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Original Article

Mycorrhiza and Common Mycorrhizal Network Regulate the Production of Signal Substances in Trifoliate Orange (*Poncirus trifoliata*)

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Abstract

Common mycorrhizal networks (CMNs) connecting two or more neighbouring plants are confirmed to transfer signals, whereas little information about CMNs effects on the signal substances production is known. In this study, a two-chambered rootbox separated by 37 μm nylon mesh was used to establish donor and receptor chambers. Two chambers both were planted with trifoliate orange (*Poncirus trifoliata*) and then only donor chamber inoculated with *Diversispora versiformis*, *Paraglomus occultum* and *Rhizoglomus intraradices*. The roots of the donor and receptor plants both were mycorrhizated suggesting that CMNs were established between donor and receptor seedlings. Moreover, the AMF association dramatically increased plant height, stem diameter, leaf numbers, and shoot and root biomass in both the donor and receptor seedlings. The AMF inoculation in the donor plants and the subsequent mycorrhizal colonization by CMNs in the receptor plants significantly increased root calmodulin (CaM) and salicylic acid (SA) concentrations, while considerably decreased root nitric oxide (NO) and jasmonic acid (JA) concentrations. This was accompanied by down-regulated expression of three JA synthetic genes (*PtLOX*, *PtAOS* and *PtAOC*), regardless of donor and receptor seedlings. These results thus suggest that CMNs between trifoliate orange seedlings manifestly promote plant growth and affect the production of signal substances.

Keywords: calmodulin, jasmonic acid, nitric oxide, salicylic acid

Introduction

Arbuscular mycorrhizal fungi (AMF), a kind of beneficial soil microorganism, colonize roots of ~80% of terrestrial plants and further form arbuscular mycorrhiza (AM) (Parniske, 2008). The host plant provides photosynthates to maintain AM formation and development, in addition to abundant external hyphae in soils (Leake *et al*., 2004). On the other hand, the external hyphae of AMs capture more water and nutrients from the soil and supply them to the host (Smith and Smith, 2011). The developed mycorrhizal external hyphae can colonize and further connect neighbouring plants of same or different species to form common mycorrhizal networks (CMNs) (Barto *et al*., 2012). AMF inoculation only impacts a narrow area which can be enlarged by mycorrhizal hyphal amalgamation (Giovannetti *et al*., 2004) and CMNs formation. Earlier works suggested that the CMN could develop between plants that are 12-20 cm apart (Song *et al*., 2010; Barto *et al*., 2011; Babikova *et al*., 2013).

CMNs benefit hosts in many ways, for instance improving seedling establishment and influencing plant and microorganism community composition (Van Der Heijden, 2004; Van Der Heijden and Horton, 2009). Zhang *et al*. (2014) demonstrated that the CMN with *Diversispora spurca* was established between trifoliate orange and white clover and improved growth of the non-inoculated plant (receiver plant). Furthermore, CMNs can be considered as conduits for the interplant nutrient and signal transduction (He *et al*., 2003; Johnson and Gibert, 2015). Song *et al*. (2014) established CMNs with *Funneliformis mosseae* between herbivore-attacked and healthy tomato plants and found that CMNs transferred the jasmonic acid (JA) signaling to active defensive enzymes in receptor seedlings. CMNs regulate different physiological processes by transferring signal substrates to adapt to different stresses (Barto *et al*., 2011).

Apart from the JA, there are many other signal substances performing the regulation function, such as nitric oxide (NO), calmodulin (CaM), and salicylic acid (SA). As a signal molecule, NO activates protective enzymes in response to

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biotic and abiotic stresses (Besson-Bard *et al.*, 2009). CaM as the second messenger always responds to various stimulations and also regulates massive cellular functions (Berridge *et al.*, 2000). The SA and JA-dependence signaling pathways play a crucial role in plant defense reactions (Yang *et al.*, 2015), and the subject has gained more attention in the recent past. Allene oxide synthase (*AOS*), 13-lipoxygenase (*LOX*), and allene oxide cyclase (*AOC*) are key enzymes in JA biosynthesis pathway, the relative genes expression level which significantly influences JA concentration (Kombrink, 2012). Nevertheless, the effect of CMNs on signal substrates in trifoliate orange [*Poncirus trifoliata* (L.) Raf.] seedlings is little known.

