



Research article

Nitric oxide induced modulations in adventitious root growth, lignin content and lignin synthesizing enzymes in the hypocotyls of *Vigna radiata*Sangeeta Sharma^a, Harminder Pal Singh^{b,*}, Daizy Rani Batish^a, Ravinder Kumar Kohli^{a,c}^a Department of Botany, Panjab University, Chandigarh, 160014, India^b Department of Environment Studies, Panjab University, Chandigarh, 160014, India^c Central University of Punjab, Mansa Road, Bathinda, 151001, India

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ABSTRACT

The present study evaluated the role of nitric oxide (NO) in mediating adventitious root (AR) growth, lignification and related enzymatic changes in the hypocotyls of *Vigna radiata*. To meet the objectives, the changes in AR growth, lignin content, and the activities of enzymes – peroxidases, polyphenol oxidases, and phenylalanine ammonia lyases – with NO donor and its scavenger were monitored. Hypocotyls were cultivated in aqueous solution supplemented with different concentrations of SNP (sodium nitroprusside, NO donor compound) and its scavenging compound (2,4-carboxyphenyl-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide; cPTIO). Specifically, at low concentrations, SNP induced AR growth, increased the total lignin content and altered the activities of related oxidoreductases- peroxidases, polyphenol oxidases and phenylalanine ammonia lyases- which are involved in lignin biosynthesis pathway. At higher concentrations, a decline in AR growth and lignification was noticed. We analysed the function of NO in AR formation by depleting the endogenous NO using scavenging compound cPTIO. Hypocotyls grown in a medium supplemented with scavenger cPTIO exhibited significant decline in AR growth and the activities of lignin synthesizing enzymes. Application of NO scavenger showed that stimulatory properties on root lignification may be owing to NO itself. In addition, changes in AR growth were significantly correlated with these modified biochemical activities. Our analysis revealed that NO supplementation induces prominent alterations in lignin level during AR formation and this might be due to an alteration in the activity of lignin biosynthetic enzymes, which further affected the polymerization of monolignols and AR growth.

1. Introduction

Nitric oxide (NO) is a gaseous secondary messenger molecule that plays a key role in various growth and development processes in plants (Astier et al., 2018). It has been regarded as an important signalling molecule regulating root growth and architecture in plants (Corpas and Barroso, 2015). Plants produce NO via arginine-dependent (catalysed by nitric oxide synthase) and nitrate/nitrite-dependent pathways (catalysed by nitrate reductase); however, recent studies have suggested that nitrite reduction is the main source of its production (Jeandroz et al., 2016).

NO is involved in regulation of various biotic and abiotic stress responses in plants (Corpas et al., 2011; Groß et al., 2013). It contributes to redox homeostasis in cells through the improvement of the antioxidant capacity by alleviating oxidative stress generated by excessive ROS (Singh et al., 2009; Groß et al., 2013; Correa-Aragunde

et al., 2015). NO reacts with free radicals and transition metals present in its microenvironment, which contributes to its regulatory mode of action (Thomas, 2015). NO induces major alterations in the phenylpropanoid pathway to confer tolerance mechanisms against stress conditions (Li et al., 2017).

The process of lignification is a significant step during root growth because it involves sealing of cells by lignin deposition. Lignin, a phenolic heteropolymer comprising of polysaccharides and proteins, is a component of secondary cell wall and provides strength and rigidity (Vanholme et al., 2010). It is synthesized via phenylpropanoid pathway that involves well-coordinated actions of phenylalanine ammonia lyases (PAL), peroxidases (POD), polyphenol oxidases (PPO) and many other related enzymes. PAL acts as a precursor molecule of lignin biosynthesis and is the first-rate limiting enzyme of lignin synthesis (Vanholme et al., 2010). PAL catalyses the formation of *trans*-cinnamic acid from the amino acid, phenylalanine (Bonawitz and Chapple,

