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PRODUCTION AND INFECTIVITY OF INOCULUM OF ARBUSCULAR MYCORRHIZAL FUNGI MULTIPLIED IN A SUBSTRATE SUPPLEMENTED WITH TRIS-HCL BUFFER

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ABSTRACT

The effect of adding Tris-HCl buffer on production and infectivity of AMF inoculum was investigated. Sporulation of *Glomus etunicatum*, *Acaulospora longula* and *Gigaspora albida* was improved in solution with buffer. The infectivity of *G. etunicatum* increased after storage, what suggests that the inoculum of this isolate is benefited by storage.

Key words: mycorrhiza, organic buffer, sporulation, storage

Due to the obligate biotrophism, the production of inoculum is one of the obstacles for application of arbuscular mycorrhizal fungi (AMF) to benefit plant crops of economic importance. Among the methods for inoculum production, one is cultivation in sand and vermiculite, supplemented with nutrient solutions containing organic buffers in its formulation; this allows high production of spores (13).

Organic buffers have the property of minimizing the alterations of the H^+ ion concentration and are used for maintaining the pH of the AMF growth media (14). The effect of a buffer on growth of AMF depends on its constitution (14) and concentration in the culture medium (12). Researches related with use of organic buffers for maintenance of pH and for stimulating mycelial growth are restricted to a few species of AMF, mostly *Glomus* (3,14).

The AMF inoculum should be produced in high density, and maintain the infectivity and effectivity for a long period of time. Although not much information regarding storage of AMF is available (20), the maintenance of the inoculum in the conditions under which it was produced is recommended (7). It has also been mentioned that maintenance of AMF inoculum at low temperature stimulates germination and spore development (22,24); however, the ideal temperature for storage of each AMF isolate should be determined.

In this work the most suitable concentration of Tris-HCl buffer for production of spores of AMF and for maintenance of the infectivity of the inoculum, under different temperatures, was investigated.

Experiment 1. Effect of addition of Tris-HCl buffer on sporulation of AMF. Seeds of *Panicum miliaceum* L. (50 per pot) were disinfected with Sodium Hypochlorite (0.05% /10 min) and washed with distilled water before being planted. The substrate used was washed river sand (pH_{H2O} 7.1) and vermiculite of medium granulation (1:1 v/v). Both were autoclaved (30 min./1 atm/120ºC), in two consecutive days, and used 15 days after sterilized. Spores of *Glomus etunicatum* Becker and Gerd. (UFPE 06), *Gigaspora albida* Schenck and Smith (UFPE 01), *Scutellospora heterogama* (Nicol. and Gerd.) Walker and Sanders (UFPE 19) and *Acaulospora longula* Spain and Schenck (UFPE 21) were inoculated as suspensions (50 spores of each AMF), in plastic pots (400 mL) with 300 g of substrate, below the host seeds. The pots were maintained in a greenhouse for 85 days; temperature ($T_{min.}$ 22.81ºC; $T_{max.}$ 32.39ºC), humidity ($RU_{min.}$ 45.62%; $RU_{max.}$ 81.13 %) and light were not controlled. The pots were irrigated every other day with nutrient solution [Hoagland and Arnon 1950 modified by Jarstfer and Sylvia (8)] supplied with 0, 10, 25, 50 or 75 mM of Tris – HCl buffer (pH 6.5). The experiment was evaluated 85 days after the inoculation.

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The AMF spores were extracted from the soil by wet sieving (4) and sucrose centrifugation (9), placed on Petri dishes and quantified using a stereomicroscope. The experimental design was entirely at random in a factorial arrangement of: 4 species of AMF x 5 concentrations of Tris-HCl buffer, with 5 replicates (100 experimental units).

