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Phosphorus amendment inhibits hyphal branching of the VAM fungus *Gigaspora margarita* directly and indirectly through its effect on root exudation

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Abstract The effect of solution phosphorus (P) concentration upon growth of pregerminated spores of the vesicular-arbuscular mycorrhizal fungus *Gigaspora margarita* was examined *in vitro*. P at 1 mM significantly inhibited branching of the primary germ tube. The number of branches and the total hyphal length were both significantly inhibited at 10 mM P. In addition, germinated spores exposed to exudates produced by Ri T-DNA-transformed roots of *Daucus carota* L. grown in the presence of P showed significantly less hyphal branching than those exposed to exudates produced by P-stressed roots. These phenomena could contribute to the observed inhibition of mycorrhiza formation by high P.

Key words *Gigaspora margarita* · Phosphorus · Exudation · Hyphal branching

Introduction

High levels of phosphorus are known to be inhibitory to the development of vesicular-arbuscular mycorrhizae (Mosse 1973). The two mechanisms proposed to explain this phenomenon are that soil P acts directly upon the vesicular-arbuscular mycorrhizal (VAM) fungus in the soil or indirectly through the increased P status of the host plant.

Most research points to host-plant mediation of the effects of P upon VAM fungus colonization. This has been shown clearly in split-root experiments in which application of P to one root compartment decreased colonization in roots on the other side (Menge et al. 1978; Thomson et al. 1991). The mechanism of host-mediated limitation of colonization is thought to concern root carbohydrate levels (Schwab 1982; Same et al. 1983) or decreased membrane permeability and exudation (Ratnayake et al. 1978; Graham et al. 1981; Schwab et al. 1983a), which would limit the ability of the fungi to form secondary penetrations. Root exudates of Ri T-DNA-transformed roots of *Daucus carota* were shown to stimulate the growth of *Gigaspora margarita* (Bécard and Piché 1989), while those of transformed *Pisum sativum* inhibited hyphal growth (Balaji et al. 1995). Citrus root exudates increased both germ tube length and branching of *Glomus epigaeum* (Graham 1982). In addition, exudates of host roots grown with high P produced less growth from spores of VAM fungi than exudates from roots grown in low P (Elias and Safir 1987; Tawaraya et al. 1996).

There is conflicting evidence regarding an effect of soil P directly upon the growth of VAM fungi. P levels of 37.5 mg kg^{-1} and above decreased germination and hyphal growth of *Scutellospora heterogama* and *Glomus etunicatum* (Miranda and Harris 1994), whereas addition of 20 mg l^{-1} $CaHPO_4$ increased germination and hyphal growth of *Gigaspora margarita* in water agar (Siqueira et al. 1982). Germination and hyphal growth of *Glomus caledonium* and *Glomus mosseae* on agar supplemented with 30 mM K_2HPO_4 were lower than controls (Hepper 1983). However, this was due to the salt, since addition of K_2SO_4 had the same effect and P amendments up to 982 mg P kg^{-1} soil had no effect upon germination or hyphal growth. P concentrations in soil in Petri plates of up to 200 mg kg^{-1} did not inhibit germination of *Glomus epigaeum* (Daniels and Trappe 1980) or *Glomus fasciculatum* (Schwab et al. 1983b).

We present evidence here of both direct and indirect effects of media P concentration upon the growth of hyphae from pregerminated spores of *Gigaspora margarita*.

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Materials and methods

Spores

Azygospores of *Gigaspora margarita* Becker & Hall (DAOM 194757) were produced in pot cultures with *Paspalum notatum* Flugge in a greenhouse. Spores were collected by wet sieving and centrifuging through 40% (w/v) sucrose, and then surfaced sterilized (Bécard and Fortin 1988). Spores were germinated for experiments by aseptic insertion into Petri plates of M medium (Bécard and Fortin 1988) followed by vertical incubation at 32 °C in 2% CO_2 for 2–4 days. The M medium used for this study was modified as indicated in Table 1.

