PLANT-MICROORGANISM INTERACTIONS

Nitric oxide generated by *Piriformospora indica*-induced nitrate reductase promotes tobacco growth by regulating root architecture and ammonium and nitrate transporter gene expression

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ABSTRACT
Nitric oxide (NO) is involved not only in the regulation of plant growth, development, and stress responses but also in the regulation of plant-microbe interactions. Here, we demonstrate that *Piriformospora indica* can induce tobacco nitrate reductase to produce a NO signal in roots which enhances nitrogen uptake capacity by inducing the expression of ammonium and nitrate transporter genes and the development of lateral root and root hair, thereby promoting tobacco growth. In addition, the NO signal induced by *P. indica* is significantly different from that induced by the pathogen *Phytophthora nicotianae*. Inoculation with *P. indica* did not produce H2O2 and maintained high expression of *Phytoglobin 1* in roots, resulting in a significantly lower NO level than in the roots inoculated with *P. nicotianae*. These findings suggest that an appropriate NO level is the likely basis of plant-*P. indica* symbiosis, which promotes the growth of host plants.

1. Introduction

Nitric oxide (NO) is an important signaling molecule in plant physiology, participating in regulating a wide range of plant growth and developmental processes, such as seed germination, root development, and nitrogen uptake (Pagnussat et al. 2002; Bethke et al. 2007; Sanz et al. 2015; Sun et al. 2015; Lombardo and Lamattina 2018). Bethke et al. (2007) suggested that the interaction of NO and ABA breaks the seed dormancy of *Arabidopsis* and promotes seed germination. As an important component of the auxin signaling pathway, NO can promote the development of lateral and adventitious roots (Pagnussat et al. 2002). A study on *Arabidopsis* confirmed that NO can work with ABA to promote root hair growth and ectopic root hair formation by modulating cytoskeleton organization (Lombardo and Lamattina 2018). Nitric oxide produced by nitrate reductase (NR) enhances nitrogen uptake by inducing lateral root formation and inorganic nitrogen uptake under partial nitrate nutrient in rice (Sun et al. 2015). Furthermore, NO can modulate plant responses to some abiotic stresses, such as hypoxia, salinity, and heavy metals (Kolbert et al. 2021).

The role of NO in plant immune systems has attracted a great deal of research interest over the past two decades (Bogdan 2001; Gupta et al. 2011; Astier and Lindermayr 2012; Kumari et al. 2019; Martinez-Medina et al. 2019a; Martinez-Medina et al. 2019b; Singh et al. 2020). During the immune response of plants to fungal pathogens, a burst of NO causes reprogramming of gene expression, synthesis of secondary metabolites with antimicrobial properties, and elicitation of hypersensitive responses (Bogdan 2001; Martinez-Medina et al. 2019a; Martinez-Medina et al. 2019b). In addition, NO is produced when interactions between plants and symbiotic fungi are established (Kumari et al. 2019; Singh et al. 2020). In the process of plant recognition of symbiotic fungi, epidermal cells, cortical cells, and root hairs in the root produce a large amount of NO, which is thought to be triggered by the host plant sensing the presence of bioactive molecules in fungal secretions (Martinez-Medina et al. 2019a). Recent evidence suggests that tight control of NO level is necessary to control mycorrhizal symbiosis (Kumari et al. 2019; Martinez-Medina et al. 2019a; Martinez-Medina et al. 2019b). During this process, for example, plant phyto-globins can act as NO dioxygenases to catalyze the metabolism of NO to nitrogen, and particular concentrations of NO activate the symbiotic regulatory pathways DMI1, DMI2, and DMI3 to provide further space for fungus growth (Martinez-Medina et al. 2019a). These findings have cemented the position of NO as a key messenger in plant-microbe interactions.

Interactions between beneficial soil microorganisms and plant roots can promote plant growth and development and improve plant resistance to multiple environmental challenges. *Piriformospora indica*, isolated from the Indian Thar desert, is an endophytic fungus that can colonize plant roots (Strehmel et al. 2016). This endophytic fungus has been well studied in more than 30 plant families. It promotes the growth and development of host plants, stimulates the accumulation of secondary metabolites, and enhances...
the resistance of host plants to biotic and abiotic stresses (Sherameti et al. 2005; Franken 2012; Pedrotti et al. 2013; Strehmel et al. 2016; Blesa et al. 2021; Li et al. 2021). Sherameti et al. (2005) suggested that stimulation of NR and the starch-degrading enzyme glucan-water dikinase (SEX1) by \textit{P. indica} promotes the growth of \textit{Arabidopsis} and tobacco. Inoculation of chickpea and black lentil with \textit{P. indica} significantly increases plant uptake of nitrogen, phosphorus, and potassium (Franken 2012). \textit{P. indica} also promotes primary root growth and induces lateral root formation in \textit{Arabidopsis} (Pedrotti et al. 2013). Here, we found that \textit{P. indica} not only promoted the growth of tobacco but also induced an NO signal in the root. In light of NO’s role as a signaling molecule for plant-microbe interactions and in plant growth and development, it is interesting to explore the role of NO in the endophyte-plant symbiosis.

