EFFECT OF ORGANIC SUBSTRATES ON GERMINATION AND GERM TUBE GROWTH OF VESICULAR-ARBUSCULAR MYCORRHIZAL FUNGUS SPORES IN VITRO[1]

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ABSTRACT - Vesicular-arbuscular mycorrhizal (VAM) fungi are obligate biotrophs and have not been grown in the absence of living roots. Pure culture of these fungi constitutes a critical prerequisite for intensive studies on their practical application. The effect of additional nutritional factors on spore germination and germ tube growth of surface disinfested spores of VAM fungi *in vitro* is reported. A range of organic substrates was tested. Most of them were inhibitory or had no effect on spores. However, small concentrations of D-galacturonic acid (1 g l^{-1}) enhaced spore germination and 4 g l^{-1} sucrose and 1% soil extract agar improved germ tube growth. The beneficial effect of soil extract is concentrated in the protein fraction, which appears to account for the limited saprophytic ability of these fungi in soil.

Index terms: mycorrhizae, spore germination, axenic culture.

EFEITO DE SUBSTRATOS ORGÂNICOS NA GERMINAÇÃO E CRESCIMENTO DO TUBO GERMINATIVO DE ESPOROS DE FUNGOS MICORRÍZICOS VESICULAR-ARBUSCULARES IN VITRO

RESUMO - Os fungos formadores das micorrizas vesicular-arbusculares (MVA) são essencialmente biotróficos e ainda não foram cultivados na ausência de raízes vivas. Cultura pura destes fungos constitui um pré-requisito crítico para estudos visando à utilização em larga escala destes organismos. É apresentado o efeito de fatores nutricionais na germinação, e crescimento do tubo germinativo, *in vitro*, de esporos desinfestados superficialmente. Vários substratos orgânicos foram testados, e a maioria deles não teve efeito ou inibiu a germinação dos esporos. Entretanto, ácido D-galacturônico (1 g l^{-1}) aumentou a germinação de esporos de *Gigaspora margarita*, ao passo que sucrose (4 g l^{-1}) e extrato de solo-ágar a 1% aumentaram o crescimento do tubo germinativo. Fracionamento molecular do extrato de solo mostrou que o fator determinante da resposta está concentrado na sua fração protéica. Este efeito benéfico, associado à fração protéica do extrato de solo, possivelmente está relacionado com a limitada capacidade saprofítica destes fungos no solo.

Termos para indexação: micorrizas, germinação de esporos, cultura axênica.

INTRODUCTION

Over the last three decades, the study of vesicular-arbuscular mycorrhizal (VAM) associations has passed from a largely unindisciplinary descriptive phase to multidisciplinary experimental phase. Although much information has been gained on their ecology, physiology and host-fungus relationship, Menge (1983) stated that "there is still a need to optimize the activities of these fungi to benefit agriculture".

The VAM fungi are ecologically obligate biotrophs and have not been cultured *in vitro*. This fact presents the most challenging and critical prerequisite for intensive studies of these fungi and their associations. However, if axenic culture succeeds, more ambitous objectives may be possible and the benefits of VAM fungi could be exploited on a large scale.

Studies to determine requirements for spore germination and germ tube growth of VAM fungi *in vitro* have met with variable results (Siqueira et al. 1982). Cultura conditions, especially nutrition, constitute one of the main factors affecting VAM fungal spore germination and germ tube growth either in agar media or in soil. In a previous publication (Siqueira et al. 1982) the effect of soluble salts, pH, thiamine and a range of carbon sources on either spore germination or germ tube

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growth in the absence of living plant roots was reported. Here, additional studies examining the effect of defined and undefined organic substrates on spore germination and germ tube growth of VAM fungi *in vitro* are published.

MATERIAL AND METHODS

The species of VAM fungi selected for use in this study were *Gigaspora margarita* Becker & Hall, and *Glomus mosseae* Gerdemann & Trappe obtained from stock cultures of Dr. N.C. Schenck of the Plant Pathology Department, University of Florida-USA. Spores were multiplied in bahiagrass (*Paspalum notatum* Flugge) pot cultures grown for five months, and stored in dry soil with 2% moisture (W/V) at 4^oC - 6^oC until required for use. The spores were separated from soil by wet sieving and decanting (Gerdemann & Nicolson 1963) followed by centrifugation in deionized water to separate the organic debris, and in 25% Ficoll to separate the mineral fraction. Spores were collected on a sieve (120 mesh) and transferred to a fresh disinfesting solution 3% (W/V) chloramine T plus 200 ppm (W/V) streptomycin for 5 min. The disinfestant was removed from spores by repeated washing with deionized water. Spores were transferred to 1% agar medium containing 20 mg l^{-1} Ca H_2PO_4 and organic substrates as indicated in Table 1. The effect of glucose was examined on acid washed, sterilized sand plates because of the high incidence of contaminants on glucose agar plates. Spores were transferred to 47 mm Gelman metricel membrane filters (0,45 μm) by Pasteur pipete with each membrane receiving 15 spores. Another membrane was used to cover the spores and they were placed between two 1 cm layers of sand in glass Petri dishes. Glucose aqueous solution at concentrations ranging from 0.4 to 4 g l^{-1} was used to bring sand moisture to optimum for germination (Siqueira 1983).