In this work, a two-chambered rootbox was used to establish CMNs, and four signal substrates including NO, CaM, SA, and JA and the relevant JA synthetic genes expression were evaluated to clarify this mycorrhizal and CMN effect.

Materials and Methods

Experimental design

A complete and random design was used in the experiment, with the AMF treatments taken as a single factor. Four AMF treatments were tested separately: *Diversispora versiformis*, *Paraglomus occultum*, *Rhizoglomus intraradices* and the non-AMF control. Each treatment had four replicates, totaling to 16 rootboxes.

Mycorrhizal inoculum

Diversispora versiformis (P. Karst.) Oehl, G.A. Silva & Sieverd, *Paraglomus occultum* (Walker) Morton & Redecker, and *Rhizophagus intraradices* (N.C. Schenck & G.S. Sm.) C. Walker & A. Schüßler were used here. The inoculum of these AMF species was provided by the Bank of Glomeromycota in China. Mycorrhizal inocula were propagated with both identified fungal spores and white clover (*Trifolium repens*) for 16 weeks in pots. The inoculums consisted of sands, spores and infected root segments.

At plant transplanting stage, approx. 1500 spores of each AM fungus were inoculated into the donor chamber of the rootbox, and the receptor chamber of the rootbox received the same amount of autoclaved inoculant plus 2 mL of mycorrhizal inoculum (25 μm filter) to minimize differences in other microbial communities.

Plant materials and culture

Seeds of trifoliate orange were sterilized with 70% of ethyl alcohol solution and germinated in autoclaved (0.1 MPa, 121 °C, 1 h) sands at 25 °C. After 30 days, three-leaf-old seedlings without mycorrhization were transplanted into a rootbox (Fig. 1). The two-chambered rootbox was made of plexiglasses, with length, width and height of 10, 8 and 18 cm, respectively. The rootbox was divided into two equal chambers using two layers of 37-μm nylon mesh, and an air gap of 1 cm width was created to avoid additional diffusion between the chambers. The 37-μm nylon mesh could allow mycorrhizal hyphae, other than roots, to move from one chamber into the other. One chamber of the rootbox was inoculated with AMF, hereby defined as root + hyphae chamber (donor). The hyphae of root + hyphae chamber went into the other chamber, forming root-free hyphae chamber (receptor). Each chamber was planted with two non-mycorrhization seedlings with three leaf and supplied with a 1.4 kg autoclaved growth substrate (soil: vermiculite = 5:1, volume / volume).

The seedlings were grown in a glass house at the Yangtze University campus for 16 weeks. The temperature was 25/19 °C day/night with 85% average relative humidity and the photo flux density varied from 721 to 967 $\mu mol^{-1} m^{-2} s^{-1}$.

Variable determinations

Before harvesting, plant height, stem diameter and the number of leafs per plant were recorded. Each seedling was divided into shoot and root, and the biomass was weighed.

Approx. five root segments per seedling with 1-2 cm long were collected and stained by 0.05% trypan blue solution in lacto glycerol, as described by Phillips and Hayman (1970). A total of 40 root segments in each treatment were used to calculate AMF colonization. The AMF colonization in roots was expressed as the percentage of AMF-colonized root length versus total observed root length. The soil hyphal length was determined using the protocol of Bethlenfalvay and Ames (1987). The hyphal length of nylon mesh was assayed by Zou *et al.* (2015) in 0.05% trypan blue solution.

