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Evaluating Effectiveness of Four Inoculation Methods with Arbuscular Mycorrhizal Fungi on Trifoliate Orange Seedlings

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ABSTRACT

Citrus plants are highly dependent on arbuscular mycorrhizal fungi (AMF). However, mycorrhizal colonization of citrus is relatively low in field. Mycorrhization of seedlings becomes a feasible tool to elevate the colonization. The present experiment was conducted to evaluate the effectiveness of four inoculation methods with *Glomus mosseae* on trifoliate orange [*Poncirus trifoliata* (L.) Raf.] seedlings. At the time of seedlings transplant, the mycorrhizal inoculum was respectively placed as one layer, two layers and core and mixed with the growth substrates. After 152 days of the inoculation, root colonization ranged from 46–65%, and the one-layer mycorrhizal inoculation exhibited the highest mycorrhizal colonization of trifoliate orange. In general, all the mycorrhizal treatments except two-layer inoculation significantly improved some traits of growth and root system architecture (RSA) in addition to marked increase in leaf chlorophyll and soluble sugar concentrations of leaf and root. It suggests that AM symbiosis induced soluble sugar accumulation to sustain growth of both AMF and roots through increasing leaf chlorophyll concentration. The one-layer mycorrhizal inoculation is the best for mycorrhization of trifoliate orange.

Key Words: Arbuscular mycorrhizal fungi; *Glomus mosseae*; Mycorrhizal inoculation methods; Root system architecture; Trifoliate orange

INTRODUCTION

Citrus is an important commercial fruit tree in south regions of China. In citrus rhizosphere, there are various kinds of soil microorganisms such as arbuscualr mycorrhizal fungi (AMF), which can form mutualistic symbiosis with the roots of citrus plants (Hartmann *et al*., 2009; Wu & Zou, 2011). The AM symbiosis helps the host to increase uptake of relatively immobile mineral elements and water through the extraradical mycorrhizal mycelium; in return, the symbiosis receives photosynthetic carbohydrates from the host for sustaining its development (Gosling *et al*., 2006; Javaid, 2009).

In field, citrus plants exhibit fewer root hairs, and its growth strongly depends on AMF (Davies & Albrigo, 1994; Wu & Xia, 2006). Large numbers of potted and field experiments have shown that inoculation with AMF can increase both growth and nutrient uptake of citrus plants, enhance adverse tolerance such as drought and salt stress, and improve fruit quality (Wu & Zou, 2009; Wu *et al*., 2010a, 2011b). Therefore, AM is the "normal" condition of roots in citrus trees. In general, mycorrhizal colonization of citrus plants in field is lower in China than in other countries such as Japan, USA, etc. (Wu *et al*., 2010c). Therefore, increasing mycorrhizal colonization will be a stringent task for Chinese citrus cultivation.

Grafting is a main type of citrus propagation where an excellent citrus cultivar as the scion is inserted into a rootstock. It seems that artificial inoculation in nursery would be a feasible procedure for better AM colonization. Seedlings are grown in sterilized or unsterilized growth substrates inoculated with effective AMF in small nursery beds or containers and planted out when the mycorrhizal colonization is well formed (Bagyaraj, 1992). It is well known that methods of applying AMF inoculum include mixing inoculum with the growth substrate, placing inoculum as one layer, applying it as a core below the seeds, and dipping roots of seedlings in a viscous suspension containing AMF propagules (Habte & Osorio, 2001). Until now, it is not clear which method of inoculation with AMF is efficient for seedlings mycorrhization in citrus nursery.

Trifoliate orange (*Poncirus trifoliata* L. Raf.), a close relative to *Citrus,* is widely used as a citrus rootstock in China. The aim of the present study was to select an effective method of AMF inoculation on trifoliate orange seedlings under the conditions of pot in terms of mycorrhizal development, growth, root system architecture (RSA), chlorophyll and soluble sugar.

MATERIALS AND METHODS

Experimental design: The experimental design was

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completely randomized in a factorial arrangement, consisting of five inoculated methods with AMF: (i) Mixing inoculum with growth substrates (Mix inoculation); (ii) Placing the inoculum as one layer at 8 cm depth below the growth substrates (One-layer inoculation); (iii) Placing the inoculum as two layers respectively at 11 cm and 5.5 cm depth below the growth substrate (Two-layer inoculation); (iv) Applying the inoculum as three cores at 11 cm depth below the growth substrate (Core inoculation); (v) Non-AMF as the control (Non-AMF). Each treatment had three replicates, resulting in a total of 15 pots.

