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GERMINATION AND HYPHAL GROWTH OF *GLOMUS MOSSEAE IN VITRO*: EFFECTS OF RHIZOSPHERE BACTERIA AND CELL-FREE CULTURE MEDIA

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Summary—Germination and growth of chlamydospores of the vesicular–arbuscular (VA) mycorrhizal fungus *Glomus mosseae* on water–agar medium under axenic conditions were compared after treatment with washed cells, cell-free supernatants and complete bacterial cultures. Spore germination was not affected by bacterial treatments, but the additions of complete bacterial cultures and cell-free supernatants significantly ($P \leqslant 0.05$) stimulated hyphal growth and the number of new vegetative vesicles formed per germinated resting spore.

INTRODUCTION

Cell-free culture media of rhizosphere bacteria were able to improve the formation of vesicular–arbuscular mycorrhizas (VAM) between *Glomus mosseae* and several host plants (Azcón *et al.*, 1978). This effect, which may be accounted for by the ability of soil microorganisms to excrete biologically active substances (Lynch, 1976; Strzelczyk and Pokojska-Burdziej, 1984), can be exerted indirectly through the host plant or directly on the fungus. Recent studies indicate that soil microorganisms can improve independent hyphal growth from *G. mosseae* spores at the pre-infection stages (Azcón–Aguilar and Barea, 1985). Furthermore, isolated free-living microorganisms were able to stimulate the axenic growth of *G. mosseae* on water agar (Azcón–Aguilar *et al.*, 1986; Mayo *et al.*, 1986). This effect was achieved even without contact between microorganisms. A logical next step is to test the effect of cell-free supernatants from free-living microorganisms on the axenic development of *G. mosseae*. The aim of this paper was to ascertain whether a soil bacterium which enhanced mycorrhizal formation and function in previous studies, is able to stimulate spore germination and hyphal growth *in vitro*.

MATERIALS AND METHODS

Spores of *Glomus mosseae* (Nicol. dx Gerd.) Gerd. and Trappe (Gerdemann and Trappe, 1974) were isolated from sporocarps. These sporocarps were obtained from the rhizospheres of alfalfa plants inoculated with pure *G. mosseae* spores and grown in stock-plant cultures under greenhouse conditions for 4 months. These mycorrhizal rhizosphere samples were stored for several months in polyethylene bags at 5°C. The sporocarps were collected from these samples by wet-sieving and decanting (Gerdemann and Nicholson, 1963). Uniform and undamaged *G. mosseae* spores were selected after excision from these sporocarps. Spores were surface-sterilized with the solution described by Mosse (1962). This consisted of 2% wv chloramine T plus 200 μg streptomicin ml^{-1} and a drop of Tween 20. The sterilant was applied for 20 min and the spores washed several times in sterile deionized water prior to transferring them to water-agar (10 g l^{-1} of Difco Bacto agar) at pH 7. Spores were placed individually in Petri dishes at approximately 2 cm from a central disc of Whatman No. 1 filter paper of 1 cm diam which had been previously sterilized. One ml of the following bacterial culture preparations were added to the discs: Complete bacterial culture, culture medium free from bacterial cells or washed bacterial cells. The sterile growth medium used for growing the bacteria was applied diluted (10^{-1}) or undiluted to discs (1 ml per disc) in the control plates. There were ten replicate plates per treatment with 6–9 single spores per replication. Plates were sealed with parafilm and incubated in the dark at 25°C.

The test bacterium, as yet unidentified, were isolated by standard procedures (see Barea *et al.*, 1976) from rhizospheric soil, and grown in a rotatory shake culture at 28°C for 14 days in 250 ml flasks containing 50 ml of medium (nutrient broth (BBL) plus soil extract, 8 g l^{-1}). L-Tryptophan (0.2 g l^{-1}) was filter sterilized (Millipore 0.2 μm pore size) and added to the medium. Water extracts from soil were obtained using 100 g 100 ml^{-1} of soil–water mixture, shaken 4 h and filtered through Whatman No. 1 filter paper. The cell-free bacterial medium was obtained by centrifuging bacterial culture at 2680 *g* for 30 min. This supernatant was sterilized. Bacterial pellets, in the bottom of the centrifuge tubes were resuspended and washed five times in sterile water and subsequently centrifuged under sterile conditions at 2680 *g* for 15 min. Bacteria were resuspended to a final concentration of 10^8 cells ml^{-1}.

Determinations of the germination, formation of vegetative vesicles and mycelial growth were made after 4, 6, 8, 11, 13, 15, 21 and 26 days of incubation.

Table 1. Effect of cell-free supernatants, washed bacterial cells, and complete bacterial cultures of a soil bacterium on germination of *Glomus mosseae* spores

Treatments	Percentage germination (days)[1] 4	6	8	11	13	15	21	26
T	36.5 ± 23	53.5 ± 26	64.6 ± 18	70.5 ± 14	74.8 ± 11	85 ± 5	85 ± 5	85 ± 5
C	31.0 ± 16	49.6 ± 11	67.0 ± 13	77.4 ± 9	81.0 ± 6	87 ± 8	87 ± 8	87 ± 8
S	35.8 ± 18	62.5 ± 18	74.5 ± 12	88.3 ± 5	88.5 ± 4	92 ± 7	92 ± 7	92 ± 7
C + S	44.6 ± 23	68.4 ± 19	84.5 ± 20	87.4 ± 21	94.0 ± 31	95 ± 12	95 ± 12	95 ± 12

[1]Days after *G. mosseae* spores were inoculated into the Petri dish on water agar medium.
Standard errors of the mean are given. ($P = 0.05$). T = Control. S = Cell-free supernatants of the bacterial cultures. C = Washed bacterial cells. C + S = Complete bacterial cultures.

