Transmembrane electric potential difference of germ tubes of arbuscular mycorrhizal fungi responds to external stimuli

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SUMMARY
Measurements of the electric potential difference across the hyphal wall and the cell membrane were made on external hyphae of three species of arbuscular mycorrhizal fungus Gigaspora margarita, Scutellospora calospora and Glomus coronatum and on germ tubes of G. margarita. The values of transmembrane electric potential difference recorded (≈ −40 mV) are less negative than those previously reported from hyphae of arbuscular mycorrhizal fungi closely associated with roots and from filamentous fungi. The external hyphae of arbuscular mycorrhizal fungi grown in soil had similar values of electric potential difference to those grown in soil-less culture, and to germ tubes. Thermodynamic calculations showed that despite these low values of electric potential difference, efficient high-affinity uptake of phosphate is possible. The transmembrane electric potential difference of germ tubes of G. margarita became more negative when plant root extract was added to the medium, showing for the first time that the early stages of interaction between plant and fungus occur via direct effects on the plasma membrane rather than via effects on gene expression. Addition of K+ reversibly depolarized the transmembrane electric potential difference of germ tubes of G. margarita, indicating that despite the low electric potential difference the fungus has control over the permeability of the plasmamembrane to K+.

Key words: electric potential difference (p.d.), germ tube, arbuscular mycorrhizal (AM) fungi, external hyphae, Gigaspora margarita.

INTRODUCTION
Arbuscular mycorrhizal (AM) fungi can increase the rate of nutrient uptake, particularly of phosphorus (P), by plants growing in nutrient-deficient soils. The improved nutrition is often reflected in increased biomass of the mycorrhizal plants compared with similar but non-mycorrhizal plants. Other benefits to the plant that are associated with colonization by AM fungi include improved water relations and disease resistance (Smith & Read, 1997).

Despite the obvious importance of improved nutrition for crop growth we still have little detailed knowledge of the physiological processes involved in nutrient uptake by, and transfer within, mycorrhizal roots (Smith et al., 1998). Almost all our present knowledge comes from experiments in which comparisons are made between mycorrhizal and non-mycorrhizal plants grown in soil. No laboratory has successfully grown AM fungi in pure culture (i.e. without a host plant); as highlighted by Marschner & Dell (1994) this is an important limitation to experimentation and to understanding the role of AM fungi in plant nutrition.

The spores of many species of AM fungi germinate in vitro and several hyphae may be produced, in turn, exploring the medium surrounding the spore. In the absence of a suitable host, hyphal elongation ceases after a few days and the cytoplasm is withdrawn from the hyphae. Cessation of growth is thought to occur before spore reserves are completely exhausted (Lei et al., 1991). We do not know if the lack of continuing growth indicates that some substance is lacking in the medium or whether the germ tube is unable to take up nutrients until it has been stimulated by a signal from a host.
There have been only two direct attempts to determine whether germ tubes of AM fungi are able to absorb mineral nutrients actively, and these have produced conflicting results. Thomson et al. (1990) measured $^{32}$P uptake by germ tubes of Gigaspora margarita and identified two P transport systems, with $K_m$ of 2–13 and 10 200–11 300 $\mu$M and $V_{\text{max}}$ of $\sim$3.4 and 440–900 nmol mg protein$^{-1}$ h$^{-1}$, respectively. They suggested that the high affinity system was likely to be a 2H$^+\cdot$H$_2$PO$_4^-$ co-transporter while the low affinity system could be facilitated diffusion of P (Smith & Smith, 1990). By contrast, Lei et al. (1991) found, using microautoradiography, that germ tubes of Gi. margarita were only able to absorb $^{32}$P if root exudates and volatiles were present. Their results suggested that unstimulated germ tubes were unable to absorb nutrients.

