

The effect of Dual Application of Arbuscular Mycorrhizal Fungi and Polyamines upon Growth and Nutrient Uptake on Trifoliolate Orange (*Poncirus trifoliata*) Seedlings

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Abstract

The experiment was carried out to study the dual application effects of Arbuscular Mycorrhizal Fungi (AMF) and polyamines on growth and nutrient uptake of trifoliolate orange (*Poncirus trifoliata*) seedlings. The seedlings were colonized by *Glomus versiforme* and irrigated with 320 mL 100 mg/L putrescine, spermidine and spermine, respectively. Two months after exogenous polyamines treatments, both putrescine and spermine applications significantly increased the mycorrhizal colonization, whereas spermidine supplement did not alter the colonization rate. The sole AMF inoculation significantly increased total dry weight, leaf P, K, Ca, Mg, Fe, Cu and Mn contents and root P, K, Ca, Fe, Cu and Zn contents of the seedlings, compared to the non-AMF control. Compared to the sole AMF inoculation, additional putrescine and spermine markedly increased total dry weight, and elevated leaf P and K contents and root P, Mg, Fe and Zn contents. These increases were more significantly in the mycorrhizal seedlings supplied with putrescine than in the mycorrhizal seedlings supplied with spermine. All these polyamines applications did not affect root Cu and Mn contents, but enhanced leaf Mn uptake and root Ca uptake. Spermidine treatment had almost no effects on nutrient uptake and growth of the seedlings. These results suggest that dual application of *G. versiforme* and putrescine could be a feasible procedure for better citrus cultivation.

Keywords: *Arbuscular mycorrhiza*, citrus, nutrient uptake, polyamine, putrescine, trifoliolate orange

Introduction

Arbuscular mycorrhizal (AM) symbiosis are unique associations formed among more than 80% of all terrestrial plants and soil-borne fungi (arbuscular mycorrhizal fungi, AMF) of the phylum Glomeromycota (Kapoor *et al.*, 2008; Quilambo, 2003). The main contribution of the these symbiosis is to assist the host plants with the acquisition of mineral nutrients due to the fine exploration of the rhizosphere by the external hyphae, while AMF obtain 100% of their carbon from the plants (Harrison, 2005). Increasing evidences have shown that AM colonization is initiated by chemical molecules including polyamines (PAs) (Requena *et al.*, 2007).

PAs, mainly diamine putrescine (Put), triamine spermidine (Spd) and tetraamine spermine (Spm), are low molecular mass polycations found in all living organisms (Kumar *et al.*, 1997). In higher plants, they have been proposed to be registered as a new category of plant growth regulators, involved in a wide range of biological processes, including growth, development and stress responses (Liu *et al.*, 2007). In addition, sole PA treatment might regulate nutrient uptake of plants. For example, foliar K concentrations are found to correlate with the accumulation of diamines in the leaves of silver birch and

downy birch (Tytti and Seppo, 2002). El Ghachtouli *et al.* (1995) observed that PAs significantly increased the frequency of mycorrhizal colonization in the myc+ pea lines and the number of appressoria formed in the mycelium. Optimal concentrations (50-200 mg/L) of exogenous PAs had significantly positive effects on spore germination rate and hyphal growth of *Glomus mosseae* and *Gigaspora margarita* in vitro culture conditions (Zhang *et al.*, 2003). So, PAs may act as regulatory factors in plant-AM fungus interactions. However, our knowledge is very limited about the role of PAs in plant-fungal symbiotic interactions.

Trifoliolate orange (*Poncirus trifoliata* (L.) Raf.), a close relative to Citrus, has relatively short root hairs in field (Poerwanto *et al.*, 1989) and are strongly dependent on Arbuscular mycorrhizae (Wu and Xia, 2006). The study was designated to evaluate the effects of dual application of AMF and PAs on mycorrhizal colonization, growth and nutrient uptake of trifoliolate orange seedlings.

Materials and methods

Experimental design

The experiment was conducted in a plastic greenhouse with no heating equipment at the College of Horticulture and Gardening, Yangtze University, Jingzhou, China, be-

tween March and July, 2008. The experiment consisted of five treatments: (1) seedlings inoculated without *Glomus* and exogenous PA (non-AMF + non-PA); (2) seedlings inoculated with *Glomus* only (AMF + non-PA); (3) seedlings inoculated with *Glomus* and additional Put (AMF + Put); (4) seedlings inoculated with *Glomus* and exogenous Spd (AMF + Spd); and (5) seedlings inoculated with *Glomus* and exogenous Spm (AMF + Spm). Three pots per treatment were used, and therefore a total of 15 pots were analyzed (three seedlings per pot). These seedlings were set in completely randomized designs.