We examined the source of the root NO signal induced by \textit{P. indica} and analyzed the regulatory role of such NO signaling in tobacco root development and nitrogen uptake. Further, by comparing differences in the NO signal after inoculation with \textit{P. indica} and pathogen \textit{P. nicotianae}, we revealed the role of NO induced by \textit{P. indica} in promoting the growth of tobacco. These results enrich our understanding of the mechanism of NO signaling in plant-microbe interactions.

2. Materials and methods

2.1. Materials and root colonization

\textit{Piriformospora indica} was provided by Dr. Mingfang Zhang, Laboratory of Germplasm Innovation and Molecular Breeding, Institute of Vegetable Science, Zhejiang University. The fungi were cultured on potato dextrose agar (Coolaber, Beijing). The co-cultivation of tobacco and \textit{P. indica} in a pot experiment was carried out with reference to the description of Li et al. (2021). \textit{P. indica} was cultured in potato dextrose broth (Coolaber, Beijing) for 20 days, and the mycelium was collected by centrifugation at 10,000 rpm. The subsequently harvested fungi were rinsed five times in ddH2O to remove residual medium, and the fungal pellets resuspended in ddH2O were homogenized by grinding. Twelve-day-old tobacco seedlings (\textit{Nicotiana tabacum} L. cv. MS Yunyan 87), which were grown under natural light in a greenhouse at day/night temperatures of 28°C/21°C, were irrigated with fungal pellet suspension adjusted to an OD600 value of 0.02. Meanwhile, the control seedlings were irrigated with the same volume of ddH2O.

After 30 days of co-culture, tobacco roots were stained with 0.05% trypan blue under a light microscope (Olympus, Tokyo, Japan) according to the method of Michal Johnson et al. (2011). Symbiosis was determined by detecting \textit{P. indica} spores colonized in tobacco roots under a light microscope with a 10x magnification objective. The colonization rate was estimated by microscopic visualization of stained chlamydospores covering the area of the root.

To investigate the effect of nitric oxide on root dry weight, nitrogen accumulation, and expressions of genes involved in nitrogen uptake in \textit{P. indica}-colonized roots, 20 μM of sodium nitroprusside (SNP, an NO donor) was applied to uncolonized tobacco, 200 μM of 2-(4-carboxyphenyl)−4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO, an NO scavenger) was applied to \textit{P. indica}-colonized tobacco, and ddH2O-treated uncolonized tobacco plants served as control. Root samples were collected and subsequently used to determine physiological and biochemical parameters.

2.2. Co-cultivation experiment in Petri dishes and estimation of plant growth

According to the method of Vadassery et al. (2010), tobacco seedlings grown on an MS medium for 10 days were transferred to filter paper matted on modified plant nutrient medium in Petri dishes. Each medium cultivated one seedling. After 24 h, all seedlings grown in Petri dishes were divided into eight treatment groups, in one of which a fungal plug of 8 mm diameter was placed 1 cm from the root. The remaining seven treatment groups were: (1) placed a fungal plug and added 200 μM of cPTIO every 24 h; (2) placed a fungal plug and added both 200 μM of cPTIO and 20 μM of SNP every 24 h; (3) placed a fungal plug and added 100 μM of tungstate (Tu, NR inhibitor) every 24 h; (4) placed a fungal plug and added both 100 μM of Tu and 20 μM of SNP every 24 h; (5) added 20 μM of SNP every 24 h; (6) added 20 μM of SNP and 200 μM of cPTIO simultaneously every 24 h; (7) control group without any treatment. All the plates were incubated at 23°C/18°C under a 14 h light/8 h dark photoperiod. After 20 days of treatment, root samples were collected and inductively analyzed. A. U. Three roots were used for the independent analysis of each treatment.

2.3. Measurement of NO in tobacco roots

According to the method of Fernández-Marcos et al. (2011), the content of nitric oxide in root samples was detected using fluorescent probe 3-Amino,4-aminomethyl-2,7'-difluorescein, diacetate (DAF-FM DA, Beyotime Bio-technology, China). Root samples were immersed in 10 μM DAF-FM dye prepared in 20 mM HEPES-NaOH buffer (pH 7.5). After incubation in the dark for 30 min, the roots were rinsed three times with HEPES-NaOH buffer and immediately observed under an Olympus FV1200 laser scanning confocal microscope (Olympus; excitation 488 nm, emission 495–575 nm). Fluorescence intensity was expressed in arbitrary fluorescence units (A.U.). Three roots were used for the independent analysis of each treatment.