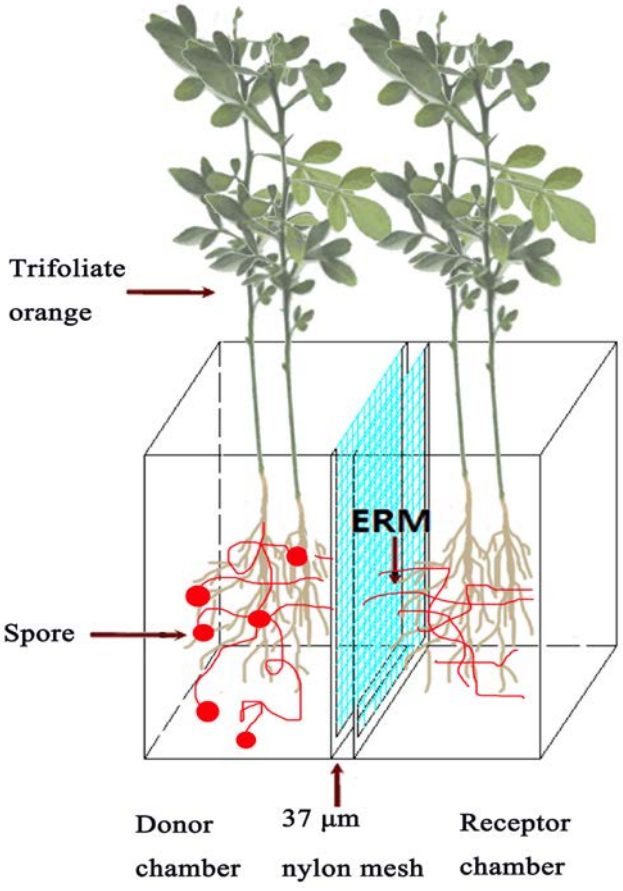

Fig. 1. The schematic diagram of the two-chambered rootbox. The rootbox was divided into two equal chambers by 37-μm nylon mesh and two trifoliate orange seedlings were planted in donor and receptor chamber respectively. The donor chamber was inoculated with AMF, forming root + hyphae chamber. The hyphae, other than roots, forming in donor chamber passed through the nylon mesh into receptor chamber, and hence the receptor chamber was free root + hyphae chamber. ERM represented extraradical mycelia of mycorrhizal fungi

Root samples were homogenized with phosphate buffer (pH 7.0) for extraction of NO, SA and JA, and with 0.6% NaCl solution for CaM extraction. The root NO concentration was determined according to the nitric acid reductase method with NO kits in ELISA in accordance with the user handbook (A012, Nanjing Jiancheng Bioengineering Institute, China). Root CaM, SA and JA concentrations were evaluated by double antibody sandwich-elisa kits in ELISA as per the user handbook (Shanghai Enzyme-linked Biotechology Co., Ltd, China).

The root sample was ground by liquid nitrogen, and root total RNA was extracted using a EASY spin Plus plant RNA kit (RN 38, Aidlab Biotecnolohies CO. Ltd, China), following the manufacturer's instruction. Thereafter, the total RNA was reversely transcribed to cDNA using the PrimeScript™ RT reagent kit with gDNA eraser (PK02006, Takara Bio. Inc, Japan), as per the manufacturer's instruction. Quantification real-time PCR (qRT-PCR) amplifications were carried out on a CFX96 Real Time PCR Detection System (BIO-RAD, USA) under the following compositions: 3.5 μL sterile water, 0.5 μL cDNA, 5 μL SYBR GREEN PCR Master Mix (Applied Biosystems, CA, USA), 0.5 μL forward prime and 0.5 μL reverse prime. These primers for selected genes (*PtLOX*, *PtAOS*, and *PtAOC*) were designed based on *Citrus sinensis* data and shown in Table 1. The relative fold change in gene expression was calculated following the $2^{-\Delta\Delta Ct}$ method (Kenneth and Schmittgen, 2001) in which the reference gene acted as the control. The measured transcripts were normalized to the relative expression value in non-AM plants. Three independent biological replicates and three technical replicates for each sample were examined.

Statistical analysis

Data (means ± SD, $n = 4$) were processed using the one-way ANOVA (SAS, version 8.1), and the Duncan's multiple range test (DMRT) was used to compare the significance of the difference among treatments at $P < 0.05$.

Results

Mycorrhizal status

As shown in Table 2 and Fig. 2, hyphae of nylon meshes and soils were observed in inoculated treatments, and the donor chamber had more hyphal length than receptor chamber except for the soil hyphae inoculated with *R. intraradices* in the soil. All the seedlings were colonized regardless of donor and receptor seedlings, indicating that hyphae of donor chamber passed through the nylon mesh into receptor chamber and that CMNs were established between trifoliate orange seedlings grown in the two-chambered rootbox. The root mycorrhizal colonization of donor seedlings varied from 31.8% to 66.4%, and that of receptor seedlings was from 26.8% to 61.0% (Table 2). In addition, a relatively higher root AM colonization was observed in the donor than in the receptor plants under three AMF species condition. Three AMF species had different affinities with trifoliate orange and ranked as *R. intraradices* > *P. occultum* > *D. versiformis* in both donor and receptor chamber.

