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ORIGINAL PAPER

Synergistic interactions between *Glomus mosseae* and *Bradyrhizobium japonicum* in enhancing proton release from nodules and hyphae

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Abstract Soybean (*Glycine max* L. Merr.) seedlings were inoculated with *Glomus mosseae* (GM) and *Bradyrhizobium japonicum* (BJ) together or separately to study the effect of interactions on net H^+ effluxes of nodules or extraradical hyphae by in vivo vibrating electrode techniques. GM promoted three-fold the H^+ effluxes of nodules on mycorrhizal lateral roots and BJ increased eight-fold the net H^+ effluxes of hyphae developing in the vicinity of nodules on lateral roots. Increments in plant P content were positively and linearly correlated with the net H^+ efflux of nodules and hyphae. It is concluded that increased H^+ effluxes of nodules resulted from enhanced nitrogenase activities induced by the presence of the AM fungus in lateral roots. The results point to additive effects of interactions between mycorrhizal fungi and rhizobia in increasing the extent of acidification of the "nodulesphere" and the hyposphere.

Keywords *Bradyrhizobium japonicum* · *Glomus mosseae* · Soybean · Proton release · Nodulesphere · Hyphosphere

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Introduction

The majority of legumes form symbiotic associations with both phosphorus (P) acquiring arbuscular mycorrhizal (AM) fungi and nitrogen (N_2) fixing rhizobia, which are of agronomic and ecological importance (Vance 2001; Scheublin and van der Heijden 2006). The mechanism of efficiently acquiring nutrients by plant–AM fungi–rhizobia tripartite symbioses has attracted much attention. Previous studies have suggested that AM fungi interact positively with rhizobia so that both atmospheric N_2 fixation and P acquisition by legumes from the soil are increased (Asimi et al. 1980; Bethlenfalvay and Yoder 1981; Bethlenfalvay et al. 1982). Nodule formation results in physiological changes such as the establishment of a complex amino-acid cycle in the plant–rhizobium interface (Lodwig et al. 2003), which may induce changes in the cation/anion balance in roots (Sas et al. 2001; Vance 2001), with an excess of cations being balanced by proton (H^+) excretion from roots (Raven and Smith 1976; Raven et al. 1990; Day et al. 2001).

Acidification of the rhizosphere or the mycorrhizal hyphosphere is a typical response of roots or mycorrhizal hyphae to P-deficient conditions, and which can result in mobilization of insoluble phosphate in calcareous soils (Hinsinger et al. 2009). It has also been reported that N_2

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fixation in *Medicago truncatula* can increase plant P acquisition by enhancing H^+ release of roots, and that H^+ release of the root system increases with the amount of N_2 fixed (Tang et al. 2001). However, whether or not nodules can directly release H^+ to acidify soil and how AM fungi may affect this process in mycorrhizal roots is not yet clear. In this work, we have used an H^+-specific vibrating probe to measure H^+ fluxes around both nodules (nodulesphere) and mycorrhizal hyphae (hyphosphere) at the flowering stage of soybean. We analyzed possible effects of AM fungi and/or *Rhizobium* inoculation on H^+ release, and where more P could be absorbed by soybean seedlings from rock phosphate as a consequence of greater nodulesphere or hyphosphere acidification during N_2 fixation.

Materials and methods

Plant growth conditions

Seeds of soybean (*Glycine max* L. Merr.) were supplied by Quzhou Experimental Station, China Agricultural University, Beijing. Seeds were disinfected by 10% (v/v) H_2O_2 for 10 min and 70% (v/v) ethanol for 3 min and then rinsed eight times with sterile deionized water. After germination at 27°C in the dark for 2 days, four germinated seeds were sown in plastic pots (diameter, diameter, and height= 15 cm×15 cm×20 cm) and thinned to two seedlings 3 days after sowing. The growth media was quartz sand (1 kg per pot), autoclaved at 121°C for 45 min. Each pot was supplied with 200 mg rock phosphate [15.7% total P, 2.46% P extractable with 2% (v/v) citric acid].

Seedlings in each pot were fertilized daily with 30 ml of nutrient solution (pH 6.5): 0.75 mM NH_4NO_3, 0.1 mM KH_2PO_4, 2.5 mM $CaCl_2$, 2 mM K_2SO_4, 2 mM $MgSO_4$, 90 μM Fe–EDTA, 46 μM H_3BO_3, 9.1 μM $MnCl_2$, 0.32 μM $CuSO_4$, 0.76 μM $ZnSO_4$, and 0.56 μM Na_2MoO_4, pH 6.5. The pots were leached with 1 l of deionized water every 2 days to minimize salt accumulation. Fresh nutrient solution was added immediately after each leaching. The experiment was conducted in a glasshouse (light intensity 480 μmol m^{-2} s^{-1}, temperature 24–30°C, relative humidity 50–60%).

