Plant Soil (2016) 405:231–241
DOI 10.1007/s11104-015-2779-8

REGULAR ARTICLE

Phosphate utilization by the fungal root endophyte *Piriformospora indica*

B. Ngwene · S. Boukail · L. Söllner · P. Franken · D. R. Andrade-Linares

Received: 5 March 2015 / Accepted: 14 December 2015 / Published online: 29 December 2015
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Abstract
Background and aim The root endophytic fungus *Piriformospora indica* increases plant resistance and tolerance to stress and promotes plant growth, but its ability to support plant nutrition is still controversially discussed. Irrespective of a potential nutrient transport towards the plant, the fungus might release P from sources unavailable for plant usage by transformation to available forms.
Methods To test this hypothesis, sterile solid and liquid in vitro cultures of *P. indica* supplied with different organic and inorganic P sources were established. Cultures were investigated for growth, solubilised P, enzyme activities, RNA accumulation of the four genes encoding phosphate transporters and the two genes for acid phosphatases and phytases respectively found in *P. indica* genome, and for pH values in the media.
Results P. indica growth was higher in the presence of inorganic P than in organic P sources. Significant amounts of P were solubilised by *P. indica* from $Ca_3(PO_4)_2$ and rock phosphate. However, no relevant intra- or extracellular enzymatic activity was detected despite RNA accumulation of related genes. In general, the genes were all repressed by higher amounts of inorganic P and were expressed the most when the fungus received phytate. We observed a decrease in medium pH in the presence of *P. indica* irrespective of the P source.
Conclusions P. indica is able to solubilise phosphate from inorganic, but not from organic P sources. This P solubilisation is not due to enzymatic activities but rather to the lowering of the medium pH.

Keywords Phosphate · Root endophyte · *Piriformospora indica* · Enzyme activity · PT-genes

Responsible Editor: Andrea Campisano.

Electronic supplementary material The online version of this article (doi:10.1007/s11104-015-2779-8) contains supplementary material, which is available to authorized users.

B. Ngwene (✉) · S. Boukail · L. Söllner · P. Franken
Leibniz-Institute of Vegetable and Ornamental Crops, Theodor-Echtermeyer-Weg 1, 14979 Großbeeren, Germany
e-mail: ngwene@igzev.de

D. R. Andrade-Linares
Institute of Biology, Plant Ecology, Free University of Berlin, Altensteinstr. 6, 14195 Berlin, Germany

Introduction

Root colonizers such as arbuscular mycorrhizal (AM) fungi support plant mineral nutrition in exchange for photosynthetic carbon (Smith and Read 2008). Many other rhizosphere fungi are able to colonize the plant and impact plant performances (Harman et al. 2004; Rodriguez et al. 2009). This impact ranges from positive to negative depending on the partners involved and on rhizosphere conditions (Mayerhofer et al. 2013). Such associations should therefore be incorporated into plant-soil interactions studies for a comprehensive understanding and for more reliable recommendations in plant production (Franken 2012). The root endophytic fungus *Piriformospora indica* is an anamorphic strain of the

Sebacinales (Basidiomycota), an order with many plant-colonizing organisms including ecto-, ericoid, and orchid mycorrhizal fungi (Weiss et al. 2004; Selosse et al. 2007). This fungus isolated in the Thar Desert of India (Verma et al. 1998) is able to establish associations with roots of a wide range of plants (Varma et al. 1999). The isolation of other *P. indica*-like strains has been mentioned (e.g., Varma et al. 2001), but was never confirmed and such strains have not been deposited in any collection. Other species belonging to Sebacinales group B showed also plant growth-promoting abilities (Deshmukh et al. 2006; Basiewicz et al. 2012; Riess et al. 2014).

Recent studies have demonstrated a multifunctional role of *P. indica* in enhancing plant performance. Promotion of vegetative growth and the induction of resistance against pathogens as the most common effects have been repeatedly shown (Franken 2012). In addition to positive effects on plant vegetative growth and resistance, *P. indica* colonization can also improve crop yield and product quality (e.g., Waller et al. 2005; Dolatabadi et al. 2011; Satheesan et al. 2012; Andrade-Linares et al. 2013). The fact that this fungus can be cultivated on different media without a host (Verma et al. 1998), easily producing large quantities of fungal biomass, encouraged researchers to propose its application in future plant production systems (Varma et al. 1999; Yadav et al. 2010; Franken 2012). In contrast to AM fungi, *P. indica* is able to colonize roots and promote plant growth independent of phosphate concentrations in the soil (Yadav et al. 2010).