2.4. Sample measurement and analysis

After drying in an oven at 65°C for 48 h, the dry mass of the roots was determined. The primary root length of the tobacco seedlings was measured with a ruler, and lateral root (LR) density was determined as the number of LRs per cm of root length. Roots of randomly selected samples were observed with a stereomicroscope (Olympus Optical Co. Ltd, Tokyo, Japan), and root hair length was analyzed with Image J software.

2.5. Determination of total nitrogen content and nitrate reductase activity

Total nitrogen content was determined according to the modified Chinese Tobacco industry standard (YC/T 161, 159-2002) (Li et al. 2017). A sample (0.1 g tissue powder, 0.1 g CuSO4, and 1 g K2SO4) was mixed with 5 ml of concentrated sulfuric acid. The mixture was kept at 150°C (30 min),
250 °C (30 min), and 370 °C (2 h) using a furnace (CWF11/13, Carbolite, UK). After cooling the mixture, 10 ml of deionized water was added to the sample and shaken thoroughly. The mixture was then filtered, and the total nitrogen content in the supernatant was determined with flow-injection analysis (Bran + Luebbe, Hamburg, AA, Germany).

Nitrate reductase activity was measured as described by Sagi and Lips (1998). Approximately 1 g of fresh tissue was homogenized in 30 mM phosphate buffer (pH 7.5) and centrifuged for 15 min at 4,000 rpm. 50 µl of the supernatant was collected and added to 250 µl of reaction buffer (30 mM phosphate buffer (pH 7.5), 25 mM KNO₃, and 0.25 mM NADH). After incubation at 28°C for 15 min, 50 µl of a 1:1 (v/v) mixture of 1 M zinc acetate and 0.3 mM phenazine methosulphate was added to the reaction solution and then vigorously mixed to remove residual NADH. Nitrite production was determined by reading the absorbance at 540 nm after the addition of 1 ml of a 1:1 (v/v) mixture of 1 M zinc acetate and 0.3 mM phenazine methosulphate was added to the reaction solution and then vigorously mixed to remove residual NADH. Nitrite production was determined by reading the absorbance at 540 nm after the addition of 1 ml of a 1:1 (v/v) mixture of 1% (w/v) sulphanilamide in 3 M HCl and 0.02% (w/v) N-540 nm after the addition of 1 ml of a 1:1 (v/v) mixture of 1 M zinc acetate and 0.3 mM phenazine methosulphate was added to the reaction solution and then vigorously mixed to remove residual NADH. Nitrite production was determined by reading the absorbance at 540 nm after the addition of 1 ml of a 1:1 (v/v) mixture of 1% (w/v) sulphanilamide in 3 M HCl and 0.02% (w/v) N-naphthyl-(1)-dihydrochloride. One unit of NR activity is defined as the production of 1 µg nitrite per hour per fresh weight (µg NO₂⁻·g⁻¹·FW·h⁻¹).

2.6. Quantitative PCR analysis

Total RNA was isolated from root material as described by Li et al. (2016). One microgram of RNA was digested with gDNA Eraser, and total RNA was used as a template for first-strand cDNA synthesis with PrimeScript™ RT reagent Kit (Takara, Japan). The qRT-PCR reactions were performed on a Viia™ 7 Real-Time PCR System from Applied Biosystems (California, United States) with TB Green® Premix Ex Taq™ II (Takara, Japan) to monitor the synthesis of double-stranded DNA. The reaction was carried out with three biological replicates under the following conditions: in the first stage, the sample was pre-denatured at 95°C for 30 s, in the second stage, the sample was incubated at 95°C for 5 s and at 60°C for 30 s, repeated 40 times, and in the third stage it was dissociated. The housekeeping gene NACTIN served as the internal standard to normalize the target gene expression data. Relative expression was defined as 2^(ΔΔCt (target gene) − Ct (control gene))?Livak and Schmittgen 2001). Primers for qRT-PCR are shown in Supplementary Table 1.

2.7. Infection assay with P. indica and pathogen

P. indica and P. nicotianae were cultivated on a potato dextrose agar (Coolaber, Beijing) medium at 25°C for 6 and 8 days, respectively. Roots from 20-day-old tobacco plants were inoculated with mycelial discs (1 cm in diameter) of the fungus and placed in Petri dishes containing filter paper saturated with sterile Hoagland’s solution (Coolaber, Beijing). The samples were incubated at 23°C/18°C under a 14 h light/8 h dark photoperiod. After 96 h of treatment, samples were collected for H₂O₂ detection.