Experimental design

The experiment was a completely randomized design of four treatments with eight replicates per treatment: (1) no AM fungus or rhizobial inoculation as the control (C), (2) *Glomus mosseae* (Nicol. & Gerd.) Gerdemann & Trappe inoculation (GM), (3) *Bradyrhizobium japonicum* (BJ) inoculation, and (4) dual inoculation (GM/BJ). Plants were harvested 56 days after sowing (R3 stage, flowering), and four replicates of each treatment were harvested to determine fresh and dry weights, nodule number, nitrogenase activity, and N and P concentrations. The remaining four replicates were used to measure net H^+ efflux and AM fungal colonization.

Inoculum of *G. mosseae* (BEG 167) was kindly supplied by AM Fungi Culture Center in the Institute of Plant Nutrition and Resource Science, Beijing Academy of Agriculture and Forestry Sciences. Spores were isolated by wet-sieving method and 1,000 spores were inoculated to each pot for the mycorrhizal treatments. *B. japonicum* (CCBAU15711) was obtained from the *Rhizobium* Collection Center, China Agricultural University. *B. japonicum* cultures were prepared by inoculating cells from a solid culture into 1,000-ml Erlenmeyer flasks containing 400 ml of nutrient solution [mM—K_2HPO_4 2.87, NaCl 1.71, $CaSO_4$ 1.47, $MgSO_4$ 0.81, $MnSO_4$ 6.67×10^{-5}, Na_2MoO_4 4.88×10^{-5}, H_3BO_3 1.62×10^{-4}, $C_6H_5O_7Fe$ 2.96×10^{-5}, $CH_2OH(CHOH)_4CH_2OH$ 27.45, sucrose 14.61, yeast extract 1 gl^{-1}] and propagated on a rotary shaker at 28°C for 72 h. Germinated seeds were dipped in 100 ml of rhizobial suspension containing approximately 1.0×10^9 colony-forming units per milliliter. After planting, 10 ml rhizobial suspension was added close to tap roots. The control (no inoculation) and GM alone treatments received 10 ml autoclaved (121°C, 45 min) rhizobial suspension.

Plant analyses

At harvest, plants were washed with deionized water and divided into shoot, taproot, nodulated lateral roots, and non-nodulated lateral roots. Nodules from lateral roots were separated from those from the taproot. Nodule fresh weight, dry weight, and number of each group were measured, and the total weight or number of nodules per plant was calculated. Plant tissues were oven-dried at 65°C for 72 h, weighed, and ground to pass a 1-mm nylon mesh sieve. Ground plant tissue was digested in a H_2SO_4–H_2O_2 solution at 370°C for 2 h. Total N was determined by the Kjeldahl method (Shi 1994) and total P by the molybdenum blue method (Allen 1989).

Fresh taproots and lateral roots with or without nodules were stained with trypan blue and the percentage mycorrhizal colonization was quantified on 30 root segments, as described by Trouvelot et al. (1986).

Nitrogen-fixing activity

Roots were washed and kept between moist tissue papers. Nodules were collected within 0.5 h after harvest. Nitrogen fixation was determined with five selected and weighed nodules from taproots or lateral roots and repeated four times for each treatment. Nitrogenase activity in nodules

was assayed by reduction of acetylene to ethylene following the method described by Hardy et al. (1968). Ethylene was measured in a gas chromatograph (GC-17A; Shimadzu, Kyoto, Japan) equipped with a flame ionization detector (FID) at 160°C and activated alumina column (GDX 502; Tianjing No. 2 Chemical Reagents Factory, Tianjing, China).

Measurement of net H^+ efflux using a non-invasive micro-test technique

In a preliminary experiment, we found that most active nodules were approximately 8–10 cm from the root apex (Fig. 1a). Three nodules from both taproots and lateral roots were selected for measurement of H^+ efflux at three positions on each nodule: the tip, the middle, and the base (Fig. 1b). Extra-radical hyphae connected to the host root approximately 0.5 cm from each nodule were collected and H^+ efflux measured in subapical (10–40 μm) regions (Fig. 1c, d) in active hyphae that exhibited cytoplasmic streaming (Ramos et al. 2008). Mean H^+ effluxes were calculated for hyphae or nodules on taproots or lateral roots.

H^+ net effluxes were measured with a non-invasive micro-test technique (NMT system BIO-001A; Younger USA Sci. & Tech. Corp., Amherst, MA, USA) (Supporting Information S1). The term NMT has been used interchangeably with SIET (selective ion electrode technique), MIFE (microelectrode ion flux estimation technique), and SERIS (self-reference ion selective electrode technique).

