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Effects of the particle size of soil-less substrates upon AM fungus inoculum production

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Abstract Production of inoculum of the arbuscular mycorrhizal fungus *Glomus intraradices* was examined in a locally available sand graded by particle size, planted with *Zea mays* and fertilized with a nutrient solution. Plants in sand with particle sizes of 0.50–0.78 mm had higher root fresh weights, spore production and percent mycorrhizal colonization than with other particle sizes. Production of spores and infectious propagules was enhanced by a nutrient solution without P. Plants were also inoculated with *G. intraradices* in pots containing clay-brick granules, charcoal, coalmarl, sand or perlite of the optimal particle size (0.50–0.78 mm). Percent root length colonized by *G. intraradices* and production of infectious propagules were 40–50 % higher for plants grown in clay-brick granules and sand than in the other media.

Key words Sand · Perlite · *Glomus intraradices* · Charcoal · Coalmarl

Introduction

The arbuscular mycorrhizal (AM) symbiosis plays a major role in plant mineral nutrition (Gianinazzi-Pearson and Gianinazzi 1983) and general plant health (Dehne 1982) and has high potential for agriculture (Menge 1983) and land reclamation (Sylvia 1990).

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The broad application of AM fungi has been limited by the difficulties of obtaining large quantities of pure inoculum from obligate symbionts. Mass production of AM inoculum has been technically feasible only since the introduction of the pot culture technique of Mosse and Gerdemann (Wood 1985), cultured with plant hosts on substrates such as sand, peat, expanded clay, perlite, vermiculite, soilrite (Mallesha et. al. 1992), rockwool (Heinzemann and Weritz 1990) and glass beads (Redecker et. al. 1995). In addition to temperature (Furlan and Fortin 1973), light (Ferguson and Menge 1982), pot size (Ferguson 1981) and soil fertility (Menge et al. 1978), the particle size of the growth substrate also affects inoculum production in pots. Optimal soil aeration is a prerequisite for AM fungus establishment and metabolic activity (Saif 1981); however, little information is available on the influence of particle size of soil-less media on AM fungus inoculum production. Mycorrhizae development in relation to soil texture has been investigated (Dakessian et al. 1986; Land and Schonbeck 1991).

Since nutrient availability, organic matter etc. vary with soil texture, we compared different particle sizes under uniform texture conditions using sterile sand as a growth substrate We studied the effect of particle size on AM fungus inoculum production using locally available river sand. Other substrates with different physical properties but with the optimal particle size determined in the first experiment were then compared for inoculum production.

Materials and methods

Preparation of inocula

Glomus intraradices Schenck & Smith (DAOM 181602, Biosystemics Research Centre, Ottawa, Canada), the AM inoculum, was multiplied for 1 year in 7-kg capacity, earthenware pots filled with soil. The physical and chemical characteristics of the soil were as follows: sandy loam, pH = 8.2, available

P = 0.53 mg/kg (Olsen et. al. 1954), NO_3N = 124 mg/kg and available K = 124 mg/kg (Jackson 1967), with *Sorghum bicolor* var. sudanense (sorghum) as the host plant. At harvest, the tops were removed and soil in the pots was allowed to dry for 1 week at 25 °C. The roots were then finely chopped and the dried root/soil mixture was thoroughly mixed to obtain an homogenous inoculum. Spores were isolated by wet sieving and decanting (Gerdemann and Nicolson 1963) and counted on filter paper (Gaur and Adholeya 1994). Percent root colonization in the inocula was assessed as described by Biermann and Linderman (1981).

Preparation of texture grades

Experiment I

Sand was collected from the Sind River of West Bundelkhand region (Uttar Pradesh, Northern India), autoclaved (121 °C, 1.2 kg/cm^2) for 3 h and then graded into four texture grades using a nest of sieves (British Standard Size, BSS): 10 (1.70 mm), 20 (0.78 mm), 30 (0.50 mm) and 60 (0.25 mm). The following particle size classes were produced: grade A (retained on 20 BSS sieve) 1.70–0.78 mm; grade B (retained on 30 BSS sieve) 0.78–0.50 mm; grade C (retained on 60 BSS sieve) 0.50–0.25 mm; grade D (particles passing through 60 BSS sieve) smaller than 0.25 mm.

