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COMMON MYCELIUM NETWORKS WITH *Paraglomus occultum* INDUCE BETTER PLANT GROWTH AND SIGNAL SUBSTANCE CHANGES BETWEEN TRIFOLIATE ORANGE SEEDLINGS

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ABSTRACT

Mycorrhizal mycelium can simultaneously colonize two and more neighboring plants to form common mycelium network (CMNs), whereas the information regarding CMN effects on endogenous signal substances is limited. In this study, a rootbox was separated by 37- or 0.45-μm mesh to establish donor chamber (the presence of roots and hyphae) and receptor (hyphae presented or not, free of roots) chamber, where an arbuscular mycorrhizal (AM) fungus *Paraglomus occultum* was inoculated into trifoliate orange seedlings of donor chamber to illustrate the underground communications of signal substances by CMNs. Mycorrhizal colonization resulted in better plant growth performance and greater root morphology in donor and receptor plants. AM inoculation increased significantly the root nitric oxide (NO) and calmodulin (CaM) levels of donor plants, regardless of 37- and 0.45-μm mesh, and subsequent CMNs induced higher root NO and CaM levels in receptor plants. Mycorrhizal colonization did not produce significant changes in root zeatin riboside (ZR) levels of donor plants, but CMN hyphae modulated lower root ZR levels of receptor plants. Mycorrhizal inoculation and subsequent CMN hyphae induced lower root gibberellin levels of donor and receptor plants, and only CMN hyphae produced lower root methyl jasmonate concentrations of receptor plants. Our results first reveal the underground communication of CaM, NO, and ZR by CMNs with *P. occultum* between trifoliate orange seedlings.

Key words: calmodulin, CMNs, mycorrhiza, nitric oxide, underground communication

INTRODUCTION

Phytohormones play an indispensable role in maintaining and coordinating the plant growth and development, especially during plant physio-biochemical processes, and are responsible for the stimulus of external environments [Huang et al. 2014, Wu et al. 2017]. Phytohormones can be strongly influenced by environmental factors, substrate nutrients and soil microbes, including arbuscular mycorrhizal fungi (AMF) [Wu et al. 2014, Caroline 2014].

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Arbuscular mycorrhizal fungi, one of the soil inhabited microorganisms, can colonize roots of most terrestrial plants to establish a mutual and beneficial association with. arbuscular mycorrhizas (AMs). Studies in the past showed the AM roles in the improvement of soil structure and modulating the physiological metabolisms in host plants, as well phytohormones [Zhang et al. 2015, Wu et al. 2017]. It is well documented that varieties of signal substances were synthesized or induced in the process of association and growth of AMF [Qi et al. 1997, Meixner et al. 2005]. Huang et al. [2014] reported that inoculation with an AM fungus, *Funneliformis mosseae*, could improve leaf calmodulin (CaM) synthesis in trifoliate orange seedlings exposed to drought stress. Zhang et al. [2013] indicated that *F. mosseae* helped the roots of *Trifolium repens* L. plants to produce nitric oxide (NO) for inducing the synthesis of root phenolic resin. Regarding phytohormones, AMF have diverse responses in different plants. The study of Qi et al. [1997] showed that inoculation with AMF significantly improved gibberellin (GA) and abscisic acid (ABA) concentrations in micropropagated apple seedlings. Liu et al. [2000] also indicated that inoculation with *F. mosseae*, *Gigaspora rosea* and *Glomus versiforme* significantly increased the levels of GAs in maize and cotton plants under drought conditions, but reduced ABA concentrations.

