Effect of Nitric Oxide and Hydrogen Peroxide on Adventitious Root Development from Cuttings of Ground-Cover Chrysanthemum and Associated Biochemical Changes

Wei-Biao Liao · Hong-Lang Xiao · Mei-Ling Zhang

Abstract It is well known that plant adventitious root formation can be stimulated by the application of nitric oxide (NO) and hydrogen peroxide (H_2O_2) exogenously but the mechanism of this physiological response is still unclear. Ground-cover chrysanthemum (*Dendranthema morifolium* 'Beiguozhicun') was used to understand the effects of NO and H_2O_2 on the rooting of plant cuttings and the associated biochemical changes of the rooting zone during the rhizogenesis process. The results showed that the effect of NO or H_2O_2 on rooting of ground-cover chrysanthemum cuttings was dose-dependent, with a maximal biological response at 50 μM of NO donor sodium nitroprusside (SNP) or 200 μM H_2O_2. There was a synergistic effect between NO and H_2O_2 on mediating rooting. NO and H_2O_2 treatments at the proper dosage might increase the activities of polyphenol oxidase (PPO) and indoleacetic acid oxidase (IAAO) and the content of water-soluble carbohydrate (WSC) and total nitrogen, while decreasing the total polyphenol content of ground-cover chrysanthemum cuttings. In addition, rooting percentage was significantly correlated with these biochemical constituent activities or contents. Together, these results indicated that NO and H_2O_2 treatments enhanced adventitious root development synergistically and independently by stimulating the activities of PPO and IAAO enzymes and the content of carbohydrate and nitrogen and simultaneously repressing the production of polyphenol.

Keywords Nitric oxide (NO) · Hydrogen peroxide (H_2O_2) · Ground-cover chrysanthemum (*Dendranthema morifolium* 'Beiguozhicun') · Adventitious root development · Biochemical changes

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Introduction

Previous studies have shown that both nitric oxide (NO) and hydrogen peroxide (H_2O_2) play crucial roles in the modulation of several physiological processes during plant life, mediating systemic signal networks in plants (Delledonne and others 1998; Pei and others 2000; Bright and others 2006; Neill and others 2008). In recent years, there has been much research about the presence of NO or H_2O_2 and their physiological roles in plant adventitious root development. The involvement of NO in promoting root growth was first observed in *Zea mays* by Gouvêa and others (1997). They found that NO induced cell elongation in a similar way to auxin. Pagnussat and others (2002) reported that NO was required for the molecular events involved in auxin-induced adventitious root development in *Cucumis sativus*. Recently, Correa-Aragunde and others (2004) demonstrated that NO production played an essential role during the formation of tomato (*Lycopersicon esculentum* Mill.) lateral root (LR) primordia and the emergence of LRs from the parent root. In lettuce (*Lactuca sativa*), NO is a critical molecule in the process leading to

root hair formation (RHF) and is involved in the auxin-signaling cascade leading to RHF (Lombardo and others 2006). The participation of NO in gravitropic bending in soybean (*Glycine max*) roots has also been described by Hu and others (2005). They found an asymmetric accumulation of NO in the primary root in response to gravistimulation.

H_2O_2 is a messenger in plant root formation and development. Joo and others (2001) reported that auxin-induced reactive oxygen species (ROS) might function as a downstream component in auxin-mediated signal transduction and play a role in root gravitropism. Some findings point to the existence of a ROS signaling pathway as a regulator of *Arabidopsis* root hair development that depends on a number of distinct enzymes such as NADPH oxidase and OXI1 kinase (Foreman and others 2003; Rentel and others 2004). H_2O_2 accumulation in the extremity of the growing *Arabidopsis* root appears to be involved in *Arabidopsis* growth restriction and RHF (Dunand and others 2007). H_2O_2 is also likely to play an important role in the development of LRs in soybean (Su and others 2006) and in the formation and development of adventitious root in *Cucumis sativus* (Li and others 2007).

Cross-talk between NO and H_2O_2 in plants, a topic of debate, has been extensively studied (Bright and others 2006; Neill and others 2008). NO and ROS are both reported to be important signaling molecules in plant-pathogen interactions. It is well known that NO can react with H_2O_2 to form the reactive molecule peroxynitrite (ONOO$^-$), which may affect cellular processes such as cell death and disease resistance (Delledonne and others 1998). NO functions in pathogen resistance, depending on salicylic acid or collaboration with H_2O_2 (Delledonne and others 1998). NO can scavenge ROS and protect plant cells from damage; therefore, it also can affect ROS generation or interact with ROS during cell death. In addition, their signal interactions in stomatal movement have also been investigated. It is well established that H_2O_2 may induce NO synthesis and accumulation in *Vicia faba* guard cells (She and others 2004). Abscisic acid (ABA)-induced NO accumulation depends on H_2O_2 production and mediates ABA- and H_2O_2-induced stomata closure in *Arabidopsis* guard cells (Desikan and others 2002; Bright and others 2006). There has also been some evidence that NO may modulate H_2O_2 levels in *V. faba* guard cells (She and others 2004); on the other hand, other experiments do not observe this phenomenon (Bright and others 2006; Zhang and others 2007).

The rooting of stem cuttings is one of the best and economically viable methods of vegetative propagation in horticulture and forestry, to obtain plants of desired genetic types or get high multiplication rates. It is well known that different phases of adventitious root formation in plants are based on physiology. Some enzymes such as peroxidase (POD), polyphenol oxidase (PPO), and indoleacetic acid oxidase (IAAO) are known to be intimately involved in the process of adventitious root formation (Gove and Hoyle 1975; Smart and others 2003). In addition, the interdependent physiological stages of the rooting process are associated with changes in endogenous enzyme activity (Rout 2006). The changes in metabolic constituents such as carbohydrate, nitrogen, and polyphenol are also known to occur during the rooting process and it has been suggested that some of these metabolic constituents may be involved in root formation (Li and Leung 2000; Smart and others 2003).