Experiment II

Five different substrates, perlite, river sand, charcoal, coal marl, and clay-brick granules were tested in this experiment. The sterile substrates were crushed into small pieces and sieved successively through 20 BSS and 30 BSS sieves. The particles retained on the 30 BSS sieve (0.50–0.78 mm) were autoclaved as above and used as the growth substrate.

Seed sterilization

Seeds of *Zea mays* L. (maize) were graded by weight (0.25–0.30 g) and surface sterilized with 10 % H_2O_2 for 5 min. Subsequently, the seeds were washed repeatedly with sterile water and kept for germination on moist cotton layers in sterile Petri plates at 30 °C in the dark for 48 h.

Experimental design

Portions of sand (450 ml) of each particle size (experiment I) were transferred to plastic pots (5 × 9 cm). There were four particle size treatments each replicated 15 times. In experiment II, five different substrates were replicated four times using the same pot size as in experiment 1.

Inoculation was carried out by mixing 50 ml of freshly produced inoculum of *G. intraradices,* consisting of soil, extraradical spores, hyphae and infected sorghum root pieces in each pot. Five seedlings of maize were selected for uniformity at 3 days and transplanted to pots. The pots were arranged on a greenhouse bench in a completely randomized design and maintained at 30 ± 5 °C with 65 % relative humidity. Plants were watered every other day with 50-ml aliquots of distilled water. All pots were also given half-strength Hoagland solution (Hoagland and Arnon 1938) once a week for the first 12 weeks (in both experiments). Thereafter, one set of replicate pots for each treatment was provided with Hoagland solution without P up to 17 weeks (experiment 1 only).

Harvest and analysis

Experiment I

Five plants from each treatment were harvested at 12 and 17 weeks. At each harvest, shoots were cut just above the crown and roots were washed free of substrate on a mesh (300 BSS). The roots were then cleared and stained (Phillips and Hayman 1970) and examined for mycorrhizal colonization (Biermann and Linderman 1981). The substrate from each pot was allowed to dry in a greenhouse for 1 week at 25 °C, homogenized and quantified for spore density (Gaur and Adholeya 1994) and number of infectious propagules (IP) (Gaur et al. 1998). Both spore density and IP were expressed per 100 ml substrate.

Experiment II

Plants were harvested after 12 weeks growth. The shoots were severed just above the crown, weighed fresh, rinsed in distilled water, dried at 70 °C for 48 h, weighed, ground to pass a 40-mesh (0.5 mm) screen and digested in H_2SO_2/H_2O_2. The P and N content of the digest was determined using the methods of Kitson and Mellon (1944) and Kjeldahl, respectively.

Roots were washed free of sand, cut into 1-cm segments and mixed thoroughly. Their total fresh weight was determined after drying thoroughly between soft filter papers. A subsample of root segments was taken for analysis of mycorrhizal colonization. Percent AM colonization was determined on 100 1-cm root segments per sample. Roots were stained using the method of Phillips and Hayman (1970). Root pieces were examined at a magnification of × 100 with a compound microscope for AM fungus hyphae, arbuscules, vesicles and spores, according to Biermann and Linderman (1981). The mean percent colonization from each replicate pot was applied in subsequent analysis. Spore density and infectivity were determined as described in experiment I.

Statistical analyses

Results were subjected to one-way analyses of variance (ANOVA) using a completely randomized design. The differences between the treatments were examined by Duncan's multiple range test using Costat Statistical software (Cohort, Berkeley, Calif.) at a significance level of 95 %.

Results

Experiment I

The percent root length colonized by *G. intraradices*, spores density and IP varied with substrate particle size. The highest root colonization at 12 weeks was found in substrates with grade B and C particle sizes, followed by grades A and D (Table 1). Similar results were obtained for IP and root fresh weight at 12 weeks with maximums recorded in grade B (Figs. 1, 3). Spore density at 12 weeks was at a maximum with grade C particles and a minimum with grade A (Fig. 2). In contrast, spore density at 17 weeks was highest in substrate B. IP was significantly (P<0.005) correlated with percent colonization and fresh root weight (r = 0.75 and 0.80, respectively), but not with spore count (r = 0.30). The correlation between percent colonization and root fresh weight was highly significant (r = 0.98) (Table 2).

