

Effects of Arbuscular Mycorrhizal Fungi on the Growth and Antioxidant Enzymes of Micropropagated Citrus *

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Abstract Inoculation effects with the arbuscular mycorrhizal (AM) fungi, *Glomus versiforme* (Karsten) Berch and *G. mosseae* (Nicol. & Gerd.) Gerdemann & Trappe, on the growth, carbohydrate and antioxidant enzymes were analyzed in micropropagated trifoliate orange (*Poncirus trifoliata* (L.) Raf.) in pots under greenhouse conditions. After 300 days' inoculation, the root colonization and the numbers of arbuscules, vesicles and entry points were the highest in the 2nd lateral roots inoculated with *G. versiforme* and in the 1st lateral roots colonized by *G. mosseae*. Stem diameter, leaf area, leaf number per plant, root volume, shoot and root dry weights, chlorophyll and carotenoid contents markedly increased under inoculated conditions. Inoculated plants showed the greater soluble sugar and total non-structural carbohydrate (NSC) contents in leaves and roots, and superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) activities were higher in mycorrhizal plants than those in non-mycorrhizal plants. However, a severe depression was found in the soluble protein contents of mycorrhizal leaves and roots. *G. versiforme* was more effective than *G. mosseae* in growth and carbohydrate increments, while *G. mosseae* was more effective than *G. versiforme* in antioxidant enzymes increments. Tab 4, Ref 26

Keywords arbuscular mycorrhizal fungi; citrus; antioxidant enzyme; carbohydrate

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丛枝菌根真菌对柑橘组培苗生长和抗氧化酶的影响 *

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摘要 在温室条件下以枳 [*Poncirus trifoliata* (L.) Raf.] 组培苗为试材,研究了接种 *Glomus versiforme* 和 *G. mosseae* 对其生长、碳水化合物和抗氧化酶的影响。结果表明,接种 *G. versiforme* 的组培苗和接种 *G. mosseae* 的组培苗分别在第二级侧根和第一级侧根中观察到最高的菌根侵染率、泡囊数、丛枝数和侵入点。两种丛枝菌根真菌都显著提高了茎粗、叶面积、叶片数、根系体积、地上部干重、地下部干重、叶绿素和类胡萝卜素含量。两种丛枝菌根真菌显著促进了叶片和根系可溶性糖以及总的非结构碳水化合物含量。丛枝菌根真菌也提高了叶片和根系中 SOD、POD 和 CAT 活性,但显著抑制了叶片和根系中可溶性蛋白含量。*G. versiforme* 对柑橘组培苗生长和碳水化合物的促进效果较好;*G. mosseae* 对组培苗抗氧化酶的促进效果较好。表 4 参 26

关键词 丛枝菌根真菌; 柑橘; 抗氧化酶; 碳水化合物

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The arbuscular mycorrhizal (AM) symbiosis is a universal symbiotic association of AM fungi and the roots of terrestrial plants. The ancient fungi could infect approximately 90% of the earth's land plant species [1]. It has been well documented that arbuscular mycorrhizal fungi (AMF) greatly enhance the growth of host plant by improving mineral nutrition, especially phosphorus, and water uptake or transport [2-5]. Fidelibus *et al.* [6] reported that four different *Glomus* species stimulated root growth (dry weight and length) in citrus (*Citrus volkameriana*) seedlings, and leaf phosphorus was higher in AM plants than in non-mycorrhizal

plants. AM fungal inoculation might affect antioxidant enzymatic activity of host plants under well-watered or water stress conditions [7-10]. The roots of mycorrhizal soybean (*Glycine max*) showed higher glutathione reductase (GR) activity than non-mycorrhizal roots [10]. However, superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) activities in nodules were lower in mycorrhizal soybean plants than those in non-mycorrhizal plants under water stress conditions [10].