Chrysanthemum (*Dendranthema morifolium*) is globally the second-most economically important floricultural crop following rose. It is one of the most important ornamental species, with the production value increasing exponentially as a result of the rapid improvement of living conditions and a greater enjoyment of life. Commercial chrysanthemum cultivars, especially potted plant species, are usually cultivated by vegetative cuttings.

The above-mentioned data suggest that adventitious root development can be stimulated by application of NO and H_2O_2 exogenously but the mechanism of this physiological response is still unclear. In addition, previous studies have also shown that H_2O_2 and NO are both synthesized in roots and they both mediate root organogenesis and development independently. However, the interactions of H_2O_2 and NO in adventitious root development of plants are still unknown. Therefore, the aim of the present study was to determine (1) the effects of NO and H_2O_2 on the adventitious root development of stem cuttings of ground-cover chrysanthemum solely or synergistically, and (2) the changes of the enzyme activities and the biochemical constituent content of cuttings treated with NO and H_2O_2 during adventitious root development.

Materials and Methods

Plant Material and Growth Conditions

Stem cuttings of ground-cover chrysanthemums (*Dendranthema morifolium* 'Beiguozhicun') from the basal portion of an annual twig, measuring about 7–9 cm in length and 2.5 mm in diameter, were obtained from Lanzhou Botanical Garden, Lanzhou, China. After collection, cuttings were immediately placed in plastic bags containing moist paper towels, sealed, and stored at 3°C. Unwanted leaves and buds were removed from the cuttings resulting in two nodes with two to three leaves in each segment. The cuttings were planted in 50-hole trays filled with a sieved blend of moist perlite and vermiculite (1:1).

The trays were then transferred onto shaded rooting benches of the No. 6 greenhouse in Lanzhou Institute of Garden Research, Lanzhou, China. During rooting, the cuttings were in intermittent mist or fog during the daylight hours and allowed to dry at night; the temperature was about 18–22°C. Cuttings were watered with various Hoagland solutions containing different chemicals once every 2 days. Fungicide treatment was also done at 10-day intervals to prevent the development of pathogens.

Chemicals and Treatments

Sodium nitroprusside (SNP, Merck, Darmstadt, Germany) was used as a NO donor, and 2-(4-carboxy-2-phenyl)-4, 4, 5, 5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO, Sigma, St. Louis, MO, USA) was used as a NO scavenger. A stock solution of 100 mM SNP was prepared in complete darkness and immediately diluted to the demanded concentrations. Catalase (CAT, Sigma) was used as an H_2O_2 scavenger. The SNP analog sodium ferrocyanide [$Na_3Fe(CN)_6$, Sigma] was used as an additional control. The solutions were stored in darkness at 2°C in brown bottles, and the time between solution preparation and use did not exceed 3 days. Unless stated otherwise, the remaining chemicals were of analytical grade from Chinese companies.

Several test Hoagland solutions were used; they contained (1) different concentration of SNP (0, 10, 50, 100, 200, 500 μM) and H_2O_2 (0, 50, 100, 200, 500, 1000 μM); (2) 200 μM c-PTIO; (3) 100 μM CAT; (4) 100 μM $NaNO_2$; (5) 100 μM $Na_3Fe(CN)_6$. All experiments were replicated three times independently with 200 cuttings in each replication.

Rooting Character Recordings

Any cutting that had at least one root was classified as rooted. The data collected were rooting percentage (RP), root number per cutting (RN), and root length per cutting (RL, the mean length of 20 roots per cutting and only roots >1 mm were considered). Observations on these morphological characteristics were recorded 25 days after planting.

Enzyme Assays

Rooting area from cuttings was measured at 2 h and 5, 10, 15, 20, and 25 days after planting to determine enzyme activity and metabolic constituent content analysis; all experiments were performed at 4°C.

For POD activity determination, 0.1 ml of the enzyme extract was added to 3 ml of substrate mixture containing 0.05 M potassium phosphate buffer, 50 mM guaiacol, and 7.5 mM of 2% H_2O_2. The formation of the oxidized tetraguaiacol polymer was monitored at 470 nm for 3 min. The levels of enzyme activity were expressed as the optical density (OD) difference per minute per milligram protein (Wang and others 1991).

For PPO extraction, 1 g fresh tissue was homogenized in 15 mM ß-mercaptoethanol, 20 mM Tris–HCl (pH 7.8), 20% glycerol, 1 mM phenylmethyl sulfonyl fluoride (PMSF), and 1% (v/v) Triton X-100. PPO activity was measured using 30 mM catecol in sodium acetate buffer (pH 4.5). The reaction was initiated by the addition of the enzyme extract containing 50 mM phosphate buffer (pH 7.0) at 400 nm and assessed by the enzymatic oxidation of catecol (Ferrer and others 1988).

To determine IAAO activity, the reaction mixture [0.2 ml enzyme extracts, 0.78 ml of 50 mM potassium-phosphate buffer (pH 6.0), 0.01 ml of 5 mM $MnCl_2$, 0.01 ml of 5 mM 2, 4-dichlorophenol, and 0.02 ml of 2.5 g l^{-1} IAA] was incubated at 30°C for 15 min. The Salkowski reagent (2 ml) was then added and the destruction of IAA was determined by measuring the absorbance at 535 nm after 30 min (Beffa and others 1990). One unit of IAA oxidase activity was equivalent to an OD_{535} of 1.0 for 1 mg of protein in 30 min.