Plant culture: Six germinated seeds of trifoliate orange were moved into a plastic pot (17.5 cm upper mouth diameter × 16.5 cm depth × 13 cm bottom mouth diameter) containing 2.8 kg of autoclaved growth substrates (xanthi-udic ferralsols/vermiculite/sphagnum, 5/1/1, v/v/v) at March 27, 2010. Fifteen gram inoculum of *Glomus mosseae* (Nicol. & Gerde.) Gerdemann & Trappe was applied into the designed pot at the time of transplant. The inoculum contained the infected root segments of *Sorghum vulgare*, spores, extraradical hyphae, and river sand. One month after transplant, every pot was thinned three seedlings. All the seedlings were placed in a non-environmentally controlled plastic greenhouse at Jingzhou, China. The experiment ended at August 26, 2010.

Parameter determinations: Plant height, stem diameter and leaf number per plant were directly determined before plant harvest. Shoots and roots were harvested and then the intact root systems were placed on a glass slide previously supplied with distilled water and scanned with Epson Perfection V700 Photo Dual Lens System (J221A, Indonesia). The scanned images were analyzed by professional WinRHIZO software in 2007 (Regent Instruments Inc., Quebec, Canada) and the traits of RSA were automatically obtained. Once all the roots were scanned, a small quantity of 1-cm root segments from each plant were cleared with 10% KOH and stained with trypan blue (Phillips & Hayman, 1970). The mycorrhizal colonization was quantified using the method described by Wu *et al*. (2008). All the shoots and the remanent roots were oven-dried (75°C, 48 h) and weighted. Soluble sugar concentrations of leaf and root were determined by the anthrone method using sucrose as the standard (Yemn & Willis, 1954). Leaf chlorophyll was extracted with 80% acetone and analyzed at 646 nm and 663 nm (Lichtenthaler *et al*., 1983).

Statistical analysis: Data were analyzed statistically using one-way analysis of ANOVA with SAS. Fisher's protected least significant difference was used to compare the means at 5% level.

RESULTS AND DISCUSSION

Mycorrhizal development: After 152 days of inoculated treatments, root mycorrhizal colonization in AMF seedlings ranged from 46.1% to 65.1%, whilst non-AM structures were found in non-AMF seedlings (Fig. 1). The AMF seedlings with one-layer inoculation exhibited the highest mycorrhizal colonization and those with two-layer inoculation showed the lowest mycorrhizal colonization. The mycorrhizal colonization was not significant differences between the mycorrhizal seedlings with either one-layer inoculation and mix inoculation or two-layer inoculation and core inoculation. The results suggest that one-layer inoculation is an effective method for mycorrhizal trifoliate orange seedlings.

Growth performance: Most studies have confirmed that inoculation with AMF increases growth performance of citrus plants (McGraw & Schenck, 1980; Wu *et al*., 2011b). In the present work, *G. mosseae* generally significantly increased growth performance, including plant height, stem diameter, leaf number, shoot, root and total dry weights, irrespectively of inoculation methods (Table I). It seems that *G. mosseae* presented dramatic effects on growth of trifoliate orange. Herein, mycorrhizal inoculation with one lay showed the best effectiveness on growth improvement. It is possible that since the highest mycorrhizal colonization was in mycorrhizal seedlings with one-lay inoculation, extraradical mycelia as the main nutrient/water-absorbing interface of the plant-soil-mycorrhiza system would uptake more water and nutrients from soil to the host (Leake *et al*., 2004), thus resulting in the growth improvement of the host.

Root system architecture (RSA): RSA, the spatial configuration of a root system in soil, is used to describe the shape and structure of root systems and will determine the ability of a plant to secure edaphic resources (de Dorlodot *et al*., 2007). In general, RSA shows a high degree of plasticity and can be regulated by various abiotic and biotic factors, including AMF (Lequeux *et al*., 2010). Our present study showed that all the mycorrhizal treatments except two-layer inoculation generally significantly increased root length, root projected area, root surface area, root volumn, and numbers of both branches and crossings in trifoliate orange seedlings (Table II). In addition, the mycorrhizal inoculation

Fig. 1: Root mycorrhizal colonization of trifoliate orange seedlings inoculated with different mycorrhizal inoculations. Means±SD (*n*=3) followed by the same letter above the bars are not significantly different at *P*<0.05

