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Title:

Nitric Oxide Is Involved in Ethylene-Induced Adventitious Rooting in Marigold (*Tagetes erecta* L.)

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Abstract: The plant hormone, ethylene, and the gaseous signaling molecule, nitric oxide (NO), are involved in numerous plant growth and development processes. However, the mechanisms by which their interaction affects adventitious root development in plants is still not adequately studied. In this experiment, the interaction of ethylene and NO in the adventitious rooting process of marigold (*Tagetes erecta* L. 'Marvel') was investigated. Treatments with different dosages of ethylene-releasing ethrel significantly affected the formation of adventitious roots. The greatest rooting ability was observed in 10 μ M ethrel-treated explants. It was further shown that the effect of ethylene could be blocked by the specific NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt (cPTIO), nitric oxide synthase (NOS) enzyme inhibitor N^G-nitro-L-Arg-methyl ester (L-NAME) and nitrate reductase inhibitor (NaN₃). Moreover, ethrel treatments induced an increase in the endogenous NO levels and significantly improved activities of nitric oxide synthase (NOS) and nitrate reductase (NR) during rooting. Thus, the induction of adventitious roots by ethylene may be through enhancing the levels of NO. Enzymatic pathway NOS and NR could be responsible for ethylene-induced NO production. Furthermore, ethylene and NO treatments at the appropriate dosage may increase the activities of indoleacetic acid oxidase, peroxidase and polyphenol oxidase. Our work suggests that the stimulation of adventitious roots by ethylene relies on internal generation of NO.

Keywords: ethylene, nitric oxide, enzyme activity, adventitious root formation

Introduction

Adventitious rooting is a multistage developmental process and a key step in vegetative propagation, which is affected by many internal and external cues (Geiss et al., 2009) including environmental stimuli (Dech and Maun, 2006) such as waterlogging and wounding (Abu-Abied., 2012). Other factors that influence adventitious root formation include phytohormones (Kumar 2013), signaling molecules, such as auxin (Bai et al., 2012), ethylene (Pan et al., 2002), nitric oxide (Liao et al., 2011), carbon monoxide (Xuan et al., 2008), hydrogen peroxide (Liao et al., 2009), Ca^{2+} ions, calmodulin (Liao et al., 2012), hydrogen sulfide (Lin et al., 2012), hydrogen (Lin et al., 2014) and methane (Cui et al., 2015). Adventitious root formation and development in cuttings occurs in four phases including cell de-differentiation, induction of cell division, development of root primordial, and root emergence; De Klerk et al., 1999). Although many studies about signal transduction in plants have been conducted, the molecular mechanisms and intermediates in plants adventitious root formation remains a major issue for scientific researchers to explore.

Ethylene as a classical phytohormone may regulate various physiological and morphological responses in plants by interacting with other signaling molecules (Khan et al., 2015), such as in seed germination and in breaking dormancy (Corbineau et al., 2014), root hair and root nodule formation, maturation, elicited kiwifruit (*Actinidia deliciosa*) ripening (Minas et al., 2016), responses to light (Weller et al., 2015) and toxic metals (Montero- Palmero et al., 2014). Ethylene was firstly reported to play a crucial role in adventitious root formation in plants in 1933 (Zimmerman and

Hitchcock, 1933). The use of an ethylene releasing compound, ethephon (trade name ethrel) was able to promote adventitious rooting in mung bean (*Vigna radiata*; Robbins et al., 1983). However, the mechanism of ethylene-induced adventitious root development needs to be fully investigated.

NO as a gaseous signaling molecule generally interacts with other endogenous molecules and plant hormones during early growth and development in plants (Sanz et al., 2015). NO is involved in the acceleration of seed germination (He et al., 2014), cotyledon and leaf greening (Abbas et al., 2015), hypocotyl elongation (Lin et al., 2012), dormancy release (Krasuska et al., 2014), vegetative and reproductive growth (Wang et al., 2015). It also plays a role in root organogenesis (Duan et al., 2015), flower development, flowering time or pollen tube growth (Yu et al., 2014), enhancing antioxidant enzyme activities and in photosynthesis (Zhang et al., 2016). Exogenous NO was shown to have played a role in the induction of root tip elongation (Kopyra and Gwóźdz, 2003) and the formation of lateral roots (Correra-Aragunde et al., 2004). Pagnussat et al. (2002) were the first to show that NO donor sodium nitroprusside (SNP) accelerated adventitious root formation in cucumber (*Cucumis sativus*) explants.