Experiment 2. Evaluation of the infectivity of the AMF inoculum produced in a substrate irrigated with nutrient solution supplemented with Tris-HCl buffer. The infectivity potential of the inocula produced in the first experiment was evaluated by the MIP method – mean percentage of infection (7). The inocula that produced higher density of spores per gram of substrate in the previous experiment were chosen for this assay (inoculum of *G. albida*, *A. longula* and *G. etunicatum* produced in the treatments supplied respectively with 75 mM, 50 mM and 10 mM of Tris-HCl buffer). The inoculum was constituted of spores, hypha and colonized roots. The infectivity of each inoculum was estimated before and after storage for 120 days, at 28ºC (room temperature) and at 4ºC (refrigerator). Samples of the material produced in the first experiment were diluted (1:10 v/v) in sand, using corn (*Zea mays* L. cv Assum preto) as the host plant. After 30 days, roots were clarified and stained with Trypan blue (0.05%) in lactoglycerol (15); the percentage of colonization was estimated by the intersect method (5). For each AMF the experimental design was completely at random with 3 treatments of storage and 7 replicates (= 21 experimental units). The pots were maintained in a greenhouse for 30 days after seedling emergence. The inoculum was considered viable, from the commercial point of view, when roots of the host presented at least 25% of mycorrhizal colonization (7).

For both experiments the data were transformed ($\sqrt{x} + 0.5$) for variance analysis and the means compared by Tukey (for sporulation) and LSD at 5% probability (for infectivity), using the Statistica Program (19).

Different responses on sporulation of the AMF were found in relation to buffer concentrations (Table 1), except for *S. heterogama*. The incorporation of >10 mM of Tris-HCl to the substrate favored the sporulation of *G. etunicatum*. *Gigaspora albida* presented significant increase on sporulation when the substrate received concentrations higher than 25 mM of buffer. Maximum production of spores of *A. longula* was obtained in substrate receiving concentrations ≥50 mM of buffer.

The sporulation of *G. etunicatum* increased in all treatments with Tris-HCl comparing with the control (without buffer). Differently from the observed with *A. longula*, sporulation of *G. albida* was positively correlated with the increase of buffer concentrations in the nutrient solution ($P < 0.05$; $r = 0.90$). Higher production of spores was attained by *G. etunicatum*, differing from those reached by the other species in all treatments, with and without buffer. Conversely, *S. heterogama* did not multiply well, in comparison with the other fungi, independently of presence of buffer in the growth medium.

The storage at 4ºC preserved the infective potential of the AMF inocula in relation to the treatment maintained at 28ºC (Table 2). With the exception of *G. albida*, the preservation of the others AMF under environmental conditions significantly reduced the infectivity of the inoculants. The infective potential of *G. etunicatum* increased when the inoculum was stored at 4ºC, while that of *G. albida* was not affected by the storage treatments.

The addition of Tris-HCl, in any concentration, promoted high sporulation of *G. etunicatum*. The benefit of adding low concentrations of buffers in the medium, for sporulation of AMF was mentioned for some *Glomus* species. Douds Jr. (3) observed that 10 mM of Tris favored the germination and the mycelial growth of *Glomus mosseae*.

Species of *Gigaspora* form a high amount of soil biomass, when compared to other AMF (6). This may result in high amounts of CO_2 (from respiration); the reaction of CO_2 with water reduced the pH of the rhizosphere (21). This could justify the efficiency of the buffer in higher concentrations, once that it would be needed high amounts of the buffer to make the pH stable. Conversely, *G. etunicatum* develops hypha that are thinner than those of *G. albida*, presenting lower respiration values; thus, the 10 mM concentration would be enough to stabilize the alterations on $[H^+]$.

Table 1. Production of spores (g^{-1} substrate) of *Acaulospora longula*, *Gigaspora albida*, *Glomus etunicatum* and *Scutellospora heterogama* after 85 days associated with *Panicum miliaceum*, irrigated with nutrient solution [Hoagland and Arnon, 1950, modified by Jarstfer and Sylvia, 1992] and supplemented with Tris-HCl buffer.

