# Nitric Oxide Is Required for Root Organogenesis[1]

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In this report, we demonstrate that nitric oxide (NO) mediates the auxin response leading the adventitious root formation. A transient increase in NO concentration was shown to be required and to be part of the molecular events involved in adventitious root development induced by indole acetic acid (IAA).

The discovery of signal molecules involved in the intricate network that triggers root formation remains a major goal for a large number of biotechnological procedures. Adventitious rooting involves the development of a meristematic tissue after removal of the primary root system. The plant hormone auxins promote this process through the dedifferentiation of cells to reestablish the new apical meristem. Although a variety of components of auxin transport and signal transduction were identified, the molecular mechanism underlying the initiation of new root meristems is poorly understood (Doerner, 2000; Berleth and Sachs, 2001).

NO is a diffusible multifunctional second messenger first described in mammals, where it plays variable functions ranging from dilation of blood vessels to neurotransmission and defense during immune response (Gow and Ischiropoulos, 2001). Several researches have shown the presence of NO in plants and have attributed novel roles to this gas in the plant kingdom (Beligni and Lamattina, 2001a and refs. therein).

Of late, and contemporary to genomics and proteomics, it is interesting to note the revival of pharmacological and surgical techniques in the field of plant developmental biology (Nemhauser et al., 2000; Reinhardt et al., 2000). In this communication, we demonstrate through pharmacological and surgical approaches that NO is required for root organogenesis.

Two NO donors, sodium-nitroprussiate (SNP) and *S*-nitroso, *N*-acetyl penicillamine (SNAP), applied to hypocotyl cuttings (primary roots removed) of cucumber (*Cucumis sativus*) were able to mimic the effect of the auxin IAA in inducing de novo root organogenesis (Fig. 1). In addition, NO- and IAA-induced roots presented similar anatomic structure when they were analyzed by optic microscopy (not shown). This NO-mediated effect was prevented when the specific NO-scavenger carboxy-PTIO (cPTIO) was added with SNP or SNAP (Fig. 1). Result of treatments performed with different SNP concentrations confirmed that the effect was dose dependent, with a maximal biological response at 10 $\mu$M SNP (Fig. 2). Within 3 d after removal of the primary root system, adventitious root development was detected in the explants treated with IAA, SNP, or SNAP. Two parameters of root growth were considered, and length and number of adventitious roots exhibit similar behavior among these treatments (Fig. 3, *t* test, $P < 0.05$). In control experiments, when hypocotyl cuttings were kept in water or in $NO_2^-$/$NO_3^-$ (normal products of NO decomposition, not shown), adventitious roots emerged 4 d after primary root removal, and they reached only 22% of the length obtained from NO- or IAA-treated explants (Fig. 3). The treatment of hypocotyls with SNP or SNAP plus IAA resulted in an increased response, displaying roots longer than those from hypocotyls treated with the NO donors or IAA alone in a factor of 1.6 (Figs. 1 and 3). The specific NO scavenger, cPTIO, delayed adventitious root emergency and significantly reduced the root length and number of the IAA-treated explants (Figs. 1 and 3, *t* test, $P < 0.05$). It is interesting that this inhibitory effect of cPTIO was reversible because adventitious root emergency was triggered by the addition of 200 $\mu$M SNP after cPTIO treatment (not shown). In addition, a direct inactivation of IAA by cPTIO per se may be discarded because a mixture of IAA plus cPTIO was able to induce epinasty, an IAA-mediated effect, at the same extent as IAA alone (not shown).

Previous results reported by Gouvêa et al. (1997) showed that NO-releasing substances induced root tip elongation. Although a nitric scavenger prevented the growth induced by NO-releasing substances, it had no effect on IAA-induced cell expansion (Gouvêa et al., 1997). In contrast, our results using the specific NO scavenger, cPTIO, strongly indicated a role of endogenous NO in IAA-mediated root organogenesis. Consequently, the endogenous