Plant growth performance

AMF-seedlings grew better than non-AMF seedlings (Table 3). For donor plants, inoculation with *D. versiformis*, *P. occultum* and *R. intraradices* increased the plant height by 71.1%, 133.5%, and 153.8%, and the leaf number by 36.8%, 63.2%, and 68.4%, respectively, as compared with the non-AMF treatment. Likewise, root colonization by CMNs with *D. versiformis*, *P. occultum* and *R. intraradices* notably increased plant height by 40.3%, 51.3%, and 53.3%, and leaf number by 35.3%, 41.2%, and 47.1%, respectively, in receptor plants, as compared with the non-CMN-colonized treatment. In comparison with non-AMF seedlings, the AMF-treatment had thicker stem diameter expect for the receptor plant inoculated with *R. intraradices*. The AMF inoculation and

Table 1. The specific primers designed for real time quantitative PCR amplification

Gene name	Gene ID	Sequence (5'-3')-forward	Sequence (5'-3')-reverse
Actin	*Cs1g05000*	CCGACCGTATGAGCAAGGAAA	TTCCTGTGGACAATGGATGGA
PtLOX	*Cs3g13930*	GCATCCTTTATTGATCGGTTTC	GGCAGGCTCGCCATG
PtAOS	*Cs3g24230*	ATCAAACGGCGGCAAAGTG	GTATTCTAACGCTACGGGTGG
PtAOC	*Cs6g18900*	AGATCGTGGCAGTCCAGCTT	GCTAAAAGGGACAAGATCACCAA

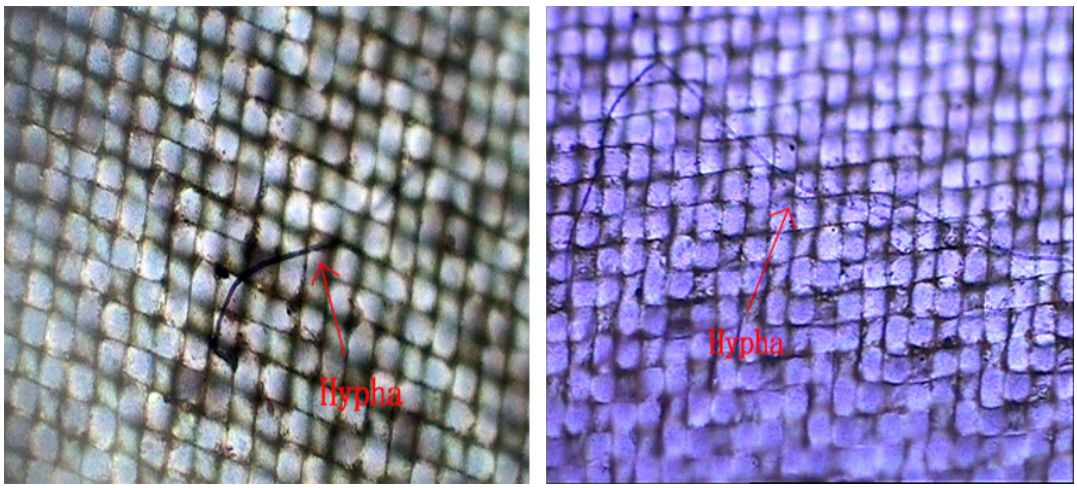

Fig. 2. The hypha on nylon mesh stained by trypan blue

Table 2. The mycorrhizal status of *Diversispora versiformis*, *Paraglomus occultum* and *Rhizogolmus intraradices* colonized trifoliate orange seedlings grown in a two-chambered rootbox

Treatments	Hyphal length of nylon mesh (mm/cm^2)		Hyphal length of soil (cm/g)		Mycorrhizal colonization (%)	
	Donor	Receptor	Donor	Receptor	Donor	Receptor
Non-AMF	0±0d	0±0d	0±0d	0±0d	0±0d	0±0d
D. versiformis	3.4±0.2c	2.9±0.3c	32.9±0.9c	29.3±7.5c	31.8±1.5c	26.8±1.3c
P. occultum	5.0±0.3b	3.7±0.1b	54.2±5.1b	39.5±2.5b	38.5±1.4b	33.7±2.4b
R. intraradices	5.7±0.6a	4.6±0.3a	70.79±3.4a	77.6±4.1a	66.4±2.9a	61.0±3.8a

