Citrus R&T (e-ISSN 2236-3122)
http://citrusrt.centrodecitricultura.br
doi: 10.5935/2236-3122.20150001

Artigo/Article

Effects of plant root extracts on growth of Trifoliate orange rootstocks inoculated with arbuscular mycorrhizal fungus under salinity conditions

Andre Freire Cruz*[1]

SUMMARY

This research aimed to evaluate how the plant extracts and arbuscular mycorrhizal fungi (AMF) can alleviate the salinity negative impacts on rootstocks growth. Targeting on this, two experiments were carried out in a greenhouse. In the first one 3 levels of NaCl (0, 75 and 150 mM) and 3 treatments (no inoculation, inoculation with AMF, and AMF with millet root extracts - MRE) were evaluated. The trifoliate orange (*Poncirus trifoliata* Raf.) was used as host plant and *Gigaspora margarita* as inoculum. The AMF colonization, total fresh weight (TFW) and root fresh weight (RFW) of trifoliate orange roots were benefited by the addition of MRE. The second experiment was carried out in the same conditions, except for NaCl levels (0, 300 and 600 mM), and the treatments (no inoculation, inoculation with AMF, and AMF with bahiagrass root extracts - BRE). The alleviation effects of BRE were observed in the TFW and RFW at 0 and 300 mM of NaCl. A third experiment was carried out *in vitro* to evaluate the effect of these extracts on the hyphal growth of AMF under salinity conditions. The petri dishes contained 3 treatments (agar only, with MRE and with BRE) with 3 levels of NaCl (0, 75, and 150mM). Increases in NaCl concentration induced decreases in the hyphal growth. Treatment with MRE and BRE, however, alleviated the effects of NaCl on the inhibition of AMF hyphal growing.

Index Terms: Bahiagrass, Hyphae, Millet, Mycorrhiza.

Efeito de extratos de raízes de plantas no crescimento de *Poncirus trifoliata* inoculado com fungos micorrízicos arbusculares sob condições de salinidade

RESUMO

Este trabalho objetivou avaliar a amortização dos efeitos negativos da salinidade no crescimento de porta-enxertos por extratos radiculares e fungos micorrízicos arbusculares (FMA), e para isso dois experimentos foram conduzidos em casa de vegetação. No primeiro foi avaliado três concentrações de NaCl (0, 75 e 150 mm) e três tratamentos (não-inoculado, inoculado com fungos micorrízicos arbusculares - FMA, e FMA com extratos de raiz milheto

[1] Kyoto Pref. Univ., 1-5 Shimogamohangi-cho, Sakyo-ku, Kyoto 606-8522, JAPAN
*Corresponding author - E-mail: andre@kpu.ac.jp

- MRE). Mudas de trifoliata (*Poncirus trifoliata* Raf.) foram utilizadas como planta hospedeira e o fungo *Gigaspora margarita* como inóculo. A colonização por FMA, matéria fresca total (TFW) e matéria fresca da raiz (RFW) da trifoliata foram beneficiados com a adição de MRE. Um segundo foi feito nas mesmas condições, com diferentes concentrações de NaCl (0, 300 e 600 mm), e os tratamentos (não-inoculado, inoculado com fungos micorrízicos arbusculares - FMA, e FMA com extratos de raiz bahiagrass - BRE). O efeito amortizador do BRE foram observados no TFW e RFW a 0 e 300 mM de NaCl. Um terceiro experimento foi realizado *in vitro* para avaliar o efeito de MRE e BRE sobre o crescimento de hifas do AMF sob condições de salinidade. O MRE e BRE foram fracionados por cromatógrafo de coluna, e os esporos *G. margarita* foram utilizados neste experimento. As placas de Petri continham 3 tratamentos (apenas ágar, ágar com MRE e ágar com BRE) com 3 níveis de NaCl (0, 75, e 150 mM). O aumento da concentração de NaCl induzida a diminuição do crescimento das hifas. O tratamento com MRE e BRE, no entanto, amortizou os efeitos do NaCl sobre a inibição do crescimento de hifas AMF.

