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Original Article

Exogenous Phytohormones and Mycorrhizas Modulate Root Hair Configuration in Trifoliate Orange

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Abstract

The study was carried out in a two-chambered rootbox separated by 37-µm nylon mesh to establish root+hyphae chamber carrying trifoliate orange (*Poncirus trifoliata*) as the test plant inoculated with *Diversispora versiformis* and hyphae chamber (without roots). This objective is to evaluate whether exogenous phytohormones regulate mycorrhizal effects on root hairs. Indole butyric acid (IBA), abscisic acid (ABA), and jasmonic acid (JA) (each at 0.1 µM concentration) were weekly applied into hyphae chamber, in total of six times before plant harvest. Mycorrhization strongly stimulated plant growth performance, and exogenous phytohormones, especially IBA, further magnified the mycorrhizal-stimulated growth response. Three exogenous phytohormones significantly decreased mycorrhizal colonization in taproot and first-order lateral roots, but increased in second- and third-order lateral roots, compared with non-phytohormone treatment in mycorrhozal plants. These phytohormones also increased hyphal length in nylon mesh and soil, irrespective of root+hyphae or hyphae chamber. Mycorrhizal inoculation significantly increased root hair density in different root classes, and exogenous hormones further strengthened the mycorrhizal effect. Average root hair length was stimulated by mycorrhization, but all exogenous phytohormones weakened the mycorrhizal response. Mycorrhization in combination with exogenous phytohormones showed no response on root hair diameter. Hence, the result suggested that application of exogenous phytohormones in hyphae chamber strengthened the *D. versiformis*-induced change in root hair density but weakened in root hair length in trifoliate orange grown in root+hyphae chamber.

Keywords: arbuscular mycorrhizal fungi, hormones, mycorrhizal hyphae, root system, symbiosis

Introduction

Trifoliate orange (*Poncirus trifoliata*) is one of the commercial citrus rootstocks (Srivastava *et al.*, 2008), predominantly used in tropical regions for extensive cultivation of world's premier cultivar, 'Satsuma' mandarin (*Citrus reticulata* Blanco). Trifoliate orange is highly dependent on arbuscular mycorrhizas (AMs) due to presence of coarse roots and lesser density of root hairs (Cao *et al.*, 2013; Wu *et al.*, 2013). Root properties are considered as a major driver to elevate nutrient-use-efficiency (Srivastava *et al.*, 1994). Meanwhile, root hairs as tubular structural expansions from epidermal cells of plant roots can extend the surface of root system for nutrient uptake (Janiak *et al.*, 2012). Root hair development can be highly influenced by a number of external or internal factors, such as nutrient reserves and microbial diversity within the rhizosphere, endogenous phytohormones, etc. (Vandamme *et al.*, 2013; Ngullie *et al.*, 2015). Of these, phytohormones are associated with root hair elongation and density as well, participating in root hair development (Libault *et al.*, 2010). The inductive response of exogenous auxin application on growth of root hairs was reported (Libault *et al.*, 2010), due to its ability of transport from non-hair cells to hair cells to maintain root hair development (Jones *et al.*, 2008). According to Zhu *et al.* (2006), jasmonic acid (JA) also promoted proliferation in root hair branches, thus, regulating root hair growth and

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altering root hair morphology. On the other hand, exogenous abscisic acid (ABA) is reported to display a lower magnitude of response on root hair elongation of clover plants (Katsumi *et al.*, 2000). Moreover, ABA accumulation maintained root hair development by proton secretion through the ABA-mediated auxin transport in root tip (Xu *et al.*, 2013). Nitric oxide (NO) is an important signaling molecule involved in root hair development in *Arabidopsis* by acting downstream auxins (Lombardo *et al.*, 2006).

Arbuscular mycorrhizal fungi (AMF), a kind of beneficial microorganism, can establish symbiosis with roots of ~80% terrestrial plants, namely, arbuscular mycorrhizas (AMs) (Smith and Read, 2008). In root systems, both mycorrhizal hyphae and root hairs coexist together (Hill *et al.*, 2010). Studies in the past had revealed the reduction in root hair density after inoculation with *Gigaspora rosea* in *Alnus glutinosa* plants (Orfanoudakis *et al.*, 2010) and *Funneliformis mosseae* and *Rhizoglomus intraradices* in *Sorghum bicolor* plants (Sun and Tang, 2013). However, previous studies showed higher root hair density and lower average root hair length in trifoliate orange seedlings colonized with *Claroideoglomus etunicatum*, *Diversispora versiformis*, *F. mosseae*, and *R. intraradices*, as compared to non-mycorrhizal seedlings (Wu *et al.*, 2016). In addition, mycorrhizal stimulated plant growth is attributed to higher root hair density (Zangaro *et al.*, 2005; Hill *et al.*, 2010) and lower root hair length (Manjunath and Habte, 1991; Declerck *et al.*, 1995).

Many phytohormones are secreted by the plants into the rhizosphere, thus, stimulating spore germination, hyphal branching, and metabolic activity of AMF (Carbonnel and Gutjahr, 2014), evident from strongly positive response of exogenous application of GA_3, IAA, and 6-BA on mycorrhizal hyphal length (Yang *et al.*, 2005; Ludwig-Müller, 2010). Many plants including trifoliate orange possess lesser root hair density (Cao *et al.*, 2013), which seriously restricted the nutrient absorption by these plants. Modulating root hair configuration is, therefore, vital for these plants to improve plant nutrition. In this background, the study was carried out to evaluate the effect of mycorrhizal inoculation, in combination with three exogenous phytohormones, on root hair configuration of trifoliate orange seedlings for it better growth.