Plant and fungal materials

The mature seeds of trifoliate orange were disinfected in 70% ethanol alcohol for 5 minutes, rinsed four times with distilled water and sowed into an autoclaved mix of red soil, vermiculite and perlite (2:1:1, v/v/v). Forty days after sowing, three uniform five-leaf seedlings were transplanted into 20-cm mouth diameter plastic pots, containing the same soil mix. The soil had 5 cm depth in all containers; each mycorrhizal treatment received 15 g (dry weight) *Glomus versiforme* (Karsten) Berch inoculum (BGC NM04B, a mixture of soil, spores, hyphae and root material from Institute of Plant Nutrition and Resources, Beijing Academy of Agriculture and Forestry Sciences). The control sample received the same amount of autoclaved *G. versiforme* inoculum plus 10 mL fungi-free inoculum filtrate.

Exogenous PAs treatments

According to Zhang *et al.* (2003), 100 mg/L Put, Spd and Spm were used as treatment concentration. Exogenous PAs treatments started 16 days after AM inoculation to allow the AM fungus to infect successfully the roots. Three hundred and twenty mL of Put, Spd and Spm fed designed pots. At the same time, the other treatments were irrigated with 320 mL of distilled water.

Parameter measurement

The present experiment ended two months after exogenous PAs treatments, and total dry weights (48 h at 75 °C) were recorded. One-cm length of root pieces, from the middle part of the roots, was cleaned with 10% KOH and stained with 0.05% trypan blue in lactophenol (Phillips and Hayman, 1970). AM colonization was microscopically examined using biological microscope (XSZ-3G, Chongqing, China) and quantified according to the following formula (Wu *et al.*, 2008):

$$\text{AM colonization (\%)} = \frac{\text{root length infected}}{\text{root length observed}} \times 100$$

The leaf and root dry tissues were ground to pass through a 0.5-mm sieve and preserved for the determination of mineral nutrients. Approximate 100 mg of leaf and root dry tissues was used for determining of mineral nutrients. P was analyzed by vanadate-molybdate-yellow method (Chapman and Pratt, 1961) using a UV/visible spectrophotometer, K by flame photometry, and Ca, Mg,

Fe, Cu, Mn and Zn by AI 1200 atomic absorption spectrophotometer (Aurora Instruments Limited, Canada).

Statistical analysis

Data from quantitative parameters were analysed using ANOVA (SAS 8.1). Fisher's Protected Least Significant Difference ($p < 0.05$) was used to compare means. Percentage values were arcsine transformed before statistical analysis.

Results and discussion

Plant roots release a wide range of compounds, which are involved in complex communication processes in the rhizosphere (Steinkellner *et al.*, 2007). Considering the wide array of plants capable of forming AM symbioses, multiple stimulatory compounds might take part in symbiosis establishment (Harrison, 2005). The present study shows that exogenous PAs obviously altered mycorrhizal colonization (Fig. 1). Hereinto, both Put and Spm applications significantly increased mycorrhizal colonization, whereas Spd did not influence the parameter. The results suggested that polyamine species presented different effects on mycorrhizal colonization of trifoliate orange seedlings. Similar results were also noted by El Ghachtouli *et al.* (1995) on pea. The stimulatory effect could be reduced by a polyamine biosynthesis inhibitor (α -DL-difluoromethylornithine) (El Ghachtouli *et al.*, 1996). These results implied that PAs were an important regulatory factor in plant-AM fungus interactions.

The data presented in Fig. 2 indicates that the sole AMF inoculation significantly increased total dry weight of trifoliate orange seedlings compared to the non-AMF + non-PA treatment. Improved growth of plants because of AMF inoculation has been reported earlier (Srinath *et al.*, 2003). Compared to the sole AMF inoculation, additional Put and Spm markedly increased total dry weight. In contrast, total dry weight was similar among each other,

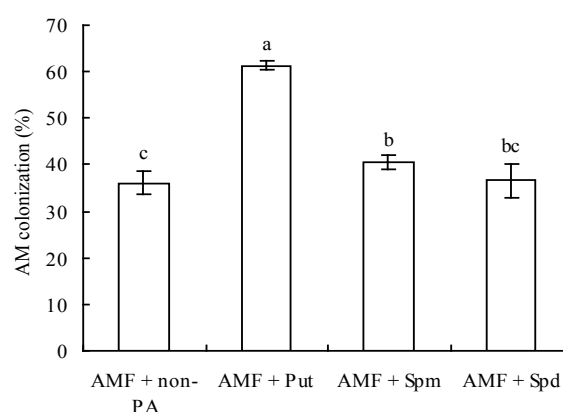


Fig. 1. Effect of *Glomus versiforme* and/or exogenous PAs on AM colonization of trifoliate orange seedlings. Values with different letters are significantly different (LSD test, $p < 0.05$).

