Association and mycorrhizal dependency in *Jatropha curcas* L. seedlings under salt stress[1]

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ABSTRACT

The cultivation of *Jatropha curcas* L. for biodiesel production is possible in salinized areas; however, biomass production is limited in these soils. Arbuscular mycorrhizal fungi (AMF) are a promising alternative for bioremediation in salinized soils. Yet, salinity also affects the AMF at the time of colonization and, in this case, the symbiosis is not always established. Therefore, the aim of this study was to test the hypotheses that three AMF species commonly found in saline soils are associated with *J. curcas* and if seedlings previously inoculated with these AMF are more tolerant to salt stress. Two trials were performed: the first one was carried out in a completely randomized design with five treatments (control, *Rhizophagus intraradices, Gigaspora albida, Claroideoglomus etunicatum,* and the three species together) and six repetitions to investigate the formation of symbiosis among species; and the second trial was carried out in randomized blocks in a 4 × 2 factorial scheme (2, 5, 8, and 10 dS m^{-1}, with and without mycorrhizae) with eight repetitions to verify the development and mycorrhizal dependency (MD) of the seedlings previously inoculated, in salinized environment. The three species of AMF are associated with *J. curcas* both alone and together. Mycorrhizal dependency increased with salinity, indicating that *J. curcas* is a facultative species. The pre-colonized seedlings with AMF are an alternative to the establishment of *J. curcas* in salinized soils.

Key words: arbuscular mycorrhizal fungi; symbiosis; salinity; physic nut.

RESUMO

Associação e dependência micorrízica em mudas de *Jatropha curcas* L. sob estresse salino

O cultivo de *Jatropha curcas* visando a produção de biodiesel é uma possibilidade para o uso de áreas salinizadas, no entanto a produção de biomassa nesses solos é limitada. Os fungos micorrízicos arbusculares (FMAs) são uma alternativa promissora para a biorremediação em solos salinizados. Porém, a salinidade também afeta os FMAs, no momento da colonização e, neste caso, nem sempre a simbiose se estabelece. Portanto, este estudo teve como objetivo testar as hipóteses de que três espécies de FMAs comummente encontradas em solos salinos se associam à *J. curcas* e, se mudas previamente inoculadas com esses FMAs são mais tolerantes ao estresse salino. Foram realizados dois experimentos, o primeiro, em delineamento inteiramente casualizado com cinco tratamentos (controle, *Rhizophagus intraradices, Gigaspora albida, Claroideoglomus etunicatum* e as três espécies em conjunto) com seis repetições, com o objetivo de investigar a formação da simbiose entre as espécies, e um segundo, realizado em blocos casualizados, em esquema fatorial 4 x 2 (2, 5, 8 e 10 dS m^{-1}, com e sem micorrizas), com oito repetições, para a verificação do desenvolvimento e dependência micorrízica (DM) das mudas previamente inoculadas, em ambiente salinizado. As três espécies de FMAs se associam a *J. curcas*, isoladamente e em conjunto. A DM aumentou com a salinidade, indicando que *J. curcas* é uma espécie facultativa. As mudas pré-colonizadas com FMA são uma alternativa para o estabelecimento de *J. curcas* em solos salinizados.

Palavras-chave: fungos micorrízicos arbusculares; simbiose; salinidade; pinhão manso.

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INTRODUCTION

Jatrophas curcas L. is a Euphorbiaceae species with potential for biodiesel production (Nass *et al*., 2007). Its average grain productivity may reach 2.37 t ha^{-1} $year^{-1}$ (Rocha *et al*., 2016). The average oil content of appropriately dry seed on mass basis is 34% (Achten *et al*., 2008; Siqueira *et al*., 2012). It stands out in relation to other oleaginous plants due to its vast edaphoclimatic adaptation, perenniality, and abundance of oil in its seeds (Achten *et al*., 2008). The areas with perspective for the cultivation of this oleaginous plant increases (Ohland *et al*., 2014), as well as the oil production for biodiesel. Cultivation of *J. curcas* is aimed, in particular, to small properties, but not to replace crops or occupy productive areas (de Arruda *et al*., 2004). This species has also been considered as an alternative for revegetation and recovery of abandoned areas, as well as for the occupation of dry and salinized areas (Gübitz *et al*., 1999). An example of such areas is the Pernambuco state in the Brazilian northeast. The area has potential for the production of *J. curcas* (Rocha *et al*., 2016), but is one of the regions that most suffers with salinization (Oliveira *et al*., 2002).