Metabolic Constituent Content Estimation

Water-soluble carbohydrate (WSC) was extracted for 12 h from water at 70°C after the cuttings had been homogenized; its content was estimated using the anthrone method (Van Handel 1968). The carbohydrate extract was analyzed by reacting 0.25 ml of the supernatant with 3 ml of freshly prepared anthrone reagent [0.06% (w/v) anthrone in 95% H_2SO_4] and placing it in a boiling water bath for 10 min. After cooling to room temperature, the absorbance at 625 nm was measured. Starch in the rooting zone of the cuttings was estimated by amyloglucosidase digestion followed by enzymic analysis of the released glucose, with the imidazole buffer at pH 8.0 (Paul and others 1991). The total carbohydrate content was determined by WSC plus starch content.

Nitrogen levels in the cuttings were determined using the micro-Kjeldahl method as described by Knowles and Ries (1981). The plant samples were solubilized by adding digestion mixture (4 ml for every 20 mg dry weight) and heating until the solutions cleared. The digestion mixture contained 1800 ml H_2SO_4, 40 ml $HClO_4$, 6 g SeO_2, and 160 ml H_2O. After cooling, the samples were diluted with distilled H_2O (6 ml for every 4 ml of digestion mixture) and poured into plastic cups which were placed on an Auto-Analyzer (FAN Corp., Germany, NOI-7) for automated Kjeldahl analysis. Ammonium was detected spectrophotometrically at 623 nm by an alkaline-phenol color

Table 1 Effect of NO and H_2O_2 on rooting of ground-cover chrysanthemum cuttings

Treatments	Rooting percentage (%)	Root number per cutting	Root length per cutting (cm)
SNP (μM)			
0 (CK)	84.7 ± 1.3 c	55.1 ± 1.6 bc	0.52 ± 0.07 b
10	88.2 ± 0.9 b	53.3 ± 4.1 bc	0.67 ± 0.07 b
50	93.6 ± 1.3 a	65.6 ± 2.7 a	1.02 ± 0.05 a
100	90.3 ± 0.9 b	57.5 ± 1.7 b	1.05 ± 0.17 a
200	81.8 ± 1.5 d	50.7 ± 0.7 cd	0.51 ± 0.10 b
500	74.5 ± 2.1 e	47.0 ± 1.8 e	0.25 ± 0.07 c
H_2O_2 (μM)			
0 (CK)	84.7 ± 1.3 c	55.1 ± 1.6 c	0.52 ± 0.07 bc
50	89.4 ± 1.1 b	60.7 ± 2.3 b	0.55 ± 0.14 b
100	92.8 ± 0.3 a	58.5 ± 0.8 b	1.14 ± 0.12 a
200	94.2 ± 2.4 a	66.4 ± 0.9 a	1.26 ± 0.18 a
500	83.3 ± 1.2 c	50.3 ± 1.5 d	0.41 ± 0.05 bc
1000	74.7 ± 2.5 d	46.0 ± 0.3 e	0.30 ± 0.12 c

The values (mean ± SE) are the average of three independent experiments. Values not sharing the same letters in a column within SNP or H_2O_2 treatment were significantly different by Duncan's multiple-comparison test ($P < 0.05$)

reaction. The carbohydrate:nitrogen (C:N) ratio was derived using the respective values of carbohydrate and nitrogen.

Total polyphenols were determined according to the procedures of Chandler and Dodds (1983). Fresh tissue (1 g) was homogenized in 5 ml of 80% ethanol using a chilled pestle and mortar, with subsequent centrifugation at 10,000 g for 20 min. The supernatant was preserved and residue re-extracted with 2.5 ml of 80% ethanol and centrifuged, then the supernatants were pooled and evaporated to dryness. The residue was dissolved in 5 ml of distilled water. In a test tube, 3-ml aliquots were taken, then 0.5 ml Folin-Ciocalteau's reagent (1 N) was added and kept for 3 min. Then 2 ml of 20% freshly prepared Na_2CO_3 solution was added to each tube and mixed thoroughly. The solution was boiled in a water bath for exactly 1 min, cooled, and then the absorbance was measured at 650 nm against a reagent as a blank. From the standard curve that was prepared using 10–100 μg of catechol, the concentrations of phenols in the unknown samples were calculated.

Statistical Analysis

Statistical analysis was conducted using Statistical Package for Social Sciences (SPSS) for Windows (ver. 13.00). In the analysis of variance (ANOVA) for studied parameters, the mean values of each replication and the standard error (SE) of the mean were estimated. One-way analysis of variance (ANOVA) and Duncan's multiple-range test or Student's t test were applied to determine the significance of the results between different treatments at the $P < 0.05$ level. The correlations between RP and WSC, starch content, total carbohydrate content, total nitrogen, C:N ratio, and total polyphenol content were also analyzed by Spearman correlations.

Results

Effect of Exogenous H_2O_2 and NO on Adventitious Root Development

To understand the effects of exogenous NO and H_2O_2 on rooting, ground-cover chrysanthemum cuttings were treated with increasing concentrations of SNP and H_2O_2 (Table 1). Compared with the control (0 μM), both SNP and H_2O_2 had significant effects on cutting rooting, and the effects were dose-dependent. SNP and H_2O_2 increased the rooting percentage (RP), root number per cutting (RN), and root length per cutting (RL) at low concentrations (10–100 μM SNP and 50–200 μM H_2O_2), whereas high levels of SNP (200, 500 μM) and H_2O_2 (500, 1000 μM) both resulted in decreased rooting characteristics. Among the different concentrations of SNP, the maximum RP (93.6%) and RN (65.6) were observed with the 50-μM treatment, and the maximum RL (1.05 cm) was achieved with the 100-μM treatment. Among the different concentrations of H_2O_2, cuttings treated with 200 μM obtained the maximum RP (94.2%), RN (66.4), and RL (1.26 cm) (Table 1). Therefore, the promotion of root development was maximal at 50 μM SNP or at 200 μM H_2O_2, and these concentrations were used for further studies during the rooting process.