The external hyphae which grow out from the root are able to absorb and transport nutrients (Cox & Tinker, 1976), and a gene encoding a high-affinity phosphate transporter (GvPt) has been identified from external hyphae of Glomus versiforme (Harrison & van Buuren, 1995). Thus, if germ tubes which have not been stimulated by root exudates or volatiles are unable to take up nutrients major changes must take place in the physiology of and/or patterns of gene expression in the fungal hyphae associated with colonization.

Electrophysiological measurements have helped to elucidate the mechanisms involved in nutrient transport in many organisms (e.g. Higinbotham et al., 1970). In this article we describe for the first time measurements of electric potential difference (p.d.) made on external hyphae of three species of AM fungi and on germ tubes of Gi. margarita.

Materials and Methods

External hyphae

External hyphae were from both soil-grown plants and plants growing in soil-less culture.

Leek (Allium porrum L.) seedlings were grown in pots containing a soil: sand mix (1:9 w/w) with inoculum of either Scutellospora calospora (Nicolson & Gerdemann) Walker & Sanders (WUM 12 (2)) or Glomus coronatum ‘Giovannetti’ (formerly ‘City Beach’) (WUM16). The pots were maintained at 12% moisture content by watering to weight and fertilized weekly with a dilute nutrient solution (Smith et al., 1994). For measurement of electric potential difference (p.d.), roots were carefully washed free of soil and transferred to Petri dishes containing a simple salt solution (2 mM K$_2$SO$_4$; 1.5 mM MgSO$_4$; 4 mM CaCl$_2$). Segments of root were mounted on modified microscope slides and bathed in the same solution, which was continuously replenished (\~1 ml min$^{-1}$).

Leek seedlings plus spores (details of which appear later in this section) of either Gigaspora margarita (Becker & Hall) or G. coronatum were grown on agarose-coated microscope slides, as described in Clarkson et al. (1988). Briefly, leek seed was germinated on damp filter paper; when the emerging radicle was c. 3 mm long the seed was transferred onto a microscope slide coated with 0.7%, agarose so that the developing root grew between the glass and the agarose. Two or three spores were placed close to each root. The slides were placed in a perspex box at an angle of \~60°, and continuously irrigated (\~1.5 ml min$^{-1}$) with dilute nutrient solution (see previous paragraph). Most preparations were used after 20–30 d, when appressoria and external hyphae were visible. Measurements of p.d. were made after inverting the entire preparation onto a modified microscope slide and perfusing with the same salt solution (Clarkson et al., 1988; Ayling et al., 1997).

Several unsuccessful attempts were made to grow S. calospora on the agarose-coated slides, so that a comparison with the soil-grown external hyphae of S. calospora could be made, but germination of the spores was erratic and the germ tubes did not elongate.

It was difficult to obtain reliably enough material for experiments with external hyphae therefore germ tubes were used to study the electrophysiological response to changes in the bathing medium.

Germ tubes

Gi. margarita was chosen for these experiments because its spores are large (>100 $\mu$m diameter) and because it has been used in other studies of germ-tube physiology (Thomson et al., 1990; Lei et al., 1991; Berbara et al., 1995).

Freshly isolated spores of Gi. margarita were aseptically transferred to Petri dishes (40 mm diam.) containing minimal (M) medium (Bécard & Fortin, 1988) made up in 0.7% (w/w) agarose. Five or six spores were placed in each dish, just below the surface of the agarose, so that the negative geotropism of the hyphae resulted in growth on or just below the surface where the hyphae were accessible with microelectrodes. The Petri dishes were sealed with Parafilm (Parafilm Laboratory Film, American National Ca®, Chicago, IL, USA) and incubated at 21°C, in the dark, in an atmosphere containing 2% CO$_2$. Germination occurred over a period of 4–24 d.

For electrophysiological observations, the sides were carefully broken off the Petri dishes and the surface of the agarose moistened with $c$. 200 $\mu$l of solution containing 1.1 mM KNO$_3$; 1 mM Ca(NO$_3$)$_2$; 0.3 mM MgSO$_4$ and 5 mM MES (pH 6) to ensure good electrical continuity.