In situ demonstration of acidification around nodules and roots

Intact GM/BJ inoculated plants were gently rinsed free of sand with deionized water 56 days after sowing. Root systems were rinsed in 1 mM $CaSO_4$ solution for 2 min and rinsed with distilled water to a pH of 6.5 in the washout. Acidification around nodule and roots was observed as described by Li et al. (2007) by placing root systems 0.9% agar gel (w/v, pH 6.5) containing 0.1 g/l pH indicator (bromocresol purple) and the plant nutrient solution without P and N. The color change around the root system was observed by eye after 12 h.

Statistical analysis

All data were subjected to a two-way ANOVA using the SAS™ software (SAS Institute Inc. 1989). Treatment means were compared by the least significant differences (LSD) at $P=0.05$.

Results

Root colonization by GM and/or BJ and nodule biomass

AM fungal colonization was higher in the lateral roots than in the tap root ($P<0.05$), regardless whether GM was inoculated or dually with BJ (Table 1). Colonization levels in taproots were not different between the GM and GM/BJ treatments, while they were significantly higher with nodules than without nodules in lateral roots. Dual GM/BJ inoculation significantly increased the number of nodules on both taproots and lateral roots compared to inoculation with *B. japonicum* (BJ) alone (Fig. 2), and total nodule numbers per root system were significantly higher in the dual GM/BJ treatment ($P<0.05$). Mycorrhizal development increased nodule biomass (dry weight) on lateral roots but not on taproots compared to the BJ treatment alone (Fig. 2).

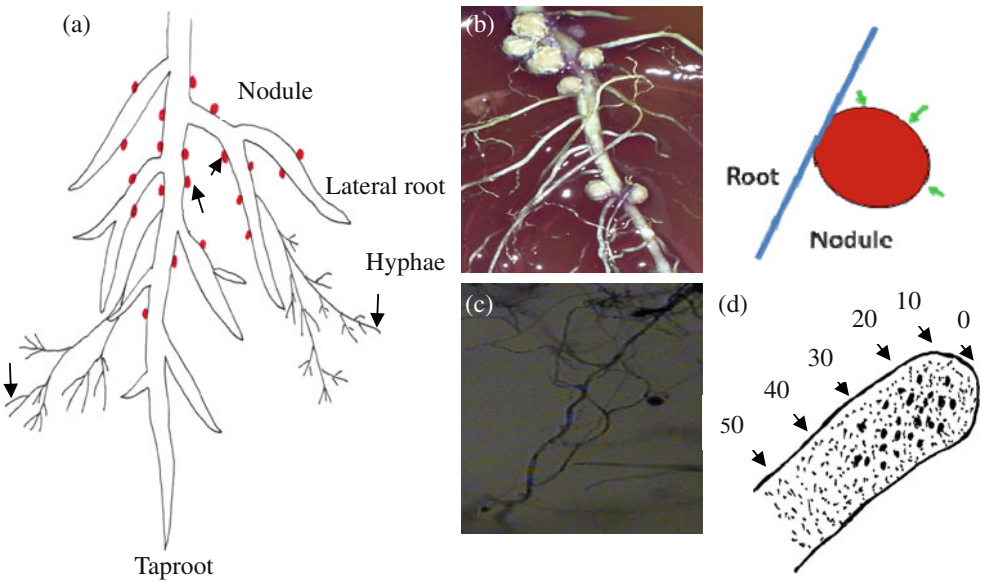

Fig. 1 Different positions of H^+ effluxes measured in the nodule and hyphae on taproots or lateral roots using an H^+-specific vibrating probe. **a** Graphical representation of the whole root. *Arrows* indicate the positions of the H^+ flux in each region, including nodules and hyphae. **b** Graphical representation of H^+ effluxes detected at the tip, the middle, and the base of nodule (*arrow*). **c** Hyphae detected is representative of three branched hyphae. **d** Graphical representation of H^+ effluxes detected on branched hyphae. *Black arrowheads* indicate the probe positioning, and the *numbers* describe the distance from the hyphal tip (μm): apical (0–10 μm), subapical (10–40 μm)

Table 1 Effects of *G. mosseae* (GM) and/or *B. japonicum* (BJ) inoculation on soybean biomass (g DW plant^{-1}), mycorrhizal colonization (%), and nodule activity (C_2H_4 μmol/DW/h) at 56 days after sowing

Inoculation	Plant biomass		Mycorrhizal colonization		Nodule activity	
	(g DW plant^{-1})		(%)		(C_2H_4 μmol/DW/h)	
	Shoots	Roots	TR	LR	TR	LR
Control	1.62±0.04c	0.35±0.04c	0b	0d	–	–
BJ	2.05±0.07b	0.56±0.02b	0b	0d	1.76±0.2b	1.94±0.15b
GM	2.20±0.11b	0.61±0.04b	4.7±0.2a	39.9±1.4c	–	–
GM/BJ	3.48±0.07a	0.93±0.02a	4.9±0.4a	63.4±4.1b[a] 78.2±2.7a[bc]	2.22±0.1a	3.48±0.34a[d]