Evidence exists about the interplay of ethylene and NO during plant developmental processes in apple (*Mallus domestica* Borb. cv. Antonówka) embryos (Gniazdowska et al., 2007), tobacco (*Nicotiana tabacum* L. cv BelW3) leaf discs (Ederli et al., 2006) and in tobacco leaves (Mur et al., 2008). Moreover, NO was reported to have enhanced ethylene production, and the two had synergistic effect in

alleviating some abiotic stresses, such as UV light (Wang et al., 2006) and salinity (Wang et al., 2009).

Biochemical changes were good indicators of rooting in peony (*Paeonia Suffruticosa*) (Jana and Jeong, 2013). Peroxidase (POD), polyphenol oxidase (PPO), and indoleacetic acid oxidase (IAAO) are useful biochemical markers for analysis of rooting phases for correlation with tissue morphological changes (Rout, 2006). Higher activities of POD and PPO in cuttings served as good markers for rooting ability and provided ample indications of better rooting ability in whip grass (*Hemarthria compressa*) cuttings (Yan et al., 2014). Coban (2007) reported that the PPO activity increased during the early stages after planting in cuttings of some grape (*Vitis vinifera* L) varieties.

Previous results showed that ethylene or NO play crucial role in promoting adventitious root development in cucumber (Pagnussat et al., 2002) and marigold (Liao et al., 2011). However, the relationship and interaction between ethylene and NO associated with the regulation of adventitious root development remains unclear. The aim of the study was to further reveal the relationships between ethylene and NO in the control of adventitious root development in marigold.

Materials and Methods

Plant material and growth conditions

The experiments were conducted in the Ornamental Horticulture Laboratory (Latitude, 36°5'27"N, Longitude, 103°41'37"E, Altitude 1540 m) in Gansu Agriculture University, Lanzhou, Gansu Province, PR China. The seeds of marigold

(*Tagetes erecta* L. var. 'Marvel') were washed in distilled water and surface-sterilized in 5% (w/v) sodium hypochlorite for 10 min, then were germinated on filter paper moistened with distilled water in Petri dishes and maintained at $25^{\circ} \pm 1^{\circ}\text{C}$ for 5 d with a 14-h photoperiod at a photosynthetically-active radiation intensity of $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ in a growth chamber (Shanghai Yuejin Medical Instruments CO., Ltd). Primary roots of 5-d-old marigold seedlings were removed and the explants were then maintained under the same temperature and photoperiod for another 5 d in the presence of different media as indicated below.

Experiment 1: After removing the primary roots, 10 explants were put per petri dish. The control treatment involved 6 ml of distilled water. The other treatments were different concentrations of ethylene-releasing compound, ethrel (Shanghai Chemical Reagent Co. Ltd.), at 0, 0.1, 0.5, 1, 10 and 50 μM . The 0, 0.1, 0.5, 1 and 10 μM were used based on previous experiments while the 50 μM was added to the treatments based on (Pan et al., 2002). The treatments were replicated three times, given a total of 18 petri dishes arranged in a completely randomized design in the growing chamber. The 10 μM ethrel treatment, which gave the highest root number and root length in experiment 1 was used in the subsequent experiments.

Experiment 2: In experiment 2, seven (7) treatments were used. These were : (i) Fifty (50) μM sodium nitroprusside (SNP; Merck, Darmstadt, Germany; a donor of NO) alone, (ii) Fifty (50) μM sodium nitroprusside (SNP; Merck, Darmstadt, Germany; a donor of NO) plus Ethrel, (iii) Optimum concentration of ethrel (10 μM), which was determined in experiment 1. (iv) 200 μM

2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO, Sigma, USA) plus Ethrel, (v) 25 μ M N-nitro-L-arginine methyl ester (L- NAME, Sigma, USA) plus Ethrel (vi) 10 μ M NaN_3 and (vii) Control (Distilled water). Each of these treatments was replicated three times in a completely randomized design, given a total of 21 petri dishes. Each petri dish contained ten (10) explants. The concentrations of these chemicals were selected based on results from previous experiments conducted in our laboratory (Liao et al., 2009). Unless otherwise stated, the remaining chemicals were of analytical grade from Shanghai Lorderan Scientific Instruments Co., Ltd, China.