Note: Data (mean ± SD, n = 4) followed by the different letters in a column are the significantly difference (P < 0.05) according to DMRT

Table 3. Effects of *Diversispora versiformis*, *Paraglomus occultum* and *Rhizogolmus intraradices* inoculation on plant growth performance of trifoliate orange seedlings grown in a two-chambered rootbox

Treatments	Plant height (cm)		Stem diameter (mm)		Leaf numbers (#/plant)		Shoot biomass (g FW/plant)		Root biomass (g FW/plant)	
	Donor	Receptor	Donor	Receptor	Donor	Receptor	Donor	Receptor	Donor	Receptor
Non-AMF	15.88±0.77d	15.69±0.77b	2.62±0.13c	2.24±0.10b	19±1 c	17±1b	0.90±0.06d	0.83±0.03c	0.99±0.08b	0.99±0.04b
D. versiformis	27.33±1.33c	22.02±1.98a	3.32±0.25b	3.32±0.20a	26±1b	23±2a	1.98±0.18c	1.63±0.09b	0.99±0.08b	1.09±0.07ab
P. occultum	37.08±2.34b	23.74±1.66a	3.91±0.25a	3.26±0.22a	31±2a	24±2a	3.15±0.19b	1.72±0.15ab	1.40±0.07a	1.17±0.04a
R. intraradices	40.31±1.94a	24.05±2.13a	3.02±0.15b	2.40±0.13b	32±1a	25±1a	3.51±0.28a	1.80±0.10a	1.43±0.11a	1.04±0.01b

Note: Data (mean ± SD, n = 4) followed by the different letters in a column are the significantly difference (P < 0.05) according to DMRT

CMN infection increased shoot and root biomass. Compared with the non-AMF treatment, *D. versiformis*, *P. occultum* and *R. intraradices* increased by 120%, 250%, and 290% in shoot biomass and 0%, 41%, and 72% in root biomass. The seedlings infected by CMN of *D. versiformis*, *P. occultum* and *R. intraradices* had 96%, 107%, and 117% more in shoot biomass and 10%, 18%, and 5% higher in root biomass in comparison with non-CMN seedlings.

Root CaM concentration

AMF inoculation or subsequent CMN infection significantly elevated root CaM concentrations in comparison with non-AMF inoculation, regardless of the donor and receptor seedlings. Compared with non-AMF treatment, the root CaM concentrations were 9.1%, 11.8%, and 20.9% higher in donor seedlings, and 14.0%, 25.7%, and 40.2% higher in receptor seedlings under inoculation with *D. versiformis*, *P. occultum* and *R. intraradices* conditions, respectively (Fig. 3).

Root NO concentration

Three AMF species all decreased root NO accumulation, but the restraining level was slightly different among treatments (Fig. 4). Compared with non-AMF treatment, the root NO concentrations decreased by 21.1%, 60.7%, and 24.1% in donor plants and 18.3%, 25.4%, and 48.0% in receptor plants inoculated with *D. versiformis*, *P. occultum* and *R. intraradices*, respectively.

Root SA concentration

In contrast with the non-mycorrhizal seedlings, the mycorrhizal trifoliate orange seedlings showed significantly higher root SA concentrations: 29.6% and 36.7% in donor and receptor seedlings when inoculated with *D. versiformis*, 9.9% and 12.5% under mycorrhization with *P. occultum*, and 11.7% and 25.5% under mycorrhization with *R. intraradices*, respectively (Fig. 5).