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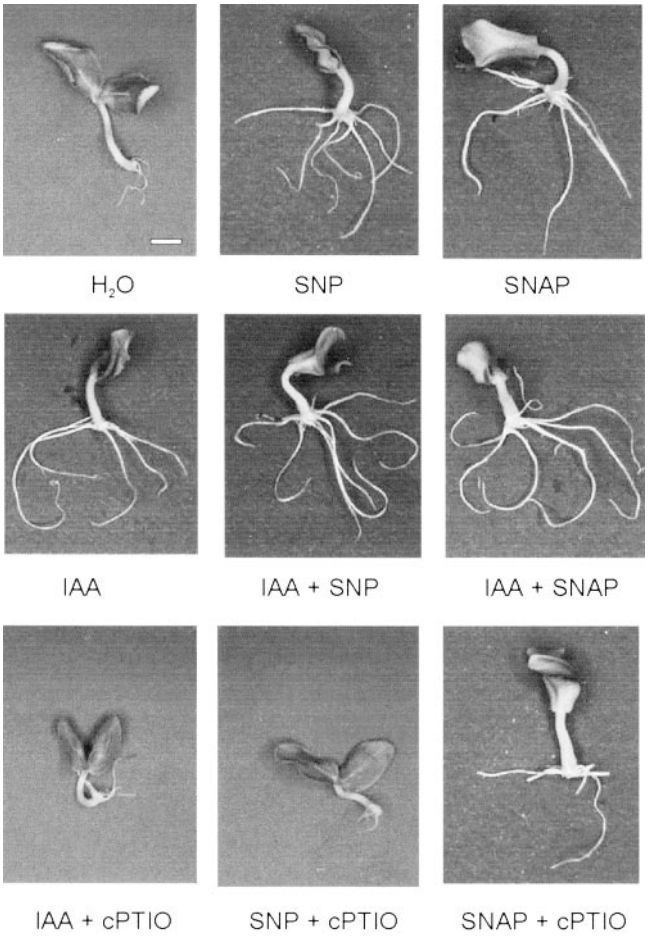


**Figure 1.** NO donors induce adventitious root development in cucumber explants. The primary root system was removed from hypocotyls of 10-d-old germinated cucumbers. Explants were incubated with water, 10 $\mu$M each of NO donors (SNP or SNAP), NO donors plus 200 $\mu$M cPTIO, or 10 $\mu$M IAA. The treatments were also done with a combination of IAA with SNP, SNAP, or the NO scavenger cPTIO. Photographs were taken after 5 d of treatments. Bar = 5 mm.

NO level was determined by electron paramagnetic resonance (EPR) in explants treated with IAA or water (control). High levels of NO were detected at $T_0$ in both treatments, probably reflecting a response to wounding after removing the primary root. However, a transient increase of NO (60 nmol $g^{-1}$ fresh weight) could be measured in explants after 24 h of treatment with IAA. NO concentration remained at detectable levels (20 nmol $g^{-1}$ fresh weight) until 96 h of treatment. On the contrary, in control explants, the level of endogenous NO dramatically shut down at 24 h (Fig. 4A). We also detected the presence of endogenous NO by using a fluorescent probe. Explants were exposed to the permeable and specific NO-sensitive fluorophore 4,5-diamino-fluorescein diacetate, which allows the detection of NO presence in animal and plant cells (Kojima et al., 1998; Foissner et al., 2000). The IAA-treated explants displayed 4-fold more fluorescence than control explants (medium pixel intensity: 138 versus 39; Fig. 4, B and C, respectively).

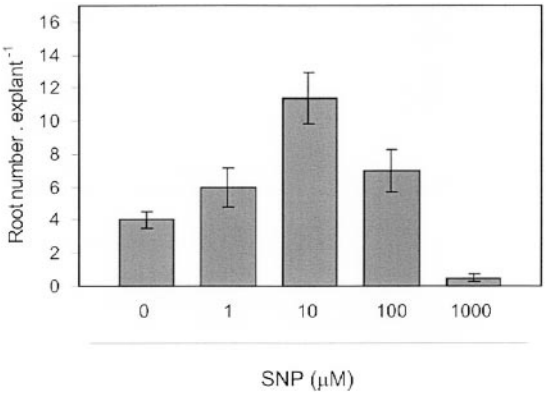


**Figure 2.** The effect of the NO donor on the induction of adventitious root formation is dose dependent. The Primary root system was removed from hypocotyls of 10-d-old germinated cucumbers. Explants were incubated with water or different concentrations of SNP as indicated. Adventitious root numbers were quantified after 5 d of treatment and are expressed as mean ± SE ($n$ = 15 explants from at least three independent experiments).

It still remains to be elucidated if NO alone has the capability to trigger root formation in the complete absence of IAA. Preliminary results in IAA-depleted cucumber hypocotyls showed that they were able to