The walls of the germ tubes were quite tough and it was not easy to make transmembrane measurements without disturbing the hyphae.
Fig. 1. Measurement of electric potential difference (p.d.) from germ tubes of *Gigaspora margarita* growing out from spores placed in agarose/M-medium. (a) Lateral impalement of a germ tube. (b) Impalement at a branch point. Bar, 100 μm.

Ideally, a flowing solution would have been used, as in the measurements of external hyphae; however flowing solution washed away the agarose, leaving the germ tubes unsupported so that stable recordings could not be made. Higher concentrations of agarose held the hyphae more securely but provided too much mechanical resistance to the fine-tipped micro-electrodes. It was often easier to obtain a stable reading if the electrode impaled a branch point in a hypha rather than the tip or side of an isolated hyphal branch. Fig. 1a shows an impalement in the side of an isolated germ tube, and Fig. 1b shows an electrode approaching a branch point.

Once a stable p.d. was recorded, 100–200 μl of plant root extract (described later in the section) or drops of KCl solution were applied to the medium bathing the fungus; after a new stable p.d. was recorded several ml of the original solution were added to dilute the test solution.

All hyphae growing out from the spore are referred to as germ tubes. External hyphae are those hyphae attached to and growing out from a root. Associated hyphae (as in Ayling *et al.*, 1998) are those on the root surface or within the epidermal layer; they are intimately associated with the root and are wider (c. 8–10 μm) than the external hyphae observed close to the root (5–6 μm). These two types of hyphae are grouped together here, because it is not always easy to distinguish them *in vivo* under the light microscope.

**Electrophysiology**

Electric potential differences (p.d.) were measured using 200 mM or 2M KCl-filled glass micropipettes (tip resistance < 100 M Ω) attached via a Ag/AgCl half cell to a high impedance electrometer unit (WPI, New Haven, CT, USA) (Purves, 1981). There was no effect of KCl concentration in the micropipette on the values recorded (data not presented). Measurements were made on external hyphae of *G. coronatum*, *S. calospora* and *G. margarita*, and on germ tubes and spores of *G. margarita*. Measurements of wall p.d. were made with the electrode tip pressing against the cell wall; when the electrode was advanced the wall flexed and the electrode tip entered the cell; transmembrane p.d. was then recorded. Cytoplasmic streaming sometimes momentarily ceased when the electrode tip entered the cell. This was also observed in root hairs by Clarkson *et al.* (1988). AM fungi have only small vacuoles (S. M. Ayling & S. Dickson, unpublished observations of *G. margarita* germ tubes stained with Oregon Green (Molecular Probes, Eugene, OR, USA)) therefore the transmembrane measurements are likely to come from impalements in which the electrode tip was located in the cytoplasm. Values of p.d. are presented as mean ± SE; comparisons were made using Student's *t*-test.

**Plant root extract**

Plant root extract was prepared by grinding 0.25 g f. wt of sterile tomato (*Lycopersicon esculentum* Mill. cv.76R; Peto Seed Co., CA, USA) root in 5 ml sterile filtered reverse-osmosis water. The extract was used immediately.

**Isolation of spores**

Spores of *G. margarita* or *G. coronatum* were isolated by wet-sieving from cultures raised on clover (*Trifolium subterraneum* L.) grown in pots containing a soil:sand mix. The spores were carefully cleaned under a dissecting microscope and sterilized by washing for 10 min in 2% Chloramine T, followed...
by three rinses in sterile water, before transfer to a sterile solution containing 200 ppm streptomycin and 100 ppm gentomycin. The spores were normally stored overnight in this antibiotic solution at 5°C (Smith & Dickson, 1998). Occasionally spores which had been stored in antibiotic for several weeks were used. These were still viable but germination was so slow (>30 d) that it was not practical to use them routinely in experiments.