Aqueous extract was obtained from and acid soil incubated for three months with 12 meq $Ca/MgCO_3$/ 100 g of soil. After incubation, soil analysis showed pH (in water) = 6.4; Al (KCl 1N) = 0.17 meq/100 g of soil and the following amounts of Mehlich I extractable nutrients in ppm: K = 13; Ca = 1.100; Mg = 550; P = 3.3; Zn = 0.63; Mn = 1.4; Fe = 1.0 e Cu = 0.12. The extract was prepared according to Ko & Hora (1972) and was fractionated using a P-10 Biorad gel column with a molecular range of 1500-2000 K. The mounted column was 25 cm in height and 5 ml of sample were eluted with deionized water at a flux rate of 4.8 ml/hr. Four ml fractions were collected in a Gilson sample collector. All fractions, including the void volume, were assayed for their protein and sugar contents and their effects on spore germination and germ tube growth. Protein content was determined by absorption at 280 mm using bovine serum albumin as standard. Total sugar content was determined by the anthrone method (Ashwell 1957). For the assay, chlamydospores of *Glomus mosseae* (25 spores/ml of extract) were soaked in the extract fractions for 72 hours and then transferred to a 47 mm membrane filter on 1% agar plates for germination.

Each experiment had three replications per treatment and was repeated at least twice. After the incubation period of ten to fifteen days, spores were stained with 0.01% acid fuchsin and assessed for germination and germ tube growth according to Siqueira et al. (1982).

TABLE 1. Effect of organic substrates on spore germination and germ tube (GT) growth of *Gigaspora margarita* azygospores incubated at 28°C on agar plates.

Substrate	Conc.	Germination	GT growth
	g/1	Control (%)[1]	
Sucrose	1.0	99	86
Sucrose	4.0	86	125
Sucrose	16.0	58	40
Fructose	12.0	49	72
L-Arabinose	1.2	45	81
Aspartic acid	2.0	39	54
Succinic acid	2.0	18	0
Tartaric acid	5.0	25	30
D-Gal. acid	0.5	98	33
D-Gal. acid	1.0	141	65
D-Gal. acid	4.0	27	30
Na-Acetate	0.3	118	95
Na-Acetate	0.6	68	71
Na-Acetate	1.2	0	-
Clycerol	4.0	50	0
Ca-Phytate	1.0	77	64
Casein hydrolysate	1.0	75	95
Peptone	1.0	100	55
1% soil extract agar	-	93	139
1% root exudate agar	-	64	103

[1] Control with 20 mg CaH_2PO_4 + 0,01 mg thiamine-HCl per liter = 100% for germination and GT growth.

RESULTS AND DISCUSSION

Surface disinfested resting spores of *Glomus mosseae* and *Gigaspora margarita* germinated readly within three to five days when placed on a suitable medium. The azygospores of *G. margarita* germinated by producing one or several germ tubes directly through the spore wall near the bulbous attachment as ilustrated in Fig. 1. The onset of germination and the following morphological events have been well studied (reviewed by Siqueira 1983 and Siqueira et al. 1985). Chlamydospores