Micropropagation is a good tool for rapid propagation of young plants. The technique has been used for propagating fruit trees and has become a standard procedure in many commercial nurseries. Whereas, micropropagated plants usually lacked of AM symbiosis in roots [11]. AMF have been successfully applied to many micropropagated fruit plants, such as grapevine, kiwifruit, persimmon, apple, pear, peach, plum, oil palm, banana, pineapple, pista-

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chios, plum, avocado and olive^[11-14]. Main positive effects include the avoidance of transplanting shock, shorter weaning phase, higher plant growth and modifications of root morphology^[11].

Most citrus varieties, such as sour orange, trifoliate orange, carrizo citrange, swingle citrumelo and cleopatra mandarin, have rare and short root hairs, and are strongly dependent on AMF, which mostly belong to *Glomus* species^[15-17]. Trifoliate orange is a major citrus rootstock used in China. However, interaction between AMF and micropropagated trifoliate orange has not been reported until now. Only Quatrini *et al.*^[11] reported the influences of native AMF and *Glomus mosseae* on acclimatization and development of micropropagated *Citrus limon* (L.) Burm.

The objective of this study is to evaluate the effects of two AMF fungi, *Glomus mosseae* and *G. versiforme*, on the growth and carbohydrates of micropropagated trifoliate orange under greenhouse conditions. Meanwhile, antioxidant enzymes are also determined in order to discuss the avoidance of transplanting shock.

1 Material & Methods

1.1 Plant culture, plant inoculation and growth conditions

Mature seeds of trifoliate orange (*Poncirus trifoliata* (L.) Raf.) were surface-sterilized with mercuric chloride (HgCl_2 , 0.1%) for 15 min. After 3~4 rinses in sterile distilled water, the seeds were aseptically inoculated into the MT culture medium^[18] for germination, and placed in a growth chamber at $(25 \pm 1)^\circ\text{C}$ under 16 h photoperiod. 28-day-old germinated plantlets *in vitro* were transplanted into plastic pots (15 cm \times 20 cm) filled with 2.950 kg autoclaved mixture of yellow soil, vermiculite and sphagnum (2 : 2 : 1, V/V/V) at pH 6.3, and with 15.0 g kg^{-1} organic matter, 22.38 mg kg^{-1} available phosphorus, $300.90 \text{ mg kg}^{-1}$ alkali hydrolyzable nitrogen, $141.72 \text{ mg kg}^{-1}$ available potassium, and 25.9% most field soil capacity. Two mycorrhizal species, *Glomus versiforme* (Karsten) Berch and *G. mosseae* (Nicol. & Gerd.) Gerdemann & Trappe, obtained from the Institute of Plant Nutrition and Resources, Beijing Academy of Agriculture and Forestry Sciences, China, were placed 5 cm below roots. Inoculation dosage was approx. 2 000 spores per pot. Experimental pots were placed in a greenhouse under natural light conditions that had no temperature control. The average day/night temperature was 24/17 $^\circ\text{C}$ during the experiment; the relative humidity was 60%~95%.

1.2 Experiment design

Plants were arrayed in a randomized complete block design of three treatments (*G. mosseae*, *G. versiforme* and non-mycorrhizal control (NM)). Each treatment was replicated nine times (or pots). Each pot was transplanted with five micropropagated citrus plantlets.

1.3 Measurements

After 300 days' transplantation, mycorrhizal and non-mycorri-

zal plants were harvested from pots and the substrate was removed thoroughly from roots. Plant height, stem diameter, leaf number, single leaf area and root volume were recorded. Half of the plants were separated from shoot and root, and oven dried at 75°C for 48 h. The dry weights of shoot and root were recorded.

