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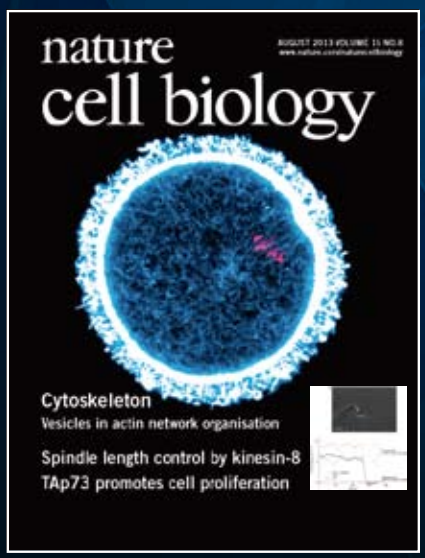

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Proton (H^+) flux signature for the presymbiotic development of the arbuscular mycorrhizal fungi

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Summary

• Ion dynamics are important for cell nutrition and growth in fungi and plants. Here, the focus is on the relationship between the hyphal H^+ fluxes and the control of presymbiotic growth and host recognition by arbuscular mycorrhizal (AM) fungi.

• Fluxes of H^+ around azygopores and along lateral hyphae of *Gigaspora margarita* during presymbiotic growth, and their regulation by phosphate (P) and sucrose (Suc), were analyzed with an H^+-specific vibrating probe. Changes in hyphal H^+ fluxes were followed after induction by root exudates (RE) or by the presence *Trifolium repens* roots.

• Differential sensitivity to P-type ATPase inhibitors (orthovanadate or erythrosin B) suggests an asymmetric distribution or activation of H^+-pump isoforms along the hyphae of the AM fungi. Concentration of P and Suc affected the hyphal H^+ fluxes and growth rate. However, further increases in H+ efflux and growth rate were observed when the fungus was growing close to clover roots or pretreated with RE.

• The H^+ flux data correlate with those from polarized hyphal growth analyses, suggesting that spatial and temporal alterations of the hyphal H^+ fluxes are regulated by nutrient availability and might underlie a pH signaling elicitation by host RE during the early events of the AM symbiosis.

Key words: arbuscular mycorrhiza, *Gigaspora margarita*, H^+-specific vibrating probe, pH signatures, presymbiosis.

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Introduction

Arbuscular mycorrhizas (AM) are the most common underground symbiosis. Colonizing roots of a wide variety of land plants, fungi of the Glomeromycota family transfer phosphate and other minerals scavenged from the soil to host cells. In turn, the host plant supplies carbon to the fungal partner, an obligate biotroph incapable of accomplishing its life cycle in the absence of a host plant (Smith & Gianinazzi-Pearson, 1988; Smith *et al.*, 2003). In plants, sucrose (Suc) is the major form in which fixed carbon is translocated through the phloem to the roots. Once in the root apoplast Suc is hydrolyzed to hexoses by exogenous invertases and then becomes available to AM fungi (Shachar-Hill *et al.*, 1995; Smith & Read, 1997). During this symbiosis, hexose is taken up only by intra-radical hyphae, but additional uptake of glucose and fructose was observed in germ tubes before their first contact with the host root. However, at this presymbiotic stage, the AM fungi live on a basal carbon metabolism (Bago *et al.*, 1999, 2002; Pfeffer *et al.*, 1999). High Suc concentrations, on the other hand, can elicit negative effects on the development of both presymbiotic and symbiotic AM fungal growth (Mosse, 1959; Mugnier & Mosse, 1987). In addition, high phosphate (P) concentrations in soil can also readily inhibit mycorrhizal interaction (Gianinazzi-Pearson & Gianinazzi, 1983 and references therein). The physiological and cellular bases of the response of P and Suc, however, are not well understood.

It is now well established, however, that before infection, germinated AM fungi respond to host root exudates by switching to an active presymbiotic growth phase, which leads to intense hyphal branching in the vicinity of the root (Giovannetti *et al.*, 1994; Giovanetti, 1997; Buee *et al.*, 2000). Little is known about the early physiological changes that precede the initial fungal–plant contact, in particular the nature of the molecular/cellular dialog that is required for recognition of the fungal partner and subsequent successful infection. Hyperpolarization of the plasma membrane and increase on cytosolic pH of *Gigaspora margarita* hyphae seem to be an early response to host root exudates, suggesting that the earliest stages of signaling during AM interaction occur via direct effects on the hyphal membrane rather than via gene expression (Jolicoeur *et al.*, 1998; Ayling *et al.*, 2000). This is in accordance with the notion that immediately downstream of the initial elicitor-receptor recognition, the activation of ion flux is one of the primary responses of the cells (Blumwald *et al.*, 1998, Fromm & Lautner, 2007). Involvement of ion dynamics in cell nutrition and growth in fungi and plants is primarily linked to the regulation of electrical and pH gradients generated in their cell membranes by P-type H^+-ATPases (Feijó *et al.*, 1999; Portillo, 2000). In this context, a relevant and recent observation is that the expression of different fungal H^+-ATPases isoforms can be regulated by the presymbiotic and symbiotic status of the nutrients P and Suc (Requena *et al.*, 2003).

In this work, we analyzed the H^+ ion flux profile around the *G. margarita* lateral hyphae and azygospores during their presymbiotic development using an H^+-specific vibrating probe. Our main goal was to test if the extracellular pH is important for a possible ionic dialogue between the partners of the AM symbiosis and subsequent AM fungal growth. In addition, we attempted to determine what relationships exist between the hyphal H^+ flux oscillations and the control of presymbiotic growth process; what defines the site where polarized growth begins; how hyphal development is regulated depending on the main nutrients exchanged during the symbiotic phase; and whether the changes in AM hyphal H^+ fluxes and growth could be modulated by recognition of host root exudates. Our data demonstrate that H^+ fluxes in specific hyphal domains are strongly influenced by supply of P and Suc and signals derived from host roots, a phenomenon that seems to be related to a differential plasma membrane H^+-ATPase activation. The implications of these findings to the elucidation of the early signaling events involved in the AM fungal cell growth and plant–fungus interaction are discussed.

Materials and Methods

Biological material

Spores of *Gigaspora margarita* Becker & Hall (BEG 34) were purchased from Biorize (Dijon, France). This AM fungal species was chosen because its spores are large (> 150 μm diameter) and it has been used in the previous physiological studies of presymbiotic fungal development (Berbara *et al.*, 1995; Jolicoeur *et al.*, 1998; Ayling *et al.*, 2000 and references therein).