of *G. mosseae* germinated by regrowth of the old hyphal attachment as described by Mosse (1956, 1959) and shown in Fig. 2. These two species of VAM fungi exhibit different vegetative growth patterns *in vitro*, but they are equally affected by nutrition. In general, sugars and other organic substrates showed no benefit on either spore germination or germ tube growth (Table 1). With the exception of 1 g l^{-1} D-galacturonic acid and 0.3 g l^{-1} Na-acetate, all carbon sources examined showed either no effect or deleterious effect on spore germination. Germ tube growth of *G. margarita* was improved by 4 g l^{-1} sucrose and soil extract agar 1% (Table 1). On sand plates 0.6 g l^{-1} of glucose improved spore germination, but concentrations higher than that exhibited inhibitory effect. The germ tube growth was equally affected at concentrations ranging from 0.4 to 0.8 g l^{-1} (Fig. 3). The inhibitory effect of sugars on VAM fungal spores has been reported elsewhere (Siqueira 1983). Surprisingly, carbohydrate deficiency is the most common limiting factor for fungal spore germination (Gottlieb 1978), and sugars are the triggering factor for spore germination in some fungal species, (Ekundayo & Carlisle 1964). The beneficial effect of small amounts of sugars reported here may be associated with the mechanism of water and nutrient uptake as discussed by Siqueira (1983). The germination promoting effect of D-galacturonic acid appears to be related to the ability of VAM fungi to produce cell wall degrading enzymes and seems to be a promising carbon source for these fungi in the absence of living root cells. Although it has been biochemically demonstrated that germinating VAM fungal spores can incorporate ^{14}C-acetate into their carbon metabolism (Beilby & Kidby 1982), Na-acetate at 0.3 g l^{-1} had little effect on spore germination and no effect on germ tube growth *in vitro*, but concentrations higher than that were highly inhibitory to both germination and germ tube growth of *G. margarita* azygospores. It is suggested that small concentrations of acetate may be crucial to initiate the metabolism of germinating spores. However, once the lipid catabolism has been activated, large amounts of acetate and other short-chain fatty acids will be available in the spore, as proposed by Siqueira (1983), and these compounds would not benefit germ tube growth, as it is reported.

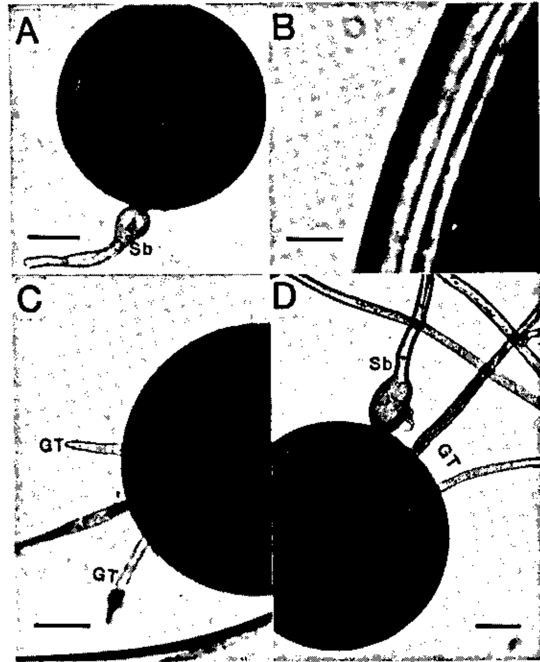

FIG. 1. Light photomicrograph of ungerminated and germinated azygospores of *Gigaspora margarita* stained with trypan blue.
A - Ungerminated azygospore (Sb - subtending hyphae) bar = 40 µm. B - view of spore wall layers, bar = 15 µm. C - germinated azygospore with two stunted germ tube (GT) bar = 50 µ. D - germinated azygospore showing two growing germ tubes (GT) and subtending hyphae (Sb). bar = 50 µm.

Soil extract has been found to improve germ tube growth of VAM fungal spores (Mosse 1959, Siqueira 1983). Because of the chemical complexity of soil extracts, no speculation has been made to explain such an effect. In the present experiment, soil extract fractions eluted around 40 ml showed very high activity on the enhancement of germ tube growth of *G. mosseae* spores, and this activity followed the extract protein content to some extent (Fig. 4). None of the fractions showed detectable sugar by the anthrone method. Data from this experiment suggest that protein is the enhancing factor in soil extracts and it may be related to the limited saprophytic ability of these fungi in

soil (Warner & Mosse 1980, Siqueira 1983). This view can be supported by recent findings that aminoacids can improve germ tube growth by pre-germinated spores of VAM fungi in the absence of their host (Hepper & Jakobsen 1983).

