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REGULAR ARTICLE

Piriformospora indica enhances phosphorus absorption by stimulating acid phosphatase activities and organic acid accumulation in *Brassica napus*

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Abstract

Aims The root endophytic fungus *Piriformospora indica* (*P. indica*) colonizes the roots of a wide range of higher plants and promotes growth, disease resistance and stress tolerance of the hosts. We investigated the role of *P. indica* for phosphate (P) mobilization in soils enriched with different P sources and for P uptake into *Brassicae napus* (*B. napus*) plants.

Methods Seedlings of *B. napus* colonized by *P. indica* were cultivated in pots with sterilized-sands supplied with Ca_3 $(PO_4)_2$ [Ca_3-P], $AlPO_4$ [Al-P] or $FePO_4$ [Fe-P]. The growth of the seedlings, P content, phosphatase activities, amount of organic acids, and expression of the genes *BnACP5* for a phosphatase and *BnPHt1;4* for a P transporter were investigated.

Results Piriformospora indica promotes growth of *B. napus* and the accumulation of P in roots and shoots when P was supplied as Ca_3-P, Al-P or Fe-P in the soil. The endophyte stimulated the P availability for the plant by higher phosphatase activities and higher expression of *BnACP5* in roots exposed to soil with Ca_3-P, Al-P or Fe-P as main P source. The amounts of oxalic, malic and citric acids increased in rhizosphere soil with *P. indica* colonized by *B. napus* seedlings. Thus, root-colonization by *P. indica* promotes the accumulation of organic acids in the rhizosphere. Stronger up-regulation of *BnPht1;4* in colonized vs. non-colonized roots demonstrates the involvement of the fungus in counteracting P deficiency by promoting its uptake.

Conclusion P. indica promotes the mobilization of P from inorganic sources and P uptake into the roots of *B. napus*. This is a combined effect of the stimulation of the P solubilizing phosphatase activity in the symbiotic interaction, the production of organic acids as well as the stimulation of the *BnPht1;4* and *BnACP5* genes under P limitation conditions.

Keywords Acid phosphatase · Organic acids · Insoluble phosphorus · *Piriformospora indica* · *Brassica napus*

Meiyan Wu and Qiao Wei contributed equally to this work.

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Introduction

Phosphorous is one of the seventeen essential nutrients for plant growth (Raghothama 1999) and the second most important macronutrient after nitrogen for crop production. The plant dry weight contains up to 0.5%

phosphorus since it is required in huge amounts for nucleic acids, and it also involves in an array of other processes such as photosynthesis, or phospholipid functioning (Baleni and Negisho 2012). However, phosphorous is the least accessible macronutrient and hence often limiting in fertilizers used in agriculture. Its availability is not only low in soil but also in many agriculturally used fertilizers. Under acidic soil conditions, phosphate (P) forms scarcely soluble complexes with aluminum and iron and under alkaline soil conditions with calcium and magnesium (Baleni and Negisho 2012). Thus, for most of the soils on earth, accessibility of P limits crop production.

Rapeseed (*B. napus L.*) is one of the most important oil crops word-wide. It is estimated that the majority of the planting areas for *Brassica napus* in Asia are currently phosphorus deficient (Zhang et al. 2009; Yao et al. 2011). As a consequence, the P fertilizer application for *B. napus* planting is much higher than for the other crop species. Since excessive P fertilization entry into the soil pollutes the water and accelerates eutrophication (Yao et al. 2011), it is important to explore other ways to improve the access of *Brassica napus* to phosphorus.

Plants acquire P from the soil through direct uptake or indirectly via symbiotic mycorrhizal associations when they are formed (Lum and Hirsh 2003; Yadav et al. 2010; Gill et al. 2016). Mycorrhizal hyphae can penetrate the soil more efficiently than roots (Tinker et al. 1992; Tinker and Nye 2000; Lambers et al. 2008), and arbuscular mycorrhiza (AM) fungi also supports plant's P uptake by hydrolyzing organic P compounds through acid phosphatases which they release into the soil (Baleni and Negisho 2012). It is estimated that the contribution of AM fungi to P uptake can increase from 49% under high P conditions to 77% under P limitation conditions (Thingstrup et al. 2000).

Piriformospora indica, an axenically cultivable root-colonizing endosymbiotic fungus, shares many features with AM fungi, it can colonize roots of a wide range of higher plants including those which cannot from symbioses with AM fungi and promotes nutrient uptake, disease resistance, stress tolerance and growth of their hosts (Gill et al. 2016; Unnikumar et al. 2013; Xu et al. 2017; Hosseini et al. 2017; Hussin et al. 2017; Zhang et al. 2018). These features open the possibility for many applications in the field by using the fungus as a biofertilizer, bioregulator, growth and yield stimulator as well as a biocontrol agent (Malla et al. 2004). Growth promotion induced by *P. indica* has been related to changes in the production and signaling of phytohormone, such as ethylene, auxin, gibberrelin or cytokinin (Vadassery et al. 2008; Camehl et al. 2010; Sirrenberg et al. 2007), however, the contribution of the fungus to the nutrient uptake into the host's root is still not clear. Yadav et al. (2010) and Kumar et al. (2011) showed that the fungal P transporter PiPT participates in the promotion of maize growth by transferring P to the host under P-deprived condition. Shahollari et al. (2005) have shown that the uptake of radio-labelled P was improved by *P. indica* in Arabidopsis seedlings. In contrast, in barley and green gram, *P. indica* stimulated growth but not the overall amount of P in the hosts (Achatz et al. 2010; Ray and Valsalakumar 2010). The different observations can be caused by the different hosts, or different experimental conditions used for these studies. Here, we analyzed the role of *P. indica* for the P uptake in *B. napus* which was grown on media with different P sources.