Isolation and germination of spores

Spores were selected by size and morphological assessment and then sterilized as described by Bécard & Fortin (1988) with minor modifications. After sterilization, five to seven spores were placed on coverslip bottom Petri dishes (4.5 cm diameter; Willco Wells BV, Amsterdam, the Netherlands) filled up with 1.35 ml of solidified M medium (Bécard & Fortin, 1988) and stored at 5°C in a cold chamber. Before analysis the dishes were removed from the cold chamber and then incubated in the dark at 26°C for 5–7 d. A fungal germination rate above 90% was observed. In order to repeat the conditions used for the measurements of cytosolic pH of *G. margarita* germ tubes, we used M medium solidified with 0.25% Phytagel (Sigma-Aldrich, Gillingham, UK) as reported by Jolicoeur *et al.* (1998). Phytagel produced a clear and colorless medium, but the preparation of the thinnest gel film was required for a good resolution during the imaging and ion flux measurements, where a very thin electrode tip (*c.* 1.5 μm tip diameter) was vibrated on the hyphal surface.

Measurements of H^+ fluxes using the ion-selective vibrating probe system

A detailed description of the experimental setup of the H^+-selective vibrating probe technique utilized in this study is described in the Supplementary Material, Text S1. Further details on the vibrating probe system can be found in Kühtreiber & Jaffe (1990), Kochian *et al.* (1992), Feijó *et al.* (1999), Shipley & Feijó (1999) and Kunkel *et al.* (2006).

H^+ fluxes measurements from azygospore and presymbiotic hyphae

The H^+ fluxes around the *G. margarita* spores were analyzed before and after germination. Spores stored at 4°C were incubated at 26°C for 5–7 d to induce the germination before analysis. To study the stage before germination, the spores were incubated at 26°C for only 1 h, and after this time we obtained metabolically active nongerminated spores. Those few spores incubated at 26°C for 5–7 d that did not germinate, but were metabolically active, exhibited a similar H^+ flux profile to those incubated for 1 h (data no shown).

All analyses were performed in M medium, at pH 5.8–5.9, as described in previous studies on *G. margarita* (Lei *et al.*, 1991; Jolicoeur *et al.*, 1998; Ayling *et al.*, 2000). Briefly, AM fungi were grown on coverslip bottom dishes containing solid M medium. For nutritional studies, the medium was

supplemented with, or depleted by, 35 μM phosphate (P) and 19.8 mM (1%) sucrose (Suc), depending on the treatment (+P + Suc; +P – Suc; −P + Suc or −P – Suc). Before each analysis in the vibrating probe system, 2 ml of the respective liquid M medium was added to the dishes (covering the AM fungal structures) to adapt the fungi to the new conditions for the experiments. Following this, the cultures were incubated for 20 min before performing measurements of the extracellular H^+ gradients, the data collection always starting on lateral hyphal tips. We chose lateral hyphae for root factor tests, because these hyphae are more accessible than primary hyphae (single germ tube). All analyses were done in an average period of 40–50 min, and after that new dishes with new fungi were taken. The same protocol was used in the measurements of the ion fluxes in hyphae growing near clover roots (Fig. S1) and after treatment with clover root exudates.

For the pharmacological tests, a predetermined volume, based on the dose–response test (Fig. S2), of each pharmacological agent, was carefully added to the liquid M medium covering the fungi and gently agitated, and after 10 min of incubation the analysis was restarted. The P-Type H^+-ATPase inhibitors, 5 μM sodium orthovanadate (Sigma-Aldrich), and 350 μM of erythrosin B (pH 6.0; Sigma-Aldrich), were prepared and placed at room temperature (25°C) before use to maintain the same conditions as for AM fungus. Thus, distillated water (pH 5.8) instead of inhibitors was added to the control. Under the latter conditions, no changes in the H^+ fluxes were detected. Background references (mV correspondent to the M medium) were taken at 2 mm distance from the fungal lateral hyphae or spores and the values were subtracted from the fungal surface measurements. The pH of the medium as continuously monitored ranged from 5.8 to 5.9.

Root exudate extraction

About 200 seeds of *Trifolium repens* L. were surface-sterilized in 70% ethanol for 1 min, and 5% sodium hypochloride for 5 min, and rinsed abundantly with distilled water. Afterwards, the seeds were germinated in pots of 24 l containing sterilized sand, placed in a growth chamber (day : night cycle – 16 h, 23°C : 8 h, 19°C) under 250 μmol m^{-2} s^{-1} of photosynthetic photon flux density, and irrigated twice a week with Clark's solution (Clark, 1975). Forty days after germination, the seedlings were harvested and their roots were washed to remove all sand and soaked in distilled water to obtain the root exudates as described by Buee *et al.* (2000) and Tamasloukht *et al.* (2003) with a few modifications. First, the AM fungus was incubated several times with the root exudates before the H^+ flux analysis, and after this optimization it was incubated for 1 h before analysis in order stabilize the fluxes. After 24 h of incubation with clover root exudates, the medium was changed and the measurements of proton flux in AM hyphae were restarted.

Statistical analysis

All data were analyzed by one-way or two-way ANOVA, which was validated by an appropriated residuals analysis and, when necessary, combined with Tukey's test for multiple comparison. Exclusively, in the case of the estimation of the effects of orthovanadate on hyphal growth, and for the H^+ flux profile around the spores, Student's *t*-test was used. The results are expressed as means with respective standard error, and the numbers of repetitions are given in each figure legend. For the correlation analysis Pearson's correlation coefficient was used. All statistical analyses were conducted in the R program and the level of significance was set at 5% (Ihaka & Gentleman, 1996).

Results

Polarization of H^+ Flux in the azygospore

The analyses of H^+ fluxes around azygospores were carried out in nongerminated and germinated spores. A clear polarized distribution of H^+ fluxes was found in spores, with H^+ influxes in the opposite region of the germ tube emergence. By contrast, H^+ effluxes occur at the site of germ tube emergence (Fig. 1a). The magnitude of the H^+ fluxes in nongerminated spores was much higher than that detected in germinating spores (Fig. 1b), indicating that such polarized H^+ flux is a phenomenon related to the early stages of germ tube emergence. No H^+ flux was detected in senescent and metabolically inactive spores (data no shown).