Soil salinization is a growing problem worldwide (Shabala & Munns, 2012). This problem is aggravated in arid and semi-arid regions, where the lack or poor distribution of rainfall causes the evaporation of the low water content of the soil, taking the salt to the root zone (Oliveira *et al*., 2002). Moreover, the toxic effect of excess absorption of ions leads to the rupture of membranes and organelles, besides causing cellular osmotic disequilibrium, which may render the area improper for cultivation (Munns & Tester, 2008).

The association of plants with arbuscular mycorrhizal fungi (AMF) is an alternative to improve crop tolerance to adverse environments (Evelin *et al*., 2009). The AMF penetrate plant roots and their network of hyphae spreads in the soil, highly increasing the surface area for absorption (Silva *et al*., 2001; Latef & Miransari, 2014; Bonfante & Desirò, 2015). However, under saline conditions, the difficulty in obtaining water affects the germination of AMF spores and the symbiosis is not always established (Sheng *et al*., 2008; Aggarwal *et al*., 2012).

Many studies on mycorrhizae associated with *J. curcas* in saline conditions are performed with native fungi without precise definitions of the species present in the inoculums (Kumar *et al*., 2009; Kumar *et al*., 2010; Kumar *et al*., 2015). This leads to a generalization of results and statements on the unspecificity of AMF, indicating that they can associate with almost all plant species. However, studies on the establishment of the relationship between fungi and plants have revealed the opposite, relating different association responses to different species (Kiers *et al*., 2011; Anuroopa & Bagyaraj, 2015). It is observed that the AMF in the soil are not always associated with the crop or promote the best mutualistic benefit (Cesaro *et al*., 2008; Oliveira *et al*., 2011).

The association efficiency between AMF and plants is quantified by the mycorrhizal dependency (MD) index, which is calculated based on the difference between the dry matter of inoculated and uninoculated plants (Gerdemann, 1975). This index can be defined as how much the plant depends on fungus partner to obtain its best growth or production (Azcon & Ocampo, 1981). The MD is an essential characteristic of the plant being classified as non-mycorrhizal when the symbiosis does not occur, mandatory when the plant survival depends on this association, or facultative, when the relationship is intensified in adverse situations (Habte & Manjunath, 1991). This symbiosis also varies according to the age, physiological and nutritional status of the plant, and each species of fungus (Balota *et al*., 2011).

Therefore, this study aimed to verify the association of *J. curcas* with three species of AMF (*Rhizophagus intraradices*, *Gigaspora albida*, and *Claroideoglomus etunicatum*), commonly found in saline soils and test the hypothesis that *J. curcas* L. seedlings, previously inoculated with these AMF, are more tolerant to saline stress by evaluating the development and MD index.