The extraradical mycelia of AM plants simultaneously colonize neighboring plants to form commom mycelium network (CMNs) between two and more neighboring plants in soils [Bücking et al. 2015, Zhang et al. 2015]. CMNs play a significant role in plant ecosystems [Bücking et al. 2015]. CMNs not only change rhizospheric soil properties between plants, but also provide the communications of nutrients, water, and disease resistive materials [Zhang et al. 2015, Yao et al. 2016, Wu et al. 2017]. It is noted that CMN presence was highly dependent on donor and receptor species [Meding and Zasoski 2008, Zhang et al. 2015]. In pot condition, a strong CMN was formed between white clover and trifoliate orange seedlings, which considerably improved soil structure and absorption of nutrients between plants [Zhang et al. 2015]. Nevertheless, the information regarding the CMN effect on endogenous signal substances is still limited.

Trifoliate orange (*Poncirus trifoliata* L. Raf.) is widely used in the citriculture as the rootstock, which heavily depends on AMs. Previous works showed the presence of CMNs between citrus plants [Zhang et al. 2015, Yao et al. 2016]. Therefore, the present work is to simulate a CMN with *Paraglomus occultum* through a two-chambered rootbox to clarify whether CMNs affect the root endogenous signal substances and plant growth between trifoliate orange seedlings.

MATERIAL AND METHODS

Preparation of two-chambered rootbox. The two-chambered rootbox (Fig. 1) was made of the plexiglass, with 18.5 cm length, 12 cm width, and 16 cm height, respectively. The rootbox was divided into two equal chambers (donor and receptor chamber) by 37-μm or 0.45-μm nylon mesh (Hangzhou Daheng Filter Cloth CO., Ltd., China), with an air gap of 1.5 cm width to eliminate any substance communications between each chamber. Plant roots cannot pass through the 37- and 0.45-μm nylon mesh, whereas mycorrhizal extraradical mycelium (ERM) can pass through the 37-μm nylon mesh, but not the 0.45-μm nylon mesh, from the donor to receptor chamber.

Experimental setup. Seeds of trifoliate orange were scrubbed with 1 mol/L NaOH solutions, disinfected with 70% alcohol solutions for 10 min, washed with distilled water 3–4 times, and germinated in autoclaved (120°C, 0.11 MPa, 1.5 h) sands in 26/20°C day/night temperature, 16:8 photoperiod, and 85% relative air humidity. Each chamber was supplied with 1.5 kg autoclaved (120°C, 0.11 MPa, 1.5 h) ferralsol (FAO system) collected from a citrus orchard of Yangtze University Campus.

Two four-leaf-old trifoliate orange seedlings without mycorrhization were transplanted into each chamber of the rootbox. The AMF strain *Paraglomus occultum* (C. Walker) J.B. Morton & D. Redecker used here was provided by the Bank of Glomeromycota in China and propagated with white clover (*Trifolium repens* L.) in pots. The 120 g inoculum including spores (approx. 1500 spores), sands, and infected root segments was inoculated into rhizosphere of trifoliate orange seedlings as donor plants, and the other seedlings without *P. occultum* in recep-

