washed twice with 0.5 mL of extraction buffer and centrifuged as before. The two supernatants were combined and 0.05 g of activated charcoal was added. After vortexing and filtration, the filtrate was leached and collected. A mixture of 0.5 mL of filtrate and 0.5 mL of Greiss reagent (Fluka) was incubated at room temperature for 30 min before absorbance was determined at 540 nm. NO content was calculated by comparing our data with a standard curve for $NaNO_2$.

Ascorbate (AsA and DHA) content was estimated per Takahama and Oniki (1992). Roots were ground in liquid nitrogen and the frozen powder was homogenized in 1 mL of 5% (w/v) *m*-phosphoric acid containing 1 mM diethylenetriamine pentaacetic acid (DTPA). Following centrifugation at 10,000×*g* for 15 min at 4°C, the supernatant was used for analysis of ascorbate and dehydroascorbate (extinction coefficient of 14.3 mM^{-1} cm^{-1}). For AsA, the initial absorbance of a 50 μL aliquot of extract was recorded at 265 nm in 60 mM potassium phosphate (pH 6.3), then re-measured following the addition of 1 unit ascorbate oxidase (EC 1.10.3.3; Sigma-Aldrich, USA). Dehydroascorbate content

was determined by subtracting AsA from the total ascorbate measured in a second aliquot after the addition of a reductant (2 mM D/L- DTT).

Root tissues (1.0 g) were homogenized in 10.0 mL of 5.0% (w/v) TCA and centrifuged at 10000 *g* for 5 min. Aliquots (0.5 mL) of the homogenates were mixed with Ellman reagent (0.5 mL of 0.01 M DTNB solubilized in 1.0 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 an extinction coefficient of 13.1 mM^{-1} cm^{-1}.

Enzyme Extraction and Protein Estimation

Fresh root tissues (1.0 g) were powdered in liquid nitrogen in a chilled pestle and mortar, then homogenized in 4.0 mL of chilled 50 mM potassium phosphate buffer (pH 7.0) containing 1.0% (w/v) insoluble polyvinylpolypyrrolidone and 1.0 mM phenylmethylsulfonylfluoride, 1.0 mM EDTA, 1.0 mM dithiothreitol (DTT), and 0.2% (v/v) Triton X-100. The homogenate was filtered through two layers of muslin and centrifuged at 10,000×*g* for 10 min at 2°C. The supernatant was stored at 2°C and used within 4 h. To assay for APX activity, 5.0 mM ascorbic acid (AsA) was included in the extraction medium. Protein in the root extract was estimated according to the method of Bradford (1976).

Assays of Enzymes

Superoxide dismutase (SOD; EC 1.15.1.1) activity was assayed by measuring 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 NBT, 2.0 μM riboflavin, 50 μL of enzyme extract, 250 μL of water, and 15 μL *N,N,N′,N′*-tetramethylethylenediamine (TEMED) (modified from the protocol of Beauchamp and Fridovich, 1971). While one set of reaction mixture was kept under darkness (and considered as corresponding blanks), the other set was exposed to light (350 μmol m^{-2} s^{-1}) for 15 min. SOD activity was expressed as units min^{-1} per milligram of protein. The enzyme extract that corresponded to 50% inhibition of the reaction was considered to be one enzyme unit.

Peroxidase (POD; EC 1.11.1.7) activity was determined by monitoring the formation of tetraguaiacol (extinction coefficient of 6.39 mM^{-1} cm^{-1}) at 436 nm during the enzymatic reaction from initial substrate guaiacol in the presence of H_2O_2, based on the method of Pütter (1974). The reaction mixture (3.18 mL) contained 100 mM potassium phosphate buffer (pH 7.0), 0.3 mM guaiacol, and plant extract. This reaction was initiated by adding 0.1 mM H_2O_2.

Ascorbate peroxidase (APX; EC 1.11.1.11) activity was measured in 3 mL of a reaction mixture containing 50 mM phosphate buffer (pH 7.0), 0.5 mM AsA, 0.1 mM H_2O_2, 0.1 mM EDTA, and a suitable quantity of enzyme extract. Both minus tissue-extract and minus H_2O_2 blanks were run; changes in absorbance every 15 s were read at 290 nm (Nakano and Asada, 1981). APX activity was calculated in terms of units (μmol of ascorbate oxidized min^{-1}) per milligram of protein.

