

Research Paper

Optimization of the production of mycorrhizal inoculum on substrate with organic fertilizer

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Abstract

The system for production of inoculum of arbuscular mycorrhizal fungi (AMF) using sand and vermiculite irrigated with nutrient solution is promising. However, organic amendments added to the substrate can stimulate sporulation of AMF and replace the nutrient solution. The aim of this study was to maximize the production of AMF (*Acaulospora longula*, *Claroideoglossum etunicatum*, *Dentiscutata heterogama* and *Gigaspora albida*) using selected organic substrates (vermicompost, coir dust and Tropstrato) together with sand and vermiculite. The production of spores varied among the tested AMF and according to the organic source added to the substrate. The vermicompost promoted higher sporulation of *A. longula* in relation to the other AMF and substrates. The Tropstrato[®] inhibited the sporulation of *D. heterogama* while the reproduction of *C. etunicatum* was not affected by the organic compounds. The inoculum of *A. longula* also showed a high number of infective propagules and promoted biomass accumulation in maize plants. The system of inoculum production using sand and vermiculite + 10% vermicompost favors the production of infective inoculum of *A. longula* with the fungus benefiting growth of corn plants.

Key words: AMF, biofertilizer, vermicomposto, inoculum.

Introduction

Many studies have described the importance of arbuscular mycorrhizal fungi (AMF) for agriculture as well as for reforestation programs of degraded areas (Caravaca *et al.*, 2002; Douds Jr *et al.*, 2007; Ijdo *et al.*, 2011; Jarstfer and Sylvia, 1992; Souza *et al.*, 2010). Brazil offers a great potential for use of this biotechnological tool (Souza *et al.*, 2006); however, one of the obstacles for the application of AMF and the large-scale production of mycorrhizal inoculum is the obligate biotrophy of these fungi.

Various methods have been tested for the production of mycorrhizal inoculum, such as: aeroponics, hydroponics, *in vitro* cultivation, “pot cultures” and on-farm (Ijdo *et al.*, 2011). Traditionally these fungi are multiplied in “pot cultures”, in association with roots of a host plant in a determined substrate (Gaur and Varma, 2007).

The chosen substrate for the cultivation of AMF can directly influence the production and the infectivity of the inoculum (Baby and Manibhushanrao, 1996; Jayaratne and Siriwardene, 2011), and should contain minimal nutrients to guarantee the survival of the host plant so that the fungus can sporulate and multiply (Jarstfer and Sylvia 1992; Silva *et al.*, 2005).

A promising system is the use of sand and vermiculite, which favors the production of glomerospores with increased infectivity (Silva *et al.*, 2005; Silva *et al.*, 2007). However, the addition of nutrient solution to the substrate is a barrier to the system because it is not simple and economical (Gianinazzi and Vosátka, 2004). The use of organic sources and the efficiency of the produced isolates are important aspects to define the quality of the inoculums (Singh *et al.*, 2011). The infectivity can be estimated by observing fungal structures in the root with various tech-

niques that will indicate the viability of the inoculum (Feldmann and Idczak, 1994; INVAM, 2010). However, the efficiency of the isolates is not always related to the degree of mycorrhizal colonization (Corkidi *et al.*, 2004), making it necessary to test the effectiveness of the inoculum as well.

Large-scale production of infective and efficient AMF inoculum using low-cost and easily accessible materials continues being a necessity to make viable the application of these fungi in agriculture and for environmental recovering programs. The objective of this study was to select organic substrates that maximize the production of mycorrhizal inoculum of high quality.

Materials and Methods

Three experiments were carried out: in the first, an organic substrate to produce AMF inoculum was selected; in the second, the infectivity of the inoculum that in the previous experiment produced more spores was determined; in the third, the efficiency of this inoculum to increase the growth of corn plants was evaluated. All the experiments were carried out in a greenhouse at the University of Pernambuco, PE. The initial inocula were produced in organic substrates (topsoil and organic compost), using folder millet (*Panicum miliaceum* L) as the host plant and stored at 4 °C until being used (Silva, 2006).