The pattern of H^+ flux in the presymbiotic hyphae

The H^+ fluxes were analyzed in lateral hyphae of *G. margarita* 5–7 d after germination in three hyphal regions: apical (0–5 μm), subapical (10–40 μm) and distal (60–200 μm). Active hyphae exhibited cytoplasmic streaming and an average growth rate of *c.* 1.6–2.2 μm min^{-1}. Maximal H^+ effluxes of *c.* 0.96 pmol cm^{-2} min^{-1} were localized on the subapical region, and much lower values (*c.* 0.36 pmol cm^{-2} min^{-1}) were detected in the distal region (Fig. 2a).

Time-course analysis of the internal movements of the germ tube and branched hyphae revealed some vesicles and cytoplasmic inclusions similar to the structures previously described by Jolicoeur *et al.* (1998), which were moving around a narrow subapical region, between 5 and 35 μm from hyphal apex (Fig. 2c,d; Video S1).

A role of H^+-ATPase in the hyphal H^+ flux oscillations

In order to reveal the involvement of H^+-ATPase in the hyphal H^+ fluxes, 350 μM of erythrosin B or 5 μM orthovanadate were added to the culture medium. Different degrees of inhibition with erythrosin B were seen mainly between 10

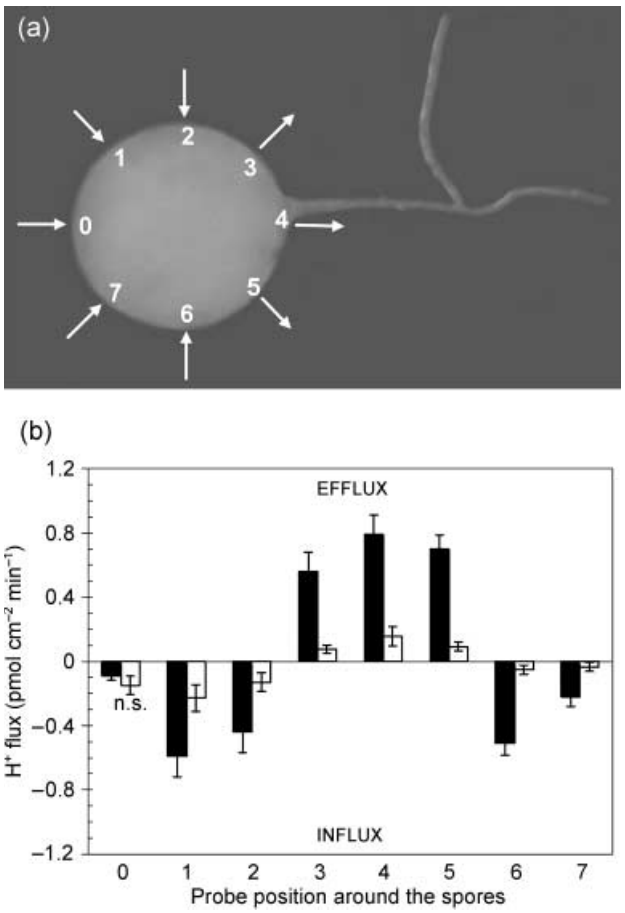


Fig. 1 Measurements of H^+ ion flux in spores of *Gigaspora margarita* using an H^+-specific vibrating probe. (a) Micrograph of a germinated azygospore. Arrows indicate the direction of the H^+ flux in each region. (b) Graphical representation of average values of H^+ fluxes detected at eight different sites (0–7) around the nongerminated (closed bars) and germinated spores (open bars) ($n = 6$). Each site around the spore is representative of four points. Site 4 represents the region of emission of germ tubes exhibiting the highest H^+ efflux (error bars represent average ± SE). n.s., expresses no statistically significant difference by *t*-test at 5%.

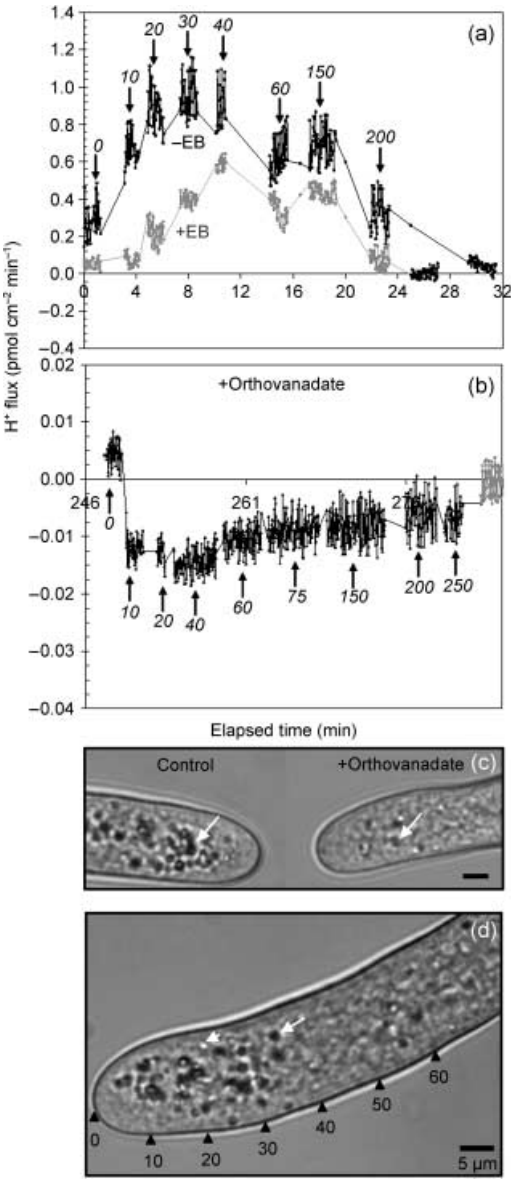


Fig. 2 H^+ flux along the growing presymbiotic hyphae of *Gigaspora margarita*. (a) A representative graphical display of the standard output showing the H^+ flux profile along lateral arbuscular mycorrhizal (AM) hyphae in the presence (+EB) or absence of erythrosin B (–EB). Arrows indicate the probe positioning along the *G. margarita* hyphae growing in M medium ($n = 10$). (b) H^+ fluxes measured in the presence of 5 µM orthovanadate, a specific inhibitor of the plasma membrane H^+-ATPase. The negative values correspond to the influx of H^+ and the positive ones are effluxes. The last points at the end of the curves (a) (+EB, –EB) and (b) represent background signals. (c) Differential interference contrast (DIC) microscopy (magnification ×40) images of a hypha exhibiting a conspicuous presence of dense granules or 'Spitzenkörper-like' structures (white arrow, left), and another hypha after incubation with orthovanadate for 30 min (white arrow, right). Bar, 5 µm. (d) DIC microscopy (magnification ×60) images showing the apical, subapical and hyphal distal regions and their 'Spitzenkörper-like' structures (white arrow) and some vesicles (white arrowhead) distributed along the hyphae. Black arrowheads indicate the probe positioning, and the numbers describe the distance from the hyphal tip (µm).

