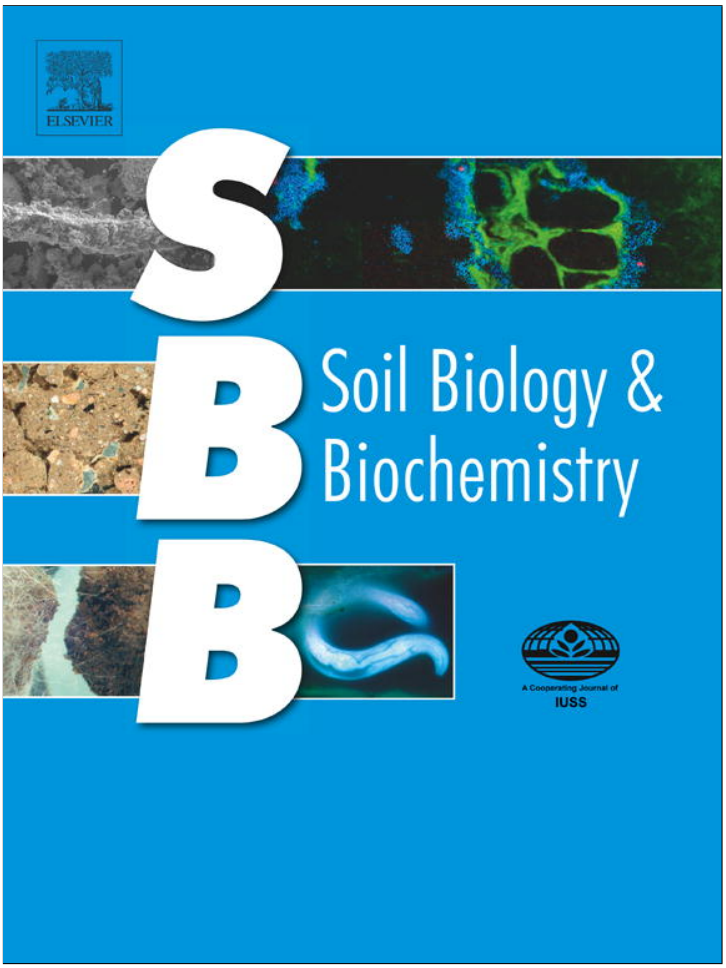

Soil Biology & Biochemistry 65 (2013) 69–74

Contents lists available at SciVerse ScienceDirect

Soil Biology & Biochemistry

journal homepage: www.elsevier.com/locate/soilbio

Arbuscular mycorrhizal fungal hyphae mediating acidification can promote phytate mineralization in the hyphosphere of maize (*Zea mays* L.)

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ARTICLE INFO

Article history:
Received 29 December 2012
Received in revised form
2 May 2013
Accepted 16 May 2013
Available online 31 May 2013

Keywords:
Ammonium
Mycelium
Phosphatases
Organic phosphate
Hydrolysis
Phytin

ABSTRACT

Mycorrhizal and non-mycorrhizal maize (*Zea mays* L.) plants were grown in two-compartment rhizoboxes, in order to study the effect of mycorrhizal hyphae-mediated acidification on organic P mineralization in the hyphosphere. The soil in the two compartments was supplemented with either KNO_3 or $(NH_4)_2SO_4$, and phytin (0 or 75 mg P kg^{-1}) was added to the hyphal compartment. P content in the shoots was significantly higher for the NH_4^+ treatment than for the NO_3^- treatment, but only in the combined presence of phytin and AM fungal mycelium (*Rhizophagus intraradices*). NH_4^+ treatment under these conditions also led to a decrease in hyphosphere pH, enhanced phosphatase activity in the hyphosphere and accelerated mineralization of phytin compared to the NO_3^- treatment. The results show that hyphosphere acidification induced by absorption of NH_4^+ by the AM fungal mycelium leads to an increase in phosphatase activity, and consequently enhances mineralization of phytin and improves maize uptake of P from phytin-P.

1. Introduction

Organic phosphate represents up to 80% of the total phosphorus (P) in a range of soils (Dalal, 1977; Turner et al., 2005). This constitutes a significant reserve of P that is potentially available to plants after hydrolysis by phosphatases (Pases), and it makes a considerable contribution to plant P nutrition (Fransson and Jones, 2007). Organic phosphate in the soil is originally derived from microbial, plant or animal residues, and is present in a variety of chemical forms, including phosphate esters, phosphonates and phosphoric acid anhydrides (Turner et al., 2005).