Experiment 1 - Selection of organic substrates for the production of mycorrhizal inoculum

Four substrates to produce AMF inoculum were tested together with sand and vermiculite (1:1 v/v): (a) nutritive solution = control; (b) 10% of vermicompost; (c) 10%

of coir dust, (d) 10% of Tropstrato[®], a commercial substrate compound of vermiculite, vegetable coal and pinus chip. The sand, from river, was washed and, just as the vermiculite, sterilized in autoclave (120 °C, 30 min) for two consecutive days. After preparing the mixtures of substrate, samples from each one of them were taken for chemical and physical analysis at Embrapa Semi-Árido (Table 1). For the experiments the substrates were placed in pots of 400 mL capacity. The organic substrates were commercially acquired and are widely used in agriculture.

Folder millet was used as host. The seeds were disinfected (0.5% sodium hypochlorite for 3 min) and washed with distilled water.

Four AMF isolates were tested: *Acaulospora longula* Spain & Schenck (UFPE 21), *Claroideoglosum etunicatum* (W.N. Becker & Gerd.) C. Walker & A. Schüßler (UFPE 06), *Dentiscutata heterogama* (T.H. Nicolson & Gerd.) Sieverd., F.A. Souza & Oehl (UFPE 19) and *Gigaspora albida* Schenck & Smith (UFPE 01).

For each pot suspensions containing 100 spores of each AMF isolate were deposited under 50 folder millet seeds and the pots were kept at the greenhouse (28 °C ± 2 °C) for 60 days. All pots were irrigated every two days; the control treatment received a nutrient solution, modified by Jarstfer and Sylvia (1992) and supplemented with Tris-HCl (Silva *et al.*, 2005) while the other treatments were irrigated with distilled water.

For each AMF isolate the experimental design was completely randomized with four substrate treatments and five replicates (80 experimental units).

The AMF spores were extracted from the substrates by wet sieving (Gerdemann and Nicolson, 1963) and water

Table 1 - Physical and chemical characterization of the substrates used to produce AMF inoculum

Characteristics	Substrates (base: sand + vermiculite)			
	Base	+ 10% of vermicompost	+ 10% of coir dust	+ 10% of Tropstrato [®]
OM (g/kg)	0.72	3.10	5.59	7.45
P (mg/dm ³)	17.85	61.50	30.45	50.78
pH (H ₂ O - 1:2.5)	6.30	6.80	6.90	6.20
CEC (cmol/dm ³)	6.41	7.39	6.37	6.71
K (cmol/dm ³)	0.17	0.36	0.38	0.23
Fe (cmol/dm ³)	46.30	31.80	44.30	49.60
Zn (cmol/dm ³)	1.00	3.80	1.30	1.60
Na (cmol/dm ³)	0.25	0.44	0.23	0.33
Total sand (g/kg)	957.82	942.76	963.20	950.80
Silt (g/kg)	22.27	54.84	35.31	42.65
Clay (g/kg)	19.91	2.41	1.49	6.55
Porosity (%)	41.02	40.47	41.94	39.92
Apparent density (km/dm ³)	1.51	1.53	1.44	1.49
Real density (km/dm ³)	2.56	2.57	2.48	2.48

OM = organic matter; CEC= cation-exchange capacity.

and sucrose centrifugation (Jenkins 1964), and quantified in a stereomicroscope (40x).

The number of spores was transformed to $\log x + 1$, submitted to ANOVA and the means compared by the Tukey test (5%), using the Statistica program (Statsoft, 1997).