AMF	Concentrations de Tris-HCl (mM)				
	0	10	25	50	75
A. longula	11.72 bB	17.53 bB	14.23 cB	43.80 bA	35.00 bcAB
G. albida	33.09 bC	42.30 bBC	56.34 bAB	53.56 bAB	65.10 bA
G. etunicatum	104.73aB	161.28 aA	198.44 aA	175.69 aA	292.01 aA
S. heterogama	4.62 bA	9.94 bA	10.80 cA	14.37 cA	15.05 cA

Means followed by the same small letter (column) and capital letter (line) do not differ by Tukey ($P < 0.05$).

Table 2. Infectivity (%) of the inocula of AMF produced in a sand vermiculite substrate irrigated with Hoagland and Arnon nutrient solution modified by Jarstfer and Sylvia (1992) and supplemented with Tris-HCl buffer, calculated at time 0 (before storage) and after maintenance for 120 days at 4ºC and at room temperature (28ºC).

Treatments	Inocula		
	Gigaspora albida 75 mM*	*Acaulospora longula* 50 mM*	*Glomus etunicatum* 10 mM*
Without storage	63.14 a	23.24 a	11,36 b
4ºC	61.82 a	18.81 ab	27.58 a
28ºC	63.35 a	7.31 b	14.61 b

*75, 50 and 10 mM= concentrations of Tris-HCl added to the nutrient solution. Means followed by the same letter (column) do not differ by the LSD test (P<0.05).

Species of *Gigaspora* were not successfully cultivated in aeroponic systems. The supply of nutrient solution with Tris-HCl may constitute an alternative for large scale production of this fungus. Millner and Kitt (13) obtained high production of spores of *G. margarita* using 0.5 mM of MES in the nutrient solution; in the same way, Silva *et al.* (17) observed high sporulation of *G. margarita* when adding 50 mM of Tris-HCl to the nutrient solution.

There are recommendations for preservation of the inoculum in the conditions under which it was produced (11). However, for inoculum produced in tropical conditions, as in this work, the maintenance at 4ºC was better for preserving the infective potential of the tested AMF, in comparison with storage at environmental temperature (28ºC). This can be due to the uniformity of germination (16), as well as to higher longevity of the structures at low temperatures, when metabolism is reduced. The results obtained agree with those that recommend maintenance of AMF inoculum at low temperatures (22). Juge *et al.* (10) mentioned that low temperatures can reduce the mortality of spores.

The only infective unit of Gigasporaceae is the spore (1), which is more resistant than the hypha (18). This may explain why spores of *G. albida* maintained its infectivity after storage. The percentage of colonization by *A. longula* was reduced after 120 days at room temperature, what could be explained by loss of hyphal viability as well as to the dormancy of the spores, a characteristic of the genus *Acaulospora* (2).

The inoculum of *G. etunicatum* maintained at 4ºC produced higher mycorrhizal colonization than that stored at room temperature. Wagner *et al.* (24) observed increase in number of infective propagules of *Glomus claroideum* when stored at 4ºC in comparison with maintenance at room temperature (24ºC). In this work, the increase on infectivity of *G. etunicatum* maintained at 4ºC might be related to inactivation of inhibitors or activation of germination promoters as suggested by Louis and Lim (11).

Only *G. albida* and *G. etunicatum* attained the quality control patterns for production of commercial inoculum, forming at least 25% of mycorrhizal colonization (7). Production in large scale of inoculum of these isolates can be obtained by using the methods here described, which include addition of Tris-HCl buffer in the nutrient solution and storage at 4ºC.

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RESUMO

Produção e infectividade de inóculo de fungos micorrízicos arbusculares multiplicados em substrato suplementado com tampão Tris-HCl

O efeito da adição do tampão Tris-HCl na produção e infectividade de inóculo foi investigado. A esporulação de *Glomus etunicatum*, *Acaulospora longula* e *Gigaspora albida* foi incrementada utilizando solução com tampão. A infectividade de *G. etunicatum* aumentou após estocagem, sugerindo que o inóculo deste isolado é beneficiado pelo armazenamento.

Palavras-chave: esporulação, estocagem, micorriza, tampão orgânico

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