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2010). NO supplementation at higher concentrations reduced root formation with declined lignin content and PAL activity (Böhm et al., 2010). In contrast, at lower concentrations, NO induced root growth and enhanced the activities of cell wall bound PPO and PAL (Böhm et al., 2010). POD are widely distributed throughout the plant kingdom and involved in various processes including lignification, healing of infectious wounds, and auxin catabolism (Hiraga et al., 2001). POD catalyses the polymerization of monolignols (coniferyl alcohol, sinapyl alcohol, paracoumaryl alcohol) to form lignin, whereas PPO utilize phenolic compounds as substrate in lignin biosynthesis pathway (Boudet et al., 2003). A rise in the activity of POD is considered as a signal inducing root initiation, whereas role of PPO in root initiation has been linked to phenolic metabolism (Liao et al., 2010). NO acts as an important upstream signalling molecule in the monolignol assembly, a part of lignin biosynthesis pathway (Ferrer and Barceló, 1999) and has regulatory function during xylem vessel lignification (Barceló et al., 2004; Gabaldón et al., 2005). NO inhibited the activity of POD, and affected the activity of lignin synthesizing enzymes and increased the transcription of their genes (Gabaldón et al., 2005). Endogenous NO controls the lignin composition in roots of *Helianthus annuus* (Monzón et al., 2014). NO regulates the expression of genes (4-coumarate-CoA ligase, cinnamyl alcohol, Caffeoyl-CoA O-methyltransferase, etc.) involved in lignin biosynthesis pathway and treatment with NO scavenger cPTIO results in differential lignin composition (Monzón et al., 2014). Smart et al. (2003) suggested that oxidoreductases like POD, PPO and IAAO (Indole acetic acid oxidase) involved in IAA degradation play a variety of roles in root organogenesis. Similar mechanisms operate during the formation of adventitious roots (AR), where POD and IAAO modify auxin content in the cells (Rama and Prasad, 1996). Treatment of NO and H₂O₂, independently as well as synergistically, improved AR formation in *Chrysanthemum* by inducing the activities of PPO and IAAO (Liao et al., 2010).

Adventitious rooting is an ecologically and economically important physiological mechanism that plays a significant role under normal and stressed environment (Steffens and Rasmussen, 2016). However, not much is known about the role of NO in mediating AR formation through modulation of lignin and enzymes involved in lignification *per se*. We therefore, explored the participation of NO in mediating AR growth, lignification and related enzymatic changes in the hypocotyls of *Vigna radiata*. To meet the objectives, changes in AR growth, lignin content, and the activities of enzymes – POD, PPO, and PAL – with NO donor and its scavenger (cPTIO) were monitored. The experimental conditions chosen for the present study were selected because the process of AR formation shows high metabolic rate and lignin formation initiates during the initial phases of root formation (Steffens and Rasmussen, 2016).

2. Materials and methods

2.1. Materials

Seeds of *Vigna radiata* (L.) R. Wilczek (var. SML 668; hereafter mung bean) were procured from the local market. These were surface-disinfected with sodium hypochlorite (0.1%, w/v), followed by rinsing in distilled water. NO was given in the form of sodium nitroprusside (SNP; MW = 297.95 g; purity = 98.5%) procured from Thomas Baker Chemical Ltd., Mumbai, India. 2,4-Carboxyphenyl-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (cPTIO) was purchased from Sigma-Aldrich (USA).

2.2. Experimental setup and treatments

Seeds were soaked overnight in distilled water and laid on enamel trays coated with moist cotton and filter sheets in a plant growth chamber at 26 ± 1 °C and 75 ± 1% relative humidity and a continuous light of ~240 μmol m⁻² s⁻¹ PFD. After a week, uniform and

similar-sized seedlings were selected and the hypocotyls, 3.5 cm below the cotyledon node, were cut by keeping the region of epicotyl completely intact. The cotyledons were carefully removed from each hypocotyl. The hypocotyls were transferred to glass vials and subjected to different NO treatments. In all, there were six treatments: i) distilled water alone (as control); ii) 0.5 μM SNP; iii) 1 μM SNP; iv) 2.5 μM SNP; v) 5 μM SNP; and vi) 0.5 μM SNP + 1 μM cPTIO. The glass vials were filled with respective solutions and top of each glass vial was covered with aluminium foil. These were placed in a growth chamber set at 25 ± 1 °C and a light intensity of ~240 μmol m⁻² s⁻¹ PFD. Each treatment was replicated five times and arranged in a completely randomized design. The volume of treatment solution including that of control was maintained by supplementing the loss caused due to transpiration by adding the respective treatment solution. Adventitious roots (AR) emerge from epidermis within 3 days after the excision of primary roots from hypocotyls. After 3rd (root induction stage), 5th (root initiation stage) and 7th (root expression stage) day of treatment, AR were excised, washed and used for biochemical estimations.