Production of exudates

The clone of Ri T-DNA-transformed roots of *Daucus carota* produced by Bécard and Fortin (1988) was propagated in M medium with 2 g l^{-1} gellan (ICN Biochemicals, Cleveland, Ohio) as gelling agent. After 14 days growth in solid medium at 25 °C, 15–20 roots were each cut into three pieces and aseptically transferred into 125 ml of M medium in 250-ml Erlenmeyer flasks without gelling agent, with or without 35 μM P as KH_2PO_4. These roots were grown in a shaking water bath (125 rpm, 25 °C) for 7–10 days. The media containing exudates were then collected and added to an equivalent volume of freshly autoclaved and cooled (50 °C) M medium with 8 g l^{-1} gellan, and poured into 9-cm-square Petri plates. The final gellan concentration in plates containing germinated spores therefore was 0.4%. Controls were prepared by adding an equal volume of sterilized M medium minus gellan to autoclaved M medium containing 8 g l^{-1} gellan.

Experimental media and growth conditions

A single pregerminated spore was axenically transferred within a core of medium to a square Petri dish (9 cm) containing M medium amended with exudates or, for other experiments, KCl, K_2HPO_4, or K_2HPO_4/KH_2PO_4, TRIS (TRIZMA HCl/TRIZMA base, Sigma), HEPES/MES, or sodium acetate/acetic acid buffers (incorporated before autoclaving) at various concentrations and pH levels. Plates of five to seven replicates of each treatment were incubated vertically at 32 °C and 2% CO_2 for 5–9 days. Since the primary germ tube exhibited a strong negative geotropic response, plates were turned when needed to redirect the tip away from the edges of the Petri dish. The surface pH of the media was determined at the end of each experiment using an Orion surface electrode.

Table 1 Composition of M medium

Compound	Concentration (mg l^{-1})
$MgSO_4 \cdot 7H_2O$	731
KNO_3	80
KCl	65
KH_2PO_4	4.8
$Ca(NO_3)_2 \cdot 4H_2O$	288
NaFeEDTA	8
KI	0.75
$MnCl_2 \cdot 4H_2O$	6
$ZnSO_4 \cdot 7H_2O$	2.65
H_3BO_3	1.5
$CuSO_4 \cdot 5H_2O$	0.13
$Na_2MoO_4 \cdot 2H_2O$	0.0024
Glycine	3
Thiamine hydrochloride	0.1
Pyridoxine hydrochloride	0.1
Nicotinic acid	0.5
Myo-inositol	50
Sucrose	10000
Gellan (root culture)	2000
Gellan (spores)	4000

Fungal growth measurements

Overall hyphal length was measured by using a 2-mm grid and counting hyphal intersections (Newman 1966). The primary germ tube (the main hypha, exclusive of its branches) was measured directly. The number of hyphal branches off the primary germ tube (secondary branches) and the number of clusters of auxiliary cells were counted directly under the dissecting microscope (×20). Tertiary branching was quantified on the first two secondary branches nearest the spore.

Data were analyzed using analysis of variance ($\alpha = 0.05$). Apparently significant treatment effects found were characterized further using Tukey's method of multiple comparisons. All experiments were replicated and representative results are presented.

Results

Effect of media P upon growth of *Gigaspora margarita*

Addition of P as K_2HPO_4 to M media affected growth of hyphae from pregerminated spores of *Gigaspora margarita* (Table 2). P at 1 mM significantly decreased the number of branches rising from the primary germ tube without affecting germ tube length, total hyphal growth, or number of auxiliary cells. P at 10 mM inhibited both hyphal growth and branching. This was shown not to be a salt effect because concentrations of KCl up to 10 mM in gels at pH 6 had no significant effect on any parameter measured (Table 3). The pH dependence of this effect was studied using 10 mM potassium phosphate buffer (Fig. 1). All potassium phosphate buffer treatments significantly decreased both the number of secondary branches and the number of tertiary branches off of the first two secondary branches ($Pr > F = 0.0159$, data not shown) relative to control M medium at pH 5.5. Total hyphal length at pH 5.0, 6.5, and 7.0 was lower than the controls (Fig. 1).

