

# Nitric oxide effect on root architecture development in *Malus* seedlings

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## ABSTRACT

The time-dependent production of nitric oxide (NO) in roots induced by indole-3-butyric acid (IBA) and the effect of sodium nitroprusside (SNP) on root architecture development were investigated, using *Malus hupehensis* Rehd. seedlings. Following IBA application, a very rapid increase in NO formation and a subsequent second wave of NO burst was observed, which was related to the induction of lateral roots (LRs) and the organogenesis of lateral root primordia (LRP), respectively. The first NO burst was correlated with the second and the two peaks of NO burst induced by IBA were totally abolished by 3,3',4',5,7-pentahydroxyflavone (quercetin). Exogenous NO promoted the emergence and elongation of LR and inhibited the elongation of primary root (PR) in a dose-dependent manner: low concentrations of SNP promoted both the amounts and the elongation of LR but inhibited the elongation of LR and PR at higher concentrations. It was concluded that (i) the rapid production of NO induced by IBA was correlated with the IBA-induced initiation of LR; (ii) quercetin inhibition of IBA-induced LR formation was correlated with the quercetin inhibition of IBA-induced NO biosynthesis, and (iii) exogenous NO affects the development of root system architecture in a dose-dependent manner.

**Keywords:** indole-3-butyric acid; quercetin; root; sodium nitroprusside

Root system of a plant is the site of water and nutrients uptake, a sensor of biotic and abiotic stress, a structural anchor to support the whole plant, and the storage organ in some species (Nibau et al. 2008). In the development of plant root system architecture (RSA), the initiation and development of LRs is one of the most important. Indole-3-butyric acid (IBA), a naturally occurring auxin besides the major indole-3-acetic acid (IAA), is effective in inducing new roots when applied exogenously. It was reported that the signal transduction pathway for IBA is at least partially different from that for IAA, suggesting that IBA is *per se* an auxin and does not act simply via the conversion to IAA (Chhun et al. 2004). However, in contrast to IAA, the crosstalk between IBA and nitric oxide (NO) in the initiation and development of LR was less understood (Lanteri et al. 2006).

Quercetin is a flavonoid widely distributed in plants that has multiple functions both in animal and plant cells. In animals, quercetin has been found to have anti-inflammatory and antioxidant

properties. In plants, quercetin is widely accepted as an auxin transport inhibitor and a signaling molecule in the formation of nodules, root knot nematode galls, LRs, and shoots branching (Peer and Murphy 2007, Wasson et al. 2009).

NO is a versatile signaling molecule that functions in numerous physiological and biological processes during plant development and stress response. The role of NO in root initiation and development was widely investigated, which evidenced that NO play central roles in auxin-induced LR development (Correa-Aragunde et al. 2004, Pagnussat et al. 2004). NO is mainly located in actively growing tissues, especially new roots (Gao et al. 2010). However, in auxin-induced rooting process, the upstream events concerning how NO is generated was little known. Exogenous NO affects the development of root architecture in many ways.

In this research, we investigated the effects of IBA and quercetin on the development of LR in *M. hupehensis* Rehd. seedlings, with special em-

phasis on the time-dependent production of NO following IBA application. In addition, the effect of exogenous NO on root architecture development was also investigated. The results will contribute to a better understanding of the multiple roles of NO, IBA and quercetin as well as their crosstalk in root architecture development.

## MATERIAL AND METHODS

**Plant material and growth conditions.** Seeds of *M. hupehensis* Rehd. var. *pinyiensis* Jiang were surface-sterilized in 50% (v/v) NaClO (5% active Cl<sub>2</sub>) for 10 min, rinsed extensively and were laminated by sand under 4°C for 35–40 days. Then, the seeds were sowed in plastic pots filled with perlite under room conditions (natural light, 15–25°C). After germination, seedlings with radicles about 2.0 cm long were selected and pre-cultured in Hoagland solution for 48 h, then were used immediately in the following treatments.