Termos de indexação: Bahiagrass, Hifa, Milheto, Micorriza.

INTRODUCTION

Saline soils inhibit or reduce plant survival and development. In these soils the soluble salts accumulate on the surface of the soil, and plant growth is adversely affected. The main problem that faces plant growing in saline soils may be due to the high concentration of salts in the soil solution, which produces a high osmotic concentration (and correspondingly low soil water potential).

AMF may improve the growth of plants under a variety of stress conditions (Allen and Boosalis, 1983), including salinity (Pfeiffer and Bloss, 1988; Poss et al., 1985). In places with relatively low Na contents and diverse plant cover, AMF spores and colonization levels were found to be high (Juniper and Abbott, 1993), the origin of that AMF is from edaphic conditions (Brundrett, 1991). Juniper and Abbott (1992) reported that the increase of NaCl concentration in the soil solution reduced the hyphae growth arisen from spores of *Gigaspora decipiens*, but increased the diameter of these hyphae.

Growth promoting effects of AMF in saline environments have been related to mycorrhiza-mediated enhancements of host plant P nutrition (Poss et al., 1985). Also mycorrhizae effects are not only related to changes in plant P status, but also to mycorrhiza mediated uptake of other nutrients (Graham and Sylvertsen, 1989; Copeman et al., 1996), and improvement of water use efficiency (Baker et al., 1995). Some species and isolates of AMF have demonstrated large variations in salt tolerance (Rosendahl and Rosendahl, 1991).

Exudates and volatile compounds eluted from growing roots promote hyphal growth from spores (Ishii et al., 1997). Some studies have been directed to the role of AMF in salt tolerance (Baker et al. 1995; Gupta and Krishnamurthy, 1996), or to the role of AMF stimulatory compounds in the AMF formation in orchards (Aikawa, 2000; Rutto, 2000). Several chemical compounds in root exudates identified as flavonoids have stimulated the formation of AMF under natural conditions (Siqueira et al., 1991, Ishii et al., 1997) and such compounds occur widely in nature. The saline soils are ideal for attempting to apply AMF and AMF stimulatory substances in order to improve the farming efficiency. Once these substances promote the AMF formation, it suggests that they can indirectly contribute to alleviate the effects of salinity. Hence the present studies were undertaken in an attempt to improve the survival and growth of trifoliate orange rootstocks in saline soil condition by using AMF and their stimulatory substances, which were originated from the millet and bahiagrass root extracts (MRE and BRE), respectively.

MATERIALS AND METHODS

Experiment 1: Effects of MRE on salinity tolerance of trifoliate orange rootstocks

The first experiment was carried out under green house conditions, where seeds of trifoliate orange were sown in trays and after three months the seedlings were transplanted to the pots containing 3.5kg of sand substrate. The substrate was sterilized with methanol and amended with 140 mg kg^{-1} of N, 10 mg kg^{-1} of P, 140 mg kg^{-1} of K and 50 mg kg^{-1} of Mg. The plants were inoculated by adding 5g of an inoculum containing approximately 70 spores of *Gigaspora margarita.* Four weeks after transplanting, plants were watered once a week with a solution containing Sodium Chloride (NaCl), and another solution containing MRE at a level of 40 µl (1g fresh weight equivalent). The MRE was obtained according to the procedures of Ishii et al. (1997). The experimental design was 3 x 3 x 3, factorial experiment, which examined 3 levels of NaCl (0, 75 and 150mM) and 3 treatments (no inoculation, inoculation with AMF , and AMF with MRE) with 3 replicates. Three months later the experiment was harvested. The roots were washed, stained according to the methods of Phillips and Hayman (1970), and observed by using a light microscope to rate the degree of root colonization by AMF (Ishii and Kadoya, 1994). The plant biomass was evaluated by measuring the total fresh weight (TFW) and root fresh weight (RFW).