and 40 µm from the tip, and also between 150 and 200 µm (Fig. 2a). The H^+ effluxes in the regions at a greater distance from the hyphal apex were almost unaffected by this inhibitor, but were fully inhibited by orthovanadate (Fig. 2a,b). On the other hand, 5 µM orthovanadate fully inhibited the H^+ effluxes along the hyphae, except at the hyphal tip, where we detected an H^+ efflux (Fig. 2b). The hyphal growth was maximal in the absence of an H^+-ATPase inhibitor (Video S2), while the addition of orthovanadate dramatically reduced the growth velocity (Video S3) to approx. 96% (*t*-test, $P < 0.01$). Hyphal growth velocity was not completely stopped in the presence of erythrosin B and its color (dark pink) precluded further analysis to determine the extent of growth.

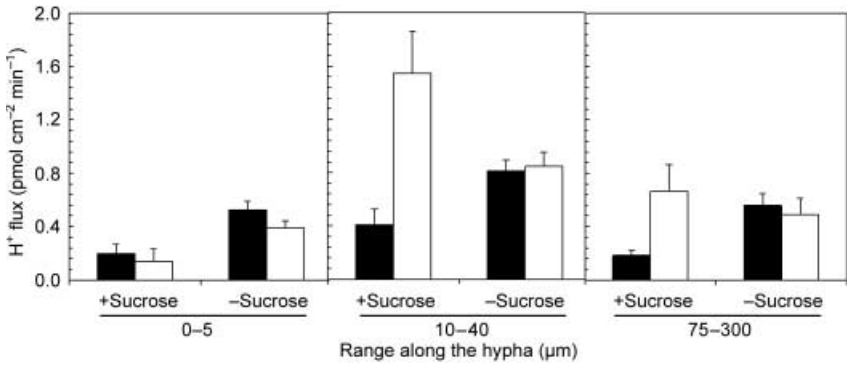


Fig. 3 Effects of phosphate (P) availability under two sucrose (Suc) states on H^+ fluxes measured in the surface of lateral growing hyphae of *Gigaspora margarita*. Secondary and nonseptated hyphae were chosen as standard for the studies because of the easy access in the microscope. Each treatment is representative of seven repetitions. Closed bars, +P; open bars, –P. By two-way ANOVA combined with Tukey's test, in the apical region there was just the significant effect of Suc ($P < 0.001$); in the subapical there was significant interaction between P and Suc ($P < 0.01$), and individual effects of P ($P < 0.001$) and Suc ($P < 0.01$). The distal region showed a similar behavior to the subapical region, where there was significant interaction between P and Suc ($P < 0.05$), and individual effects of P ($P < 0.001$) and Suc ($P < 0.01$).

Table 1 Effects of phosphate (P) and sucrose (Suc) availability on branching, maximal hyphal growth and hyphal average volume of *Gigaspora margarita* calculated from stereoscopic observations of at least six germinating spores for each condition

Treatment	Branching (no)	Maximal hyphal length (μm)	Hyphal average volume (μm^3)
+P + Suc	3.74 c	440.8 c	46411.0 c
–P + Suc	8.56 b	1253.3 a	121141.2 a
+P – Suc	13.74 a	866.6 b	77226.8 bc
–P – Suc	9.52 b	1066.6 ab	100492.6 a
P value (P × Suc)	0.005	0.05	0.01

The fungal images were acquired by stereoscope and the hyphal growth length and volume were measured using MetaMorph software, version 4.55 (Universal Imaging, West Chester, PA, USA). The means followed by the same letter, in a column, are not significantly different by Tukey's test at $P < 0.01$ ($n = 15$). *P* value refers to statistical significance of the interaction between P and Suc.

Effects of phosphate and sucrose on hyphal H^+ flux oscillations

The effects of P and Suc on H^+ fluxes were investigated using germinating spores, which were cultured in either complete M medium or in the same medium lacking one or both nutrients. The standard concentrations of P and Suc in the M medium were 35 μM and 29.2 mM (1% or 10 g l^{-1}), respectively. The exclusion of P induced no significant changes in the hyphal apex H^+ efflux, while it increased the H^+ efflux in all regions behind the hyphal apex, particularly in the subapical region (Fig. 3). In this region, the absence of Suc induced increases in H^+ efflux, but without any significant effect of P. In summary, Suc induced H^+ efflux, but only if P was absent in the M medium. The results showed that the subapical and distal hyphal regions have qualitatively similar behavior in terms of the effect of P and Suc (Fig. 3).

Relationship between hyphal growth and the H^+ flux behavior as a function of phosphate and sucrose

Growth parameters (branching, maximal hyphal length and volume) were analyzed as a function of P and Suc status of the medium and in relation to the H^+ flux activation profile. As occurred with the H^+ flux, the rate of fungal growth was sensitive to P and Suc supply (Table 1). However, no statistically significant effects of the interaction of P and Suc supply were observed on the number of emerged germ tubes and septa (data not showed). A significantly enhanced branching, along with the formation of new, thinner hyphae, was positively correlated with the highest rate of hyphal growth in response to P and Suc starvation (0.89, $P < 0.03$). The exclusion of any of these nutrients from the culture medium led to a stimulation of hyphal growth and branching. Furthermore, hyphal growth (hyphal length and volume) was lower in a medium supplemented with both P and Suc, and formation of hyphal

