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Endophytic fungus *Serendipita indica* increased nutrition absorption and biomass accumulation in *Cunninghamia lanceolata* seedlings under low phosphate

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ABSTRACT

Cunninghamia lanceolata is important forest tree species in southern China, and its successive plantations resulted in degradation of soil fertility in pure stands, causing decline in forest productivity. How to improve productivity in *C. lanceolata* pure stands is a tough task. Usage of mycorrhizal fungi might be a plausible access to the task. The objective is to study the possibility of the endophytic fungus *Serendipita indica* (named formerly as *Piriformospora indica*) in culture of *C. lanceolata*. Seeds were sowed in plastic pots with river sand. When seedlings had two true leaves, hyphae suspension solution of *S. indica* was added to near the roots of seedlings in each plastic pot. Such pots with seedlings were placed in a greenhouse and normal management was carried out for the seedlings. Symbiosis effects on root development, nutrition uptake and allocation, and biomass accumulation of *C. lanceolata* seedlings under low phosphate were investigated. The results showed that *S. indica* could symbiose with *C. lanceolata*. The symbiosis did not result in significant changes in root system architecture under low phosphate, but significantly increased nitrogen and phosphorus levels in leaves under low phosphate. Although the symbiosis did not significantly increased nitrogen allocation in leaves under low phosphate, it significantly increased phosphorus allocation in leaves. The interaction between *S. indica* and *C. lanceolata* resulted in increase in total biomass under low phosphate and changes in biomass allocation between shoots and roots. The results suggested that *S. indica* helps host plants to absorb more nutrients under low phosphate and to allocate more nitrogen and phosphate to leaves, promoting plant growth; the fungus might be used in pure stands of *C. lanceolata* because of its large-scaled axenic culture.

1. Introduction

Phosphate is a major macronutrient needed by plants for their development, health, and productivity, but available phosphate is very low in soil all over the world because most phosphate in soil is insoluble and unavailable forms, with an average of available phosphate of ~ 2 $\mu mol \cdot L^{-1}$ in the soil solutions, which is several orders of magnitude lower than that in plant tissues (5–20 mM) [1]. In highly weathered soils, sandy soils and alkaline soils, available phosphate levels are commonly <1 $\mu mol \cdot L^{-1}$, and even in the most fertile soils, available phosphate levels in soil solution rarely exceed 8 $\mu mol \cdot L^{-1}$ [2]. Furthermore, diffusion rate of phosphate is very slow in soil (10–12 to 10–15 $m^2 \cdot s^{-1}$) [3], thus there are often depletion zones of phosphate around plant roots (i.e., rhizosphere) in phosphate-deficient soil. Therefore, Plant growth and development is limited by the phosphate availability in most natural ecosystems.

Plants have evolved to possess two distinct models to enhance absorption of phosphate from soil. The direct model is that plants use their own phosphate transporters in roots and carry out changes in root morphology, biochemistry, and physiology, especially great increase in the numbers of lateral roots [4] and root hairs [5–7], secretion of organic acids [8], and formation of cluster [9, 10] and dauciform roots [11–14] under phosphate deficiency. The indirect model is that plants symbiose with mycorrhizal fungi and other microorganisms [15–17]. In the symbiosis system, host plants provide the mycorrhizal fungi with carbohydrates, and the latter helps their hosts to absorb nutrients, especially phosphate.

Serendipita indica (formerly known as *Piriformospora indica*, [18]) is endophytic fungus found in an arbuscular mycorrhizal fungal spore from a low-nutrient desert soil in Rajasthan, India [19], and shows the same functions as mycorrhizal fungi did, especially increase in nutrition uptake and utilization [20–22]. *S. indica* has some unique properties, such as artificial propagation by axenic cultures [23, 24] and broad host plants, including *Arabidopsis thaliana* [25–32]. Usually, *A. thaliana* is regarded as non-mycorrhizal [33–36]. Therefore, its properties will

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be very useful in sustainable agriculture and forestry, especially under stress environment [37–39].

Cunninghamia lanceolata (Chinese fir) is an important commercial tree species in southern China with large plantation area, however its successive plantations resulted in degradation of soil fertility, causing decline in forest productivity [40–45]. How to maintain soil fertility and productivity in pure stands of *C. lanceolata* is a very important scientific question to foresters in southern mountains. In recent decades, some attempts have done to maintain soil fertility in pure stands of Chinese fir. After the vegetation change from evergreen broad-leaved stands to pure plantations of Chinese fir, the microbial biomass carbon and the quantities of culturable bacteria and actinomyces decrease, and structure of microbial communities showed great changes in pure plantations of Chinese fir, compared to evergreen broad-leaved stands [46]. The total number of microbes in the in the second-rotation pure stands of Chinese fir was less than that of the first-rotation pure stands [47]. The change resulted in reduction in litter mineralization rates and nutrition uptake in pure stands of Chinses fir. The application of biofertilizers should be a good approach to increase litter decomposition rates and nutrition uptake. For the aim, some rhizospherical microbes were isolated and identified for Chinese fir, such as plant growth-promoting rhizobacteria [48] and identification of mycorrhizal fungi [49]. Few studies were involved to the effects of microbial inoculation on survival rate and growth of seedlings of Chinese fir in field [50]. However, more detailed researches have not carried out, especially effects of rhizospherical microbes on nutrition uptake and growth and development of Chinese fir, and usage of biofertilizers has not carried out in pure plantations of Chinese fir and seedlings nurseries. In the present article, our aim is: (1) to investigate the effects of *S. indica* colonization on growth, nutrition absorption and allocation of *C. lanceolata* seedlings under different phosphate levels in order that the fungus might be used in pure stands of *C. lanceolata* to increase stand productivity; (2) to reveal the reason why *S. indica* promotes plant growth.