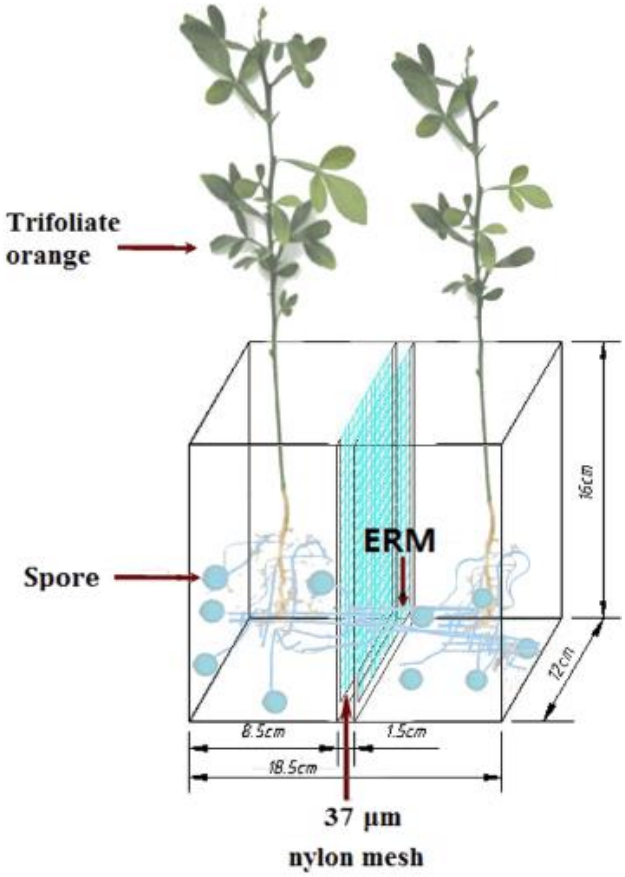

Fig. 1. Schematic diagram of the two-chambered rootbox. Here, mycorrhizal donor plants with *Paraglomus occultum* developed extraradical mycelium (ERM), which can pass through 37 μm nylon mesh into receptor chamber to colonize receiver plants

tor chamber were designed as the receptor plants, which might be colonized by hyphae of CMNs from donor plants. During the seedlings acclimated, a small amount of water was irrigated every three days to keep the soil moisture and avoid the water-logging in the rootbox.

All the rootboxes were placed in a glass greenhouse of the Yangtze University campus from March 28, 2014 to August 16, 2014, where photosynthetic photon flux density was 880 μmol/m^2/s, day/night temperature 26/20°C and relative air humidity 80%.

Experimental design. The study was designed with single factor in a completely randomized arrangement, including: (1) two chambers of the rootbox were separated by 37-μm mesh and were not inoculated with *P. occultum* in donor and receptor chamber (TO^-/37/TO^-); (2) two chambers of the rootbox were separated by 37-μm mesh and were inoculated with *P. occultum* only in donor chamber (TO^+/37/TO^-); (3) two chambers of the rootbox were separated by 0.45-μm mesh and were inoculated with *P. occultum* only in donor chamber (TO^+/0.45/TO^-). Each treatment had four replicates, resulting in a total of 12 rootboxes.

Variable determinations. The plant height, stem diameter and leaf number of trifoliate orange seedlings were determined at harvesting. And the root systems were collected, scanned by an Epson Perfection V700 Photo Dual Lens System (Seiko Ep-

son Corp, Japan), and analyzed by a professional WinRHIZO software in 2007b (Regent Instruments Inc., Quebec, Canada). Leaves and roots collected were frozen immediately at –80°C for the analysis of biochemical variables.

Root AMF structures were stained according to the protocol of Phillips and Hayman [1970], and root mycorrhizal colonization was calculated as the percentage of AMF colonized root lengths against total root lengths. Hyphal length in the soil and the mesh was determined according to the protocol of Bethlenfalvay and Ames [1987] and Zou et al. [2015], respectively.

Root endogenous ABA, GA, indole-acetic acid (IAA), methyl jasmonate (Me-JA), zeatin riboside (ZR), and brassinosteroids (BR) were extracted following the protocol of Chen et al. [2009] and determined by the Enzyme-Linked Immunosorbent Assay (ELISA) in the Crop Chemical Control Center, China Agricultural University, Beijing, China.

Root nitric oxide (NO) was measured by the nitrate reductase method, in which NO is converted into both NO_2^- and NO_3^- [Xie et al. 2016]. The NO kit (A012) was provided by the Nanjing Jiancheng Bioengineering Institute, Nanjing, China. Root CaM was assayed by the double antibody sandwich method, in which the compound involved in antibody, antigens, and enzyme-labeled antibody was dyed by tetramethylbenzidine, and the gradation of the color was positively correlated with CaM levels. The Plant CaM kit (YAD001) was produced by the Beijing Dingguochangsheng Biotechnology CO., Ltd. All the measured procedures were followed by the user's guide.

Statistical analysis. The data were statistically analyzed with single-factor ANOVA in SAS (8.1) software, and the Duncan's multiple range tests were used to compare the significant differences between treatments at $P < 0.05$.

