

## SPORULATION OF ARBUSCULAR MYCORRHIZAL FUNGI USING TRIS-HCl BUFFER IN ADDITION TO NUTRIENT SOLUTIONS

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### ABSTRACT

The production of inoculum is one of the hindrances in the large scale application of arbuscular mycorrhizal fungi (AMF). The objective of this work was to evaluate the effect of nutrient solutions with or without Tris-HCl buffer, on sporulation of AMF. The experiment was carried out in a greenhouse, using a substrate with sand and vermiculite (1:1 v/v). Fifty spores of *Gigaspora margarita*, *Scutellospora heterogama*, and *Glomus etunicatum* were inoculated in *Sorghum vulgare* (sorghum) or *Panicum miliaceum* (fodder millet). The substrate received the following nutrient solutions: Hoagland with 3 µM P (S1); Long Ashton II with 15.9 µM P (S2) and Hoagland with 20 µM P (S3), with or without 50 mM of Tris-HCl buffer (pH 6.5); the control treatment, consisting of a soil + sand + vermiculite (2:1:1 v/v) substrate was irrigated with deionized water. Ten weeks after the beginning of the experiment sporulation did not differ in treatments with sorghum. *Panicum miliaceum* promoted higher sporulation of the AMF than sorghum, and differences among treatments with nutrient solutions were observed. Production of spores of *G. margarita* and *S. heterogama* increased significantly after addition of buffer in S1 and S2, while that of *G. etunicatum* was improved when the substrate was irrigated with S1 + buffer and S3 solutions. Solution S1 + buffer benefited sporulation of the three fungi. However, as observed, each AMF, host, and substrate system should be studied separately for establishment of the most favorable conditions for inoculum production.

**Key words:** *Glomus etunicatum*, *Gigaspora margarita*, *Scutellospora heterogama*, inoculum production, nutrient solution

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### INTRODUCTION

Arbuscular mycorrhizal fungi (AMF) are obligate symbionts that need a compatible plant host to complete their life cycle and produce spores, the main source of propagules used for application in crop production. The AMF are not considered as host specific. However, some plants are more susceptible than others in relation to development of the symbiosis. Because of that, several studies have been carried out to determine the best combinations of AMF and host species, in order to increase spore production (2,17,34).

Several systems for inoculum production of AMF have been developed, such as the aeroponic (18), hydroponic (16) and the

monoxenic cultivation (32). Although these systems have the advantage of producing spores suitable for studies of ontogeny (40), ultrastructure (25), and molecular biology (30), in general they have the disadvantage of high cost of installation and maintenance (26). In some cases, reduction of the germination rate (18) and production of spores with smaller diameter (27) have been described, compared with the traditional methods, where the plant is cultivated in containers with different substrates. Gaur and Adholeya (11) obtained maximum production of AMF inoculum in sand, which may have allowed better soil aeration, drainage, oxygen supply and root growth than other substrates tested. Another advantage of pot culture in sand is the low maintenance cost (37).

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The choice of the best method for inoculum production should consider the species of AMF, host combination, and soil characteristics (37). Among these factors, pH can affect directly the AMF, determining the occurrence of species (1) and the germination of spores (13). It also may indirectly affect the redox potential of the soil (7). Some species of AMF are found only in acid or alkaline soils and many do not grow when transferred to soils with a different pH (28). Bartolome-Esteban and Schenck (4) mentioned that pH does not directly affect the mycorrhizal association, but acts through interference in the activity of soil macro and micronutrients.

Due to the importance of pH for development of the mycorrhizal symbiosis, some organic buffers have been used in addition to nutrient solutions (26) and in the medium of the monoxenic culture (8,21,27). Vilarinho *et al.* (39) suggested that buffer stimulates growth of external hyphae of AMF, contributing to increase sporulation.

The objective of this work was to evaluate the effect of nutrient solutions with Tris-HCl buffer on sporulation of AMF.