Mycorrhizal developments of different morphological roots were investigated. Firstly, only plant roots were separated at four levels, that is, taproots, 1st lateral roots, 2nd lateral roots and 3rd lateral roots. The 1st lateral roots here were defined all the first embranchments from taproots. Similarly, the 2nd lateral roots referred to all the second embranchments from taproots or the first embranchments from the 1st lateral roots, and the 3rd lateral roots all the third embranchments from taproots or the first embranchments from the 2nd lateral roots. Secondly, the roots were immersed into tap water for 6 h, washed carefully by tap water, cut into 1 cm long root pieces and fixed by formalin-acetic acid-alcohol (FAA) solution (at least 24 h). Whereafter, root samples were cleared with 10% (w/V) KOH solution and stained with 0.05% trypan blue in lactic acid^[19]. The root colonization was observed under microscope. At the same time, the numbers of AM fungi entry points, vesicles and arbuscules were calculated in the infected roots. Finally, the root colonization was counted by the following formula: the root colonization (%) = root length infected / root length observed $\times 100$.

Soluble sugar and soluble starch contents of leaves and roots were measured by anthrone method^[20-21]. The total non-structural carbohydrates (NSC) were the sum of soluble sugar and soluble starch. Chlorophyll, carotenoid, soluble protein, SOD and peroxidase (POD) were measured using the methods described by Li^[20]. CAT activity was determined using the method of Chen and Wang^[21] with slight modification. 0.4 g fresh sample was homogenized in 5 mL of phosphate buffer (0.1 mol L^{-1} , pH 7.8) and transferred into 25 mL volumetric flask with phosphate buffer. Homogenate was centrifuged at $4\,200 \times g$ for 10 min at 4°C , and the supernatant was used for the CAT assay. The reaction mixture (3 mL) consisted of 0.2% (V/V) H_2O_2 , distilled water and 0.1 mL supernatant. Variation in the absorbance was determined at 240 nm during 1 min incubation. Blank reactions consisted of reaction mixture with 0.1 mL phosphate buffer instead of the supernatant. The absorbance decreased 0.01 was defined an enzymatic activity unit.

1.4 Statistical analysis

Variations of the experimental data were analyzed by ANOVA using Statistical Analysis System (SAS) 8.1 software. Averages were compared by least significant difference test ($P < 0.05$).

2 Results

2.1 Mycorrhizal colonization

The roots of non-mycorrhizal plants were observed after 300 days' transplantation, indicating the absence of AM (Table 1). However, the *G. mosseae*-inoculated and *G. versiforme*-inoculated roots of micropropagated plants were infected (Table 1). The

average root colonization varied from 18.90% to 24.94%. The AM colonization in different morphological roots was variance. In *G. versiforme* – inoculated roots, the AM colonization and the numbers of entry points, vesicles and arbuscules in different morphological roots were the highest in the 2nd lateral roots and lowest in taproots. The development of *G. mosseae* in roots was the high-

est in the 1st lateral roots and lowest in the 3rd lateral roots. The root colonization was higher in all the different morphological roots colonized by *G. versiforme* than that in the corresponding *G. mosseae* – colonized roots. The result indicated that *G. versiforme* was more infective than *G. mosseae* for micropropagated citrus plantlets.

Table 1 Effect of AMF on the AM fungal development in different morphological roots of micropropagated trifoliate orange after 300 days' inoculation

Root level	Root colonization (r/%)		Entry points (n/cm ⁻¹ root length)		Vesicles (n/cm ⁻¹ root length)		Root colonization (r/%)	
	<i>G. versiforme</i>	<i>G. mosseae</i>	<i>G. versiforme</i>	<i>G. mosseae</i>	<i>G. versiforme</i>	<i>G. mosseae</i>	<i>G. versiforme</i>	<i>G. mosseae</i>
Taproot	15.27b	13.26b	1.5a	1.6a	0.9c	0.7bc	1.5a	1.3b
1 st lateral root	33.85a	31.86a	3.9a	4.5a	2.4ab	2.0a	4.4a	5.6a
2 nd lateral root	35.72a	19.64b	4.2a	6.6a	2.6a	1.9ab	4.5a	3.9a
3 rd lateral root	14.94b	10.84b	3.6a	1.9a	1.2bc	0.2c	3.3a	1.1a
Average value	24.94	18.90	3.3	3.7	1.8	1.2	3.4	3.0