2.3. Lignin determination

Lignin was semi-quantitatively estimated by following the method given by Sancho et al. (1996). About 100 mg of tissue was thoroughly rinsed in hot water and after centrifugation the insoluble particles were pelleted out and rinsed in 100% ethanol. The dry residue thus obtained was solubilized for 2.5 h in a solution of 2.5 ml of HCl/ethanol. Further, 10 μl of 20% of phloroglucinol-HCl was mixed in 1 ml of previous solution. After 30 min of incubation, the absorbance of the mixture was recorded at 540 nm.

2.4. Polyphenol peroxidases (PPO)

Activity of PPO was monitored using the methodology previously described by van Lelyveld (1973). Root tissue (100 mg) was homogenized in 10 ml of 100 mM phosphate buffer (PO₄³⁻ buffer; pH = 7.0) and homogenate was centrifuged at 15,000 g for 25 min at 4 °C. Afterwards, the supernatant was collected and used for enzymatic estimations. The protein content present in the samples was spectrophotometrically quantified as per Bradford (1976). The enzyme extract (0.5 ml) was mixed with 3 ml catechol prepared in 100 mM PO₄³⁻ buffer (pH = 6.0). The change in absorption of solution was measured at 495 nm for 1 min. The enzyme activity was represented as *kat* sec⁻¹ mg⁻¹ protein.

2.5. Peroxidases (POD)

The activity of POD was evaluated as per the procedure described by Batish et al. (2006). Enzyme extract (0.5 ml; as prepared for PPOs) was incubated in a mixture of 3.5 ml of 0.1 M PO₄³⁻ buffer (pH = 6.5), 0.1 ml of 0.1% *o*-dianisidine and 0.2 ml of 200 mM H₂O₂. The rise in optical density was monitored for 1 min at 430 nm. The amount of enzyme was represented as *kat* sec⁻¹ mg⁻¹ protein.

2.6. Phenylalanine ammonia lyases (PAL)

PAL activity was determined as per the method described by Ke and Saltveit (1986). The homogenate was prepared by crushing 1 g of fresh root tissue with 4 ml of 50 mM borate buffer (pH = 8.5) along with 0.4 g PVP and 5 mM 2-mercaptoethanol. The mixture was filtered and centrifuged at 17,930 × g for 20 min. The reaction mixture consisted of 0.3 ml of the supernatant along with 0.7 ml of 100 mM phenylalanine and 3 ml of 50 mM borate buffer (pH = 8.5). It was incubated for 1 h at 40 °C and the reaction was terminated by adding 0.1 ml of 5 mol l⁻¹ of HCl. The activity of PAL was calculated based on the cinnamic acid production by monitoring the absorbance at 290 nm.

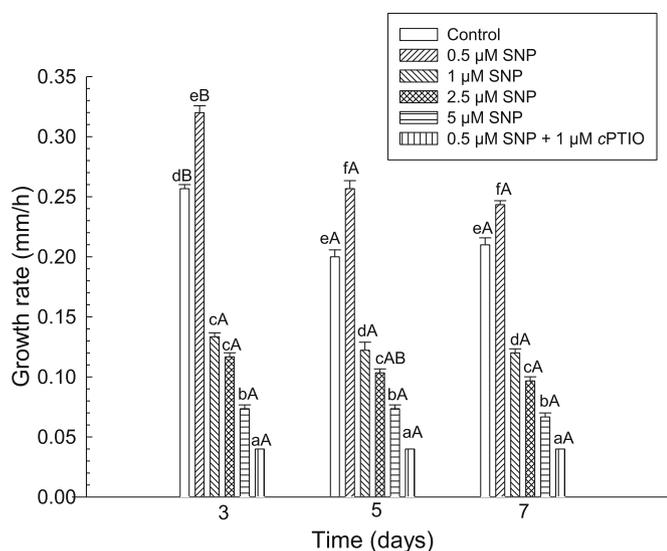


Fig. 1. Changes in growth (mm/h) of the adventitious roots (AR) on hypocotyls of *Vigna radiata*, measured on 3rd, 5th and 7th day after treatment with NO (as SNP) and NO scavenger (cPTIO). Values presented as mean \pm SE of five replicates. Upper and lower-case letters represent the significant difference in values with increasing time and concentration, respectively, at $p \leq 0.05$ applying Tukey's test.