## MATERIALS AND METHODS

### Substrate and containers

A substrate with sand (pH 6.9) and vermiculite (medium granulation, pH 7.4), in a 1:1 proportion (v:v), was used 15 days after sterilization in autoclave (120°C for 1h at 1 atm) for two consecutive days. For control treatment a mixture of soil: sand: vermiculite (2:1:1, v/v), similar to the natural substrate for AMF was used after sterilized as described. The soil was a Red Yellow Podzol, with pH 5.4 and 3 mg dm<sup>-3</sup> of P, 1.4; 0.8; 0.12; 0.15 and 0.20 cmol dm<sup>-3</sup> of Ca<sup>2+</sup>, Mg<sup>2+</sup>, Na<sup>+</sup>, K<sup>+</sup>, and Al<sup>3+</sup> respectively, sterilized with methyl bromide (98% of methyl bromide and 2% of chloropicrine), and used after 15 days. The soil pH was adjusted to 6.0 with dolomitic limestone. Plastic pots (8.5 x 10 cm) were filled with 300 g of substrate.

### Plant hosts

Fifty seeds of sorghum (*Sorghum vulgare* L.) and fodder millet (*Panicum miliaceum* L.) were disinfected with sodium hypochlorite (NaOH) 0.5% for 15 min, and germinated in each pot.

### AMF isolation and inoculation

Spores of *Gigaspora margarita* Becker and Hall (UFPE 02), *Scutellospora heterogama* (Nicolson & Gerdemann) Walker & Sanders (UFPE 19) and *Glomus etunicatum* Becker & Gerdemann (UFPE 06) from the Laboratório de Micorrizas da Universidade Federal de Pernambuco (UFPE) were maintained in association with fodder millet in a greenhouse and stored at 28°C

for three months before being used. For each inoculation treatment, fifty spores were distributed in the pots immediately below the seeds of each host. The pots were maintained in the greenhouse with natural photoperiods, temperature range of T<sub>min.</sub> 20.87°C; T<sub>max.</sub> 32.66°C, and relative air humidity between RH<sub>min</sub> 44 %; RH<sub>max</sub> 81.78%.

### Nutrient solutions

The following nutrient solutions were applied: S1= Hoagland modified by Jarstfer and Sylvia (19), with 3 µM P; S1B = Hoagland modified by Jarstfer and Sylvia (19) with 3 µM P+buffer Tris-HCl, S2 = Long Ashton II modified by Liyanage (22), with 15.9 µM P; S2B = Long Ashton II modified by Liyanage (22) with 15.9 µM P+Tris-HCl buffer, S3 = Hoagland modified by Millner and Kitt (26), with 20 µM P and without the (2-[N-morpholino] ethanesulfonic acid) MES buffer and S3B = Hoagland modified by Millner and Kitt (26), with 20 µM P and without the (2-[N-morpholino] ethanesulfonic acid) MES buffer + Tris-HCl buffer.

The pH of buffer (50 mM) was 6.5 and after adding it, the hydrogenionic concentration of solutions S1, S2 and S3 changed respectively from 4.95 to 6.32; 5.4 to 5.83; 4.78 to 6.40. The pots were irrigated every other day with the nutrient solutions and once a week with distilled deionized water, to avoid accumulation of salts. The control treatment was irrigated only with distilled deionized water (Table 1).

**Table 1.** Nutrient solutions: S1- Hoagland modified by Jarstfer and Sylvia (19), S2- Long Ashton II modified by Liyanage (22), and S3- Hoagland modified by Millner and Kitt (26)