The concept of Pase-mediated biological transformation or turnover of organic phosphate is not new. It is generally recognized that organic phosphate mineralization is driven by plant root-excreted Pases in the rhizosphere under P-deficient soil conditions, or by microbial-derived Pases in bulk soil, where soil microbes transform organic phosphate to microbial biomass phosphorus (Lambers et al., 2009; Richardson and Simpson, 2011). The process of Pase-mediated organic phosphate mineralization in soil depends on both the Pase activity itself, which is strongly correlated with soil pH (Turner and Haygarth, 2005) and on the organic P substrate availability (George et al., 2008). The ability to release Pase has been considered as a crucial adaptation strategy for plants to cope with P deficiency stress (Marschner, 1995). However, organic P is not always readily available to plants, since soil components like iron and aluminum oxides have a high capacity to retain phosphates (Celi et al., 1999; Ognalaga et al., 1994; Shang et al., 1990, 1992), while in calcareous soil, Ca^{2+} or Mg^{2+} may combine with organic phosphates to form insoluble salts (Turner et al., 2005). Such chemical reactions in soil may reduce the solubility of organic phosphate, and reduce its availability to Pase hydrolysis.

However, these effects can be countered in neutral or alkaline soils by rhizosphere acidification, which can promote mobilization of sparingly soluble inorganic phosphates (Marschner, 1995). Recent studies have shown that a decrease of rhizosphere pH from >7 to ~ 6 both enhanced Pase activity and increased substrate availability to Pases by promoting dissolution of phytin, thus improving P uptake of plant from phytin both under axenic conditions and in soil (Ding et al., 2011). This significant observation

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http://dx.doi.org/10.1016/j.soilbio.2013.05.010

suggests that rhizosphere acidification may play a crucial role in mineralizing organic P, and could be considered an important aspect of how plants solubilize organic phosphates from soil.

In conjunction with plant roots, arbuscular mycorrhizal (AM) fungi are also important in organic phosphate transformation or turnover. These fungi promote mineralization of organic phosphates, acquire P and deliver it to host plants (Feng et al., 2003; Joner and Jakobsen, 1995; Koide and Kabir, 2000), in a pathway thought to be mediated by enhanced acid phosphatase activity at the hyphae–soil interface (hyphosphere) (Feng et al., 2002; Joner and Johansen, 2000). When the soil was supplied with NH_4^+, reduction of soil pH and development of a P depletion zone were detected at both the root-soil and the AM fungal hyphae–soil interfaces (Li et al., 1991b). The acidification in the hyphosphere promoted the mobilization of sparingly soluble calcium phosphates, thus increasing P uptake and growth of clover plants (Yao et al., 2001). However, there is no information on whether acidification in the hyphosphere promotes the mineralization of insoluble phytates.

In this study, we examined the hypothesis that acidification induced by NH_4^+ combined with hyphae of AM fungi in the hyphosphere would enhance Pase activity for accelerating mineralization of phytin-P and consequently improve maize uptake of P from phytin-P.

2. Materials and methods

2.1. Soil and microcosms

The soil used in this study was collected from Tai'an, Shandong Province, China and was a clay soil with the following physico-chemical properties: pH (soil:H_2O 1:5) 6.4, organic matter 5.19 g kg^{-1}, mineral nitrogen 7.2 mg kg^{-1}, Olsen-P [($NaHCO_3$)-extractable] 4.9 mg kg^{-1}, and NH_4Cl-exchangeable potassium 117.3 mg kg^{-1}. The soil was air-dried, sieved (2 mm) and then irradiated before use to eliminate indigenous microorganisms (10 kGy ^{60}Co γ-rays, Beijing Radiation Application Research Center).

The microcosms used were acrylic boxes constructed to permit spatial separation of soil zones for root and hyphal growth. The boxes had two compartments, one for root growth including mycorrhizal structures (root compartment), and a second compartment (buffer zone and hyphal compartment), separated from the first by 30 μm nylon mesh through which hyphae but not roots could pass (Fig. 1). Soil density was adjusted to 1.2 g cm^{-3}, and added to the boxes in the following amounts: 800 g in the root compartment, 320 g in the buffer zone, and 480 g in the hyphal compartment.