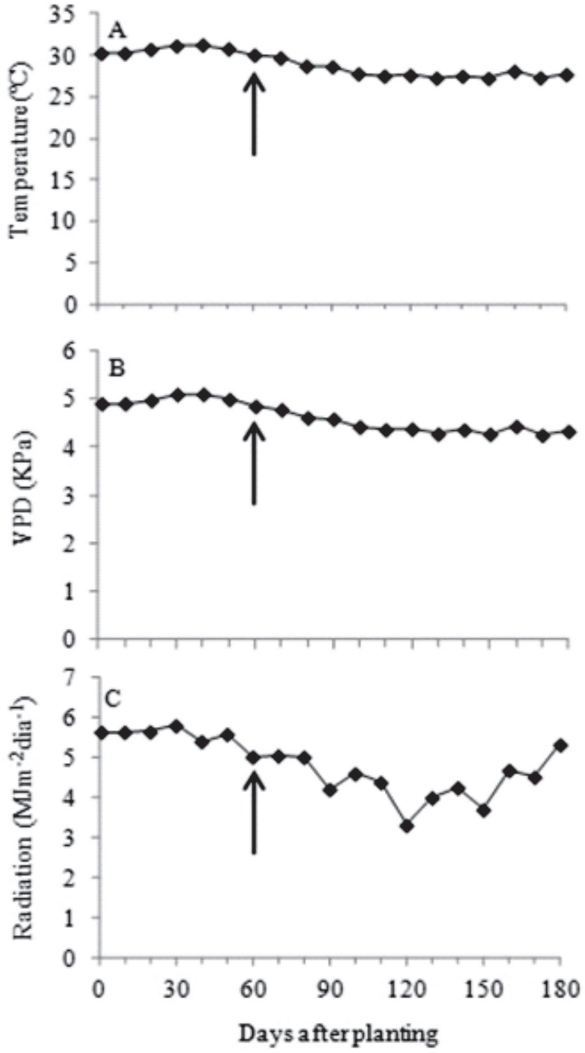
MATERIAL AND METHODS

Study area and soil analysis

This study was carried out in a greenhouse (09°28'S; 35°49W and 127 m a.s.l.). The environmental conditions within the greenhouse were monitored by a WS-GP1 automatic weather station (Delta-T Devices Ltd, Cambridge, England). Data of temperature and relative air humidity were collected every 5 min and the insolation every 10 sec. The vapor pressure deficit was calculated based on the temperature and relative air humidity (Allen *et al*., 1998). During the experiment, the following wheater characteristics were recorded: temperature from 27 to 31 °C, vapor pressure deficit from 4.2 to 5.1 KPa, and radiation from 3.3 to 5.6 MJ m^{-2} day^{-1} (Figure 1).

A sandy-clay Latosol was used in this study (Table 1), which was sieved and autoclaved at 120 ºC for 60 min for the production of seedlings of the first and second trials. To salinate the soil for the second test, part of the same soil was separated and NaCl was added at four different doses: 0.0, 0.6, 1.2, and 2.4 g NaCl kg^{-1} of dry soil. Portions of 17 kg dry soil were arranged in sixty-four 20 L pots. The salt doses were dissolved in water and added to the soil in the pots. Afterwards, the pots were irrigated to the field capacity for better homogenization of the salt. The pots were covered and stored. Monthly, the material of each

pot was mixed to ensure the homogenization of the salt. Soil was incubated in this way for four months. The incubation promoted the increase of the electrical conductivity (EC) of soil saturation extract from 2 to 5, 8, and 10 dS m^{-1} at doses of 0.6, 1.2, and 2.4 g NaCl kg^{-1} of soil, respectively (Table 1).



Averages recorded every 10 days.
The arrow indicates the beginning of salt stress.

Figure 1: Temperature (a), vapor pressure deficit (VPD) (b), and radiation (c), recorded in the greenhouse during the experiment.

The soil EC was estimated according to Richards (1954). The salinized soil was sterilized in a forced ventilation oven at 105 ºC for 48 h. The material was tested to verify the elimination of mycorrhizal fungus propagules according to Miranda (2008). Subsequently, the pots were covered with plastic film and stored in the greenhouse until the transplanting of seedlings.

First trial: mycorrhizal association with J. curcas

The association between fungi and plants was verified by a completely randomized design (first experiment) with five treatments: non-inoculated, inoculated with each of the three AMF species isolated (*Rhizophagus intraradices*, *Gigaspora albida*, and *Claroideoglomus etunicatum*), and inoculated with a mixture of the three fungi (MIX), with six repetitions each.

Jatropha Curcas L. seeds, from an experimental plantation area (09º28'02''S; 35º49'43''W), were washed and disinfected with a sodium hypochlorite solution (2.5%) for 4 min and rinsed three times with distilled water. The isolated cultures of AMF were acquired from the International Collection of Glomeromycota Culture (CICG FURB, Blumenau, SC, Brazil, www.furb.br/cicg) and consisted of: *Rhizophagus intraradices* SPL301A (Schenck and Smith) Walker and Schüâler, *Gigaspora albida* SCT200A (Schenck and Smith), and *Claroideoglomus etunicatum* SCT101A (Becker and Gerd) Walker and Schüâler. About six grams of sorghum roots colonized by each treatment were placed five centimeters below the seeds in 1 L plastic bags containing previously autoclaved non-saline soil.