The mechanisms behind the plant growth promoting effects of *P. indica* are still a matter of debate, particularly whether there is any contribution to plant P uptake by the fungus as seen in the AM symbiosis (Achatz et al. 2010; Kumar et al. 2011). Growth promotion in *P. indica*-colonized plants has been linked to changes in the production and signaling of phytohormones like ethylene, auxin, gibberellin and cytokinin (Barazani et al. 2007; Vadassery et al. 2008; Schäfer and Kogel 2009; Camehl et al. 2010). These effects however do not exclude a plant nutritional effect and there is some indication that *P. indica* can contribute to plant P nutrition (Yadav et al. 2010; Kumar et al. 2011).

Yadav et al. 2010 cloned and analysed a P transporter encoding fungal gene (PiPT) in *P. indica* and observed that maize plants colonized by a PiPT-knocked down *P. indica* mutant had less total phosphate content and biomass than the *P. indica* wild-type–colonized plants. By quantitative RT-PCR analysis, they observed that PiPT transcripts were 18-fold enriched in extra-radical mycelium (ERM) than in intra-radical mycelium (IRM), indicating that the main site of PiPT expression is the ERM. From these observations, the authors suggested that phosphate plays a role in the increased plant yield or biomass, and PiPT is actively involved in the phosphate transportation. In accordance to these results, uptake of radio-labelled P was strongly enhanced in Arabidopsis in the presence of *P. indica* (Shahollari et al. 2005). Despite this strong indication of a direct contribution of *P. indica* to plant P nutrition, experimental results on agricultural crops have been inconsistent. While in tobacco, barley and green gram, colonization by *P. indica* did not increase P content of plants despite plant growth promotion (Barazani et al. 2007; Achatz et al. 2010; Ray and Valsalakumar 2010), chickpea and black lentil plants showed higher P content (Nautiyal et al. 2010; Kumar et al. 2012). This inconsistency may be partly explained by differences in experimental conditions (Fakhro et al. 2010) and highlights the need for further research.

The interaction between *P. indica* and its host plant in relation to P nutrition might be either direct by fugal transport of P through the hyphae from the soil to the host plant, or indirect by fungal activity mobilising P from the surrounding substrate for plant root uptake. The release of P will depend among other factors, on the form of P available (Richardson and Simpson 2011). To get more insights into the role of *P. indica* in plant P phosphate nutrition it is imperative to understand first how *P. indica* can utilize different forms of P for growth, and second the ability of *P. indica* to release P from these different sources into the surrounding media. We therefore investigated the physiological and molecular changes related to P dynamics in *P. indica* supplied with different forms of organic and inorganic P.

Material and methods

In vitro growth and P solubilisation activity of *P. indica* supplied with different P forms

Cultivation of P. indica

Substrate composition for in vitro cultivation of *P. indica* (strain DSM 11827) was prepared based on the Pikovskaya medium (Pradhan and Sukla 2005) with

the following compounds per litre: 13 g glucose, 0.5 g NH_4SO_4, 0.2 g NaCl, 0.1 g $MgSO_4.7H_2O$, 0.2 g KCl, 0.5 g yeast extract, 0.002 g $MnSO_4.H_2O$, 0.002 $FeSO_4.7H_2O$. Based on the results of a preliminary experiment (Fig. S1), phosphorus (P) was supplied at a concentration of 200 or 500 mg/l as monopotassium phosphate, 0.879 g/l (KH_2PO_4); lecithin, 4.258 g/l (L-α-Phosphatidylcholine, Sigma Aldrich, Germany); phytate, 0.709 g/l (phytic acid sodium salt hydrate, Sigma Aldrich, Germany); tricalcium phosphate, 1.0 g/l ($Ca_3(PO_4)_2$) (Sigma Aldrich, Germany); rock phosphate, 1.389 g/l (natural Moroccan phosphorite, Sigma Aldrich, Germany) in the agar medium, and in the form of phytate, $Ca_3(PO_4)_2$ or rock phosphate in the liquid medium. All compounds were mixed in distilled water and the pH was adjusted to 7.2 using KOH.

Assessment of fungal growth on P-sources and potential P solubilisation of P. indica

To assess the potential of *P. indica* to produce extracellular enzymes, the fungus was cultivated on agar plates supplemented with the different P-sources: KH_2PO_4 (KP), $Ca_3(PO_4)_2$ (CP), rock phosphate (RP), lecithin (L) or phytate (Phy) as detailed above (see Cultivation of *P. indica*). A fungal disc (0.5 cm) of *P. indica* freshly propagated on potato dextrose agar (PDA, VWR, Berlin, Germany) was transferred into the middle of the Petri dish. The plates were incubated at 25 °C. Each treatment consisted of 4 replicates. Every 5 days, the diameter of the colony in the Petri dish was measured and the hydrolysis halo around the colony was recorded. As growth parameter, the specific growth rate of the colony was estimated from the colony diameters. The growth rate was calculated as: GR = (colony diameter at 15 days—colony diameter at 5 days)/incubation time. Additionally the density of fungal mycelium and morphology of the colonies grown on CP, RP, L or Phy-agar were compared with those grown on KP-agar (used as control of growth).

