



Contents lists available at ScienceDirect

Soil Biology & Biochemistry

journal homepage: www.elsevier.com/locate/soilbio

Short communication

Aqueous biphasic system to extract arbuscular mycorrhizal spores from soils

M. Salvador-Figueroa^a, L. Adriano-Anaya^a, S. Tzusuki-Calderón^a, M.E. Gavito Pardo^b, J.A. Ocampo^{c,*}^aÁrea de Biotecnología, Facultad de Ciencias Químicas, Universidad Autónoma de Chiapas, Carretera a Puerto Madero Km. 2, Tapachula, Chiapas, Mexico^bCentro de Investigaciones en Ecosistemas, UNAM, Antigua Carretera a Patzcuaro No. 8701, Morelia, Michoacán, Mexico^cDepartamento de Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín, CSIC, Prof. Albareda 1, E-18008, Granada, Spain

ARTICLE INFO

Article history:

Received 17 January 2008

Received in revised form 26 May 2008

Accepted 30 May 2008

Available online 25 June 2008

Keywords:

Ammonium sulphate

Aqueous biphasic system

Arbuscular mycorrhizas

PEG

Spores extraction

ABSTRACT

The aqueous biphasic system (ABS) method using PEG and ammonium sulphate improved the recovery of spores from tropical soils as compared with the sucrose gradient method. The ABS was not affected by low temperatures. The optimal recovery of spores was achieved when 100% PEG and 50% ammonium sulphate concentration and PEG₈₀₀₀ instead of PEG₄₀₀₀ were used. Under these conditions an optimal interface formation which facilitates the localization and recovery of spores was obtained. Both phases of the ABS are aqueous and did not affect either the viability of spores or the level of root colonization reached by these spores. Soil composition did not affect the number of spores obtained by the ABS method either.

© 2008 Elsevier Ltd. All rights reserved.

Spores are the main propagules of the arbuscular mycorrhizal (AM) fungi. Despite the fundamental importance of extraction of spores from soils, there have been few reports of new methods for the isolation of these propagules. The method of wet sieving and decanting has been the most common to extract spores from soil (Gerdemann and Nicolson, 1963). However, from tropical soils with high clay and organic matter content the extraction of spores has been very difficult and only the sucrose gradient method developed by Jenkins (1964) seems to be effective (Sieverding, 1983; Brundett et al., 1999; Martin et al., 2001).

Aqueous biphasic system (ABS) has been widely used for protein, virus and cell recovery (Diamond and Hsu, 1990). In ABS, two separate phases are formed when polymers such as polyethylene glycol (PEG) are mixed with salts such as ammonium sulphate in particular concentrations. The equilibrium distribution (partitioning) of a substance in ABS depends not only on its own surface properties such as charge and hydrophobicity but also on the physicochemical properties of the two phases which can be manipulated by adjusting factors such as the polymer molecular weight and concentration, and salt concentration (Baskir et al., 1989).

To study the efficiency of ABS the method, spores were isolated from four soils located in the Ejido Raymundo Enriquez (ERE) field, Tapachula, Mexico. The texture, pH and organic matter were determined (Walkley and Black, 1934; Bouyoucos, 1951) (Table 1). Three replicate root-soil samples were collected from each soil. Each sample consisted of five bulked subsamples taken from the top 10 cm of soil. In order to calibrate the optimal ABS conditions, the sandy-loam soil cultivated with *Theobroma cacao* was used. AM spores were isolated from 100 g of each soil sample by the wet sieving and decanting technique (Gerdemann and Nicolson, 1963), counted in a Doncaster dish (Doncaster, 1962) and considered as 100% of spore recovery. The ABS was made by mixing the same quantities (V/V) of PEG₈₀₀₀ or PEG₄₀₀₀ aqueous solutions (from 10 to 100% of saturation) with ammonium sulphate aqueous solutions (from 10 to 100% of saturation). The isolates from the sieves were suspended in 15 ml of ammonium sulphate solutions and, after 1 min agitation, they were mixed with an equal volume of PEG. The mixture was centrifuged for 1 min at 500 rpm and the spores were placed in the interphase and were separated with a pipette, rinsed with distilled water and counted in a Doncaster dish. The ABS with 100% PEG₈₀₀₀ and 50% ammonium sulphate concentration was tested at temperatures of 28 and 4 °C. The sucrose gradient method (Jenkins, 1964) was carried out with the isolates from the sieves which were centrifuged at 3500 rpm for 2 min in a 33, 50 or 69% (W/V) of sucrose. The spores obtained in the interphase of the gradient were separated with a pipette, rinsed with distilled water and counted in a Doncaster dish. For each of the ABS and sucrose

* Corresponding author. Tel.: +34 58 121011; fax: +34 958 129600.