Nutrient Composition	Final concentration / L		
	S1	S2	S3
<i>Macronutrients</i>			
KNO <sub>3</sub>	1.5. 10 <sup>-3</sup> M	0.4. 10 <sup>-3</sup> M	2.5. 10 <sup>-3</sup> M
Ca(NO <sub>3</sub> ) <sub>2</sub> .4H <sub>2</sub> O	1.5. 10 <sup>-3</sup> M	0.27. 10 <sup>-3</sup> M	2.5. 10 <sup>-3</sup> M
MgSO <sub>4</sub> .7H <sub>2</sub> O	0.3. 10 <sup>-3</sup> M	0.15. 10 <sup>-3</sup> M	1. 10 <sup>-3</sup> M
KH <sub>2</sub> PO <sub>4</sub>	3. 10 <sup>-6</sup> M	-	20. 10 <sup>-6</sup> M
NaH <sub>2</sub> PO <sub>4</sub> .H <sub>2</sub> O	-	15.9. 10 <sup>-6</sup> M	-
FeNa EDTA	45. 10 <sup>-6</sup> M	4.9. 10 <sup>-6</sup> M	50. 10 <sup>-6</sup> M
NaCl	30. 10 <sup>-6</sup> M	9.9. 10 <sup>-6</sup> M	-
<i>Micronutrients</i>			
MnSO <sub>4</sub> .H <sub>2</sub> O	-	1.3. 10 <sup>-6</sup> M	-
ZnSO <sub>4</sub> .7H <sub>2</sub> O	0.7. 10 <sup>-6</sup> M	0.1. 10 <sup>-6</sup> M	1. 10 <sup>-6</sup> M
CuSO <sub>4</sub> .5H <sub>2</sub> O	0.3. 10 <sup>-6</sup> M	0.1. 10 <sup>-6</sup> M	0.5. 10 <sup>-6</sup> M
NiSO <sub>4</sub> .6H <sub>2</sub> O	-	-	0.2. 10 <sup>-6</sup> M
MnCl <sub>2</sub> .4H <sub>2</sub> O	9.5. 10 <sup>-6</sup> M	-	2. 10 <sup>-6</sup> M
Na <sub>2</sub> MoO <sub>4</sub> .2H <sub>2</sub> O	0.066. 10 <sup>-6</sup> M	-	0.2. 10 <sup>-6</sup> M
HCl3N	-	-	25. 10 <sup>-6</sup> M
H <sub>3</sub> BO <sub>3</sub>	46.2. 10 <sup>-6</sup> M	5. 10 <sup>-6</sup> M	10. 10 <sup>-6</sup> M

### Experimental design

Two experiments were performed in a completely randomized design, one with *P. miliaceum*, the other using *S. vulgare*, in order to evaluate the effect of nutrient solution (with or without buffer) on sporulation of AMF. Each experiment was in factorial of 7x3: 6 nutrient solutions (S1, S1B, S2, S2B, S3 and S3B) + 1 control (without nutrient solution) x 3 AMF isolates (*G. margarita*, *G. etunicatum* and *S. heterogama*) in 3 replicates, in a total of 63 experimental parcels for each host.

### Evaluation and statistical analysis

Seventy days after the beginning of the experiment, spores of AMF were extracted from the substrates, by wet sieving and decanting (12) and water and sucrose centrifugation (20), placed in Petri dishes and counted in a stereomicroscope (40x).

For variance analysis the factors: 1) AMF and 2) Nutrient solution and interaction between them, were employed. Data

were transformed in  $\sqrt{x+1}$  and media compared by the Tukey test at 5% probability, using the Statistica (33) and Sanest (41) programs.

## RESULTS

Higher sporulation of AMF was observed in the treatment with *P. miliaceum* where in absolute numbers density of spores varied from 3.7 g<sup>-1</sup> substrate (*S. heterogama*) to 152.8 g<sup>-1</sup> substrate (*G. etunicatum*); in the rhizosphere of *S. vulgare* sporulation was from 0.03 g<sup>-1</sup> substrate to 1.54 g<sup>-1</sup> substrate considering *G. etunicatum*. Production of spores did not differ in the experiment with *S. vulgare* using the nutrient solutions (Table 2). However, comparing among the fungi, the sporulation differed in the treatments control, S3 and S3B. *Gigaspora margarita* produced more spores in media with these solutions, differing from *S. heterogama* in S3 and from *G. etunicatum* in S3B. In the control

**Table 2.** Production of spores (n<sup>o</sup> g<sup>-1</sup> substrate) of *Gigaspora margarita*, *Glomus etunicatum* and *Scutellospora heterogama*, after 70 days associated to fodder millet (*Panicum miliaceum*) and sorghum (*Sorghum vulgare*), in substrates irrigated or not with nutrient solution and supplemented or not with Tris-HCl buffer (pH 6.5).