Statistical Analysis

Results represented the means of three independent experiments. A one-way analysis of variance (ANOVA) was applied to our data. Statistical assessments of differences between mean values were performed by a least significant difference (LSD) test at P 0.05, using the SAS program (SAS Institute, Inc., USA).

RESULTS AND DISCUSSION

SNP Supply Enhances Endogenous NO and Growth of Adventitious Roots

In our experiments, we found that the supply of sodium nitroprusside, a producer of nitric oxide, at 250 μM induced increases in the dry weight of adventitious roots from *Echinacea purpurea* (Table 1). We also observed a rise in the endogenous level of NO in those roots when exogenous SNP was applied (Fig. 1). Gouvêa et al. (1997) also have reported that NO induces cell elongation in a manner similar to auxin. Moreover, a transient increase in NO and auxin concentration has been shown to be involved in adventitious root development (Pagnussat et al., 2003). Because exogenous application of NO enhances the activity of NADPH oxidase (an enzyme responsible for $O_2^{\cdot-}$ generation) and the production of $O_2^{\cdot-}$ in ginseng adventitious roots (Tewari et al. 2007), one might infer that ROS is associated with the growth and development of roots and root hairs, as has been suggested from other research (Sagi and Fluhr, 2006; Liszkay et al., 2004; Gapper and Dolan, 2006). Although we could not estimate NADPH oxidase activity here, we did observe upregulation in the activity of SOD, a substrate-inducible enzyme responsible for the removal of $O_2^{\cdot-}$ (Rao et al., 1995). This implies the generation of $O_2^{\cdot-}$ is improved in SNP-treated adventitious roots.

Table 1. Effect of elicitation with NO producer (SNP) on root growth, phenolics, and production of flavonoids in the adventitious roots of *Echinacea purpurea*. Data were collected after 5 weeks of culturing in a 5-L bioreactor containing 4 L of 1/2 MS medium.

SNP supply (μM)	Biomass (g L^{-1})		Growth ratio*	Phenolics	Flavonoids
	Fresh mass	Dry mass		(mg g^{-1} DW)	
0.0 (Control)	70.1 ± 0.25a	11.17 ± 0.09b	13.5	57.88 ± 0.12b	37.27 ± 0.28b
50	70.4 ± 1.08a	11.33 ± 0.26b	13.7	57.41 ± 0.23b	37.39 ± 0.14b
100	69.8 ± 0.70a	11.31 ± 0.22b	13.7	61.07 ± 0.33a	39.93 ± 0.22a
250	69.4 ± 1.92a	11.99 ± 0.04a	14.6	53.62 ± 0.17c	35.64 ± 0.15c

*Growth ratio is the quotient of the dry weight after culturing and the dry weight of the inoculum.
Data are means of 3 experimental replicates. Values not followed by the same letters within a column are statistically different ($P<0.05$).

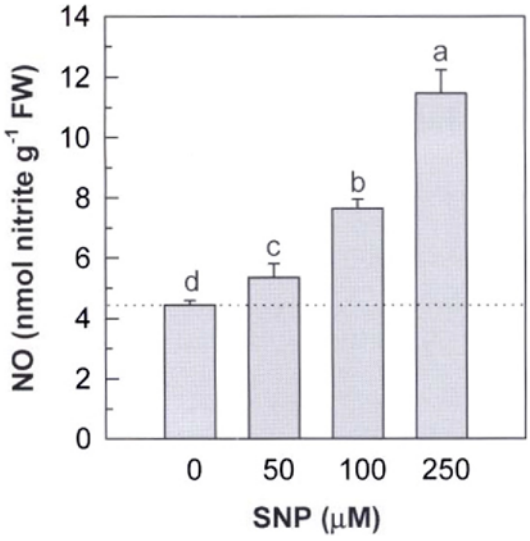

Figure 1. Nitric oxide (nitrite) concentrations in adventitious roots of *Echinacea purpurea* elicited with 0, 50, 100, or 250 μM SNP in liquid suspension of 1/2 MS medium. Values represent mean ± SE (n=6) for 3 experimental replicates. Bars not labeled with same letters are significantly different at $P \leq 0.05$ (DMRT).