Effect of $NaNO_2$, $Na_3Fe(CN)_6$, and H_2O_2 or NO Scavenger on Adventitious Root Development

As shown in Table 2, there were no significant differences in RP, RN, and RL between the control and treatment with the 100-μM $NaNO_2$ solution (normal products of NO decomposition, as control for NO decomposition).

Table 2 Effect of NaNO$_2$, Na$_3$Fe(CN)$_6$, and H$_2$O$_2$ or NO scavenger on rooting of ground-cover chrysanthemum cuttings

Treatments	Rooting percentage (%)	Root number per cutting	Root length per cutting (cm)
Ck	84.7 ± 1.3 c	55.1 ± 1.6 b	0.52 ± 0.07 c
SNP	94.2 ± 2.4 b	66.4 ± 0.9 a	1.26 ± 0.18 ab
H$_2$O$_2$	93.6 ± 1.3 b	65.6 ± 2.7 a	1.02 ± 0.05 b
NaNO$_2$	83.9 ± 1.7 c	51.0 ± 1.2 bc	0.64 ± 0.11 c
Na$_3$Fe(CN)$_6$	82.5 ± 1.9 c	54.3 ± 2.9 b	0.59 ± 0.11 c
SNP + cPTIO	83.6 ± 1.2 c	47.1 ± 3.0 c	0.44 ± 0.07 c
H$_2$O$_2$ + CAT	84.1 ± 2.4 c	52.1 ± 5.5 bc	0.49 ± 0.15 c
H$_2$O$_2$ + SNP	97.9 ± 1.5 a	69.0 ± 2.9 a	1.48 ± 0.32 a

SNP, H$_2$O$_2$, NaNO$_2$, Na$_3$Fe(CN)$_6$, cPTIO, and CAT were used at 50, 200, 100, 100, 200, and 100 μM, respectively. The values (mean ± SE) are the averages of three independent experiments. Values not sharing the same letters among different treatments were significantly different by Duncan's multiple-comparison test ($P < 0.05$)

Moreover, 100 μM sodium ferrocyanide [Na$_3$Fe(CN)$_6$], an analog of SNP that does not release NO, had no effect on adventitious root development (Table 2). Thus, the remarkable results suggested that NO released from SNP might be responsible for the promotion of rooting.

To be sure that the NO-releaser SNP- or H$_2$O$_2$-induced rooting in cuttings was specific to NO or H$_2$O$_2$ further, a specific NO scavenger cPTIO or H$_2$O$_2$ scavenger CAT was analyzed on the same experimental system. If 200 μM cPTIO was added to the SNP solution simultaneously, the effect of SNP was reversed. The promotive effect on rooting of H$_2$O$_2$ was also largely depressed by the simultaneous presence of 100 μM CAT (Table 2). This further confirmed the specific roles of NO and H$_2$O$_2$ in promotion of adventitious root development.

Although there was no significant difference in RN among SNP, H$_2$O$_2$, and SNP + H$_2$O$_2$ treatments, RP and RL of cuttings treated with NO + H$_2$O$_2$ were significantly higher than those of cuttings treated with either NO or H$_2$O$_2$ alone (Table 2, Fig. 1), showing that H$_2$O$_2$ may act synergistically with NO to enhance adventitious root development.

To determine whether the SNP and H$_2$O$_2$ treatments affected adventitious root outgrowth or initiation, an anatomical study was performed. We analyzed the transverse sections of the rooting area of ground-cover chrysanthemum cuttings treated for 12 days with water, SNP, H$_2$O$_2$, or SNP + H$_2$O$_2$, respectively (not shown). At the end of the induction phase (upon 12 days), cell proliferation and differentiation into root primordia (RP) were clearly detected in SNP-, H$_2$O$_2$-, and SNP + H$_2$O$_2$-treated cuttings (not shown). At the same time, cell proliferation was only barely detected and lower RP was observed in the control treatment. Therefore, this might suggest that the treatments (H$_2$O$_2$/SNP) affect adventitious root initiation.

Fig. 1 Effects of NO donors SNP and H$_2$O$_2$ on adventitious root development from ground-cover chrysanthemum cuttings. Cuttings were watered with water (**a**), H$_2$O$_2$ (**b**), SNP (**c**), or H$_2$O$_2$ + SNP (**d**) as indicated. SNP and H$_2$O$_2$ were used at 50 and 200 μM, respectively. Photographs were taken after 25 days of treatments