Experiment 2 - Infectivity of the mycorrhizal inoculum produced in substrates with organic fertilizer

Samples of the inoculum of *A. longula*, *C. etunicatum*, *D. heterogama* and *G. albida* multiplied in substrate with 10% vermicompost were used immediately after being produced. This inoculum was selected in the previous experiment and produced more spores than the other treatments. To evaluate the infectivity of this inoculum, two methods were used:

A) NMP = samples of the inoculum consisting of spores, colonized hyphae and roots were diluted (1:10; 1:100; 1:1000 v/v) with autoclaved sand (121 °C, 1 h) using corn seeds disinfected (*Zea mays* L. cv. Assum Preto) as host plant. After 30 days in the greenhouse, the plants were harvested and the roots washed, clarified with 10% KOH, stained with Trypan blue (0.05%) (Phillips and Hayman, 1970), and the presence of mycorrhizal structures observed to estimate the most probable number (MPN) of infective propagules using the Cochran's table (Feldmann and Idczak, 1994).

B) MIP = The mean percentage of infection (INVAM, 2010) of the inoculum was evaluated in roots cultivated in the dilution 1:10 (inoculum:disinfested sand) (experiment 2A) with the colonization determined by the gridline intersect method (Giovannetti and Mosse, 1980).

Experiment 3 - Effectiveness of AMF multiplied in substrates with organic fertilizer

The soil used in this trial was collected in an area of native Caatinga close to km 152, in the municipality of Petrolina-PE, and taken for chemical analysis at the Embrapa Semi-Arido, presenting the following characteristics: 3 mg dm⁻³ of P; 2.48 g kg⁻¹ of organic matter; 9.62 cmol dm⁻³ of cation-exchange capacity and pH 4.9. Pots with a 2 L capacity were used for the experiment.

Corn seeds were disinfected with 0.5% sodium hypochlorite for three minutes and washed with distilled water. Inocula (10 mL) of *G. albida*, *A. longula*, *C. etunicatum* e *D. heterogama*, multiplied in sand and vermiculite + 10% vermicompost, obtained from experiment 1 were separately placed below four corn seeds in pots of 2 L capacity. After germination, thinning was carried out, leaving only one plant per pot. The pots were kept in the greenhouse and irrigated every other day with filtered water.

Seventy days after inoculation fresh and dry matter of the aerial part and the total fresh and dry matter of the plants were evaluated. The dry matter was determined after leav-

ing the plants in an oven (40 °C) until constant weight. The growth increase was calculated based on the total dry biomass (Weber *et al.*, 2004).

The biomass data were submitted to ANOVA and the means compared by the Tukey test (5%), using the Statistica program (Statsoft, 1997).

Results and Discussion

In the first experiment the organic substrates affected AMF spores production ($p < 0.05$). The addition of these substrates favored sporulation, but the benefits depended on the organic source added to the basic substrate (Table 2).

The vermicompost added to sand + vermiculite promoted higher sporulation of *A. longula* in relation to the other AMF and substrates. The Tropstrato[®] inhibited the sporulation of *D. heterogama* while the reproduction of *C. etunicatum* was not affected by the organic compounds. These results confirm that some species benefit more than others from organic fertilization (Silva 2006). Oehl *et al.* (2004) reported a high abundance of Acaulosporaceae species and a low frequency of occurrence of *Gigaspora* and *Scutellospora* species in areas with organic fertilization. The sporulation of the isolate of *C. etunicatum* did not differ in terms of substrate; this was probably due to the functional plasticity of the species (Weissenhorn *et al.*, 1994).

Organic sources applied to the soil can increase production of AMF (Douds Jr *et al.*, 2006; Gaur and Adholeya, 2005) and the addition of these residues to the medium used to produce inoculum increases sporulation (Silva *et al.*, 2005), as registered in this study (Table 2). Baby and Manibhushanrao (1996) tested 12 organic sources and reported that the type of organic source influences the production of spores, because some substances present in the organic compost can have a phytotoxic effect and/or inhibit the development of AMF (Martín *et al.*, 2002).

The nutrient solution used in the basic substrate can be substituted by organic sources, and depending on the AMF isolate and the type of organic source used, the production of spores can increase in relation to the use of the solution. Sporulation of *A. longula* increased 2420% when

Table 2 - Production of AMF spores (GA- *Gigaspora albida*; CE- *Claroideoglomus etunicatum*; AL- *Acaulospora longula*; DH- *Dentiscutata heterogama*) in substrates with a sand and vermiculite basis to which organic substrates were added, using proso millet as host, 60 days after inoculation under protective cropping.