The means in a column followed by different letters are significantly different at 5% level (LSD test). The same below

2.2 Effect of AMF on plant growth

Table 2 shows that leaf number, stem diameter, leaf area, root volume, root dry weight and chlorophyll in mycorrhizal plants were notably higher than those in non-mycorrhizal plants, though no marked difference was observed between *G. versiforme* – inoculated and *G. mosseae* – inoculated plants. Plant height was higher in *G. versiforme* – inoculated plants than that in the other treated plants, while no significant difference was detected between *G. mosseae* – inoculated and non-mycorrhizal plants. *G. versiforme* and *G. mosseae* treatments significantly increased the shoot dry weight, the total weight and the carotenoid content, compared with non-mycorrhizal treatment. *G. versiforme* – inoculated plants had

higher shoot and total dry weights than *G. mosseae* – inoculated plants. All of the results suggested that *G. versiforme* was more effective than *G. mosseae* in increasing growth variates.

2.3 Effect of AMF on plant carbohydrate status

The mycorrhizal inoculations significantly increased the soluble sugar content of leaves and the NSC contents of leaves and roots (Table 3). The soluble sugar and soluble starch contents of roots were higher in *G. versiforme* – inoculated plants than those in non-mycorrhizal plants, but there was no difference between *G. mosseae* – inoculated and non-mycorrhizal plants. The soluble starch and NSC contents of leaves were higher in *G. versiforme* – inoculated plants than those in *G. mosseae* – inoculated plants.

Table 2 Effect of AMF on the vegetative characteristics of micropropagated trifoliate orange after 300 days' inoculation

Treatment	Plant height (h/cm)	Leaf no. per plant	Stem diameter (d/cm)	Leaf area (A/cm ²)	Root volume (V/cm ³)	Shoot dry weight (m/g)	Root dry weight (m/g)	Total dry weight (mt/g)	Chlorophyll content (r/%)	Carotenoid content (r/%)
<i>G. versiforme</i>	36.43a	33a	0.283a	4.71a	2.37a	0.72a	0.30a	1.02a	3.02a	0.46b
<i>G. mosseae</i>	30.10b	30a	0.273a	4.60a	2.17a	0.61b	0.28a	0.89b	3.12a	0.50a
NM	26.66b	22b	0.250b	3.66b	1.70b	0.50c	0.23b	0.73c	2.80b	0.43c

Table 3 Effect of AMF on the carbohydrate status of leaves and roots in micropropagated trifoliate orange after 300 days' inoculation

Treatment	Soluble sugar (w/g kg ⁻¹ , FW)		Soluble starch (w/g kg ⁻¹ , FW)		NSC (w/g kg ⁻¹ , FW)	
	Leaves	Roots	Leaves	Roots	Leaves	Roots
<i>G. versiforme</i>	64.4a	57.1a	103.5a	27.6a	167.6a	84.6a
<i>G. mosseae</i>	62.9a	47.1ab	70.3b	24.4ab	133.2b	65.1a
NM	46.7b	14.9b	51.9b	23.5b	98.6c	38.4b

2.4 Effect of AMF on the antioxidant enzymatic activities and the soluble protein content

Two mycorrhizal fungal inoculations markedly decreased the soluble protein contents of leaves and roots, but no difference was observed between *G. versiforme* – colonized plants and *G. mosseae* – colonized plants (Table 4). SOD activity was 22% ~ 67% higher in the mycorrhizal leaves than that in the non-mycorrhizal leaves. Although SOD activity in the mycorrhizal roots was 11% ~

16% higher than that in the non-mycorrhizal roots, no significant difference was observed among all treatments. The POD and CAT activities of leaves were significantly higher in mycorrhizal plants than those in non-mycorrhizal plants. *G. versiforme* – inoculation did not affect the POD activity of roots, while *G. mosseae* – inoculation notably increased the POD activity of roots. AM inoculations did not affect the CAT activity of roots.