Table 1 Percentage root length of maize grown in sand of four different particle sizes colonized by *Glomus intraradices*. *LSD* Least significant difference. Values are means of five replicates ± standard deviation. Mean values at 12 weeks or 17 weeks followed by same letter are not significantly different ($P \leq 0.05$). *A–D* represent particle sizes of 1.70–0.78 mm; 0.78–0.50 mm, 0.50–0.25 mm, and smaller than 0.25 mm, respectively

Sand particle size	Time (weeks)		
	12	17	
		+P	−P
A	34.0 ± 1.1b	35.3 ± 1.5ab	37.5 ± 1.6a
B	49.1 ± 1.8a	35.4 ± 1.6ab	37.4 ± 0.9a
C	50.0 ± 1.42a	35.4 ± 1.9ab	36.1 ± 1.5ab
D	31.1 ± 2.00c	34.4 ± 1.5b	36.6 ± 1.8ab
LSD	2.2	2.0	

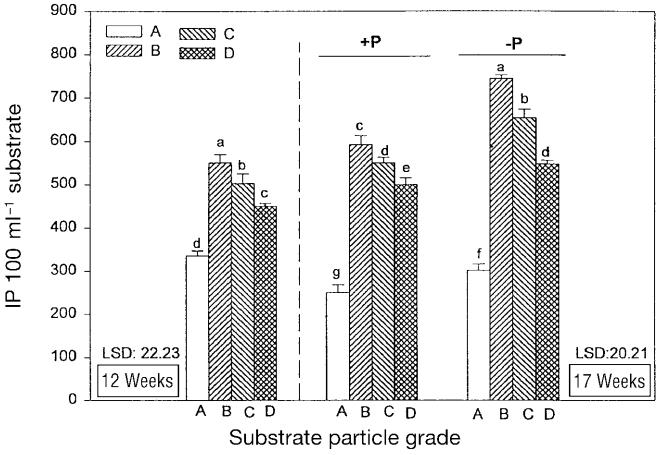

Fig. 1 Number of infectious propagules (*IP*) of *Glomus intraradices* produced in pot cultures with maize in sand of four different particle sizes. Columns with different letters within a sample time are significantly different ($P \leq 0.05$). *Bars* denote standard deviations of the means of five observations. *A*, *B*, *C* and *D* represent particle sizes 1.70–0.78 mm, 0.78–0.50 mm, 0.50–0.25 mm, and smaller than 0.25 mm, respectively

Spore density and IP increased markedly from 12 weeks until harvest at 17 weeks in substrates B and C, even though percent colonization was not significantly different between treatments. Maximal spore counts and IP at 17 weeks were again recorded in sand with particles of grade B and the minimum with grade A (Figs. 1, 2). The effects on spore density and IP were greatest for each particle size when no P was applied in the nutrient solution. Mycorrhizal colonization was similar in all pots and unaffected by reduced P fertilization (Table 1). At 17 weeks, IP was significantly correlated to spore density ($r = 0.83$) but not to percent colonization ($r = 0.09$) (Table 2).

Experiment II

The highest AM fungus inoculum production occurred in sand and clay-brick granules (Fig. 4). Overall AM colonization ranged from 29.35 % (in perlite) to 71.28 % (in clay-brick granules) (Table 3). Sand was the best for spore production, followed by charcoal, clay-brick granules, coal marl and perlite.

Mycorrhizal plants grown in clay-brick granules and sand produced the highest dry shoot weight. Root growth was higher in clay-brick granules than in sand, charcoal, coal marl and perlite. Root/shoot ratios were similar for plants grown in clay-brick granules, charcoal and coal marl but were lower in sand and perlite (Table 3).

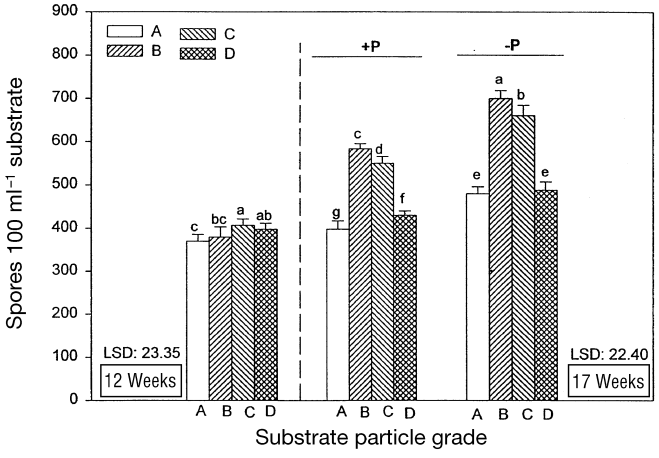

Fig. 2 Density of spores produced in pot cultures with maize inoculated with *G. intraradices* in sand of four different particle sizes. Columns with different letters within a sample time are significantly different ($P \leq 0.05$). *Bars* denote standard deviations of the means of five observations. Abbreviations as in Fig. 1)