2.2. Host plants and mycorrhizal inoculum

Maize (*Zea mays* L., Nongda 108) seeds were surface sterilized with 10% H_2O_2 for 10 min, thoroughly washed 5–8 times with distilled water and then germinated on moistened filter paper at 26 °C. Two seeds were initially sown in each root compartment and thinned to one after emergence.

The inoculum of *Rhizophagus intraradices* (formerly *Glomus intraradices*, BEG 141, kindly supplied by Prof. Vivienne Gianinazzi-Pearson, INRA, France) was propagated on maize and clover, and consisted of spores, mycelium, root fragments and soil. Mycorrhizal treatments received 15 g inoculum and non-mycorrhizal treatments received an equivalent amount of sterilized inoculum together with 5 ml filtrates of unsterilized inoculum to provide a similar microflora except for the absence of the mycorrhizal fungus. The inoculum was mixed uniformly with the soil of the root compartment before sowing of the germinated seed.

2.3. Experimental design

The experiment was set up in a randomized block design with three factors: (1) two different nitrogen forms, nitrate and ammonium; (2) two different organic P levels; (3) two AMF levels, inoculated with or without *Rhizophagus intraradices*. N was supplied either as KNO_3 (200 mg N kg^{-1} soil) or as $(NH_4)_2SO_4$ (200 mg N kg^{-1} soil) and was added uniformly to all compartments. Organic P was added (0 or 75 mg P kg^{-1} soil) as phytin (TCI, Tokyo) and was applied only to the hyphal compartment. The nitrification inhibitor 3, 4-dimethylpyrazole phosphate (DMPP), a commercial inorganic solution product named "ENTEC flüssig" produced in Germany, was added to KNO_3 and $(NH_4)_2SO_4$ at a rate of 1% (w/w) of nitrogen applied. Soil moisture samplers (Rhizosphere Research Products, Wageningen) were inserted carefully into the hyphal compartment

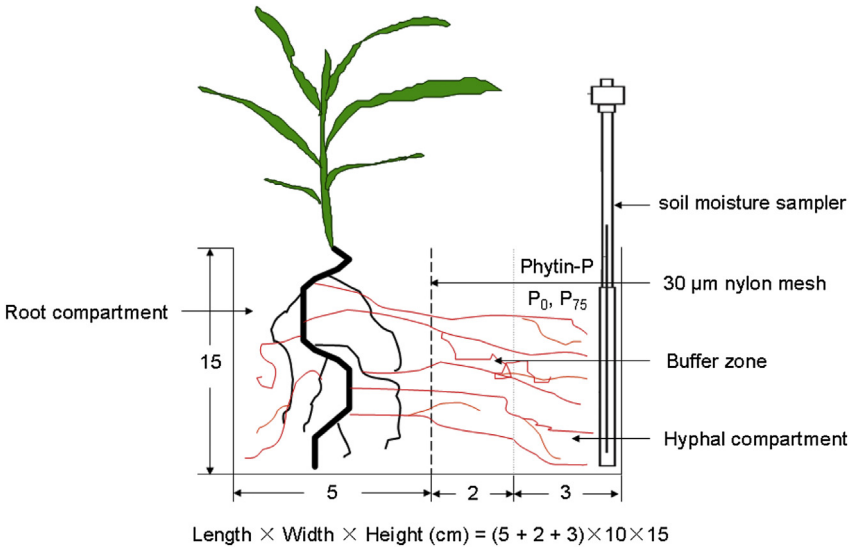

Fig. 1. Schematic drawing of the two compartments cultivation system (rhizobox) used in this study. The rhizoboxes were divided into a root compartment and a hyphal compartment, separated by a 30 μm nylon mesh and a buffer zone. Overall dimensions were 10 × 10 × 15 cm.

close to the wall of the compartment (Fig. 1). The experiment was carried out in three-fold replication, and the 24 boxes were arranged in randomized block design in the greenhouse, with the position of each box re-randomized every week. Distilled water was supplied to all compartments to maintain the soil moisture level close to field capacity (about 20% w/w) during the period of growth.