2. Materials and methods

2.1. Growth conditions of fungus and plant

Endophtic fungus *Serendipita indica* was obtained from profressor Ralf Oelmüller in Friedrich-Schiller-University Jena, Germany. The growth medium and growth condition of fungus were carried out according to the description by Johnson et al. [51].

In order to investigate the effects of colonization of *S. indica* on root system architecture of *C. lanceolata* seedlings, seeds were sterilized using 0.1% (*w*/*v*) $HgCl_2$ and sowed in plastic pots filled with river sands of the same weight. The river sands were cleaned and sterilized before they were placed in the plastic pots (high 10 cm and diameter 10 cm). The plastic pots were cultivated in a greenhouse (16 h ligh/8 h dark, 25 °C).

2.2. Experimental design

After the seeds germinated and the seedlings had two true leaves, 3 mL of 10 $g \cdot L^{-1}$ hyphae suspension solution of *S. indica* was added to near the roots of seedlings in each plastic pot, and the control was added the same volume of sterilized hyphae suspension solution. After a week, four seedlings were selected in a plastic pot and other seedlings were picked out for investigation on colonization of *S. indica*. Another three days later, seedlings were provided with the same volume of MS solutions with different phosphate levels every three days. There were four treatment ways: high phosphate (1 $mmol \cdot L^{-1}$) with *S. indica* colonization (i.e., HP + P), high phosphate without *S. indica* colonization

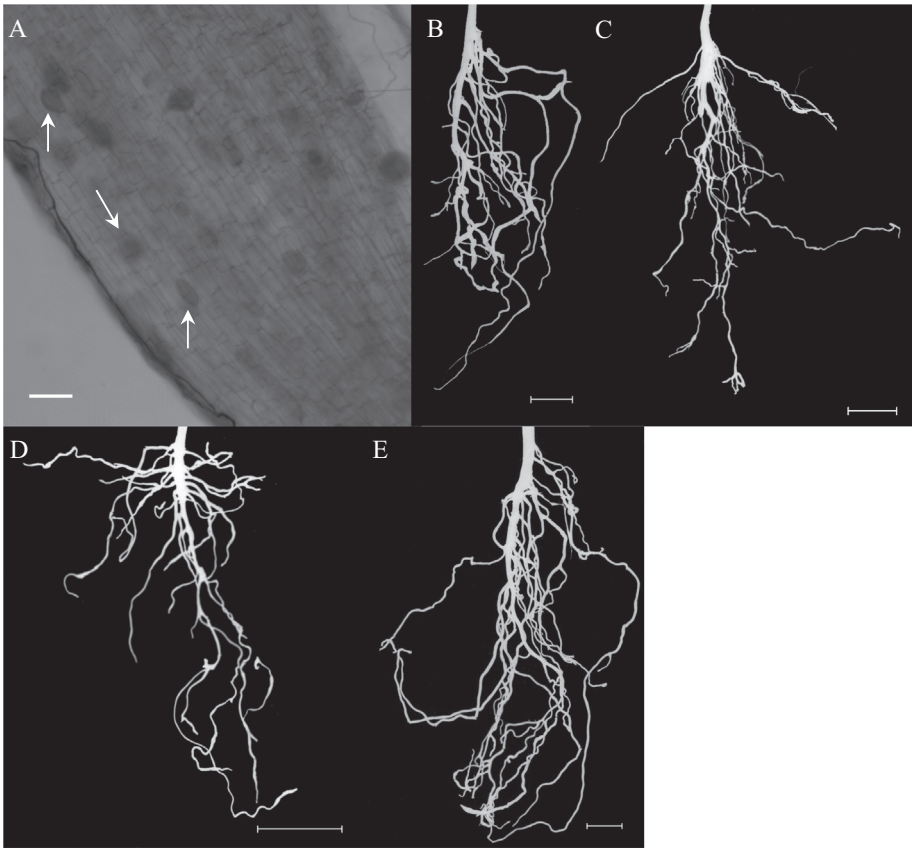

Fig. 1. *S. indica* infection and effects of *S. indica* infection on root systems of *C. lanceolata* seedlings under different phosphate levels. A: *S. indica* infection in roots of *C. lanceolata* seedlings. Arrows indicate the spores of *S. indica* in the cortex of roots, and the bar = 100 μm. B–E. Root architectures of the seedlings under the condition of HP + P, LP + P, HP − P, LP − P, respectively, and the bar = 0.5 cm.