Total P uptake by plants was significantly higher in clay-brick granules and sand than in charcoal, perlite or coal marl (Table 3). Significant interrelationships

Table 2 Correlation coefficients of the relationships between plant and fungal parameters in the experiment with sand of different particle sizes. Root fresh weight and IP are dependent variables (*y*). *IP* Infectious propagules

Time (months)	Parameters		Regression parameter (*y*)	Correlation coefficient
3	% Colonization	Root fresh wt.	3.7 + 0.88x	0.9767***
	% Colonization	IP	1.76 + 0.07x	0.7485***
	Spore density	IP	0.29 + 1.10x	0.2934 ns
	Fresh root wt.	IP	1.35 + 0.08x	0.7958***
4	Spore density	IP	1.70 + 1.28x	0.8295***
	% Colonization	IP	2.38 + 0.08x	0.0847 ns

Table 3 Influence of different types of substrates on plant parameters and mycorrhizal colonization. Within columns, numbers followed by the same letters are not significantly different ($P = 0.05$) by Duncan's multiple range test. Values represent means of four replicates

Treatments	Root dry wt. (g)	Shoot dry wt. (g)	R/S ratio	% Mycorrhizal colonization	Shoot minerals	
					P (mg/g)	N (%)
Perlite	1.41e	3.78d	0.36c	29.35e	1.3d	2.23e
Coal Marl	1.85d	3.62e	0.51a	41.0d	0.74e	2.52d
Sand	2.65b	6.01b	0.45b	70.05a	1.7a	3.4c
Charcoal	2.01c	4.03c	0.51a	55.43b	1.6b	3.9b
Clay-brick granules	5.05a	6.56a	0.51a	71.28a	1.7a	4.75a
LSD	0.022	0.057	0.014	1.754	0.032	0.159

between fungal and plant variables were observed. Percent AM colonization was positively correlated with shoot P and shoot weight ($r = 0.71$ and 0.84, respectively). The correlation was more significant between IP and percent AM colonization ($r = 0.70$) than spore count ($r = 0.50$) (Table 4).

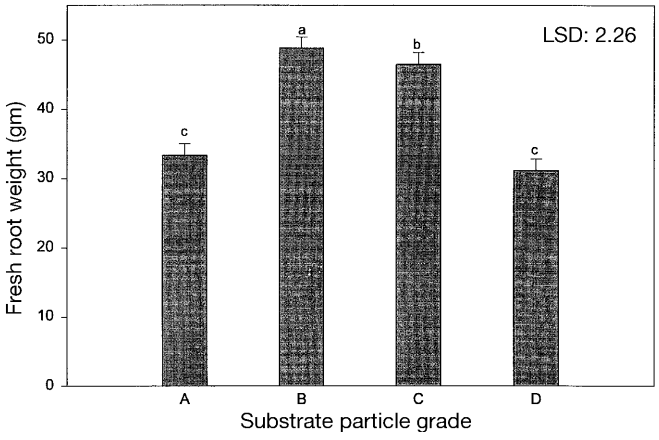

Fig. 3 Root fresh weights of mycorrhizal maize plants grown for 12 weeks in sand of four different particle sizes. Columns with different are significantly different ($P \leq 0.05$). *Bars* denote standard deviations of the means. Abbreviations as in Fig. 1

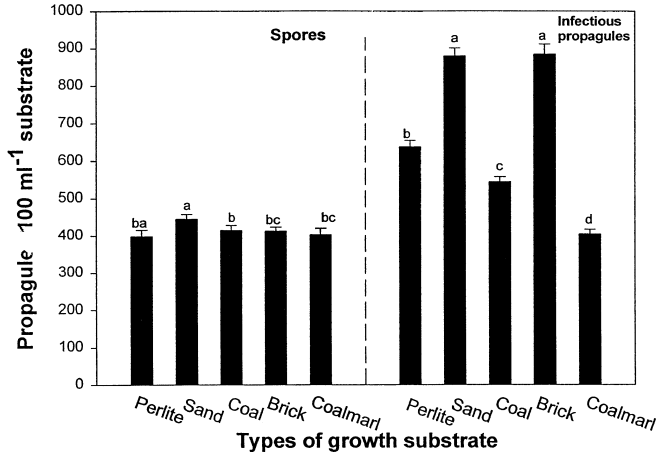

Fig. 4 Number of AM propagules produced after 12 weeks in pot cultures with maize as host plant grown in different substrates with the same particle size. Different letters on the top of bars denote significantly different values ($P \leq 0.05$). *Bars* denote standard deviations of the means of five observations