3. Results

3.1. Effect on AR growth

NO supplementation significantly ($p \leq 0.05$) affected AR growth in a concentration-dependent manner. At low dose (0.5 μ M), it promoted the AR growth, whereas at higher doses ($\geq 1 \mu$ M), a decline in AR growth was noticed (Fig. 1). Addition of cPTIO (NO scavenger) had a negative impact on AR growth and reversed the impact of NO on AR growth. AR growth (mm/h) was increased by $\sim 24\%$, $\sim 28\%$ and $\sim 15\%$ ($p \leq 0.05$) over the control on 3rd, 5th and 7th day, respectively, in response to 0.5 μ M of SNP. However, upon addition of cPTIO, it declined ($p \leq 0.05$) by $\sim 84\%$, $\sim 80\%$ and $\sim 80\%$, respectively, over the control (Fig. 1). AR growth declined ($p \leq 0.05$) by $\sim 48\%$, $\sim 53\%$ and $\sim 71\%$ at 1, 2.5 and 5 μ M of SNP treatment, respectively, on 3rd day (root induction stage), over that of control. A similar trend of reduction ($p \leq 0.05$) was observed on 5th (initiation stage; 33%, 48%, 63% decline) and 7th (expression stage; 36%, 54%, 68% decline) day of treatment. The decline in AR growth was the greatest when NO scavenger cPTIO was used (Fig. 1).

3.2. Effect on lignin content

Treatment with 0.5 μ M SNP enhanced ($p \leq 0.05$) lignin content by $\sim 85\%$, $\sim 26\%$ and $\sim 36\%$ on 3rd, 5th and 7th day, respectively, over the control (Fig. 2). SNP treatment at $\geq 1 \mu$ M caused a reduction in lignin content. Lignin content declined by $\sim 13\%$, $\sim 33\%$ and $\sim 45\%$ on 3rd day, $\sim 31\%$, $\sim 41\%$ and $\sim 53\%$ on 5th day and $\sim 91\%$, $\sim 93\%$ and $\sim 94\%$ on 7th day of treatment, respectively, in response to 1, 2.5 and 5 μ M SNP treatment, over that in the control (Fig. 2). However, cPTIO application reversed the promotory effects of SNP on lignin content. cPTIO reduced ($p \leq 0.05$) the lignin content by $\sim 57\%$, $\sim 70\%$ and $\sim 96\%$ on 3rd, 5th and 7th day, respectively, over the control (Fig. 2).

3.3. Effect on lignin synthesizing oxidoreductase enzymes

SNP significantly affected the activities of POD, PAL and PPO in AR. In general, 0.5 μ M SNP enhanced the activities of POD, PPO and PAL, whereas these were reduced at $\geq 1 \mu$ M SNP (Fig. 3 a-c).

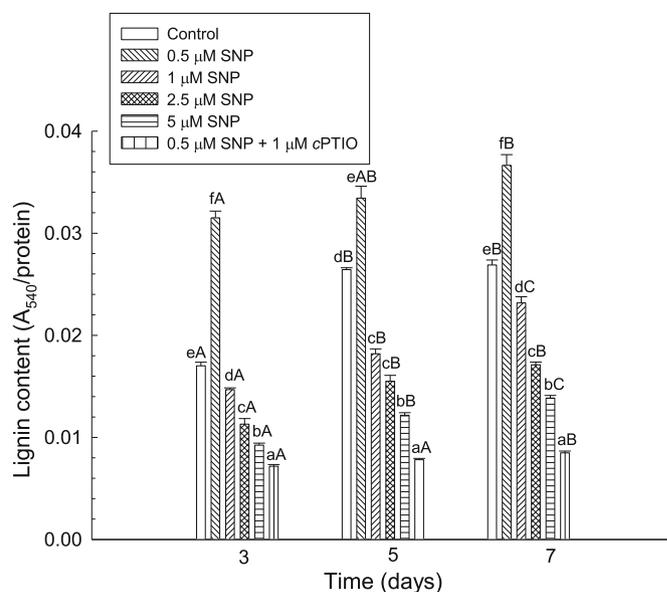


Fig. 2. Changes in lignin content (Absorbance_{540nm}/protein content) in the adventitious roots (AR) on hypocotyls of *Vigna radiata*, measured on 3rd, 5th and 7th day of treatment with NO (as SNP) and NO scavenger (cPTIO). Values presented as mean \pm SE of five replicates. Upper and lower-case letters represent the significant difference in values with increasing time and concentration, respectively, at $p \leq 0.05$ applying Tukey's test.