Changes in POD, PPO, and IAAO Activities of Cuttings During Rooting

The activities of enzymes play important roles during the rooting process in cuttings; consequently, the effects of
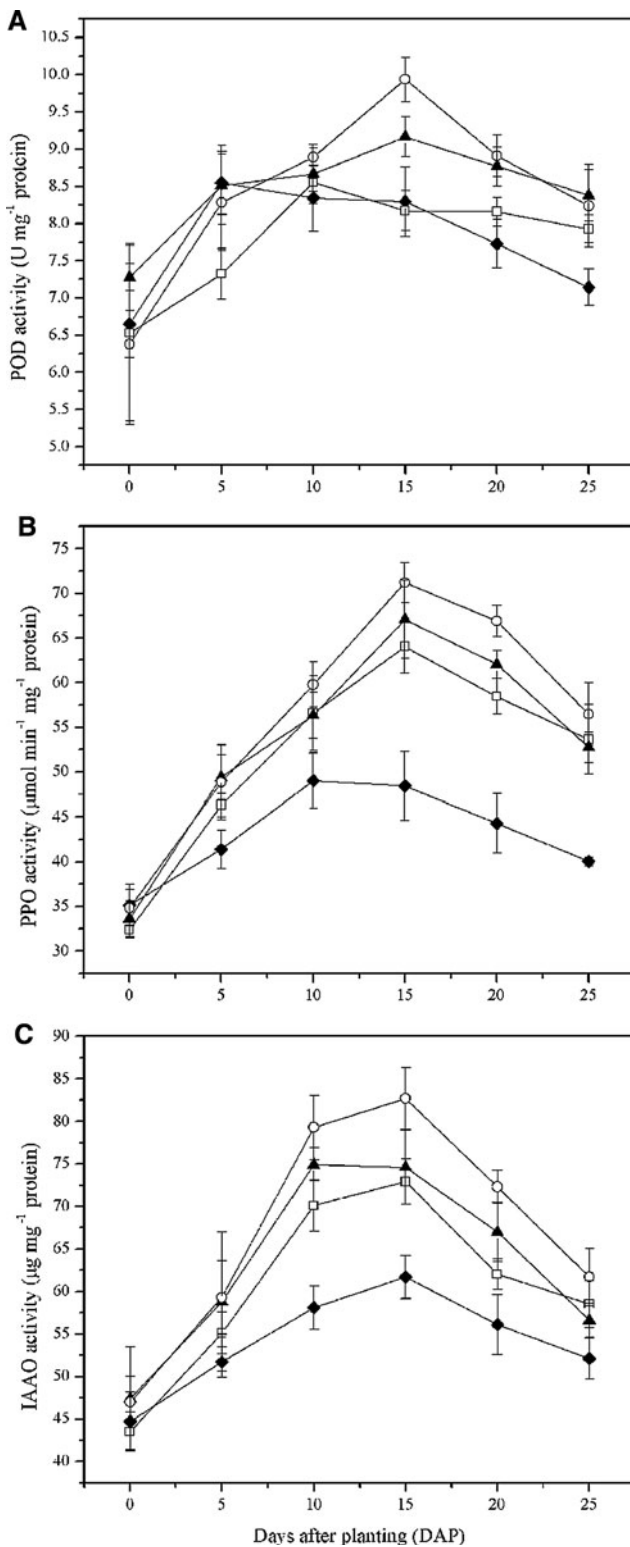
Fig. 2 Changes of POD, PPO, and IAAO activities in the rooting region of cuttings during the rooting process. Cuttings were watered with water (*diamond*), H_2O_2 (*square*), SNP (*triangle*), or H_2O_2 + SNP (*circle*) as indicated. SNP and H_2O_2 were used at 50 and 200 μM, respectively. Vertical bars represent mean ± SE value from three independent experiments

H_2O_2 and SNP on POD, PPO, and IAAO activities of cuttings were investigated. According to the observation of the morphological changes in the basal parts of cuttings, adventitious rooting occurred in three distinct phases: induction (0–12 days), initiation (12–15 days), and expression (15–25 days).

The activity of POD in H_2O_2 + SNP-, H_2O_2-, or SNP-treated cuttings clearly increased up to the expression phase and then decreased, whereas in control cuttings it reached its highest on day 5 and then decreased the following day (Fig. 2a). As shown in Fig. 2a, during the initiation phase POD activity of cuttings treated with SNP or H_2O_2 + SNP was enhanced compared with that of controls, especially on day 15. PPO activity reached a peak level on day 10 in controls but on day 15 for the other treatments. Then the enzyme activity declined during the expression period (Fig. 2b). Similarly, Fig. 2b also shows that higher PPO activity in H_2O_2 + SNP-, H_2O_2-, or SNP-treated cuttings compared to controls was obtained during the induction and initiation phases. The highest PPO activity was observed in the H_2O_2 + SNP treatment, which was 46.9% higher than that of controls on day 15 (Fig. 2b). The activity of IAAO changed almost coordinately with PPO activity. Its activity in all treatments increased during induction and initiation phases (0–15 days) and decreased during the expression phase (15–25 days) (Fig. 2c). The IAAO activity during the induction and initiation periods was higher in H_2O_2 + SNP-, H_2O_2-, or SNP-treated cuttings compared with that of controls. Among the three treatments, the maximum enhancement occurred with H_2O_2 + SNP treatment, particularly on day 15 (Fig. 2c). Therefore, we conclude that SNP or H_2O_2 significantly increased the activities of POD, PPO, and IAAO, and the combination of SNP with H_2O_2 increased these enzyme activities up to the level of SNP or H_2O_2 treatment alone.

Correlation analysis revealed that RP was (highly) significantly and positively correlated with PPO and IAAO activities ($r = 0.825$, $P < 0.05$ and $r = 0.908$, $P < 0.01$). It was not correlated with POD activity ($r = 0.530$,

Table 3 Correlation analysis of rooting percentage and enzyme activity in ground-cover chrysanthemum cuttings

Item	Rooting percentage (RP)	POD activity	PPO activity	IAAO activity
RP	1.000			
POD activity	0.530	1.000		
PPO activity	0.825*	0.231	1.000	
IAAO activity	0.908**	0.406	0.819*	1.000

Spearman correlation coefficients were used for analyses ($n = 20$)

* Correlation was significant at the 0.05 level (2-tailed); ** correlation was significant at the 0.01 level (2-tailed)

$P > 0.05$; Table 3). It seems that the PPO and IAAO activities were closely associated with adventitious root development.

Changes in Metabolic Constituent Contents from Cuttings during Rooting

Water-soluble carbohydrate (WSC), starch, nitrogen, carbohydrate/nitrogen (C:N) ratio, and polyphenol have been reported to influence adventitious rooting in plant species. Thus, the changes of these constituents of cuttings during the rhizogenesis process were investigated in this study.