All treatments received basal mineral nutrients which were added uniformly to all compartments at rates of 200 mg kg^{-1} K as K_2SO_4, 50 mg kg^{-1} Mg as $MgSO_4{\cdot}7H_2O$, 5 mg kg^{-1} Zn as $ZnSO_4{\cdot}7H_2O$, 5 mg kg^{-1} Mn as $MnSO_4{\cdot}H_2O$ and 2 mg kg^{-1} Cu as $CuSO_4{\cdot}5H_2O$. In addition, 10 mg kg^{-1} P was applied as KH_2PO_4 to the root compartment.

2.4. Harvest and sample analysis

Soil solution was extracted from each microcosm immediately before harvest using the soil moisture sampler, and the pH of the soil solution was determined for the different treatments. The plants were harvested after eight weeks of growth. The roots were washed from soil with tap water, and cut into 1-cm-long segments in order to determine the percentage of root length colonized by AM fungi, as previously described (Giovannetti and Mosse, 1980). The remainder of the plant material was dried in a forced-air oven at 70 °C for 48 h and weighed. Samples were then milled with a pulverizer prior to elemental analysis and ground plant materials were digested in a H_2SO_4–H_2O_2 mixture at 370 °C for 2 h. Plant P concentration was determined by the standard vanado–molybdate method (Murphy and Riley, 1962).

After harvest, the rhizoboxes were dismantled, separated into root and hyphal compartments and the top 2 cm of soil was removed. To obtain thin slice of hyphosphere soil, the soil block of the hyphal compartment was placed in an acrylic holder of similar dimensions, and then lifted slightly by pushing an acrylic plate (10 mm thickness) underneath. The soil projecting from the holder was then sliced off with a sharp knife. The sliced 10 mm thickness soil was mixed well and regarded as hyphosphere soil used for measuring phosphatase activity and hyphal density.

Pase activity in each hyphosphere soil was assayed using *p*-nitrophenyl phosphate (Sigma St. Louis, MO, USA) as the substrate, in acetate buffer (200 mM) that had been adjusted to the respective hyphosphere pH determined above for each sample. The reaction was incubated for 30 min at 30 °C, and terminated by addition of 0.5 M NaOH and centrifugation for 5 min at 1500 × *g*. Production of *p*-nitrophenol was measured spectrophotometrically at 405 nm (Joner and Johansen, 2000). The hyphal density of hyphosphere soil was measured using a modified membrane filter technique (Jakobsen et al., 1992).

2.5. Statistical analysis

Statistical analyses were performed with SPSS software, version 16.0 (SPSS Inc.). All data were checked for homogeneity of variances using Levene's tests ($P > 0.05$). AM colonization and external hyphae data were analyzed using two-way analysis of variance (ANOVA), with N form and P level as fixed factors in the mycorrhizal treatments. Data for pH and phosphatase activity in the hyphosphere, plant biomass and P uptake were subjected to a three-way analysis of variance (ANOVA). Treatment means were compared by *T*-test at $P < 0.05$ to respectively determine whether differences were significant between NH_4^+ and NO_3^- treatments within each P level or between P_0 and P_{75} treatments within each N form during plant inoculated with two AMF levels for mycorrhizal or non-mycorrhizal.

3. Results

3.1. AM colonization and external hyphae production

The plants were well colonized in the treatments inoculated with *R. intraradices* (Fig. 2A), with root length infection ranging from 55% to 65%. No significant difference in colonization was found between NH_4^+ and NO_3^- treatments when no P was added (P_0). When phytin was supplied (P_{75}), root colonization rate was significantly higher in the NH_4^+ treatment than in the NO_3^- treatment.

In the absence of an added mycorrhizal inoculum there was very little AM fungal colonization observed on the roots, and no AM fungal hyphae seen in the hyphal compartment. By contrast, when the plant was inoculated with *R. intraradices*, the hyphae proliferated more extensively into the hyphal compartment in the NH_4^+ treatments than in the NO_3^- treatments, independently of phytin-P addition (Fig. 2B, Table S1). The P_{75} treatments produced *c.* 1.2 times greater hyphal length density than the P_0 treatments (Table S1).