Assessment of fungal growth, P solubilisation and pH change by P. indica in liquid medium

To determine pH change and P release, the fungus was cultivated in liquid medium. Based on the agar plates experiments, 150 ml medium, supplemented with 500 mg/l of P as CP, RP, or Phy were poured into 300 ml Erlenmeyer flasks and then autoclaved. A fungal disc (0.5 cm) of *P. indica* freshly propagated on potato dextrose agar (PDA, VWR, Berlin, Germany) was transferred into the media. Negative controls received an empty PDA agar plug. In addition, a treatment without P supplementation served as a control for pH assessment. Cultures were incubated at 25 °C by shaking at 110 rpm (GFL 3033 Shaking Incubator, GFL mbH, Burgwedel, Germany). Each treatment consisted of 4 replicates. The fungal cultures were grown for 4 weeks. Subsamples (5 ml) were taken from the liquid culture media every 5 days for measurements of soluble P concentration at 436 nm wavelength after staining with ammonium molybdate-vanadate solution (Gericke and Kurmies 1952) with the EPOS 5060 analyser (Eppendorf, Hamburg Germany). After 14 days of incubation, 5 ml subsamples were collected from the liquid medium to measure pH by a laboratory pH meter (inoLab pH 720, WTW GmbH, Weilheim, Germany). Fungal biomass as fresh and dry weights and C content in fungal mycelium were determined after incubation.

Analyses of enzymatic activities and RNA accumulation

P. indica was cultivated in liquid medium, prepared as described above, at a P concentration enabling minimal growth (0.02 mg/l P) for 20 days and then transferred to new media containing CP, RP or Phy at low and high P concentrations (0.25 or 500 mg/l P). Negative controls with no additional P were also included.

Analysis of phytase and phosphatase activities

For measurements of extra- and intracellular enzyme activities, 5 ml subsamples were taken from C, and Phy media after 1 and 5 days. The subsamples were centrifuged at 1200 rpm for 2 min to separate medium from mycelium debris. The supernatant was collected in new tubes for extracellular enzyme activity assay. The mycelium samples were washed three times with deionized water, grounded in liquid nitrogen and then deionized water was added to a total volume of 45 ml (Malla et al. 2004). The solution was centrifuged at 1200 rpm for 20 min (Centrifuge 5415 R, Eppendorf AG, Hamburg, Germany) and the supernatant was collected in new tubes for enzyme activity assay. The precipitated mycelium was dried, and the dry weight recorded for reference. Intra- and extraradical enzyme activities (phytase and phosphatase) were assayed from the

respective supernatants, which were maintained during the extraction process under cold conditions (4 °C). The phytase activity was measured following the method of Yadav and Tarafdar (2003), while acid phosphatase activity was measured using an assay kit according to the manufactures' instructions (Acid Phosphatase Assay Kit, Sigma Aldrich, Germany). The selected samples included the Phy (organic source) and the CP (inorganic source) treatments at 500 mg/l P.

RNA accumulation analysis of P. indica genes

To clarify whether the results for phytase and phosphatase activity can be explained by *P. indica* genome composition, the database published by Zuccaro et al. (2011) was screened for the corresponding genes. For both activities each two full size genes could be detected which were called *PiPHY1*, *PiPHY2*, *PiAPH1* and *PiAPH2* (Table S1). It may not be excluded that these genes are not expressed or that the gene expression does not respond to the presence of the P sources and therefore the genes were analysed by qRT-PCR with gene specific primers. The four genes encoding phosphate transporters (*PiPT1-4*) detected in the *P. indica* genome (Table S1) were also included in the analysis.

