



## Effect of auxin inhibitor and AMF inoculation on growth and root morphology of trifoliolate orange (*Poncirus trifoliata*) seedlings

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### ABSTRACT

Auxins are considered as one of the important plant hormones coordinating the signals in regulation of plant development including the transformation in root morphology. Arbuscular mycorrhizal fungi (AMF) produce small amount of auxins, the role of which is yet not known in root and mycorrhizal development. The present study was undertaken to evaluate the effects of AMF (*Glomus versiforme* Karst.) and an auxin inhibitor (P-Chlorophenoxyisobutyric acid, PCIB, 10 mM) on plant growth and root development of four-month-old mycorrhized trifoliolate orange [*Poncirus trifoliata* (L.) Raf.] seedlings. Root mycorrhizal colonization and number of entry points and vesicles were significantly inhibited by exogenous PCIB treatment, suggesting that auxins are involved in establishment of AMF-host plant symbiosis. Application of PCIB significantly decreased the plant height, stem diameter, leaf number, and shoot and root fresh weight. On the other hand, AMF colonization increased the plant height, shoot and root fresh weight, thereby, suggesting that AMF colonization alleviated the negative effects of PCIB. Similarly, PCIB application significantly decreased taproot length, root average diameter, roots projected area, surface area of roots, root volume, root length under 0–1 cm category, and total root length. These root traits significantly improved upon inoculation with AMF. The study, hence, suggested that inoculation with AMF negated the adverse effects of PCIB through AMF-induced auxin production that actively participated in both root and mycorrhizal plant development.

**Key words:** Arbuscular mycorrhizal fungi, P-Chlorophenoxyisobutyric acid, Plant growth, Root morphology, Trifoliolate orange

Arbuscular mycorrhizal fungi (AMF) are probably most abundant in agricultural soils, and can form a symbiotic association (arbuscular mycorrhiza, AM) with the roots of ~80% of terrestrial plants (Srivastava *et al.* 2002). The AM-symbiosis obtains plant carbohydrates to complete its life cycle, and AMs would enhance plant growth and development by acquisition of phosphate and other mineral nutrients from the soil (Porrás-Soriano *et al.* 2009, Srivastava 2009, Srivastava and Ngullie 2009). Inoculation with AMF by and large affects the root traits of host plant, including taproot length, number of lateral roots, and root volume (Wu *et al.* 2008; Wang *et al.* 2014). Colonization with *Glomus intraradices* on the other hand, is reported to significantly increase the number of lateral roots in rice (Gutjahr *et al.* 2009). AMF colonization also promoted the formation of lateral roots of trifoliolate orange seedlings, induced more fine roots and less coarse roots according to Yao *et al.* (2009).

Auxins play an important role in plant development by

inducing branching in lateral roots, responsible for providing water and nutrients (Zandonadi *et al.* 2010). Auxins are produced by spores of AMF (Splivallo *et al.* 2009), which help the host plant to improve the configuration of root system (Ludwig-Muller 2010). The AM-induced auxin production is anticipated to trigger the modification of root systems of the host plant. Until now, the relevant mechanisms responsible for AM-induced root modification are poorly known. P-Chlorophenoxyisobutyric acid (PCIB), an inhibitor of indole-3-acetic acid (IAA), has been widely used to inhibit auxin action by means of competing with auxin at the binding site of the auxin receptor (Xie *et al.* 2000, Oono *et al.* 2003). Studies showed that PCIB notably inhibited the formation of *Arabidopsis* root, including lateral root development, gravitropic response, and primary root growth (Oono *et al.* 2003). Biswas *et al.* (2007) further revealed that PCIB inhibited *Arabidopsis* root growth by reducing the size of root meristem. While, Zandonadi *et al.* (2010) reported that PCIB decreased the lateral root formation and the primary root length of *Zea mays* by 50–55%. These studies offered a strong clue that could be used to clarify the roles of auxin development in root systems and further the possible impact of AMF. In recent years, massive efforts have been made worldwide to study the interaction effect of AMF on plant root development.

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However, there are still hammering gaps in our knowledge on the integrative effect of PCIB and AMF on root traits of the host plant.

The aims of the present study were to: i. evaluate the effects of both AMF and PCIB on the growth performance and root traits development of trifoliolate orange [*Poncirus trifoliata* (L.) Raf.] seedlings and ii. to establish the role of AM-induced auxins on root development of host plant.