Materials and Methods

Plant culture

Seeds of trifoliate orange were surface-sterilized with 70% ethanol for 10 min, rinsed with distilled water, and germinated and then grown on sterilized sand at 28°C for one month. And then, five-leaf-old seedlings were used as the plant material.

A two-chambered rootbox was made with polyvinyl chloride, having the dimension of 20 cm long × 10 cm width × 18 cm height. The rootbox was divided into two equal chambers, separated through 37-μm nylon mesh, capable of allowing extraradical mycorrhizal hyphae to crossover, but not plant roots, from one chamber to the other chamber. There is an air gap of 0.5 cm separated by two layers of the nylon mesh. Two five-leaf-old trifoliate orange seedlings were transplanted into one chamber as root+hyphae chamber, where 2300 spores of *Diversispora versiformis* (P. Karst.) Oehl, G.A. Silva & Sieverd were mixed with 1.5 kg of autoclaved (121°C, 0.11 MPa, 2 h) sand. And, the other chamber of the rootbox defined as hyphae chamber, included extraradical hyphae only. Non-AMF treatment was supplied with the same amount of autoclaved (121 °C, 0.11MPa, 2h) inoculum plus 2 mL filtrate (25 μm filter) of inocula to minimize differences in other microbial communities. The AM fungus, *D. versiformis*, commercially provided by the Bank of Glomeromycota in China, was isolated from the rhizosphere of *Astragalus adsurgens* in Ejin Horo Banner, Inner Mongolia Autonomous Region, China. The AMF strain was propagated with the identified spores, in combination with white clover as a host plant for 16 weeks in pots.

All the seedlings were grown in an environmentally controlled greenhouse of Yangtze University campus at average photosynthetic photon flux density of 880 μmol/m^2/s, day/night temperature 28/21 °C, and relative humidity 85% during March-August, 2014.

Experimental design

The experiment consisted of five treatments replicated three times in a completely randomized design. The five treatments included: (i) no application of both AMF and phytohormones (non-AMF), (ii) application of AMF (AMF), (iii) application of both AMF and 0.1 μM IBA (AMF+IBA), (iv) application of both AMF and 0.1 μM ABA (AMF+ABA), and (v) application of both AMF and 0.1 μM JA (AMF+JA). The concentration of 0.1 μM IBA, ABA and JA used was selected according to the result of Zhang *et al.* (2013).

After transplanting of the seedlings into the root+hyphae chamber, 50 mL of the Hoagland nutrient solution was applied at every two days interval for 3 months. The hyphae chamber was treated with 50 mL distilled water only. Subsequently, 50 mL IBA, ABA, and JA (at 0.1 μM concentration each) were applied weekly into the hyphae chamber, whilst root+hyphae chamber was still kept with 50 mL Hoagland nutrient solutions. In total, six times of exogenous phytohormone applications were given. The seedlings were harvested after two weeks in the end of exogenous phytohormone application (August 18, 2014).

Measurements of variables

Growth parameters such as plant height, stem diameter, and leaf numbers were determined following the time of harvesting. The fresh weight of shoot and roots was determined separately. The whole root system was placed in a test-bed, and the numbers of lateral root were counted artificially. Root AMF colonization was assayed by clearing 1-cm-long root fragments with 10% KOH at 90 °C for 1.5 h and stained with 0.05% trypan blue for 5 min (Phillips and Hayman, 1970), and expressed as the percentage of AM infected root lengths against total root length.

The nylon mesh was collected from root-box and cut into 2 × 2 cm size, stained with 0.05% trypan blue in lactophenol for 3 min, and observed in an optical microscope (Zou *et al.*, 2015). Soil hyphal length in a 0.5 g soil sample was determined by the protocol described by Bethlenfalvay and Ames (1987).

Root NO was extracted with 0.2 g fresh root samples in 5 mL phosphate buffer (pH, 7.0) at 4°C and centrifugated at 10000 g for 20 min. The supernatant was immediately used to

determine NO level, which was assayed by the enzyme-linked immunosorbent assay (ELISA) in NO kit (A012, Nanjing Jiancheng Bioengineering Institute, Nanjing, China) as per the supplier's guide.

For phytohormone assay, 0.2 g of fresh roots was frozen in liquid nitrogen, crushed with 4 mL 80% methanol containing 1 mM 2,6-ditertbutyl-4-methylphenol, incubated for 4 h at 4 °C, and finally centrifuged at 4,000 *g* for 10 min at 4 °C (Chen *et al.*, 2009). The supernatants were isolated by an AccuBond C18 solid phase extraction cartridge (Agilent Technologies Inc., USA). The residues were utilized to analyze the IAA, MeJA, ABA, and zeatin riboside (ZR) concentration according to ELISA, manufactured by the Crop Chemical Control Center, China Agricultural University, Beijing.