The pH of the unbuffered M media was difficult to control. Measurement of pH after autoclaving and setting of the gel yielded values inconsistent with those before autoclaving (eg. 5.0 before autoclaving and 5.4 after, 7.0 before autoclaving and 6.2 after). A variety of buffers was examined to determine the effect of pH upon the inhibition of branching and/or hyphal growth.

Buffering systems composed of sodium acetate/acetic acid, TRIZMA HCl/TRIZMA base, and HEPES/MES were tested at 10 mM. Sodium acetate completely stopped the growth of pregerminated spores shortly after transfer to experimental dishes (data not shown). TRIS buffer stimulated hyphal growth, auxiliary cell

Table 2 Growth of pregerminated spores of *Gigaspora margarita* after 5 days in the presence of K_2HPO_4/KH_2PO_4 (*KP*) buffer at pH 6.0. The control pH at the end of the experiment was 5.7. The data are the means of seven observations. Numbers in the same column followed by the same letter are not significantly different ($\alpha=0.05$)

Treatment	Hyphal length (cm)		Number of auxiliary cells	Branches	
	Germ tube	Total		Secondary[a]	Tertiary[b]
Control	4.5 a	15.1 a	3.3 a	14.8 a	1.5 a
0.1 mM KP	4.8 a	16.7 a	4.4 a	16.0 a	0.9 a
1.0 mM KP	4.0 a	12.1 ab	3.6 a	9.3 b	0.4 a
10.0 mM KP	3.2 b	8.1 b	2.7 a	4.9 b	0.1 a
Pr>F	0.0001	0.0003	0.1076	0.0001	0.0720

[a] Number of branches off the primary germ tube
[b] Mean number of tertiary branches off the first two secondary branches of the primary germ tube

Table 3 Effect of KCl concentration upon the growth of pregerminated spores of *Gigaspora margarita* after 5 days. The data are the means of six observations ±SEM. The pH of the gels at the conclusion of the experiment was 6.0, 5.9, and 5.9 for control, 1 mM and 10 mM KCL, respectively

Treatment	Hyphal length (cm)		Number of auxiliary cells	Branches	
	Germ tube	Total		Secondary[a]	Tertiary[b]
Control	4.5±0.1	14.1±0.8	3.7±0.5	13.5±0.7	1.7±0.2
1.0 mM KCl	4.6±0.1	14.9±0.3	4.2±0.4	14.5±0.6	1.7±0.3
10.0 mM KCl	4.5±0.1	15.4±1.1	4.7±1.0	12.7±0.6	1.4±0.4
Pr>F	0.8310	0.5307	0.5775	0.1443	0.9217

[a] Number of branches off the primary germ tube
[b] Total number of tertiary branches

Table 4 Effect of 10 mM TRIS buffer upon the growth of pregerminated spores of *Gigaspora margarita* after 6 days. The data are the means of seven observations. The control pH was 5.0 before autoclaving and 5.4 after the setting of the gel. Numbers in the same column followed by the same letter are not significantly different ($\alpha=0.05$)

Treatment	Hyphal length (cm)		Number of auxililary cells	Branches	
	Germ tube	Total		Secondary[a]	Tertiary[b]
Control	4.3 b	11.6 b	2.7 c	14.4 a	0.2 b
TRIS pH 5.0	4.9 ab	16.7 b	6.4 ab	15.6 a	0.4 b
TRIS pH 6.0	4.5 b	14.6 b	4.3 bc	14.3 a	0.7 b
TRIS pH 7.0	5.2 a	24.4 a	9.0 a	11.7 a	5.2 a
Pr>F	0.0011	0.0001	0.0001	0.0673	0.0001

[a] Number of branches off the primary germ tube
[b] Mean number of branches off the first two secondary branches of the primary germ tube

production, and tertiary branching at pH 7.0, but did not affect the growth or branching of the primary germ tube (Table 4). No parameter measured for spores grown in TRIS pH 6.0 was significantly different from controls, indicating that the P effect shown in Table 1 was not a buffered pH effect. The reason for the stimulation of hyphal growth and branching with TRIS at pH 7 is unknown. HEPES-MES had little effect on hyphal growth except to decrease branching at pH 6.0 and 7.0 (Table 5). MES has been shown to inhibit the growth of another VAM fungus, *Glomus mosseae* (D.D. Douds, unpublished work).