Mycelium samples were collected of all treatments 5 days after incubation, immediately frozen in liquid nitrogen and stored at −80 °C for gene expression analyses. Total RNA was extracted from frozen mycelium in liquid nitrogen using the RNeasy® Plant Mini Kit (QIAGEN Sample & Assay Technologies GmbH; Hilden, Deutschland) and DNase-treated with RNase-free DNase (Promega kit RQ1, Mannheim, Germany) according to the protocol of the manufacturers. Absence of DNA contaminations was checked by standard PCR (95 °C for 1 min, 35 cycles of 30 s at 95 °C, 30 s at annealing temperature, 1 min at 60 °C; 20 μL volume) in a Primus thermocycler (MWG Biotech, Ebersberg, Germany) with the primer pairs indicated in Tab. S1 and 0.5 U *Taq* polymerase (Peqlab, Erlangen, Germany) using the RNA as template. RNA concentration (ng/μl) was measured with NanoDrop 1000 Spectrophotometer (Thermo Fischer SCIENTIFIC; Wilmington, USA). One μg RNA was reverse transcribed in 10 μL total volume with an M-MLV reverse transcriptase system using oligo-dT primers (Promega). The resulting cDNA (0.1 μL) was used as a template for standard PCR (see above) with gene-specific primers that were designed for the selected genes encoding phosphate transporters, acid phosphatases and phytases (Table S1). Amplification products were cloned and sequenced in order to verify that they were derived from the corresponding genes. Quantitative real time RT-PCR (qRT-PCR) was carried out using the 7500 Fast Real-Time PCR System and Power SYBR Green (Applied Biosystems, Warrington, UK) with the following temperature programme: 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 15 s at 95 °C, 1 min at 60 °C followed by a melting curve analysis (heating from 56 to 95 °C with a rate of 0.5 °C per 10 s). The melting curve indicated the specificity of the primer pairs. RT-PCR reactions were conducted in three biological replicates with three technical repetitions. For estimating relative expression levels the translational elongation factor gene of *P. indica* (*PiTEF1*) was used as reference gene (Table S1). Relative gene expression values were calculated as 2-ΔCt.

Statistics

All data were tested for normal distribution and subjected to analyses of variance (ANOVA). Colony growth rate data were log transformed to get a normal distribution and significant differences between treatments were analysed by a pairwise comparison and Duncan test. Mean values were compared by a one-way ANOVA (Tukey (HSD) test), or a *T*-test where appropriate. Two-way ANOVA was used to estimate whether single factors alone or in interaction, had a significant influence on the mean values. Differences were considered significant when *p*-values were below 0.05. Statistics were performed using the SigmaStat 3.5 program (STATCON Germany).

Results

In vitro growth and P solubilisation activity of *P. indica* supplied with different P forms

Assessment of fungal growth on P-sources and potential P solubilisation of P. indica

The growth rate of *P. indica* on different P substrates in Petri dish was significantly higher with KP, CP and RP supplied than with both organic P sources (Fig. 1a), in which the colonies grew about 5 mm per day.

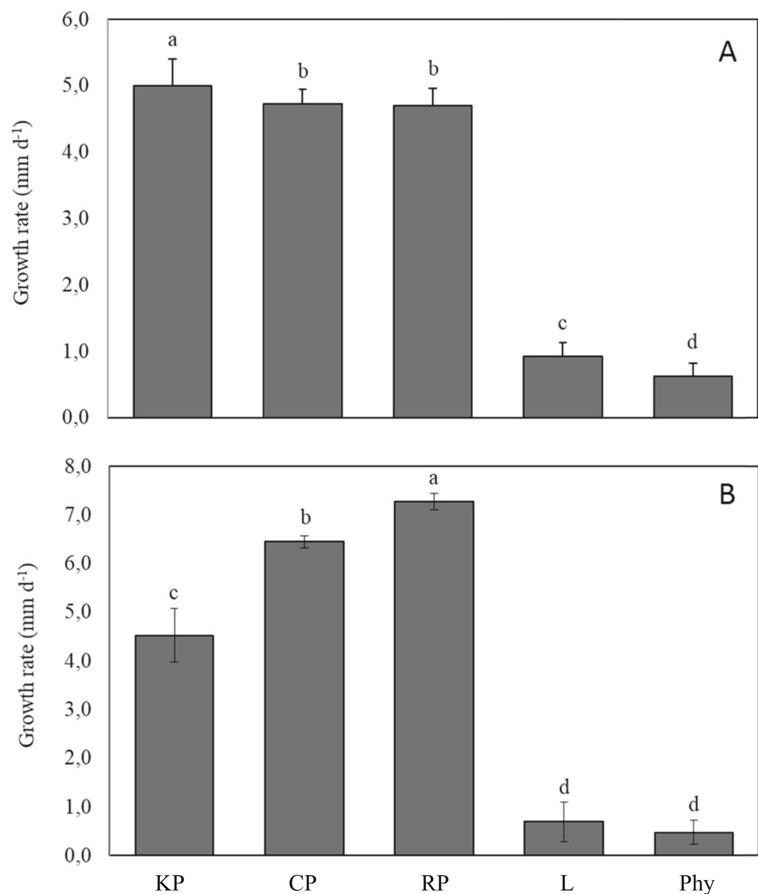

Fig. 1 Growth rate of *P. indica* colonies in different P sources. Colony diameters were measured 5, 10 and 15 days after incubation at 25 °C. A basal medium was supplied with 200 mg/l (**a**) or 500 mg/ml (**b**) P as KH_2PO_4 (KP), $Ca_3(PO_4)_2$ (CP), rock phosphate (RP), lecithin (L) or phytate (Phy). Data were log transformed and analyzed by one factorial ANOVA ($p = 0.05$, $n = 4$). Different *letters* indicate significant differences between treatments as analyzed with a pairwise comparison and Duncan test

Nevertheless, the highest growth rates were observed at 500 mg/ml P for RP and CP agar plates (Fig. 1b). The minimal fungal growth rate was in L and Phy. No hydrolysis halo was observed in any of the treatments and mycelium thickness of colonies grown on CP, RP and KP with the same concentration of glucose were similar (data not shown). In the organic P sources (L and Phy) the fungal growth was significantly reduced up to 4 fold compared to the inorganic sources (Fig. 1a and b).