Experiment 2: Effects of BRE on salinity tolerance of trifoliate orange rootstocks

The second experiment was carried out under green house conditions. The procedures of seedling, transplanting and inoculation were the same as those in the experiment 1. The seedlings were transplanted to plastic pots filled with 1:1:2 mixtures of vermiculite, zeolite and perlite. The fertilization, inoculation and sodium chloride (NaCl) application also followed the procedures of the experiment 1. Once a week BRE, previously extracted (Ishii et al. 1997), at 1g dry weight equivalent were applied to the plants. The design was a 3 x 3 x 4, factorial experiment, which examined 3 levels of NaCl (0, 300 and 600mM) and 3 treatments (no inoculation, inoculation with AMF, and AMF with BRE) with 4 replicates. Three months later the experiment was harvested. AMF colonization was determined by the same methods described in the. TFW and RFW were measured in this experiment by using the same methods of the experiment 1.

Experiment 3: Effects of MRE and BRE on AMF hyphal growth under NaCl stress

This experiment was done *in vitro* conditions with the AMF fungus *Gigaspora margarita.* The sterilization and spores transfer were done according to the procedures of Cruz et al. (2003, 2004). The experimental design was as follows: 1) Agar only, 2) Agar with MRE, and 3) Agar with BRE. Each one was treated with 3 levels of NaCl (0, 300, and 600mM). Two weeks later, hyphal growth was observed by using an image-processing system equipped with a light microscope and a personal computer (Ishii and Kadoya, 1994).

For all experiments above the statistical analysis were proceeded by calculate the mean and standard error, considering the significant effect at 95%.

RESULTS AND DISCUSSION

Experiment 1:

The AMF colonization of trifoliate orange roots in the pots with and without MRE decreased with the increasing of NaCl concentration. At all levels of NaCl the presence of MRE increased the AMF colonization as compared to the single treatment of AMF. At the highest concentration of NaCl (150 mM) the AMF colonization were inhibited in both treatments. Even though the salinity depressed the AMF colonization, at the highest concentration of NaCl in both treatments it could be considered with a satisfactory level, i.e. more than 25% (Table 1).

The results of TFW indicated that the growth of plants reduced from 0 to 150 mM of NaCl in all of the treatments and the rate of reduction was more severe in the plot with no AMF. At 75 and 150 mM of NaCl there was significant effect of the AMF inoculation compared to the control treatment (no AMF). And effect of MRE was positive at 0 and 75 mM of NaCl as compared with the plants with only AMF (Table 1). The increasing of

Table 1. AMF colonization, Total fresh weight and Root Fresh Weight of trifoliate orange as affected by 25% MeOH eluates of millet root extract (MRE) and AMF at 3 levels of NaCl

NaCl (mM)	AMF colonization (%)			Total Fresh Weight (g)			Root Fresh Weight (g)		
	No AMF	AMF[*]	AMF+ MRE	No AMF	AMF	AMF+ MRE	No AMF	AMF	AMF+ MRE
0	0	78.5 (7.2)	81.4 (9.4)	6.1 (0.5)	6.8 (0.4)	7.8 (0.7)	3.5 (0.3)	3.9 (0.2)	4.4 (0.4)
75	0	62.5 (5.9)	64.3 (6.1)	4.7 (0.5)	5.7 (0.5)	7.1 (0.6)	2.9 (0.3)	3.6 (0.4)	3.9 (0.3)
150	0	43.4 (3.1)	44.1 (4.8)	3.7 (0.4)	4.6 (0.4)	4.5 (0.5)	2.4 (0.3)	2.7 (0.3)	3.1 (0.3)

[*] Mean (standard error – SE).

salinity level induced the decreasing of RFW. In the AMF plants the RFW were higher than no AMF plants at all levels of NaCl. And the AMF+MRE treatments had a significant increase compared to the AMF plants in all levels of NaCl.

The results from this study agree with previous data (Baker et al., 1995) showing that mycorrhizal symbiosis increases the growth of plants under low levels of salinity. The mechanisms of mycorrhizal effects under low levels of salinity were shown to be in large part due to an indirect P-mediated effect (Azcón and El-Atrash, 1997). The plant growth and AMF colonization diminished as salinity in the medium increased. By the same way, the hyphal growth decreased with the increase of salinity in the petri dish experiment.