Table 2. Effect of cell-free supernatants, washed bacterial cells and complete bacterial culture of a soil bacterium on the formation of vegetative spores on mycelium arising from resting spores of *Glomus mosseae*

Treatments	Number of vegetative spores per germinated resting spore[1] (days) 4	6	8	11	13	15	21	26
T	0	0	0.06 ± 0.085	0.16 ± 0.21	0.40 ± 0.21	0.70 ± 0.21	1.21 ± 0.28	2.10 ± 0.71
C	0	0	0.05 ± 0.078	0.29 ± 0.17	0.40 ± 0.21	0.50 ± 0.35	1.10 ± 0.50	1.70 + 0.50
S	0	0	0.35 ± 0.35	0.85 ± 0.35	1.40 ± 0.50	2.30 ± 0.57	3.34 ± 0.57	4.30 ± 0.71
C + S	0	0	0.96 ± 0.42	1.50 ± 0.42	2.00 ± 0.57	2.70 ± 0.28	3.44 ± 0.42	4.30 ± 0.42

[1]Conventions as in Table 1.

A spore was considered germinated when the germ-tube was clearly seen (at 40×) under a disection microscope.

RESULTS

Glomus mosseae spore germination occurred within 4 and 15 days of incubation (Table 1). Germination was unaffected by the treatments assayed. The greater percentage of germination was reached when complete bacterial cultures was applied but it was not statistically different from the other treatments. A significative increase in secondary vesicle formation occurred when cell-free supernatant and complete bacterial cultures were added to filter discs (Table 2). The first new vegetative vesicles were observed after 8 days of spore incubation (Table 2). As the number of vesicles formed on the mycelium was previously found to be closely related with the amount of mycelium (Azcón-Aguilar *et al.*, 1986) this was subjectively quantified into categories (see conventions in Table 3). A stimulation of hyphal growth was also clearly observed by complete bacterial cultures and cell-free supernatant applications (Table 3).

Complete bacterial cultures produced the highest stimulation of *G. mosseae* spores as evaluated by spores germination, mycelia development and formation of vegetative vesicles. Its effect was more relevant in the first observations.

No contact was observed between spores and bacterial preparations applied to the membranes.

Table 3. Effect of cell-free supernatants, washed bacterial cells and complete bacterial cultures of a soil bacterium on mycelial (hyphal) growth from resting spores of *Glomus mosseae*

Treatment	Hyphal development[1] (days) 4	6	8	11	13	15	21	26
T	—	—	a	a	a	a	b	b
C	—	—	a	a	b	b	b	b
S	—	—	b	b	c	c	c	c
C + S	—	b	b	c	c	c	c	c

[1]Conventions as in Table 1. The three categories established, i.e. a, b and c, indicate the dimensions of mycelial development; (a) slight: The largest dimension was less than 5 mm; (b) moderate: between 5 and 10 mm (c) extensive: greater than 10 mm.

DISCUSSION

Glomus spores are known to store genetic information for the development of biosynthetic processes involved in germination (McDonald and Lewis, 1978; Hepper, 1979; Beilby and Kidby, 1982). Hence, it is logical that these spores readily germinate independently from exogenous applications. Nearly 90% germination was obtained axenically corroborating previous results on water-agar (Hepper and Smith, 1976; Azcón-Aguilar *et al.*, 1986). This last paper also indicates that inoculation of free-living microorganisms in the germinating medium hardly affected the final percentage of germination although they hastened the germination rate.

The most noticeable results in this study is the ability of cell-free preparation of bacterial cultures to improve mycelial development from germinated *G. mosseae* spores in comparison with the growth medium as applied either undiluted or diluted. This indicates the involvement of water-soluble diffusible substances of microbial origin as was suggested by Azcón-Aguilar *et al.* (1986).

Mycorrhizal fungi, as typical rhizosphere organisms, might be stimulated by some organic substances present in the root zone, that can be of microbial origin (Lynch, 1976). For example the beneficial effect of some soil bacteria on colonization rates of plants infected with *G. mosseae* was attributed to hormonal substances produced by these bacteria (Azcón *et al.*, 1978). Moreover vitamins and amino-acids had also been reported (Hepper, 1979 and 1983; Hepper and Jakobsen, 1983; Siqueira *et al.*, 1982) to stimulate VAM fungal spores grown *in vitro*.

The similar effect by complete bacterial cultures

and cells-free supernatants on *G. mosseae* development suggest that bacteria acted through their metabolites in the supernatant. Since the test medium is very poor in nutrients the bacterial cells did not continue forming active products during the assay. The positive effect of the complete bacterial cultures over the cell-free preparations in the first observations could be due to the bacteria still having some nutrients as supplied with the inoculum.

The results presented here could be taken as a direct evidence of a positive soil microorganism–VAM fungus interaction, which may be of interest in rhizosphere biology.

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