Assessment of fungal growth, P solubilisation and pH change by P. indica in liquid medium

When CP was applied to the liquid medium (500 mg/ml), P was steadily released into the medium in significantly higher amounts in the presence of *P. indica* compared with the control treatment (Fig. 2). A similar result was observed when RP instead of CP was applied, but to a lower extent (Fig. 2). In contrast to the inorganic P sources, the addition of phytate did not lead to P solubilisation by the fungus. In this case, the initial level of soluble P was maintained throughout the study, and there was no difference in the concentration of soluble P in the medium between the *P. indica* treatment and the control (Fig. 2).

The fungal biomass in terms of fresh and dry weights, and P content of fungal mycelium were also determined after incubation (Table S2). However these results could have confounding effects due to the fixation of the fungal mycelium to these two inorganic sources that made it not possible to recover the mycelium completely free of them. Therefore we do not emphasize on this result. We however measured fungal C content as an estimate for fungal growth (Table S2). Although we observed significant higher growth rates on Petri dishes supplemented with 500 mg/ml P as CP or RP compared to KP (Fig. 1b), the mycelium C content was significantly less (Table S2).

There was a consistent significant decrease in the pH in the presence of *P. indica* (Fig. 3), with or without P

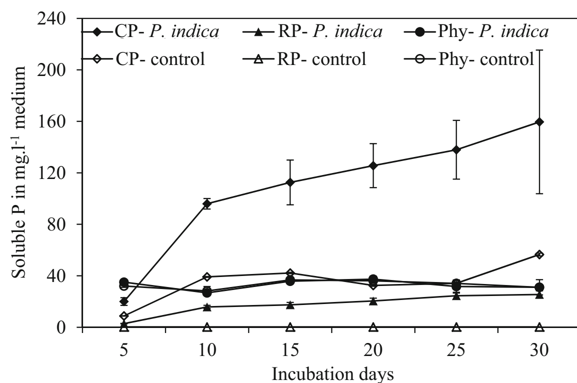

Fig. 2 Concentrations of soluble P in liquid medium supplied with $Ca_3(PO_4)_2$ (CP), rock phosphate (RP), or phytate (Phy) (500 mg/l P) after 5 to 30 days incubation at 25 ° C with or without *P. indica*. Shown are mean values ± standard deviations. ($p = 0.05$, $n = 4$)

addition, irrespective of the P source, compared to the cultures where *P. indica* was absent. The change of the pH for medium with phytate (not shown) was similar to that of no additional P. There was a significant interaction ($p < 0.001$) between the two factors (P source and fungal treatment) on the medium pH.

Enzymatic and phosphate transport activities

Analysis of phytase and phosphatase activities

No intra- or extracellular phytase activity was observed in any of the *P. indica* cultures, while a different fungus

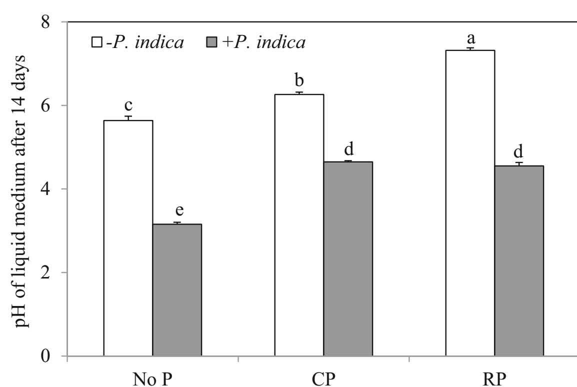

Fig. 3 pH of liquid medium supplied with no additional phosphate (No P), $Ca_3(PO_4)_2$ (CP), or rock phosphate (RP) (500 mg/l P) after 14 days of incubation at 25 °C with or without *P. indica*. Shown are mean values ± standard deviations. ($n = 4$). The two *P. indica* treatments were compared by Student *T*-test ($p = 0.05$) and significant differences are indicated by *asterisks*

from our collection (*Leptodontidium orchidicola*) (Helotiales, Ascomycota; accession number AM944358) gave positive results (data not shown). We observed no intracellular and very little extracellular acid phosphatase activity (Fig. 4) in the tested cultures compared to the positive control (about 1 Unit/ml). The activity was not significantly affected by the P source or the duration of growth (Two-way ANOVA, $p = 0.05$).