As shown in Fig. 3a, the content of WSC in all treatments decreased during the first 10 days of planting (induction phase), and then increased until the end of rooting. However, it should be noted that during the induction phase the WSC content decrease was much steeper in the control cuttings than in other cases. On day 10, the WSC content was significantly lower in the controls than in other treatments ($P < 0.05$). Similarly, it appeared that the WSC content was lower in the controls than in other treatments throughout the rooting process (Fig. 3a), suggesting that WSC may be associated with rooting.

In all treatments, the starch content decreased slightly during the induction and initiation phases, and then increased sharply during the expression phase (Fig. 3b). The differences in starch content among different treatments were not as striking as in the WSC content, and the different treatments did not result in significant differences in the starch content during the rhizogenesis process.

The total carbohydrate content showed a decrease at the beginning and started to increase after day 10 for all treatments except the H_2O_2 treatment (Fig. 3c). In the H_2O_2 treatment, the decrease in total carbohydrate content continued through to day 15. It reached the lowest level on day 15 and then increased. Compared with the control, the total carbohydrate content of the other treatments was significantly higher during the rooting process ($P < 0.05$), especially after day 10 (Fig. 3c).

Similar to the content of WSC, a sharp decrease in the total nitrogen content was observed in all treatments during the induction phase, reached its lowest level on day 10, and then increased in the following days (Fig. 3d). Higher levels of nitrogen accumulated in cuttings treated with $H_2O_2 + SNP$, H_2O_2, and SNP than in the control cuttings. Another point to note was that more nitrogen did seem to accumulate in $H_2O_2 + SNP$-treated cuttings than in SNP or H_2O_2 treatments alone, particularly from day 15 to day 25 (Fig. 3d).

Results presented in Fig. 3e show that the C:N ratio changes in cuttings exhibited high variability. It decreased initially in all treatments (0–15 days) and then increased

subsequently in all the cases at 15 day. The C:N ratio of cuttings treated with $H_2O_2 + SNP$, SNP, or H_2O_2 compared to that of controls was less at day 5, higher at day 10, and equal at day 20 (Fig. 3e).

In all treatments, the content of total polyphenol was primarily decreased during the induction phase, which might be due to an activation of self-induction in plants responding to wounding. However, it gradually rose after that period, then decreased again on day 20 (Fig. 3f). Figure 3f showed that $H_2O_2 + SNP$, H_2O_2, and SNP treatments all alleviated the content of total polyphenol, and $H_2O_2 + SNP$ treatment showed the maximum decrease in total polyphenol accumulation compared with that of the control. On day 10, the total polyphenol content of H_2O_2, SNP, and $H_2O_2 + SNP$ treatments decreased by 28.7, 38.9, and 45.1%, respectively, compared with the control (Fig. 3f). This confirmed that H_2O_2 and NO could reduce the content of total polyphenol which may play an important inhibition role in cutting rooting.

Statistically significant relationships were determined between RP and WSC content, starch content, total carbohydrate content, total nitrogen content, C:N ratio, and total polyphenol content (Table 4). The results showed that there were likely significant (high) correlations between RP and WSC, total nitrogen, and total polyphenol content, but no obvious relationship between RP and starch content, total carbohydrate content, and C:N ratio. Coefficient analyses indicated that WSC and total nitrogen had a positive direct effect on RP of ground-cover chrysanthemum cuttings ($r = 0.923$, $P < 0.01$ and $r = 0.846$, $P < 0.05$, respectively). However, total polyphenol content had a negative direct effect on cutting RP ($r = -0.722$, $P < 0.05$).