Fungi	Nutrient Solutions <sup>1</sup>						
	<i>P. miliaceum</i>						
	S1	S1B	S2	S2B	S3	S3B	Control
<i>G. margarita</i>	13.8abAB	43.6aB	4.7bA	29.9abA	21.3abB	30.3aB	19.1abA
<i>G. etunicatum</i>	31.2bA	108.0aA	18.6bA	36.8bA	152.8aA	94.7aA	27.2bA
<i>S. heterogama</i>	5.4bB	44.5aB	4.8bA	32.5aA	3.7bB	20.5abB	20.0abA
Factors	D.F.	S.S.	M.S.	F value	Prob.>F		
AMF (F)	2	139.92170	69.960850	41.8092	0.00001		
Solutions (S)	6	139.84303	23.307173	13.9425	0.00001		
F x S	12	115.18231	9.598526	5.7377	0.00005		
Residue	42	70.58384	1.680567				
CV%	23.83						
	<i>S. vulgare</i>						
	S1	S1B	S2	S2B	S3	S3B	Control
<i>G. margarita</i>	0.63aA	0.04aA	0.31aA	0.08aA	0.49aA	0.44aA	0.44aB
<i>G. etunicatum</i>	0.22bA	0.04bA	0.08bA	0.03bA	0.22bAB	0.10bB	1.54aA
<i>S. heterogama</i>	0.24aA	0.08aA	0.13aA	0.09aA	0.07aAB	0.10aAB	0.28aB
Factors	D.F.	S.S.	M.S.	F value	Prob.>		
AMF (F)	2	16.973223	8.486618	7.4479	0.00211		
Solutions (S)	6	90.717230	15.119538	13.0574	0.00001		
F x S	12	63.983180	5.3319317	4.7649	0.00020		
Residue	42	48.157387	1.1466045				
CV%	32.12						

Means followed by the same small letter (line) and capital letter (column) does not differ ( $P < 0.05$ ). <sup>1</sup>S1= Hoagland modified by Jarstfer and Sylvia (19), S1B= Hoagland modified by Jarstfer and Sylvia (19)+buffer Tris-HCl, S2= Long Ashton II modified by Liyanage (22), S2B= Long Ashton II modified by Liyanage (22) + Tris-HCl buffer, S3= Hoagland modified by Millner and Kitt (26) and S3B= Hoagland modified by Millner and Kitt (26) + Tris-HCl buffer; Control: soil+sand+vermiculite.

treatment, *G. etunicatum* produced higher amount of spores, differing from the other fungi (Table 2). In the experiment with *P. miliaceum*, statistical difference among sporulation of the AMF was observed in the treatments S1, S1B, S3 and S3B, with *G. etunicatum* producing the highest amount of spores.

Higher sporulation of *G. etunicatum* in *P. miliaceum* was found in the treatments S3 (increment of 92,425%), S3B and S1B, and all of them differed from the control. The production of spores of *S. heterogama* increased with addition of buffer in substrates receiving nutrient solutions S1 and S2 (Table 2), when compared with samples without buffer, although not differing of the control, while sporulation of *G. margarita* increased with addition of Tris-HCl, but did not differ from treatments with solutions free of buffer. Thus, presence of buffer significantly stimulated sporulation ( $p < 0.001$ ), promoting increase of about eight and six times, respectively, in relation to the S1 and S2 solutions for *S. heterogama*, and increase of five times for *G. margarita* maintained in substrate receiving the S2B solution. In this case, however, the average of spore production did not significantly differ from that of the treatment using solution S2 (without buffer). Although the use of buffer in all solutions had promoted higher sporulation than the treatments buffer free, the results did not significantly differ (at the level of 5%) from the control (Table 2), with exception of *G. etunicatum*. Solution S2 (Long Ashton II without buffer) did not stimulate the sporulation of the AMF, which was similar to that produced by the control.