RESULTS

Resting electric potential difference

The p.d.s recorded from external hyphae of *G. coronatum*, *Gi. margarita* and *S. calospora* are shown in Table 1. Some of the measurements made on *S. calospora* are already published (Ayling et al., 1998) but they have been reanalysed in line with the new measurements and are included here for the sake of completeness. There was very little difference in either transmembrane p.d. or cell wall p.d. between the three species or between values recorded from external hyphae on soil-grown roots and external hyphae on roots grown on agarose-coated microscope slides.

Table 1. Electric potential differences (p.d.) recorded from the external hyphae of AM fungi colonizing roots of leek (*Allium porrum* L.) seedlings grown either in soil or on agarose-coated microscope slides; attempts to grow Scutellospora calospora on agarose-coated slides were unsuccessful

<table>
<thead>
<tr>
<th>Species</th>
<th>Glomus coronatum</th>
<th>Gigaspora margarita</th>
<th>Scutellospora calospora</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trans-membrane</td>
<td>Cell wall</td>
<td>Trans-membrane</td>
</tr>
<tr>
<td>Soil-grown</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (mV)</td>
<td>-44</td>
<td>-21</td>
<td>-34</td>
</tr>
<tr>
<td>n</td>
<td>6</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>SE (mV)</td>
<td>3.71</td>
<td>4.49</td>
<td>4.24</td>
</tr>
<tr>
<td>Agarose-coated slides</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (mV)</td>
<td>-38</td>
<td>-17</td>
<td>-35</td>
</tr>
<tr>
<td>n</td>
<td>4</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>SE (mV)</td>
<td>3.68</td>
<td>4.55</td>
<td>1.73</td>
</tr>
</tbody>
</table>

Measurements of wall p.d. from the associated hyphae of AM fungi gave values between −35 and −45 mV (soil-grown *G. coronatum*, −45 mV (n = 2), soil-grown *S. calospora*, −36 mV (n = 9), *Gi. margarita* grown on agarose-coated slides, −35 mV (n = 2)).

Table 2 shows values of p.d. measured in spores and germ tubes of *Gi. margarita* growing in agarose/M medium. There was quite large variation in the values of wall p.d. and transmembrane p.d. recorded even between hyphae emerging from the same spore. Fig. 2 shows values of wall p.d. and one value of transmembrane p.d. recorded from a representative spore of *Gi. margarita*. The values of intraspore p.d. and spore wall p.d. from nongerminated spores were slightly more negative than those from germinated spores (Table 3); this difference was not statistically significant.

Effect of plant root extract

Application of plant root extract to germ tubes in which cytoplasmic streaming was visible caused a small but significant hyperpolarization of the membrane in nine of 12 experiments (t = 2.67, 11 df; Fig. 3 and Table 4). In nonstreaming germ tubes no p.d.
was recorded \((n = 3)\) and the extract had no effect (Fig. 3 and Table 4). The velocity of cytoplasmic streaming increased following application of the root extract.

**Effect of changing external \(K^+\) concentration**

Spores and germ tubes of *Gi. margarita* were rapidly and reversibly depolarized by KCl (Fig. 3; Tables 4 and 5). A larger depolarization was produced by 2M KCl than by 0.2 M. When the \(K^+\) was diluted by adding more of the original solution the p.d. recovered almost completely; if the \(K^+\) was diluted merely by diffusion into the agarose, recovery was only partial. Germinated and nongerminated spores were sensitive to \(K^+\). Only germ tubes in which cytoplasmic streaming was visible showed a response to increased concentrations of \(K^+\) in the medium. When \(K^+\) was added, the dominant direction of streaming, normally towards the apex, rapidly reversed before the velocity of streaming decreased, or in the germ tubes treated with 2M KCl, ceased. As the \(K^+\) was diluted streaming gradually resumed and the original pattern was re-established.