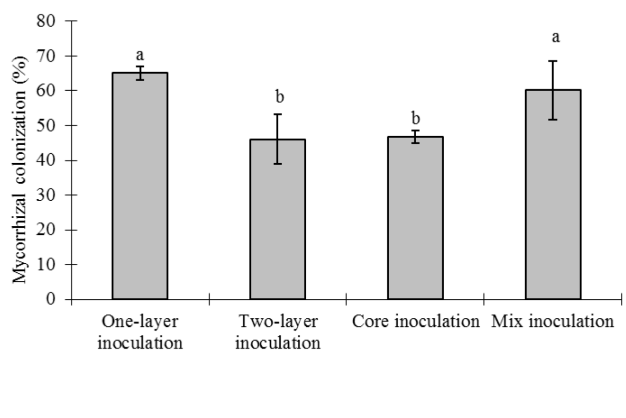


Table I: Influence of different inoculated methods on plant performance of trifoliate orange seedlings

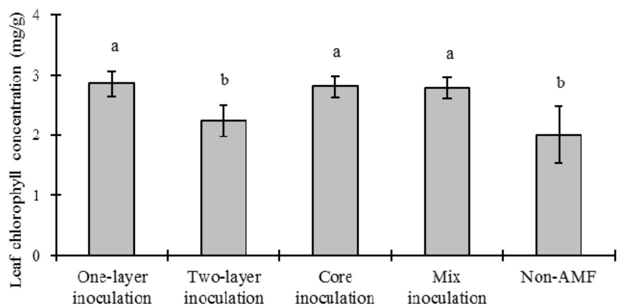
Inoculation method	Plant height (cm)	Stem diameter (cm)	Leaf number per plant	Dry biomass (g/plant)		
				Shoot	Root	Total
One-layer inoculation	33.2±1.7a	0.394±0.004a	26.4±1.4ab	1.04±0.02a	0.60±0.02a	1.64±0.01a
Two-layer inoculation	32.3±1.4a	0.353±0.006b	27.7±1.2a	0.88±0.04b	0.40±0.02d	1.28±0.03bc
Core inoculation	29.0±1.0b	0.352±0.014b	24.4±2.0bc	0.84±0.04b	0.48±0.02b	1.32±0.05b
Mix inoculation	29.4±2.3b	0.360±0.017b	22.9±1.3c	0.84±0.07b	0.45±0.02c	1.29±0.08b
Non-AMF	23.2±0.6c	0.328±0.006c	18.9±1.3d	0.75±0.02c	0.44±0.02c	1.19±0.02c

Table II: Influences of different inoculated methods on some traits of root system architecture of trifoliate orange seedlings

Inoculated method	Root length (cm)	Root projected area (cm^2)	Root surface area (cm^2)	Average diameter (mm)	Root volume (cm^3)	Branches	Crossings
One-layer inoculation	610±15a	30.0±2.3a	94.1±7.2a	0.49±0.03a	1.16±0.05a	3144±365a	642±35a
Two-layer inoculation	475±25c	20.0±1.8c	62.7±5.6c	0.42±0.02b	0.68±0.04c	2237±103b	521±9b
Core inoculation	571±3b	24.1±0.3b	75.7±1.0b	0.42±0.01b	0.80±0.02b	2487±206b	551±24b
Mix inoculation	613±9a	25.0±0.9b	78.6±2.7b	0.41±0.02b	0.81±0.07b	2396±232b	514±51b
Non-AMF	478±20c	20.7±0.6c	64.9±2.0c	0.43±0.03b	0.71±0.07c	1765±37c	327±25c

Note: Means±SD (*n*=3) followed by the same letter within a column are not significantly different at $P<0.05$

Fig. 2: Leaf chlorophyll concentration of trifoliate orange seedlings inoculated with different mycorrhizal inoculations. Means±SD (*n*=3) followed by the same letter above the bars are not significantly different at *P*<0.05