Activity of POD was increased ($p \leq 0.05$) by 11% and 17% on 3rd and 5th day of 0.5 μ M SNP treatment, respectively. It declined by 10%, 40% and 50% ($p \leq 0.05$) on the 3rd day in response to 1, 2.5 and 5 μ M SNP treatment, respectively (Fig. 3a). POD activity declined further, and 10–20% ($p \leq 0.05$) and 30–40% ($p \leq 0.05$) reduction was observed on 5th and 7th day, respectively, in response to 2.5–5 μ M SNP. The induction of POD was the greatest during root initiation stage (5th day). cPTIO caused greater decline ($p \leq 0.05$) in the POD activity, which was less ($p \leq 0.05$) by 59%, 65%, and 51% on 3rd, 5th and 7th day, respectively, over the control (Fig. 3a).

In response to 0.5 μ M SNP, the activity of PAL was increased ($p \leq 0.05$) by 10%, 7%, and 14% over the control on 3rd, 5th and 7th day, respectively (Fig. 3b). In contrast, 1, 2.5 and 5 μ M of SNP declined ($p \leq 0.05$) PAL activity by 12%, 22% and 38% over the control on 3rd day and by 5%, 9% and 14% on 7th day. It declined further when cPTIO was supplemented, and a decrease ($p \leq 0.05$) of 46%, 53% and 52% was observed on 3rd, 5th and 7th day, respectively (Fig. 3b).

The activity of PPO was increased ($p \leq 0.05$) by 15%, $\sim 10\%$ and 18% at 0.5 μ M of SNP, after 3rd, 5th, 7th day of treatment relative to the control (Fig. 3c). NO supplementation at higher doses ($\geq 1 \mu$ M) down-regulated the induction levels of PPO. Exposure to 1, 2.5 and 5 μ M SNP declined ($p \leq 0.05$, except at 1 μ M on 3rd day) PPO level by $\sim 5\%$, 16% and 29% on 3rd day, 7%, 14% and 42% on 5th day, and 8%, 21% and 30% on 7th day, respectively, relative to the control (Fig. 3c). PPO activity declined further with cPTIO supplementation and 37%, 54%, and 57% reduction was observed over the control on 3rd, 5th, and 7th day, respectively (Fig. 3c).

4. Discussion

The important inference that can be drawn from the present study is that NO supplementation in the form of SNP has significant and dual (promotory or inhibitory) impact on AR growth and lignification in mung bean. At low concentration, NO induced AR growth and lignification, whereas high NO concentration and its scavenger (cPTIO) reduced AR growth and lignification. These observations are corroborated by similar findings in other plant species. For example, NO at low