2.8. Determination of H₂O₂ in tobacco roots

The content of H₂O₂ was measured according to Bellincampi et al. (2000). Briefly, after grinding the sample using liquid nitrogen, 0.2 ml HCLO₄ was added, and the extract was centrifuged to obtain the supernatant. The supernatant was added to 500 µL of assay reagent (500 µM ferrous ammonium sulfate, 50 mM H₂SO₄, 200 µM xylenol orange, and 200 mM sorbitol). After 45 min of incubation, H₂O₂ measurements were performed based on the determination of peroxy- mediated Fe²⁺ to Fe³⁺ oxidation by measuring the absorbance of the Fe³⁺-xylene orange complex at 560 nm.

2.9. Statistical analysis

Results are expressed as means ± standard error. Tukey’s test (p < 0.05) and one-way ANOVA in GraphPad Prism version 4 were used to analyze the data.

3. Results

3.1. P. indica colonization induces NO accumulation in tobacco roots

Figures 1(A and B) demonstrate that P. indica can colonize the roots and improve the growth of tobacco seedlings. After 30 days of co-cultivation, the colonization rate of P. indica was 55.4% (Figure 1(D)). The dry weight of P. indica-colonized roots was 2.2-fold higher than that of uncolonized plants, and the primary root length of P. indica-colonized plants was 35% higher than that of uncolonized plants (Figure 1(E)). Furthermore, tobacco roots inoculated with P. indica significantly increased nitrogen content by 12.9% compared to the control (Figure 1(E)). It is noteworthy that inoculation of tobacco roots with P. indica increases root differentiation comparatively more than did the control (Figure 1(E)). Inoculation with P. indica significantly increased the density of lateral roots and promoted the development of root hair, suggesting that P. indica participated in the regulation of the root architecture.

Nitrogen is a major limiting factor of plant growth, and NO is considered to be a key signal in the nitrate sensing pathway. Considering the role of NO as a signaling molecule for plant-microbe interactions and the role of NO in plant growth and development, we measured the NO signal in the roots of P. indica-colonized and uncolonized plants. The NO-associated green fluorescence increased in P. indica-colonized roots, and quantification of DAF fluorescence intensities indicated an ~162% increase in NO content in P. indica-colonized roots compared with the control (Figure 1(C, E)). These results show that NO production in the roots of P. indica-colonized tobacco was higher than in the uncolonized tobacco plants. It is interesting to speculate whether there is any connection between NO signaling and growth promotion by P. indica.

3.2. Nitric oxide participated in root architecture formation induced by P. indica colonization

In order to explore whether NO participated in the regulation of root architecture by P. indica colonization, we used a NO donor and scavenger (SNP and cPTIO) to examine the effect of NO on lateral root density and root hair development in the P. indica-colonized tobacco plants. The application of 10 µM SNP significantly induced NO accumulation in roots (Figure 2(A-B)). The DAF fluorescence inten-
and the lateral root density and root hair length of roots treated with SNP were also significantly increased compared with the control (Figure 2(B)). In comparison, the root architecture of SNP-treated plants was similar to that of *P. indica*-colonized plants (Figure 2(A-B)). There was no significant difference in root dry weight and lateral root density between the two treatment groups. Only the root hair length of the SNP treatment group was slightly lower than that of *P. indica*-colonized tobacco plants.

We observed that treatment with the NO scavenger cPTIO resulted in inhibition of SNP- and *P. indica*-induced root architectures. After 14 days of cPTIO application, the NO-associated green fluorescence markedly decreased in *P. indica*-colonized roots and SNP-treatment roots (Figure 2(A-B)). The application of cPTIO erased the effects of *P. indica* colonization and SNP treatment on root dry weight, lateral root density, and root hair development (Figure 2(A-B)).

We then used tungstate (Tu), an inhibitor of NR, to detect the effect of NR activity on the root architecture induced by *P. indica* colonization. After 14 days of Tu application, the NO-associated green fluorescence markedly decreased in *P. indica*-colonized roots (Figure 2(A-B)). Correspondingly, the root dry weight, lateral root density, and root hair length of the *P. indica*-colonized roots decreased by 30.6%, 52.7%, and 49.5%, respectively, after Tu treatment, indicating that the inhibition of NR activity by Tu restricted the root architecture induced by *P. indica* colonization (Figure 2(B)). Furthermore, supplementation with SNP then relieved the inhibitory effect of Tu on the promotion of root dry weight, lateral root density, and root hair length by *P. indica* colonization. These results suggest that the *P. indica*-induced NO accumulation is derived mainly from the enzymatic pathway by NR.