The accessibility of P for the hosts in the soil depends on the available P form, the exudation of organic acids and/or protons and of phosphatase enzyme activities in the rhizosphere (Richardson and Simpson 2011). Ngwene et al. (2016) showed that *P. indica* solubilizes P from inorganic, but not organic P sources, and P solubilisation was not caused by enzymatic activities but rather decreasing pH in the medium. In contrast, Swetha and Padmavathi (2016) reported that a cell membrane preparation of the fungus has phosphatase activity in the presence of zinc phosphate. Also Malla et al. (2004) showed intracellular acid phosphatase activities of the fungus. That is, *P. indica* solubilizes P from inorganic P sources under in vitro conditions. However, it was not tested whether such an activity is symbiosis-specific or even stimulated in symbiotic interaction of *P. indica* with hosts. Therefore, we hypothesis that *P. indica* contributes to the P uptake of *B. napus* when different insoluble inorganic P forms were supplied, and then, we conducted the pot experiments with *P. indica* cultures to explore the ability of *P. indica* to release P from three different sources Ca_3-P, Al-P or Fe-P into the symbiotic system of *P. indica* with *B. napus*. It is important to improve the utilization rate of phosphate fertilizer and reduce the environmental pollution due to the loss of phosphorus in rapeseed production.

Materials and methods

P. indica co-cultivation with *B. napus* and treatments with different insoluble P forms

The *P. indica* fungus was cultivated in a 250 ml Erlenmeyer flask with Aspergillus (ASP) medium. Cultures were incubated at 26 °C in the dark by shaking at 150 rpm. After 14 days, the liquid was removed by filtration and excess culture medium was carefully removed from the mycelia.

Seeds of *B. napus* (97,009 cultivars) were washed with distilled water for 30 min, then surface-sterilized with 75% ethanol for 1 min and 2% NaClO for 15 min, and finally rinsed 5 times with distilled water. The seeds were distributed evenly on a wet double-layer filter paper (sterilized at 121 °C), placed in a light incubator (GTOP-500Y, Beijing, China) at 28 °C in the dark for 2 days, and then cultured in the light for 1 day. Germinated seeds were inoculated by adding 3 ml of a spore suspension from *P. indica*. As control 3 ml sterilized spore suspension solution without the fungus was used. These seeds in plates were kept in a growth chamber at 25 °C in the light for 14 days. After 14 days, the root systems of the colonized seedlings were examined by staining with trypan blue and analyzed under a Leica microscope (DM5000B, Germany). The rate of colonization was calculated by the ratio of the length of infected root segments to the total length of checked root segments.Colonized and non-colonized (control) seedlings were transplanted in plastic pots (6.5 cm × 8 cm × 7 cm) (1 plant/pot) with 250 g sterilized sand, which was supplemented with different P sources: no P (control), tri-calcium phosphate (Ca_3-P), iron phosphate (Fe-P) and aluminum phosphate (Al-P) at 1.0 g/kg P, respectively. For all 8 treatments, 16 replicates were performed. The pots were placed into a growth chamber at 25 °C with a 16 h-light and 8 h-dark photoperiod, and watered with 50 ml distilled water every second day and with 50 ml P-deficient Hoagland nutrient solution (Hothem et al. 2003) every 4th day. After 30 days, the aerial parts and roots were harvested separately.

Root scanning and P content analysis

After 30 days, the leaf areas of the second leaf from the top were determined for each plant by the plant image analyzer LA-S (Hangzhou, China). The roots were carefully washed with water and analyzed with the root scanner device and software WinRHIZO (Canada) for their total length, total surface area, total volume, average diameter and root tip number. After that, the shoot and root of the plants were dried at 65 °C in an oven for the determination of the dry weight. From these samples, the P content was determined with the SFA Segmented continuous flow analyzer (Alliance-Futura II, AMS, France).

Analysis of phosphatase activities in shoots and roots of *B. napus* and in the rhizospheric sand

Twelve seedlings per treatment removed from their pots and the sand attached to the root surface was collected and stored in sealed plastic bags at -20 °C. One-third of the sand was used for phosphatase activity assays, one-third for the determination of the organic acids, and the last part for P analysis. The assays were repeated 4 times. Phosphatase activities were determined spectrophotometrically using para-nitrophenol (pNPP) as substrate, according to Fornasier et al. (2011). Additionally, leaves and roots of *Brassica napus* seedlings were washed and immediately frozen in liquid nitrogen for RNA extraction.

RNA preparation and analysis of *BnACP5* and *BnPHt1;4* expression by RT-qPCR

Total RNA was extracted with TRIzol (Invitrogen) according to manufacturer's instructions. One microgram of total RNA was subjected to first-strand cDNA synthesis using the Prime-Script RT reagent kit with gDNA Eraser (Takara). RT-qPCR was performed by the SYBR green method using the StepOne Plus Real-time PCR system (Applied Biosystems). Real-time quantitative reverse transcription PCR and the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) were used to calculate the cycle threshold value of each sample. Data are means ± SD ($n = 4$) of four replicates. RT-qPCR primers designed by Primer 5 are listed in Table 1.