**DISCUSSION**

**Resting electric potential differences**

The values of hyphal wall p.d. recorded from the external hyphae and the germ tubes are similar to those reported from *Neurospora crassa* \((-10\) to \(-20\)

**Table 3. Electric potential differences (p.d.) recorded from germinated and nongerminated spores of Gigaspora margarita in Petri dishes containing agarose/M-medium**

<table>
<thead>
<tr>
<th></th>
<th>Germinated</th>
<th>Nongerminated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intraspore</td>
<td>Spore wall</td>
</tr>
<tr>
<td>Mean (mV)</td>
<td>-88</td>
<td>-31</td>
</tr>
<tr>
<td>(n)</td>
<td>11</td>
<td>5</td>
</tr>
<tr>
<td>SE (mV)</td>
<td>2.83</td>
<td>3.95</td>
</tr>
</tbody>
</table>
Table 4. The effect of adding plant root extract and KCl to the bathing medium on the transmembrane electric potential difference (p.d.) recorded from germ tubes of Gigaspora margarita

<table>
<thead>
<tr>
<th>p.d. (mV)</th>
<th>Resting</th>
<th>After root extract</th>
<th>Change</th>
<th>K-sensitive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streaming</td>
<td>−18</td>
<td>−40</td>
<td>22</td>
<td>Not tested</td>
</tr>
<tr>
<td></td>
<td>−52</td>
<td>−62</td>
<td>10</td>
<td>Not tested</td>
</tr>
<tr>
<td></td>
<td>−8</td>
<td>−12</td>
<td>4</td>
<td>Not tested</td>
</tr>
<tr>
<td></td>
<td>−24</td>
<td>−60</td>
<td>36</td>
<td>Not tested</td>
</tr>
<tr>
<td></td>
<td>−45</td>
<td>−50</td>
<td>5</td>
<td>Not tested</td>
</tr>
<tr>
<td></td>
<td>−32</td>
<td>−45</td>
<td>13</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>−42</td>
<td>−39</td>
<td>−3</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>−25</td>
<td>−29</td>
<td>4</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>−17</td>
<td>−16</td>
<td>−1</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>−39</td>
<td>−50</td>
<td>11</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>−53</td>
<td>−52</td>
<td>−1</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>−43</td>
<td>−46</td>
<td>3</td>
<td>Yes</td>
</tr>
<tr>
<td>Nonstreaming</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>No</td>
</tr>
<tr>
<td>p.d. of agarose M-medium</td>
<td>0</td>
<td>−3</td>
<td>−3</td>
<td>No</td>
</tr>
</tbody>
</table>

Table 5. The effect of KCl on the electric potential difference (p.d.) recorded from germ tubes and spores of Gigaspora margarita germinating in Petri dishes containing agarose/M-medium

<table>
<thead>
<tr>
<th>Tissue</th>
<th>[K+] added (M)</th>
<th>Change in p.d. after K+ (mV)</th>
<th>n</th>
<th>Washed</th>
<th>Extent of recovery (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spore</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nongerminated</td>
<td>2</td>
<td>70</td>
<td>1</td>
<td>No</td>
<td>Partial 35</td>
</tr>
<tr>
<td>Germinated</td>
<td>2</td>
<td>70</td>
<td>1</td>
<td>No</td>
<td>Partial 45</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>80</td>
<td>1</td>
<td>Yes</td>
<td>Full 80</td>
</tr>
<tr>
<td>Germ tube</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transmembrane</td>
<td>2</td>
<td>52</td>
<td>2</td>
<td>Yes</td>
<td>Partial 41</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>25</td>
<td>6</td>
<td>Yes</td>
<td>Full 24</td>
</tr>
<tr>
<td>Wall</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

KCl was added dropwise to the surface of the medium.

mV; Levina et al., 1995) but smaller than those recorded from the associated hyphae of AM fungi (−35 to −45 mV) and from leek root cell walls (~ −37 mV; Ayling et al., 1998). Differences in structure and composition between different types of hyphae (Bonfante-Fasolo & Grifoniolo, 1982), could be reflected in differences in the charge held on the wall (Ayling et al., 1998).