3.3. *P. indica*-induced NO accumulation is derived mainly from the enzymatic pathway by nitrite reductase

In higher plants, NR and Nitric Oxide Synthase (NOS) are considered to be two key enzymes leading to NO production. Here, we detected the expressions of NR and NOA1 (a homologue of NOA1 in *Arabidopsis*) in tobacco roots inoculated with *P. indica*. NR expression in the root of *P. indica*-colonized tobacco increased ~375% compared with that of uncolonized plants, while no significant difference in NOA1 expression was observed between the roots of *P. indica*-colonized and control tobacco plants (Figure 3(A)). Furthermore, analysis of NR activity in the roots showed that *P. indica* colonization could significantly improve NR activities (Figure 3(B)). We then used tungstate (Tu), an inhibitor of NR, to detect the effect of NR activity on the root architecture induced by *P. indica* colonization. After 14 days of Tu application, the NO-associated green fluorescence markedly decreased in *P. indica*-colonized roots (Figure 2(A-B)). Correspondingly, the root dry weight, lateral root density, and root hair length of the *P. indica*-colonized roots decreased by 30.6%, 52.7%, and 49.5%, respectively, after Tu treatment, indicating that the inhibition of NR activity by Tu restricted the root architecture induced by *P. indica* colonization (Figure 2(B)). Furthermore, supplementation with SNP then relieved the inhibitory effect of Tu on the promotion of root dry weight, lateral root density, and root hair length by *P. indica* colonization. These results suggest that the *P. indica*-induced NO accumulation is derived mainly from the enzymatic pathway by NR.

3.4. Nitric oxide affected the expressions of nitrate and ammonium transporter genes during *P. indica* colonization

Although NR is a rate-limiting enzyme for nitrogen assimilation, it is also interesting to determine whether NO
generated by NR enhances nitrogen uptake during the growth promotion of tobacco by *P. indica* colonization. We compared the effects of *P. indica* colonization, cPTIO, and SNP application on root dry weight, nitrogen accumulation, and the expression of genes involved in nitrogen uptake in tobacco roots. Application of cPTIO reduced the nitrogen content and root dry weight of *P. indica*-colonized roots by 5.1% and 18.7%, respectively, compared to the untreated *P. indica*-colonized roots (Figure 4(A)). In turn, SNP treatment increased the nitrogen content and dry weight of tobacco roots by 10% and 215%, respectively, compared with the control (Figure 4(A)). Further qRT-PCR analysis of genes involved in nitrogen uptake revealed that inoculation with *P. indica* and application of SNP were able to significantly induce the expressions of four nitrate transporter genes (*NRT1.1*, *NRT1.2*, *NRT2.1*, and *NRT2.2*) compared with the control (Figure 4(C)). In addition, the application of SNP induced transcript levels of *AMT1.1*, *NRT1.1*, *NRT1.2*, *NRT2.1*, and *NRT2.2* genes.
AMT1.2, AMT1.3, and AMT2.1. The expressions of AMT1.1 and AMT1.2 were also significantly increased by *P. indica* inoculation (Figure 4(B)). Conversely, the application of cPTIO could inhibit the induction of NRT1.1, NRT1.2, NRT2.1, NRT2.2, and AMT1.2 expressions in the root by *P. indica* colonization (Figure 4(B-C)). These results suggest that NO is likely involved in the induction of most nitrate and ammonium transporter genes by *P. indica* inoculation.

**Figure 3.** Effect of *P. indica* colonization on expressions of NR and NOA1, and on NR activity in tobacco roots. (A) Differences in expressions of NR and NOA1 in *P. indica*-colonized (+*P. indica*) and non-colonized (-*P. indica*) tobacco plants. (B) Differences in NR activity in *P. indica*-colonized (+*P. indica*) and non-colonized (-*P. indica*) tobacco plants. Asterisks (*) indicate the statistical significance of the difference between the experimental and the control groups. (Student’s t-test, *P* < 0.05, **P** < 0.01).

**Figure 4.** Effect of NO on nitrogen accumulation and expressions of ammonium and nitrate transporter genes in *P. indica*-colonized tobacco plants. (A) Effect of NO on nitrogen content and root dry weight in *P. indica*-colonized tobacco plants. Different lowercase letters indicate significant differences among treatments (One-way ANOVA test, *P* < 0.05). (B-C) qRT-PCR analysis of ammonium and nitrate transporter genes in tobacco plants treated with *P. indica*, SNP, and cPTIO. Data are means ± standard error (SE). Asterisks (*) indicate the statistical significance of the difference between the experimental and the control groups. (Student’s t-test, *P* < 0.05, **P** < 0.01).
3.5. Nitric oxide accumulation in tobacco roots differs in response to a fungal pathogen and *P. indica* during the early infestation

Several reports have pointed out that NO signaling is involved in plant defense against pathogens. To investigate whether the NO signal in tobacco roots differs in response to *P. indica* and a pathogen, we examined the NO content in tobacco roots in response to *P. indica* and pathogen *P. nicotianae*, respectively, during the first four days of early infestation. As shown in Figures 5(A and B), the NO-associated green fluorescence in tobacco roots increased continuously after *P. indica* colonization and peaked at the 48th hour, followed by a slight decrease. In roots inoculated with *P. nicotianae*, the DAF fluorescence increased gradually over 96 h, and its value at the 96th hour was 77.5% higher than that in *P. indica*-colonization roots.