Data are means±SE (n=4). Different letters in the same column denote significant differences at P=0.05

[a] Mycorrhizal colonization of non-nodule lateral root (LR−)

[b] Mycorrhizal colonization of noduled-lateral root (LR+)

[c] Significant difference at P=0.05 in mycorrhizal colonization between both TR to LR and LR− to LR+ in the GM/BJ treatment by t test

[d] Significant difference at P=0.05 in nodule activity between TR and LR in the GM/BJ treatment by t test

Effects of GM and/or BJ inoculation on plant growth, and N and P acquisition

Biomass production of both shoots and roots was greatest in dually (GM/BJ) inoculated plants, intermediate with GM or BJ alone, and least in the non-inoculated treatment (Table 1). The same trend was observed for the P and N content (Table 2) or concentration (Supporting Information S2) of shoots, roots, and nodules.

Effects of GM on nodule nitrogenase activity

Overall nodule nitrogenase activity was significantly higher in the dual GM/BJ treatment than the sole BJ treatment, indicating that GM promoted nodule nitrogenase activity (Table 1). Nitrogenase activity was similar between nodules from the lateral roots and taproot in the BJ treatment, but significantly higher in nodules from lateral roots than from the taproot in the dual GM/BJ treatment.

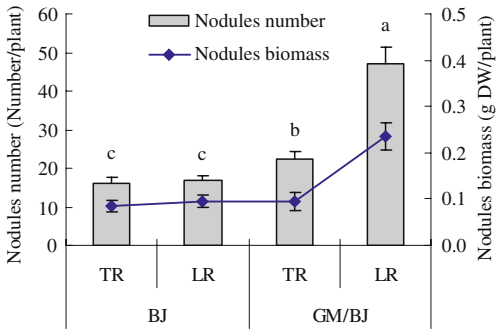

Fig. 2 Effects of *G. mosseae* (GM) and/or *B. japonicum* (BJ) inoculation on the number and biomass of nodules developing on the taproot and lateral roots of soybeans at 56 days after sowing. Values are means±SE (n=4). Different *letters* indicate significant differences at P=0.05. *TR* taproot, *LR* lateral roots, *GM* inoculated with *G. mosseae* alone, *BJ* inoculation with *B. japonicum* alone, *GM/BJ* dual inoculated with *G. mosseae*+*B. japonicum*

Effects of GM and/or BJ inoculation on net H^+ effluxes of roots, nodules and extraradical hyphae

H^+ acidification indicated by yellowing of the pH indicator was observed around roots or nodules in the entire root systems of dual GM/BJ inoculated soybean seedlings (Fig. 3). Neither GM nor BJ inoculation significantly affected tap root H^+ efflux, but this was increased along lateral roots in the presence of both microorganisms (Supporting Information S3). H^+ efflux measurements of nodules from the taproot showed no differences between inoculation treatments, while nodules growing on dual inoculated (GM/BJ) lateral roots had significantly higher net efflux levels than with BJ inoculation alone (Fig. 4a). Nodule development significantly affected the proton release activity of hyphae. H^+ effluxes of hyphae collected from nodulated taproot and lateral roots were significantly higher after dual GM/BJ inoculation than GM inoculation alone (Fig. 4b). H^+ effluxes of hyphae were enhanced eight-fold in the vicinity of nodules on lateral roots. No significant differences in H^+ efflux were detected in hyphae from the two treatments in the absence of nodulation. Overall, H^+ efflux was highest in hyphae from nodulated lateral roots, intermediate with taproots, and lowest with lateral roots without nodules.

The relationship between H^+ effluxes in nodule or hyphae and P uptake of plant

Net H^+ efflux of nodules growing on lateral roots was linearly correlated (r=0.9602, n=8; P<0.05) with the

Table 2 Effects of *G. mosseae* (GM) and/or *B. japonicum* (BJ) inoculation on N and P content (mg/plant) in shoots, roots, and root nodules of soybeans at 56 days after sowing

Inoculation	N and P content (mg/plant)					
	Shoots		Roots		Nodules	
	N	P	N	P	N	P
Control	15.1±0.3d	0.72±0.03d	2.4±0.2d	0.19±0.02d	–	–
BJ	37.6±0.8b	1.06±0.06c	5.2±0.4b	0.38±0.02c	35.1±1.6b	0.42±0.01b
GM	32.9±0.2c	1.95±0.02b	5.3±0.2b	0.58±0.02b	–	–
GM/BJ	79.4±3.9a	3.26±0.21a	16.3±1.2a	0.95±0.06a	76.1±1.3a	1.80±0.03a

Data are means±SE (n=4). Different letters in the same column denote significant differences at P=0.05

increment in plant P content in between dual GM/BJ and sole BJ treatments (Fig. 5a). The nitrogenase activity of nodules was linearly correlated (r=0.8663, n=16; P<0.05) with the net H^+ efflux of nodules (Fig. 6). Increment in plant P content between the GM/BJ and the GM treatments was also linearly correlated (r=0.9665, n=8; P<0.05) with the net H^+ efflux of hyphae (Fig. 5b).