Substrates	AMF (spores/20 mL)			
	GA	CE	AL	DH
Control	9.4ab	6.4a	7.8bc	8.2a
Tropstrato [®]	4.2b	4.2a	2.8c	2.2b
Coir dust	4.6b	3.0a	9.4b	5.2ab
Vermicompost	45.4a	8.8a	196.6a	6.8a

Means followed by the same letter do not differ by the Tukey test (5%).

the isolate was maintained in the substrate with vermicompost in relation to the control. This increase is probably due to the soil cation-exchange capacity and to the nutrients present in the vermicompost (Table 1). The soil properties benefit from the use of vermicompost (Cavender *et al.*, 2003), favoring the availability of nutrients in relation to other organic sources (Samaranayake and Wijekoon, 2010). These data are in accordance with those obtained by Silva (2006) when using organic sources to produce mycorrhizal inoculum.

The greatest spore production was observed in substrates with 10% vermicompost. Possibly, the value of P affected the outcome since this nutrient plays an important role in the regulation of production of AMF propagules (Posada *et al.*, 2008). However, Douds Jr (1994) observed that the addition of P to the solution used to irrigate diminished the production of spores. It is likely that the isolates multiplied in the present study were more adapted to the conditions of high fertilization (especially P) once they were produced in organic substrate with high P-levels (Silva, 2006).

The negative effect of Tropstrato[®] on sporulation of *D. heterogama* was possibly due to the chemical composition of the substrate, with high levels of Mn, Ca and Fe (Table 1). High concentrations of Mn and Fe in the substrate can inhibit the germination of spores and mycelial growth (Moreira and Siqueira 2002). Cardoso *et al.* (2002) observed that an isolate of *D. heterogama* was more tolerant than others in relation to the Mn doses tested. The Ca can interfere in the permeability of the membranes and consequently in the colonization of the roots (Moreira and Siqueira, 2002).

Besides the composition, the granulometry of the medium can influence the production of propagules; however, the substrates used in this study did not show a significant granulometric difference (Table 2). Gaur and Adholeya (2000) observed that there was more sporulation of AMF in soil with 0.78 - 0.50 mm particles and a greater weight of the roots in relation to soils with other particle sizes. However, a mixture of materials with particles of different sizes also stimulated sporulation in relation to other mixtures (Verma *et al.*, 2008). The use of organic substrates is advantageous because it reduces the apparent density and increases the porosity (Caravaca *et al.*, 2002). The decrease in the density of the material is also pointed out as one of the objectives to produce low cost inoculum for commercial purposes (Jayaratne and Siriwardene, 2000; Silva, 2006). In the present study, the addition of organic sources did not cause any significant differences in the density or porosity; thus, in this case spore production was not determined by the physical characteristics of the substrates.

Considering the tested isolates, the high percentage of colonization and the low number of infective propagules of *G. albida* can be the result of the biology of the fungus. Spores of *Gigaspora* species may produce many germ

tubes, establishing in this way various possibilities for colonization of the host (Maia *et al.*, 1993). In general, members of the Gigasporaceae family produce few spores (Souza *et al.*, 2005) and are incapable of colonizing from hyphae fragments (Klironomos and Hart, 2002), as happens with *Acaulospora* (Hart and Reader, 2002). However, it has been shown that *G. albida* can have a greater infective capability than *A. longula* (Silva *et al.*, 2005).

The inoculum of *A. longula* produced a high number of infective propagules, although spores of this species are known to have a long dormancy period (Tommerup, 1983); it is possible that the hyphae play a more important role than the spores in the colonization process (Silva *et al.*, 2005). The colonization produced by the AMF was not related with the number of infective propagules (Table 3), as also mentioned by Sreenivasa and Bagyaraj (1988).