#### MATERIALS AND METHODS

The proposed study was executed through 2×2 factorial randomized block design experiment including inoculation with or without *Glomus versiforme* (Karst.) Berch and PCIB treatment at 0 and 10 mM concentration. Each treatment had four replicates with a total of 16 pots. The surface of seeds of trifoliolate orange was disinfected with 70% alcohol for 10 min, rinsed with distilled water, and sown into a plastic pot (17 cm upper diameter × 12 cm below diameter × 16 cm height) carrying 2.4 kg of autoclaved (121°C, 0.11 Mpa, 2 h) Xanthi-Udic Ferralsols (FAO system) soils collected from a Citrus orchard of Yangtze University campus. An inoculum of 15 g *G. versiforme* carrying 300 spores were inoculated with the growth substrates for AMF treatments at the time of sowing. Non-AMF pots were supplied with 15 g of the sterilized inoculums as the control. Two months after mycorrhizal inoculation, 200 mL of 0 and 10 mM PCIB was added into the designed pots at an interval of three days. During the PCIB treatments no additional water was supplied. The experiment was conducted during April–August, 2012 under plastic greenhouse of Yangtze University campus with day/night temperature of 26/20°C, photosynthetic photon flux density of 820 μmol/m<sup>2</sup>/s, and relative air humidity of 85%.

After seven times of PCIB treatments, the AM and non-AM seedlings were harvested and divided into shoots and roots, whose fresh weights were recorded. Plant height, stem diameter, and leaf number per plant were also determined before harvest. The whole root system was immediately scanned with an Epson Perfection V700 Photo Dual Lens System (Seiko Epson Corp, Japan). The obtained images of root system were analyzed by a professional WinRHIZO software in 2007 (Regent Instruments Inc., Quebec, Canada), and the traits of root morphology, including length, projected area, surface area, volume, average diameter and the 0–1, 1–2, 2–3, 3–4 and >4 cm classed root lengths were automatically obtained. Taproot length was determined using a vernier caliper, and the number of different order lateral roots was artificially quantified.

A small quantity of root segments (1 cm long) were cleared by 10% KOH and stained with 0.05% trypan blue in (Phillips and Hayman 1970). Root AM colonization was calculated as the percentage of colonized root lengths against total root lengths, and mycorrhizal structures such as entry points, vesicles were microscopically examined and expressed as the number per cm root length (Wu *et al.* 2008). Data were subjected to analysis of two-factor variance

Table 1 Effect of exogenous PCIB on mycorrhizal development of *Glomus versiforme*-colonized trifoliolate orange (*Poncirus trifoliata*) seedlings

PCIB levels (mM)	Inoculation treatments	Root colonization (%)	Vesicle (num./cm root)	Entry point (num./cm root)
0	Non-AMF	0.00±0.00c	0.00±0.00c	0.00±0.00c
	AMF	57.78±7.23a	5.33±1.53a	3.77±0.59a
10	Non-AMF	0.00±0.00c	0.00±0.00c	0.00±0.00c
	AMF	33.93±3.32b	0.20±0.05b	1.86±0.56b
<i>Significance</i>				
AMF		**	**	**
PCIB		**	**	**
AMF×PCIB		**	**	**

Note: Data (mean ± SE, n=4) followed by different letters within a column mean significant differences among treatments at P<0.05.

(ANOVA) with SAS v8.1, and the significant differences among treatments were used to compare with the Duncan Multiple range test at P < 0.05.

#### RESULTS AND DISCUSSION

##### *Mycorrhizal development*

Mycorrhizal structures were conspicuously observed in the roots of the trifoliolate orange seedlings inoculated with AMF solely or in combination with PCIB (Table 1). Mycorrhizal development was significantly restricted by exogenous PCIB application. At 10 mM PCIB treatment, root colonization and number of vesicles and entry points in AM seedlings decreased by 41.3, 96.2, and 50.7% respectively, as compared to 0 mM PCIB. The results suggested that auxin is an important factor in regulating AM development. In fact, spores of AMF, such as *G. intraradices*, produced small amounts of indole-3-acetic acid for spore germination and hyphal length according to Ludwig-Müller *et al.* (1997). It was concluded that PCIB treatment inhibited auxin production in spores, finally resulted in reduction of root AM colonization. Results were reported of similar kind in the wild and mutant peas (Müller 1999). Ludwig-Müller (2010) observed that additional auxin could increase spore germination and hyphal growth. In contrast, Xie *et al.* (1997) reported that root colonization of *Lablab purpureus* by *G. mosseae* was significantly increased after treatment by an auxin inhibitor triiodobenzoic acid (TIBA). It suggested that different auxin inhibitors such as PCIB and TIBA could exhibit differential roles in the process of AM development.

##### *Plant growth performance*

AMF alone induced significant promotion in plant performance compared to the non-AMF control with regard to plant height, stem diameter, leaf number, and fresh weight of shoot and root (Table 2). Porras-Soriano *et al.* (2009) reported that improvement in plant growth performance due to elevated water and nutrient uptake by extraradical

Table 2 Effect of *Glomus versiforme* alone or in combination with exogenous PCIB on growth performance of trifoliolate orange (*Poncirus trifoliata*) seedlings

PCIB levels (mM)	Inoculation treatments	Plant height (cm)	Stem diameter (cm)	Leaf number per plant	Shoot fresh weight (g)	Root fresh weight (g)
0	Non-AMF	17.8±1.89b	0.258±0.020b	17.8±1.8b	1.14±0.59b	0.99±0.39b
	AMF	30.2±1.83a	0.294±0.040a	25.2±1.4a	2.08±0.62a	1.48±0.49a
10	Non-AMF	12.9±2.03c	0.199±0.016d	12.3±1.7d	0.50±0.07d	0.42±0.14d
	AMF	16.3±1.53b	0.219±0.022c	15.2±1.9c	0.96±0.05c	0.76±0.08c
<i>Significance</i>						
AMF		**	**	**	**	**
PCIB		**	**	**	**	**
AMF×PCIB		**	NS	*	**	NS

Note: Data (mean ± SE, n=4) followed by different letters within a column mean significant differences among treatments at  $P<0.05$ .

