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Original Article

Mycorrhizal Fungi Regulate Root Responses and Leaf Physiological Activities in Trifoliate Orange

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Abstract

Plant responses to mycorrhization are mediated through secretion of certain signal molecules deposited in mycorrhizosphere in response to environmental stimuli. Responses of four arbuscular mycorrhizal fungi (AMF), namely *Claroideoglomus etunicatum*, *Diversispora versiformis*, *Funneliformis mosseae*, and *Rhizoglomus intraradices* on root morphology, lateral root (LR) number, and leaf carbohydrates, nitric oxide (NO), and calmodulin (CaM) changes were studied using trifoliate orange. Inoculation response of *D. versiformis*, *F. mosseae*, and *R. intraradices* registered significantly higher plant growth performance (plant height, stem diameter, leaf number, and shoot and root biomass), root morphological traits (total length, projected area, surface area, and volume), and LR number (first-, second-, third-, and forth-order), compared to un-inoculated response. Higher concentrations of CaM, NO, glucose, and fructose and lower sucrose level in leaves were observed in AMF-seedlings than in non-AMF seedlings. Correlation studies further revealed, root morphological traits and LR numbers were significantly negatively correlated with sucrose whereas positively correlated with glucose, fructose, NO, and CaM level in leaves. These results suggested, AMF-induced root modification is routed through sucrose cleavage and partly through changes in NO and CaM.

Keywords: calmodulin, nitric oxide, root morphology, symbiosis, sucrose cleavage

Introduction

Arbuscular mycorrhizal fungi (AMF) are reported to establish symbiotic association, with roots of ~80% of terrestrial plants (Kiers and van der Heijden, 2006). Such symbiosis derives ~20% of photosynthetic carbohydrates from the host plant on account for mycorrhizal growth, and in return, AMF provide the host plant, a greater access to nutrients and water absorption (Smith and Read, 2008; Parniske, 2008). The essential roles of AMF in crops like citrus, litchi, strawberry, lettuce, pepper etc are well documented (Borowicz, 2010; Ortas *et al*., 2011). Trifoliate orange [*Poncirus trifoliata* (L.) Raf.] is a rootstock used in citriculture in Asia (Srivastava *et al*., 2008). Citrus is usually considered as a crop severely lacking in root hair (Srivastava and Singh, 2009; Wu *et al*., 2016), and thus depends heavily on AMF for meeting the nutrient requirement.

Root systems play a significant role in acquisition of nutrients from within a given soil volume (Yao *et al*., 2009). Different microbial communities play an important role in growth and developmental responses in host plants (Sorgona *et al*., 2007; Jung *et al*., 2013). Of them, AMF have shown to regulate root system architecture through enhanced mineral nutrient absorption (Smith and Read, 2008). Earlier studies showed that AMF-inoculation strong stimulated morphological modification in root features like root length, surface area, and volume to varying proportions (Schellenbaum *et al*., 1991; Wu *et al*., 2011, 2015a). AMF-induced root modification is reported to have strong relations with metabolism of endogenous polyamines (Wu *et al*., 2010). However, such relationship is independent of signaling of common symbiotic transactions (Gutjahr *et al*., 2009). On the other hand, Isobe *et al*. (2002) reported a negative effect of AMF inoculation on the length and the number of tap roots and lateral roots (LRs) in *Phaseolus vulgaris*. Root morphological alteration depends on AMF species and plant genotypes (Yao *et al*., 2009; Wu *et al*., 2011; Li *et al*., 2013). These studies suggested number of mechanisms involved in describing AMF-induced LR development such as, excretion of AMF spore germination, phosphorus (P) nutrition

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improvement, changes in hormone levels, sugar signals, synthesis of nitric oxide (NO) as a signaling molecule, and active involvement of calmodulin (CaM) as Ca^{2+} receptors (Zhao *et al*., 2007; Yang *et al*., 2010; Chen and Kao, 2012; Zhang *et al*., 2013; Fusconi, 2014).

In this background, the present study evaluated the responses of different AMF species on root colonization, root morphology, LR number, and leaf carbohydrates, NO, and CaM changes to understand the underlying mechanistic insights involved.