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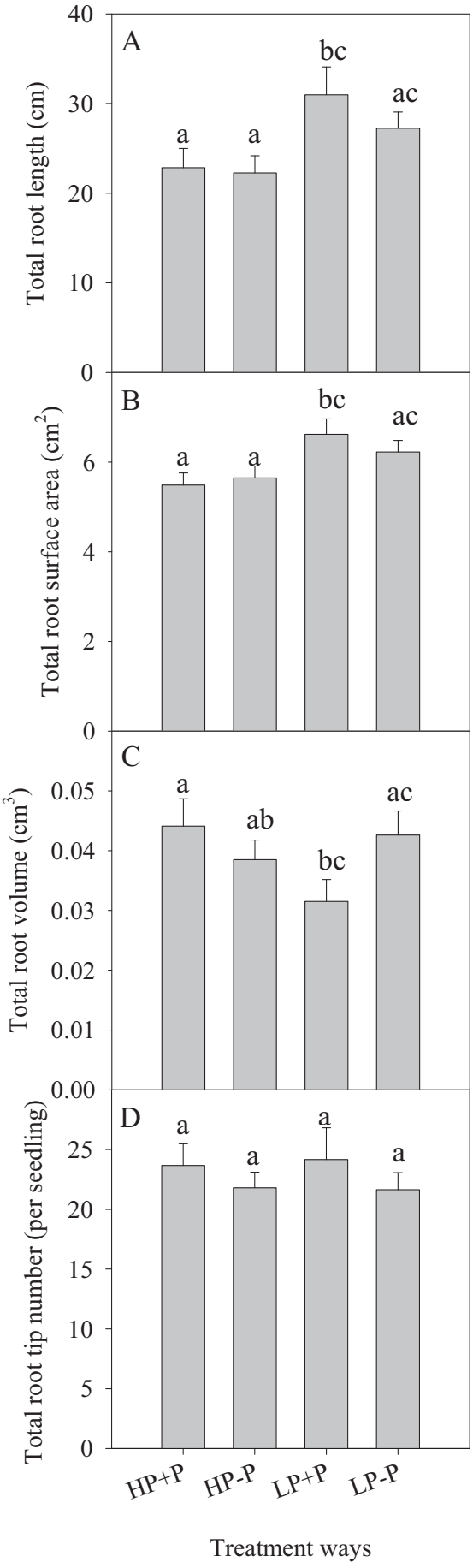

(i.e., HP − P), low phosphate (5 μmol·L^{-1}) with *S. indica* colonization (i.e., LP + P), and low phosphate without *S. indica* colonization (i.e., LP − P). 15 pots for each treatment and 60 seedlings in each treatment. The seedlings were used for investigation on the effect of the endophytic colonization on root system architecture under phosphate deficiency.

In order to investigate the effects of the colonization of *S. indica* on nutrition states of *C. lanceolata* seedlings, six month-old seedlings were also planted in plastic pots filled with the cleaned and sterilized river sands, each pot (high 20 cm and diameter 20 cm) with four seedlings. The pots were transported into a greenhouse immediately, and were provided with 3 mL of 10 g·L^{-1} hyphae suspension solution of *S. indica* after a week, the control was provided with 3 mL of sterilized hyphae suspension solution with the concentration of 10 g·L^{-1}. The seedlings were provided with MS solutions with different phosphate levels as mentioned above, 40 seedlings for each treatment, 10 pots for each treatment.

All experimental materials were in a glasshouse for culture, and MS solutions with different phosphate levels were provided each two days, 100 mL each time for a pot.

2.3. Staining of S. indica spores and micrograph

Twenty days after transplanting, few seedlings incubated by the fungus were selected randomly, and their roots were separated from the aerial parts and washed with tap water. These roots were put into 10% KOH solution overnight and subsequently 1% HCl for 10 min. Roots were stained in 0.05% trypan blue for 30 min and washed in distilled water for 1 min. Finally the stained root was mounted on glass slide with 50 μL GL solution and covered with glass cover [52], and micrography was carried out (Nikon, DS-Ri2).

2.4. Root scanning, biomass determination, and allometric growth analysis

After treatment in eight months, thirty seedlings from seeds were chosen randomly and excavated and washed with tap water. The whole root systems were cut from the individual plant, and subsequently moisture was removed with absorbent paper. The resulting dried roots were scanned on WinRhizo root scanner (Regent Instruments, Quebec, Canada). Images from the scanner were processed by using the software Image-Pro Plus (v.6.0, Media Cybernetics, USA), and root system parameters (total root length, total root surface, total root volume, and number of root tips) were obtained using the software WinRhizo (V5.0, Regent Instruments, Quebec, Canada).