## DISCUSSION

The high sporulation of the AMF with fodder millet, in this experiment, was similar to the results obtained by Bagyaraj and Manjunath (2) using *Panicum maximum* Jacq. and *Glomus fasciculatum* (Thaxter) Gerd. & Trappe emend Walker & Koske. The authors observed that the association of this fungus with *P. maximum* produced about 730 spores  $50 \text{ g}^{-1}$  of soil, after 75 days, while with sorghum the sporulation was 50% lower (346 spores  $50 \text{ g}^{-1}$  of soil). Using the same fungus associated with *Sorghum vulgare* var. *sudanense* (Piper) Hitch., Hetrick and Bloom (17) observed higher production of spores of *G. fasciculatum* than that obtained with other plant species; however the experiment was concluded in approximately 120 days. It is possible that the duration of the experiment positively affected the sporulation, as also observed by Luedders *et al.* (24). Struble and Skipper (34) observed an increase in the sporulation of several species of AMF associated with sorghum with the increase of culture duration. Thus, the low results on sporulation of the AMF associated with sorghum in this study might be related to the short duration of the experiment (70 days).

The production of hormones by the host is not equal throughout its phenological cycle, and this can affect sporulation (35,36), establishment, and effectiveness of the

mycorrhizal symbiosis (3). When the host is in the flowering stage, high consumption of energy is required, which may decrease the flow of carbohydrates to the AMF (5). This could be a sign to stimulate the production of spores as a strategy for survival. Thus, the flowering of fodder millet, which occurred 60 days after the beginning of the experiment, may have contributed to stimulate sporulation of the AMF in this host. The same did not occur with sorghum, which was in vegetative growth through the 70 days of the experiment; in this case, production of spores was not stimulated.

Although in AMF host specificity seems not to exist (15), the difference in the sporulation of the species associated with fodder millet vs. sorghum, reinforces Sanders and Fitter's (29) hypothesis of the existence of an "optimum" host-AMF combination, as well as differences in the cycles and strategies of life of the AMF species (6). It has been suggested that AMF differ in growth, sporulation and establishment of the symbiosis as a function of the host, due to differential perception of signs emitted by the root (14,17). Abe *et al.* (1) considered the possibility of the specificity between *Elymus mollis* Trin. (Gramineae) and species of *Glomus* to justify the predominance of *Glomus* species in the rhizosphere of this plant.

Hoagland's solution, modified by Jarstfer and Sylvia (19) and supplemented with Tris HCl buffer (S1B) promoted higher sporulation of *G. etunicatum* and *S. heterogama* than the treatment without buffer supply. One of the factors contributing to this result was probably the low concentration of P in this nutrient solution ( $3 \mu\text{M}$ ). High concentration of P can reduce hyphal growth (23), as well as colonization and sporulation of AMF (10), while the reduction can improve spore production (9,38). On the other side, high sporulation of *G. etunicatum* was obtained in substrate irrigated with S1B or with S3, what indicates possible plasticity of this isolate in relation to wide values of pH and P concentration in the nutrient solution.