Nitric oxide accumulation can be regulated by plant phytoglobins which act as NO dioxygenases to catalyze the metabolism of NO to nitrate. Here, we analyzed the expression of tobacco phytoglobin gene PHYTOGB1 in roots inoculated with *P. indica* and *P. nicotianae* (Figure 5(C)). qRT-PCR analysis showed that the expression of PHYTOGB1 could be continuously induced at 24, 48, 72, and 96 h after *P. indica* inoculation. In contrast, the pathogen *P. nicotianae* caused an initial increase in PHYTOGB1 expression at 24 h post-inoculation but a strong decrease in the subsequent time period.

3.6. H2O2 production in tobacco roots differs in response to a fungal pathogen and *P. indica*

Nitric oxide and reactive oxygen species (ROS) act as signals in plant innate immunity. Both are closely linked in plant defense against pathogens, particularly in the establishment of a hypersensitive response (HR). Hypersensitive cell death is activated following the interaction of NO with H2O2, rather than O2− (Delledonne et al. 2002). Thus, we investigated whether tobacco roots produce different H2O2 in response to a pathogen and *P. indica* (Figure 6). Inoculation with *P. indica* did not stimulate the production of H2O2 in tobacco roots compared to the control. However, inoculation with *P. nicotianae* significantly induced H2O2 production in the tobacco roots, and the H2O2 content in *P. nicotianae*-treatment group was 6.81-fold higher than that in *P. indica*-colonization roots. This suggests that the ROS signal, involved in plant innate immunity, is different in response to *P. indica* and the oomycete pathogen *P. nicotianae*.

4. Discussion

In recent decades, numerous attempts have been made to utilize soil beneficial microorganisms (*Arbuscular mycorrhiza*, *Bacillus*, *Pseudomonas*, *Trichoderma* sp., etc.) to sustainably promote plant growth and development by increasing nutrient efficacy, enhancing nutrient cycling, accelerating seed germination, and building inducible systemic resistance (Jeffries et al. 2003; Jain et al. 2012; Singh et al. 2014; Pii et al. 2015; Giles et al. 2016; Pii et al. 2016; Sagliola et al. 2016). Unlike most rhizosphere microorganisms, which are obligate biotrophs and cannot be cultured without the plant, *P. indica* is a cultivable endophyte that colonizes plant roots and can migrate with its host to new environments (Strehmel et al. 2016). Consistent with reports in *Arabidopsis*, tomato, sunflower, rice, and pineapple (Sherameti et al. 2005; Cruz et al. 2013; Moreira et al. 2015; Anith et al. 2018; Eliaspour et al. 2020), inoculation of tobacco with *P. indica* in our study significantly promoted root growth and nitrogen accumulation. In addition, inoculation with *P. indica* significantly increased the density of lateral roots and promoted the development of root hair, which suggested that *P. indica* participated in the regulation of the root architecture. A recent study has shown a similar phenomenon; inoculation of *P. indica* significantly increased root length, root surface area, root volume, and the number of root tips in treated plants as compared to an uninoculated control (Singh et al. 2022). Several studies have shown that the development of lateral root and root hair greatly increases the surface area of the root system for nutrient uptake and consequently promotes plant growth (Bloom et al. 2002; Datta et al. 2011; José et al. 2003; Magalhaes et al. 2017; Stephanie and Iye 2012). This suggests that *P. indica* can expand the channels of nutrient uptake by regulating root architecture to facilitate crop growth.

Interestingly, our study found that inoculation with *P. indica* could significantly induce NO generation in tobacco roots. The addition of the NO scavenger cPTIO to *P. indica*-colonized plants resulted in a decrease in NO content and an inhibition of the pro-growth effect of *P. indica* on tobacco. NO is a key regulator of early nitrate perception in maize roots (Manoli et al. 2014), and an increasing number of studies have indicated that the NO signal can promote plant growth and development (Krouk et al. 2010). Therefore, we suggest that NO is also one of the important signals of endophyte-plant symbiosis and is involved in the pro-growth effect of endophyte on plants. This is similar to the importance of NO signaling in rhizome formation, where NO can be produced throughout the rhizobium-legume symbiosis (Singh et al. 2020).