Twenty two-cm-long root segments per treatment from first- or second-order lateral root were selected to analyze root hair morphology. Root hairs in lateral roots were measured by the protocol proposed by Zhang *et al.* (2013) after pre-fixing the roots with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), dehydrated by alcohol with the increasing concentrations, and dried by the critical-point drying (CPD). Photographs of root hairs were collected with a scanning electron microscope (JSM-6390LV, JEOL Co., Japan) at 100 and 400 magnifications. Root hair density, length, and diameter in 30 photographs of root hair per root segment were measured using the Image J software (National Institutes of Health, Maryland, USA).

Statistical analysis

Data were subjected to the analysis of the one-factor variance (ANOVA) with SAS software, and the significant differences among the treatments were compared with the Duncan's multiple range test ($P<0.05$).

Results

Mycorrhizal status

Roots of trifoliate orange seedlings were colonized by *D. versiformis* (Fig. 1a). Exogenous application of phytohormones into hyphae chamber altered the intensity of root mycorrhizal colonization in AM seedlings (Fig. 2). In AM plant, exogenous ABA, IBA, and JA dramatically reduced root mycorrhizal colonization by 35%, 39%, and 41% in taproot part and by 43%, 33%, and 41% in first-order lateral roots, respectively, as compared with non-phytohormone treatment. On the other hand, ABA, IBA, and JA treatments accelerated the root mycorrhizal colonization in second-order lateral roots by 7%, 44%, and 20%, respectively. Mycorrhizal seedlings treated with exogenous IBA and JA showed 13% and 9% significantly higher root colonization in third-order lateral roots, as compared with AM seedlings only. Interestingly, application of JA into hyphae chamber showed no effect on average root colonization, while ABA and IBA treatments strongly reduced average mycorrhizal colonization.

Extraradical mycelium were found in the 37-μm of nylon mesh, and rest of the external hyphae passed through the nylon mesh from root+hyphae to hyphae chamber (Fig. 1b, 1c), confirming the formation of an external hyphal network between two different chambers. Compared with sole AMF treatment, ABA, IBA, and JA application markedly increased hyphal length of nylon mesh by 51%, 26% and 84% in root+hyphae chamber and by 139%, 103% and 197% in hyphae chamber, respectively (Fig. 3a, 3b). Soil hyphal length was 22%, 14% and 40% significantly higher in root+hyphae chamber and 88%, 67% and 103% higher in hyphae chamber after ABA, IBA, and JA application, compared with alone AMF treatment (Fig. 3a, 3b).

Plant growth performance

Inoculation with AMF significantly promoted the growth performance of the host plants in terms of plant height, stem diameter, leaf number and the whole plant biomass production, irrespective of exogenous phytohormones applied into hyphae chamber or not (Table 1). And exogenous application of phytohormones into hyphae chamber like ABA, IBA, and JA further stimulated significant increase in growth performance of AMF seedlings. Of them, IBA application exhibited the highest magnitude of the response.

Number of lateral roots

Lateral roots of trifoliate orange were dominated by second order (Table 2). Sole AMF treatment did not alter number of first-order lateral roots but significantly decreased number of second-order and third-order lateral roots, as compared with non-AMF treatment. In addition, application of exogenous ABA and IBA into hyphae chamber considerably increased number of second- and third-order lateral roots, and JA significantly increased number of second-order lateral roots and decreased number of third-order lateral roots.

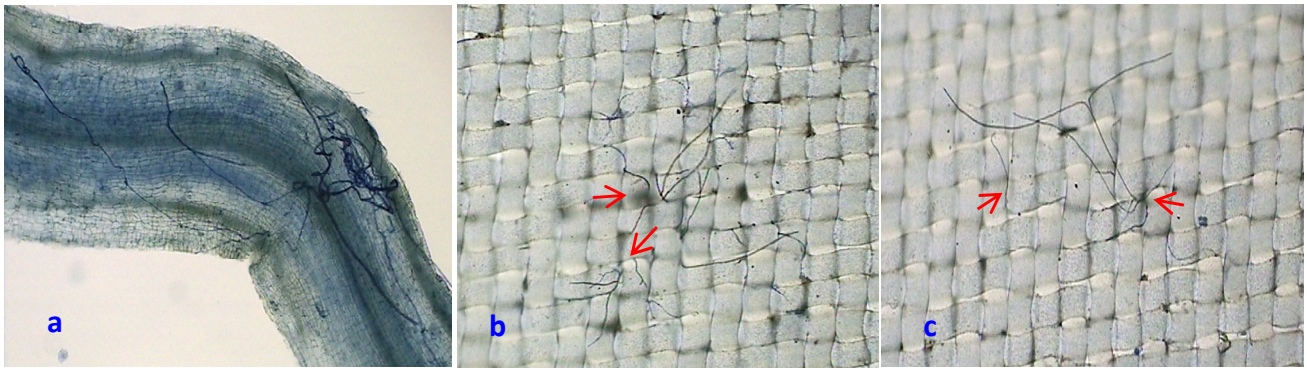

Fig. 1. Root mycorrhizal colonization (a) and extraradical mycelium (b in root+hyphae chamber; c in hyphae chamber. Red arrow shows the entry of extraradical mycelium into 37-μm mesh) in 37-μm mesh separated between root+hyphae and hyphae chambers in trifoliate orange (*Poncirus trifoliata*) seedlings inoculated with *Diversispora versiformis*