Effects of exudates upon growth of *Gigaspora margarita*

Exudates produced in the presence or the absence of P did not affect the growth of the primary germ tube of *Gigaspora margarita* (Table 6). Total hyphal length and branching characteristics were equivalent for the control and exudate with P treatments; however, exudate produced in the absence of P significantly increased branching and hyphal growth.

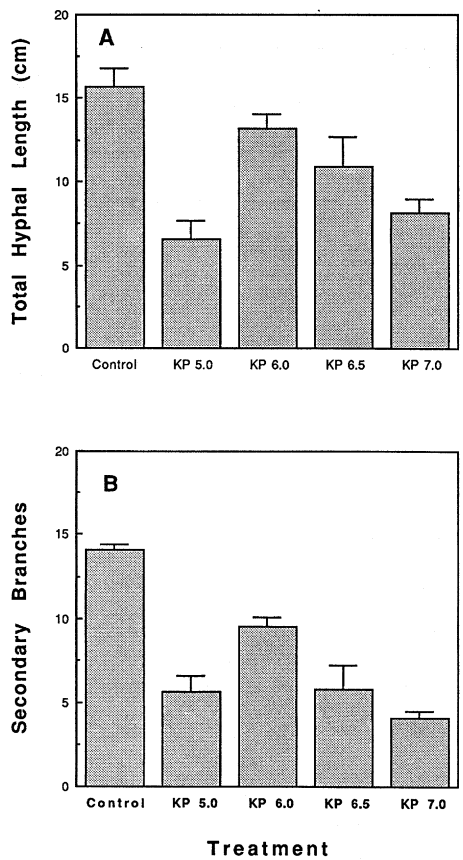

Fig. 1 The effect of initial pH on hyphal growth (**A**) and production of secondary branches (**B**) from pregerminated spores of *Gigaspora margarita* in the presence of 10 mM potassium phosphate buffer or control M medium after 6 days. The data are the means of seven observations ± SEM

Discussion

Four mechanisms have been proposed to explain the inhibition or limitation of mycorrhizal development by high P. First, anatomical or physiological changes may occur in roots which limit the intraradical spread of the fungus (Menge et al. 1978; Amijee et al. 1989, 1990; Koide and Li 1990; Braunberger et al. 1991; Li et al. 1991; Thompson et al. 1991). Second, recent evidence, utilizing the production of spores as a measure of the host's partitioning of carbon to the fungus suggests a lower carbon flow through a unit of colonized root under high P than under low P conditions (Douds 1994). Third, there may be qualitative or quantitative changes in root exudation, which somehow affect the growth of the extraradical mycelium and thereby the development of secondary penetrations (Ratnayake et al. 1978; Graham et al. 1981; Graham 1982; Schwab 1982; Schwab et al. 1983a; Elias and Safir 1987; Tawaraya et al. 1994; Tawaraya et al. 1996). Lastly, P may directly inhibit the growth of the fungus in the soil (Hepper 1983; Louis and Lim 1988; Miranda et al. 1989; Thompson et al. 1991; Miranda and Harris 1994).

We present evidence for a mode of action consistent with mechanisms three and four above. The *in vitro* method of Bécard and Piché (1992) has allowed researchers to visualize and study the interaction of host and fungus prior to and upon colonization. One common observation is the formation of areas of prolific branching when the fungus "senses" the presence of a root (Bécard and Fortin 1988; Giovannetti et al. 1993). Hyphal branching would be an effective long- and short-distance fungal strategy to efficiently explore a volume of medium/soil to ensure contact with a root. We have shown here that P added to the growth medium can inhibit hyphal branching directly, and that the quantity/quality of root exudates, regulated by host P status, also affects hyphal branching.