RESULTS

Mycorrhizal status in roots, meshes, and soils. In the donor chamber, *P. occultum* exhibited 37.7% and 58.7% root mycorrhizal colonization (Fig. 2a), 10.3 and 30.6 cm/g hyphal length in soil, and 1.88 and 2.17 cm/cm^2 hyphal length (Fig. 2b, c) in mesh under 37 μm and 0.45 μm mesh, respectively (Tab. 1). Moreover, the mycorrhizal status was significantly higher under 0.45 μm mesh condition than under 37 μm mesh condition. In the receptor chamber, the trifoliate orange seedlings only formed symbiosis with AMF exposed to 37 μm mesh but not 0.45 μm mesh, because the extraradical hyphae of donor chamber could not enter into receptor chamber under 0.45 μm mesh condition.

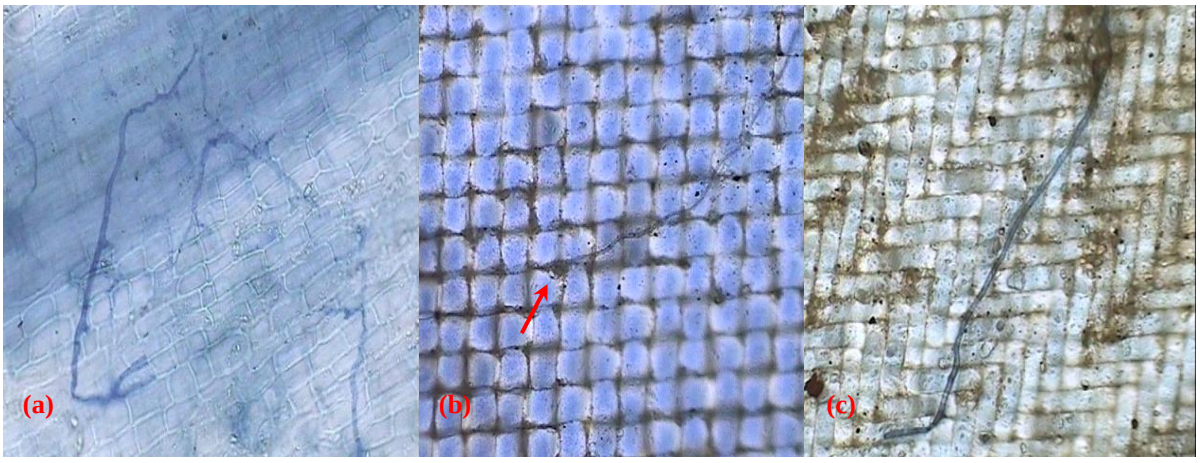

Fig. 2. Root mycorrhizal colonization (a) and mycorrhizal extraradical hyphae in 37-μm (b) and 0.45-μm (c) nylon mesh in trifoliate orange colonized by *Paraglomus occultum* in a two-chambered rootbox. Red arrow in b-subfigure shows the entry of extraradical mycelium into 37-μm nylon mesh

Table 1. Mycorrhizal status in roots, soils, and meshes in *Paraglomus occultum*-inoculated trifoliate orange seedlings grown in a two-chambered rootbox

Treatments	Donor chamber			Receptor chamber		
	hyphal length in soil (cm/g soil)	hyphal length in mesh (cm/cm^2)	root AM colonization (%)	hyphal length in soil (cm/g soil)	hyphal length in mesh (cm/cm^2)	root AM colonization (%)
TO^+/37/TO^-	10.3 ±0.9b	1.88 ±0.16b	37.7 ±1.8b	10.25 ±0.87a	1.34 ±0.22a	30.4 ±2.2a
TO^+/0.45/TO^-	30.6 ±2.0a	2.17 ±0.10a	58.7 ±1.8a	0 ±0b	0 ±0b	0 ±0b
TO^-/37/TO^-	0 ±0c	0 ±0c	0 ±0c	0 ±0b	0 ±0b	0 ±0b