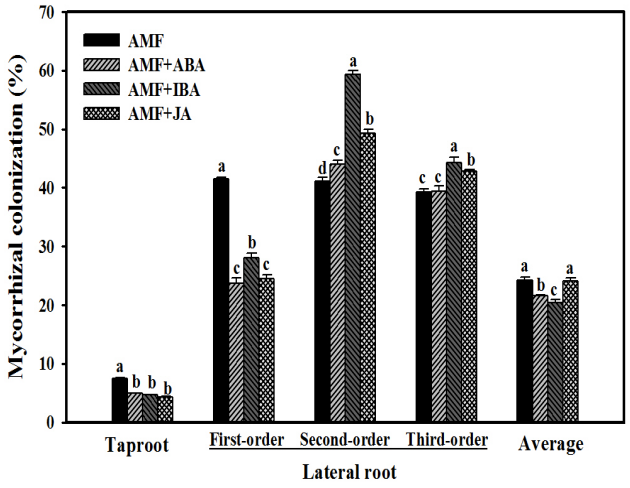

Fig. 2. Effect of *Diversispora versiformis* and exogenous phytohormones on mycorrhizal colonization of different root classes in trifoliate orange (*Poncirus trifoliata*) seedlings grown in a two-chambered rootbox. Data (means ± SD, n = 3) followed by different letters above the bars indicate significant differences between treatments with the Duncan's multiple range test at 5% level. Here, the data of non-AMF treatment were not shown due to non-mycorrhizal colonization. Abbreviations: same as for Table 1

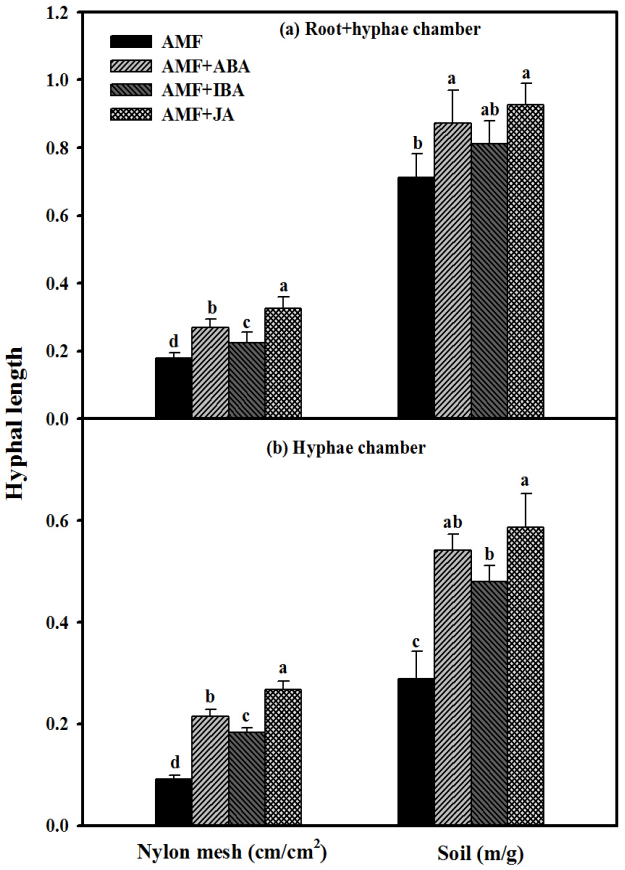

Fig. 3. Effect of *Diversispora versiformis* and exogenous phytohormones on hyphal length in 37-μm nylon mesh and soil of trifoliate orange (*Poncirus trifoliata*) seedlings grown in a two-chambered rootbox. Data (means ± SD, n = 3) followed by different letters above the bars indicate significant differences between treatments with the Duncan's multiple range test at 5% level. Here, the data of non-AMF treatment were not shown due to non-mycorrhizal hyphal formation. Abbreviations: same as for Table 1

Changes in root endogenous hormones

Compared with non-AMF treatment, AMF-inoculation alone or in combination with ABA, IBA, and JA produced a significant increase in root ZR concentration by 15%, 59%, 38%, and 69% and in root ABA concentration by 30%, 64%, 96%, and 53%, respectively (Fig. 4).

AMF inoculation showed no significant effect on root IAA concentration. However, AMF inoculation in combination with exogenous ABA, IBA, and JA application into hyphae chamber significantly elevated root IAA concentration, as compared to non-AMF treatment. The root MeJA concentration was significantly increased by 19% with AMF inoculation. ABA and JA treatments in AM seedlings markedly increased root MeJA concentration by 79% and 49%, compared with non-AMF treatment. However, AMF+IBA treatment induced a significantly lower root MeJA concentration, as compared with non-AMF or AMF treatment alone.

Changes in root NO level

Compared with non-AMF treatment, sole AMF treatment significantly decreased root NO concentration by 63%. However, the dual treatment of AMF inoculation and exogenous phytohormones (ABA, IBA, JA) application into hyphae chamber were dramatically increased the root NO concentration of trifoliate orange seedlings by 90, 157, and 91%, respectively (Fig. 4).