Discussion

The results presented in this work are significant for both fundamental and applied plant biology. This work strongly argues that H_2O_2 or the NO donor SNP, when applied exogenously, enhanced adventitious root development of cuttings of ground-cover chrysanthemum. The effects of SNP and H_2O_2 were both dose-dependent, with a maximal biological response at 50 μM SNP and 200 μM H_2O_2, respectively (Table 1). In addition, endogenous NO or H_2O_2 also played specific roles in the development of adventitious roots of cuttings (Table 2). The involvement of NO in promoting root growth of Zea mays was first observed by Gouvĕa and others (1997). They found that NO induced cell elongation in a similar way to auxin, and Beligni and Lamattina (2001) suggested that NO was a phytohormone with nontraditional hormone properties. Furthermore, Pagnussat and others (2002) provided the first
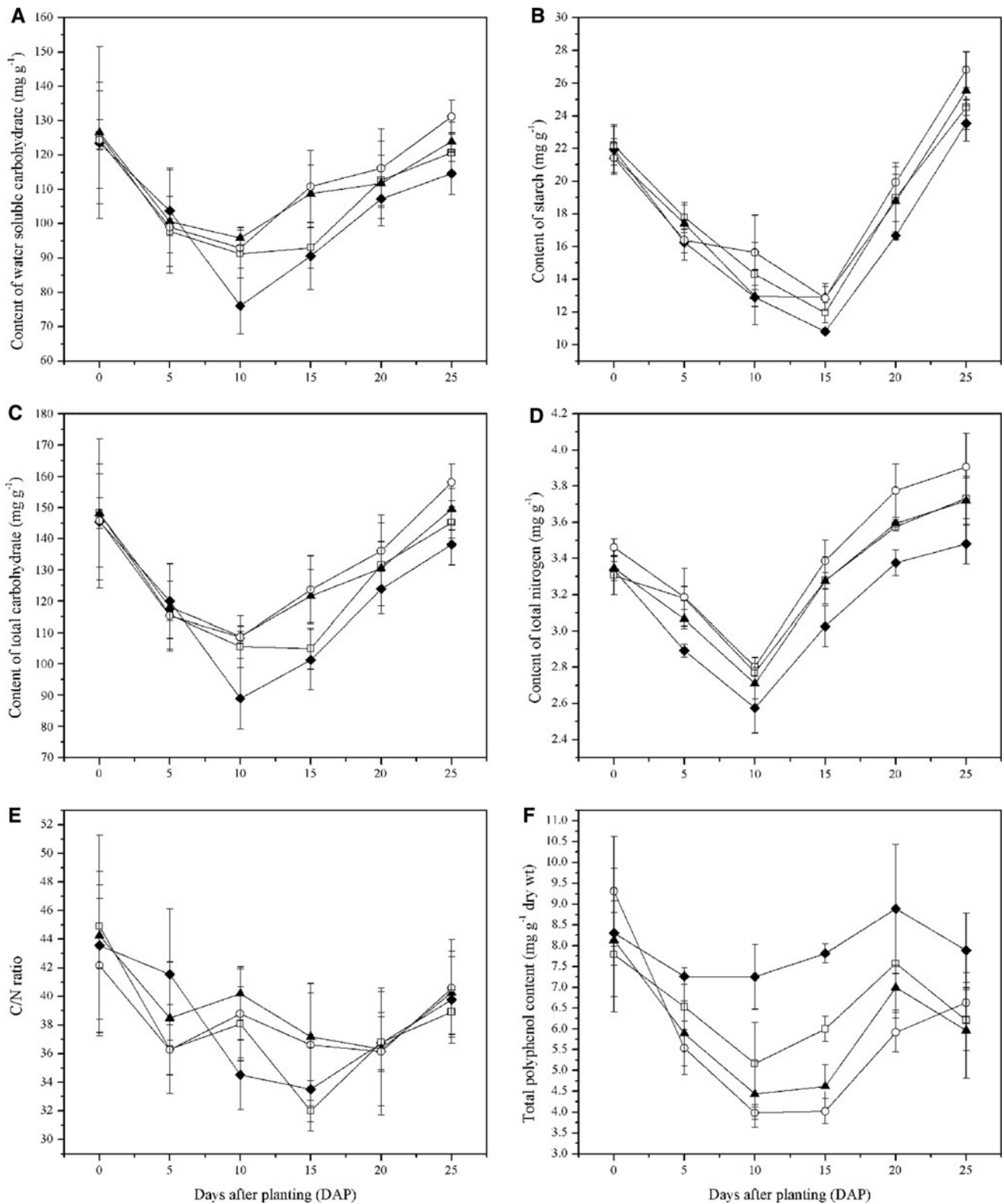
Fig. 3 Changes of biochemical constituents in the rooting region of cuttings during the rooting process. Cuttings were watered with water (*diamond*), H_2O_2 (*square*), SNP (*triangle*), or H_2O_2 + SNP (*circle*) as indicated. SNP and H_2O_2 were used at 50 and 200 μM, respectively. Vertical bars represent mean ± SE value from three independent experiments

Table 4 Correlation analysis of rooting percentage and metabolic constituents in ground-cover chrysanthemum cuttings

Item	RP	Water-soluble carbohydrate content (X_1)	Starch content (X_2)	Total carbohydrate content (X_3)	Total nitrogen content (X_4)	C:N ratio (X_5)	Total polyphenol content (X_6)
RP	1.000						
X_1	0.923**	1.000					
X_2	0.322	0.212	1.000				
X_3	0.455	0.497	0.340	1.000			
X_4	0.846*	0.774*	0.523	0.291	1.000		
X_5	0.238	0.337	0.318	0.164	0.535	1.000	
X_6	−0.722*	−0.656	−0.073	−0.344	−0.751*	−0.490	1.000

Spearman correlation coefficients were used for analyses ($n = 20$)

* Correlation was significant at the 0.05 level (2-tailed); ** correlation was significant at the 0.01 level (2-tailed)

evidence on NO and auxin (indole acetic acid, IAA) cross-talk during adventitious root formation in *Cucumis sativus*. Then another report suggested a novel role for NO in the regulation of tomato LR development, probably operating in the auxin 1-naphthylacetic acid (NAA) signaling transduction pathway (Correa-Aragunde and others 2004). The participation of NO in the process leading to RHF of lettuce has been described by Lombardo and others (2006). The authors found that NO was also involved in the auxin-signaling cascade leading to RHF. In this report, we provide new evidence of NO participation in the adventitious root development of stem cuttings of ground-cover chrysanthemum and NO-stimulated cutting rooting in a dose-dependent manner. It may, however, be noted that the optimum concentration of SNP for rooting in various plant species was not the same.

H_2O_2 plays various physiological roles in both plants and animals. As mentioned before, the involvement of H_2O_2 in plant adventitious root development had been reported recently. Several studies have indicated that H_2O_2 appears to be involved in root hair development of *Arabidopsis* (Foreman and others 2003; Rentel and others 2004; Dunand and others 2007), in the development of LRs of soybean (Su and others 2006), and in the formation and development of adventitious roots in *Cucumis sativus* (Li and others 2007). Moreover, it has been claimed that the generation of ROS is involved in gravitropism of *Zea mays* (Joo and others 2001). Based on the work reported here, we discovered that H_2O_2 was able to mimic the effect of NO in a dose-dependent manner, inducing adventitious root development in ground-cover chrysanthemum cuttings. In addition, the H_2O_2 and NO treatments both affected adventitious root initiation but not outgrowth, which agrees with previous reports by Pagnussat and others (2004) and Li and others (2007).

As far as we know, NO and H_2O_2 are both involved in plant cutting rooting. However, up to now there has been no suggestion of cross-talk between H_2O_2 and NO during plant adventitious root organogenesis. Our data show that RP and RL of cuttings treated with both H_2O_2 and NO were significantly higher than those of cuttings treated with H_2O_2 or NO alone (Table 2, Fig. 1), which suggested that NO and H_2O_2 may act synergistically to mediate adventitious root generation and development. It is clear that both NO and H_2O_2 are generated under similar stress situations and similar kinetics, and they can react with each other to form the reactive molecule $ONOO^-$. It is suggested they both impact the same or related signaling pathways and thereby lead to additive and possibly synergistic responses (Neill and others 2008). This is the first report to show that NO and H_2O_2 exhibit the same regulatory roles in the plant adventitious root developmental process, and they may enhance each other's levels by their mutual amplification in that process.