Sampling for data collection

In the determination of root number and root length, 10 cuttings were used per replication. And in the determination of the activity of nitrate reductase activity and other physiological indicators, we used 0.2 g per replication, given a total of 0.6 g per treatment.

Root number and root length

Any explant that had at least one root was classified as rooted. The adventitious root length was measured from the root tip to the junction between the adventitious root and the hypocotyl with a ruler for each explant. The length of adventitious roots was determined by finding the average length of the rootlets per explant. For each treatment, 30 explants were used for evaluation of the number of roots and root length.

Determination of NO content

The content of NO was determined once every 6 h according to the method of Liao et al (2011). Hypocotyls (0.5 g) were frozen in liquid nitrogen and then ground in a mortar with pestle in 3 mL of 50 mM cool acetic acid buffer (pH 3.6, containing 4% (w/v) zinc diacetate). The homogenates were centrifuged at 10,000 rpm for 15 min at 4°C and the supernatants were collected. The pellets were washed using 1 mL of extraction buffer and centrifuged as before. The two supernatants were combined and 0.1 g of charcoal (Shanghai Chemical Reagent Co, Ltd.) was added. After vortex mixing and filtration, the filtrate was leached and collected. The mixture of 1 mL of filtrate and 1 mL of Greiss reagent was incubated at room temperature for 30 min to convert nitrite into a purple azo-dye. The absorbance was then determined at 540 nm (TU-1900; Beijing Purkinje General Instrument Co., Ltd.).

Determination of NOS and NR activity

Nitric oxide synthase activity was determined according to the manufacturer's recommendations with a nNOS kit (Nanjing Jiancheng Biological Co.). Fresh hypocotyls from explants (0.2 g) were ground in liquid N₂. There are two main types of NOS that are included in the T-NOS (total NOS): cNOS (constitutive NOS) and iNOS (inducible NOS). The kit is capable of detecting the activity of iNOS and T-NOS. The enzymatic activity was represented using $\mu\text{ mg}^{-1}$ protein, where one unit is defined as the yield of brown substance produced per unit of protein per unit time when it interacts with NO. The simulated inoculation was used as a control, and three replicates were set for each of the reactions. The protein absorbance was measured at 530 nm according to Bradford (1976).

Nitrate reductase activity was assayed in accordance with Hageman and Reed (1980) with some modifications. Hypocotyls (0.2 g) from marigold explants were frozen in liquid nitrogen, ground to a powder, then homogenized with 0.9 mL of an extraction medium (25 mM potassium phosphate, pH 7.8, 5 mM KNO₃, 1 mM cysteine, 5 mM EDTA, 25 µM FAD) and were then centrifuged at 4000 rpm for 10 min. Two mL of supernatant was collected and mixed with 1 mL of a reaction reagent (20 mM KNO₃, 50 mM potassium phosphate, pH 7.5, and 0.8 mM NADH) for colorimetric determination. Each treatment was replicated three times. The absorbance at 540 nm was determined using spectrophotometry.

Determination of IAAO, POD, PPO activity

Fresh tissue (0.2 g) was ground to homogenate with a mortar and pestle in 10 mL of 100 mM pre-cold phosphate buffer (pH 6.0) containing 1 % (w/v) dithiothreitol and centrifuged (4°C, 10,000 rpm, 20 min). The supernatant was recovered and used as a crude enzyme extract for the enzyme activities assay.

IAAO activity: The reaction mixture was made of the 0.2 mL enzyme extracts, 0.78 mL of 50 mM potassium-phosphate buffer (pH 6.0), 0.01 mL of 5 mM MnCl₂, 0.01 mL of 5 mM 2, 4-dichlorophenol, and 0.02 mL of 2.5 g l⁻¹ IAA. After 15 min at 30°C in darkness 2 mL Salkowski reagent was added and the degradation of IAA was monitored-via spectrophotometry at 535 nm after 30 min.

POD activity: After 0.1 mL enzyme extract was added into in 3 mL reaction solution (0.05 M potassium-phosphate buffer, 7.5 mM of 2% H₂O₂, 50 mM guaiacol), and the oxidation of guaiacol was followed by the increase of absorbance at

470 nm for 3min.