Analysis of organic acids and pH in the rhizosphere sands

The organic acids in the rhizospheric sand were analyzed by adding 10 ml of 5% 0.01 M KH_2PO_4 solutions

(pH 2.73) to 3.0 g sands. The slurry was sonicated for 15 min and centrifuged at 4000 rpm for 10 min. The supernatant was then filtered through a 0.45 μm filtration membrane and 10 μl was subjected to Agilent 1200 Series HPLC (Agilent Technologies Inc., CA, USA) using a C18 column with 100% methanol for 5 min followed by 15 min 95% methanol and 5% 0.01 M KH_2PO_4, pH 2.73. The flow rate was maintained at 0.8 ml/min (Yin et al. 2015). Peaks were quantified using the following standards in 0.01 M KH_2PO_4, pH 2.73: oxalic (10.05 μg/ml), malic (102.3 μg/ml), citric (112.8 μg/ml), acetic (103.9 μg/ml) and tartaric (50.25 μg/ml) acids. Non-inoculated media was used as the control. The organic acid content was calculated by using the external standard peak area. The pH value was measured by a laboratory pH meter (FE28, Mettle Toledo, Swiss). The supernatant was adding 25 ml sterilized distilled water to 10 g sands. The pH of the sands before planting was 6.78.

Statistical analysis

The data obtained from four independent experiments were analyzed using SAS 9.2 and Microsoft Excel. All data were subjected to analyses of variance (ANOVA). Significant differences between treatments were analyzed by Duncan test. Differences were considered significant when *p*-values were below 0.05. Each value represents the mean of four independent experiments performed in triplicate.

Results

Growth of *B. napus* cultivated with or without *P. indica* and analysis of the root structure under different insoluble P forms

Microscopic inspection of *B. napus* roots inoculated with *P. indica* and stained with trypan blue showed that the fungus colonized successfully the root cortex (Fig. 1). The colonization rate with 80.2% was quite high, and we often found spores within the root cells (Fig. 1). *P. indica* stimulated positively the leaf areas and shoot dry weights of *B. napus* seedlings already when the seedlings were watered with media without any additional P source (Table 2, S1 Fig). The stimulatory effects of the fungus were even stronger when the seedlings were treated with media containing Ca_3-P, Al-P or Fe-P. Also the root growth parameters were significantly greater for *P. indica* colonized plants, and the highest values for the total root lengths, surfaces, average diameters, total volumes and number of root tips were observed for seedlings exposed to Ca_3-P solution (Table 3).

Table 1 Primer sequences of P stress-responsive genes in *B. napus* probed in RT-PCR assays

Gene name	Sequences
BnACP5	F: 5′-ttcgtagtcaacgcagagttagtcg-3’
	R: 5′-tggattgttggtcgtcgggtc-3’
BnPht1;4	F: 5′-gtaccggcggagatcttcccagc-3′
	R: 5′-ctacacaatggggaccgttc-3’

F Forward primer; *R* Reverse primer

Effect of *P. indica* on the P concentration in shoot and root of *B. napus* and rhizosphere sand supplied with different P sources

The P concentration in shoots and roots of colonized and non-colonized *B. napus* by *P. indica* was grown on the soil supplemented with the different insoluble P sources (Fig. 2a, b). In all cases, the amounts of P in shoots and roots were higher in the presence of *P. indica*. The highest amount was measured for roots and shoots of plants exposed to Ca_3-P and *P. indica.* Furthermore, the detectable P content in the rhizosphere sand samples was also higher when *P. indica* was present compared to samples from non-colonized plants, the values

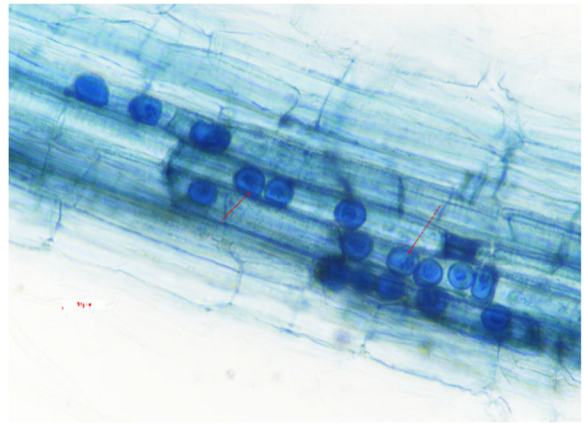

Fig. 1 Colonization of *B. napus* roots by *P. indica.* After inoculation of the roots with a spore suspension of *P. indica* as described in the Methods part, co-cultivation of both symbionts was performed in soil for 14 days

Table 2 Leaf area of *B. napus* plants grown with (+) or without (−) *P. indica* on different inorganic phosphate sources

Treatments	Leaf area (cm^2)	Shoot dry weight (g)	Root dry weight (g)
C-	11.0 ± 1.6^{e}	0.082 ± 0.008^{f}	0.042 ± 0.004^{c}
C+	19.5 ± 1.3^{d}	0.094 ± 0.004^{e}	0.044 ± 0.002^{c}
Ca_3-P-	29.9 ± 0.9^{c}	0.145 ± 0.006^{d}	0.050 ± 0.004^{bc}
Ca_3-P+	47.2 ± 1.5^{a}	0.173 ± 0.009^{cd}	0.060 ± 0.004^{ab}
Al-P-	39.8 ± 5.7^{b}	0.301 ± 0.010^{b}	0.069 ± 0.004^{a}
Al-P+	48.6 ± 2.9^{a}	0.334 ± 0.008^{a}	0.072 ± 0.003^{a}
Fe-P-	29.7 ± 0.9^{c}	0.152 ± 0.005^{d}	0.039 ± 0.002^{c}
Fe-P+	$40.1 \pm 1.0_{b}$	0.204 ± 0.012^{c}	0.058 ± 0.002^{b}