Different letters between treatments indicate significant differences (Duncan test, P < 0.05). Donor: the plant inoculated with mycorrhizal fungi. Receptor: the plant inoculated without mycorrhizal fungi but infected by mycorrhizal mycelium of another inoculated plant

Table 2. Effects of inoculation with *Paraglomus occultum* into donor chamber of a two-chambered rootbox on plant growth and root morphology of trifoliate orange seedlings

Chamber	Treatments	Plant height (cm)	Stem diameter (mm)	Leaf number (per plant)	Total plant Biomass (g FW/plant)	Root length (cm)	Root surface area (cm^2)	Root volume (cm^3)
Donor chamber	TO^+/37/TO^-	42.46 ±4.03b	0.37 ±0.05a	34 ±6a	7.10 ±0.50a	504 ±14b	86.6 ±3.3b	1.19 ±0.03b
	TO^+/0.45/TO^-	49.29 ±2.84a	0.38 ±0.02a	34 ±4a	7.00 ±0.51a	611 ±18a	104.5 ±3.4a	1.43 ±0.03a
	TO^-/37/TO^-	27.79 ±1.69c	0.26 ±0.03b	27 ±2b	3.34 ±0.15b	267 ±6c	43.0 ±3.8c	0.55 ±0.04c
Receptor chamber	TO^+/37/TO^-	39.13 ±1.36a	0.39 ±0.03a	32 ±1a	6.40 ±0.29a	445 ±10a	73.2 ±4.2a	0.97 ±0.07a
	TO^+/0.45/TO^-	27.86 ±1.07c	0.28 ±0.03c	26 ±1c	3.70 ±0.15c	350 ±9b	58.9 ±3.9b	0.79 ±0.04b
	TO^-/37/TO^-	33.16 ±1.45b	0.34 ±0.03b	27 ±1b	4.84 ±0.14b	363 ±14b	59.4 ±3.4b	0.79 ±0.02b

Different letters between treatments from donor or receptor chamber indicate significant differences (Duncan test, $P < 0.05$). Donor: the plant inoculated with mycorrhizal fungi. Receptor: the plant inoculated without mycorrhizal fungi but infected by mycorrhizal mycelium of another inoculated plant.

Plant growth responses. Inoculation with *P. occultum* significantly promoted the plant height, stem diameter and leaf number in donor plant, regardless of 37-μm or 0.45-μm mesh (Tab. 2). In receptor chamber, plant height, stem diameter, leaf number, and total plant biomass were significantly promoted by 18%, 15%, 16%, and 32% under TO^+/37/TO^- treatment, whereas they were notably decreased by 16%, 18%, 4%, and 24% under TO^+/0.45/TO^- treatment, as compared with TO^-/37/TO^- treatment, respectively.

As shown in Table 2, *P. occultum* inoculation also significantly promoted root length, surface area, and volume in donor plants, regardless of 37- and 0.45-μm mesh (Tab. 2). Moreover, relatively greater root morphology of donor trifoliate orange plants was under 0.45-μm mesh than under 37-μm mesh conditions. In receptor chamber, root length, surface area, and volume were dramatically higher under TO^+/37/TO^- treatment conditions than under TO^+/0.45/TO^- and TO^-/37/TO^- treatment conditions.

Changes in concentrations of root endogenous signal substances. Significantly higher root NO and CaM concentrations were ranked as the trend of TO^+/0.45/TO^- > TO^+/37/TO^- > TO^-/37/TO^- in donor plants and TO^+/37/TO^- > TO^-/37/TO^- > TO^+/0.45/TO^- in receiver plants (Fig. 3, 4).