The individual root systems and their corresponding shoots were sealed in the same envelopes and were dried in an air oven at 80 °C for 24 h before weighing. The dried roots, stems, and leaves were left for nutrition analyses as mentioned below.

Twelve seedlings with initial age of six months were randomly chosen for allometric growth analysis. Allometric growth analysis was carried out as described by Hunt [53].

2.5. Nutrition analyses

Eight months after hyphae suspension solution was added, three seedlings with initial age of six months were randomly chosen, and total nitrogen, phosphate, and potassium in their roots, stems, and leaves were determined. Total potassium was determined by flame AES, and total nitrogen and phosphorus were determined by spectrophotometry as described [54].

Fig. 2. Effects of *S. indica* infection on root systems of *C. lanceolata* seedlings under different phosphate levels. For the same root parameter, the bars with different letters show significant differences ($p < 0.05$).

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2.6. Statistical analysis

The data were analyzed by using one-way ANOVA (SPSS, version 17.0) to compare the differences in root system architecture, nutrition uptake and allocation, and biomass accumulation, with means being compared by LSD at the 5% level. Regression analysis was used allometric growth.

3. Results

3.1. Endophytic colonization and changes in root system architecture

S. indica has a wide range of hosts, and it also colonized in the cortex of the roots of *C. lanceolata* (Fig. 1A), and such colonization affected the root system architecture (Fig. 1B–E).

Under high phosphate condition (1 mmol·L^{-1}), total root length of *C. lanteolata* seedlings did not significantly increased whether these seedlings were infected by *S. indica* or not ($p > 0.05$, Fig. 2A). In the colonized root systems, the total root length of seedlings under low phosphate treatment was significantly increased, compared with high phosphate ($p < 0.05$, Fig. 2A). Under low phosphate, whether *S. indica* colonized in roots of *C. lanceolata* seedlings or not, their total root length did not change significantly ($p > 0.05$, Fig. 2A). The similar pattern was also observed in total root surface area (Fig. 2B).

The total root volumes of seedlings treated with HP + P were significantly higher than those treated with LP + P ($p < 0.05$, Fig. 2C), and there were no significant differences between other treatments ($p > 0.05$, Fig. 2C). There were no significant changes in total root tip numbers among all the four treatments ($p > 0.05$, Fig. 2D).

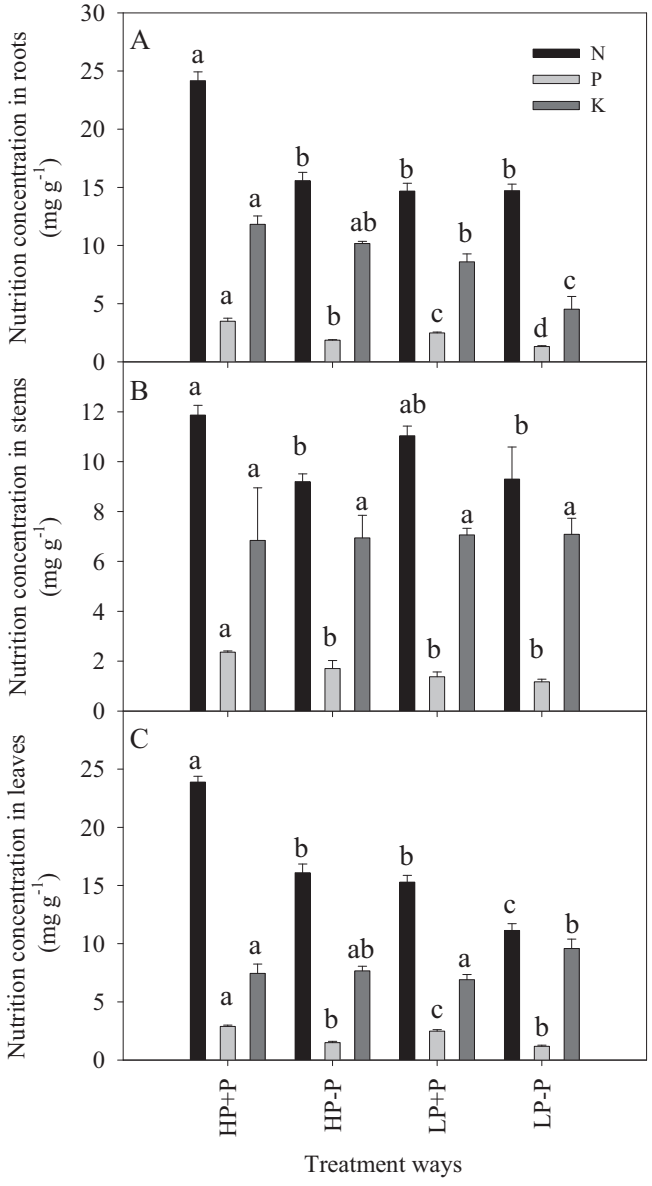

Fig. 3. Effects of *S. indica* colonization on nutrition concentrations in roots (A), stems (B) and leaves (C) of *C. lanceolata* seedlings under different phosphate levels. The results are shown as mean ± SE ($n = 3$). For the same element in each small figure, the bars with different letters show significant differences ($p < 0.05$).