Materials and Methods

Experimental setup

The experiment was carried out during March-August, 2013 at Yangtze University, Jingzhou, China. On March 30, 2013, five-leaf-old seedlings (~6 cm height) without mycorrhization were transplanted into a 4.8-L pot filled with 4.5 kg autoclaved (121 °C, 2h) sands. As many four AMF species viz., *Claroideoglomus etunicatum* (W.N. Becker & Gerd.) C. Walker & A. Schüßler, *Diversispora versiformis* (P. Karst.) Oehl, G.A. Silva & Sieverd, *Funneliformis mosseae* (T.H. Nicolson & Gerd.) C. Walker & A. Schüßler, and *Rhizoglomus intraradices* (N.C. Schenck & G.S. Sm.) C. Walker & A. Schüßler were tested. As a result, the experiment had five treatments, namely, *C. etunicatum*, *D. versiformis*, *F. mosseae*, *R. intraradices* and non-AMF control. Each treatment had four replicates, for a total of 20 pots (three seedlings per pot).

At the same time, approx. 1000 spores of each AM fungus used were mixed along with sands for AMF treatment. The non-AMF control received the same amount of autoclaved inoculums plus 2 mL filtrate (25 μm filter) of mycorrhizal inoculums to minimize the differences in other microbial communities. Seedlings were grown in a controlled environment characterized by 27/20 °C (day/night) temperature, 982 μmol/m^2/s photonflux density, and 80% relative air humidity. A 150 mL Hoagland solution (1/10 P strength) was applied into each pot at an alternate day. The AMF- and non-AMF seedlings were harvested on August 17, 2013.

Observations and analysis

Plants following their harvest, were divided into shoots and roots, and recorded the fresh biomass. The root system from each pot was scanned with the Epson Perfection V700 Photo Dual Lens System (Seiko Epson Corp, Japan). Root images were then analyzed through the WinRHIZO software (Regent Instruments Incorporated, Canada), to obtain different root traits viz., length, surface area, volume, and diameter. The root of seedlings was divided into taproot and LRs, to count the number of LRs artificially on a test-bed.

Root mycorrhizal colonization was determined using clearing with 10% KOH at 90 °C for 1.5 h, then staining with 0.05% trypan blue for 5 min (Phillips and Hayman, 1970).

NO concentration in leaves was estimated with the ELISA assay with NO kit (A012, Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and CaM using the Plant CaM ELISA Kit (YAD 001, Beijing Dingguochangsheng Biotechnology Co., Ltd., Beijing, China). Carbohydrate forms such as fructose, glucose and sucrose concentrations in leaves were assayed through the protocol as described by Wu *et al*. (2015b).

Statistical analysis

Data (means ± SD, $n = 4$) were analyzed by one-way variance (ANOVA). Significant differences between treatments were compared with the Duncan's multiple range tests at $P < 0.05$. Pearson correlation coefficients between variables were carried out with Proc Corr procedure. All the statistical analyses were performed using the SAS software 8.1v.

Results and Discussion

Root mycorrhizal colonization and plant growth performance

Mycorrhizal colonization is associated with different plant growth parameters through an increase in root absorptive surface area (Aguín *et al*., 2004). Root AMF colonization was observed to vary from 17.64% to 29.69%, following the order of *F. mosseae* ≈ *D. versiformis* > *R. intraradices* > *C. etunicatum* in the decreasing order (Table 1). With the exception of *C. etunicatum*, other three AMF species showed a significant increase in different growth attributing parameters viz., plant height, stem diameter, leaf number, and shoot and root biomass, compared to non-AMF treatment (Table 1). Studies in the past showed strong positive effect of AMF colonization on growth of the host plant (Aguín *et al*., 2004). Our studies showed, except *C. etunicatum*, inoculation with *F. mosseae*, *D. versiformis*, and *R. intraradices* produced significantly better plant growth performance, which is possible due to improved nutrients and the compatibility between AMF and host plants (Yao *et al*., 2009).

Response on root traits

AMF-inoculation was observed to alter different root traits of trifoliate orange seedlings (Fig. 1; Table 2). Out of four AMF species, three species viz., *F. mosseae*, *D. versiformis* and *R. intraradices* significantly increased root total length, projected area, surface area, and volume; however, *C. etunicatum* increased root total length only. Root average diameter also, remained unaffected with all the four AMF species. Likewise higher LR number was recorded in different order than non-AMF-

Table 1. Effects of different AMF species (*Claroideoglomus etunicatum*, *Diversispora versiformis*, *Funneliformis mosseae*, and *Rhizoglomus intraradices*) on plant growth and mycorrhizal development of trifoliate orange (*Poncirus trifoliata*) seedlings