3.2. Plant growth and P uptake

Inoculation with *R. intraradices* increased plant shoot biomass significantly, N forms or phytin-P supplementation did not affect shoot dry weight (Fig. 3A). Mycorrhizal plants took up more P than did non-mycorrhizal plants, and this mycorrhizal effect was accentuated by phytin supplementation (Fig. 3B, Table S2).

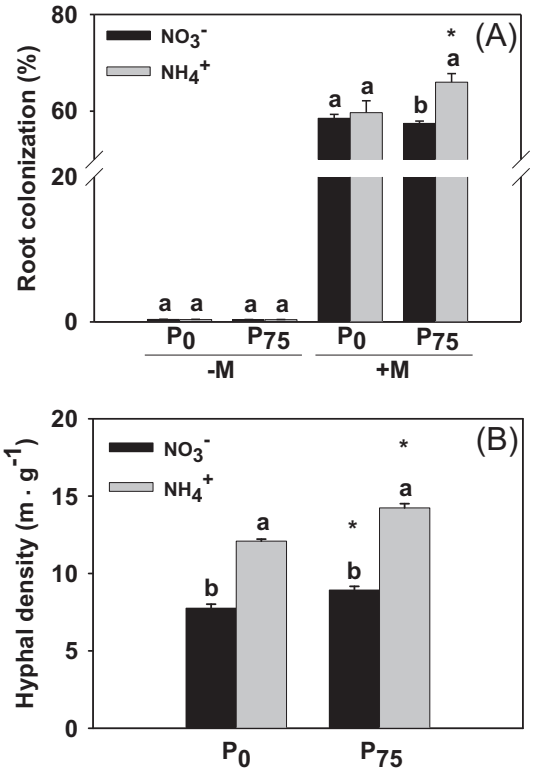

Fig. 2. Root colonization and hyphal density of maize plants treated with NH_4^+ or NO_3^-. (A) Root colonization of plants inoculated with *R. intraradices* (+M) or not inoculated (-M). (B) Hyphal density of *R. intraradices* in the hyphal compartment after inoculation with *R. intraradices*. Two P treatments were applied to the hyphal compartment, P_0 and P_{75}. For each P treatment, letters (a, b) denote significant differences between NH_4^+ and NO_3^- treatments, and asterisks denote significant differences between P_0 and P_{75} treatments for a given nitrogen treatment. Bars represent means + SE. Significance was determined using *T* test, $p < 0.05$.

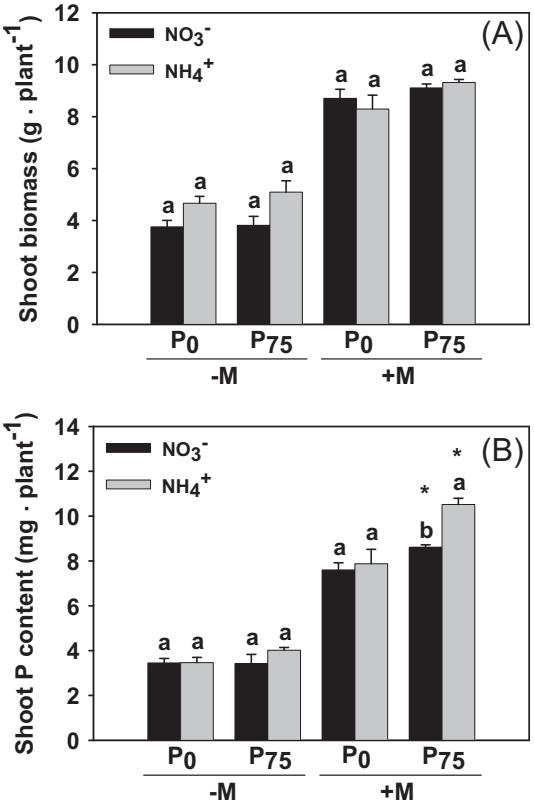

Fig. 3. Shoot dry weight and P content of maize plants treated with NH_4^+ or NO_3^-. (A) Shoot dry weight of plants inoculated with *R. intraradices* (+M) or not inoculated (-M). (B) P content of plants inoculated with *R. intraradices* (+M) or not inoculated (-M). Two P treatments were applied to the hyphal compartment, P_0 and P_{75}. For each P treatment, letters (a, b) denote significant differences between NH_4^+ and NO_3^- treatments, and asterisks denote significant differences between P_0 and P_{75} treatments for a given nitrogen treatment. Bars represent means + SE. Significance was determined using *T* test, $p < 0.05$.