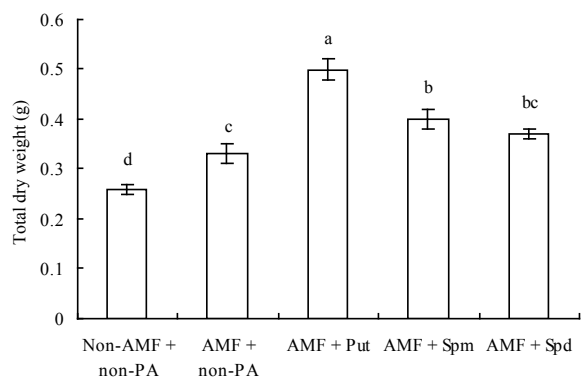


Fig. 2. Effect of *Glomus versiforme* and/or exogenous PAs on total dry weight of trifoliolate orange seedlings. Values with different letters are significantly different (LSD test, $p < 0.05$).

between the sole AMF and the dual AMF + Spd treatment. These results may suggest that the mycorrhizal plant growth promoted by application of PAs depends on exogenous PAs species and is mainly due to enhanced primary root length and the extension of branching (Bais *et al.*, 2000). Additionally, PAs effects on endogenous

use even as sensitive biochemical markers to distinguish the K levels for grapevines (Geny *et al.*, 1997). However, there is not enough data about the effect of AMF and PAs combination on nutrient contents of hosts. From Tab. 1, it clearly showed that the sole AMF inoculation significantly increased leaf P, K, Ca, Mg, Fe, Cu and Mn contents and root P, K, Ca, Fe, Cu and Zn contents. These results are in agreement with the previous studies on citrus (Ortas *et al.*, 2002; Wu and Zou, 2009). Compared to the sole AMF inoculation, three PAs all enhanced leaf Mn uptake and root Ca uptake. Additional Put and Spm significantly increased leaf P and K contents and root P, Mg, Fe and Zn contents of the seedlings, when compared to the sole AMF inoculation. These nutrient improvements due to exogenous Put and Spd applications may result from two explains. One explain is that exogenous Put and Spd treatments stimulates the colonization of trifoliolate orange seedlings by *G. versiforme* (Fig. 1), thus helping hosts to absorb mineral from soils (Ortas *et al.*, 2002; Wu and Zou, 2009). The other explain is that PAs applications, especially Put, took part in root formation and growth (Bais and Ravishankar, 2002), wherein PAs generally influence root growth and differentiation of *Beta vulgaris*

Tab. 1. Effects of *G. versiforme* and/or exogenous PAs on nutrient uptake (mean \pm S.E. in mg/g) of trifoliolate orange (*Poncirus trifoliata*) seedlings