Treatments	Plant height (cm)	Stem diameter (mm)	Leaf number	Biomass (g FW/plant)		AMF colonization (%)
				Shoot	Root	
Non-AMF	36.6±3.6c	3.57±0.29c	32±2b	2.69±0.34c	1.83±0.35d	0d
C. etunicatum	36.1±6.7c	3.55±0.16c	32±3b	2.64±0.68c	1.72±0.35d	17.64±0.63c
D. versiformis	66.5±3.2a	4.79±0.39a	41±2a	7.06±0.51a	3.86±0.31b	29.58±1.36a
F. mosseae	67.6±7.5a	5.08±0.40a	43±2a	7.60±0.93a	4.32±0.53a	29.69±2.20a
R. intraradices	56.5±6.0b	4.37±0.33b	40±3a	5.11±0.94b	2.83±0.45c	22.68±2.61b

Note: Data (means ± SD, $n = 4$) followed by different letters indicate significant differences ($P < 0.05$) between treatments

Table 2. Effects of different AMF species (*Claroideoglomus etunicatum*, *Diversispora versiformis*, *Funneliformis mosseae*, and *Rhizoglomus intraradices*) on root morphological traits and lateral root (LR) number of trifoliate orange (*Poncirus trifoliata*) seedlings

Treatments	Total length (cm)	Project area (cm^2)	Surface area (cm^2)	Average diameter (mm)	Volume (cm^3)	Number of LR (#/plant)			
						First-order	Second-order	Third-order	Forth-order
Non-AMF	373±16e	58.4±7.4b	183.4±5.6b	1.50±0.24a	6.57±0.86b	49±3c	138±10c	21±5c	1±0c
C. etunicatum	436±11d	59.6±9.9b	187.2±8.9b	1.38±0.13a	6.94±0.89b	51±3bc	150±12c	29±6c	4±1b
D. versiformis	552±21c	81.5±8.0a	256.0±8.2a	1.53±0.26a	10.08±0.68a	58±5a	226±11b	65±7a	9±1a
F. mosseae	761±27a	91.6±8.5a	287.6±7.7a	1.25±0.24a	9.24±0.77a	60±3a	298±6a	61±5a	4±1b
R.intraradices	629±9b	84.3±7.6a	264.7±9.2a	1.39±0.28a	9.25±0.69a	56±3ab	206±13b	46±5b	4±1b

Note: Data (means ± SD, n = 4) followed by different letters indicate significant differences ($P < 0.05$) between treatments

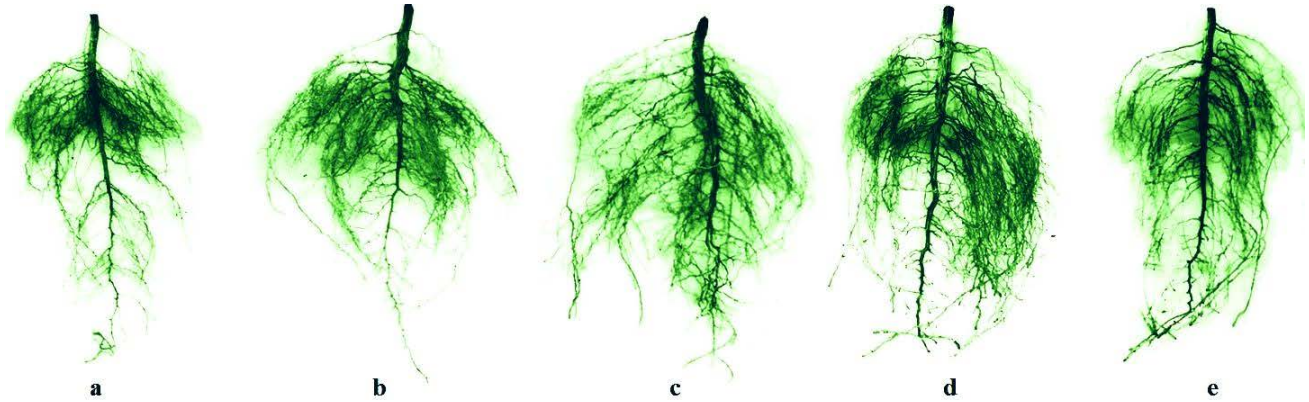

Fig. 1. Root morphology of trifoliate orange (*Poncirus trifoliata*) seedlings treated with a) non-AMF; b) *Claroideoglomus etunicatum*; c) *Diversispora versiformis*; d) *Funneliformis mosseae*; e) *Rhizoglomus intraradices*

colonized seedlings (Table 2). *F. mosseae* showed significantly superior response on these root-related traits than other three AMF species. These observations are in agreement with our previous study in trifoliate orange (Wu *et al.,* 2015a).