**Treatments.** The pre-cultured seedlings were transferred to glass beakers containing 30 mL of various treatment solutions and were incubated under room conditions (natural light, 18–25°C). To investigate the effect of IBA, NO and quercetin on lateral root development, the treatment solutions are (i) Hoagland solution (control); (ii) 100 µmol indole-3-butyric acid (IBA), and (iii) 100 µmol IBA plus 100 µmol 3,3',4',5,7-pentahydroxyflavone (quercetin), based on the results of preliminary experiment. The roots of the seedlings were sampled at 0.25, 0.5, 1, 3, 8, 24, 48, and 96 h after treatment, respectively, and were frozen with liquid nitrogen for the determination of NO. In addition, roots were additionally sampled at 8 h and 96 h after treatment for the determination of lateral root primordia (LRP). To investigate the effect of exogenous NO on the development of RSA, the treatment solutions are Hoagland solution (control), 10, 50, 100, and 500 µmol SNP, respectively, and the development of root system (the number of LR per seedling, the length of LR and PR) was investigated after 14 days of incubation. The above two experiments were both performed in triplicate (10–12 seedlings per replicate).

**Assay of NO generation.** NO production was estimated by the luminol-H<sub>2</sub>O<sub>2</sub> chemiluminescence (CL) method according to our previous studies (Gao et al. 2010). Samples were ground in an ice bath at a ratio of 200 mg fresh mass per cm<sup>3</sup> of cold deoxygenated water (freshly prepared by boiling distilled deionized water for 1 h). The extracts

were centrifuged at 12 000 × g for 20 min and the supernatant was immediately used for CL detection using a BPCL ultra-weak CL analyzer (Institute of Biophysics, Academia Sinica, P.R. China) by quickly injecting four volumes of 'illumination buffer' containing 50 mmol sodium carbonate-sodium bicarbonate, pH 9.72, 10 µmol luminol, 20 mmol H<sub>2</sub>O<sub>2</sub>, 2 mmol ethylenediamine tetraacetic acid (EDTA) at 30°C. Relative NO production was expressed as CL counts per microgram of protein [CL counts/µg (protein)].

**Lateral root primordia (LRP) determination.** The PR was cut and fixed with Carnoy's Fluid (3 alcohol + 1 acetic acid) at 4°C for 24 h, then was stained using Feulgen staining method (Vuylsteker et al. 1998). The LRP was counted by light microscopy at 50 amplifications.

Protein content was determined according to Bradford (1976), using bovine serum albumen (BSA) as standard.

**Statistical analysis.** The statistical analysis was done by the Duncan's multiple range test ( $P \leq 0.05$ ) using the SPSS software (SPSS 16.0 for Windows).

**Reagents.** The reagents used were manufactured by Sigma (St. Louis, USA).

## RESULTS AND DISCUSSION

**Time-dependent production of NO induced by IBA in roots of *M. hupehensis* Rehd. seedlings and the participation of quercetin in the NO generation.** Figure 1 shows that in comparison with control seedlings, treatment with 100 µmol IBA alone triggered a transient increase in NO production very rapidly (as early as 0.25 h after treatment) in roots. This transient increase in NO accumulation culminated at 0.5 h after treatment and quickly attenuated from 1 h to 24 h after treatment to even below control level; then, a second wave of NO burst can be detected at 48 h and 96 h after IBA treatment. However, this two-peak burst of NO induced by IBA was totally abolished by quercetin.

The number of LRP at 8 h and 96 h after different treatments was determined in order to investigate whether the second wave of NO generation induced by IBA was due to the formation of LR. Figure 2 shows that there was no significant difference ( $P \leq 0.05$ ) among the three treatments in the number of LRP per seedlings at 8 h after treatment. However, at 96 h after treatment, treatment with IBA alone significantly ( $P \leq 0.05$ ) induced LRP formation in comparison with control or IBA plus