Experiment 2:

The AMF colonization of trifoliate orange roots in the pots with and without BRE reduced responding to the concentrations of NaCl from 0 to 600 mM. At 0 and 600 mM of NaCl the presence of BRE increased significantly the AMF colonization. At the highest concentration of NaCl (600 mM) the AMF colonization were severely inhibited in both treatments, in particular the colonization was very low and could not be considered with a satisfactory level, i.e. less than 25% (Table 2).

The plant growth represented by TFW and RFW was severely inhibited by NaCl treatment in this experiment as compared to the experiment 1. The TFW in all treatments of trifoliate orange rootstocks decreased with the increase of NaCl concentration. Also the growth of no AMF plants was lower in all levels of NaCl compared with AMF and AMF+BRE plants. And in the plants with BRE, the TFW was higher than the plants with only AMF at 0 and 300 mM of NaCl (Table 2). The increasing of NaCl levels led to suppress the growth of RFW. AMF and AMF+BRE treatments were higher than that of the plot with no AMF at 0 and 300 mM of NaCl, and at the same levels, the addition of BRE to the roots induced a positive response compared to the plants with only AMF.

The decrease of mycorrhizal colonization in response to the increase in salinity level suggests that the effects of salinity on mycorrhizal colonization were evident, and at high levels the AMF colonization were severely inhibited (experiment 2). Baker et al. (1995) showed similar results in *Prosopis juliflora* where as the NaCl concentration increased the plant dry mass and AMF colonization decreased. Graham and Syvertsen (1989) reported citrus root colonization by *Glomus intraradices* was unchanged by salinity level, while Pfeiffer and Bloss (1988) reported AMF colonization decreased as salinity increased. The plant growth was affected by salinity treatments, however the presence of AMF reduced the effects of the salt stress, and the MRE and BRE contributed to increase the function of AMF. One possible explanation of this tolerance is that the presence of an AMF fungus alters the osmotic balance in root tissues, since AMF have found to influence the compositions of amino acids and carbohydrates in host plants under salt stress conditions (Rosendahl and Rosendahl, 1991). Maas (1986) found similar results where the tomato growth under field conditions was reduced 50% by salinity.

According to Copeman (1996) and Saranga et al. (1993) mycorrhizal effects could be attributed to improve long-term plant survival under salinity stress by limiting

Table 2. AMF colonization, Total fresh weight and Root Fresh Weight of trifoliate orange as affected by 25% MeOH eluates of Bahiagrass root extract (BRE) and AMF at 3 levels of NaCl

NaCl (mM)	AMF colonization (%)			Total Fresh Weight (g)			Root Fresh Weight (g)		
	No AMF	AMF*	AMF+ MRE	No AMF	AMF	AMF+ MRE	No AMF	AMF	AMF+ MRE
0	0	60.0 (6.2)	71.4 (7.4)	1.5 (0.2)	2.1 (0.3)	3.7 (0.3)	0.7 (0.2)	1.3 (0.2)	1.7 (0.2)
300	0	38.5 (3.9)	42.3 (4.1)	1.4 (0.2)	1.9 (0.2)	2.4 (0.1)	0.6 (0.1)	0.8 (0.1)	1.5 (0.3)
600	0	19.4 (2.1)	21.1 (2.8)	1.2 (0.1)	1.5 (0.1)	2.1 (0.2)	0.5 (0.1)	0.6 (0.1)	1.3 (0.2)

* Mean (standard error – SE).

the Cl^- concentration. Also, it can act in the improvement of water relations and nutrient concentrations. The increase of water resistance to water loss, together with osmotic adjustment, may enable salinized plants to keep the turgor under drought conditions (Azcón and El-Atrash, 1997). Studies on the effect of salinity on AMF plants have shown that AMF roots have higher Na concentrations but also have higher K concentrations and thus maintain a high K/Na ratio, and consequently a osmotic adjustment (Poss et al., 1985). It means that the improvement of tolerance to salinity stress by AMF is due to other mechanisms besides the P nutrition.