Ninety days after planting, samples of 1 g of fine roots were collected in each repetition, clarified with 1 M KOH, and stained with trypan blue for the preparation of microscope slides (Miranda, 2008), which were photographed with an image capture system coupled to a BioVídeo microscope (BEL, Brazil).

Second trial: mycorrhizal dependency in J. curcas *seedlings under salt stress.*

The seeds were previously inoculated with mycorrhizae outside the saline environment. After the

Table 1: Chemical characterization of the soil used in the experiment

NaCl	EC	OP	pH	ESP	CEC	Na^+	Al^{3+}	K^+	Ca^{2+}	Mg^{2+}	SB	P
ad.	dSm^{-1}	Mpa		%	$mmol_c/dm^3$							mg/dm^3
0.0	2	-0.1	6	2	100	1	0	3	76	8	87	18
0.6	5	-0.3	6	4	146	6	0	2	114	13	135	17
1.2	8	-0.7	6	11	133	13	0	2	92	14	121	18
2.4	10	-1.2	6	22	154	31	0	3	92	17	142	21

NaCl ad. - NaCl per kg of dry soil; EC - electrical conductivity; OP - osmotic potential; ESP - exchangeable sodium percentage; CEC - cation exchange capacity; SB - sum of bases.

establishment of the symbiosis, the stress was imposed. The design was performed in randomized blocks in a 4 × 2 factorial scheme. The factors were soils with four salinity levels (2, 5, 8, and 10 dS m^{-1}) and pre-inoculated (with mix) and non-inoculated plants, with eight replications.

According to the methodology already described, the seeds were disinfected, inoculated with sorghum roots colonized by the mix of the three species, and planted in fifty plastic bags containing non-saline autoclaved soil. Other fifty plastic bags with noninoculated treatments received the same amount of soil and seeds and no inoculum, a total of hundred bags of seedlings.

To confirm pre-inoculation, microscope slides were prepared (Miranda, 2008). Only the seedlings that showed abundant mycorrhizal colonization in the roots were transplanted to the salinized soil as pre-colonized seedlings. Likewise, only the seedlings that showed absence of fungal structures were considered non-colonized. The same symbiosis verification procedure was repeated at the end of the experiment, to check if there was no change in the colonization status of the seedlings.

Sixty days after planting the seeds, salt stress was imposed. The seedlings were acclimatized for nine days and submitted to the addition of small doses of salt (1/3 of the final concentration) in the irrigation water at intervals of three days between each dose to avoid osmotic shock. After reaching the same concentration of the salts in the pots containing salinized soil, the seedlings were transplanted. The experiment was irrigated only with distilled water and the drained water was collected and put back into the pots through a manifold present in each pot. The humidity in soil was always kept close to field capacity.

The survival of seedlings was monthly checked. After 120 days under saline stress, the plants were collected, separated into root, stem, and leaves, and dried in forced ventilation oven at 65 ºC until constant weight. The mycorrhizal dependency was calculated according to Gerdemann (1975), by the difference between the dry matter of pre-colonized and non-colonized plants, divided by the dry matter of the pre-colonized plants, and expressed as percentage.

Data were subjected to two-way factorial ANOVA with a block effect. Salinity levels were analyzed by regression adjustment, for mycorrhizae factor. They were also categorized and analyzed by the Tukey test, in a factorial scheme, as the presence or absence of mycorrhizae ($P < 0.05$).

RESULTS AND DISCUSSION

The three AMF species were associated with the roots of *J. curcas* in 100% of the inoculated seedlings and a large number of vesicles, arbuscules, and hyphae were observed (Figure 2). The non-inoculated plant roots showed a complete absence of any fungal structure (Figure 2 A), evidencing that the sterilization method was efficient. The *G. albida* arbuscules (Figure 2 B) showed irregular contour, distinguishable from the large globular vesicles present in roots colonized by *R. intraradices* (Figure 2 C) and from the long conspicuous hyphae formed by *C. etunicatum* (Figure 2 D). The same pattern was observed in Schenck & Smith (1982), as well as in records of the international collection of culture of arbuscular mycorrhizal fungi - INVAM (Morton, 2002). The roots inoculated with mix showed a large number of fungal structures with characteristics of the three species (Figure 2 E). Therefore, it is believed that AMF occurred together in the mix treatment.