An increase in spore density of AMF when the buffer was added to the solutions probably occurred due to the maintenance of the pH in the substrate. Vilarinho *et al.* (39) used Tris-HCl and MES buffers for sporulation of *Glomus intraradices* Schenck & Smith; pH was reduced in both substrates, but only the MES buffer promoted increase of extraradical hyphae and sporulation of the AMF. The authors concluded that this occurred due to the activity of soil microorganisms stimulated by MES buffer, possibly through liberation of sulphur in this compound. On the other hand, Douds Jr. (8) observed improved hyphal growth of *Glomus mosseae* (Nicolson & Gerd.) Gerd. & Trappe in a medium supplemented with 10 mM Tris, in comparison to the addition of MES buffer. Our results showed that the sporulation of the AMF in the control treatment was in general similar to that obtained in the treatment using solutions without buffer, what indicates the importance of the buffer capacity of the soil in the control mixture. Sporulation of *G. etunicatum* in the treatments with solutions



S1B, S3 and S3B differed from the control. However, effect of addition of buffer, among the solutions, was observed only in the S1 treatment.

The results of Millner and Kitt (26) were similar to those obtained in this work with *G. margarita* and *G. etunicatum*; however, the amount of the initial inoculum used by these authors was, respectively, seven and forty times higher than that employed here, indicating that it is possible to obtain high sporulation with a low initial amount of inoculum.

Although the hosts were in different developmental stages at the 85th day, it was shown that each one had a singular effect on sporulation, confirming previous studies (31) that indicate a host effect on production of spores of AMF. Thus, in a same period of time, fodder millet was a better host than sorghum, promoting sporulation of the AMF.

The solutions S1 and S2 receiving buffer improved the sporulation mainly of *S. heterogama*, but it is not possible to say if this was due to pH or to the own organic compounds present in the solutions.

In conclusion, the use of buffer enhanced sporulation of the AMF, especially that of *S. heterogama* e *G. etunicatum*. Studies involving a new cycle of multiplication of AMF in sorghum and other hosts, can also confirm or not the decisive role of phenologic stages in the sporulation process. In the same way, studies regarding the effect of Tris-HCl buffer on germination, hyphal growth and mycorrhizal colonization should be made so that the behavior of other AMF species in relation to buffer supply can be understood.

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#### RESUMO

##### **Esporulação de fungos micorrízicos arbusculares usando solução nutritiva adicionada de tampão Tris HCl**

A produção de inóculo é um dos entraves na aplicação de fungos micorrízicos arbusculares (FMA) em larga escala. O objetivo deste trabalho foi avaliar o efeito de soluções nutritivas associadas a tampão Tris-HCl sobre a esporulação dos FMA. Foram conduzidos dois experimentos em casa de vegetação, um para cada hospedeiro, painço (*Panicum miliaceum*) e sorgo

(*Sorghum vulgare*), usando como substrato areia:vermiculita (1:1 v/v), onde foram aplicados cinquenta esporos de *Gigaspora margarita*, *Scutellospora heterogama* e *Glomus etunicatum*. O substrato foi irrigado com as seguintes soluções nutritivas: Hoagland com 3 µM P (S1), Long Ashton II com 15,9 µM P (S2) e Hoagland com 20 µM P (S3), com ou sem 50 mM de tampão Tris-HCl (pH 6,5); o tratamento controle, que consistiu de solo: areia:vermiculita (2:1:1 v/v) recebeu água deionizada. Após 10 semanas não houve diferença na esporulação, nos tratamentos com sorgo, exceto para *G. etunicatum*. Porém, no experimento com painço os tratamentos com solução nutritiva diferiram significativamente. A esporulação de *S. heterogama* aumentou após adição do tampão nas soluções S1 e S2, enquanto a de *G. etunicatum* foi incrementada no substrato irrigado com S1 + tampão e S3, independente da presença do tampão. A produção de esporos de *G. margarita* foi maior nas soluções S1 e S3, ambas com tampão, porém diferenças significativas foram observadas somente em relação à solução S2, sem tampão. A utilização de soluções nutritivas e a adição de tampão podem contribuir para o aumento da esporulação de FMA; porém, como demonstrado, cada sistema substrato, FMA e hospedeiro deve ser avaliado separadamente, para estabelecimento das condições mais favoráveis para produção de inóculo em larga escala.

**Palavras-chave:** *Glomus etunicatum*, *Gigaspora margarita*, *Scutellospora heterogama*, produção de inóculo, solução nutritiva

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