The transmembrane p.d.s (~ −40 mV) indicate that the germ tubes and external hyphae of AM fungi are weakly polarized compared with those of other fungi, for example, filamentous hyphae of N. crassa, −127 mV (Slayman & Slayman, 1962) and −200 mV (Miller et al., 1990), Achlya bisexualis, −160 mV (Kropf et al., 1984), and compared with our own limited data for associated hyphae of soil-grown G. coronatum (~−132 mV, n = 2) and S. calospora (~−77 mV, n = 3). However, values ~ −64 mV have been reported from the apical 20 μm of growing hyphae of N. crassa (Lew, 1998). Initially we rejected the values recorded from external hyphae on soil-grown roots as being too low (i.e. insufficiently negative) for physiologically active hyphae, and assumed that the hyphae had been damaged during harvesting. However, the similarity between the p.d. recorded from external hyphae on soil-grown roots and that from external hyphae on plants grown on agarose-coated slides or germ tubes grown on agarose suggests that these values are genuine.

Using a vibrating probe, Berbera et al., (1995) measured a small current (mean density 2 nA cm−2) at the hyphal tip of germ tubes of G. margarita. This current was less than one tenth that found in other fungi, leading Berbera et al. (1995) to suggest that the current was not coupled to proton-driven nutrient uptake but reflected metabolic activities involving the nutrient reserves of the spore. Although this explanation could be extended to the
present results for *Gi. margarita* germ tubes (Table 2), it is difficult to envision how it could be applied to the results for external hyphae (Table 1) unless nutrient uptake by AM fungi involves mechanisms very different from those found in other fungi. This seems unlikely (see Smith et al., 1998).

**Effects of plant root extract**

Hyperpolarization of the plasma membrane often reflects stimulation of membrane-bound H⁺-ATPases. Using cytochemical methods, Lei et al. (1991) showed that ATPase activity only occurred in germ tubes which had been stimulated by root extracts and volatiles. The increase in p.d. seen in germ tubes (Table 4) following application of root extract could reflect such a stimulation. The reaction between plant and AM fungus is a compatible one, therefore we would not expect a large disruption of the metabolism of either symbiont (Harrison & Dixon, 1994; Ayling et al., 1998). The present results, a small increase in p.d. and an increase in the velocity of cytoplasmic streaming, support the idea that perception of a host stimulates the metabolic activities of the fungus. Unlike earlier studies, in which measurement of effects on the hyphae took place several days after stimulation (e.g. Bécard & Piché, 1990; Lei et al., 1991) measurement of p.d. allows us to show that the fungus responds almost instantaneously to stimulation. This suggests that the earliest stages of interaction between plant and fungus occur via direct effects on the plasma membrane rather than via effects on gene expression.

**Effect of changing external [K⁺]**

The experiments with K⁺ were carried out because the low p.d. values could have been an indication that the plasma membrane was ‘leaky’, so that the fungus had poor control over its ionic composition. K⁺ is a major determinant of the p.d. in many plants and fungi (Clarkson, 1974). M-medium contains approx. 2 mM K⁺ (Bécard & Fortin, 1988). If [K⁺] in the cytoplasm is assumed to be around 100 mM, as in many other organisms (Leigh et al., 1994) the calculated potassium diffusion potential is −98 mV. Only the spores of *Gi. margarita* approach this value (Table 3). The calculated cytoplasmic [K⁺] for a potassium diffusion potential of −60 mV is 22 mM; at −40 mV cytoplasmic [K⁺] will be 10 mM. Thus, for K⁺ to be close to electrochemical equilibrium across the plasma membrane, cytoplasmic [K⁺] concentration in the germ tubes must be low.