After 120 days under salt stress, the seedlings of *J. curcas* showed 100% survival in all treatments. In similar assays, using mycorrhizal seedlings of *J. curcas*, the survival values ranged from 38 to 44% in non-mycorrhizal plants and from 45 to 66% in inoculated plants, when exposed to a salinity of 6.4 dS m^{-1} and 7.20 dS m^{-1} for 90 and 60 days, respectively (Kumar *et al.*, 2010; Kumar *et al.*, 2015). Our study suggests that the planting of seedlings in non-saline environment improves the survival of *J. curcas* in saline soils. It is likely that, regardless of the presence of mycorrhizae, seedlings that develop without stress have the possibility to better establish their root system and aboveground part, better tolerating future stress.

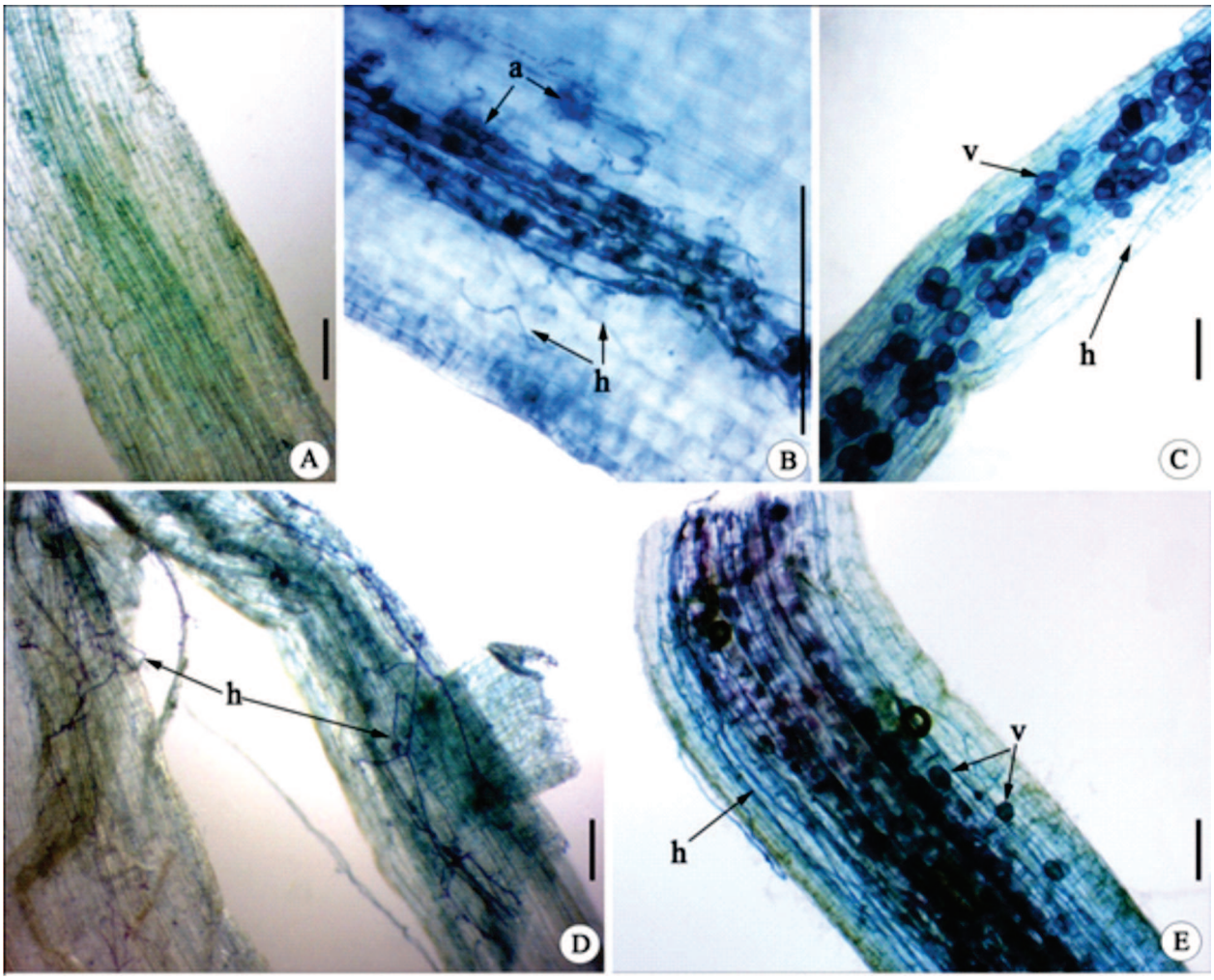
In non-colonized plants, the increase in salinity caused the reduction of the dry weight of leaves, stem, roots, and in the whole plant (Figure 3 A, C, E, G and Table 2). At the highest level of the salinity used (10 dS m^{-1}), the decrease in dry weight was 40% in the plant, 41% in the leaves, 40% in the stem, and 42% in the roots compared with the control treatment. The lower production of dry weight, in function of salinity, is probably because of the physiological drought due to the reduction of the osmotic potential of the soil solution, since the roots were the most affected. The difficulty of water absorption also affects the nutrient uptake, which compromises seedling development (Maia *et al*., 2014). In another study, *J. curcas* seedlings had a decrease of 75% in total dry weight when irrigated with NaCl solution of 4.5 dS m^{-1} (de Oliveira *et al.*, 2010). Growth inhibition is a common response to salt stress and can be used as an indicator of plant tolerance to this condition. According to the classification proposed by Mass (1986), our study suggests that *J. curcas* crop is sensitive to salinity and is not suitable for planting in saline areas.