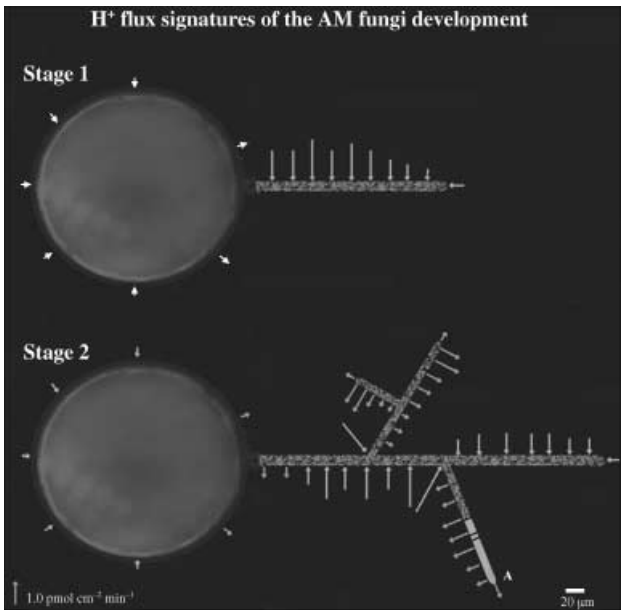


Fig. 4 Diagrammatical representation of the H^+ flux signature related to presymbiotic development of *Gigaspora margarita* fungus growing in complete M medium. Stage 1 was measured when the fungus presented just a single germ tube; therefore stage 2 was the maximal state of fungal differentiation (5–7 d old). In stage 1, a consistent H^+ influx in the primary hyphae is kept the same after branching. This figure is a summary of all experiments realized in our laboratory, where the H^+ flux profile was characterized in all kinds of hyphae and structures of the arbuscular mycorrhizal (AM) fungus *G. margarita*. The length of the arrows (scaled as indicated in the figure) is representative of the magnitude of the flux in each probe position.

branching was also affected (Table 1). Supplied together, P and Suc induced a dramatic inhibition in apical H^+ efflux (Fig. 3), as well as an inhibition of hyphal growth and branching (Table 1). By contrast, in the absence of both nutrients, stimulation on H^+ efflux and growth was observed (Fig. 3, Table 1). Figure 4 is a diagrammatic representation summarizing the H^+ flux pattern developed in each presymbiotic AM fungal structure. It was possible to draw a polarized pattern where H^+ effluxes are located at the tip and subapical regions of growing lateral hyphae, while a strong H^+ influx occurs on the extreme opposite side of these hyphae, at the branching corners and also in primary hyphae.

Activation of the hyphal H^+ fluxes by root factors

Both the hyphal H^+ flux profile and total growth of *G. margarita* hyphae were clearly stimulated when cultured in the presence of a host root or when incubated with root exudates (RE) (Fig. 5; Table 2). In the presence of white clover roots (*Trifolium repens*), significant increases in hyphal H^+ effluxes of 80–90% were observed in the apical (0–5 µm) and subapical (10–40 µm) regions (Fig. 5a). Even greater stimulation in the apical region (*c.* 130%) was obtained when the fungus was pretreated with root exudates (1 : 10 dilution) of clover roots (Fig. 5a). Much lower increases in H^+ effluxes were observed with both treatments in distal regions (70–200 µm). The time-course experiment with root exudates showed an initial decrease in the apical H^+ efflux followed by stimulations after 15 min of incubation (Fig. 6).

Discussion

In the absence of a host root, even when AM spores are germinated in a rich nutrient medium, hyphal growth of AM fungi ceases after a few days or weeks, depending on the fungal species and on the culture conditions. This strict obligate biotrophic habit of AM fungi has impeded analysis of their presymbiotic developmental stage, although Ca^{2+} and H^+ ions are indicators for transducing chemical and environmental signals in fungi and plant cells. In order to characterize the early physiological alterations related to the presymbiotic developmental stage of AM fungi, we have investigated H^+ ion flux dynamics in *G. margarita* hyphae using a vibrating ion-selective microelectrode, a procedure which is noninvasive and allows the detection of real-time changes in specific ion activity on the surface of living cells. This parameter has proved to be more biologically relevant than the concentration measurements taken by other, more familiar invasive techniques (Miller, 1995; Kunkel *et al.*, 2006).

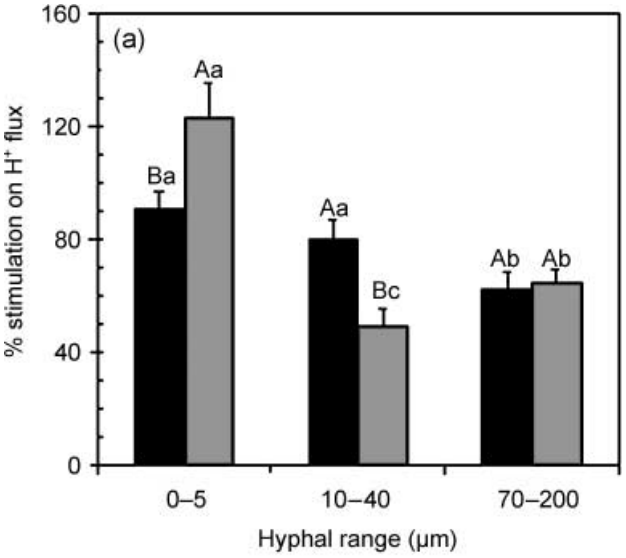
H^+ flux profiles and their sensibility to P-type ATPase inhibitors

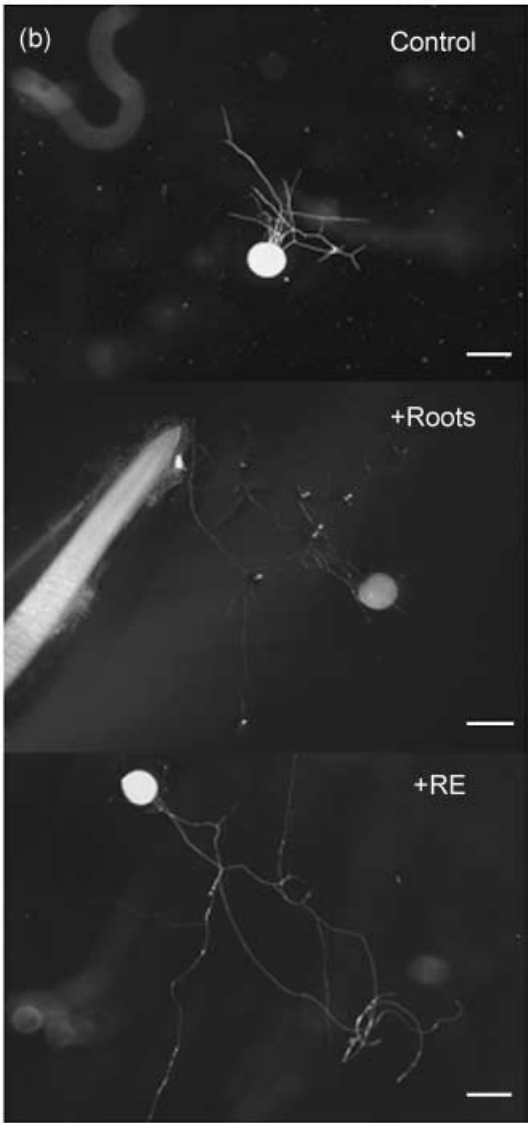
An asymmetric electrochemical gradient was formed around active spores, a situation that enabled the reproducible prediction of the site of germ tube emergence (Fig. 1). Although, the