Table 1. Effect of *Diversispora versiformis* and exogenous phytohormones on plant growth performance of trifoliate orange (*Poncirus trifoliata*) seedlings grown in a two-chambered rootbox

Treatments	Plant height (cm)	Stem diameter (cm)	Leaf number per plant	Fresh weight (g FW/plant)	
				Shoot	Root
Non-AMF	13.8±0.7e	0.22±0.05c	15±1c	0.70±0.12d	0.87±0.05c
AMF	18.1±0.9d	0.28±0.03bc	16±1c	1.02±0.09c	0.88±0.08c
AMF+ABA	26.6±0.9b	0.35±0.04a	23±1b	1.66±0.07b	1.32±0.07b
AMF+IBA	35.2±0.8a	0.35±0.03a	26±1a	2.43±0.11a	1.84±0.04a
AMF+JA	22.7±0.8c	0.29±0.03ab	23±1b	1.54±0.07b	1.35±0.09b

Data (means ± SD, n = 3) followed by different letters among treatments indicate significant differences with the Duncan's multiple range test at 5% level. Abbreviation: non-AMF: inoculation without *Diversispora versiformis*; AMF: inoculation with *Diversispora versiformis*; AMF+ABA: treatment with *Diversispora versiformis* and ABA; AMF+IBA: treatment with *Diversispora versiformis* and IBA; AMF+JA: treatment with *Diversispora versiformis* and JA.

Table 2. Effect of *Diversispora versiformis* and phytohormones on number of lateral roots in trifoliate orange (*Poncirus trifoliata*) seedlings grown in a two-chambered rootbox

Treatments	Numbers of lateral root (#)		
	First-order	Second-order	Third-order
Non-AMF	34±4c	113±9d	10±1c
AMF	36±4bc	98±9e	8±2d
AMF+ABA	40±5abc	145±6b	14±2b
AMF+IBA	42±4ab	195±8a	49±3a
AMF+JA	45±5a	129±6c	6±1de

Data (means ± SD, n = 3) followed by different letters among treatments indicate significant differences with the Duncan's multiple range test at 5% level. Abbreviations: same as for Table 1

Table 3. Correlation coefficients between root endogenous ABA, IAA , JA, ZR, NO and average root hair variables , AM colonization, hyphal length in root chamber and hyphae chamber (n=12)

	Average root hair density	Average root hair length	Average root hair diameter	Average root AM colonization	Soil hyphal length	
					Root+hyphae chamber	hyphae chamber
ABA	0.51	-0.83**	0.24	-0.77*	0.32	0.45
IAA	0.38	-0.40	0.23	-0.39	0.54	0.54
MeJA	0.01	0.20	-0.28	0.42	0.43	0.34
ZR	0.77 **	-0.63*	-0.05	-0.03	0.88**	0.92**
NO	0.81*	-0.89**	0.18	-0.70*	0.53	0.74**

* P<0.05; **P<0.01.

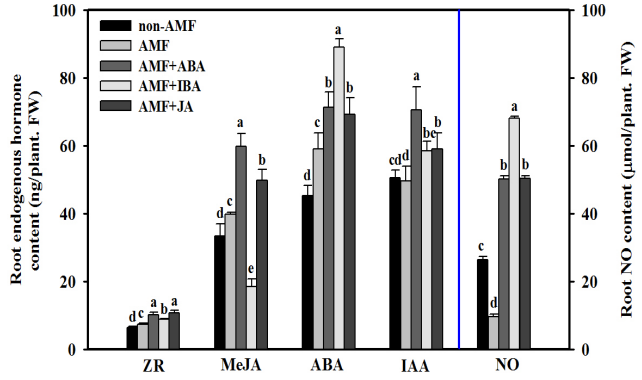

Fig. 4. Effect of *Diversispora versiformis* and exogenous phytohormones on root endogenous hormone and NO concentrations of trifoliate orange (*Poncirus trifoliata*) seedlings grown in a two-chambered rootbox. Data (means ± SD, n = 3) followed by different letters above the bars indicate significant differences between treatments with the Duncan's multiple range test at 5% level. Abbreviations: same as for Table 1

Changes in root hair development

Inoculation with AMF significantly increased root hair density of first-, second-, and third-order lateral roots by 114%, 37%, and 37%, respectively, compared with non-AMF treatment (Fig. 5a). AM seedlings supplied with exogenous phytohormones like ABA, IBA, and JA into hyphae chamber showed a considerably lower root hair density in first-order lateral roots and higher root hair density in second- and third-order lateral roots, as compared with AMF seedlings without phytohormone treatments. The response of different treatments on average root hair density was rated as AMF+JA > AMF+IBA > AMF+ABA > AMF > non-AMF in decreasing order.

The treatments like AMF and AMF+ABA showed no significant response on root hair length in first-order lateral roots compared to non-AMF treatment. Nevertheless, AMF+IBA and AMF+JA treatments produced a significantly lower root hair length than either non-AMF treatment or AMF treatment alone (Fig. 5b). Compared with non-AMF treatment, AMF inoculation alone or in combination with exogenous ABA, IAA, and JA resulted in a significantly higher root hair length by 13%, 27%, 4%, and 35% in second-order lateral roots and 221%, 83%, 98%, and 111% higher in third-order lateral roots, respectively. The magnitude of response on average root hair length could be rated as AMF > AMF+ABA ≈ AMF+JA ≥ AMF+IBA > non-AMF in decreasing order.