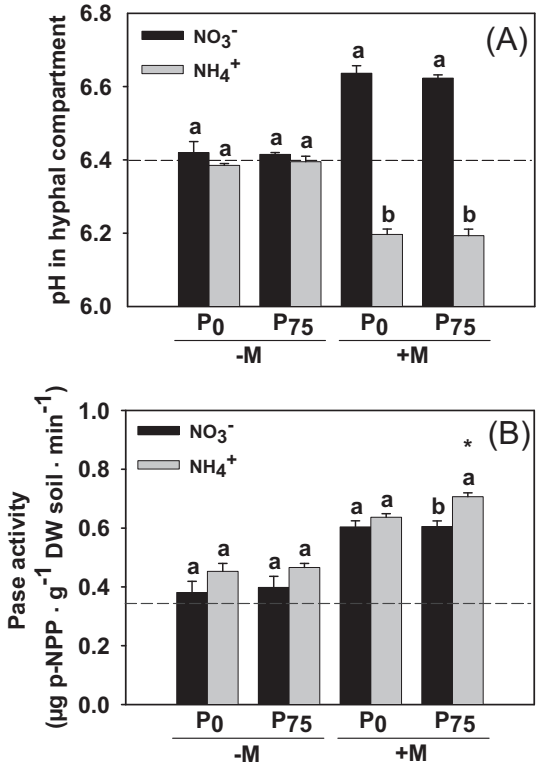

Fig. 4. Soil pH (A) and Pase activity (B) in the hyphosphere of the hyphal compartment from maize plants treated with NH_4^+ or NO_3^-. Plants were either inoculated with *R. intraradices* (+M) or not inoculated (-M), and two P treatments were applied to the hyphal compartment, P_0 and P_{75}. The black dotted line respectively represents the initial pH value of the soil (A) and Pase activity of soil in the absence of plant (B). For each P treatment, letters (a, b) denote significant differences between NH_4^+ and NO_3^- treatments, and asterisks denote significant differences between P_0 and P_{75} treatments for a given nitrogen treatment. Bars represent means + SE. Significance was determined using *T* test, $p < 0.05$.

No significant differences in shoot P content was observed between NH_4^+ and NO_3^- treatments at P_0 or P_{75} in the absence of mycorrhizae. In the mycorrhizal treatments, P content in the shoots did not differ between NH_4^+ and NO_3^- treatments when phytin was not added, but was significantly higher in the NH_4^+ treatment than in the NO_3^- treatment when phytin was supplied to soil (Fig. 3B). The interaction effect between phytin level and N form was not significant ($P = 0.058$) (Table S2).

3.3. Hyphosphere pH and Pase activity

Soil pH in the hyphal compartment was basically consistent with the initial pH under the non-mycorrhizal conditions (Fig. 4A). When *R. intraradices* was inoculated, hyphosphere pH decreased in the NH_4^+ treatment but increased in the NO_3^- treatment relative to the initial soil pH. The difference in hyphosphere pH values between NH_4^+ and NO_3^- treatments was significant ($P < 0.001$) (Table S3). The interaction between N form and mycorrhizal status on the soil pH in hyphal compartment was significant ($P < 0.001$) (Table S3).

There was no significant difference in Pase activity between NH_4^+ and NO_3^- treatments at P_0 or P_{75} when plant was not inoculated with *R. intraradices*. Higher Pase activity in the hyphal compartment was observed in mycorrhizal treatments compared to non-mycorrhizal treatments (Table S3). Hyphosphere Pase activity was significantly higher in the NH_4^+ treatment than in the NO_3^- treatment when phytin-P was added in the presence of mycorrhizae (Fig. 4B). The effects of interaction between any two of or among all three of the factors, mycorrhizal status, phytin level and N form, on Pase activities in the hyphal compartment were not significant (Table S3).