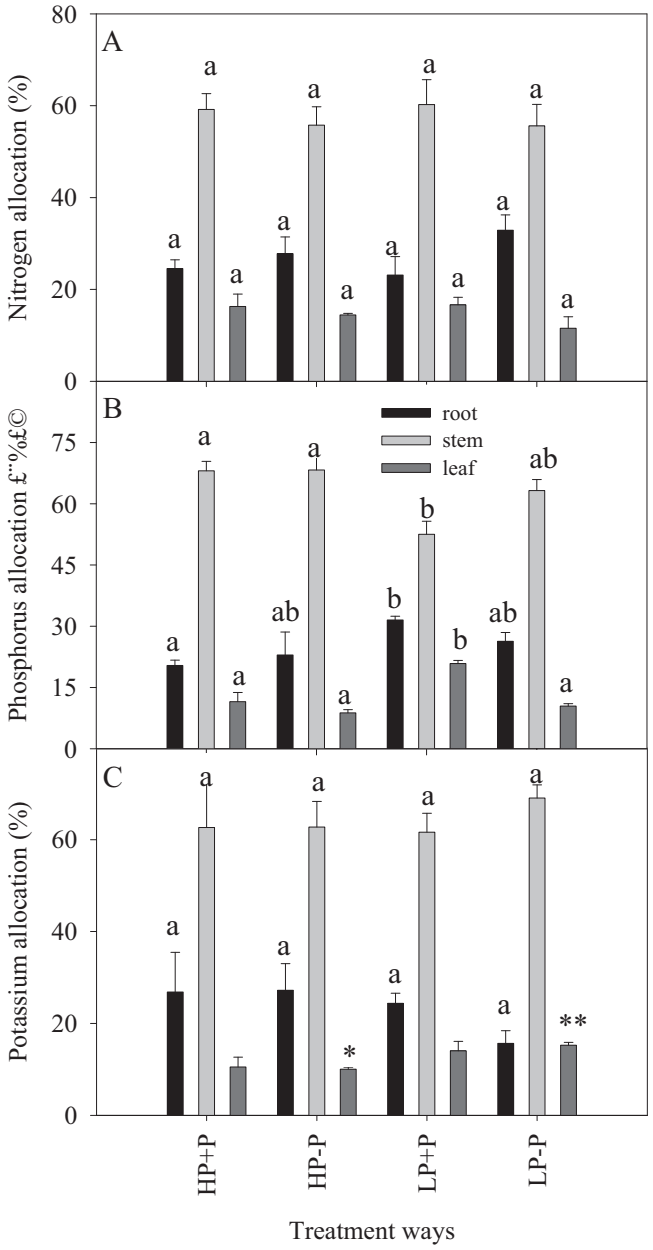

Fig. 4. Effects of *S. indica* colonization on allocation of nitrogen (A), phosphorus (B), and potassium (C) in roots, stems, and leaves of *C. lanceolata* seedlings under different levels. The results are shown as mean ± SE (n = 3). For the same organ, the bars with different letters show significant differences ($p < 0.05$). In C, for potassium allocation in leaves, the different numbers of asterisks show significant difference ($p < 0.05$), and the other comparisons show no significant differences ($p > 0.05$).

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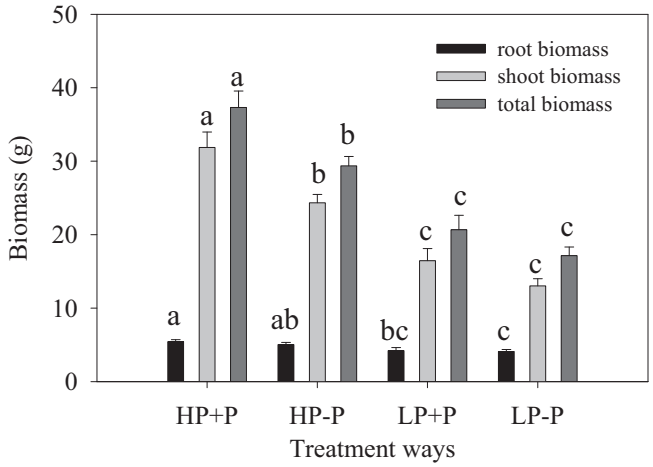

Fig. 5. Effects of *S. indica* colonization on biomass accumulation of *C. lanceolata* seedlings under different phosphate levels. The bars with different letters show significant differences ($p < 0.05$).