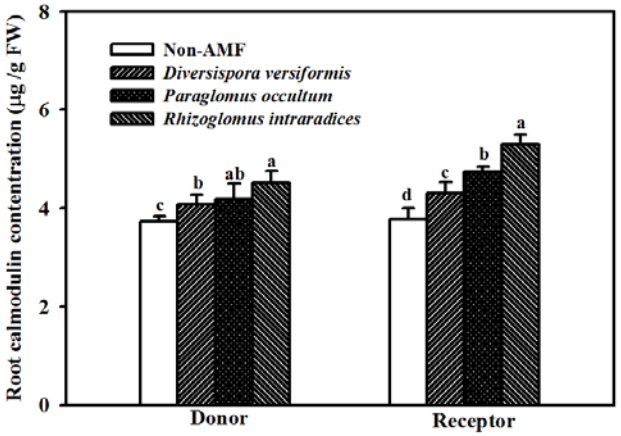

Fig. 3. Effects of *Diversispora versiformis*, *Paraglomus occultum* and *Rhizogolmus intraradices* inoculation on root calmodulin level of trifoliate orange seedlings grown in a two-chambered rootbox. Bars (means ± SD, n = 4) bearing different letters are significantly different (P < 0.05) according to DMRT

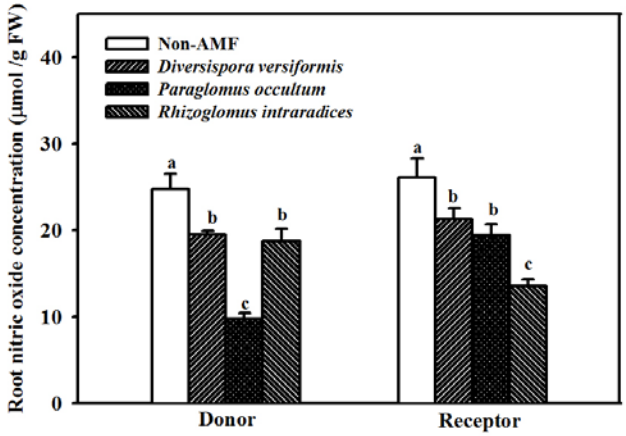

Fig. 4. Effects of *Diversispora versiformis*, *Paraglomus occultum* and *Rhizogolmus intraradices*, inoculation on root nitric oxide level of trifoliate orange seedlings grown in a two-chambered rootbox. Bars (means ± SD, n = 4) bearing different letters are significantly different (P < 0.05) according to DMRT

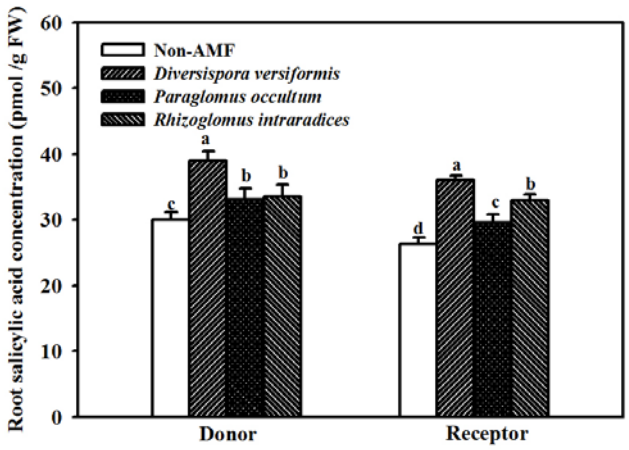

Fig. 5. Effects of *Diversispora versiformis*, *Paraglomus occultum* and *Rhizogolmus intraradices* inoculation on root salicylic acid level of trifoliate orange seedlings grown in a two-chambered rootbox. Bars (means ± SD, n = 4) bearing different letters are significantly different (P < 0.05) according to DMRT

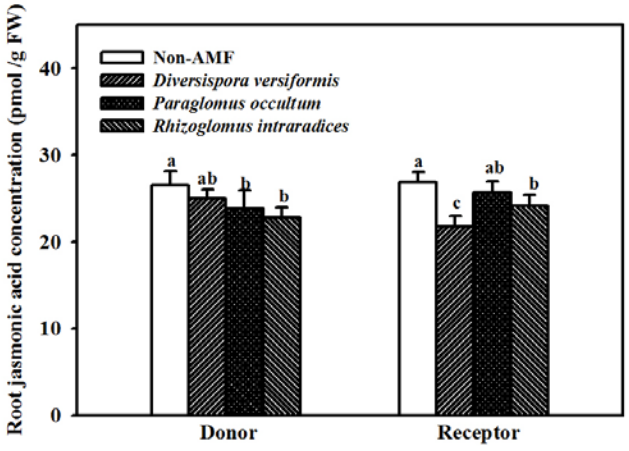

Fig. 6. Effects of *Diversispora versiformis*, *Paraglomus occultum* and *Rhizogolmus intraradices* inoculation on root jasmonic acid level of trifoliate orange seedlings grown in a two-chambered rootbox. Bars (means ± SD, n = 4) bearing different letters are significantly different (P < 0.05) according to DMRT