Nitric oxide synthase and NR are two potential enzymatic sources for NO production in plants. NOS enzymes are thought to produce NO by participating in the L-arginine pathway. However, no NOS enzymes have been identified in higher plants, and the original *Arabidopsis* NOS enzyme thought to be involved in NO synthesis was identified as a GTPase enzyme and renamed as an NO-associated enzyme (AtNOA1) (Crawford 2006; Moreau et al. 2008). Here, the expression of tobacco NOA1 (a homologue of NOA1 in *Arabidopsis*) was not affected by the inoculation of *P. indica*. It is possible that *P. indica* did not induce NO level in roots via the NOS pathway or that tobacco NOA1 does not play a role in NO synthesis. In contrast, NR gene expression, as well as NR enzyme activity in tobacco roots, could be significantly induced by *P. indica*, which is consistent with the results obtained previously (Sherameti et al. 2005). As a rate-limiting enzyme for nitrogen assimilation, NR reduces nitrate to nitrite and subsequently reduces nitrite to NO. We found that the application of an inhibitor of NR enzyme significantly reduced NO level in roots colonized by *P. indica* and that the pro-growth effect of *P. indica* on tobacco was also inhibited. When supplemented with NO donor SNP, the pro-growth effect of *P. indica* on tobacco was restored. In addition, the application of SNP on uncolonized roots produced effects similar to *P. indica* colonization in promoting tobacco root growth and development. This suggests that
P. indica colonization induces the expression and activity of NR, and the resulting NO signal is further involved in the growth and development of tobacco.

An increasing number of studies have focused on the role of NO produced by NR in response to nutrient fluctuations in regulating plant root growth and nitrogen metabolism (Krouk et al. 2010; Meng et al. 2012; Sun et al. 2015; Liu et al. 2017; Sun et al. 2017). Nitric oxide induced by partial nitrate nutrition is involved in the formation of lateral roots in rice (Sun et al. 2015). A burst of NO triggered by Mg deficiency stimulates the development of Arabidopsis root hair (Liu et al. 2017). In addition, NO plays a role as a common signaling molecule in Fe and P deficiency-induced cluster root formation in white lupine (Meng et al. 2012). In the present study, the addition of cPTIO or Tu to P. indica-colonized tobacco plants resulted in a decrease in NO content and an inhibition of the pro-growth effect of P. indica on root architecture. The root dry weight, lateral root density, and root hair development of tobacco were suppressed. Back-supplementation of SNP to Tu-treated P. indica-colonized plants restored the growth and development of the root architecture, and a similar effect was achieved by

**Figure 5.** Differences in NO levels and PHYTOGB1 expressions in roots inoculated with P. indica and P. nicotianae. (A) NO production in the roots treated with P. indica and P. nicotianae is shown as green fluorescence. Scale bar indicates 500 μm. (B) NO production in root expressed as fluorescence intensity (A.U.). (C) qRT-PCR analysis of PHYTOGB1 in roots inoculated with P. indica and P. nicotianae. Data are means ± standard error (SE).
singly increasing the NO level in uncolonized roots. This suggests that NO is involved in the regulation of tobacco root architecture by *P. indica* colonization.

On the other hand, NO had a significant effect on the uptake of organic and inorganic nitrogen in the fine roots of beech seedlings and the partitioning of nitrogen among the different nitrogen pools (Simon et al. 2009). Furthermore, rhizospheric NO in European beech improves ammonium uptake through post-translational modification of proteins rather than regulation of gene expression (Astier and Lindermayr 2012). A recent report indicated that the NO-mediated enhancement of nitrate and ammonium uptake in rice was achieved at the transcriptional level and that NO-induced nitrogen uptake in the rice variety Nan- guang contributed to improved nitrogen use efficiency (Sun et al. 2015). Here we analyzed the effect of *P. indica*-induced NO on nitrate and ammonium transporter gene expressions. Both SNP application and *P. indica* colonization increased nitrogen content and induced the expression of nitrate and ammonium transporter genes (*NRT1.1*, *NRT1.3*, *NRT2.1*, *NRT2.2*, *AMT1.1*, and *AMT1.2*) in tobacco roots, whereas removal of the NO signaling inhibited these effects. Taken together, we suggest that *P. indica* can enhance the ability of tobacco to capture external nitrogen through two pathways: one is to regulate the root architecture mediated by NO signaling to increase the surface area of the root system for nitrogen uptake, and the other is to induce the expression of ammonium and nitrate transporter genes through NO signaling to enhance nitrogen uptake.