Some commercialized inocula show 19 to 54% of colonization rate, as evaluated by the MIP (INVAM 2011). Considering the colonization produced by the tested inocula, which was within the suggested range for commercial inoculum they can therefore be recommended for use as biofertilizers.

With the exception of *G. albida* (\pm) the produced inocula incremented the production of biomass of the corn plants. The capacity of the introduced AMF being more efficient can be due to the low activity of native AMF (Douds Jr *et al.*, 2007), as observed in the control treatment. Another factor that may have contributed to the results of this study was the interaction of the fungal isolate with the plant species (Hart and Reader, 2002; Pouyu-Rojas *et al.*, 2006). Growth of corn plants was not increased by inoculation with *G. albida*; it is possible that this isolate, under the experimental conditions, consumed more Carbon from the host than that available just for the fungus (Klironomos *et al.*, 2000).

The inoculum of *A. longula* was effective in stimulating biomass accumulation in corn plants (Table 4). In other situations, inoculation with isolates of *A. longula* was beneficial to seedlings of soursop (*Annona muricata*) (Silva *et al.*, 2008), leucaena (*Leucaena leucocephala*) (Lins *et al.*, 2007) and native plants used for revegetation (Souza *et al.*, 2010).

Table 3 - Number of infective propagules in the inocula produced in substrates with a basis of sand and vermiculite to which 10% of vermicompost was added and mycorrhizal colonization in corn roots, 30 days after inoculation.

Isolates	N° of propagules (cm ⁻³ substrate)	Mycorrhizal colonization (%)
<i>Acaulospora longula</i>	> 1600	23.2
<i>Gigaspora albida</i>	180	56.9
<i>Claroideoglossum etunicatum</i>	350	31.8
<i>Denticutata heterogama</i>	32	19.8

Table 4 - Fresh and dry matter of corn plants (*Zea mays*) cultivated in native Caatinga soil, inoculated with 10 mL of soil-inoculum, and maintained in greenhouse for 70 days.

Isolates	Fresh matter (g)	Dry matter (g)	Increment (%)
Control	4.47b	0.70bc	-
<i>Acaulospora longula</i>	8.62a	2.44ab	92.8
<i>Gigaspora albida</i>	3.52b	1.22c	-21.2
<i>Claroideoglossum etunicatum</i>	8.90a	2.67a	99.1
<i>Dentiscutata heterogama</i>	7.31ab	1.94abc	63.5

Means followed by the same letter, in the column, do not differ by the Tukey test (5%).

Inoculum produced in substrates with organic fertilizer can benefit the host in a differentiated way. Douds *et al.* (2008) observed that the inoculum produced with organic sources promoted better growth of potato seedlings in relation to uninoculated plants maintained in native soil. Conversely, Silva *et al.* (2008) observed that inoculum of *A. longula* produced in soil promoted more growth of sorghum than inoculum produced in soil + 10% of vermicompost; however, the same was not observed for *G. albida*.

A system for inoculum production has quality when, besides stimulating sporulation, it provides high infectivity and efficiency (Gianinazzi and Vosátka, 2004). Thus, from the tested isolates, only *A. longula* presented such characteristics, when multiplied in substrate with vermicompost. It is important to emphasize that the sporulation of this fungus was lower than that registered in other studies (Silva *et al.*, 2005; Silva *et al.*, 2007; Zambolim *et al.*, 1992) but the fungus was infective and effective in favoring growth of corn.

The results suggest that the production of inoculum using sand and vermiculite + 10% vermicompost favours the reproduction of *A. longula* with the fungus being beneficial to the host.

Conclusions

In the system of inoculum production using sand and vermiculite, the substitution of nutrient solution by organic fertilizers can improve sporulation, but the benefits vary according to the fungus and the organic source.

The substrate on the basis of sand and vermiculite + 10% of vermicompost favors the production of inoculum of *C. etunicatum* infective and effective in increasing the biomass of corn.

Reproduction of *A. longula* can be improved in sand and vermiculite + 10% of vermicompost and the inoculum is infective and effective immediately after being produced.

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