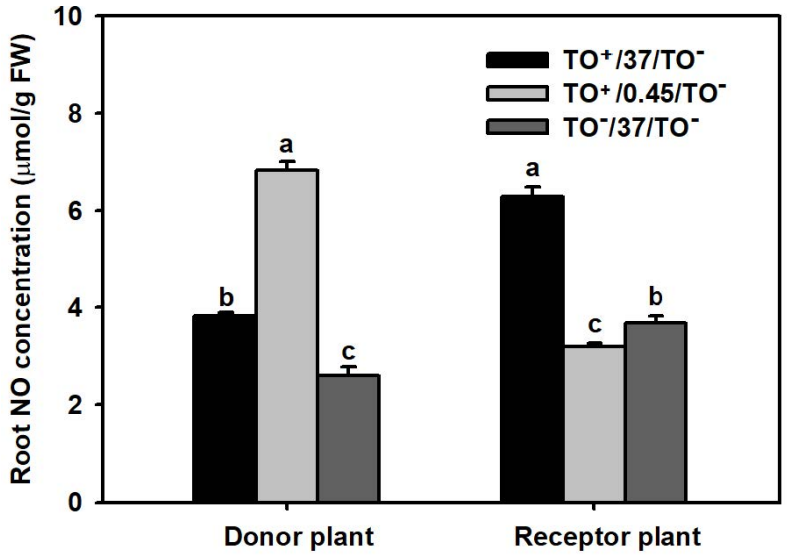

Fig. 3. Effects of inoculation with *Paraglomus occultum* into donor chamber of a two-chambered rootbox on root nitric oxide (NO) concentration of trifoliate orange seedlings. Data (means ±SD, n = 4) from donor or receptor plants followed by different letters (a, b) above the bars are significantly different at $P < 0.05$

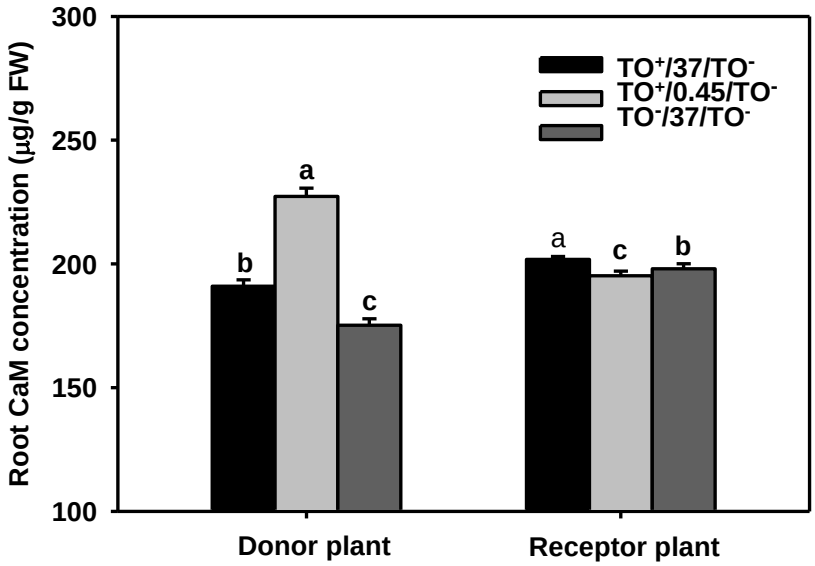

Fig. 4. Effects of inoculation with *Paraglomus occultum* into donor chamber of a two-chambered rootbox on root calmodulin (CaM) concentration of trifoliate orange seedlings. Data (means ±SD, n = 4) from donor or receptor plants followed by different letters (a, b) above the bars are significantly different at $P < 0.05$

Root ABA, IAA, and BR levels were not significantly affected by mycorrhizal inoculation or CMN hyphae, irrespective of donor and receptor plants (Fig. 5a, b). In addition, the root MeJA concentration of donor plants and the root ZR concentration of receptor plants were not significantly different between three treatments. Root GA concentration was significantly higher under $TO^-/37/TO^-$ treatment than under $TO^+/37/TO^-$ treatment in donor plants and higher under $TO^+/0.45/TO^-$ treatment than under $TO^+/37/TO^-$ treatment (Fig. 5a–b). Significantly higher root ZR level of donor plants was ranked as the trend of $TO^-/37/TO^-$ treatment > $TO^+/0.45/TO^-$ treatment ≈ $TO^+/37/TO^-$ treatment in the decreasing order, and higher root Me-JA concentration of receptor plants was listed as the trend of $TO^-/37/TO^-$ treatment ≈ $TO^+/0.45/TO^-$ treatment > $TO^+/37/TO^-$ treatment in the decreasing order, respectively (Fig. 5a, b).