4. Discussion

4.1. Utilization of phytin-P by maize plant

Comparing with non-mycorrhizal conditions, addition of phytin enhanced the P content in maize shoot when AM fungal mycelium was present in the hyphal compartment, independent of N forms (Fig. 3B). Such difference was enlarged when NH_4^+ was supplied comparing with NO_3^- treatment. However, there was no significant difference in shoot P content between NO_3^- and NH_4^+ treatments under non-mycorrhizal conditions, independent of phytin addition. It was significantly higher for NH_4^+ treatment than for NO_3^- treatment when phytin was supplied and AM fungal mycelium was present in the hyphal compartment (Fig. 3B). These results show that maize plants acquire P from phytin via the extraradical mycelium of *R. intraradices*, and that this is promoted in the presence of NH_4^+. However, the interactions between N form and P level or between N form and mycorrhizal status were not significant at 5% critical levels (Table S2). Such results imply that enhanced maize P acquisition from phytin is mainly attributed to NH_4^+ and mycorrhizal status, the joint effects among phytin level, N form and mycorrhizal status are relatively weaker.

Organic phosphorus becomes available to plants following hydrolysis and the release of free phosphate, in a process catalyzed by Pase enzymes (Turner and Haygarth, 2005). Phosphatase activities were higher in mycorrhizal treatments than in non-mycorrhizal treatments (Fig. 4B, Table S3), which is consistent with previous findings that the extraradical mycelium of the AM fungi is important in the enhancement of phosphatase activity in the hyphosphere (Feng et al., 2002). However, no convincing evidence has yet been reported to demonstrate that AM fungi are directly involved in the hydrolysis of organic P by releasing Pases to the hyphosphere. However, most of the relevant studies have been conducted without consideration for the influence of soil microbes. Using *in vitro* culturing methods, Koide and Kabir (2000) showed that extraradical hyphae of *G. intraradices* can hydrolyze organic P. However, a few reports showed that AM fungi were described as very limited in their capacities of directly exploiting organic P sources (Smith and Smith, 2011). Therefore in the present study, some other soil microbes, such as phosphorus solubilizing bacteria may have involved in the hydrolysis process of the organic P in hyphal compartment. Although the growth substrate in such studies is usually sterilized, the AM fungal inoculum itself usually contains bacteria, since bacteria are readily able to colonize AM fungal hyphae (Andrade et al., 1998; Artursson and Jansson, 2003; Toljander et al., 2006). Indeed, in a previous study from our laboratory, we isolated a range of bacterial strains from the AM fungal inoculum, which were able to excrete phosphatase and mineralize phytin (unpublished data). It is therefore very likely that phosphate solubilizing bacteria are involved in the turnover of phytin, and that the phosphatases contributing to the mineralization of phytin in the experiment were derived from both fungi and bacteria. In addition, the extensive growth of extraradical hyphae was observed in the hyphal compartment when plant was inoculated with *R. intraradices* (Fig. 2B), which explored considerably large volume of soil and hydrolyze more phytin, and then plants maybe capture more P via extraradical hyphae.

4.2. Regulation of Pase activity and phytin-P hydrolysis by hyphosphere pH

Pase activities are highly pH and substrate dependent (George et al., 2008; Turner, 2010; Turner and Haygarth, 2005). Soil Pase activities are usually measured at pH 6.5 or pH 11.0 for acid and alkaline Pase, respectively, to allow for reliable comparison of potential Pase activity between studies with different soils or from different laboratories (Carreira et al., 2000; Turner and Haygarth, 2005). However, if the pH of the samples differs from the pH used in the assay buffer, then this approach may not correctly reflect the actual Pase activity in the environmental samples. In the present experiment, the original soil pH was 6.4, but a significant pH decline in the hyphal compartment after NH_4^+ treatment, and an increase after NO_3^- treatment (Fig. 4A, Table S3), indicated that the uptake of NH_4^+ or NO_3^- by AM fungal mycelium induced an imbalance in anion/cation ratio in the fungal tissue (Li et al., 1991b). Most interestingly, Pase activities which were measured at the actual pH values determined in the hyphal compartment (P_{75} treatment) were significantly higher in the NH_4^+ treatment than in the NO_3^- treatment (Fig. 4B). The shoot P content was positively correlated with hyphosphere Pase activity, which is compatible with plant uptake of P from phytin (Fig. 3B). Such results show that a relatively lower pH supports higher Pase activity, and that acidification in the hyphosphere therefore accelerates the mineralization of phytin. Acidification also provides higher substrate availability of organic P to Pase – the solubility of phytin has been shown to be increased at lower pH in the rhizosphere (Ding et al., 2011), and our present results support this finding in the hyphosphere, an even smaller 'micro-zone' where very little is yet known about the biological processes of organic P turnover and transformation. There is the possibility that the enhanced phytin concentration due to acidification might led to increased production of Pase by the AM fungi and/or bacteria in the hyphosphere. Such a feedback loop has not been tested so far.