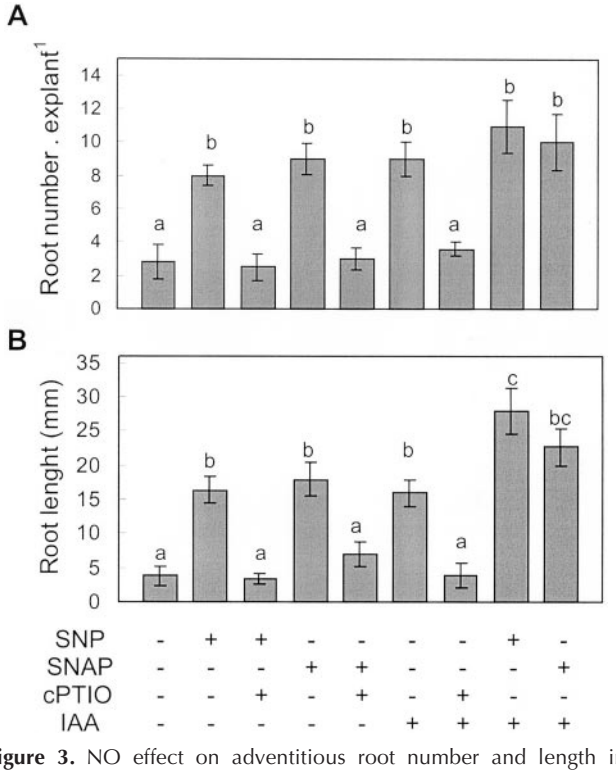


**Figure 3.** NO effect on adventitious root number and length in cucumber explants. Ten-day-old germinated cucumber seedlings were treated as described in Figure 1. Root number (A) and length (B) values are expressed as mean ± SE ($n$ = 20 explants from at least three independent experiments). Bars with different letters are significantly different with $P < 0.05$ ($t$ test).

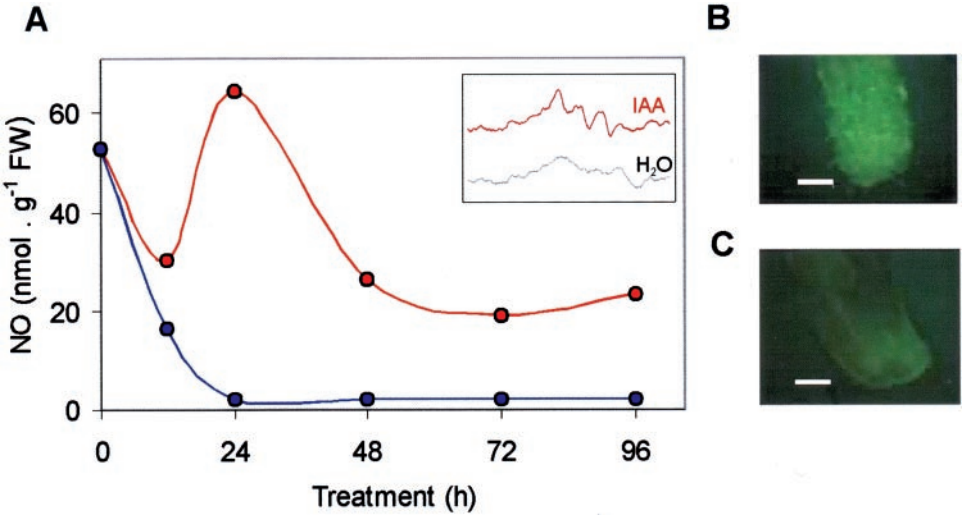


**Figure 4.** IAA induces a transient increase of endogenous NO in cucumber explants. Endogenous NO level was determined by EPR (A; MGD kit M-0010; OMRF SPIN TRAP SOURCE, Oklahoma City, OK) in explants treated with 10 $\mu$M IAA (red circles) or water (blue circles). Inset, EPR profile obtained from IAA- and water-treated explants after 24 h of incubation. B and C, 4,5-Diamino-fluorescein diacetate fluorescence detected in a longitudinal section from the tip of the hypocotyls, where new meristematic tissue and adventitious roots are formed. Pictures were taken after 24 h of treatment from an IAA-treated explant (B) and an untreated one (C). Bars = 0.5 mm.

induce the adventitious root formation after treatment with NO donors (G. Pagnussat, L. Lanteri, and L. Lamattina, unpublished data). However, further investigations are needed to conclusively confirm these observations.

Although a direct effect of NO cannot be discarded, our findings indicate that NO could mediate the auxin response during the adventitious rooting process in cucumber. Nevertheless, a serial linkage IAA $\rightarrow$ NO $\rightarrow$ rooting cannot be distinguished from a scenario in which IAA and NO could be acting in parallel on a third intermediate. The involvement of NO in this auxin-signaling pathway opens a wide field of research for every reported IAA effect in plant biology. Considering that NO has been postulated as a signal molecule during development and adaptive plant responses (Klessig et al., 2000; Beligni and Lamattina, 2001b; Garcia Mata and Lamattina, 2001), our results strongly support the idea of NO as a versatile molecule with variable functions in plants, as occurs in animal systems.

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