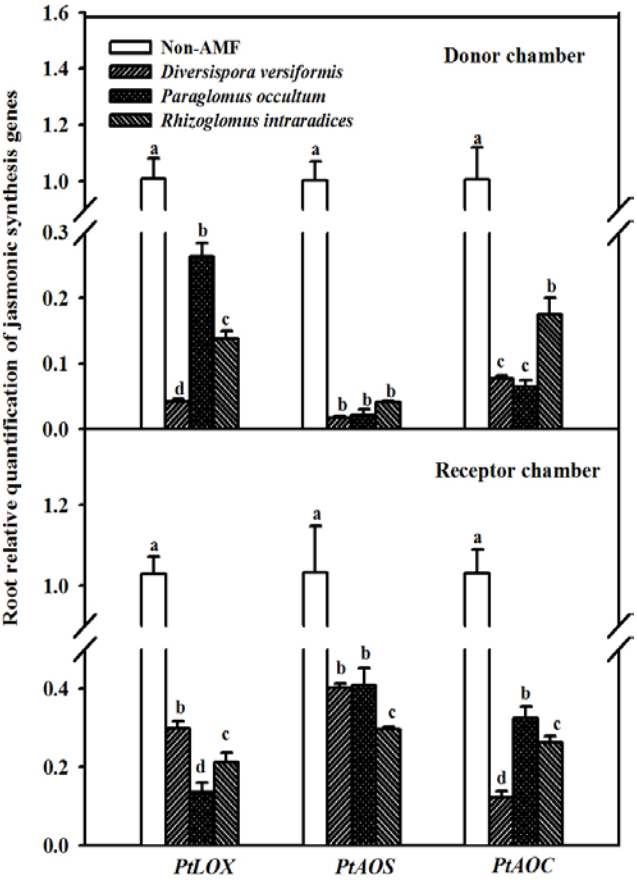

Fig. 7. Effects of *Diversispora versiformis*, *Paraglomus occultum* and *Rhizogolmus intraradices* inoculation on relative expression of root jasmonic acid synthetic genes of trifoliate orange seedlings grown in a two-chambered rootbox. The non-AMF treatment was acted as the control in which the gene expression level was 1. Bars (means ± SD, n = 4) bearing different letters are significantly different (P < 0.05) according to DMRT

Root JA concentration

Inoculation with *P. occultum* and *R. intraradices* decreased root JA concentrations by 10.3% and 14.2% in donor seedlings, respectively, whereas *D. versiformis* had no significant change in root JA concentration of donor seedlings, as compared with non-AMF treatment (Fig. 6). In receptor seedlings, CMNs with *D. versiformis* and *R. intraradices* reduced root JA concentrations respectively by 19.0% and 10.0%, while *P. occultum* had no significant effects on root JA concentrations, as compared with the non-AMF inoculation.

Relative expression of JA synthetic genes

qRT-PCR analysis revealed that AMF inoculation significantly down-regulated the expression of three JA synthetic genes in donor and receptor roots (Fig. 7). In donor plants, in contrast with non-AMF inoculation, *D. versiformis* decreased the expression of *PtLOX*, *PtAOS* and *PtAOC* genes respectively by 96%, 98%, and 92%. On the other hand, the decrease under *P. occultum* was by 74%, 98%, and 94%; and *R. intraradices* was by 86%, 96%, and 83%. Similarly, the CMN-infected receptor seedlings had 71%, 61%, and 88%; 87%, 61%, and 68%; and 79%, 71%, and 75% lower expression of (respectively) *PtLOX*, *PtAOS*, and *PtAOC* genes in roots, compared with non-CMN-infected controls.