PPO activity: The reaction mixture contained 20 mM Tris-HCl (pH 7.8), 15 mM β -mercaptoethanol, 1 mM phenylmethyl sulfonyl fluoride (PMSF), 20% glycerol, and 1% (v/v) Triton X-100. PPO activity was measured using 30 mM 4-methyl catecol in sodium acetate buffer (pH 4.5). 50 mM phosphate buffer (pH 7.0) was added to reaction solution to start reaction and the change of at 400 nm using spectrophotometry was recorded.

Statistical Analysis

Results were expressed as the mean values $\pm$ SE of at least three independent experiments with 10 explants per treatment. Statistical analysis was performed using software SPSS 17.0. Analysis of Variance (ANOVA) was done and the Duncan's multiple range test ($P < 0.05$) was used in the separation of means. The relationship between the ethrel concentrations (x) and adventitious rooting(y) was fitted by a second-order polynomial function (Hu et al., 2015).

Results

Effects of exogenous ethylene on the adventitious root development

The application of the ethylene releasing compound, ethrel, ranging from 1 to 50 μ M increased adventitious root number and root length, with the highest root number and root length obtained with the application of 10 μ M when compared with the control (Fig. 1). However, there were no differences in root number among the control, 0.1 and 0.5 μ M ethrel treatments. We also noticed that there was no difference between the control and 0.1 μ M ethrel treatments in root length. The least root

number and root length were observed at 50 μM . The regression analysis (Fig. 2) shows that there was a curvilinear relationship between ethylene concentration and root number ($R=0.95$) and root length ($R=0.89$). Increasing ethylene concentration from 0 to 10 μM led to increased growth with respect to root number and root length. The mathematical maximum response occurred at 27.3 μM , beyond which increasing the concentration resulted in reduced root number and root length.

Effects of cPTIO, L-NAME, and NaN_3 on ethylene-induced adventitious root development

Adventitious root number and length induced by ethylene treatment were suppressed when cPTIO, L-NAME or NaN_3 was added (Fig. 3). Root length of explants treated with ethrel + SNP were longer than those of explants treated with ethrel or SNP alone (Fig. 3). The results show that there was a possible inter-relationship between ethylene and NO during the adventitious rooting process.

Effects of exogenous ethylene on the endogenous NO content of explants

The time course experiments showed that, a fast burst of endogenous NO release occurred at 6 h both in the ethrel treatment and in the control (Fig. 4). The accumulation of NO reached a maximum after 24 h in ethrel treatments, followed by a gradual decrease. However, the NO content remained at low levels in the control until 48 h except at 24 h when there was a slight peak. High levels of NO were detected in both the ethrel and SNP treatments compared to the control treatment (Fig. 5). However, there was no difference between ethrel and SNP treatments. When NO scavenger cPTIO, NOS inhibitor L-NAME or NR inhibitor NaN_3 was administered to

ethrel-treated explants, it resulted in reduction of NO content.

Effects of exogenous ethylene on NOS and NR activity in marigold explants

The results showed that high levels of NOS activity were detected at 0 h in both the control and ethrel treatments, and then declined with time (Fig. 6a). NOS activity in the control remained at low levels after 18 h and increased slightly after 24 h of application of treatment. Nitric oxide synthase-like enzyme activity of the explants treated with ethrel increased rapidly after 6 h and reached the highest levels at about 24 h, but the NOS activity decreased to levels below the control after 36 h (Fig. 6a). NR activity was also similar to that of NOS activity in the ethylene treated plants during adventitious rooting in marigold explants. The time-course of ethrel treatment improved NR activity (Fig. 6b). The increase of NR activity occurred in the control and ethrel treatments within 6 h, and subsequently decreased to around 18 h after treatment. The maximal level on NR activity was observed at 24 h after ethrel treatment, and it rapidly decreased to a level below the control until 48 h (Fig. 6b).