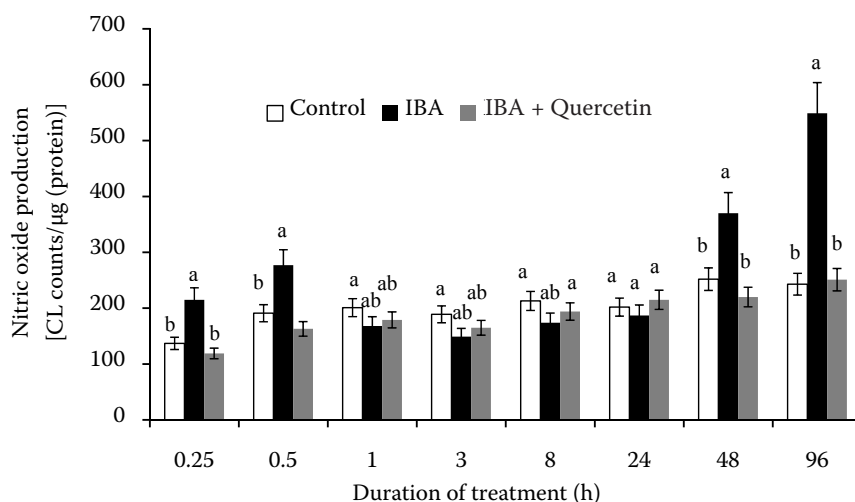


Figure 1. Indole-3-butyric acid (IBA) induced a transient increase in nitric oxide (NO) formation [chemiluminescence (CL) counts/ $\mu\text{g}$  (protein)] in roots of *Malus hupehensis* Rehd. seedlings, followed by a second wave of NO burst starting at 48 h after treatment. Results are means  $\pm$  SD ( $n = 3$ ). Different lower-case letters indicate significant differences on each sampling date at  $P \leq 0.05$  (Duncan's multiple range test). Quercetin – 3',4',5,7-pentahydroxyflavone

quercetin treated seedlings, consistent with the first and the second wave of NO burst induced by IBA treatment alone (Figures 1 and 2).

From Figures 1 and 2 we can see that the first wave of NO burst was correlated with the second burst, and that quercetin was involved in at least the first wave of NO formation induced by IBA. In *Pisum sativum*, Kolbert et al. (2008) also investigated the time-dependent generation of NO following IBA application during root growth. However, the very rapid NO burst (NO burst at 0.25 h and 0.5 h after IBA application in this research) induced by IBA was not observed, possibly due to the different IBA concentration that was used. The development of LRs consists of three stages: (i) LR initiation; (ii) LRP organogenesis, and (iii) LR emergence. In this research, it should be concluded that the first and second wave of NO burst following IBA application was correlated with the initiation of LR and the organogenesis of LRP, respectively, and that

the first burst of NO (initiation of LR) was correlated with the second NO burst (organogenesis of LRP). That is to say, the very rapid increase in NO formation induced by IBA might trigger the signal transduction pathway(s) that was required for the initiation of LR.

In *Medicago truncatula*, Imin et al. (2007) also observed that exogenous quercetin inhibited the auxin-induced rooting process. As quercetin is an auxin transport inhibitor, the authors argued that inhibition of auxin transport may prevent specific cell populations from reaching a localized auxin maximum which is required for the establishment of root primordia. However, Wasson et al. (2009) proved that flavonoid was necessary for nodulation but not necessary for LRs formation. In animals, quercetin can act as phosphatase and kinase inhibitors, as well as mitochondrial ATPase inhibitors, particularly inhibiting NO formation through inhibition of nitric oxide synthase (NOS) (Peer

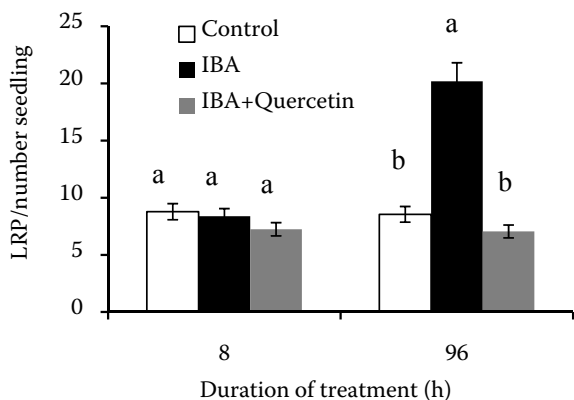


Figure 2. The formation of lateral root primordia (LRP) induced by indole-3-butyric acid (IBA) at 96 h after treatment was totally abolished by quercetin in roots of *Malus hupehensis* Rehd. seedlings. Results are means  $\pm$  SD ( $n = 3$ ). Different lower-case letters indicate significant differences on each sampling date at  $P \leq 0.05$  (Duncan's multiple range test). Quercetin – 3,3',4',5,7-pentahydroxyflavone

Table 1. Effect of nitric oxide donor sodium nitroprusside (SNP) on primary root (PR) and lateral root (LR) development during post-germination growth of *Malus hupehensis* Rehd. seedlings

	SNP concentration ( $\mu\text{mol/L}$ )				
	0	10	50	100	500
PR length (cm)	$3.48 \pm 0.26^a$	$3.36 \pm 0.28^a$	$3.02 \pm 0.21^b$	$2.87 \pm 0.17^c$	$2.31 \pm 0.12^d$
LR number/seedling	$5.74 \pm 0.36^d$	$12.52 \pm 0.76^a$	$13.48 \pm 0.87^a$	$10.81 \pm 0.55^b$	$7.96 \pm 0.43^c$
LR length (mm)	$9.36 \pm 0.86^b$	$15.43 \pm 1.03^a$	$7.78 \pm 0.78^c$	$5.86 \pm 0.61^d$	$3.34 \pm 0.39^e$
LR color	yellowish white	white	white	brownish yellow	brown