+: Inoculation with *P. indica*; −: Non-inoculation with *P. indica*. C: control; Ca_3-P: tri-calcium phosphate; Al-P: aluminum phosphate; Fe-P: iron phosphate. Data are average values ±SD ($n = 4$) calculated from four independent experiments

Statistically significant differences are marked with letters ($p < 0.05$)

increased 1.4- and 2.6-fold on soil supplemented with Ca_3-P and Fe-P, respectively, and there was no difference between *P. indica* colonized and non-colonized plants with Al-P (Fig. 2c).

The effect of *P. indica* on phosphatase activities in shoots and roots of *B. napus*

The phosphatase activities in shoots and roots of *B. napus* with *P. indica* showed different trends depending on the application of Ca_3-P, Al-P or Fe-P solutions (Fig. 3a, b). Phosphatase activity was only stimulated in shoots when *P. indica* colonized plants were grown on sand with Ca_3-P or Fe-P solutions (Fig. 3a). However, in roots, the stimulatory effect of the fungus on the phosphatase activities was the highest for Al-P, followed by Ca_3-P and Fe-P (Fig. 3b).

BnACP5 and *BnPHt1;4* expression in shoots and roots of colonized and non-colonized *B. napus* on the different P sources

P. indica had different effects on the expression of the *BnACP5* and *BnPHt1;4* in shoots and roots of *B. napus* exposed to the different P sources (Fig. 4a, b). Expression of *BnACP5* encoding a phosphatase was significantly increased by the fungus in roots exposed to Ca_3-P, Al-P or Fe-P (Fig. 4a). However, in shoots, a stimulatory effect of the fungus could only be detected for plants exposed to Ca_3-P (Fig. 4a).

The expression of *BnPHt1;4* encoding a phosphate transporter of the PHT1 family in roots was also stimulated by *P. indica* under all treatments (Fig. 4b), in addition, only Ca_3-P showed a stimulatory effect of the fungus in shoots. These results suggest that the effect of

Table 3 The effect of *P. indica* on *B. napus* root parameters treated under different inorganic phosphate sources

Treatments	Total length (cm)	Total surface Area(cm^2)	Average diameter(cm)	Total volume	Root tip number (n)
C-	771.0 ± 54.0^{d}	60.1 ± 5.2^{e}	0.202 ± 0.004^{e}	0.426 ± 0.039^{d}	1382.2 ± 87.8^{e}
C+	911.6 ± 56.6^{c}	71.2 ± 4.4^{de}	0.249 ± 0.003^{d}	0.443 ± 0.029^{d}	1985.2 ± 112.8^{d}
Ca_3-P-	839.7 ± 52.5^{cd}	97.8 ± 6.9^{bc}	0.372 ± 0.015^{a}	0.921 ± 0.090^{ab}	3001.9 ± 153.1^{b}
Ca_3-P+	1397.8 ± 30.2^{a}	145.2 ± 3.2^{a}	0.331 ± 0.006^{b}	1.205 ± 0.040^{a}	3990.2 ± 276.4^{a}
Al-P-	950.4 ± 45.1^{bc}	75.3 ± 4.1^{c}	0.251 ± 0.003^{cd}	0.476 ± 0.029^{cd}	1816.4 ± 77.2^{d}
Al-P+	972.5 ± 38.8^{b}	80.4 ± 3.9^{c}	0.262 ± 0.004^{c}	0.530 ± 0.031^{c}	2706.3 ± 109.6^{c}
Fe-P-	862.9 ± 28.5^{c}	74.9 ± 3.0^{cd}	0.276 ± 0.005^{c}	0.726 ± 0.039^{b}	2473.8 ± 150.7^{c}
Fe-P+	1016.3 ± 37.2^{b}	102.4 ± 4.7^{b}	0.320 ± 0.005^{b}	0.823 ± 0.049^{b}	2506.8 ± 274.6^{c}

+: Inoculation with *P. indica*; −: Non-inoculation with *P. indica*. C: control; Ca_3-P: tri-calcium phosphate; Al-P: aluminum phosphate; Fe-P: iron phosphate. Data are average values ±SD ($n = 4$) calculated from four independent experiments

Statistically significant differences are marked with letters ($p < 0.05$)