Changes in IAAO, POD and PPO activities of explants during rooting

The activity of IAAO in all treatments showed a similar trend during adventitious root development (Fig. 7a). They increased gradually until 12 h and then declined by the 48th h. At 12 h, the IAAO activity of ethrel + SNP, ethrel, and SNP treatments increased by 40, 21, and 13% over the control respectively (Fig. 7a). The maximum IAAO activity was found in the ethylene + NO treatment. The results also showed that the activity of POD was in a “rise- fall” trend with two peaks at 4 and 24 hrs (Fig.7b). At 24 h, the activity of POD of ethylene, NO, and ethylene + NO

treatments were 33.8%, 26.3% and 41% more than the control respectively (Fig. 7b).

The time-course of PPO activity of various treatments was the same as that of POD, and there were two peaks at 2 and 24 hrs which occurred during the rooting process (Fig. 7c). The highest PPO activity was observed in ethylene + NO treatment, which was 29.4% higher than that of the control at 2 h. At 48 h, the PPO activity in the control treatment was 25.6%, 20% and 30% lower than that in ethylene, NO, and ethylene + NO treatments, respectively (Fig. 7c).

Discussion

The development of root systems in plants is critical for the survival of the whole plant (Rogers et al., 2014). Ethylene was shown to be involved in several developmental processes and responses to biotic and abiotic stress in plants (Wang et al., 2002). For example, ethylene triggered adventitious root development on stem nodes of rice (*Oryza sativa*) (Steffens et al., 2012). The results of our study also show that exogenous ethylene promoted adventitious root development in marigold (Fig.1). The least root number and root length were observed at 50 μ M. The increased root number and root length which occurred until 10 μ M could be due to increased levels of endogenous NO observed in the ethylene treated plants. The regression analysis conducted, however, indicated that the mathematical maximum response occurred at 27.3 μ M (Fig.2). Probably, increasing the ethylene beyond 27.3 μ M led to significant decline in NO levels and thus, reduced root number and root length. Pan et al. (2002) found that treatment with ethephon at 50 μ M stimulated adventitious root formation in mung bean hypocotyl cuttings. Similar results were reported by Negi et al. (2010)

who found a positive regulatory role of ethylene in adventitious root formation in tomato (*Solanum lycopersicum*) and in mung bean cuttings (Robbins et al., 1983). Duege et al. (2014) also reported that ethylene acted as an important stimulator of adventitious roots formation in *Petunia hybrida*. Our results provide further evidence that ethylene plays a crucial role in adventitious root development in marigold.

Nitric oxide and ethylene have been reported to have both synergistic and antagonistic effect in modulating root growth and development (Freschi, 2013), inducing the expression of Fe acquisition genes (García et al., 2010), modulating ion homeostasis (Wang et al., 2009), breaking dormancy (Gniazdowska et al., 2007) and regulating fruit ripening process (Manjunatha et al., 2012). However, the crosstalk between the two molecules during adventitious rooting is still unclear. Nitric oxide scavenger cPTIO and NO inhibitors L-NAME and NaN₃ treatments significantly blocked ethylene-induced adventitious root development (Fig.3). Some reports indicated an antagonistic action of ethylene and NO during mature fruit abscission (Parra-Lobato and Gomez-Jimenez, 2011) and senescence of cut rose flowers (Liao et al., 2013). However, the cooperative and synchronized interaction between ethylene and NO in mediating cell death was reported by Yordanova et al. (2010). Mira et al. (2015) reported that ethylene is involved in NO regulation of somatic embryogenesis in vitro. Thus, the interaction between ethylene and NO in the regulation of plant growth and development seems to be very complex. It may be because the two molecules play different roles in the different physiological process. Our results suggest that NO may be required for ethylene-induced adventitious rooting and it may

be downstream signal molecules in the ethylene signaling.

In our study, a possible link between ethylene and NO during adventitious rooting was examined by monitoring endogenous NO contents in ethylene-treated explants. Exogenous ethylene increased the endogenous levels of NO indicating that ethylene may enhance the development of adventitious roots partially through enhancing the endogenous NO production. Our results of NO content showed a double peak at 6 and 24 h. The increased NO content from 0 to 6 h in both the control and the ethylene treated explants could be attributed to response to wounding which occurred during cutting. From 6-18 h could be healing period after which the ethylene treated explants had increased NO content until 24 h. The effect of ethylene is usually short-lived because of the gaseous nature. This, probably explains why NR activity declined after 24h. The results of NR activity showed a similar trend with the results of the NO content, exhibiting a double peak at 6 and 24 h after treatment. The NO production in plants was first discovered by Klepper (1975) and recently, evidence shows that endogenous NO is involved in various fundamental growth and development processes, including adventitious root development (Liao et al., 2011), vegetative and reproductive growth (Wang et al., 2015), dormancy release in bulbs (Niu et al., 2015), seed germination (Liu et al., 2009), cotyledon expansion (Yang et al., 2014), and pollen tubes growth (Šírová et al., 2011). Liao et al. (2011) reported that after removal of primary roots, the wound healing response is associated with reduced NO contents. Similar to our results, Yordanova et al. (2010) reported a tight regulation of the levels of both ethylene and NO in which ethylene stimulates NO