In first-order lateral roots, AMF inoculation alone did not alter root hair diameter, but AMF inoculation in combination with ABA and IBA into hyphae chamber induced significantly higher root hair diameter (Fig. 5c). The treatment of AMF+JA resulted in lower root hair diameter, as compared with non-AMF inoculation treatment. In second-order lateral roots, AMF colonization significantly decreased root hair diameter than non-AMF treatment. On the other hand, exogenous ABA, IAA, and JA application in combination with mycorrhization significantly increased root hair diameter than treatment involving mycorrhization alone, but without altering root hair diameter with non-AMF treatment. In third-order lateral roots, different treatments like AMF, AMF+ABA, AMF+IBA, and AMF+JA significantly increased root hair diameter by 26%, 9%, 20%, and 25%, respectively, compared to non-AMF treatment. AMF inoculation showed no effect on average root hair diameter, as compared with non-AMF treatment. And, exogenous application of ABA, IBA, and JA into hyphae chamber did not alter the average root hair diameter in AM seedlings.

Correlation studies

Soil hyphal length in either root+hyphae chamber or hyphae chamber was significantly and positively correlated with average root hair density and root ZR concentration, but negatively correlated with average root hair length (Fig. 6a-6c;

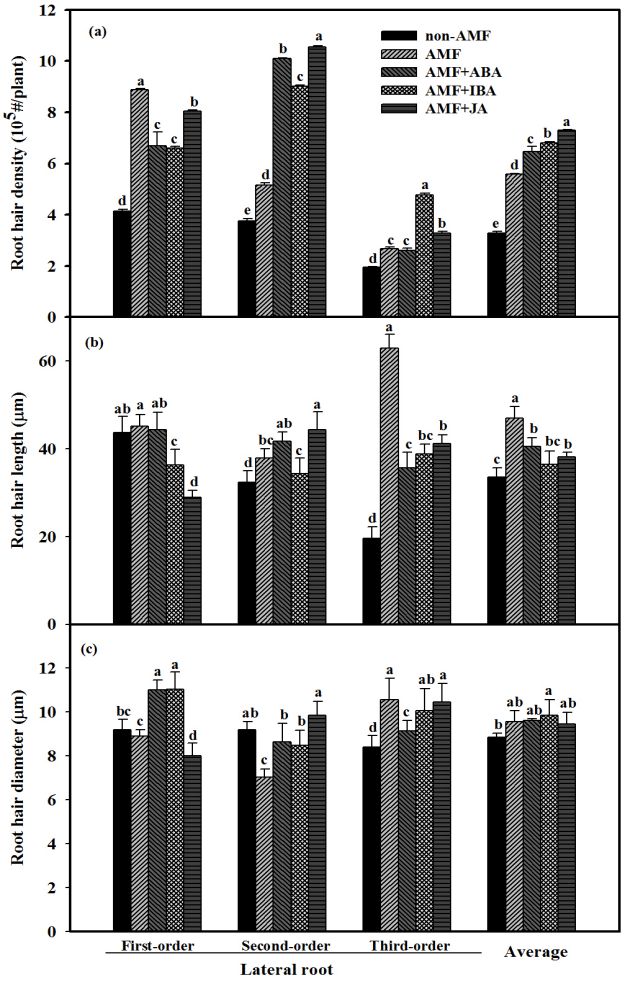

Fig. 5. Effects of *Diversispora versiformis* and exogenous phytohormones on root hair development of lateral roots of trifoliate orange (*Poncirus trifoliata*) seedlings grown in a two-chambered rootbox. Data (means ± SD, n = 3) followed by different letters above the bars indicate significant differences between treatments with the Duncan's multiple range test at 5% level. Abbreviations: same as for Table 1