Table 4 Relationships between plant and fungal parameters in the experiment involving different types of substrates. The first and the second column represent x (independent) and y (dependent) variables, respectively. *IP* Infectious propagules

Parameters		Regression parameter (y)	Correlation coefficient
Spore density	IP	$-10.93 + 4.23x$	0.5045^{*}
% Colonization	IP	$1.96 + 0.09x$	0.7016^{***}
% Colonization	Shoot P	$0.59 + 15.24x$	0.7080^{***}
% Colonization	Shoot fresh wt.	$0.99 + 0.07x$	0.8355^{***}

Discussion

Mycorrhizal fungus inoculum production was highest in sand particles of grades B and C. Enhanced production in these substrates may be related to better soil aeration, drainage, oxygen supply and root growth. (Saif 1981). The largest substrate particle size produced few IP. This may be attributable to more frequent drought conditions due to the lower water retention capacity of this grade. Redhead (1975) reported that the drastic effects of extreme fluctuation in soil moisture on external hyphae led to lower spore production. Loss of nutrient medium due to excessive leaching from this substrate would also result in decreased root biomass, thus affecting AM fungus development. Water levels allowing for the greatest plant growth would, therefore, result in the highest amount of photosynthate available for fungal growth and development. Thus, optimal water availability for plant growth would also result in the highest spore production.

Application of Hoagland solution without P enhanced both spore production and infectious AM fungal propagules, irrespective of grade size. Douds and Schenck (1990) also demonstrated that low levels of P help maintain high levels of infectious AM fungus populations. High concentrations of other nutrients increase the amount of photosynthate available for sporulation. In our study, nutrient medium without P did not influence root colonization by *G. intraradices*, though lack of P has been reported to enhance colonization of roots (Hepper 1983; Thompson 1987). Our results may be attributable to high P in roots following the application of P in the nutrient medium during the first 12 weeks.

The infectivity of the AM fungal inoculum (IP) produced at 12 weeks was directly proportional to the percent mycorrhizal colonization and root biomass produced by host plants but not to sporulation of the mycorrhizal fungi. Thus, any given mass of mycorrhizal roots produced more infectious inocula at this harvest. It is quite possible that external hyphae growing out from the surface of mycorrhizal roots and colonized root pieces were much more effective than spores at initiating new mycorrhizal colonization. In contrast, IP at 17 weeks was correlated to spore density, which may be attributable to rapid increase in sporulation in the reproductive phase of the plant. A relatively constant spore density during the vegetative phase and a sharp increase at the reproductive stage have been reported for various crops (Pozzebon et. al. 1992; Saif and Khan 1975; Sutton and Barron 1972). In our experiment, flowering and subsequent yellowing was observed at 17 weeks and it is likely that senescence and dead roots stimulate the onset of sporulation at the end of the host growing season (Baby and Manibhushanrao 1996). Thus, in our experiments, IP rather than spore density or percent colonization is the best parameter for assessing rate of inoculum production.

The particle size optimum for inoculum production in sand in our conditions was grade B. This particle size was compared with four other substrates in a subsequent experiment. Of these substrates, the highest inoculum production was recorded in sand and clay-brick granules. Both substrates were devoid of organic carbon and retained optimal moisture for root growth and AM production. This also supports the concept that the ideal substrate for AM inoculum production will be low in nutrients and carbon (Sreenivasa and Bagyaraj 1988). The infectivity of this substrate-based inoculum was directly proportional to the mass of mycorrhizal roots produced by host plants. Percent colonization also correlated with root biomass. Thus, any given mass of mycorrhizal roots produced the most infectious inocula when sand or clay-brick granules were used as growth substrate. It is likely that the ability of the AM fungus to spread and form a hyphal network in the substrates was influenced by their different physical properties such as compaction, water retention etc. Thus, substrates of similar particle size under similar growth conditions and host plant resulted in differences in AM fungus inoculum and plant biomass production. Some of the substrates tested here offer desirable and stable conditions highly suitable for mass multiplication of AM fungus inoculum.

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