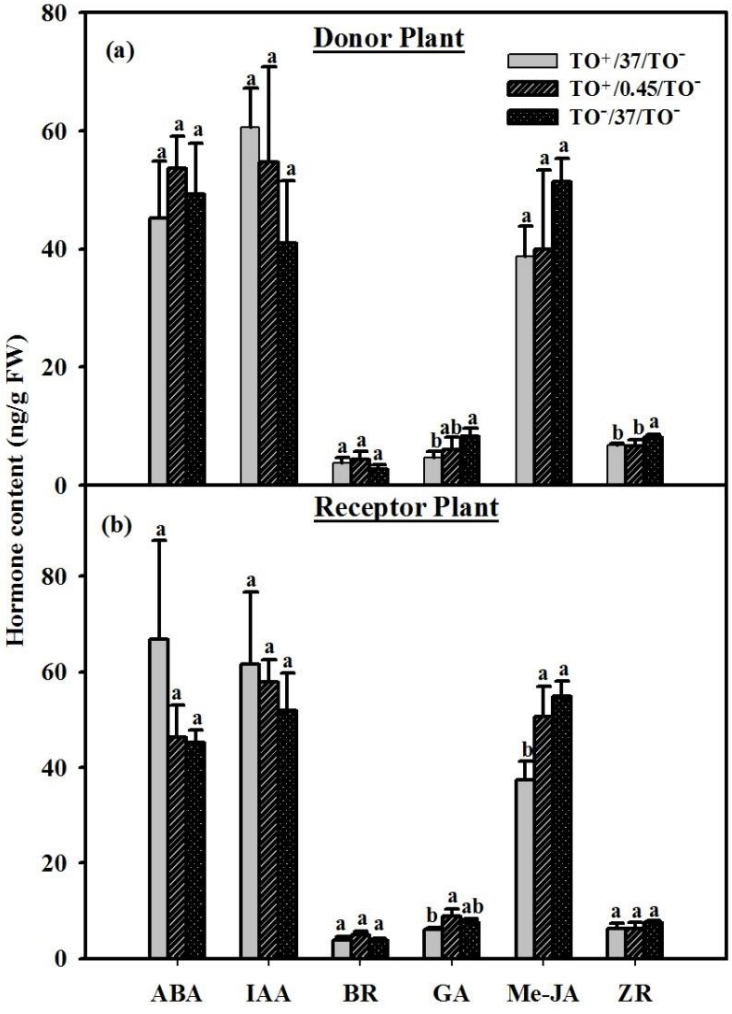

Fig. 5. Effects of inoculation with *Paraglomus occultum* into donor chamber of a two-chambered rootbox on root abscisic acid (ABA), indole-acetic acid (IAA), brassinosteroids (BR), gibberellin (GA), methyl jasmonate (Me-JA), and zeatin riboside (ZR) concentrations of trifoliate orange seedlings. Data (means ± SD, n = 4) from donor or receptor plants followed by different letters (a, b) above the bars are significantly different at $P < 0.05$

DISCUSSION

The present study showed the positive effects of *P. occultum* inoculation on plant growth performance and root morphology of trifoliate orange seedlings in donor chamber, which is consistent with Zhang et al. [2015]. The extraradical mycelium of *P. occultum* could pass through 37-μm mesh, but not 0.45 μm mesh, to colonize receptor plants, resulting in the establishment of CMNs between donor and receptor plants under 37-μm mesh conditions. Such CMNs in receptor chamber strongly improved the plant growth performance and root morphology in receptor plants under 37-μm mesh conditions. However, under 0.45-μm mesh conditions, receptor roots were not colonized by hyphae of CMNs, thereby, resulting in no significant differences with control roots under 37-μm mesh conditions. As reported by Zhang et al. [2015], CMNs transferred carbohydrates and minerals from donor to receptor plants. It suggests that extraradical mycelium of mycorrhizas could form a CMN between trifoliate orange seedlings to strongly improve the plant growth and root morphology.