Recent studies have shown that NO plays an important role in the interactions between plant roots and microorganisms and that the regulatory effect of NO on plant-microbe relationships is concentration-dependent. An appropriate NO level promotes the formation of plant-microbe symbiosis, and a high concentration of NO enhances plant immunity (Zipfel and Oldroyd 2017; Kumari et al. 2019; Martínez-Medina et al. 2019a; Martínez-Medina et al. 2019b). Both in legume-rhizobium symbiosis and arbuscular mycorrhizal symbiosis, the host plant produces a large amount of NO in recognition of the symbiotic fungi, which in turn can significantly affect the formation of rhizomes and mycorrhizal (Martínez-Medina et al. 2019a; Singh et al. 2020). In this process, NO induces the expression of the *Phytoglobin* gene. Plants are able to express *Phytoglobin* genes at low concentrations, which effectively oxidizes NO to NO$_3^-$, thereby controlling the local cellular NO concentration (Kumari et al. 2019; Martínez-Medina et al. 2019b). The reciprocal regulation of NO and Phytoglobin is essential for rhizome formation, oxygen level, and energy status in legume-rhizobium symbiosis (Kolbert et al. 2021).

In contrast, pathogens induced sustained accumulation of NO and significant down-regulation of *Phytoglobin* in host plants (Kumari et al. 2019; Martínez-Medina et al. 2019a; Martínez-Medina et al. 2019b). Plants can recognize Pathogen-Associated Molecular Patterns (PAMPs) or Danger-Associated Molecular Patterns (DAMPs) through transmembrane Pattern Recognition Receptors (PRRs) at the cell surface to trigger Pattern-Triggered Immunity (PTI) responses after pathogen infestation, during which plant cells can produce a high concentration of NO and reactive oxygen species (ROS) (Martínez-Medina et al. 2019a; Martínez-Medina et al. 2019b). A high level of NO accumulation or the reaction of NO with H$_2$O$_2$ may lead to nitration of tyrosine residues of phytoglobin, which in turn disrupts the homeostatic regulation of NO, resulting in an elevated NO level, and leading to local cell death (Delledonne et al. 2002; Martínez-Medina et al. 2019a; Martínez-Medina et al. 2019b). Our study showed a significant difference in the intensities of the root NO signal induced by *P. indica* and pathogen *P. nicotianae*. The NO signal induced by *P. indica* reached its highest value at the 48th hour after the inoculation. However, the NO signal induced by *P. nicotianae* continued to increase, and the level of NO was higher at the 96th hour after the inoculation by nearly 77.5% than that of the *P. indica* treatment. In addition, *P. indica* did not induce H$_2$O$_2$ accumulation, whereas *P. nicotianae* induced significant accumulation of H$_2$O$_2$. Further analysis of the regulatory pathway of NO level revealed that the expression of tobacco *PHYTOGB1*, which can potentially oxidize NO, was maintained at a high level in roots inoculated with *P. indica*. In contrast, the expression of *PHYTOGB1* in roots inoculated with *P. nicotianae* decreased sharply after 24 h. This implies that NO level is probably dynamically balanced at a reasonable level due to the induction of *PHYTOGB1* expression in the roots colonized by *P. indica*. In contrast, in roots inoculated with *P. nicotianae*, NO is likely to accumulate continuously due to the inhibition of *PHYTOGB1* expression. The accumulated NO might be able to further disrupt the homeostatic regulation of NO by nitration of tyrosine residues of phytoglobin with the H$_2$O$_2$, thus becoming a plant immune signal leading to local cell death.

The application of excessive exogenous NO donors or the production of excessive NO through genetic mutations can inhibit plant growth and development, while an appropriate NO level has a promoting effect (Sánchez-Vicente et al. 2019). Therefore, taken together, we speculate that *P. indica* induces NR to produce an NO signal, and the generated NO is likely to be regulated by *PHYTOGB1* to form a homeostatic NO level equilibrium. This appropriate concentration of NO is involved in regulating root architecture and nitrogen uptake, thus promoting the growth of tobacco.

![Figure 6. Differences in H$_2$O$_2$ levels in roots inoculated with *P. indica* and *P. nicotianae*. Data are means ± standard error (SE), and different lowercase letters indicate significant differences among treatments (One-way ANOVA test, p < 0.05).](image-url)
5. Conclusions

In the present study, we found that *P. indica* induces tobacco NR to produce an NO signal, and the generated NO participates in the induction of ammonium and nitrate transporter genes and the growth and development of lateral roots and root hair in a robust root system. In addition, inoculation with *P. indica* did not produce H$_2$O$_2$ and maintained high expression of *PHYTOGB1* in roots, which resulted in an NO level in the roots inoculated with *P. indica* significantly lower than that of the roots inoculated with *P. nicotianae*. This suggests that, unlike the plant defense against the pathogen, the NO induced by *P. indica* is likely to be regulated by *PHYTOGB1* to form a homeostatic NO level equilibrium, thus allowing the appropriate concentration of NO to participate in regulating the root architecture and nitrogen uptake. These results enrich the mechanism of NO signaling in plant-microbe interactions.

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