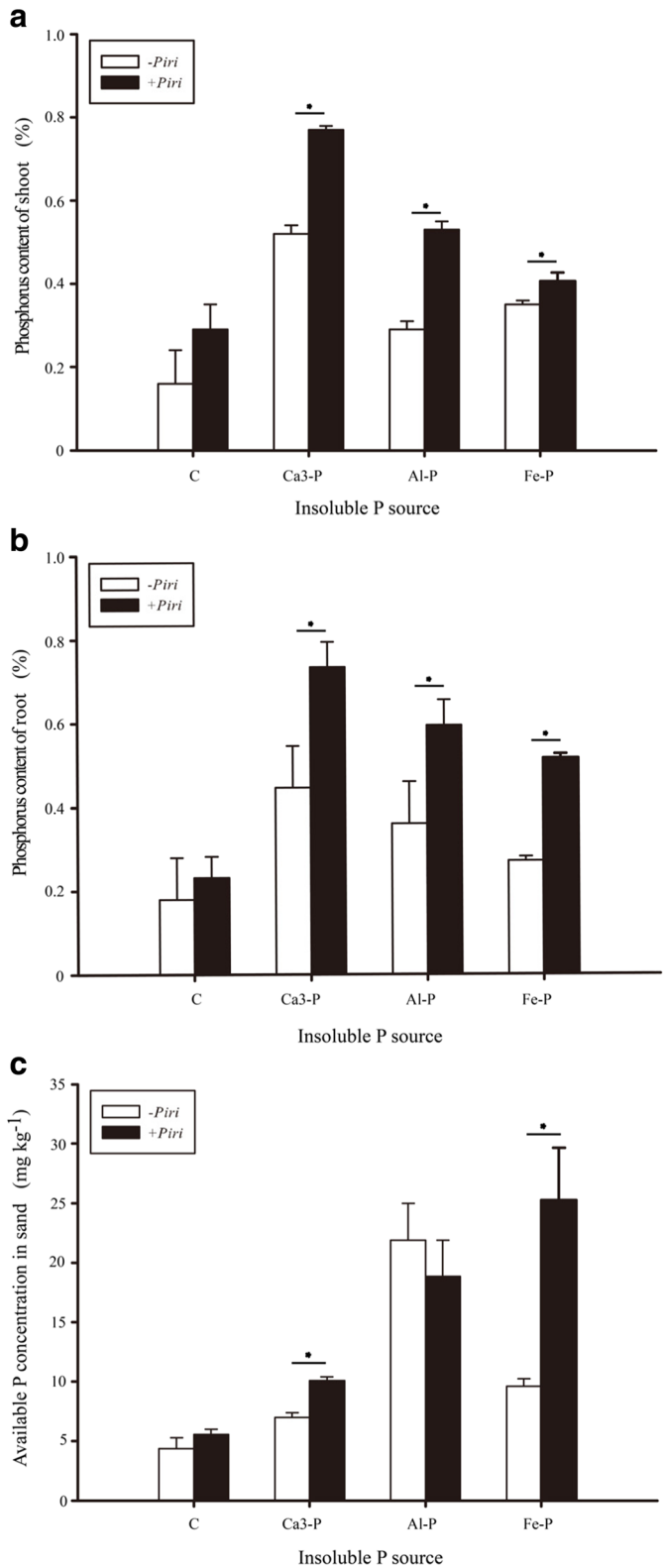

Fig. 2 Phosphorus concentration in shoot and root of *P. indica* colonized and uncolonized *B. napus* seedlings in pots watered with different insoluble phosphorus solutions (A: Shoot; B: Root; C: Rhizosphere soil). C: control; Ca_3-P: tri-calcium phosphate; Al-P: aluminum phosphate; Fe-P: iron phosphate. Data are average values ±SD ($n = 4$) calculated from four independent experiments. Asterisks indicate significant differences between *P. indica* inoculated and non-inoculated seedlings (*$p < 0.05$)