Discussion

Interactions between mycorrhizal fungi and N_2-fixing microbes is a common and crucial strategy in plant nutrient acquisition for partners to access limiting resources like N and P (van der Heijden et al. 2006; He et al. 2009). The increased nodulation and nodule nitrogenase activities of *G. mosseae*-colonized soybean plants are consistent with previous reports (see for example, Asimi et al. 1980; Bethlenfalvay and Yoder 1981; Bethlenfalvay et al. 1982, 1985). It is well known that the establishment of one type of symbiosis may influence another symbiotic association (Bonfante and Anca 2009). Increased nodulation and nitrogen-fixing activity in mycorrhizal plants has been attributed to satisfaction by AM fungi of the high P demand for these processes (Asimi et al. 1980). Stimulation of *G. mosseae* colonization in nodulated plants might be explained by the promotion of lateral root formation, which would provide new targets for mycorrhizal colonization (Olah et al. 2005). This has been strongly supported by a recent report which showed the root growth and branching of the legume *M. truncatula* was stimulated by AM fungus *G. intraradices* secreted symbiotic signals which has been identified as a mixture of sulfated and non-sulfated simple lipochitooligosaccharides (LCOs) (Maillet et al. 2011). Moreover, AM fungi have been supposed to have high N requirements (Scheublin et al. 2004; Scheublin and van der Heijden 2006), so *G. mosseae* may also be attracted by the relatively high nitrogen content in dual inoculated roots.

The excess H^+ generated in nodules by N_2 fixation (Day et al. 2001) is partly translocated to the roots in order to maintain cytoplasmic pH (Supporting Information S3) (Raven and Smith 1976; Raven et al. 1990). Furthermore, in N_2-fixing legumes, the assimilated N is transferred from the nodules into roots, and subsequently the nodulated roots absorb relatively less nitrate which causes a higher cation to anion ratio in the root system and induces H^+ release from roots (Raven et al. 1990; Sas et al. 2001). However, there has been no direct in situ evidence to show whether nodules release H^+ to acidify the surrounding soil environment. The present study has demonstrated that an acidified zone does occur around soybean nodules, although not all, which may be related to a natural variability in N-fixing activities (Scheublin and van der Heijden 2006). Moreover, in situ H^+ efflux measurements have confirmed a strong H^+ efflux around nodules which is correlated to nodule nitrogenase activities and higher in mycorrhizal roots systems, perhaps as a feedback of an increased nitrogenase activity.

Increased H^+ exudation from roots is a widely accepted plant mechanism to positively adapt to P-deficient soils and provide access to the non-soluble pool of soil P (Hinsinger

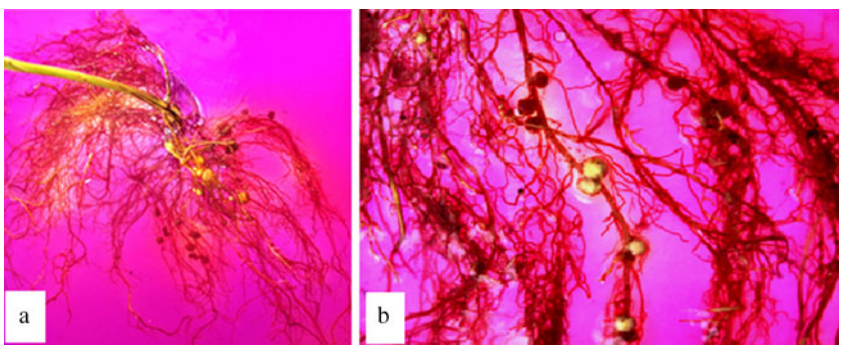

Fig. 3 Visualization of rhizosphere H^+ acidification indicated by a *yellow color* around **a** the entire root system and **b** nodules on the lateral roots (×10) in a 56-day-old dual GM/BJ inoculated soybean seedling