3.2. Effects of endophytic colonization on nutrient concentrations

S. indica colonization affected the concentrations of nitrogen, phosphate, and potassium in the roots, stems, and leaves of *C. lanceolata* seedlings (Fig. 3). Under high phosphate treatment (1 mmol·L^{-1}), *S. indica* colonization significantly increased nitrogen levels in roots, compared with the other three treatments ($p < 0.05$, Fig. 3A). Under low phosphate treatment (5 μmol·L^{-1}), the colonization did not affect nitrogen levels in roots, compared with LP − P ($p > 0.05$, Fig. 3A). Under high phosphate treatments, phosphorus level was greater in colonized roots than in the non-colonized, such changes also observed under low phosphate treatments ($p < 0.05$, Fig. 3A). In addition, potassium levels did not show significant changes in neither colonized nor non-colonized roots under high phosphate treatments ($p > 0.05$, Fig. 3A), but significantly differed under low phosphate ($p < 0.05$, Fig. 3A), with greater concentration in colonized roots.

Colonization of *S. indica* did not significantly affect nitrogen levels in stems under low phosphate treatment ($p > 0.05$, Fig. 3B), and nitrogen levels in stems did not show differences between HP + P and LP + P treatments ($p > 0.05$, Fig. 3B). Similarly, the colonization of *S. indica* did not affect phosphorus levels in stems under low phosphate treatment (Fig. 3B). There were no significant changes in potassium levels in stems among all the four treatments ($p > 0.05$, Fig. 3B).

Under low phosphate treatment, the colonization of *S. indica* increased significantly levels of nitrogen and phosphorus in leaves, compared with seedlings without colonization ($p < 0.05$, Fig. 3C). In all the four treatments, there only was a significant difference in potassium levels in leaves between HP + P and LP − P ($p < 0.05$, Fig. 3C).

Nitrogen allocation among roots, stems, and leaves of the seedlings did not show significant differences among all the four treatments ($p > 0.05$, Fig. 4A). Although *S. indica* colonization resulted in 30.80% increase in phosphorus allocation in leaves under low phosphate treatment, compared to those in leaves of seedlings without colonization, there was no significant difference between them($p > 0.05$, Fig. 4A). Phosphorus allocation in roots of seedlings under HP + P was significantly higher than that in roots of seedlings under LP + P ($p < 0.05$, Fig. 4B). Similar differences in phosphorus allocation in stems occurred (Fig. 4B). Under low phosphate treatment, colonization of the fungus significantly increased phosphate allocation in leaves ($p < 0.05$, Fig. 4B).

Potassium allocation in roots and stems did not show significant differences among all the four treatments ($p > 0.05$, Fig. 4C), and there only

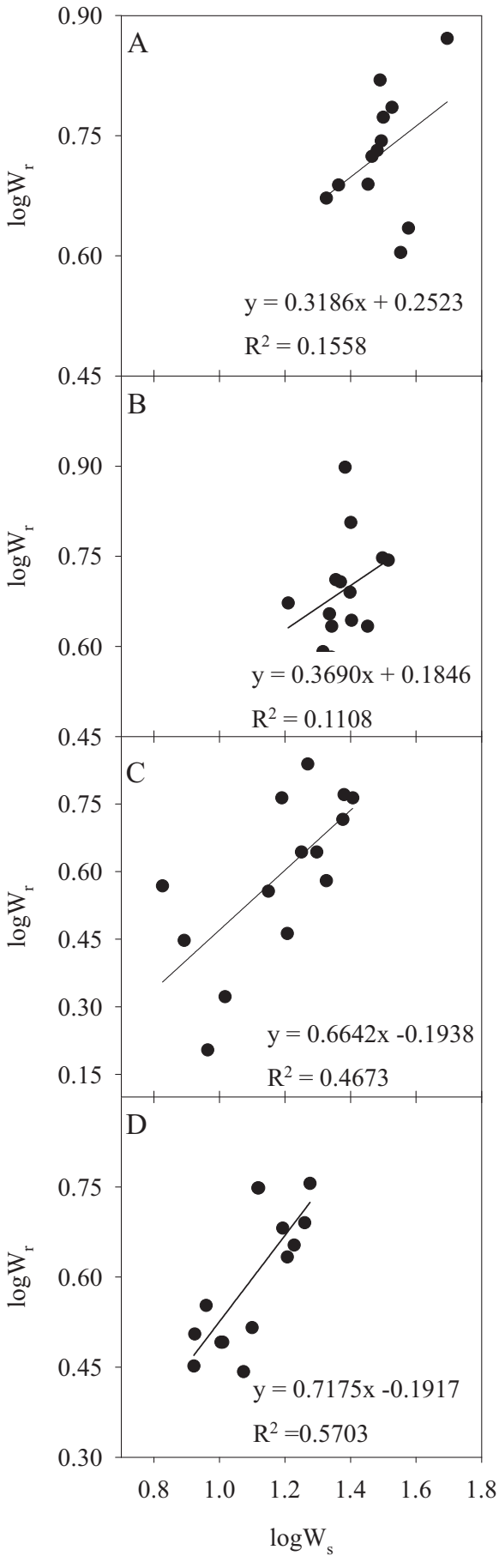

Fig. 6. Effects of *S. indica* on allometric growth of *C. lanceolata* seedlings under different phosphate levels. A: HP + P; B: HP − P; C: LP + P; D: LP − P.

was significant difference in potassium allocation in leaves of seedlings under HP − P and LP − P ($p < 0.05$, Fig. 4C).