NO is an important signal molecule and closely related with morphogenesis and physiological processes of plants [Andreas et al. 2011]. CaM is the Ca^{2+} binding protein and closely related to the signal transduction [Wu et al. 2014]. Our study showed that root NO and CaM concentrations of trifoliate orange seedlings were significantly enhanced in donor chamber, which is consistent with results of Zhang et al. [2013] and Huang et al. [2014] in white clover and trifoliate orange seedlings. Earlier studies pointed out that NO promoted the synthesis of ADP-ribose, which can be combined with the channel of Ca^{2+} in cells, thus, stronger activating the Ca^{2+} release [Zhang et al. 2014]. In addition, AMF spores and extraradical mycelium can regulate Ca^{2+} levels in host plants, and CaM and NO can help mycorrhizal colonization and responce signals in host plant growth [Huang et al. 2014, Wu et al. 2014]. It suggests that inoculation with AMF could promote the root NO production in plants, which further activated the Ca^{2+} release, resulting in the increase in root CaM. In the dual trifoliate orange–trifoliate orange system, CMNs significantly promoted the root NO and CaM concentrations in receptor plants under 37-μm nylon mesh and decreased those under 0.45-μm nylon mesh. In general, 0.45-μm nylon mesh not only hindered the AMF colonization between donor and receptor plants, but also affected the substance communications by CMNs. It suggests that NO and CaM participate in the signal transduction by CMNs [Xiong et al. 2014].

In the interval of 37-μm nylon mesh, inoculation with *P. occultum* induced relatively lower root GA concentration in donor and receptor plants. This is in line with the findings of Foo et al. [2013], who reported a negative effect of GA on mycorrhizal development. Wu et al. [2017] confirmed no involvement of GA by CMNs between white clover and trifoliate orange plants. This study also showed that root GA was not involved in underground communications by CMNs between trifoliate orange plants.

A certain concentration of Me-JA can promote AMF colonization in host plants [Herrera-Medina et al. 2008]. In this study, *P. occultum* induced a significant decrease in root Me-JA of receptor plants only in the interval of 37-μm nylon mesh. Hause and Sara [2009] reported that endogenous Me-JA promoted the increase of carbohydrates in plants, and induced nutrient swaps between AM symbiosis and host plants. Since AM symbiosis needs the expenditure of root carbohydrates, relatively lower root Me-JA level in receptor plants under 37-μm mesh conditions and in donor plants under 37-μm and 0.45-μm mesh could be explained by the AM carbon expenditure, but not CMN communication.

Torelli et al. [2000] showed that AMF promoted endogenous ZR concentration in leek plants. However, in this study, root ZR levels of trifoliate orange were significantly lower in the donor chamber under mycorrhized than under non-mycorrhized conditions. It suggests that AMF-induced ZR changes are dependent on a host plant species. In addition, no significant differences in root ZR concentrations were found between treatments in receptor chamber. It suggests that root ZR was transferred unidirectionally from donor roots to receptor roots through CMNs. This is in agreement with findings of Wu et al. [2017], who found an unidirectional communication of root ZR from supplier trifoliate orange to supplier white clover association.

CONCLUSIONS

In short, CMNs could be established between trifoliate orange seedlings by *P*. *occultum*, and such CMNs stimulated the plant growth and root modification of receptor plants. Based on the analysis of eight signal substances, we concluded that NO, CaM and ZR as signal substances might take part in the underground communication of CMNs. Further works still need to clarify such communication through molecular technique and isotope labelling.

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