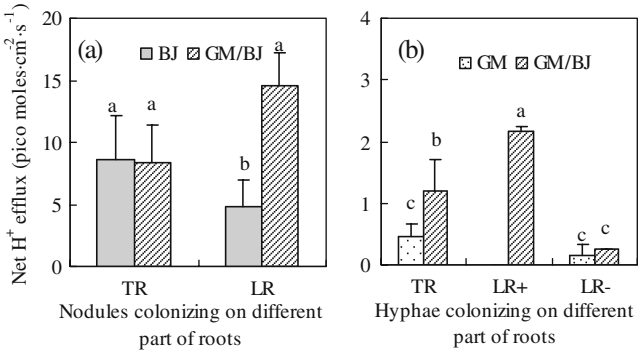

Fig. 4 Net H^+ effluxes (pmol cm^{-2} s^{-1}) of nodules (**a**) and of extraradical hyphae (**b**) associated with the taproot or lateral roots. Values are means+SE (n=4). Different *letters above the bars* indicate significant differences at P=0.05. *TR* taproot, *LR* lateral roots, *LR+* lateral roots with nodules, *LR−* lateral roots without nodules, *GM* inoculated with *G. mosseae* alone, *BJ* inoculation with *B. japonicum* alone, *GM/BJ* dual inoculated with *G. mosseae*+*B. japonicum*

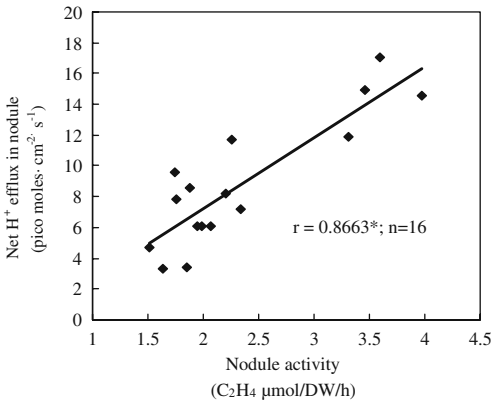

Fig. 6 Relationships between net H^+ efflux of nodules and nodule nitrogenase activity in treatments inoculated with *B. japonicum* alone (BJ) or dual inoculated with *G. mosseae*+*B. japonicum* (GM/BJ)

et al. 2009). In the present study, soybean plants were supplied with 200 mg/pot rock phosphate plus a daily supply of 30 ml low P nutrient solution to compensate P shortage. In spite of this, P concentrations in roots and shoots were only 0.46–0.94 mg/g at flowering stage (Supporting Information S2), which ranks as severe P deficiency according to Bell et al. (1995). The P content (mg/plant) was increased by dual inoculation and tissue concentrations were highest (0.94 mg/g) in these plants. The P content and concentration in nodules was significantly higher in dual inoculated plants (1.8 mg/plant, 5.46 mg/g) than in the treatment with BJ alone (0.42 mg/plant, 2.32 mg/g), and this may be related to the net increase in H^+ effluxes from the nodules.

AM fungal hyphae can extrude protons and reduce pH by up to 1 unit in the hyphosphere soil (Li et al. 1991). More recently, Ramos et al. (2008) used an H^+-specific vibrating probe to analyze the effect of phosphate (P) and sucrose on H^+ fluxes around spores and along hyphae of *Gigaspora margarita* during presymbiotic growth. High H^+ effluxes were detected in the subapical region of hyphae grown in the absence of P, and root exudates of clover stimulated the H^+ efflux rate. In the present study on *G. mosseae*, H^+ effluxes of hyphae were enhanced eight-fold in the vicinity of nodules on lateral roots of soybean. This phenomenon has not been previously described and the mechanism responsible is not clear. It is unlikely that it results from modifications in microbial populations associated with nodules or hyphae since these would not explain the constant increased flow of protons around the symbiotic structures. It may be rather attributed to changes in the root

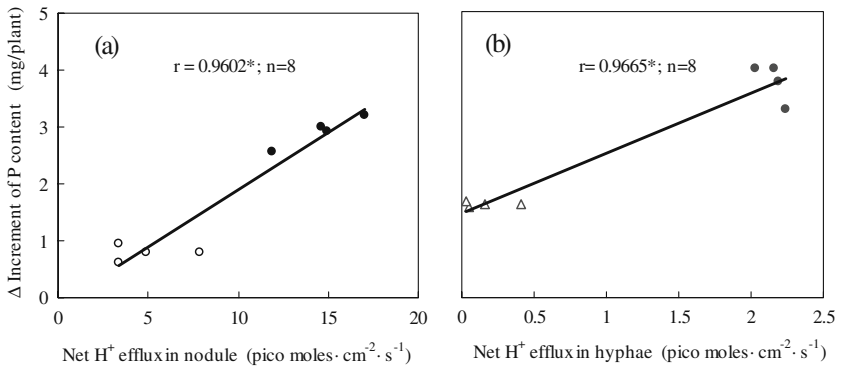

Fig. 5 Relationships between the increment of P content in plants and net H^+ efflux (pmol·cm^{-2}·s^{-1}) from **a** nodules or **b** hyphae on lateral roots in different inoculation treatments. Fig. 5a shows P uptake improvement in plants due to nodule H^+ excretion (*open circles* sole BJ inoculation treatment, *filled circles* dual GM/BJ inoculation treatment). Increments of P content were calculated by P content in GM/BJ treatment subtracted by P content in GM alone treatment, or by P content in BJ inoculation alone treatment subtracted by P content in the control. Fig 5b shows P uptake improvement in plants due to mycorrhizal hyphae H^+ excretion (*open triangles* GM inoculation alone treatment, *filled circles* dual GM/BJ inoculation treatment). Increments in P content were calculated by P content in the GM/BJ treatment subtracted by P content in BJ alone treatment, or by P content in GM inoculation alone treatment subtracted by P content in the control