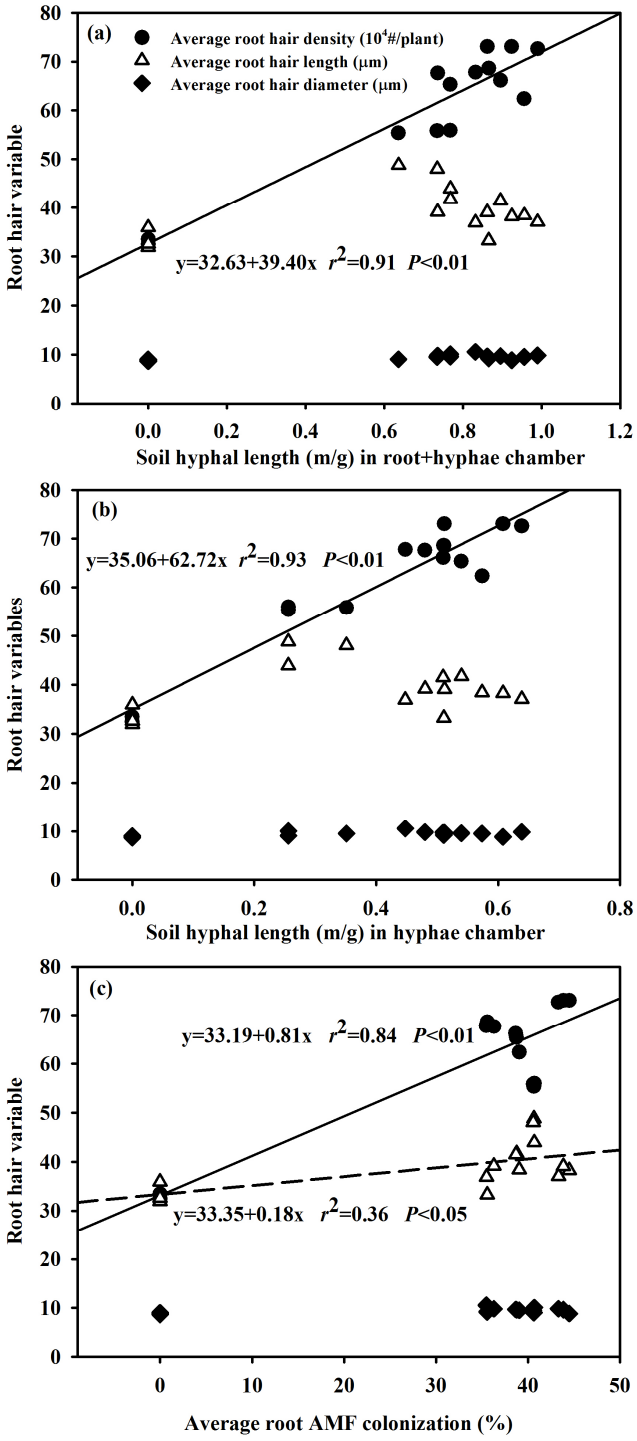

Fig. 6. Linear regression between root hair variables and soil hyphal length (a and b) or root mycorrhizal colonization (c) (n=12)

Table 3). Soil hyphal length in hyphae chamber only was positively correlated with root NO concentration (Table 3).

Average root hair density was significantly and positively correlated with root endogenous ZR and NO concentration (Table 3). While, average root hair length was significantly and negatively correlated with root ABA, ZR, and NO concentration. There was no correlation of average root hair diameter with root endogenous ABA, IAA, MeJA, ZR, and NO concentration.

Discussion

In this study, exogenous application of ABA, IBA, and JA into hyphal chamber significantly increased the growth performance and promoted the number of second-order lateral roots in trifoliate orange seedlings grown in root+hyphae chamber. Niranjan *et al.* (2007) earlier reported that exogenous application of IAA triggered the growth performance of *Dalbergia sissoo* plant colonized by *Glomus fasciculatum*. Earlier studies also showed a significant increase in number of lateral roots in *Arabidopsis* treated with exogenous auxin (Boerjan *et al.*, 1995; Laskowski *et al.*, 1995). Exogenous ABA showed a dramatic effect on root morphology via elongation of primary root, but strongly restricted the formation of lateral roots (Smet *et al.,* 2003). The present work revealed that 0.1 μM ABA applied into hyphae chamber remarkably increased the number of second- and third-order lateral roots in trifoliate orange.

Possibly, ABA was applied into hyphae chamber, which further stimulated the formation of lateral roots. Interestingly, ABA and IBA dramatically stimulated the increase in third-order lateral root number, whereas JA did not alter third-order lateral root number, implying that the change is dependent on phytohormone species.

In this study, exogenous application of IBA, ABA, and JA into hyphae chamber induced a significant reduction in root AMF colonization in taproot and first-order lateral roots, but significantly stimulated the increase of root colonization in second- and third-order lateral roots. It seems that IBA, ABA, and JA-induced AMF colonization, preferentially targeting the development of lateral root classes. Extraradical hyphae, however, influence the efficiency of mycorrhizae more strongly than root colonization in a nutrition ecosystem (McGonigle *et al.*, 1990), due to effective increase in the volume of soil explored for nutrients extending beyond the nutrient depletion zone around the roots (Allen *et al.*, 2003). In the present work, exogenous application of phytohormone (IBA, ABA and JA) into hyphae chamber markedly promoted the development of AM external hyphae in nylon mesh and soil, irrespective of chamber type, containing root+hyphae or hyphae alone. The best effect was found under JA application conditions. Auxins are involved in the host-AMF interaction, due to the pre-symbiotic exchange of signals during early stages of AM formation (Fusconi, 2014). On the other hand, JA contributed towards the life cycle of AMF, and AM formation was dependent on the concentration of JA used (Ludwig-Müller, 2010). Evidently, root endogenous IAA and MeJA concentrations were not significantly correlated with average root colonization and soil hyphal length. As proposed by Ludwig-Müller (2010), ABA is more effective towards the AM functional establishment than simply a stress signal involvement in arbuscule formation. ZR, one of cytokinins, is produced by AMF, and plays a key role in the initial phases of AM colonization. In the present study, average root AMF colonization was significantly and negatively correlated with root endogenous ABA and NO concentration, suggesting that ABA and NO could effectively participate in regulation of root mycorrhizal colonization. Positive correlation of root ZR concentration with soil hyphae length in root+hyphae and hyphae chamber, and with root NO in hyphae chamber, suggested that ZR and NO as an important modifier involved in the process of formation of AM hyphae and their proliferation .