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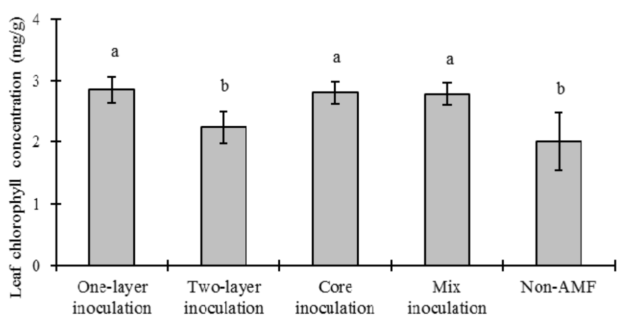

with one layer significantly increased root average diameter by 14.0% and other inoculations did not affect root average diameter, compared to the non-AMF control. The result is in agreement with the findings of Padilla and Encina (2005), who found that *G. intraradices* inoculation with mixing method increased total root length and adventitious root length of adult *Annona cherimola*. Improvement of RSA by AMF was also observed in red tangerine (*Citrus tangerine*) colonized by *G. mosseae* or *Paraglomus occultum* under salt stress conditions (Wu *et al*., 2010a) and in *G. versiforme*-colonized trifoliate orange seedlings supplied with exogenous putrescine (Wu *et al*., 2010b). In the present mycorrhizal treatments, one-layer inoculation could induce the best improvement of RSA, implying that one-layer mycorrhizal inoculation is propitious to improvement of RSA in trifoliate orange seedlings.

Leaf chlorophyll: In the present study, except that the mycorrhizal treatment with two-layer inoculation did not affect leaf chlorophyll concentration, all the mycorrhizal inoculations notably increased leaf chlorophyll concentration, compared to non-AMF control (Fig. 2). Herein, mycorrhizal treatment with one-layer inoculation exhibited the highest leaf chlorophyll concentration (2.85 mg/g). A similar result is also found in the *Pogostemon patchouli* infected with *Acaulospora scrobiculata*, *Gigaspora margarita*, *G. aggregatum*, *G. geosporum*, *G. mosseae*, *Sclerocystic pakistanika*, and *Scutellospora heterogama* (Selvaraj *et al*., 2009). Malekzadeh *et al*. (2007) proposed that increased P concentration induced by AM symbiosis positively affected leaf chlorophyll concentration. The promotion of chlorophyll formation in mycorrhizal plants may presumably reflect more photosynthesis to meet the carbon requirements of AM symbiosis (Meenakshisundaram & Santhaguru, 2011).

Soluble sugar: Since AM symbiosis must acquire photosynthetic carbohydrates of the host for sustaining its development, the symbiosis might regulate carbon allocation (Schaarschmidt *et al*., 2007). From the Fig. 3, we observed that all the mycorrhizal inoculations except two-layer inoculation significantly increased soluble sugar concentrations of leaf and root, compared to the non-AMF control. Mycorrhizal treatments with one-layer inoculation, core inoculation and mix inoculation increased soluble sugar concentrations of leaf respectively by 11.0%, 5.4% and 8.5%, and those of root by 27.3%, 16.5% and 15.3%. The result is consonant with the findings of Wu *et al*. (2011a), who observed that *G*. *mosseae* significantly increased soluble sugar concentrations of leaf and root in red tangerine. In addition, root growth is assumed to be sustained by photosynthetic carbohydrates (Eissenstat & Duncan, 1992). The present result suggests that the mycorrhizal symbiosis obviously induced the accumulation of soluble sugar to sustain growth of both AMF and roots through increasing leaf chlorophyll concentration.

The present study also showed that soluble sugar allocation to root was 48.5% in the non-AMF seedlings, 51.9%, 46.7%, 50.9% and 50.0% in the mycorrhizal seedlings with one-layer inoculation, two-layer inoculation, core inoculation and mix inoculation, respectively. Due to the highest mycorrhizal colonization occurred in the mycorrhizal seedlings with one-layer inoculation, root mycorrhizas must require a large supply of carbohdyrates. Therefore, the mycorrhizal seedlings with one-layer inoculation presented the highest soluble sugar allocation to root. A similar result is observed in *Acaulospora longula*-, *G*. *intraradix*-, and *Gigaspora margarita*-colonized *Paspalum notatum* conducted by Douds and Schenck (1990).

CONCLUSION

As stated above, using the one-layer mycorrhizal inoculation induced the highest mycorrhizal colonization of trifoliate orange seedlings. All the mycorrhizal treatments except two-layer inoculation generally improved some traits of both growth and RSA, and also notably increased leaf chlorophyll and soluble sugar concentrations of leaf and root. Our study suggests that the mycorrhizal symbiosis induced the accumulation of soluble sugar to sustain growth of both AMF and roots through increasing leaf chlorophyll concentration. The one-layer mycorrhizal inoculation is the best for mycorrhization of trifoliate orange among the four treatments.

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