Experiment 3:

In the treatments with only agar, the increasing of NaCl concentration depressed the growth of hyphae. However, in the petri dishes with MRE and BRE, the AMF hyphal growth was longer at 0 and 300 mM of NaCl, as compared to those with only agar. The hyphal growth in the petri dishes with MRE was longer than that with BRE at 0 and 300 mM. At the highest level of NaCl (600 mM) the hyphal growth was severely reduced in all of treatments and there was no significant difference between them (Figure 1).

Berta et al. (1990) demonstrated that the increase in the number of adventitious roots and branching of the roots could be a crucial factor for water relations in AMF inoculated plants, which in turn may be relevant to salt tolerance. The mycorrhizal possess specific individual traits with respect to their host tolerance to stress (Ruiz-Lozano et al., 1995). The results from this study suggest that the MRE and BRE can contribute to tolerate soil salt stress conditions by stimulating the AMF activity, and then this symbiont provide its specific benefit to the host.

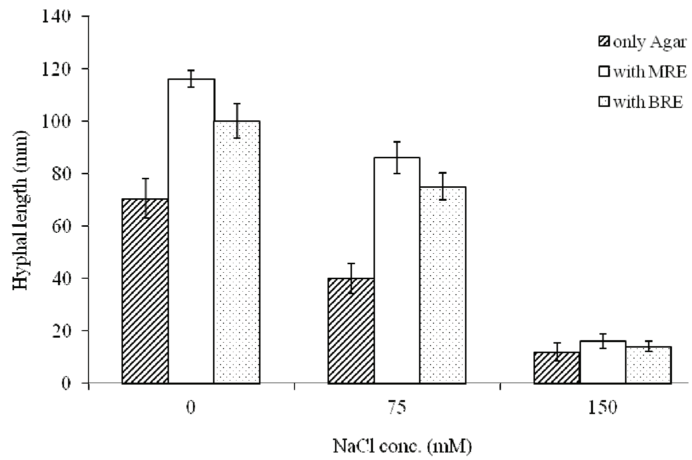

Figure 1. Hyphal lengths of Gigaspora margarita spores cultured with only agar, and 25% MeOH eluates of millet and bahiagrass root extract (MRE and BRE respectively), in media containing 3 levels of NaCl. Vertical bars indicate SE (n=3).

Therefore, the introduction of millet and bahiagrass to citrus orchards where the salinity occurs may be useful for reducing damage caused by NaCl, however, depending on the place the place the appropriate AMF specie should be selected to the rootstock (Watanarojanaporn, et al., 2011; Wang and Wang, 2014).

CONCLUSIONS

Bahiagrass and Millet root extracts were able to alleviate the negative effects of salinity on trifoliate orange growth and AMF colonization. Moreover, the Bahiagrass and Millet extracts could improve the hyphal growth of *Gigaspora margarita in vitro* under salinity conditions.

REFERENCES

Aikawa, J (2000) Studies on the utilization of growth stimulatory substances for vesicular-arbuscular mycorrhizal fungi in citrus orchards. Ph.D. Thesis. Ehime Univ., Matsuyama, Ehime, Japan.

Allen MF & Boosalis MG (1983) Effects of two species of VA mycorrhizal fungi on drought tolerance of winter wheat. New Phytologist 93: 67-76.

Azcón R & El-Atrash F (1997) Influence of arbuscular mycorrhizae and phosphorus fertilization on growth nodulation and N_2 fixation (^{15}N) in *Medicago sativa* at four salinity levels. Biology and Fertility of Soils 24:81-86.

Baker A, Sprent JI & Wilson J (1995) Effects of sodium chloride and mycorrhizal infection on the growth and nitrogen fixation of *Prosopis juliflora*. Symbiosis 19: 39-51.

Berta G, Fusconi A, Trotta A & Scannerini S (1990) Morphogenetic modification induced by the mycorrhizal fungus *Glomus* strain E3 in the root system of *Allium porum* L. New Phytologist 114: 207-215.

Brundrett M (1991) Mycorrhizas in natural ecosystems. Advances in Ecological Research 21: 171-313.