environment due to nodule exudates (Raven et al. 1990), with a feedback on hyphae following the enhanced nitrogenase activity caused by AM fungal colonization. It is known that root exudates contain active compounds which can promote hyphal growth and development of AM fungi (Akiyama et al. 2005; Steinkellner et al. 2007; Gamalero et al. 2008). Some bacteria have been reported to be able to excrete growth regulating materials that stimulate the metabolic activity and growth of AM fungal hypha (Artursson et al. 2006; Frey-Klett et al. 2007; Bonfante and Anca 2009), but the influence of nodule exudates has not been investigated. Considering the significant correlation between increased P uptake by mycorrhizal plants and H^+ efflux levels from hypha, this phenomenon may play a role in increasing mobilization of non-soluble soil phosphates (Yao et al. 2001).

In conclusion, in situ pH changes demonstrated using a pH sensitive dye or H^+ selective microelectrode detection showed protons are released from nodules and hyphae associated with soybean roots. We propose to term the H^+ gradients formed around nodules as a "nodulesphere" effect. There are many reports showing that the rhizosphere or mycorrhizal hyphosphere have distinct biochemical, chemical, and physical characteristics which differ from the bulk soil (Johansson et al. 2004; Hinsinger et al. 2005). However, to our knowledge, this is the first report of a "nodulesphere" effect and raises questions concerning the repercussions on other biological processes affecting nutrient transformation and bioavailability, as well as the multiple ecological roles of nodule–mycorrhizal cross interactions in plant adaptation to nutrient-limiting environments.

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References

Akiyama K, Matsuzaki K, Hayashi H (2005) Plant sesquiterpenes induce hyphal branching in arbuscular mycorrhizal fungi. Nature 435:824–827

Allen SE (1989) Chemical analysis of ecological materials. Blackwell, Oxford

Artursson V, Finlay RD, Jansson JK (2006) Interactions between arbuscular mycorrhizal fungi and bacteria and their potential for stimulating plant growth. Environ Microbiol 8:1–10

Asimi S, Gianinazzi-Pearson V, Gianinazzi S (1980) Influence of increasing soil phosphorus levels on interactions between vesicular–arbuscular mycorrhizae and *Rhizobium* in soybeans. Can J Bot 58:2200–2205

Bell P, Hallmark W, Sabbe W, Dombeck D (1995) Diagnosing nutrient deficiencies in soybean, using M-DRIS and critical nutrient level procedures. Agron J 87:859–865

Bethlenfalvay G, Yoder J (1981) The *Glycine–Glomus–Rhizobium* symbiosis. Physiol Plant 52:141–145

Bethlenfalvay G, Pacovsky R, Bayne H, Stafford A (1982) Interactions between nitrogen fixation, mycorrhizal colonization, and host-plant growth in the *Phaseolus–Rhizobium–Glomus* symbiosis. Plant Physiol 70:446–450

Bethlenfalvay G, Brown M, Stafford A (1985) The *Glycine–Glomus–Rhizobium* symbiosis: II. Antagonistic effects between mycorrhizal colonization and nodulation. Plant Physiol 79:1054–1058

Bonfante P, Anca IA (2009) Plants, mycorrhizal fungi, and bacteria: a network of interactions. Annu Rev Microbiol 63:363–383

Day D, Poole P, Tyerman S, Rosendahl L (2001) Ammonia and amino acid transport across symbiotic membranes in nitrogen-fixing legume nodules. Cell Mol Life Sci 58:61–71

Frey-Klett P, Garbaye J, Tarkka M (2007) The mycorrhiza helper bacteria revisited. New Phytol 176:22–36

Gamalero E, Berta G, Massa N, Glick BR, Lingua G (2008) Synergistic interactions between the ACC deaminase-producing bacterium *Pseudomonas putida* UW4 and the AM fungus *Gigaspora rosea* positively affect cucumber plant growth. FEMS Microbiol Ecol 64:459–467

Hardy R, Holsten R, Jackson E, Burns R (1968) The acetylene–ethylene assay for N_2 fixation: laboratory and field evaluation. Plant Physiol 43:1185–1207

He XH, Xu MG, Qiu GY, Zhou JB (2009) Use of ^{15}N stable isotope to quantify nitrogen transfer between mycorrhizal plants. JPE 2:107–118