Response on leaf carbohydrates

It is a known fact that root growth is strongly dependent on carbon import, and the major loss of root carbon is due to root respiration and AM growth (Walter and Nagel, 2006). AMF-seedlings showed significantly lower leaf sucrose concentration, but significantly higher leaf glucose concentration, as compared with non-AMF seedlings (Fig. 2), regardless of AMF species. Such responses were observed highly variable. Maximum reduction in leaf glucose concentration was observed with *D. versiformis* (45.97%), followed by *F. mosseae* (42.25%), *R. intraradices* (34.84%) and *C. etunicatum* (14.41%) over uninoculated control. While, mycorrhization induced significantly higher glucose concentration in leaves with *D. versiformis* and *F. mosseae* (30.00-30.70%) followed by *R. intraradices* (21.44%) and *C. etunicatum* (5.22%) over control. While, the response of AMF on fructose concentration was entirely different. Only *F. mosseae* produced an increase in leaf fructose concentration by 13.45% over non-AMF control (Fig. 2). AMF as a symbiotic fungi, rely on the host plant to provide carbohydrates for its own growth (Bago *et al*., 2003), and glucose would be preferentially absorbed and transformed in terms of sucrose cleaving (Schubert *et al*., 2004) to support the uninterrupted growth. Root mycorrhizal colonization was significantly and positively correlated with leaf glucose and fructose, and negatively with leaf sucrose (Table 3), due to sucrose cleavage (Wu *et al*., 2015a). AMF-induced root modification was significantly negatively correlated with leaf sucrose but positively with leaf fructose and glucose, indicating that mycorrhiza-induced changes in carbohydrates are associated with changes in root morphology and numbers of LR formation (Table 3). A relatively greater hexose level in AMF plants would provide greater substrates for the growth of both AMF and roots. Glucose stimulates the accumulation of a transcription factor or Auxin-mediated signalling for root initiation (Mishra *et al*., 2009; Singh *et al*., 2014). As a result, the AMF-induced root modification is so closely related to the AMF-stimulated sucrose cleavage.

Response on leaf NO and CaM

Compared with non-AMF treatment, leaf NO and CaM levels were significantly higher with mycorrhization conditions, irrespective of AMF species (Fig. 3). As much as 46.35%, 74.92%, 103.14%, and 8.75% significantly higher leaf NO concentration was observed upon inoculation with *C. etunicatum*, *D. versiformis*, *F. mosseae*, and *R. intraradices*, respectively, over non-AMF-seedlings. Likewise 28.65%, 35.15%, 27.94% and 15.01% significantly higher leaf CaM concentration was observed in *C. etunicatum*, *D. versiformis*, *F. mosseae*, and *R. intraradices*, respectively, over non-AMF-seedlings. These results are in agreement with the results of Huang *et al.* (2014) in trifoliate orange. Strong correlation of NO and CaM with root AMF colonization suggested that, root AMF colonization could be driven through NO and CaM (Huang *et al*., 2014). Leaf NO and CaM also correlated positively with root total length and number of LR in first order, indicating that AMF-induced NO and CaM as a signalling molecule are partly involved in the root development. Both NO and CaM interacted synergistically to stimulate root development and LR formation with the cross-talk of auxins (Liao *et al*., 2012). The interaction between NO and CaM/Ca^{2+} under mycorrhizaiton would further decode the underlying mechanisms involved.

Table 3. Correlation coefficients between root colonization, root morphological traits, or lateral root (LR) number and physiological variables in trifoliate orange (*Poncirus trifoliata*) seedlings colonized by *Claroideoglomus etunicatum*, *Diversispora versiformis*, *Funneliformis mosseae*, and *Rhizoglomus intraradices* (n = 20)

	Root AMF colonization	Total root length	Root project area	Root surface area	Root diameter	Root volume	LR number			
							First-order	Second-order	Third-order	Forth-order
Sucrose	-0.86**	-0.90**	-0.80**	-0.92**	0.24	-0.73**	-0.73**	-0.78**	-0.93**	-0.67**
Fructose	0.68**	0.65**	0.48*	0.59**	-0.30	0.34	0.42	0.64**	0.61**	0.36
Glucose	0.81**	0.93**	0.79**	0.94**	-0.18	0.76**	0.72**	0.85**	0.93**	0.61**
NO	0.74**	0.63**	0.41	0.54**	-0.31	0.32	0.62**	0.75**	0.39	-0.16
CaM	0.79**	0.45*	0.32	0.43	-0.27	0.29	0.46*	0.44	0.28	0.07