Inoculation with AMF improved the dry matter production of leaves, stems, roots, and plant in saline

treatments and the results were equal to those found in plants cultivated in non-saline soil (Figure 3 B, D, F, H). However, the mycorrhizae did not increase the dry matter in the control treatment plants. Arbuscular mycorrhizal fungi promote a better absorption of nutrients, compartmentalization of toxic ions, and production of osmolytes that mitigate the effects of salinity (Aggarwal *et al*., 2012). Phosphorus is one of the nutrients that have its absorption increased by the presence of AMF in the roots (Saboya *et al*., 2012). This is a probable reason for the greater development of mycorrhizal plants because P increases the development of *J. curcas* seedlings (Silva *et al*., 2015; Lima *et al*., 2011).

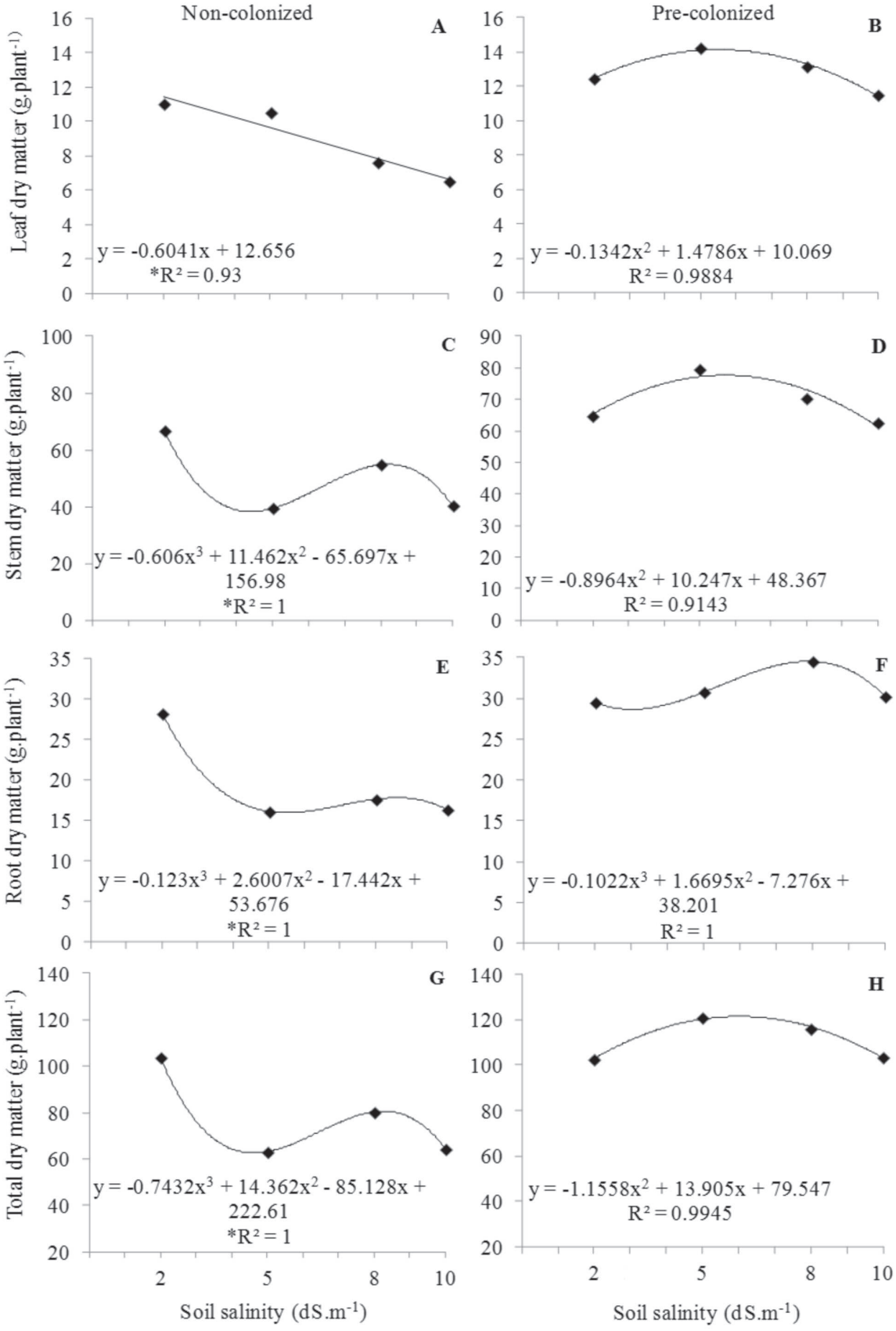
Many studies have reported that mycorrhizal seedlings of *J. curcas* subjected to salt stress have higher dry weight than non-inoculated seedlings (Kumar *et al*., 2009; Kumar *et al*., 2010; Kumar *et al*., 2015). However, these data differ from our results considering that the increment of dry matter, provided by the AMF, decreased with the increase of salinity. In those studies, the increase in biomass production was not significant in the more saline treatments with mycorrhizae (Kumar *et al*., 2009; Kumar *et al*., 2010; Kumar *et