The activity of Pase in soil is determined by three key factors: the concentration of the Pase protein itself, the activity of the enzyme (governed by environmental factors like pH and bound/free ratio) and the substrate concentration in soil solution (George et al., 2007; Giaveno et al., 2010; Hayes et al., 2000). In this experiment, NH_4^+ stimulated hyphal growth (Fig. 2B), which may increase the quantity of the enzyme produced both by the AM fungus and by bacteria colonizing on the hyphae or living in the hyphosphere soil. In addition, decline in pH was also beneficial to dissolve the metal-ions combined with phytate, which would increase substrate availability of organic P to Pase and keep higher phosphatase activity (George et al., 2008). As a consequence, phytin-P mineralization was enhanced and plant P nutrition was improved in the NH_4^+ treatment. Therefore, the results show that NH_4^+-induced hyphosphere acidification increases the Pase activity and consequently enhances mineralization of organic phosphorus and promotes maize uptake of P from phytin-P.

Moreover, ammonium sulfate was used as one of the N forms in the present study so that it may lead to effects that are ammonium-independent for a sulfate-limited soil. To avoid this possible effect, the basal mineral nutrients were added uniformly to each compartment as sulfate salts - these provide a sulfate concentration in the soil of at least 262 mg kg^{-1}, (in addition to S already present in the soil matrix) which are sufficient for sulfate requirements of bacteria and maize plant. Therefore, sulfate was not a limiting factor for bacteria and plant growth, and the increased phosphatase activities are due to ammonium rather than sulfate.

4.3. Ecological significance of organic P mineralization in the hyphosphere

Rhizosphere or hyphosphere acidification is one of several important mechanisms involved in mobilization of sparingly soluble soil phosphates by plant roots or AM fungi (Hinsinger et al., 2003; Li et al., 1991b; Richardson et al., 2009; Zhang et al., 2004). AM fungi are thought to play an important role in plant acquisition of P from soil beyond the zone accessible to the plant roots (Bucher, 2007; Smith et al., 2011). A number of mechanisms for acquiring P from soil via hyphae have been proposed (Feng et al., 2003; Li et al., 1991a, 1991c; Yao et al., 2001), but the pathway proposed here, that acidification induced by mycelial absorption of NH_4^+ accelerates the mineralization of phytin, has not previously been reported. Indeed, the mechanism whereby acidification of the hyphosphere leads to an increase of Pase activity is still not well explained. Hyphal growth is stimulated more by NH_4^+ than by NO_3^- because less energy is required for assimilation of ammonium (Bledsoe and Zasoski, 1983; Finlay et al., 1992; Jin et al., 2012; Johansen et al., 1994), but a direct relationship between increased hyphal growth and increased Pase activity has not yet been proved, since secretion of Pase by AM fungi, independently of bacteria, is still a controversial subject. Further study is therefore needed to reveal the extent to which interactions between AM fungi and phosphate solubilizing bacteria direct organic P mineralization from soil.

AM fungal mycelia are common in natural environments, even in high input and intensive crop production systems (Grigera et al., 2007; Liu, 2008). High-N fertilizer inputs have resulted in soil acidification in the major Chinese crop-production areas, and average soil pH has declined by 0.5 units from the 1980s to the 2000s (Guo et al., 2010). Consequently, acidification unquestionably possesses an

enormous potential for enhancing mineralization, transformation and turnover of organic P. The results reported here provide a new explanation for this.

Acknowledgements

The study was partly supported by the National Natural Science Foundation of China (31071871), the Ph.D. Programs Foundation of Ministry of Education of China (20120008130001; 20110008110034) and the Innovative Group Grant of the National Natural Science Foundation of China (31121062). We are grateful to Prof. Volker Römheld for supplying DMPP in the experiment.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2013.05.010.

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