POD, PPO, and IAAO are useful biochemical markers for analysis of rooting phases for correlation with tissue morphological changes (Rout 2006). Moreover, it is well known that apart from enzyme activities, the other metabolic constituents may also play important roles in the rooting of cuttings of different species (Smart and others 2003; Rout 2006). Overall, in this study, POD, PPO, and IAAO activities were lower in the control cuttings than in the cuttings treated with SNP, H_2O_2, or SNP + H_2O_2 (Fig. 2a–c). Another point to note is that enzyme activities were greater in SNP + H_2O_2-treated cuttings than in SNP- or H_2O_2-treated cuttings in this study. The higher POD, PPO, and IAAO activities in SNP-, H_2O_2-, or SNP + H_2O_2-treated cuttings during the rooting period, especially in the induction phase, appeared to be responsible for better development of adventitious roots, possibly serving as the source of free auxin. Auxin-induced changes in POD and IAAO during the rooting process have also been well reported (De Klerk 1996; Liu and others 1996). Our results showed that H_2O_2 and NO might act

synergistically to promote enzyme activities and result in mediating adventitious root development of the cuttings. These data support the contention that NO and H_2O_2 impact the same or related signaling pathways and thereby lead to additive and possibly synergistic responses (Neill and others 2008). According to correlation analysis, PPO and IAAO activities were positively correlated with RP (Table 3). These correlations suggest that high PPO and IAAO activities in cuttings treated with SNP or H_2O_2 would generally result in increased rooting ability in cuttings. This corresponds to the findings of Coban (2007), who reported increased activity of PPO in cuttings of some grape (*Vitis vinifera* L) varieties during the early stages after planting. Similar views regarding the nature of the IAAO enzyme have been put forth by other workers. Some authors have suggested that IAAO activity influences rooting by IAA catabolism (Bolduc and others 1970; Bagatharia and Chanda 1998). Our investigation also leads to the interesting observation that NO and H_2O_2 can both modify the IAAO activity of ground-cover chrysanthemum cuttings.

The results presented in this work show that the contents of WSC, total carbohydrate, and total nitrogen in cuttings treated with SNP or H_2O_2 were higher than those in the control cuttings throughout the rooting process. The highest content of these metabolic constituents was attained in the SNP + H_2O_2 treatment (Fig. 3a, c, d). The increase in metabolic constituents observed in SNP- or H_2O_2-treated cuttings might serve as a good marker for rooting ability in cuttings, for these constituents induced by SNP or H_2O_2 were expected to be consumed as energy and carbon/nitrogen skeletons for the further development of adventitious roots (De Klerk 1996; Jàsik and De Klerk 1997). Many data supported the points of view that carbohydrate levels in general and nitrogen status in particular should be important considerations in studies of adventitious root formation (Haissig and Davis 1992; Druege and others 2000). The RP was highly and positively correlated with WSC and total nitrogen content, respectively (Table 4). These findings further show that increased WSC and total nitrogen content in cuttings induced by SNP or H_2O_2 might be associated with adventitious root development of ground-cover chrysanthemum cuttings. Thus, enhancement of the formation of cutting adventitious roots by SNP or H_2O_2 is through hydrolysis and translocation, amounts and redistribution of carbohydrate and nitrogen compounds, and promotion of cell wall extensibility. Without question, this and the greater question of the mechanism of the effects of SNP and H_2O_2 on carbohydrate and nitrogen compounds in cuttings during the rooting period require further investigation.

The present results also showed that total polyphenol content was minimal in SNP + H_2O_2-treated cuttings, particularly in the initiation phase, and its content in the cuttings treated with SNP or H_2O_2 alone was lower than that in the control cuttings (Fig. 3f). The correlation showed that during adventitious root development, RP was negatively correlated with total polyphenol content (Table 4). It is possible that polyphenol played a negative role in adventitious root development of ground-cover chrysanthemum cuttings. Previous studies had shown that phenol might be involved in different steps of adventitious root formation (Berthon and others 1993). However, Rout (2006) found that phenolic compounds of cuttings of *Camellia sinensis* var. TV-20 could be used as a rooting enhancer in tea plant. There is apparent disagreement between Rout (2006) and our study, which may be attributed to the different plant species and different treatments used in the two experiments. Thus, future studies should investigate how exogenous SNP and H_2O_2 affect the polyphenol content of cuttings.

In conclusion, optimum concentrations of SNP and H_2O_2 treatments helped adventitious root development in cuttings of ground-cover chrysanthemum. There was synergistic action between H_2O_2 and NO on mediating rooting of cuttings. Among all the biochemical parameters evaluated, NO and H_2O_2 treatments at the proper dosage increased the activities of PPO and IAAO and the content of WSC and total nitrogen, while decreasing the total polyphenol content of ground-cover chrysanthemum cuttings during rooting. RP was positively correlated with the activities of PPO and IAAO and the content of WSC and total nitrogen, whereas RP was negatively correlated with the total polyphenol content. Together, these results indicated that NO and H_2O_2 treatments enhanced adventitious root development synergistically and independently by stimulating the activities of some enzymes and the content of carbohydrate and nitrogen, and simultaneously by repressing the production of polyphenol. Thus, this investigation will be useful for understanding the roles of NO and H_2O_2 in the development of adventitious roots in cuttings; future experiments should examine how NO and H_2O_2 affect the biochemical constituents in the cuttings during the rhizogenesis process.

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