	Treatments				
	AMF + Put	AMF + Spm	AMF + Spd	AMF + non-PA	Non-AMF + non-PA
Leaf					
P	2.84 \pm 0.19a	2.64 \pm 0.07a	2.37 \pm 0.08b	2.35 \pm 0.21b	1.99 \pm 0.12c
K	23.05 \pm 0.26a	23.14 \pm 1.52a	22.63 \pm 1.90ab	20.76 \pm 1.11b	17.87 \pm 0.20c
Ca	14.48 \pm 1.41bc	15.22 \pm 2.08bc	19.01 \pm 1.01a	17.01 \pm 1.48ab	13.82 \pm 1.13c
Mg	8.71 \pm 0.56a	8.20 \pm 0.18ab	7.93 \pm 0.14b	7.78 \pm 0.24b	6.75 \pm 0.16c
Fe	0.476 \pm 0.018a	0.454 \pm 0.034ab	0.448 \pm 0.015ab	0.431 \pm 0.010b	0.382 \pm 0.025c
Cu	0.083 \pm 0.003a	0.079 \pm 0.004ab	0.079 \pm 0.004ab	0.074 \pm 0.005b	0.058 \pm 0.005c
Mn	0.085 \pm 0.002a	0.082 \pm 0.001a	0.082 \pm 0.006a	0.072 \pm 0.003b	0.063 \pm 0.006c
Zn	0.089 \pm 0.009b	0.087 \pm 0.011b	0.125 \pm 0.021a	0.127 \pm 0.031a	0.143 \pm 0.015a
Root					
P	3.56 \pm 0.15a	3.43 \pm 0.16a	2.87 \pm 0.06b	2.64 \pm 0.06b	2.18 \pm 0.23c
K	20.84 \pm 0.79a	20.26 \pm 0.21ab	20.37 \pm 0.00ab	19.74 \pm 0.79b	18.68 \pm 0.15c
Ca	8.65 \pm 0.17a	8.35 \pm 0.18a	8.57 \pm 0.20a	7.43 \pm 0.05b	6.79 \pm 0.17c
Mg	7.06 \pm 0.44a	6.94 \pm 0.37ab	6.11 \pm 0.00bc	6.02 \pm 0.17c	5.75 \pm 0.51c
Fe	1.27 \pm 0.04a	1.27 \pm 0.09a	1.13 \pm 0.03b	1.13 \pm 0.05b	0.98 \pm 0.03c
Cu	0.044 \pm 0.002a	0.044 \pm 0.002a	0.043 \pm 0.002a	0.042 \pm 0.000a	0.038 \pm 0.000b
Mn	0.162 \pm 0.002a	0.169 \pm 0.006a	0.159 \pm 0.010a	0.163 \pm 0.012a	0.159 \pm 0.012a
Zn	0.085 \pm 0.002a	0.084 \pm 0.004a	0.076 \pm 0.001b	0.076 \pm 0.004b	0.066 \pm 0.001c

hormones levels of hosts should be considered, because PAs supplement increased the endogenous IAA levels of *Araucaria angustifolia* embryogenic cultures (Steiner *et al.*, 2007). Evidences gathered in recent years indicate that free PAs levels were closed associated with mineral contents of plants (Delgado *et al.*, 1989; Geny *et al.*, 1997; Tytti and Seppo, 2002; Zhang *et al.*, 2008). PAs can be

and *Tagetes patula* during hairy root cultures (Bais *et al.*, 2000). Additional Spd treatment had almost no effect on nutrient uptake, because Spd did not affect mycorrhizal colonization and plant growth (Fig. 1, Fig. 2). Further studies are to be conducted in order to study the effect of dual AMF and PAs on root morphology of hosts.

Conclusions

In short, among the three PAs, only Put application did not only enhanced mycorrhizal colonization, but also increased plant growth and nutrient uptake of trifoliolate orange seedlings, compared to the sole AMF inoculation. Therefore, mycorrhization combined with Put is to be a feasible measure for better citrus production.