SNP Supply Enhances Secondary Metabolites Accumulation

Increasing the supply of SNP up to 100 μM elevated the contents of both phenolics and flavonoids, but higher concentrations caused a decline in their accumulations (Table 1). Likewise, levels of caffeic acid derivatives (caftaric acid, chlorogenic acid, and cichoric acid) increased in response to SNP applications of up to 100 μM (Table 2). Therefore, 100 μM SNP appears to be beneficial for the accumulation of secondary metabolites. An increase in the endogenous NO level upon elicitation with methyl jasmonate (MJ) or a fungal agent has previously been reported (Wang and Wu, 2004). These elicitors also induce phenylalanine ammonia-lyase (PAL), the first enzyme in the phenylpropanoid pathway (Wang and Wu, 2004). Because this elicitor effect (i.e., PAL activity and the accumulation of secondary metabolites) is diminished when an NO scavenger is incorporated into the system, it might be that the NO-upregulated phenylpropanoid pathway (Wang and Wu, 2004) is ultimately responsible for metabolite synthesis. We found that decreases in the contents of flavonoids, phenolics, and caffeic acid derivatives in response to a higher supply of SNP appeared to be a result of the severe stress that developed due to an over-

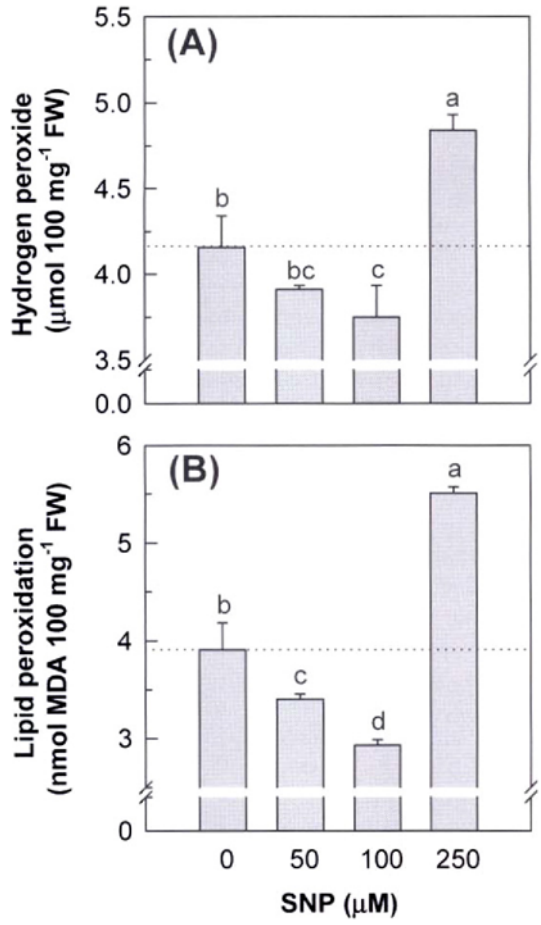

Figure 2. Levels of hydrogen peroxide (**A**) and lipid peroxidation (**B**) in *Echinacea* adventitious roots elicited with 0, 50, 100, or 250 μM SNP in liquid suspension of 1/2 MS medium. Values represent mean ± SE (n=6) for 3 experimental replicates. Bars not labeled with same letters are significantly different at $P \leq 0.05$ (DMRT).

accumulation of H_2O_2 (Fig. 2A). This was consequently manifested as damage to the membrane lipids (i.e., lipid peroxidation; Fig. 2B).

NO Acts as Both Antioxidant and Pro-oxidant Depending on Its Concentration

Lipid peroxidation, apart from development-related processes, could be an outcome of increased ROS generation because SNP induces $O_2^{\cdot -}$ accumulation in adventitious roots (Tewari et al., 2007). Therefore, we estimated H_2O_2 as

Table 2. Effects of elicitation with NO producer (SNP) on the accumulation of caffeic acid derivatives in the adventitious roots of *Echinacea purpurea*. Data were collected after 5 weeks of culturing in a 5-L bioreactor containing 4 L of 1/2 MS medium.

SNP supply (μM)	Caffeic acid derivatives (mg g^{-1} DW)			
	Caftaric acid	Chlorogenic acid	Cichoric acid	Total*
0.0 (Control)	3.22 ± 0.03c	4.73 ± 0.12ab	27.59 ± 0.16c	35.53 ± 0.07c
50	3.47 ± 0.02b	4.53 ± 0.02b	28.82 ± 0.54c	36.82 ± 0.58c
100	3.71 ± 0.03a	4.95 ± 0.04a	34.89 ± 0.32a	43.55 ± 0.31a
250	3.68 ± 0.04a	4.60 ± 0.07b	33.64 ± 0.44ab	41.93 ± 0.54b