Data were obtained after 14 days of incubation and were means  $\pm$  SD,  $n = 3$ . Different letters within each row indicate significant differences at  $P \leq 0.05$  (Duncan's multiple range test)

and Murphy 2007). In plants, there are emerging evidences showing that quercetin most likely play similar roles as in animals. In this research, we found that quercetin inhibition of IBA-induced LRs was related to inhibition of IBA-induced NO formation. Therefore, quercetin in plants may act as a protein kinase inhibitor (Pan et al. 2005), an ATPase inhibitor (Takahashi et al. 1998), and/or an electron transport inhibitor (Moreland and Novitzky 1987), thus inhibiting IBA-induced NO formation and subsequently the LRs initiation.

The roles that NO play in auxin-mediated rooting process was well documented (Lanteri et al. 2006). However, the upstream event concerning IBA-induced NO biosynthesis is not fully understood. NO can be produced either enzymatically through NO-generating enzymes, or nonenzymatically through nitrite at acidic conditions. To date, there are at least seven enzymatic pathways of NO biosynthesis that were reported in plants, with nitrate reductase being the best characterized (Gupta et al. 2011). Most recently, it was reported that protein kinase was involved in the rapid production of NO induced by multiple stimuli (Wang et al. 2010). In this research, it was found that IBA-triggered rapid NO burst was totally abolished by quercetin (Figure 1), possibly due to quercetin inhibition of protein kinase (Wang et al. 2010) or the mitochondrial electron transport (Moreland and Novitzky 1987, Gupta et al. 2011). It might be supposed that the first NO burst was generated via the phosphorylation of one or more NO-generating enzymes (or related enzymes), which was achieved by the rapid activation of protein kinase following IBA application. However, which enzymatic pathway(s) (or related enzymes) should be involved in this IBA-induced NO burst remains to be elucidated in future research. Recently, Wang et al. (2010) found that phosphorylation of nitrate reductase was responsible for the rapid NO

production through the activation of MAPK by hydrogen peroxide.

**Effect of exogenous NO on the development of root system architecture during post-germination growth of *M. hupehensis* Rehd. seedlings.** From Table 1 we can see that SNP increased the amounts and the length of LR, while decreased the length of PR in a dose-dependent manner. The promotion of LR number was the most effective at 50  $\mu\text{mol}$  SNP, followed by 10  $\mu\text{mol}$ . SNP treatment also affected LR elongation dose-dependently: concentrations between 50–500  $\mu\text{mol}$  SNP significantly inhibited LR elongation while 10  $\mu\text{mol}$  SNP greatly promoted LR growth ( $P \leq 0.05$ ). It could be concluded that exogenous NO affects root architecture development dose-dependently: low concentrations of NO promoted both the number and elongation of LR, while higher concentrations inhibited the elongation of LR and PR. Table 1 also shows that exogenous NO affected the color of LRs dose-dependently. The color of LRs in 10  $\mu\text{mol}$  and 50  $\mu\text{mol}$  SNP treated seedlings was white, while that of 100  $\mu\text{mol}$  and 500  $\mu\text{mol}$  SNP treated seedlings was brownish yellow and brown, respectively. These results indicate that apart from a signaling molecule, exogenous NO may act as a stress factor in controlling LR initiation and development, in agreement with recent findings in soybean seedlings (Böhm et al. 2010).

In soil, nitrate and nitrite can be converted to NO enzymatically or non-enzymatically (Gupta et al. 2011). In low nitrate soils, patches of high nitrate have a localized stimulatory effect on LR development. However, where nitrate levels are globally high (i.e. not growth limiting), LR growth is inhibited (Nibau et al. 2008). Whether or not this effect of nitrate on LR development is due to nitrate-originated NO is an interesting question.

In general, we found that IBA induced a very rapid increase in NO accumulation in roots of

*Malus* seedlings that was correlated with the initiation of LR and that IBA-triggered rapid NO burst was totally abolished by quercetin. However, the enzymatic pathway(s) involved in IBA-induced NO formation as well as the molecule targets of quercetin regulation in this process remain to be elucidated. Furthermore, apart from the fact that IBA can act as a signal molecule *per se*, it is also an important source of IAA. In this sense, the mechanisms underlying the rapid production of NO induced by IBA should be further studied.

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