Treatment	Branching (no.)	Hyphal average length (µm)	Hyphal average volume (µm³)
Control	4.23 c	337.5 c	26513.1 c
+Roots	8.46 b	719.2 b	56490.4 b
+RE	11.92 a	1392.0 a	109327.4 a

Table 2 Branching number, hyphal average length and volume of *Gigaspora margarita* fungus growing in the vicinity of white clover (*Trifolium repens*) roots (+Roots) or with clover root exudates (+RE) calculated from stereoscopic observations of at least 10 germinating spores for each condition

The fungal images were acquired by stereoscope and were measured using MetaMorph software, version 4.55. The means followed by the same letter, in a column, are not significantly different by Tukey's test at $P < 0.01$ ($n = 8$).





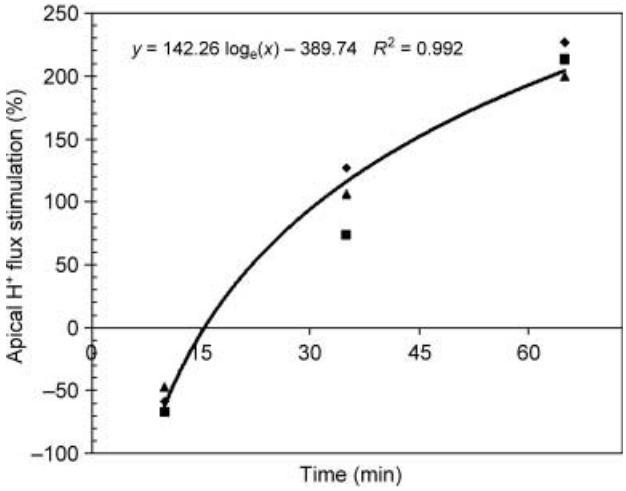


Fig. 6 Time-course analysis of the effects of root exudates (dilution 1 : 10) on H^+ efflux of *Gigaspora margarita* lateral hyphae. The graph represents the percentage of stimulation or inhibition of the proton fluxes ($n = 3$).

same polarized distribution of the H^+ fluxes was observed in both germinated and nongerminated spores, the magnitude of these fluxes was lower in germinated spores, suggesting specific activation of an H^+ current across the spore membrane before germ tube emergence, and then decreased with the metabolic activity of growing hyphae. This result is in agreement with previous data on electrical currents and electrical membrane potential of *G. margarita* spores before and after germination (Berbara *et al.*, 1995; Ayling *et al.*, 2000), and indicates that the electrical membrane changes in AM hyphae may be under the control of their H^+ transport systems. The frequent observation of these ion fluxes in the region opposite the germ tube emergence, simply by changes in the pH of the medium, suggests that passive proton fluxes could be occurring in this region. Further studies are necessary to determine when these fluxes in the azygospore are mediated by active or passive mechanisms.

The maximal H^+ effluxes were observed in the subapical region (Fig. 2a), a result consistent with that reported earlier using a ratiometric dye to estimate the intracellular pH of

Fig. 5 (a) H^+ efflux stimulation in *Gigaspora margarita* lateral hyphae growing in the vicinity of white clover (*Trifolium repens*) roots (black bars) or pretreated with clover root exudates 60 min (gray bars). For root exudate (RE) experiments, the control arbuscular mycorrhizal (AM) fungi were incubated at the same conditions with water pH 5.7 instead of RE. Bars represent the means ± SE of five independent experiments. (b) Hyphal growth representation under the experimental setup carried out for H^+ flux analysis; however, for the treatment with RE the images were acquired 24 h after incubation, and for the treatment with clover roots, 2 d after spore germination. Bars, 200 µm. The data were analyzed by ANOVA combined with Tukey's test. The bars followed by the same capital letter, in the same hyphal region, are not significantly different by Tukey's test at $P < 0.01$. The bars followed by the same lowercase letter, in different hyphal regions, are not significantly different at $P < 0.01$ ($n = 5$).

G. margarita germ tubes grown under similar conditions to the present work (Jolicoeur *et al.*, 1998). They found an enhanced cytosolic alkalinization in the regions where we found the highest H^+ effluxes. Thus the internal pH and the magnitude of the effluxes we describe decreased with distance from the tip. Previously, Ayling *et al.* (2000) had speculated that the extracellular H^+ flux in the germ tube of *G. margarita* could explain the cytoplasmic pH values detected by Joliceour *et al.* (1998) and those previously seen in other cells (Robinson *et al.*, 1996; Feijó *et al.*, 1999, 2001).

In plant and fungi, ion transport across the cell membrane is energized by ATP hydrolysis driven by H^+ pumps (P-type H^+-ATPases), which extrude H^+ out of the cell (Serrano, 1989; Portillo, 2000). Some genes encoding P-type H^+-ATPases have been isolated from AM fungi (Ferrol *et al.*, 2000; Requena *et al.*, 2003). Ferrol *et al.* (2000) isolated five gene fragments coding for homologs of H^+-ATPases in *Glomus mosseae*. Only two, however, were demonstrated to encode P-type H^+-ATPases (Corradi *et al.*, 2004).