*Total caffeic acid derivative = caftaric acid + chlorogenic acid + cichoric acid.
Data are means of 3 experimental replicates. Values not followed by the same letters within a column are statistically different ($P<0.05$).

a dismutation product of $O_2^{\cdot-}$ (Halliwell, 2006) and lipid peroxidation as an index of membrane damage in our SNP-treated *Echinacea* adventitious roots. Application of SNP at up to 100 μM caused a decline in both H_2O_2 content and lipid peroxidation, but a further increase in the supply of SNP elevated both (Fig. 2). This suggests two opposing roles for NO: 1) as an antioxidant at concentrations of 50 or 100 μM (as reflected by decreases in H_2O_2 and lipid peroxidation) and 2) as a pro-oxidant at a higher supply, e.g., 250 μM, where it induced the accumulation of H_2O_2 and lipid peroxide. For example, a concentration of 100 μM did not induce lipid peroxidation, most likely due to the lower H_2O_2 content in our NO-treated adventitious roots. Because, NO can remove Fenton active Fe^{2+} from the cellular environment (Wink et al., 1995), it can terminate the committed step of $OH^{\cdot}$ formation, thus perhaps limiting the chance for lipid peroxide generation to occur. In fact, at a lower SNP supply, NO appears to be an inhibitor of lipid peroxidation as reported before (Rubbo et al., 2000). Because an excess of NO (250 μM SNP) increased both H_2O_2 and lipid peroxidation in our experiments (Fig. 2a, b), we might conclude that this peroxidation is dependent on H_2O_2 concentration in the roots. A greater accumulation of H_2O_2 at 250 μM SNP most probably resulted due to a rate of H_2O_2 generation that was higher than the capacity of the cell to scavenge it. This was because of a decline in the activity of APX, an important H_2O_2-scavenging enzyme, relative to the 100 μM concentration of SNP (Fig. 3C). Such down regulation in the expression and activity of APX at a higher level of NO (250 μM SNP) has been reported previously (Murgia et al., 2004). We also estimated the activities of SOD (Fig. 3A) and POD (Fig. 3B) in the adventitious roots of *E. purpurea*. Whereas the former increased (Fig. 3A), the latter was diminished by SNP treatment (Fig. 2B). APX activity, however, was differently affected by that treatment (Fig. 3C). NO has also been implicated in the induced activities of SOD and APX in *Stylosanthes guianensis* leaves (Zhou et al., 2005) and rice (Uchida et al., 2002; Hung et al., 2002). Nevertheless, to the best of our knowledge, no reports have been made earlier on this particular plant organ (adventitious roots) or species (*E. purpurea*) as used in our experimental system with NO donors.