One possible explanation is that the germ tube does not absorb K⁺ but instead uses K⁺ stored in the spore, which gradually becomes diluted as the germ tube elongates. This would explain the small currents seen in *Gi. margarita* (Berbara et al., 1995) and might explain the variability in p.d. observed between germ tubes (Table 2). If a spore has a diameter of 150 μm, walls 20 μm thick and 10% of the volume of the spore is cytoplasm, the cytoplasmic volume is $4.75 \times 10^9 \text{mm}^3$. If after 10 d there are 200 mm of hyphae (Bécard & Piché, 1989) of diameter 10 μm and 10% of the volume is cytoplasm, the volume of hyphal cytoplasm is $6.28 \times 10^8 \text{mm}^3$. Assuming the initial cytoplasmic [K⁺] in the spore is 100 mM and no K⁺ is adsorbed, cytoplasmic [K⁺] in the hyphae is 43 mM. Further elongation of the hyphae would result in further decreases in cytoplasmic [K⁺]. However, it seems unlikely, given the relative constancy in cytoplasmic [K⁺] across a wide range of organisms and the requirement for K⁺ for protein synthesis (Leigh et al., 1994) that cytoplasmic [K⁺] concentration would be allowed to fall below c. 50 mM to the low concentrations (<15 mM) required to balance the Nernst relationship. Thus, if elongation growth continued for more than 10 d, or the cytoplasmic volume was >10%, K⁺ would need to be absorbed from the external medium.

The rapid depolarization followed by recovery of the p.d. (Table 5 and Fig. 3) indicates that in the case of K⁺ at least, the fungus has reasonable control over the permeability of the plasma membrane. That only hyphae which exhibited cytoplasmic streaming had measurable p.d. and were sensitive to K⁺ is evidence supporting this conclusion.

**Implications for phosphorus absorption**

The results presented allow us to make the first calculations about the thermodynamics of inorganic phosphate (Pi) absorption by hyphae of AM fungi.

First, it is clear that the Pi in the cytoplasm is far from electrochemical equilibrium. In M-medium Pi concentration is ~35 μM (Bécard & Fortin, 1988); at pH 6.33 μM of this is in the monovalent form (H₂PO₄⁻). Substituting the value of −47 mV into the Nernst equation for the equilibrium ‘diffusion’ potential for Pi gives a theoretical concentration of Pi in the cytoplasm of <0.05 μM. In a wide range of species the concentration of Pi in the cytoplasm is in the order of a few millimolar (Ratcliffe, 1994); given the importance of maintaining stable cytoplasmic Pi concentration for cellular homeostasis, it is likely that the cytoplasmic Pi concentration in the hyphae of AM fungi is also a few millimolar.

It is generally accepted that high-affinity Pi uptake is by H⁺ co-transport. Uptake of Pi in *Gi. margarita* is reduced by CCCP, an uncoupler of oxidative phosphorylation (Thomson et al., 1990). The pH in the cytoplasm of the germ tube and external hyphae of *Gi. margarita* is around 6.8 (Jolicoeur et al., 1997). Within the cytoplasm approximately half the Pi is in the monovalent form, thus [Pi]ₘₜ is 5 mM is equivalent to [H₂PO₄⁻]ₘₜ of 2.5 mM. If the Nernst relationship is expanded to take account of co-transport of Pi
with H⁺, we can substitute the known values into Eqn 1:

\[ \Delta \Psi = (58/(z+nH^+)) \left( \Delta pH + \log \left[ H_2PO_4^- \right]_{cyt} \right) \]

\[ \Delta pH = (pH_{cyt} - pK_w) = 0.8; \]

\[ pH_{cyt} = 6.8 \text{ pH}_{out} = 6; \left[ H_2PO_4^- \right]_{out} = 33 \mu M; \]

\[ \left[ H_3PO_4 \right]_{cyt} = 2.5 \text{ mM}; \Delta \Psi = -47 \text{ mV} \]  Eqn 1