al*., 2015). Probably, the pre-colonization of seedlings with AMF before the salt stress has been the main factor that promoted an increase in the dry matter when under stress. The inoculation of AMF in non-saline soil provided an abundant presence of fungi in the roots before the stress condition, resulting in a better effect of symbiosis compared with fungi directly inoculated in saline soils. This method is a simple and economical solution that can allow the planting of *J. curcas* seedlings in relatively saline areas.

The degree of association between fungi and plants indicated by the MD was low in control plants (2 dS m^{-1}), high in plants under EC of 5 dS m^{-1}, and mean in 8 and 10 dS m^{-1} treatments (Table 3). Therefore, the effect of mycorrhizal association in *J. curcas* is affected by stress. This result characterizes *J. curcas* as a facultative species



A: control; B: *Gigaspora albida*; C: *Rhizophagus intraradices*; D: *Claroideoglomus etunicatum*; E: mix.
The arrows indicate arbuscules (a), intercellular vesicle (v), and intracellular hyphae (h). Scale bars = 50μm.

Figure 2: Mycorrhizal roots of *J. curcas* seedlings stained with trypan blue, 90 days after planting.



AMF - arbuscular mycorrhizal fungi.
* Significant at $P < 0.05$.

Figure 3: Dry matter production of *J. curcas* seedlings pre-colonized and non-colonized by AMF, after 120 days of salt stress.