Note: * P<0.05; ** P<0.01

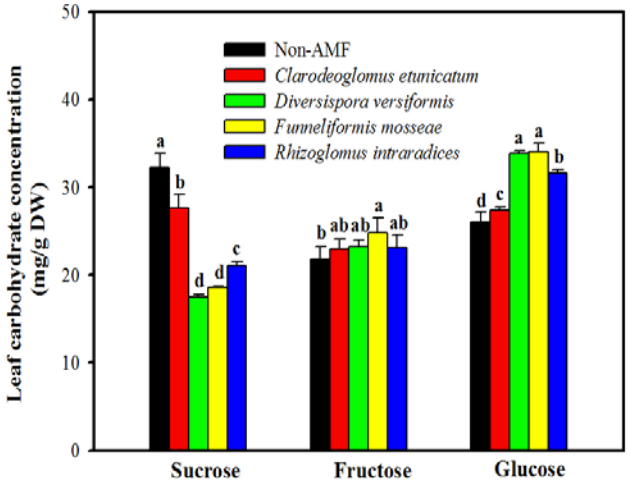

Fig. 2. Leaf sucrose, fructose, and glucose concentrations in trifoliate orange (*Poncirus trifoliata*) seedlings colonized by *Claroideoglomus etunicatum*, *Diversispora versiformis*, *Funneliformis mosseae*, and *Rhizoglomus intraradices*. Data (means ± SD, n = 4) followed by different letters above bars indicate significant differences (P < 0.05) between treatments

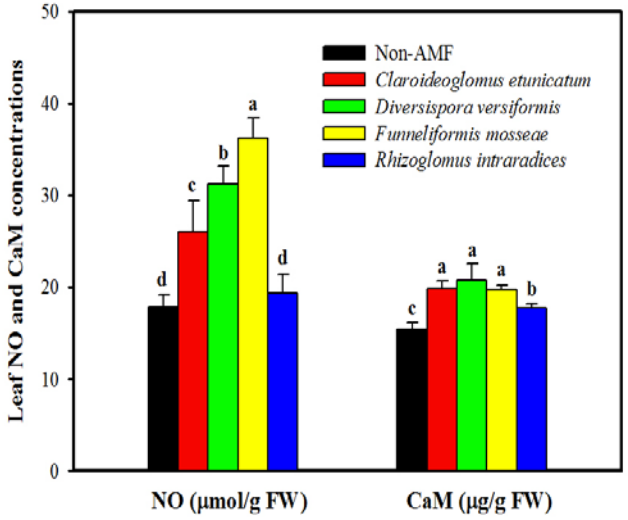

Fig. 3. Concentrations of leaf NO and CaM in trifoliate orange (*Poncirus trifoliata*) seedlings colonized by *Claroideoglomus etunicatum*, *Diversispora versiformis*, *Funneliformis mosseae*, and *Rhizoglomus intraradices*. Data (means ± SD, n = 4) followed by different letters above the bars indicate significant differences (P < 0.05) between treatments

Correlation studies

Different root traits display a significant correlation with three forms of carbohydrate, NO and CaM in leaves (Table 3). Amongst LR numbers, first-order, second-order, third-order, and fourth-order were negatively correlated with leaf sucrose concentration (r = -0.67 ~ -0.93, P < 0.01) and positively correlated with leaf glucose concentration (r = 0.61 ~ 0.93, P < 0.01), corresponding to pattern of similar response on root volume viz., root volume versus sucrose (r = -0.73, P < 0.01) and root volume cooperate with leaf glucose concentration (r = 0.76, P < 0.01). The other root related parameters like root AMF-colonization, total root length, root projected area, and root surface area were positively correlated with fructose as well as glucose concentration in leaves. With exception of root projected area, leaf NO and CaM concentration showed a positive correlation with root AMF-colonization (r = 0.74 and 0.79, P < 0.01) and total root length (r = 0.63 and 0.45, P < 0.01 and P < 0.05, respectively), and with root diameter remaining unaffected with any of the three forms of carbohydrates, NO, and CaM in leaves. These observations suggested, mycorrhizal response of trifoliate orange is strongly dependent upon leaf carbohydrate metabolism, NO, and CaM activity, as underlying mechanisms to explain the physiological responses of AMF.

Conclusions

Responses of trifoliate orange to mycorrhization are regulated through carbon metabolism coupled with root morphological changes. These changes were further partitioned, partly into sucrose cleavage and partly as NO- and CaM-induced changes.

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