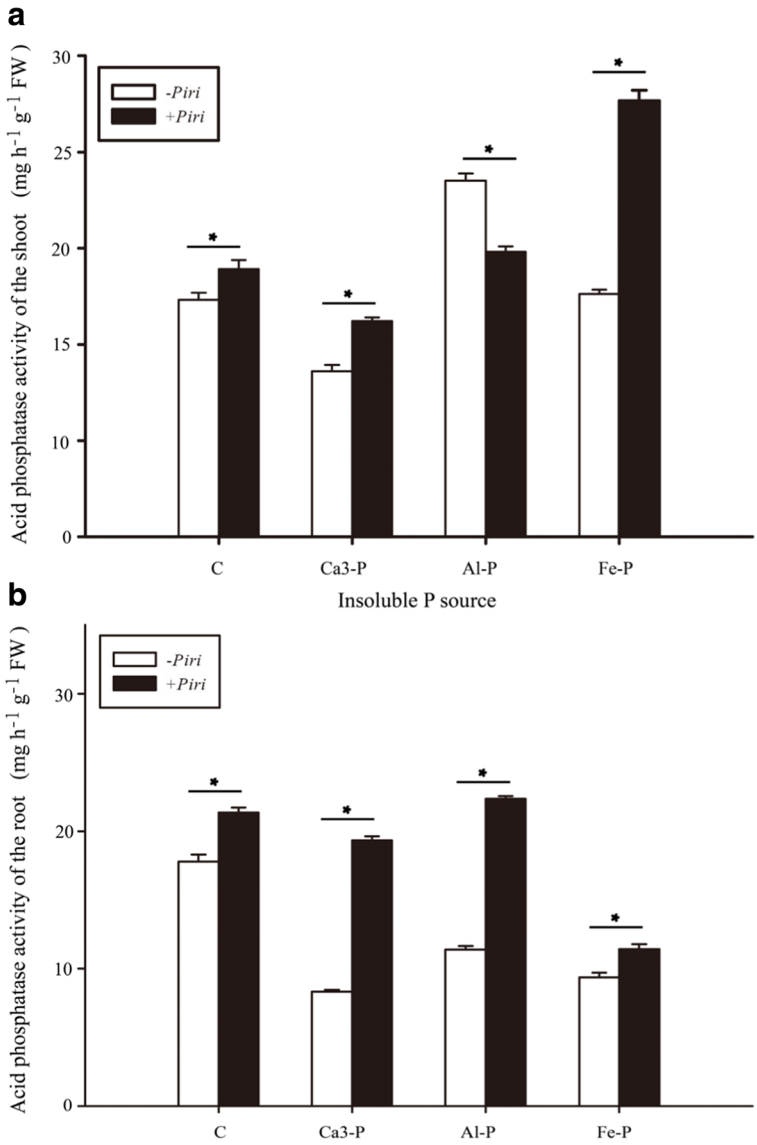

Fig. 3 Acid phosphatase activities in shoot and root of *P. indica* colonized *B. napus* seedlings grown in rhizosphere soils watered with different insoluble phosphorus solutions (A: Shoot; B: Root). C: control; Ca_3-P: tri-calcium phosphate; Al-P: aluminum phosphate; Fe-P: iron phosphate. Data are average values ±SD ($n = 4$) calculated from four independent experiments. Asterisks indicate significant differences between *P. indica* inoculated and non-inoculated seedlings ($*p < 0.05$)

the fungus on the expression of *BnACP5* and *BnPHt1;4* is greater in roots than shoots.

Effect of *P. indica* on organic acids and pH value in the rhizosphere sands

HPLC analysis and pH value of the rhizosphere sands were performed to identify and quantify the organic acids produced after treatment of the plants with different P sources (Table 4). Oxalic, malic and citric acids were secreted into the rhizosphere sands after planting *B. napus*, while tartaric and acetic acids were not detected (Table 4). Overall, the organic acid production appeared to be a common event of occurrence when the P source applied to the soil in our data, however, the types and quantities of acid produced depended on the type of phosphate source. The citric acid was the major organic acid which was produced in response to Ca_3-P or Al-P treatments whereas oxalic acid was mainly produced in response to Fe-P treatment. Furthermore, in the presence of *P. indica*, higher levels for these organic acids were found in the sand. It suggests that the fungus stimulated organic acid accumulation in the rhizosphere .

There was a significant decrease in the pH in the presence of *P. indica* (Table 4), with or without P

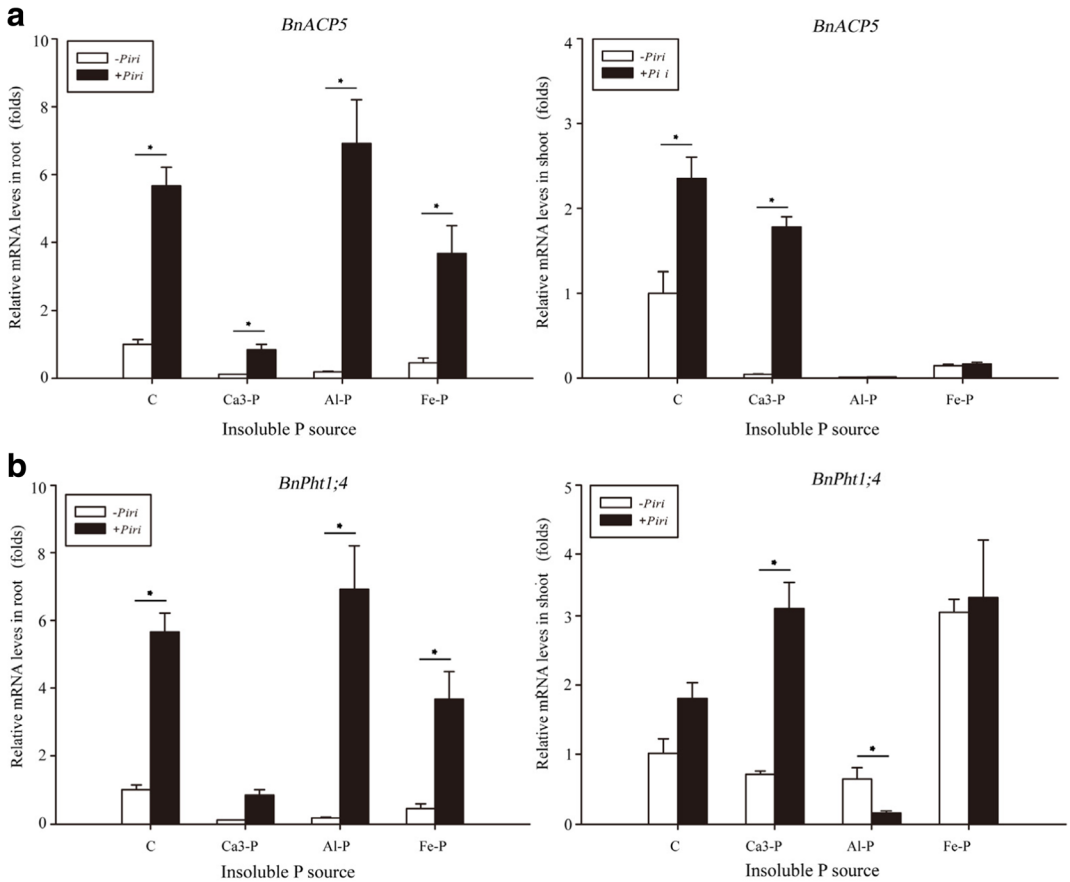

Fig. 4 Expression of *BnACP5* and *BnPHt1;4* in roots and leaves of *B. napus* grown in soil watered with different insoluble phosphorus solutions (A: *BnACP5* gene; B: *BnPHt1;4* gene; Left: Roots; Right: Leaves). C: control; Ca_3-P: tri-calcium phosphate; Al-P: aluminum phosphate; Fe-P: iron phosphate. Data are average values ±SD ($n = 4$) calculated from four independent experiments. Asterisks indicate significant differences between *P. indica* inoculated and non-inoculated seedlings (*$p < 0.05$)

addition, irrespective of the P source, compared to the cultures where *P. indica* was absent. The pH was reduced by 0.15, 0.2 and 0.33 unit for Ca_3-P, Al-P and Fe-P, respectively. The result further proved that the organic acid was accumulated in the rhizosphere with different inorganic P source.