The pharmacological analysis also showed that the extracellular H^+ fluxes around the lateral hyphae were differentially susceptible to P-type ATPase inhibitors. The H^+ effluxes in the active hyphae were inhibited by 350 μM erythrosin B, mainly at the apical region, but fully inhibited by orthovanadate at concentrations as low as 5 μM (Fig. 2a,b). Although H^+ flux localized to the hyphal apex was nearly abrogated by both inhibitors, the addition of erythrosin B only caused a relatively small inhibition of the H^+ efflux activity in the subapical region, 20 μm beyond the tip (Fig. 2a). Taken together, the susceptibility to these inhibitors is consistent with the flux being generated by different fungal H^+-ATPase isoforms distributed asymmetrically along the growing hyphae (Wach & Graber, 1991). Previously, it was reported that two yeast H^+-ATPase isoforms exhibited different sensitivities to these inhibitors (van Dyck *et al.*, 1990). It is worth noting that the H^+ flux also depends on the balance of cotransporters carrying H^+ in and out of the cell. Previously, it was found that orthovanadate enters cells via the Pi transport system and inhibits the growth of the *Neurospora crassa* fungi (Bowman, 1982; Bowman *et al.*, 1983). Thus, in the case of orthovanadate, the complete abolishment of the H^+ efflux, along with the influx observed (Fig. 2b), should represent the activity of membrane P transporters carrying H^+ and orthovanadate into the fungal cells, followed by the H^+-ATPase inhibition. In any case, the predominance of H^+ effluxes in the subapical region indicates the relative abundance of H^+-ATPases over the secondary ion transporters present in this region. This is in agreement with data from Lei *et al.* (1991), who reported a diethylstilbestrol-sensitive ATPase activity too, mainly localized at the hyphal subapical region, which correlated with the P uptake. Here, we also show that inhibiting the H^+ efflux in germinating AM hyphae by orthovanadate similarly decreases the hyphal tip growth rate (Videos S2, S3), as described for *N. crassa* by Bowman (1982).

Filamentous fungi contain specific apical bodies called Spitzenkörpers consisting of a cluster of small membrane-bound vesicles embedded in a meshwork of actin microfilaments. Not only found in pathogenic fungi, Spitzenkörpers are strictly located in the hyphal apical region and there is evidence that they play a role in the guidance control of the growth process (Bartnicki-Garcia *et al.*, 2000; Riquelme & Bartnicki-Garcia, 2004; Harris *et al.*, 2005). The vesicle supply centre (VSC) model of polarized growth in filamentous fungi proposes that the Spitzenkörper is the repository for secretory vesicles that are transported along the hyphae towards the tip (Reynaga-Peña *et al.*, 1997). Vesicles radiate from the Spitzenkörper and travel to the cell surface, where they fuse with the plasma membrane and release their cargo (Crampin *et al.*, 2005). In the case of AM fungi, dark vesicles or dense granules defined by Sward (1981) and Maia & Kimbrough (1994), by electron-microscopy analysis, indicate they could be 'Spitzenkörper-like' structures. Figure 2(d) demonstrates these structures in the hyphal subapical region of the AM fungus *G. margarita*, but their clustering tends to be in the membrane regions expressing the highest H^+ effluxes. In addition, orthovanadate also affects the accumulation of 'Spitzenkörper-like' structures at the hyphal apex, as previously described for pathogenic fungi (Lópes-Franco & Bracker, 1996), where the abundance of these structures was also correlated to the hyphal growth (Video S3; Riquelme & Bartnicki-Garcia, 2004; Harris *et al.*, 2005).

Effects of phosphate and sucrose on hyphal growth and H^+ fluxes

More recently, Requena *et al.* (2003), using a molecular approach, analyzed the impact of Suc and P on the expression of two isoforms (*GmPMA1* and *GmHA5*) of the plasma membrane H^+-ATPase from *G. mosseae*. They found that *GmPMA1* was highly expressed during fungal presymbiotic development, whereas the *GmHA5* transcript was induced at the appressorium stage. The authors reported that the *GmHA5* transcript was down-regulated by Suc and induced by P, while the *GmPMA1* was hardly affected by these nutrients. Their results were achieved using concentrations of KH_2PO_4 and Suc (35 and 29 mM, respectively) similar to those used in the present study. Therefore, it is likely that the highest H^+ effluxes found in the subapical region of hyphae upon P starvation in the presence of Suc (highest dark column in Fig. 3) could reflect a post-transcriptional regulation of *G. margarita* counterpart, for example of the *GmPMA1*, or simply an up-regulation of another H^+-ATPase isoform with a different regulation mode by a nutrient supply not yet described. Nevertheless, the experiments are consistent with the notion that the nutrient status of the fungus could regulate the H^+-ATPase activity and thus the H^+ flux control in the fungal cell membrane.

Although P and Suc are nutrients exchanged in the symbiotic phase, it is well known that high concentrations of these nutrients in soil solution or in the plant can promote inhibitory

effects on hyphal growth and root colonization (Mosse, 1973; Menge *et al.*, 1978; Siqueira *et al.*, 1982; Abbott *et al.*, 1984; Smith & Read, 1997). This phenomenon can be correlated to the lowest H^+ effluxes found in hyphae grown on complete M medium containing both Suc and P (Table 1). Indeed, under these conditions, the lowest rate of hyphal branching and growth was observed (Table 1), a finding in agreement with previous studies that reported a negative effect of Suc on germination and hyphal growth of *G. mosseae* (Mosse, 1959) and *G. margarita* (Siqueira *et al.*, 1982). However, as suggested by Requena *et al.* (2003), it is possible that the amount of Suc used exceeded the physiological amounts that the fungus usually meets in soil, since 29 mM Suc inhibited the attachment of mycorrhizal hyphae to the root surface and the formation of symbiosis. In addition, the physiological roles of high concentrations of Suc have already been reported and include osmotic balance, carbon storage, redox balance, and ion transport through hyphae (Witteveen & Visser, 1995). The high H^+ effluxes were detected in the subapical region of the hyphae grown in the absence of P but in the presence of Suc (Table 1). Solaiman & Saito (1997) demonstrated a modest Suc uptake by intraradical hyphae, and concluded that AM fungi could preferentially take up glucose after hydrolysis of Suc. Although some fungi possess cell wall-bound invertase and transporters for Suc uptake (Aked & Hall, 1993; Lam *et al.*, 1994), this is not confirmed in AM fungi. In ericoid mycorrhizal fungus *Hymenoscyphus ericae* (Read) Korf & KernanMost, 50% of an acid invertase is wall-associated, 41% forming an extracellular fraction and 9% a soluble, cytoplasmic fraction (Straker *et al.*, 1992). Studies on the detection of invertase activity, sugar uptake and isolation of the genes codifying sugar transporters in the AM germ tubes will eliminate the credence of sucrose uptake in AM fungal cells during the presymbiosis.

Although AM fungal hyphae are considered coenocytic, with septa appearing under conditions of conservation of storage reserves and essentially cutting off arms, the septation process also seems to promote a burst of hyphal expansion towards a host root (Smith & Read, 1997; Bago *et al.*, 1998). If the fungus does not find a root to colonize, at the end of the process the whole germ tube becomes septated and the spore reverts to dormancy (Bago *et al.*, 1998 and references therein). Surprisingly, the septated hyphae also exhibit H^+ efflux as high as nonseptated hyphae, even in regions with conspicuous cytoplasmic retraction (Fig. 4). Therefore, in stage 1, the H^+ influx was very similar to those found in *N. crassa* (Lew, 2007) and pollen tubes (Feijó *et al.*, 1999).