E-mail addresses: msalvad@biochiapas.org (M. Salvador-Figueroa), jocampo@eez.csic.es (J.A. Ocampo).

Table 1
Physical characteristics of soils

Plant culture	Texture	Clay (%)	Organic matter (%)	pH
<i>Mangifera indica</i>	Loam	19.8	1.70	5.69
<i>Mussa</i> sp.	Sandy-clay-loam	24.2	2.27	6.71
<i>Carica papaya</i>	Sandy-clay	38.2	2.17	5.72
<i>Theobroma cacao</i>	Sandy-loam	18.2	4.05	5.85

gradient tests, six replicates per each soil sample were made. The root colonization by the spores isolated using the different methods was assessed on *Arachis hypogaea* L. grown in 300 ml of steamed soil:sand pots inoculated with 50 spores. There were 50 replicate pots per treatment. The sandy-loam soil collected from the *T. cacao* culture located at the (ERE) field was used. The plants were kept in a controlled-climate glasshouse, and were watered with 10 ml Hewitt's nutrient solution with 25% of P, per week (Hewitt, 1952). Plants were harvested after 30 d, the root system was cleared and stained (Phillips and Hayman, 1970) and the percentage of AM root length colonization was measured (Giovannetti and Mosse, 1980). The viability of 50 spores obtained with the different procedures was assessed by the tetrazolium bromide technique (Meier and Charvat, 1993).

ABS and sucrose gradient methods were compared by extracting spores from four different soils located in the (ERE) field (Table 1). The concentration of 100% PEG₈₀₀₀ and 50% ammonium sulphate at 4 °C for ABS and 69% of sucrose for Jenking method were used. The extraction efficiency (EE), as a percentage, was determined as follows: $EE = (E_i/E_t) \times 100$; E_i = number of spores in the interphase; E_t = total number of spores ($E_i + E_s$); E_s = number of spores in the sediment.

The data were subjected to one-way ANOVA. The mean values of samples were compared using the Duncan multiple range and the standard errors of mean tests.

The two-phase formation of the ABS was observed between a mix of 100% saturated solution of PEG with >10% saturated solutions of ammonium sulphate to a mix of 10% saturated solution of PEG with >40% saturated solutions of ammonium sulphate (values of the right side of the binodal curve, Fig. 1). When we used ABS with solutions of PEG₄₀₀₀:ammonium sulphate saturation ranged between 100:100, 100:90, 100:20 and 50:90, respectively, at 28 °C. With these ABS solutions, the spore extraction efficiency from the sandy-loam soil of the *T. cacao* culture located at the (ERE) field ranged between 48.4 ± 3.8 , 47.8 ± 3.6 , 39.4 ± 4.2 and 17.6 ± 3.5 . A higher average of spore extraction efficiency was observed when the polymer of the ABS with 100% PEG and 50% ammonium

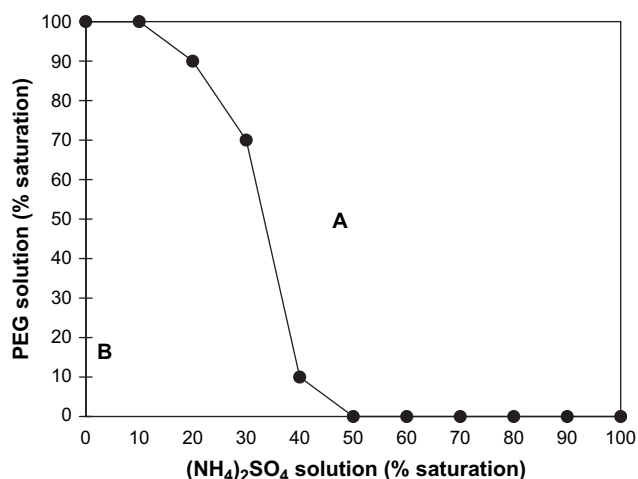


Fig. 1. Binodal curve of ABS system. A = two phases, B = one liquid phase.