Discussion

At the pot experiments, the shoot and root growth of *B. napus* grown on soil supplemented with Ca_3-P, Al-P or Fe-P as P source was clearly enhanced by *P. indica*, expect for average diameter supplemented with Ca_3-P (Tables 2 and 3), and the amount of P in both organs was increased in the presence of the fungus. Furthermore, we demonstrate that the available P concentration in the rhizosphere sand was strongly stimulated by the fungus when Ca_3-P or Fe-P was applied as additional P source (Fig. 2). These results suggest that *P. indica* stimulates the growth of the host by stimulating the P availability from the rhizosphere. The fungus releases P from P sources which are not accessible for the plant which is taken up by the roots directly or via a passage through the fungal hyphae. The performance of the fungus was mentioned in adult maize plants under P limitation conditions (Kumar et al. 2011). It was noticed that the root average diameter of *B. napus* seedlings with *P. indica* supplemented with Ca_3-P was significantly decreased in our results. The possible reason was the changes in cytosolic Ca^{2+} concentration, which plays a role in affecting root architecture under low-P conditions (Niu et al. 2013). However, we also observed that the number of lateral root and root tip were increased

Table 4 Concentration of organic acids and pH in the rhizosphere sands with respect to different inorganic phosphate sources

Treatments	Organic acids (μg/g)					pH
	Oxalic acid	Malic acid	Citric acid	Tartaric acid	Acetic acid	
C-	68.5 ± 4.32^{c}	23.1 ± 6.17^{b}	156.9 ± 6.72^{a}	–	–	6.80 ± 0.02^{bc}
C+	70.8 ± 1.13^{c}	26.2 ± 3.50^{b}	162.5 ± 7.30^{a}	–	–	6.61 ± 0.04^{d}
Ca_3-P-	32.1 ± 1.82^{f}	9.5 ± 0.38^{c}	63.9 ± 12.99^{bc}	–	–	6.98 ± 0.01^{a}
Ca_3-P+	42.2 ± 0.56^{e}	47.2 ± 6.69a	64.4 ± 7.47^{bc}	–	–	6.83 ± 0.03^{b}
Al-P-	51.1 ± 8.83d[e]	6.7 ± 1.24^{d}	59.7 ± 2.39^{c}	–	–	6.86 ± 0.02^{ab}
Al-P+	60.4 ± 1.84^{cd}	11.8 ± 1.95^{c}	74.3 ± 10.22^{b}	–	–	6.66 ± 0.03^{cd}
Fe-P-	324.4 ± 17.29^{b}	55.1 ± 14.89^{a}	49.9 ± 11.15^{c}	–	–	6.73 ± 0.02^{c}
Fe-P+	439.3 ± 8.52^{a}	11.4 ± 0.50^{c}	25.4 ± 0.85^{d}	–	–	6.40 ± 0.01^{e}

+: Inoculation with *P. indica*; −: Non-inoculation with *P. indica*. C: control; Ca_3-P: tri-calcium phosphate; Al-P: aluminum phosphate; Fe-P: iron phosphate. Data are average values ±SD ($n = 4$) calculated from four independent experiments

Statistically significant differences are marked with letters ($p < 0.05$)

significantly, which enabled the root to better explore the soluble phosphorus in the soil (Lynch and Brown 2001; Baleni and Negisho 2012; Niu et al. 2013). The contribution to the growth promoting effect of *B. napus* under field conditions needs to be further studied.

Phosphatase enzymes are believed to be important for P uptake. They have wide specificity in cleaving P ester bonds and hydrolysis of insoluble polyphosphates and organic phosphates. This has a strong influence on P uptake into roots and distribution within the cells (Swetha and Padmavathi 2016). In our data, the phosphatase activities in roots of *B. napus* were strongly stimulated by *P. indica*, when Ca_3-P, Al-P or Fe-P was applied to the symbionts, and *BnACP5* was higher expressed in roots. The phosphatase activities and *BnACP5* expression in shoots showed different trends (Fig. 4a). These results indicate that the fungus mainly stimulated the phosphatases in roots of the host. The phosphatases from either plant or fungal origin convert insoluble P forms into soluble forms and make them accessible for the plants (cf. also Singh et al. 2000). However, in vitro culture, *P. indica* could solubilize P from Ca_3-P and rock phosphate, but no relevant intra- or extracellular phosphatase activities were detected despite a stimulatory effect of the fungus on the expression of the relevant plant genes (Ngwene et al. 2016). Numerous reasons can explain this discrepancy, among which it is reasonable to assume that the fungus induces the enzymatic activities only under symbiotic conditions.