RNA accumulation analysis of P. indica genes

RNA accumulation of the four *PT* genes *PiPT1-4* was differentially regulated by the P source (Fig. 5; Table S3). The gene *PiPT3* did not respond significantly neither to the P source nor the P concentration, while the other P transporter-encoding genes were significantly regulated by both, P source and P concentration (Table S3). The expression of the gene *PiPT2* was significantly affected by the interaction between the factors P source and P concentration (Table S3). In general, all genes showed an increasing expression from CP via RP to Phy-fungal growth (Fig. 5). Moreover higher P concentrations repressed the RNA accumulation of the *PT* genes and this was significant for CP (*PiPT1-3*) and RP (*PiPT1-2*). The influence of Phy concentrations was never significant.

The general regulation pattern for genes encoding acidic phosphatase (*PiPA1* and *PiPA2*) and phytases (*PiPHY1* and *PiPHY2*.) were similar to those of the *PT* genes (Fig. 5). Interaction between the factors P source and P concentration could be observed for *PiPA1*. Significant down regulation of gene expression by increasing concentrations of CP could be observed for all four genes and by rock phosphate for all genes except *PiAP2* (Fig. 5). Increased Phy concentrations repressed only the RNA accumulation of *PiPHY2* (Fig. 5).

Discussion

The root endophytic fungus *P. indica* is able to enhance the biomass production of many different plant species under various conditions, increases plant resistance and tolerance to biotic and abiotic stresses, but its ability to support plant nutrition is a matter of debate (Oelmüller et al. 2009; Franken 2012). Concerning phosphorus, most reports show that the fungus is able to promote plant growth irrespective of P_i concentrations in fertilizers (Franken 2012). It

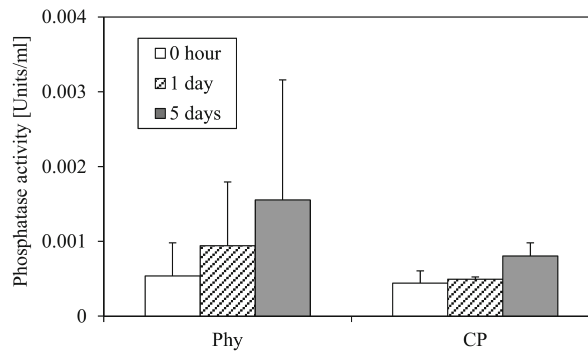

Fig. 4 Extraradical phosphatase activity. *P. indica* was cultivated at a minimal KH_2PO_4 concentration (0.02 mg/l P) and transferred to a new medium containing $Ca_3(PO_4)_2$ (CP) or phytate (Phy) at a P concentration of 500 mg/l P. Extra-radical acid phosphatase activity was measured prior to transfer into the new medium, and after 1 and 5 days. Phosphatase activity was not significant effected (Two-way ANOVA, $p = 0.05$, $n = 3$) by any of the two factors (P source, time)

is, however, possible that the fungus is able to release P from mineralized or organic sources which are unavailable for the plant, transforming them into a soluble form as orthophosphate which could be taken up by both, the roots and the root-colonizing endophyte.