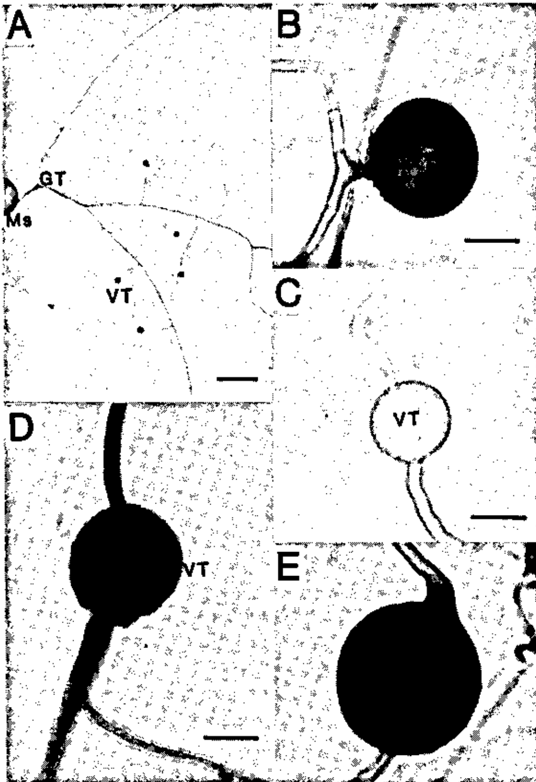

FIG. 2. Light Photomicrograph of germinated *G. mosseae* chlamydospores stained with trypan blue. A - germinated spores; note germ tube (GT) produced by the regrowth of the subtending hyphae. Note branching pattern and "vegetative spores" (VT), bar = 150 μm. B - "vegetative spore" as a continuation of the hyphae wall, bar = 5 μm. C - regrowth of very thin walled vegetative spore (VT), bar = 5 μm (unstained). D - regrowth of VT from a mother spore germinated on membrane filter in soybean rhizosphere. Note branching on the regrowth, bar = 5 μm. E - early stages of development of chlamydospore recovered from root associated mycelium, bar = 5 μm.

Spores of *G. mosseae* and *G. margarita* germinated readily when incubated on a suitable medium. Although germination and germ tube growth rates can be improved by addition of nutritional factors, the spores of VAM fungi appear to carry biological information necessary for germination and early germ tube growth in the absence of living root cells. The inability of these fungi to grown axenically for extended periods apart from the living cells is apparently associated with a metabolic deficiency exhibited during their filamentous phase, rather than by the spores themselves, which require no additional nutritional factor for germination *in vitro*, as is the case with many other obligate biotrophic fungi.

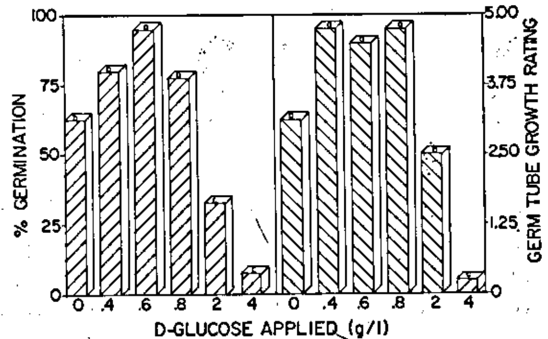

FIG. 3. Effect of D-glucose concentrations on germination and germ tube growth of *Glomus mosseae* chlamydospores on sand plates. Mean for 45 spores incubated for 12 days. Columns followed by the same letter are not different by Duncan's test at 0.05 level.

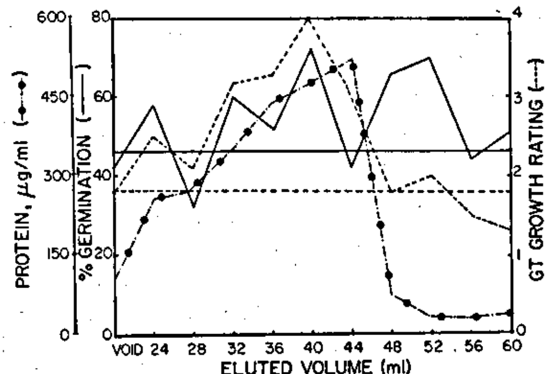

FIG. 4. Protein content and activity of different fractions of soils extracts on germination and growth of *Glomus mosseae* chlamydospores. Mean for 45 chlamydospores incubated for 10 days. The horizontal lines represent control treatments.

CONCLUSIONS

1. With the exception of D-galacturonic acid incorporated into agar medium at 1 g l^{-1} and glucose aqueous solution at 0.6 g l^{-1} applied to sand plates, most organic substrates tested were inhibitory or had no effect on spore germination *in vitro*.

2. Germ tube growth was improved by sucrose at 4 g l^{-1} on agar medium, 1% soil extract-agar and glucose aqueous solution at 0.4 to 0.8 g l^{-1} applied to sand plates.

3. Proteins appear to be the factor responsible for the enhanced germ tube growth promoted by soil extracts.

4. Emerged germ tubes can grow extensively, but sub-culture and sporulation apart from living roots have not been achieved.

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