Positive modulation of the hyphal H^+ fluxes by root exudates and host roots in the vicinity of the AM hyphae

The present data on hyphal H^+ fluxes and those with transmembrane electric potential differences (Em) in *G. margarita* were obtained under quite similar assay conditions (Ayling *et al.*, 2000) indicating that the AM fungal hyphae are weakly polarized in the absence of host roots. Such a characteristic might be explained by the fungal physiological state during the presymbiotic development, where the magnitude of the H^+ fluxes (Figs 1 and 5) and electrical membrane potential was also much lower than that observed in plant cells (Cárdenas *et al.*, 1999; Feijó *et al.*, 1999; reviewed in Kunkel *et al.*, 2006). For instance, measurements of total membrane electric potential carried out by Ayling *et al.* (2000) revealed Em values of germ tubes of *G. margarita* of approx. −40 mV, a value much lower than −200 mV in *N. crassa* (Miller *et al.*, 1990), −160 mV in *Achlya bisexualis* (Kropf *et al.*, 1984) and −132 mV in pollen tubes (Weisenseel *et al.*, 1975). On the other hand, changes in hyphal H^+ fluxes appear to be closely related to the metabolism and physiological state of AM fungi. Indeed, this situation changes as the AM fungus is getting near to the host root or after incubation with host root exudates. Clover root exudates stimulated the hyphal H^+ efflux more than intact clover roots in the vicinity of the *G. margarita* hyphae (Table 2). Nevertheless, the Pearson's coefficients showed strongly significant positive correlation between the hyphal growth and apical (0.92, $P < 0.0001$) and distal H^+ effluxes (0.91, $P < 0.002$) after treatment with root factors. Both experiments show that the apical region seems to be a critical hyphal zone of the perception of root signals and a very interesting target for further studies. In addition, electrophysiological studies during the presymbiosis of *G. margarita* demonstrated that Em became hyperpolarized when plant root extracts were added to the medium (Ayling *et al.*, 2000). In addition, Jolicoeur *et al.* (1998) reported the intracellular pH profile in *G. margarita* hyphae was more alkaline when the fungi were growing together with *Daucus carota* roots, particularly at the apical hyphal region, but extraradical hyphae of *G. intraradices* also had a high apical pH. Thus, when an AM fungus senses the signals of a near host root, a cascade of events triggered by H^+ ion currents is transmitted along the membrane surface, resulting in hyphal branching and growth towards the fastest interaction. The activity of H^+ transport systems participates in this event, as is evident from correlations, control of hyphal Em, cytosolic pH and fungal growth summarized in the Table 2.

In fact, root exudates derived from host roots in the vicinity of the AM hyphae have been shown to stimulate AM hyphal growth at early stages of fungal development (Nair *et al.*, 1991; Chabot *et al.*, 1992). Most root exudates contain several active compounds, such as peptides/proteins, flavonoids, strigolactones and others. Although flavonoid derivatives can influence the initial stages of the fungal life cycle, experiments with flavonoid-deficient mutants of maize indicate that they are not essential for the development of the AM symbiosis, as previously believed (Bécard *et al.*, 1995; Harrison, 1999, 2005; Buee *et al.*, 2000). As shown in Fig. 6, 10 min after incubation with clover root exudates a significant decrease in apical H^+ efflux can be observed. After that, however, the stimulations

were detected mainly at 65 min. One explanation for this effect has been postulated by Fromm & Lautner (2007), who proposed that ion fluxes across the plasma membrane generate electrical signals, which promote the formation of a stimulus sufficiently big to depolarize the fungal membrane. Then an action potential is generated and can be reflected in the recognition of the certain host molecule(s) by the cell. In the case of the clover exudates, the action potential formed could reflect the recognition of the exudate molecules by the AM fungal hyphae.

Further studies using an ion-selective vibrating probe system and imaging analysis will define the impact of the main AM fungal stimulators, such as strigolactones, specific flavonoids and other growth factors not only on H^+ ion dynamics, but also on the fluxes of other signaling ions, such as Ca^+ and K^+. The elucidation of these ion dynamics in AM fungal hyphae will give new insights into the role of the membrane transport systems in polarized cell growth and in signaling during AM interaction.

Conclusions

This study describes the H^+ flux profile of an AM fungus during the asymbiotic and presymbiotic development *G. margarita* and its correlation with hyphal growth, branching and host recognition. The findings are pertinent to the controversial role of extracellular and intracellular H^+ ion gradients in the control of polarized growth in plant and fungal cells (Harold & Caldwell, 1990; Gibbon & Kropf, 1991). In essence, the hyphal H^+ flux observed reveals that pH signature correlated with the growth pattern of the fungus, namely on germ tube branching and lateral hyphal formation. There is growing evidence that protons may be functionally important, as a regulatory signaling or effector ion. External pH changes have been reported as important indicators of host–pathogen interactions that correlate with fungal development (Felle, 2001). These hyphal fluxes were dramatically influenced by two stimuli: pretreatment with clover root exudates; and simply growing in the vicinity of clover roots. In this regard, it is tempting to speculate that a differential activation and distribution of AM fungal electrogenic H^+-pump isoforms could play a crucial role during AM hyphal growth and host recognition. The present work contributes to the demonstration that pH is involved in fungal growth and in the molecular dialogue between AM fungi and host plants.

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Supplementary Material

The following supplementary material is available for this article online:

Text S1 Measurements of H^+ fluxes using the ion-selective vibrating probe system.

Fig. S1 Representation of a dish containing *Gigaspora margarita* spores growing together with clover (*Trifolium repens*) roots.

Fig. S2 Dose–response test of different concentrations of orthovanadate and erythrosin B (pH 6.0) in the arbuscular mycorrhizal (AM) hyphal growth ($n = 50$).

Video S1 Time-lapse of the hyphal growth of *Gigaspora margarita* in M medium showing cytoplasmic streaming and movement of white vesicles and probably Spitzenkörper (dark spots) in the hyphal subapical region (magnification ×60).

Video S2 Time-lapse of the hyphal growth of *Gigaspora margarita* in M medium (magnification ×40).

Video S3 Time-lapse of the hyphal growth of *Gigaspora margarita* in M medium and in the presence of 5 μM orthovanadate (magnification ×40).

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