Table 2: Dry matter production of leaves, stems, roots, and total of *J. curcas* seedlings pre-colonized (M+) and non-colonized (M-) by AMF, after 120 days of salt stress

Treatment	Leaf dry matter (g plant^{-1}) S			tem dry matter (g plant^{-1})		
	M-	M+	Salinity factor	M-	M+	Salinity factor
2 dS m^{-1}	9.06A^{a}	8.42B^{a}	8.74A	66.59A^{a}	64.58A^{a}	65.59A
5 dS m^{-1}	7.72B^{b}	10.64AB^{a}	9.18A	39.30B^{b}	79.42A^{a}	59.36AB
8 dS m^{-1}	7.94B^{b}	11.23A^{a}	9.58A	54.70AB^{b}	70.19A^{a}	62.45AB
10 dS m^{-1}	7.83B^{b}	10.71AB^{a}	9.27A	40.20B^{b}	62.45A^{a}	51.32B
AMF factor	8.14^{b}	10.25^{a}		50.20^{b}	69.16^{a}	
	Root dry matter (g plant^{-1})			Total dry matter (g plant^{-1})		
	M-	M+	salinity factor	M-	M+	salinity factor
2 dS m^{-1}	28.21A^{a}	29.51A^{a}	28.86A	103.86A^{a}	102.52A^{a}	103.19A
5 dS m^{-1}	16.11B^{b}	30.79A^{a}	23.45B	63.13B^{b}	120.86A^{a}	91.99AB
8 dS m^{-1}	17.62B^{b}	34.54A^{a}	26.08B	80.26AB^{b}	115.96A^{a}	98.11AB
10 dS m^{-1}	16.35B^{b}	30.24A^{a}	23.29B	64.37B^{b}	103.40A^{a}	83.87B
AMF factor	19.57^{b}	31.27^{a}		77.9^{b}	110.68^{a}	

AMF - AMF - arbuscular mycorrhizal fungi.
Averages followed by the same letter do not differ by the Tukey test ($P \geq 0.05$; n = 8).
Uppercase letters refer to salinity levels (column), lowercase letters compare plants with and without mycorrhizae (line).

Table 3: Mycorrhizal dependency (MD) of *J. curcas* seedlings pre-colonized with arbuscular mycorrhizal fungi and subjected to 120 days of salt stress. Classification according to Habte and Manjunath (1991)

Treatment	MD (%)	Classification
2 dS m^{-1}	1	Low
5 dS m^{-1}	47	High
8 dS m^{-1}	32	Mean
10 dS m^{-1}	39	Mean

concerning the association with the mix of species of AMF used in this study. When sumitted to stress, the plant releases chemical signals that stimulate fungi that are already present in the roots and the association intensifies, reflecting on the production of biomass (Latef & Miransari, 2014; Bonfante & Desirò, 2015).

In another trial, the mycorrhizal dependency of *J. curcas* seedlings directly inoculated in saline soil was 19, 21, and 18%, when under EC of 5.6, 6.8, and 7.2 dS m^{-1}, respectively (Kumar *et al*., 2010). These MD values were lower than those found in our study, in all saline treatments, which may also be related to the pre-colonization with AMF in seedlings in non-saline environment.

In moderately saline soil (5 dS m^{-1}), the salt was sufficient to promote increase of the interaction, although it did not cause an extreme stress to the plant, resulting in plants with higher MD. Also regarding the treatments under EC of 8 and 10 dS m^{-1}, the benefit of mycorrhizae was also evident, but the most extreme salinity probably limited the fungi development, resulting in a lower MD in comparison with treatment under 5 dS m^{-1}. Thus, we suggest that, although salinity is a limiting factor at the time of colonization, moderate stress conditions can improve the mycorrhizal association already established in *J. curcas*. Therefore, the use of pre-colonized seedlings probably allows better results to cultivate this species in saline soil.

CONCLUSIONS

The arbuscular mycorrhizal fungi *R. intraradices*, *G. albida*, and *C. etunicatum* can associate with *J. curcas* roots both individually and together.

Salinity affects *J. curcas* seedlings by reducing the dry matter production.

The pre-colonization with arbuscular mycorrhizal fungi improves the performance of seedlings in saline environments, making them more tolerant.

Moderate salt stress increases the mycorrhizal dependency in *J. curcas* seedlings pre-colonized with mycorrhizal fungi.

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