Copeman RH, Martin CA & Stutz JC (1996) Tomato growth in response to salinity and mycorrhizal fungi from saline or non saline soils. Hortscience 31(3): 341-344.

Cruz AF, Ishii T, Matumoto I & Kadoya K (2003) Evaluation of the mycelial network formed by arbuscular mycorrhizal hyphae in the rhizosphere of papaya and other plants under intercropping system. Brazilian Journal of Microbiology 34 (1): 72-76.

Cruz AF, Ishii T, Matsumoto I & Kadoya K (2004) Relationship between arbuscular mycorrhizal fungal development and eupalitin content in bahiagrass roots grown in a Satsuma mandarin orchard. Journal of Japanese Society for Horticultural Science 73: 529–533.

Graham JH & Syvertsen JP (1989) Vesicular-arbuscular mycorrhizas increase chloride concentration in citrus seedlings. New Phytologist 113: 29-36.

Gupta R & Krishnamurthy KV (1996) Response of mycorrhizal and nonmycorrhizal *Arachis hypogaea* to NaCl and acid stress. Mycorrhiza 6: 145-149.

Ishii T & Kadoya K (1994) Effects of charcoal as a soil conditioner on citrus growth and vesicular-arbuscular mycorrhizal development. Journal Japanese Society for Horticultral Science 63: 529-535.

Ishii T, Narutaki A, Sawada K, Aikawa J, Matsumoto I, & Kadoya K (1997) Growth stimulatory substances for vesicular-arbuscular mycorrhizal fungi in Bahia grass (*Paspalum notatum* Flügge.) roots. Plant and Soil 196: 301-304.

Juniper S, & Abbott LK (1992) The effect of a change of soil salinity on growth of hyphae from spores of *Gigaspora decipiens* and *Scutellospora calospora*. Abs. of the Intern. Symp. on Manag. of Mycorrhizas in Agriculture, Horticulture and Forestry. University of Western Australia, Perth, Australia, p 34.

Juniper S & Abbott LK (1993) Vesicular-arbuscular mycorrhizas and soil salinity. Mycorrhizas 4: 45-57.

Mass EV (1986) Salt tolerance of plants. Applied Agriculture Research 1: 12-26.

Watanarojanaporn N, Boonkerd N, Wongkaew S, Prommanop P, Teaumroong N (2011) Selection of arbuscular mycorrhizal fungi for citrus growth promotion and Phytophthora suppression. Scientia Horticulturae. 128 (4): 423-433.

Pfeiffer CM & Bloss HE (1988) Growth and nutrition of guayule (*Parthenium argentatum*) in saline soil as influenced by vesicular-arbuscular mycorrhiza and phosphorus fertilization. New Phytologist 108: 315-321.

Phillips JM & Hayman DS (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.

Poss JA, Pond E, Menge JA & Jarrel WM (1985) Effect of salinity on mycorrhizal onion and tomato in soil with and without additional phosphate. Plant & Soil 88: 307-319.

Rosendahl CN & Rosendahl S (1991) Influence of vesicular-arbuscular mycorrhizal fungi (*Glomus* spp.) on the response of cucumber (*Cucumis sativus* L.) to salt stress. Environmental and Experimental Botany 31:313-318.

Ruiz-Lozano JM & Azcón R (1995) Hyphal contribution to water uptake I mycorrhizal plants as affected by fungal species and water status. Physiologia Plantarum 95: 472-478.

Rutto KL (2000) Studies on the axenic culture of vesicular-arbuscular mycorrhizal fungi as a contribution to sustainable agriculture. M.S. Thesis. Ehime Univ., Matsuyama, Ehime, Japan.

Saranga Y, Zamir D, Marani A & Rudich J (1993) Breeding tomatoes for salt tolerance: Variations in ion concentrations associated with response to salinity. Journal American Society for Horticultural Science 118: 405-408.

Siqueira JO, Safir GR & Nair MG (1991) Stimulation of vesicular-arbuscular mycorrhiza formation and growth of white clover by flavonoid compounds. New Phytologist 118: 87-93.

Recebido: 27/02/2015 – Aceito: 02/02/2016
(CRT 078-15)