3.3. Biomass accumulation and allometric growth

Colonization of *S. indica* did not significantly increased root and shoot biomass accumulation (compared with un-colonization) under low phosphate treatment ($p > 0.05$, Fig. 5), thus total biomass of seedlings under LP + P was not significantly higher than those of the seedlings under LP − P ($p > 0.05$, Fig. 5), although the colonization increased total biomass accumulation.

Colonization of *S. indica* in roots of seedlings of *C. lanceolata* changed allometric growth of seedlings (Fig. 6). Under high or low phosphate, allometric coefficient of the colonized seedlings (i.e., slope coefficient of the linear equation) was less than that of non-colonized seedlings (Fig. 6), suggesting the colonization of *S. indica* resulted in less biomass allocation to roots.

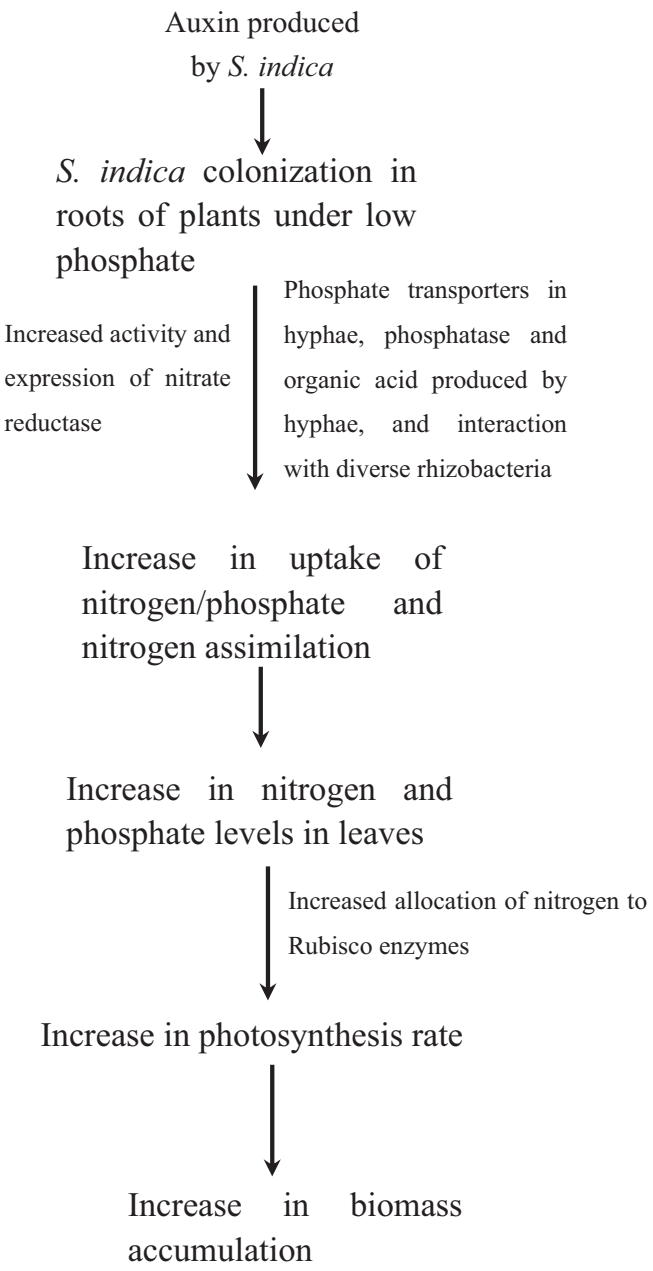

Fig. 7. Colonization of *S. indica* promotes plant growth under low phosphate.

4. Discussion

Some previous studies showed that colonization of *S. indica* improved plant growth and development [24, 32, 55–58], but the growth promotion is not induced by mycelium-synthesized auxin [57]. It is of significance to explore how colonization of *S. indica* promotes the growth of host plants.