Table 4 Effect of AMF on soluble protein contents, and superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) activities of leaves and roots in micropropagated trifoliate orange after 300 days' inoculation

Treatment	Soluble protein ($\mu\text{g mg}^{-1}$, FW)		SOD (A/U g^{-1} , FW)		POD (A/U g^{-1} , FW)		CAT (A/U g^{-1} , FW)	
	Leaves	Roots	Leaves	Roots	Leaves	Roots	Leaves	Roots
<i>G. versiforme</i>	10.15b	3.28b	280.55ab	283.46a	196.43a	145.56ab	550.60b	316.67a
<i>G. mosseae</i>	10.05b	3.34b	384.66a	296.21a	188.49a	174.44a	744.05a	416.67a
NM	10.48a	3.95a	230.33b	255.44a	122.02b	116.70b	446.43c	301.93a

3 Discussion

In our study, AM development was dissimilar in different morphological roots. AM development was the highest in the 2nd lateral roots inoculated with *G. versiforme* and in the 1st lateral roots inoculated with *G. mosseae* (Table 1). The taproot diameter was the largest in all the morphological roots and the formation of the 3rd lateral roots was the latest, resulting in the lower AM development. Yao, *et al.* [22] reported that more 1st lateral roots were observed in *G. intraradices* or *G. margarita* - inoculated litchi seedlings. Moreover, the free and bound indole-3-butyric acid (IBA) was increased at different stages of mycorrhizal maize plants, accompanying with increased percentage of lateral roots [23]. Thus, the 1st and 2nd lateral roots of micropropagated citrus plantlets should be largely induced during transplantation, in order to acquire the greater mycorrhizal development or IBA.

AM is a mutually beneficial association between AMF and host plant roots. AM is capable of providing water and minerals for host plant, and host plant transfers carbohydrate to AM. In the study, AM inoculation was able to largely accumulate soluble sugar, soluble starch and NSC contents of mycorrhizal roots (Table 3). The higher carbohydrate in AM roots was propitious to AM development. A ¹⁴C - labeling experiment showed that mycorrhizal roots accumulated 66% and 68% of the ¹⁴C - labeled photosynthates translocated to roots of sour orange and carizo citrange [24]. The distribution was independent of the status of phosphorus in leaves. Nemec and Guy [25] also reported an increase in soluble sugar, soluble starch and NSC contents of citrus leaves or roots colonized by *G. macrocarpus*. However, the transferred processes of carbohydrate from host plant to AMF were unknown. Moreover, AMF were capable of converting absorbed soluble sugar into storage compounds that were not readily available to the plant, such as glycogen, mannitol or trehalose [26].

The *G. mosseae* and *G. versiforme* - inoculations, especially *G. mosseae* - inoculation, stimulated the SOD, POD and CAT activities of leaves and roots (Table 4). The results were similar to *Retama sphaerocarpa* inoculated with *G. claroides* [7]. In micropropagated mycorrhizal *Citrus limon*, there was an increasing trend in plant survival [11]. This might be explained by the result of our study that the mycorrhizal citrus had higher antioxidant enzymatic activities than non-mycorrhizal plants. The greater antioxidant enzymatic activities in mycorrhizal plants were capable of enhancing the resistance of host plant under adverse environmental conditions, resulting in the avoidance of transplanting shock. A study

showed that the antioxidant enzymatic increments in mycorrhizal plants were closely related to increments in shoot biomass and phosphorus or nitrogen [7]. Mycorrhizal inoculation also stimulated some new SOD species production [9]. Different *Glomus* species had dissimilar increments in antioxidant enzymes, and *G. mosseae* was more effective than *G. versiforme* in micropropagated citrus plantlets.

In short, AM inoculation altered some physiological status, such as growth, mineral assimilation and antioxidant enzymes, as a result of the positive or beneficial effects in micropropagated citrus plantlets. Thus, AMF should be used as a biotechnological tool to enhance the rapid growth of *ex vitro* micropropagated citrus plantlets and the avoidance of transplanting shock during transplantation.

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