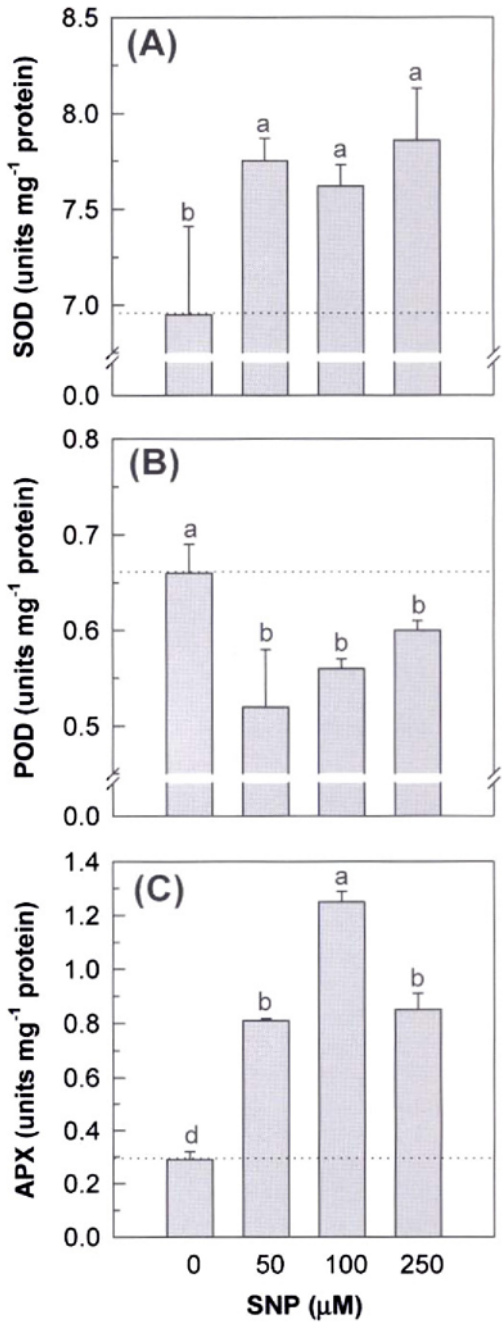

Figure 3. Activities of antioxidant enzymes SOD (**A**), POD (**B**), and APX (**C**) in *Echinacea* adventitious roots elicited with 0, 50, 100, or 250 μM SNP in liquid suspension of 1/2 MS medium. Values represent mean ± SE (n=6) for 3 experimental replicates. Bars not labeled with same letters are significantly different at *P* 0.05 (DMRT).

NO Modulates the Cellular Redox Environment by Regulating Ascorbate and Non-Protein Thiols

As a redox active molecule, NO can also react with ascorbate and thiol. Therefore, we estimated the status of ascorbate and thiols, two major redox controllers of the cellular environment. SNP supplied at 100 μM increased the total ascorbate content, with AsA being the predominant form; this was reflected by a decline in the ratio of DHA/AsA (Fig. 4A). At higher levels (250 μM SNP), that ratio increased (Fig. 4A). Thiols are a component of the ferredoxin-thioredoxin system, and they play a crucial role in the redox homeostasis of cells by regulating the activities of redox centers for various enzymes involved with sulphur and nitrogen assimilation (Heldt, 1997). Our measurements of non-protein thiols showed that their contents were diminished by SNP (NO) treatment (Fig. 4B). Because thiol and NO are reactive molecules, they might have reacted; we also observed a decrease in the amounts of non-protein thiol when the supply of exogenous NO was increased. To confirm whether *in vivo* NO status was really up-regulated by SNP supply, we estimated NO as nitrite, and observed a rise in its content (Fig. 1). These observations suggest that NO modulates the levels of both ascorbate and glutathione (a representative thiol) in *E. purpurea* adventitious roots.

To elucidate any further role of NO in antioxidant defenses within those root tissues, we investigated possible

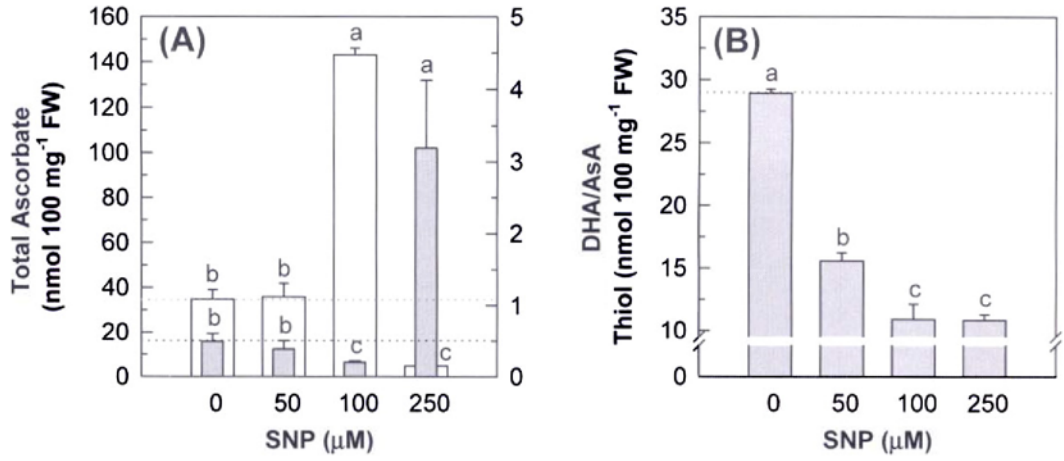

Figure 4. Molecular antioxidant concentrations of (**A**) total ascorbate (unfilled bar) and ratio of DHA/AsA (filled gray bar), and (**B**) non-protein thiol concentrations in *Echinacea* adventitious roots elicited with 0, 50, 100, or 250 μM SNP in liquid suspension of 1/2 MS medium. Values represent mean ± SE (n=6) for 3 experimental replicates. Bars not labeled with same letters are significantly different at $P \leq 0.05$ (DMRT).