BnPht1;4 is a high-affinity P transporter of the PHT1 family, and plays a crucial role in the P starvation response. In this study, *BnPHt1;4* expression was stimulated by *P. indica* in roots of *Brassica napus* with Ca_3-P, Al-P or Fe-P, while in shoots a stimulatory effect was only detectable with Ca_3-P (Fig. 4b). This suggests that *P. indica* has the potential to induce P transporter in *B. napus* roots under P deficiency condition there by facilitating *B. napus* to have better accessibility to soil phosphorus. This point was supported by the roots under P deficient conditions, and the gene may be regulated by both MYBCC and WRKY family transcription factors (Ren et al. 2014). Further studies need to show the transcription factors which are targeted by *P. indica* in the host under different inorganic P sources.

Organic anions such as citrate and malate are the major released root exudates, in response to P deficiency for mobilizing P for plant uptake (Dechassa and Schenk 2004). In our study, oxalic, malic and citric acids were accumulated in the rhizosphere sands when *P. indica* colonized *B. napus* plants were growing under Ca_3-P or Al-P treatments, and oxalic acid was produced under Fe-P treatment (Table 4). The results were in accordance with Swetha and Padmavathi (2016), who also reported the production of oxalic acid and citric acid. However, tartaric, acetic, lactic and succinic acids were not found in our experiments. This could be caused by different experimental conditions or genotypes used (Corrales et al. 2007). However, organic acids in the culture medium were not detected probably because their

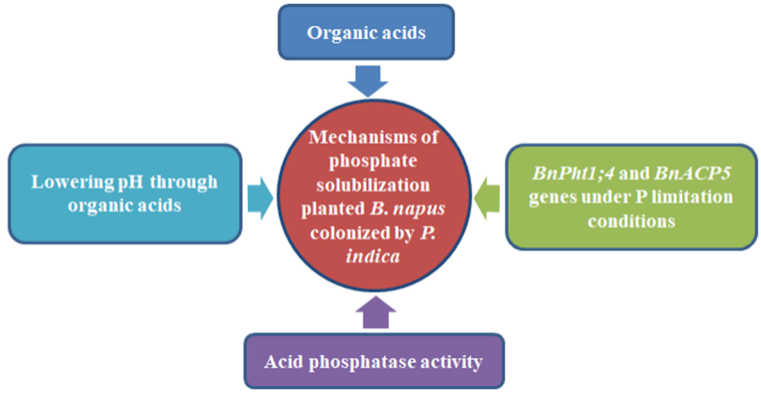

Fig. 5 Mechanisms of solubilization of inorganic P planted *B. napus* seedlings colonized by *P. indica* in pots

amounts were too low (Ngwene et al. 2016). In our data, the malic and citric acid accumulation was decreased at Fe-P treatment, and the oxalic acid content was significantly higher than other treatments (Table 4). This resulted from different types of phosphorus sources (Vyas and Gulati 2009; Mardad et al. 2013). Moreover, the synthesis of organic acids is controlled by genes. One gene for a malate synthase and three genes for citrate synthases have been identified when *P. indica* was cultivated without the plant (Zuccaro et al. 2011). Therefore, the expression of these genes during the symbiotic interaction of *P. indica* with *B. napus* roots needs to be analyzed in detail.

Phosphorus solubilization is the combined effected of both drop in pH and organic acid production (Fankem et al. 2006). We also found that the pH value was decreased significantly when *P. indica* growth in the presence of Ca_3-P, Al-P or Fe-P (Table 4). The most range of the reduced pH value was resulted from applying Fe-P and the minimum range was caused by Ca_3-P. The probable reason is a buffering effect of the dissolved Ca_3-P. Organic acids produced by the phosphate solubilizing microorganisms have been mainly involved in chelating the insoluble complexes of phosphate (Bagyaraj et al. 2000), therefore, the pH value in our experiment was above 6.0 in all treatments.

Notably, the P concentration in *P. indica* colonized *B. napus* shoots growing on soil supplemented with the different insoluble P sources was higher than in non-colonized plants. Since the alterations of the phosphatase activity and the expression of *BnACP5* and *BnPHt1;4* did show different trends in our experiments with the three different insoluble P sources, additional factors might contribute to the P transport from the roots to the shoots.

Phosphorus-solubilizing microorganisms and plants form a synergistic relationship in nature, presumably because it is beneficial for both partners. We proved that the pre-hypothesis was correct through our experiment, and secretion of organic acids and phosphatase enzymes might be crucial for such a scenario in the interaction studied here (cf. Malla et al. 2004; Singh et al. 2000). We propose that these processes participate in the promotion of plant growth and crop productivity. Solubilization of phosphate by *P. indica* interaction with *Brassica napus* occurs due to the combined effect of both phosphatase enzyme in roots and organic acid production in the rhizosphere soil. We have also presented a small model diagram of the mechanisms of solubilization of inorganic P planted *B. napus* seedlings colonized by *P. indica* in our experimental conditions (Fig 5).

Conclusion

The present study shows that the P levels in shoots and roots of *P. indica* colonized *B. napus* increases when the symbionts are growing under P limitation conditions with Ca_3-P, Al-P or Fe-P as P sources. Especially under Ca_3-P conditions, the expression of a high-affinity Pi transporter was remarkably stimulated in the roots, which indicates that the fungus mobilizes some P from inorganic sources for uptake, but higher expression of P transporter genes often demonstrates that P is still limiting. The symbionts respond to this situation by stimulating both phosphatase enzymes in the roots and the production of organic acid in the rhizosphere soil.

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