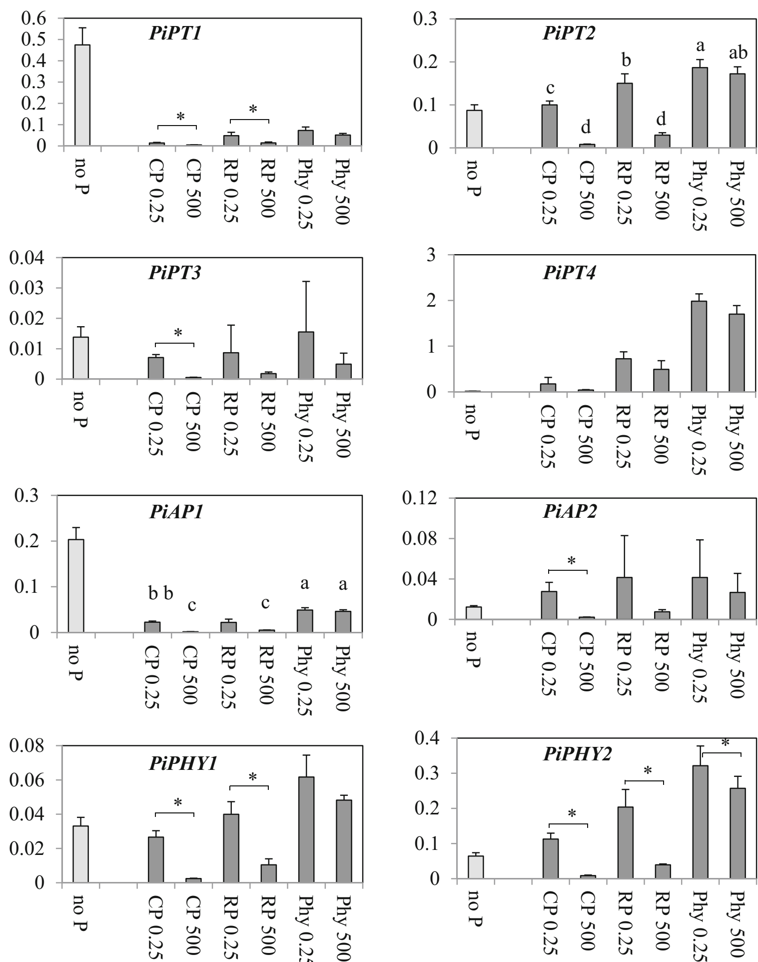

Fig. 5 Expression of *P. indica* genes. *P. indica* was cultivated at a minimal (0.02 mg/l) P concentration in form of KH_2PO_4 (no P) and transferred to a new medium containing $Ca_3(PO_4)_2$ (CP), rock phosphate (R) or phytate (Phy) at two different P concentrations (0.25 or 500 mg/l P). RNA was extracted and quantitative RT-PCR was performed with primer pairs for phosphate transporter genes (*PiPT1-4*) and genes encoding acidic phosphatase (*PiAP1-2*) or phytases (*PiPHY1-2*). Expression levels were normalized by data obtained for *PiTEF1*. Results of a two-way ANOVA ($n = 3$, $p = 0.05$) are shown in table S3. If the two factors 'P source' and 'P concentration' interact, significant differences are indicated by *letters*. If they do not interact, different concentration treatments were compared by Student *T*-test ($n = 3$, $p = 0.05$) and significant differences are indicated by *asterisks*

In vitro growth and P solubilisation activity of *P. indica* supplied with different P forms

Growth and phosphate solubilisation ability of *P. indica* was first analysed on solid agar medium supplied with organic P (lecithin or phytate), sparingly soluble inorganic P ($Ca_3(PO_4)_2$ or rock phosphate) or freely available soluble P (KH_2PO_4). The lowest colony growth was observed when *P. indica* was grown in medium supplemented with the organic P sources (lecithin and phytate). This can be principally due to the unavailability of P in this two P sources; in form of phospholipids in the lecithin (Scholfield 1981) and in complex with the inositol in the phytate molecule (Mullaney and Ullah 2007). There was also no visible hydrolysis halo around the fungal colony on the agar medium, which minimizes but not excludes the possibility of a release of extracellular enzymes. Malla et al. (2004) observed growth of *P. indica* on organic P with a similar rate as on different inorganic P sources. They used, however, glycerophosphate as organic P source. Contrary to growth in organic P forms, *P. indica* growth was evident in the presence of the less soluble inorganic P forms such as $Ca_3(PO_4)_2$ and rock phosphate. This has also been used before to estimate the solubilising capacity of microorganisms (Kang et al. 2002; El-Azouni 2008; Xiao et al. 2008).

One way to measure fungal biomass is the C content in mycelium. The C content in *P. indica* mycelium grown in $Ca_3(PO_4)_2$ or rock phosphate was significantly less than that when the fungus was cultivated in KH_2PO_4. This result together with those about growth rates on plates suggest that *P. indica* has an explorative growth behaviour under low inorganic P supply, growing faster at the hyphal tips to reach less depleted regions. This fungal growth strategy has been observed in filamentous fungi as a response to low nutrient availability (Ritz 1995; Ritz and Crawford 1999). On the other hand, when the fungus is growing in optimal or high nutrient amounts (in this case only the treatment with 500 mg/l P in the form of KH_2PO_4), the exploratory rate is lower but the biomass is higher due to its exploitative behaviour and active nutrient translocation (Bruggen et al. 2000; Falconer et al. 2005).

In liquid culture a significant amount of P was released into the medium from both inorganic P sources ($Ca_3(PO_4)_2$ or rock phosphate) by *P. indica*. The fungal growth in the medium led to a significant decrease in pH which could suggest the production of some inorganic acids by the fungus during its growth under recalcitrant P conditions (Hoberg et al. 2005; Scervino et al. 2010). Such a mechanism has already been shown for other fungal species such as *Penicillium* and *Aspergillus*. They solubilise $Ca_3(PO_4)_2$ and other forms of less soluble P by secreting organic acids into the medium (Pradhan and Sukla 2005). Up to now, it was not possible to detect any organic acids in the culture medium, probably because their amounts are very low. Citrate, oxalate and malate are discussed to be involved in phosphate solubilisation in the rhizosphere (Jones 1998). No genes encoding an oxalate synthase could be detected in the genome of *P. indica*, but one gene for a malate synthase and three genes for a citrate synthase have been identified (Zuccaro et al. 2011). All four genes are mainly expressed when *P. indica* is cultivated without the plant, which suggests that these fungal enzymes are needed for solubilising nutrients (Zuccaro et al. 2011).