Hinsinger P, Bengough A, Vetterlein D, Young I (2009) Rhizosphere: biophysics, biogeochemistry and ecological relevance. Plant Soil 321:117–152

Hinsinger P, Gobran G, Gregory P, Wenzel W (2005) Rhizosphere geometry and heterogeneity arising from root-mediated physical and chemical processes. New Phytol 168:293–303

Johansson J, Paul L, Finlay R (2004) Microbial interactions in the mycorrhizosphere and their significance for sustainable agriculture. FEMS Microbiol Ecol 48:1–13

Li L, Li SM, Sun JH, Zhou LL, Bao XG, Zhang HG, Zhang FS (2007) Diversity enhances agricultural productivity via rhizosphere phosphorus facilitation on phosphorus-deficient soils. PNAS 104:11192–11196

Li X, George E, Marschner H (1991) Phosphorus depletion and pH decrease at the root–soil and hyphae–soil interfaces of VA mycorrhizal white clover fertilized with ammonium. New Phytol 119:397–404

Lodwig E, Hosie A, Bourdes A, Findlay K, Allaway D, Karunakaran R, Downie J, Poole P (2003) Amino-acid cycling drives nitrogen fixation in the legume–*Rhizobium* symbiosis. Nature 422:722–726

Maillet F, Poinsot V, André O, Puech-Pagès V, Haouy A, Gueunier M, Cromer L, Giraudet D, Formey D, Niebel A, Martinez EA, Driguez H, Bécard G, Dénarié J (2011) Fungal lipochitooligosaccharide symbiotic signals in arbuscular mycorrhiza. Nature 469:48–64

Olah B, Briere C, Becard G, Denarie J, Gough C (2005) Nod factors and a diffusible factor from arbuscular mycorrhizal fungi stimulate lateral root formation in *Medicago truncatula* via the DMI1/DMI2 signalling pathway. Plant J 44:195–207

Ramos AC, Façanha AR, Feijó JA (2008) Proton (H^+) flux signature for the presymbiotic development of the arbuscular mycorrhizal fungi. New Phytol 178:177–188

Raven J, Smith F (1976) Nitrogen assimilation and transport in vascular land plants in relation to intracellular pH regulation. New Phytol 76:415–431

Raven J, Franco A, de Jesus E, Jacob-Neto J (1990) H^+ extrusion and organic-acid synthesis in N_2-fixing symbioses involving vascular plants. New Phytol 114:369–389

SAS Institute Inc (1989) SAS/STAT user's guide. SAS Institute, Cary

Sas L, Rengel Z, Tang C (2001) Excess cation uptake, and extrusion of protons and organic acid anions by *Lupinus albus* under phosphorus deficiency. Plant Sci 160:1191–1198

Scheublin T, Ridgway K, Young J, Van Der Heijden M (2004) Nonlegumes, legumes, and root nodules harbor different arbuscular mycorrhizal fungal communities. Appl Environ Microbiol 70:6240–6246

Scheublin T, van der Heijden M (2006) Arbuscular mycorrhizal fungi colonize nonfixing root nodules of several legume species. New Phytol 172:732–738

Shi R (1994) Determination of the total nitrogen in plant materials. In: Shi RH, Bao SD, Qin HY, An ZS (eds) Soil agriculture chemistry analysis method. China Agriculture Press, Beijing, pp 213–216

Steinkellner S, Lendzemo V, Langer I, Schweiger P, Khaosaad T, Toussaint J, Vierheilig H (2007) Flavonoids and strigolactones in root exudates as signals in symbiotic and pathogenic plant–fungus interactions. Molecules 12:1290–1306

Tang C, Hinsinger P, Drevon J, Jaillard B (2001) Phosphorus deficiency impairs early nodule functioning and enhances proton release in roots of *Medicago truncatula* L. Ann Bot 88:131–138

Trouvelot A, Kough J, Gianinazzi-Pearson V (1986) Mesure du taux de mycorhization VA d'un systeme radiculaire. Recherche de methodes d'estimation ayant une signification funvtionnelle. In: Gianinazzi-Pearson V, Gianinazzi S (eds) Physiological and genetic aspectes of mycorrhizae. INRA Press, Paris, pp 217–221

van der Heijden M, Bakker R, Verwaal J, Scheublin T, Rutten M, van Logtestijn R, Staehelin C (2006) Symbiotic bacteria as a determinant of plant community structure and plant productivity in dune grassland. FEMS Microbiol Ecol 56:178–187

Vance CP (2001) Symbiotic nitrogen fixation and phosphorus acquisition. Plant nutrition in a world of declining renewable resources. Plant Physiol 127:390–397

Yao Q, Li X, Feng G, Christie P (2001) Mobilization of sparingly soluble inorganic phosphates by the external mycelium of an arbuscular mycorrhizal fungus. Plant Soil 230:279–285