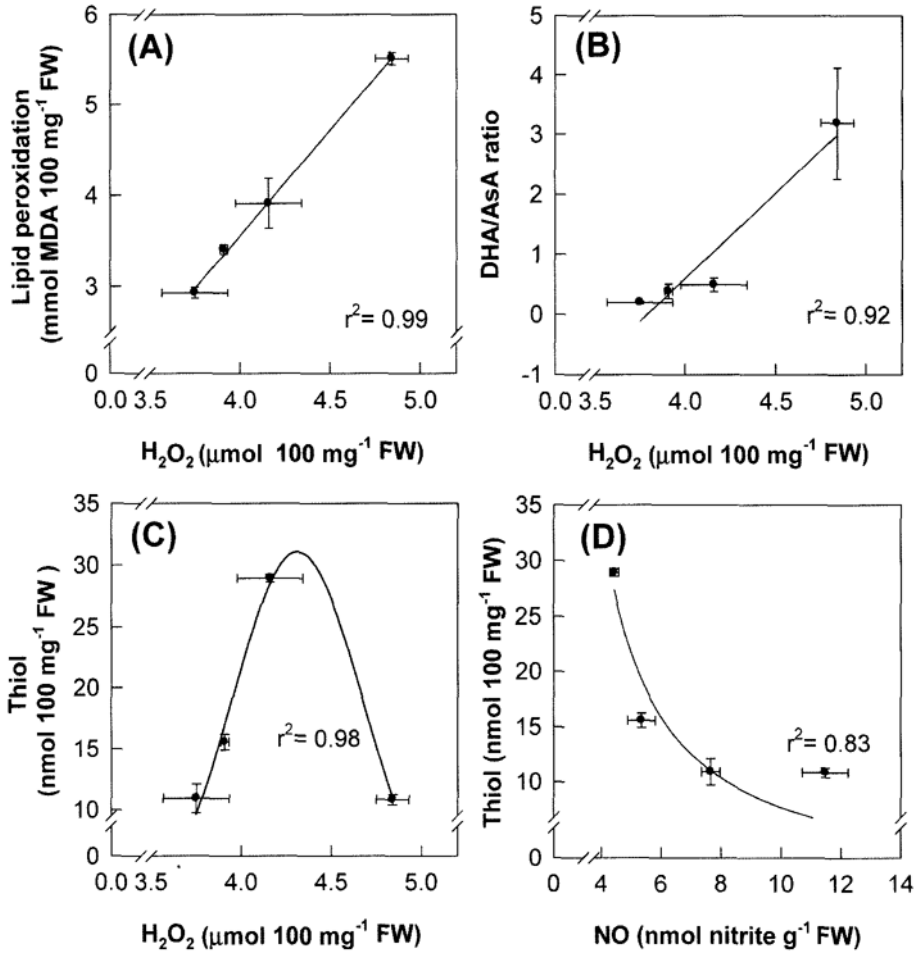

Figure 5. Relationships between H_2O_2 concentration and lipid peroxidation (**A**), H_2O_2 concentration and DHA/AsA ratio (**B**), H_2O_2 concentration and thiol concentration (**C**), and NO (nitrite) concentration and thiol concentration (**D**) in *Echinacea* adventitious roots. Each value represents mean ± SE (n=6) for 3 experimental replicates.

relationships among various parameters: H_2O_2 with lipid peroxidation, DHA/AsA ratio and the level of non-protein thiol, and NO and non-protein thiol content (Fig. 5). Correlations were linear and positive between H_2O_2 and lipid peroxidation (Fig. 5A) and between H_2O_2 and the DHA/AsA ratio (Fig. 5B). This demonstrated that, beyond a certain level, NO induces oxidative stress, modifies the cellular redox status (DHA/AsA), and enhances H_2O_2 contents. However, no consistent relationship existed between H_2O_2 and non-protein thiol, with linearity being found only up to

a certain level of H_2O_2 before severe stress conditions caused that relationship to disappear (Fig. 5C). NO content and non-protein thiol certainly showed a negative relationship (Fig. 5D), which suggests the reactivity of NO toward thiol. These observations imply that, after a certain maximum, NO becomes toxic and brings on oxidative stress probably by forming ROS, as reflected in the higher content of H_2O_2, rate of lipid peroxidation, and DHA/AsA ratio.

CONCLUSIONS

Our observations lead us to conclude that 100 μM exogenous NO (SNP supply) is beneficial for the accumulation of secondary metabolites (phenolics, flavonoids, and caffeic acid derivatives), as well as for the upregulation of antioxidant defenses in the adventitious roots of *Echinacea purpurea*. Although 50 or 100 μM SNP might act as a signal of oxidative stress, this does not really cause any damage due to the activation of antioxidant-functioning in those tissues.

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