Our results with KCl and the following calculations suggest that the direct effect of P upon hyphal growth and branching of the VAM fungus *Gigaspora margarita* is unlikely to be a salt effect, as described by Hepper (1983). Commercial gellan gum for microbiological applications at a concentration of 4 g l^{-1} contributes a minimum of 9 mmol of solutes (Doner and Douds 1995). The M nutrient media provides an additional 13 mmol mineral solutes and 27.3 mmol sucrose (Bécard and Fortin 1988). It is, therefore, unlikely that the inhibition of secondary branching caused by 1 mM KH_2PO_4 was due to a salt effect. The final concentration of P in the medium in which the assays were run was approximately 1.163 mM: 0.128 mM from gellan (P = 0.099% dry wt. according to Doner and Douds

Table 5 Effect of 10 mM HEPES-MES buffer upon the growth of pregerminated spores of *Gigaspora margarita* after 5 days. The data are the means of seven observations. The control pH at the end of the experiment was 5.8. Numbers in the same column followed by the same letter are not significantly different ($\alpha = 0.05$)

Treatment	Hyphal length (cm)		Number of auxiliary cells	Branches off germ tube
	Germ tube	Total		
Control	3.9 a	12.5 a	2.8 a	13.0 a
HEPES-MES pH 5.0	3.8 a	9.6 a	1.3 b	10.3 ab
HEPES-MES pH 6.0	3.0 ab	11.4 a	2.6 ab	6.2 b
HEPES-MES pH 7.0	2.7 b	8.8 a	3.6 a	5.6 b
$Pr > F$	0.0059	0.2149	0.0040	0.0008

Table 6 Effect of exudates produced by Ri T-DNA-transformed roots of *Daucus carota* grown in liquid culture with or without P upon the growth of pregerminated spores of *Gigaspora margarita* after 5 (experiment 1) and 6 (experiment 2) days. The data are the means of seven (experiment 1) or six (experiment 2) observations. The pH of the media were 6.0, 6.9 and 6.4 in experiment 1 and 6.0, 6.7 and 7.0 in experiment 2 for control, +P exudate, and −P exudate, respectively. Numbers in the same column followed by the same letter are not significantly different ($\alpha = 0.05$)

Treatment		Hyphal length (cm)		Number of auxiliary cells	Branches	
		Germ tube	Total		Secondary[a]	Tertiary[b]
Experiment 1	Control	4.6 a	16.0 a	5.1 a	14.7 ab	0.6 b
	Exudate +P	4.2 a	16.1 a	5.0 a	11.1 b	0.1 b
	Exudate −P	4.7 a	21.2 a	5.0 a	15.7 a	4.4 a
	$Pr>F$	0.6225	0.2008	0.9910	0.0237	0.0001
Experiment 2	Control	4.0 a	12.0 b	5.5 a	14.2 a	0.2 b
	Exudate +P	4.3 a	13.8 b	2.3 b	13.5 a	0.4 b
	Exudate −P	4.0 a	22.2 a	7.0 a	16.7 a	4.5 a
	$Pr>F$	0.1088	0.0030	0.0002	0.0722	0.0018

[a] Number of branches off the primary germ tube
[b] Mean number of branches off the first two secondary branches of the primary germ tube

1995) plus 0.035 mM from the M medium (Bécard and Fortin 1988), plus 1 mM experimental addition, much less than the 20–30 mM applied by Hepper (1983).

The average soil solution P concentration for base-saturated soils is 0.03 mg P l^{-1}, or 1 μM (Wild 1981). Hoagland's nutrient solution contains 1 mM P (Hoagland and Arnon 1938) and growth responses to mycorrhizal fungi have been noted up to 148 mg kg^{-1} (Lamar and Davey 1988). Other reports indicate an upper limit for mycorrhizal benefit of approximately 20 mg available P kg^{-1} soil (Miller et al. 1981; Hung et al. 1990). Assuming an average bulk density of 1.2 and a moisture content of 25%, this may be expressed as 3.1 mM P. Therefore, the 1 mM P inducing inhibition of hyphal branching of VAM fungi in our experiments can occur in natural situations and can contribute to decreased mycorrhization of roots.

We describe two responses which may contribute to the decreased colonization of roots by VAM fungi in the presence of high soil P. These responses would result in reduced exploration of the soil, less contact with roots, and consequently a lower colonization. Work is presently underway to identify compounds present in host exudates that trigger the hyphal branching response.

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