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References

- Bais, H. P., R. Madhusudhan, N. Bhagyalakshmi, T. Rajasekaran, B.S. Ramesh and G.A. Ravishankar (2000). Influence of polyamines on growth and formation of secondary metabolites in hairy root cultures of *Beta vulgaris* and *Tagetes patula*. *Acta Physiol. Plant.* 22:151-158.
- Bais, H. P. and G. A. Ravishankar (2002). Role of polyamines in the ontogeny of plants and their biotechnological applications. *Plant Cell Tiss. Org. Cult.* 69:1-34.
- Chapman, H. D. and P. F. Pratt (1961). *Methods of Analysis for Soils, Plants and Waters*. University of California, California, Riverside.
- Delgado, M. M., I. M. Sanchez-Calle, M. Diaz-Miguel and A. J. Matilla (1989). Effects of polyamines on the ion content and solutes excretion of isolated embryonic axes and the germination rate of seeds of chickpea. *J. Plant Nutr.* 12:509-522.
- El Ghachtouli, N., M. Paynot, J. Martin-Tanguy, D. Morandi and S. Gianinazzi (1996). Effect of polyamines and polyamine biosynthesis inhibitors on spore germination and hyphal growth of *Glomus mosseae*. *Mycol. Res.* 100:597-600.
- El Ghachtouli, N., M. Paynot, D. Morandi, J. Martin-Tanguy and S. Gianinazzi (1995). The effect of polyamines on endomycorrhizal infection of wild-type *Pisum sativum*, cv Frisson (nod⁺myc⁺) and two mutants (nod⁺myc⁻ and nod⁻myc⁺). *Mycorrhiza* 5:189-192.
- Geny, L., M. Broquedis, J. Martin-Tanguy, J.-P. Soyer and J. Bouard (1997). Effects of potassium nutrition on polyamine content of various organs of fruiting cuttings of *Vitis vinifera* L. cv. Cabernet sauvignon. *Am. J. Enol. Vitic.* 48:85-92.
- Harrison, M. J. (2005). Signaling in the arbuscular mycorrhizal symbiosis. *Annu. Rev. Microbiol.* 59:19-42.
- Kapoor, R., D. Sharma and A. K. Bhatnagar (2008). Arbuscular mycorrhizae in micropropagation systems and their potential applications. *Sci. Hortic.* 116:227-239.
- Kumar, A., M. Taylor, T. Altabella and A.F. Tiburcio (1997). Recent advances in polyamine research. *Trend. Plant Sci.* 2:124-130.
- Liu, J. H., H. Kitashiba, J. Wang, Y. Ban and T. Moriguchi (2007). Polyamines and their ability to provide environmental stress tolerance to plants. *Plant Biotech.* 24:117-126.
- Ortas, I., D. Ortakci, Z. Kaya, A. Cinar and N. Onelge (2002). Mycorrhizal dependency of sour orange in relation to phosphorus and zinc nutrition. *J. Plant Nutr.* 25:1263-1279.
- Phillips, J. M. and D. S. Hayman (1970). Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. *Trans. Br. Mycol. Soc.* 55:158-161.
- Poerwanto, R., H. Inoue and I. Kataoka (1989). Effects of temperature on the morphology and physiology of the roots of trifoliolate orange budded with satsuma mandarin. *J. Japan. Soc. Hort. Sci.* 58:267-274.
- Quilambo, O. A. (2003). The vesicular-arbuscular mycorrhizal symbiosis. *Afr. J. Biotech.* 2:539-546.
- Requena, N., E. Serrano, A. Ocón and M. Breuniger (2007). Plant signals and fungal perception during arbuscular mycorrhiza establishment. *Phytochem.* 68:33-40.
- Srinath, J., D.J. Bagyaraj and B.N. Satyanarayana (2003). Enhanced growth and nutrition of micropropagated *Ficus benjamina* to *Glomus mosseae* co-inoculated with *Trichoderma harzianum* and *Bacillus coagulans*. *World J. Microbiol. Biotech.* 19:69-72.
- Steiner, N., C. Santa-Catarina, V. Silveira, E. I. S. Floh and M.P. Guerra (2007). Polyamine effects of growth and endogenous hormones levels in *Araucaria angustifolia* embryogenic cultures. *Plant Cell Tiss. Org. Cult.* 89:55-62.
- Steinkellner, S., V. Lenzemo, I. Langer, P. Schweiger, T. Khaosad, J.-K. Toussaint and H. Vierheilig (2007). Flavonoids and strigolactones in root exudates as signals in symbiotic and pathogenic plant-fungus interactions. *Molecules* 12:1290-1306.
- Tyrti, S. and K. Seppo (2002) Potassium nutrition and free polyamines of *Betula pendula* Roth and *Betula pubescens* Ehrh. *Plant Soil* 238:141-149.
- Wu, Q. S. and R. X. Xia (2006). Arbuscular mycorrhizal fungi influence growth, osmotic adjustment and photosynthesis of citrus under well-watered and water stress conditions. *J. Plant Physiol.* 163:417-425.
- Wu, Q. S. and Y. N. Zou (2009). Mycorrhizal influence on nutrient uptake of citrus exposed to drought stress. *Philipp. Agric. Scientist* 92:33-38.
- Wu, Q. S., R. X., Xia and Y. N. Zou (2008). Improved soil structure and citrus growth after inoculation with three arbuscular mycorrhizal fungi under drought stress. *Eur. J. Soil Biol.* 44:122-128.
- Zhang, G. W., Z.L. Liu, J.G. Zhou and Y. L. Zhu (2008). Effects of Ca(NO₃)₂ stress on oxidative damage, antioxidant enzymes activities and polyamine contents in roots of grafted and non-grafted tomato plants. *Plant Growth Regul.* 56:7-19.
- Zhang, Y., L. Y. Xie, B. Q. Xiong and M. Zeng (2003). Effect of polyamine on growth and development of arbuscular mycorrhizal fungi *in vitro* culture condition. *Mycosyst.* 22:417-423.