Table 2
Average spore viability and percentage of root colonization of *Theobroma cacao* by the spores obtained with different extraction procedures

Treatment	% Spore viability	% Root length colonized
Wet sieving	70.3b	49.4b
Sucrose gradient	51.8a	30.7a
ABS	68.6b	50.3b

Column values with the same letter are not significantly different as the Duncan multiple range test ($P = 0.05$).

sulphate concentration was PEG₈₀₀₀ (59.2 ± 0.8) than when it was PEG₄₀₀₀ (48.4 ± 0.7). A higher average of spore extraction efficiency was observed with the ABS at 4 °C (69.9 ± 2.8) compared to 28 °C (60.3 ± 1.2). The spore extraction efficiency from the sandy-loam soil of the *T. cacao* culture located at the (ERE) field using the sucrose gradient method ranged between 4.7 ± 0.1 , 10.7 ± 0.4 and 46.1 ± 0.6 when the concentration of sucrose was 33, 50 and 69% respectively. A similar percentage of root length colonization of *A. hypogaea* and number of viable spores obtained from the sandy-loam soil by the method of Gerdemann and Nicolson (1963) and by the ABS methods were observed. However, both parameters were lower when the Jenkins method was used (Table 2).

The number of spores isolated by the wet sieving and decanting technique from the sandy-clay, loam, sandy-loam and sandy-clay-loam soils was 122.4 ± 16.3 , 95.2 ± 7.8 , 426.7 ± 13.4 and 241.8 ± 30.6 , respectively. Spores belonging to the *Glomeraceae* and *Acaulosporaceae* families were the most frequent (sessile spores); spores belonging to the *Gigasporaceae* family (spores with bulbous subtending hyphae) were scarce. The spore extraction efficiency obtained by the Jenkins method was increased from 4% with the sandy-clay soil, to 12–15% with the other soils, but 70% extraction efficiency was obtained by the ABS method and no significant differences in the spore extraction efficiency among the different soils were observed (Fig. 2).

The ABS method using PEG and ammonium sulphate improved the recovery of spores from tropical soils as compared with the sucrose gradient method (Jenkins, 1964). Moreover, the ABS, unlike the sucrose, was not affected by the low temperatures; the highest percentage of spore recovery at 4 °C facilitates biochemical and physiological experiments that need low temperatures. The polymer concentration and molecular weight are important in the equilibrium distribution (partitioning) of ABS (Brooks et al., 1985). In fact, the optimal recovery of spores was achieved when 100% PEG and 50% ammonium sulphate concentration and when PEG₈₀₀₀

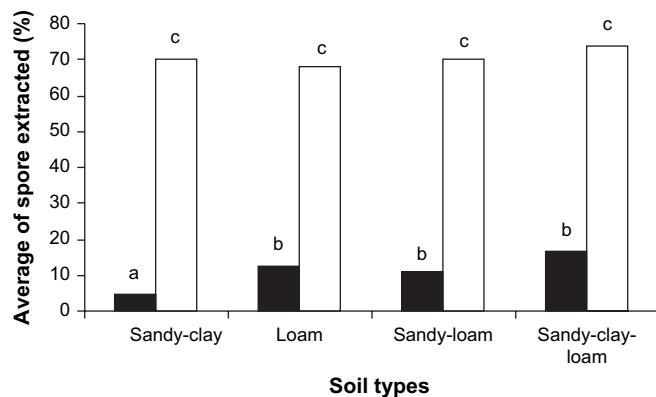


Fig. 2. Average of spores extracted by sucrose gradient (■) and by ABS (□) methods from different soils. Values followed by the same letter are not significantly different as determined by one-way ANOVA followed by Duncan multiple range test ($P = 0.05$).

instead PEG₄₀₀₀ were used. Under these conditions an optimal interface formation which facilitates the localization and recovery of spores was obtained.