This study indicated significantly higher root hair density in mycorrhizal than in non-mycorrhizal trifoliate orange seedlings, irrespective of orders of lateral roots or exogenous phytohormones applied into hyphae chamber or not. The observations also suggest that mycorrhizas possess the ability to regulate root hair configuration. These observations are in accordance with previous works in trifoliate orange seedlings infected with four different AMF species (Wu *et al.*, 2016). Greater root hair density in AM plants is a key way forward process of increasing the colonized sites by AMF (Kohls *et al.*, 1989), as evident for the significant positive correlation between average root hair density and average root colonization. On the other hand, it is further implied that AMF inoculation has a profound influence on the development of root hair density through a series of consequential processes. Possibly, AM infection triggered the root hair formation from epidermal cell outgrowth (Hill *et al.*, 2010). Exogenously applied ABA, IBA, and JA into hyphae chamber significantly increased the root hair density in AM seedlings, further suggesting that these phytohormones have a stimulatory role in development of root hairs. Positive correlation of average root hair density with root ZR and NO concentration, further strengthened these facts. However, root MeJA, ABA, and IAA concentrations were not significantly correlated with average root hair density. Possibly, JAs mainly promoted branching of root hairs, but not the root hair density (Zhu *et al.*, 2006), since IAA was involved in root hair cell fate decision (Rahman *et al.*, 2002). These observations suggested that mycorrhization stimulated root hair density, possibly through elevated synthesis of ZR and NO within the roots.

According to Zhang *et al.* (2013), root hair development of trifoliate orange was strongly promoted by exogenous IBA, which was confirmed in this study as well, since AMF inoculation markedly increased the root hair density of trifoliate orange, besides positive correlation of root AM colonization with root hair density. Moreover, root AMF colonization was significantly positively correlated with average root hair length. However, exogenous application of phytohormones considerably decreased the root hair length in AM seedlings, possibly because root hair elongation principally depends on delivery of cell wall materials to the growing tips (Assad, 2009). As proposed by Wu *et al.* (2016), both root hairs and mycorrhizal hyphae have a similar diameter, and the latter could extend beyond root depletion zones. These exogenous phytohormones into hyphae chamber are likely to be insufficiently absorbed by the mycorrhizal extraradical hyphae. In this study, root hair length was negatively correlated with root ABA, ZR, and NO concentrations. These observations are in agreement with the findings of Wu *et al.* (2016) using AMF-colonized trifoliate orange. Interestingly, soil hyphal length in root+hyphae chamber and hyphae chamber was positively correlated with root hair density, but negatively correlated with root hair length, indicating a diversified response, from development of soil mycorrhizal hyphae to root hair configuration in trifoliate orange.

NO is a sensitive signal molecule involving in plant-pathogen and plant-AMF interactions (Calcagno *et al.*, 2012). Studies showed that NO played an important role in root hair formation in Arabidopsis through regulating auxin level (Lombardo *et al.*, 2006). The present study showed a significant positive correlation between root NO concentration and average root hair density, indicating that NO was involved in the root hair formation of trifoliate orange seedlings. Calcagno *et al.* (2012) demonstrated that NO accumulated rapidly in *Medicago truncata* roots which were inoculated with the exudate from *Gigaspora margarita* spore cell walls. However, in the present work, sole AMF treatment induced a significantly lower root NO concentration, whereas AMF inoculation in combination with exogenous phytohormones significantly increased root NO concentration. And, root NO was significantly positively correlated with soil hyphal length in hyphae chamber. These results suggested that NO is involved in the establishment of mycorrhizas (Espinosa *et al.*, 2014), and is regulated by exogenous phytohormones.

It is well documented about the changes in endogenous phytohormone levels of the AM plant (Fusconi, 2014; Wu *et al.*, 2016). An increase of ABA level has been reported earlier in

maize (Danneberg *et al.*, 1992) and soybean (Meixner *et al.*, 2005). Likewise, an increase of IAA in AM onion (Torelli *et al.*, 2000), AM litchi (Yao *et al.*, 2005) and AM soybean (Meixner *et al.*, 2005) was also well documented. In AM barley and cucumber, considerably higher JA levels had been observed, compared to non-AM controls (Hause *et al.*, 2002; Vierheilig and Piche, 2002). The present study indicated the significantly higher root ZR, MeJA, and ABA concentrations in AMF seedlings than non-AMF seedlings. These observations suggested that mycorrhization triggers the endogenous phytohormone responses of the host plant (Ludwig-Müller, 2010). The significant response of exogenous ABA, IBA, and JA treatments on the increase in root endogenous ABA, IAA, ZR, and MeJA concentrations in AM trifoliate orange seedlings corroborate these facts. However, there is lower concentration of root MeJA in AMF+IBA than treatment carrying AMF alone. These observations, hence, implied that these exogenous phytohormones into hyphae chamber might either stimulate the growth of the extraradical mycorrhizal mycelium aiding in higher production of endogenous hormones, or assimilate by the extraradical mycelium. Interestingly, the lower root MeJA concentrations in AMF+IBA treatment than AMF alone support the fact that JA and auxin conjugation had completely diverse functions during AM formation (Ludwig-Müller, 2010), or JA is involved in the penetration of fungal hyphae inside plant cells (Foo *et al.*, 2013).

Conclusions

Application of exogenous phytohormones into hyphae chamber showed a reduction in root colonization by *D. versiformis*, but increased hyphal length in mesh and soil. Exogenous phytohormones strongly promoted the growth performance of *D. versiformis*-colonized seedlings as a consequential effect of increased root endogenous phytohormone levels. Mycorrhization with *D. versiformis* induced an increase in average root hair density and average root hair length, and exogenous phytohormones into hyphae chamber further strengthened the *D. versiformis*-induced change in root hair density but weakened in root hair length. Such results will activate the regulation of root hairs by mycorrhization and further by exogenous phytohormones in root-hair-deficient plants for effective absorption of nutrients in fertility depleted land ecosystem.

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