This gives an estimate of the number of protons transported for each H₃PO_4⁻ molecule of 2.33 (because M-medium has a relatively high Ca⁺⁺ content, the effective [H₃PO_4⁻] might be <33 µM, which would increase to the number of protons required). This number of protons is greater than that usually assumed to be co-transported with H₃PO_4⁻ (2:1); however, stoichiometries as high as 4:1 and 6:1 have been reported (see Schachtman et al., 1998). A high flux of protons could be the reason why the pH observed in the germ tube of *Gi. margarita* by Joliceur et al. (1997) is lower than that recorded from other fungi (e.g. Miller et al., 1990; Robson et al., 1996; Ayling et al., 1997). The estimate for cytoplasmic Pi concentration has a strong influence on the result of this calculation. If [Pi] in the cytoplasm is 1 mM, then the calculated stoichiometry is 1.47:1 with 33 µM H₃PO_4⁻ outside, or 1.74:1 with 20 µM H₃PO_4⁻ outside.

The free energy difference for Pi across the plasma membrane can also be calculated from the Nernst relationship (see Miller, et al., 1990; Equation 2).

\[ \Delta \Psi = -1F \Delta \Psi + RT \ln \left( \left[ H_3PO_4^- \right]_{cyt} \right) \]

assuming \( \Delta \Psi = -47 \text{ mV}; \) \[ F = 96.5 \text{ coulomb.mol}^{-1} \]

\[ R = 8.31 \text{ J.mol}^{-1}.K^{-1}; T = 295 K \]  Eqn 2

When \( \left[ H_3PO_4^- \right]_{cyt} \) is 2.5 mM and \( \left[ H_3PO_4^- \right]_{out} \) is 33 µM, Eqn 2 gives \( \Delta \Psi_{H_3PO_4^-} \) as 15.14 kJ.mol⁻¹; if \( \left[ H_3PO_4^- \right] \) in the cytoplasm is 0.5 mM then \( \Delta \Psi_{H_3PO_4^-} \) is 11.19 kJ.mol⁻¹. If there are 20 µM H₃PO_4⁻ outside then the calculated free energies are 16.37 and 12.42 kJ.mol⁻¹, for \( \left[ H_3PO_4^- \right] \) in the cytoplasm 2.5 and 0.5 mM, respectively. The free energy of ATP hydrolysis is c. 29 kJ.mol⁻¹ (Nicholls, 1982). This would predict a <1:1 stoichiometry for Pi absorption.

It is difficult to reconcile these two different stoichiometries. Mistrik & Ullrich (1996) concluded that all published results on Pi uptake could be explained by a 1H⁺:1 H₃PO_4⁻ stoichiometry and that any depolarization of the plasma membrane or acidification of the cytosol could be explained in terms of changes in K⁺ efflux. In that case a p.d. less negative than expected might be attributed to strong K⁺ efflux. In gram-positive bacteria the proton motive force is consistently lower in cells grown in P-rich vs minimal media; a tenfold decrease in intracellular Pi can increase the free energy of ATP hydrolysis available to proton-pumping ATPases by c. 8 kJ.mol⁻¹ (Bond & Russel, 1998). Unfortunately, attempts to determine whether there was an observable effect on p.d. of changing [H₂PO₄] in the solution were inconclusive. But whatever the mechanism involved in Pi uptake by AM fungi, the small values of wall and membrane p.d. in the external hyphae and germ tubes make absorption of negatively charged ions less energetically demanding.

In conclusion, the results show that there is no significant difference in transmembrane or hyphal wall p.d. between external hyphae and germ tubes of AM fungi and that the germ tubes of *Gi. margarita* respond to plant root extract and changes in K⁺ concentration. Therefore it seems unlikely that there are major physiological differences between these two types of hyphae. The rapid response of the germ tube transmembrane p.d. to plant root extract suggests that direct interactions with the plasma membrane are involved and that the metabolism of the fungus is poised ready for an external stimulus. Thermodynamic calculations suggest that despite the low (weakly polarized) transmembrane p.d., efficient high-affinity uptake of Pi is possible.

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**References**