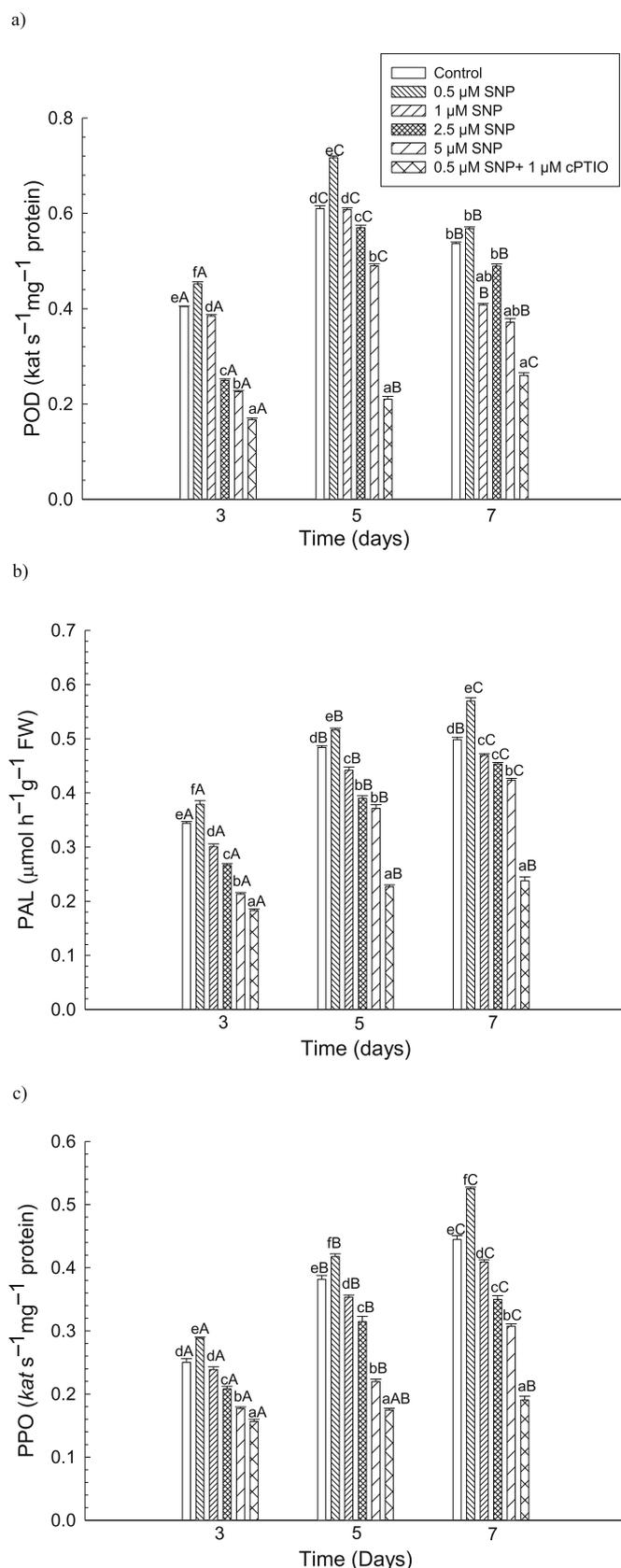


Fig. 3. Effect of exogenous NO (as SNP) on the specific activities of (a) peroxidases [POD], (b) phenylalanine ammonia lyases [PAL], and (c) polyphenol oxidases [PPO] in adventitious roots (AR) on hypocotyls of *Vigna radiata*, measured on 3rd, 5th and 7th day after treatment with NO (as SNP) and NO scavenger (cPTIO). Values presented as mean \pm SE of five replicates. Upper and lower-case letters represent the significant difference in values with increasing time and concentration, respectively, at $p \leq 0.05$ applying Tukey's test.

concentration (10^{-4} – 10^{-1} μM) induced root tip expansion in maize in a dose-dependent manner (Gouvêa et al., 1997). NO supplementation (100 μM of SNP) reduced the growth of hypocotyls in lettuce, *Arabidopsis thaliana* and potato (Beligni and Lamattina, 2000). SNP (10–100 μM) regulated auxin response, thereby inducing AR formation, whereas the induction was inhibited at 1000 μM (Pagnussat et al., 2002). Treatment of soybean roots with low doses of NO (0.1–0.75 nmol/g) promoted root expansion, whereas at higher concentrations (≥ 1 nmol/g), root elongation was inhibited (Hu et al., 2005). In the current investigation, exogenous NO affected the AR growth in mung bean and also modulated the activities of PAL, POD and PPO enzymes, and lignin content. These findings are significant in view of the role of lignin in root formation and development. In our study, lignin content declined concomitantly with reduction in AR growth at higher doses of SNP, whereas at extremely low dose (0.5 μM), SNP induced AR growth and increased lignification. This suggested a concentration-dependent effect of NO on AR growth and lignin content. Previously, microarray analysis has provided evidences that endogenous NO participates in sunflower root organogenesis along with the regulation of genes involved in lignin biosynthesis (Monzón et al., 2014). It implied that lignin biosynthetic genes are the prime target of NO-mediated gene regulation, though it was not investigated in the present study. In our study, PAL activity was increased at low NO concentration, whereas it declined at higher NO concentrations. It corroborated the earlier studies that SNP affects the activity of PAL. For example, SNP (10 μM) induced the specific activity of PAL in the cells of *Taxus yunnanensis* (Wang et al., 2006); SNP at 100 μM induced a rapid stimulation in the levels of PAL in *Pelargonium peltatum* (Floryszak-Wieczorek et al., 2006). In our study, we found that NO induced a rise in the activity of soluble POD, which can be correlated to the anti-oxidative activity of POD. It is in agreement with an earlier finding that SNP boosted POD activity in wheat seedlings (Tian and Lei, 2006). It is well-documented that plants possess multiple forms of POD, which are localized in diverse cellular sections, and these are distinctly regulated in response to several stimuli (Passardi et al., 2005). Therefore, it has been assumed that NO supplementation affects few specific isoforms of POD, thus explaining the rise in POD specific activity in treated hypocotyls. Böhm et al. (2010) observed a rise in root growth, activity of PAL and cell wall-bound POD at 5 μM of SNP in soybean roots. NO has been demonstrated to inactivate thiol and transition metal containing enzymes (Bogdan, 2001). It is speculated that NO reacts with two Cu ions present in the active center of PPO, forms copper-nitrosyl complexes (NO–Cu–PPO), thereby reducing the activity of PPO. Gabaldón et al. (2005) performed girdling experiments and revealed that NO generation and lignification of cell wall are inversely connected metabolic events during the process of xylem differentiation. NO affects lignin biosynthesis enzymes by reacting directly with enzymes or adding up to its effects upon availability of substrate or by transcriptional regulation of target enzyme (Delledonne et al., 2003). NO regulates lignin biosynthesis pathway by interacting with metalloenzymes like cinnamate-4-hydroxylase or with secondary messengers like protein kinases or guanylyl cyclase (Neill et al., 2008). Enkhardt and Pommer (2000) suggested that NO non-competitively inhibits the cinnamic acid hydroxylase formation, which governs the rate limiting step during biosynthesis of lignin. This leads to an accumulation of cinnamic acid, which participates in regulatory feedback mechanisms that work by regulating the phenylpropanoid gene transcription or by reducing the PAL content (Bolwell et al., 1986). As reported in current work, the decline in lignin content was accompanied by a reduction in root growth and PAL activity at higher doses of NO. Therefore, it can be hypothesized that the decline in PAL activity may lessen the phenolic acid content and additionally the biosynthesis of lignin. This observation explains the dual effects of NO on lignification during AR development.