At first, colonization of *S. indica* affected root development such as growth, which is known to be related to auxin concentration in root tissues [59–61]. There are two important aspects in the relationship. Firstly, auxin promotes colonization of roots by *S. indica*, which produces auxin, IAA [62, 63]. Although the growth of the host plant is not stimulated by mycelium-synthesized auxin [57, 64], auxin and indole derivatives are required for biotrophic colonization by *S. indica* [64]. Secondly, colonization by *S. indica* increases auxin levels in roots and promotes root hair development and root growth. For example, the auxin level in the Chinese cabbage roots infected by *S. indica* was twofold higher compared to un-colonized controls [65]. This increased auxin level resulted in the strong stimulation of root hair development (a bushy root phenotype) [65]. Vadassery et al. [63] showed that free IAA levels were highly reduced in Arabidopsis *sur1–1* mutant (an auxin overproducer) colonized by *S. indica*, while the conjugated IAA level was increased. It was found that fresh weight of roots of both mutant seedlings and wild type was increased upon colonization by *S. indica* [63]. In the present study, colonization of roots of *C. lanceolata* seedlings by *S. indica* significantly increased total root length and root surface under low phosphate, compared to high phosphate (Fig. 2A, B). The increase in total root length and root surface under low phosphate treatments seems not to be related to colonization of *S. indica*, because total root length and surface did not show significant changes under such conditions (Fig. 1A, B). Therefore, phosphate signaling seems to play a more important role in root system development under low phosphate [66].

Next, colonization of *S. indica* promotes nutrition absorption and assimilation. *S. indica* enhances the expression of the gene encoding nitrate reductase in roots of *A. thaliana* and also stimulates the expression of the Arabidopsis nitrate reductase (Nia2) in transgenic tobacco seedlings [67]. In addition, colonization of *S. indica* in roots of tobacco seedlings caused 50.2% increase in the plant specific NADH-dependent nitrate reductase activity in the roots, and also mediates nitrate uptake [67].

At the same time, *S. indica* promotes phosphate absorption of host plants [20, 22, 68, 69, 83]. The fungus produces high amounts of phosphatase [70], and it can indirectly solubilize soil phosphorus-reserves by interacting/communicating with diverse rhizobacteria, which have inorganic phosphate-solubilizing capabilities by virtue of production of a variety of organic acids and acid phosphatases [71–73]. For example, co-inoculation of *S. indica* with phosphate-solubilizing rhizobacteria *Pseudomonas striata* resulted in enhanced phosphate uptake and phosphate content in host plant due to better establishment of phosphate-solubilizing rhizobacteria in the mycorrhizaopshere [71]. Increased uptake of phosphorus from the medium and phosphorus translocation to the host and a sharp increase in phosphorus content in the shoot were mediated by *S. indica* [69, 74]. Moreover, hyphae of *S. indica* help phosphate absorption. In *S. indica*, a high-affinity phosphate transporter named PiPT, which is localized in the external hyphae, has been isolated and identified [22]. The phosphate transporter plays a role in phosphate transport to its host plants [20, 22, 83]. Expression of PiPT in fungal mycelia depends on the availability of soluble phosphate in soil. Real-time PCR analysis indicated that the PiPT gene is not expressed in phosphate-rich conditions, but is highly expressed under phosphate-deprived conditions [20].

These roles of colonization of *S. indica* in phosphate/nitrogen uptake and assimilation can be used to explain why nitrogen and phosphate levels in the leaves of *C. lanceolata* seedlings under low phosphate (Fig. 3C) and high phosphate allocation in leaves (Fig. 4B). Increased

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nitrogen and phosphate levels in leaves of *C. lanceolata* seedlings might be the main reason that *S. indica* promotes plant growth under low phosphate.

Under low phosphate condition, colonization of *S. indica* significantly increased nitrogen and phosphate levels in leaves of *C. lanceolata* seedlings (Fig. 3C), and phosphate can affect nitrogen allocation to Rubisco [75].

The increased nitrogen levels in leaves promote photosynthesis. A large part of leaf nitrogen is invested in photosynthetic apparatus, i.e., chloroplasts. In mature rice leaves, about 80% of the total leaf nitrogen is allocated to chloroplasts [76] as is the case with other C3 plants [77, 78]. Ribulose bisphosphate carboxylase oxygenase (Rubisco), the most important enzyme in carbon fixation, occupies the most part of soluble proteins in leaves [79–81], and phosphate can affect nitrogen allocation to Rubisco [75].Therefore, it is speculated that the leaves with high nitrogen and phosphate levels under low phosphate should have high concentrations of Rubisco, increasing photosynthesis rate, because photosynthesis rate is related to concentrations of nitrogen and Rubisco in leaves [82]. The significant increase in nitrogen and phosphate levels in the leaves of *C. lanceolata* seedlings might result in increment in photosynthesis rates, thus causing high biomass accumulation under low phosphate (Fig. 5).

According to the results and the facts mentioned above, the reason why colonization of *S. indica* promotes plant growth is outlined in Fig. 7.

Some details should be more paid on in Fig. 7: (1) ammonium is one of main nitrogen forms in soils, how about the effects of colonization of the fungus on ammonium uptake and assimilation? (2) what are the effects of the colonization on root hair formation under low available phosphate? (3) how about the activity and expression of phosphate transporters in the hyphae of the fungus under low available phosphate? Therefore, more studies are needed to answer the questions.

Conclusion: (1) *S. indica* helps host plants to absorb more nutrients under low phosphate and to allocate more nitrogen and phosphate to leaves, promoting plant growth; (2) the fungus might be used in pure stands of *C. lanceolata* because of its large-scaled axenic culture.

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