Discussion

In the two-chambered rootbox, the CMNs were formed between donor chamber inoculated with mycorrhizal and non-inoculation receptor chamber, and such treatments significantly stimulated growth performance in the donor and receptor seedlings. This is in agreement with earlier studies in trifoliate orange-white clover system (Zhang *et al.*, 2014). Since the AM association was established earlier in seedlings of the donor chamber, the effect of mycorrhizal colonization was more profound in donor seedlings than in receptor seedlings.

Meanwhile, three AMF species behaved differently as a result of the inoculation effect, possibly due to AMF specificity with the host plant (Van Der Heijden *et al.*, 1998).

The complex of calcium (Ca^{2+}) and CaM acts as the signaling to regulate physiological metabolisms (Kim *et al.*, 2009), which is employed by AMF to strengthen the signal transduction. Chabaud *et al.* (2011) indicated that exudates from AMF spores induced Ca^{2+} rapid increase and Gleason *et al.* (2006) further proved that Ca^{2+}/CaM regulated calcium and calmodulin-dependent kinase (CCaMK) and induced specific gene transcription. Huang *et al.* (2014) reported that inoculation with *F. mosseae* increased CaM level and promoted the resistance to drought stress in trifoliate orange. In this work, inoculation with AMF activated root CaM level in donor seedlings, and subsequent CMN colonization also promoted root CaM increase in receptor seedlings. It was suggested that AMF inoculation and CMN colonization had similar effect on regulating the Ca^{2+}/CaM signal pathway.

In our work, mycorrhizal colonization strongly decreased the root NO concentration, irrespective of AMF species and donor or receptor plants. Earlier studies proved that NO took part in the post-translational modification of protein involving stress, redox and signaling/regulating (Lindermayr *et al.*, 2005). It may be speculated that more NO was used to modify proteins for performing various functions under AMF inoculation conditions. Concurrently, the receptor seedlings had the same tendency with donor seedlings, which meant that the CMN-infected seedlings also employed more NO to mediate physiological functioning.

Earlier studies showed that both SA and JA could transfer the signal of wounding, pathogen, and herbivores attack to trigger the defense responses in plants (Malamy *et al.*, 1990; Wasternack and Parthier, 1997; Sanders *et al.*, 2000). After colonization, the biotrophic microorganisms including AMF trigger the system to acquire resistance, which is always accompanied with more SA accumulation (Dempsey *et al.*, 2011). The SA signals exhibit a more durable and intense response to pathogen infection (Song *et al.*, 2010). Other studies showed that inoculation with *F. mosseae* promoted the phenolic synthesis via SA signaling pathways and plant disease resistance was further enhanced (Zhang *et al.*, 2013). In the present work, AM seedlings, regardless of donor and receptor, showed significantly higher root SA levels but lower root JA concentrations accompanied with the down-regulation of three JA synthetic genes (*PtLOX*, *PtAOS*, and *PtAOC*), compared with non-AMF treatment. Therefore, it is suggested that mycorrhizal seedlings connected by CMNs might have a relatively greater capacity to tolerate pathogen attack by increasing SA concentration.

In general, SA inhibited JA biosynthesis and defense responses (Glazebrook, 2001; Robert-Seilaniantz *et al.*, 2011) and the inhibiting effect always realized by transcription factors such as *WRKY70*, *WRKY62*, *MPK4*, *MYC2*, and *NPR1* (Bari and Jones, 2009). The inhibition was also shown in this present work, with an increase of root SA but a decrease of root JA. SA accumulation was at the cost of JA synthesis depression, which was also confirmed by Spoel *et al.* (2003) and Laudert and Weiler (1998). In our work, the JA synthetic gene expression was more susceptible than JA level under mycorrhizal colonization conditions, regardless of donor and receptor seedlings. Isayenkov *et al.* (2005) also found that *AOC* transcripts had more significant change than *AOC* protein under mycorrhization condition. Perhaps there are non-known factors that affect JA protein translation. Therefore, the mechanisms by which the synthesis related genes influence JA level in roots need to be further investigated.

Conclusions

The CMNs were established between trifoliate orange seedlings and played vital roles in growth promotion and production of signal substances. Inoculation with AMF increased root and shoot biomass in both donor and receptor seedlings. Meanwhile, the signal pathways were activated by inoculating with AMF. There were more root CaM and SA but less root NO and JA along with down-regulation of JA synthetic genes (*PtLOX, PtAOS,* and *PtAOC*) under mycorrhization.

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