production and NO also stimulates ethylene production. Leshem and Haramaty (1996) also found that the application of ACC (ethylene precursor) enhanced NO emission in senescing pea (*Pisum sativum* Linn.) leaves. In addition, the enhancement of NO production induced by ethylene was suppressed by cPTIO, L-NAME or NaN_3 . Our results provide further evidence that the endogenous NO is essential for ethylene-induced adventitious rooting. Thus, we report that ethylene acts as a signal molecule in adventitious root development induced by NO in marigold. As a precursor, $^{\text{L}}$ -arginine-dependent NO synthase (NOS) and nitrate reductase using nitrite/nitrate (NR/NiR) are two main enzymatic pathways of NO biosynthesis (Corpas and Barroso, 2015). The two pathways of NO production have been shown to participate in a variety of physiological processes in plants, including seed germination, root organogenesis, plant defense responses and stress tolerance (Crawford et al., 2005). Up to date, the signal pathway regulating NOS and NR activities under ethylene conditions is still unclear. Here, ethylene treatment significantly enhanced NOS and NR activities in marigold explants, suggesting that the NO may be primarily formed through the NOS and NR enzymatic reaction pathways during ethylene-induced adventitious rooting. Our previous results also showed that both NOS and NR enzymes might be responsible for the production of NO during adventitious rooting in marigold (Liao et al., 2009). NOS as well as NR might be involved in phosphatidic acid-induced NO biosynthesis in cucumber leaves under water stress (Arasimowicz-Jelonek et al., 2009). Recent research reports indicate that NOS enzyme exists in some algae species but appeared not to be conserved in terrestrial

plants (Jeandroz et al., 2016). Therefore, NO synthesis in plants and the possible ethylene–NO interaction in response to NO-generating systems through NOS enzymes also needs further verification. It has been reported that the nitrate-regulated expression of NR played a major role during the early perception and signaling of nitrate in maize roots (Trevisan et al., 2011). Kan et al. (2016) showed that the cadmium-induced NO production primarily occurred through activation of the NR pathway. It seems that NR is the main pathway to produce NO in plants.

The activities of enzymes in the rooting zone of cuttings might provide an easy, fast and reliable means of assessing cellular differentiation into roots (Husen and Pal, 2007). In previous work, IAAO, POD and PPO enzymes activities were shown to be closely related to adventitious root development (Liao et al., 2010). It has been reported that ferulic acid treatment considerably increased the activities of POD and IAAO in growth of maize (*Zea mays* L.) seedlings (Rama et al., 1996). Furthermore, PPO catalyzed the oxidation of polyphenols and lignification in plant cells during the rooting process (Khorsheuzzaman et al., 2010). Mohamed-Yasseen and Splittstoesser (1990) reported that IAAO is similar to POD and may assist in regulating IAA content. The increase in IAAO activity caused the decrease in IAA content of *Gingo biloba* plant (Li et al., 2009). Ebrahimzadeh and Abrishamchi (2001) demonstrated that in bud and flower formation, lower levels of IAA was accompanied by higher contents of phenolic compounds and enhanced activities of IAAO, POD and PPO. Our current results showed that ethylene and NO increased IAAO, POD and PPO activities. Here, the significant variations in the activities of IAAO, POD and PPO were associated

with the induction and elongation of root primordials. Therefore, IAAO, POD and PPO activities could be used as indicators of better rooting ability and might serve as a good marker for rooting ability. González et al. (1991) confirmed that ethylene modified POD and PPO activities during rooting in hazelnut (*Corylus avellana* L. cv. Casina). In addition, NO treatment stimulated PPO and IAAO enzymes activities and enhanced adventitious root development (Liao et al., 2010). Our results also suggest that ethylene and NO probably acted synergistically and promoted IAAO, POD, PPO enzymes activities which led to improved adventitious root development in marigold.