The use of the ABS method improved the recovery of spores more than the sucrose gradient method even when 69% of sucrose was used. On the other hand, living spores are necessary to provide fungal propagules for research and practical applications. Most spores from field soils obtained by using the gradient sucrose method did not germinate (Brundett et al., 1999), possibly due to the high osmotic shock of the sucrose solution. However, both phases of the ABS are aqueous and did not affect either the viability of spores or the level of root colonization reached by these spores.

The composition and overall organic matter content of the soil affected the recovery of spores (Mohammad et al., 2003). By using the sucrose gradient method, different percentages of spore recovery from the different soils were observed, but no close relationships between the percentage of spores and the clay, organic matter and pH of the soils tested were found. However, there was no effect of soil components on the number of spores obtained by the ABS method because no significant differences in the percentage of spore recovery from the different soils were observed.

References

- Baskir, J.N., Holtton, T.A., Suter, U.W., 1989. Protein partitioning in two – phase aqueous polymer systems. *Biotechnology and Bioengineering* 34, 541–558.
- Bouyoucos, G.J., 1951. A recalibration of the hydrometer method for making mechanical analysis of soils. *Agronomy Journal* 43, 434–438.
- Brooks, D.E., Sharp, K.A., Fisher, D., 1985. Theoretical aspects of partitioning. In: Walter, H., Brooks, D., Fisher, D. (Eds.), *Partitioning in Aqueous Two-Phase Systems*. Academic Press, New York, pp. 11–13.
- Brundett, M.C., Abbott, L.K., Jasper, D.A., 1999. Glomalean mycorrhizal fungi from tropical Australia. 1. Comparison of the effectiveness and specificity of different isolation procedures. *Mycorrhiza* 8, 305–314.
- Diamond, A.D., Hsu, J.T., 1990. Protein partitioning in PEG/Dextran aqueous two-phase systems. *AIChE Journal* 36, 1017–1024.
- Doncaster, C.C., 1962. A counting dish for nematodes. *Nematologica* 7, 334–337.
- Gerdemann, J.W., Nicolson, T.H., 1963. Spores of micorrhizal *Endogone* species extracted from soil by wet sieving and decanting. *Transactions of the British Mycological Society* 46, 235–244.
- Giovannetti, M., Mosse, B., 1980. An evaluation of techniques for measuring vesicular–arbuscular mycorrhizal infection in roots. *New Phytologist* 84, 489–500.
- Hewitt, E.J., 1952. Sand water culture methods used in the study of plant nutrition. Commonwealth Agriculture Bureau. Technical communication No. 22.
- Jenkins, W.R., 1964. A rapid centrifugal flotation technique for separating nematodes from soil. *Plant Disease Research* 48, 692.
- Martin, J., Bereau, M., Louisanna, E., Ocampo, J.A., 2001. Vesicular arbuscular mycorrhizas in *Dicorynia guianensis* and *Eperua falcata* trees from primary tropical rain forest of French Guiana. *Symbiosis* 31, 283–291.
- Meier, R., Charvat, I., 1993. Reassessment of tetrazolium bromide as a viability stain for spores of vesicular–arbuscular mycorrhizal fungi. *American Journal of Botany* 80, 1007–1015.
- Mohammad, M.J., Hamad, S.R., Malkawi, H.I., 2003. Population of arbuscular mycorrhizal fungi in semi-arid environment of Jordan as influenced by biotic and abiotic factors. *Journal of Arid Environments* 53, 409–417.
- Phillips, J.M., Hayman, D.S., 1970. Improved procedures for clearing roots and staining parasitic and vesicular–arbuscular mycorrhizal fungi for rapid assessment of infection. *Transactions of the British Mycological Society* 55, 158–161.
- Sieverding, E., 1983. *Manual de Métodos para la Investigación de la Micorriza Vesículo Arbuscular en el Laboratorio*. Centro Internacional de Agricultura Tropical (CIAT), Cali, Colombia, 121 pp.
- Walkley, A., Black, T.A., 1934. An examination of the Degtjoreff method for determining soil organic matter and a proposed modification of the chromic acid titration method. *Soil Science* 37, 29–38.