To confirm the hypothesis that lower doses of NO improved lignification and content of related enzymes, we conducted experiments

with NO scavenger cPTIO. This is a widely used specific NO scavenger, which terminates NO responsive actions in plants. Spatial and temporal concentration of NO might control the expression of genes that participate in lignin synthesis and lead to formation of compounds enriched in S-monomerolignols in plants. Treatment with cPTIO decreased the levels of enzymes that participate in lignification. It demonstrated that this stimulation associates with a variance constitution of lignin that leads to an augmented G/S (guaiacyl/syringyl) relationship. Low concentrations of NO induce high lignin G/S rate, which resulted in increased crosslinking of lignin, thereby making it more susceptible to rupture. On the other hand, a high concentration of NO may lead to a reduction in the G/S rate and formation of more resistant and mechanical tissue (Monzón et al., 2014). NO regulates the lignification process by different mechanisms. Enkhardt and Pommer (2000) reported a direct effect of NO on the heme proteins that participate in lignin synthesis and inhibition of POD activity. Additionally, NO induces transcriptional activation of certain genes, regulates H₂O₂ levels and changes the activity of enzymes that participate in ROS scavenging (Clarke et al., 2000; Delledonne et al., 2001). Previously, a relationship between deposition of lignin and modulations in the root system of *Arabidopsis thaliana* has been noticed when the plant was subjected to Cu stress (Lequeux et al., 2010). Therefore, various accumulated evidences suggest a strong interaction between AR growth and lignification in hypocotyls of mung bean.

In conclusion, our findings suggest that supplementation of NO in the form of SNP induced significant modifications in AR growth and lignification in mung bean hypocotyls. At low concentrations NO showed inductive role on AR growth, lignin content and the activities of enzymes that participate in lignin biosynthesis. However, treatment with NO scavenger reversed the effects of NO on AR growth, lignin content and the activities of oxidoreductase enzymes. At higher concentration, NO behaves like a stress-inducing molecule that causes inhibition of AR growth, lignin content and a decline in the activities of POD, PPO and PAL. The study documented that the observed effect are due to NO alone. However, we did not elucidate whether the effects are due to cytotoxicity of NO itself or the by-products of NO metabolism. Nevertheless, NO controls various biological processes by covalent modification of target proteins through post-translational modifications or by affecting the gene transcription (Romero-Puertas et al., 2013). The regulatory action of NO depends upon its location and the rate of generation; and NO content further determines cytotoxic or stimulating action (Serrano et al., 2012). Thus, a precise control on NO content by switching the NO signalling seems to be a crucial event during growth and development.

Author contribution

H.P. Singh and D.R. Batish designed the work. S. Sharma conducted experiments, collected and analysed the data. H.P. Singh, D.R. Batish, S. Sharma and R.K. Kohli contributed equally to the write up of this manuscript.

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