The results of our current study provide clear evidence that external ethylene and NO could improve adventitious rooting in marigold and further demonstrate that NO might be involved in ethylene-induced adventitious root development. It was also observed that ethylene-induced adventitious rooting through mediating NO production. Finally, these results indicate that ethylene and NO treatments enhanced adventitious root development by stimulating the activities of IAAO, POD and PPO. It, therefore, provides theoretical basis for the application of both ethylene and nitric oxide in promoting adventitious rooting for commercial production in the ornamental horticulture industry. Thus, the results presented here are significant for both fundamental and applied plant biology.

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Figure captions

Fig. 1. Effects of different concentrations of ethrel on adventitious root development in marigold explants. The primary root system was removed from hypocotyls of 5-day-old germinated marigold. Explants were incubated with distilled water or different concentrations of ethrel as indicated for 5 days. Adventitious root numbers (a) and length (b) were expressed as mean $\pm$ SE (n=10 explants from three independent experiments). Bars not sharing the same letters were different by Duncan's test ($P < 0.05$). Photographs (c) were taken after 5 days of treatment.

Fig. 2. Relationship between ethylene concentration and root number and root length.

A second order polynomial function was fitted to the experimental results of relationship between ethylene concentration and root number(◆) and root length (□).

Fig. 3. Effects of ethrel, SNP, cPTIO, L-NAME and NaN₃ on adventitious root in marigold explants. Explants of marigold were incubated with distilled water, 0.5 μ M ethrel, 50 μ M SNP, 200 μ M cPTIO, 25 μ M L-NAME, 10 μ M NaN₃ as indicated for 5 days. Adventitious root numbers (a) and length (b) were expressed as mean $\pm$ SE (n=10 explants from three independent experiments). Bars not sharing the same letters were different by Duncan's test ($P<0.05$) Photographs(c) were taken after 5 days of treatment.

Fig. 4. Effects of exogenous ethrel on the endogenous NO content in marigold explants. The primary root system was removed from the hypocotyl of 5-d-old, germinated marigold seedlings. NO level of hypocotyls was determined in explants treated with distilled water (Δ) or 0.5 μ M ethrel ($\square$) for 48 h. Values (means $\pm$ SE) are the average of three independent experiments (n=10 explants from three independent experiments).

Fig. 5. Effects of exogenous ethrel, SNP, cPTIO, L-NAME, NaN₃ on endogenous NO level in hypocotyl of marigold explants treated. The primary root system was removed from the hypocotyl of 5-d-old, germinated marigold seedlings. Endogenous

NO level were determined in explants treated with distilled water (control), 0.5 μ M ethrel, 50 μ M SNP, 0.5 μ M ethrel plus 200 μ M cPTIO, 0.5 μ M ethrel plus 25 μ M L-NAME, 0.5 μ M ethrel plus 10 μ M NaN_3 . NO contents were determined after 24 h of treatment. Values were expressed as means $\pm$ SE (n = 10 explants from each of three independent experiments). Bars with different lower-case letters in each Panel were different (t-test; $P < 0.05$).

Fig.6. Effects of exogenous ethrel on NOS and NR activity in marigold explants. The primary root system was removed from the hypocotyl of 5-d-old, germinated marigold seedlings. Explants were incubated in distilled water (Δ) or 0.5 μ M ethrel ($\square$), and NOS and NR activity were measured at the indicated times. Values (means $\pm$ SE) are the average of three independent experiments (n=10 explants from three independent experiments).

Fig. 7. Changes in IAAO, POD, and PPO activities of explants during rooting. The primary root system was removed from the hypocotyl of 5-d-old, germinated marigold seedlings. Explants were incubated in distilled water ($\blacklozenge$), ethrel ($\diamond$), SNP($\square$), ethrel+SNP(Δ) in as indicated. ethrel and SNP were used at 0.5 and 50 μ M, respectively. Values (means $\pm$ SE) are the average of three independent experiments (n=10 explants from three independent experiments). * Correlation was significant at the 0.05 level (Above the bars; Duncan's multiple range tests; $P < 0.05$).

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