Enzymatic and phosphate transport activities

Based on the genomic information (Zuccaro et al. 2011), we found that *P. indica* has the potential to express four different phosphate transporters, two acid phosphatases and two phytases. Although two genes encoding acid phosphatases and two phytase genes were expressed under our conditions, neither intra- nor extracellular enzymatic activity was detected in comparison with the reaction of our positive control. Discrepancies between RNA accumulation and enzymatic activities have been observed before (e.g., Liu et al. 1999; Yun et al. 2002; Breuninger et al. 2004). Malla et al. (2004), however, showed intracellular activities of a *P. indica* acid phosphatase. The fungus seems therefore to be able to show such enzymatic activities, but a particular stimulus might have been needed to induce the enzymatic activity or avoid some catabolic regulation during the fungal growth in our P source treatments. Further studies on the rhizosphere of *P. indica*-colonized plants are necessary to confirm enzymatic activities in vivo, and to test if the fungal enzymatic production could be induced by the fungal interaction with the plant.

We identified four genes encoding phosphate transporters. One of the encoded proteins has been already characterized, the PiPT1, is a symporter located in the plasma membrane of the fungus which shows high affinity for low concentrations of phosphate (Yadav et al. 2010; Pedersen et al. 2013). Knock-down of this phosphate transporter resulted in abolishment of the

plant growth-promoting effect suggesting an important role of this PiPT1 for the interaction. At the same time, colonization of the root was not reduced. This suggests that the transport of phosphate via PiPT1 is not essential for fungal development and that it can be complemented by the other PT-encoding genes. One good candidate is *PiPT4* which is highly expressed at least on RNA level in comparison to the other three genes. Further studies need to show the precise functions and roles of these four *PT* genes.

Concerning the regulation of their expression, *PT* genes could be classified into three groups (Fig. 5). The expression of the first group with the genes *PiPT1* and *PiAP1* shows a clear inverse correlation to the P concentration in the medium. Such a P_i-dependent response to phosphate has been shown already for the phosphate transporter gene *PiPT1* (Yadav et al. 2010). The second group of genes (*PiPT4*, *PiAP2* and *PiPHY2*) responded at first positively to the shift from 0.02 mg/L of KH_2PO_4 (initial cultivation) to 0.25 mg/L P of the other P sources. High concentrations of the two inorganic P sources, however, had a negative effect. A similar response has been observed for the AM fungal gene *GiPT* also encoding a high affinity transporter (Maldonado-Mendoza et al. 2001). This gene only showed high expression if a low amount of phosphate was added to the extra-radical mycelium and low expression if no P_i or high P_i amounts were present. Gene expression of the third group with the two remaining phosphate transporter genes *PiPT2* and *PiPT3* and with the gene *PiPHY1* encoding a phytase did not clearly respond to low amounts of inorganic P. The expression of these genes were however, repressed by high Ca_3PO_4 or rock phosphate concentrations similarly to all other genes. This clear negative response to the inorganic P sources indicates that phosphate is released into the medium and this was indeed shown in the presence of the fungus and probably results from acidification of the medium.

Conclusion

In this study we found that the endophyte *P. indica* is able to grow saprotrophically on poorly soluble inorganic P forms $Ca_3(PO_4)_2$ or rock phosphate. Under our conditions, growth was not due to enzymatic activities detectable in our plate growth halo assay, although corresponding genes were present and expressed. Instead, lowering the pH, probably by releasing organic acids, appeared responsible for this fungal ability. The growth rate is similar during cultivation on KH_2PO_4 indicating that the fungus is able to solubilise phosphate in amounts sufficient for its own growth. Measuring an increase in phosphate concentration in the medium suggests that a surplus of this mineral nutrient is produced which could theoretically be taken up by roots in the vicinity of the fungus. If the fungus improves phosphate nutrition of plants growing on inorganic non-available P sources in this way, remains to be proven. How much this contributes to plant growth promotion under controlled conditions and in the field probably depends on the host plant species and the environmental conditions.

Acknowledgments This work was funded by the Ministries of Consumer Protection, Food and Agriculture of the Federal Republic of Germany, of the State of Brandenburg and Thüringen. We are also grateful to Mrs. Kerstin Fischer for technical assistance.

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