Table 3 Effects of *Glomus versiforme* alone or in combination with exogenous PCIB on root morphological traits of trifoliolate orange (*Poncirus trifoliata*) seedlings

PCIB levels (mM)	Inoculation treatments	Taproot length (cm)	Average root diameter (mm)	Projected root area (cm <sup>2</sup> )	Surface area of roots (cm <sup>2</sup> )	Root volume (cm <sup>3</sup> )	Number of lateral roots		
							1 <sup>st</sup> order	2 <sup>nd</sup> order	3 <sup>rd</sup> order
0	Non-AMF	24.4±7.0b	0.48±0.06b	18.5±1.9b	58.2±2.2b	0.74±0.37b	34±6a	165±3b	14±8b
	AMF	33.3±6.0a	0.53±0.04a	24.5±2.6a	76.9±2.5a	1.06±0.41a	35±6a	202±8a	30±7a
10	Non-AMF	12.8±4.2c	0.38±0.05c	5.2±1.2d	16.3±3.7d	0.16±0.05d	19±4c	26±7d	3±1d
	AMF	15.0±6.1c	0.38±0.06c	6.9±1.7c	19.4±1.7c	0.21±0.04c	25±3b	62±8c	7±1c
<i>Significance</i>									
AMF		*	*	**	**	**	*	**	**
PCIB		**	**	**	**	**	**	**	**
AMF×PCIB		**	*	**	**	**	*	NS	**

Note: Data (mean ± SE, n=4) followed by different letters within a column mean significant differences among treatments at  $P<0.05$ .

mycelium of AMF. But, after exogenous application of PCIB, plant growth parameters were markedly decreased, irrespective of AM or non-AM, indicating that the PCIB treatment inhibited plant growth performance. Buzzello *et al.* (2013) reported that the plant height of soybean was reduced after application of an auxin inhibitor TIBA. Mycorrhizal colonization on the other hand counteracted the negative effect of PCIB on the plant growth performance. Regardless of concentration of PCIB treatment, the AMF inoculation significantly increased plant height, stem diameter, leaf number, and shoot and root fresh weight, warranting that AMF alleviated the growth inhibition by PCIB. Since auxin is involved in the AM-host plant interaction, AMF may produce a small quantity of fungal exudates containing auxins to offer relief against the negative impact of PCIB on growth performance (Novero *et al.* 2008).

#### Root morphological traits

Compared to non-PCIB treatment, PCIB treatment significantly decreased the taproot length, average diameter, projected area, surface area, volume, and number of 1<sup>st</sup>, 2<sup>nd</sup>, and 3<sup>rd</sup> order lateral roots irrespective of AMF inoculation (Table 3, Fig 1). Earlier studies showed the an auxin-transport inhibitor, naphthylphthalamic acid applied into growth agar of tomato roots, markedly decreased the number

of lateral roots (Muday and Haworth 1994). While, application of PCIB on *Zea mays* seedlings inhibited the taproot length and decreased the lateral root development (Zandonadi *et al.* 2010). On the contrary, the AMF colonization partly eliminated the negative effects of PCIB. Under 0 mM PCIB conditions, inoculation with *G. versiforme* significantly increased taproot length, average diameter, projected area, surface area, volume, and number of 2<sup>nd</sup> and 3<sup>rd</sup> order lateral roots (Table 3, Fig 1). On the other hand, under 10 mM PCIB treatment, *G. versiforme* significantly increased root projected area, surface area, volume, and number of 1<sup>st</sup>, 2<sup>nd</sup>, and 3<sup>rd</sup> order lateral roots. Such results were earlier reported on red tangerine seedlings infected by *Paraglomus occultum* (Wu *et al.* 2012b). It is well known that root auxin can regulate the size of root apical meristem by promoting cell division as well as the cell elongation (Muday *et al.* 2012). AM-mediated root modification suggested that AMF alleviated the PCIB negative effect on root traits through better uptake of water and nutrients by extraradical hyphae and release of auxin.

#### Different classed root lengths

Trifoliolate orange seedlings mainly depended on frequency of 0–1 cm root length in root total length size (Table 4). PCIB application significantly reduced different root length sizes of 0–1, 1–2, and 3–4 cm and total root

Table 4 Effects of *Glomus versiforme* alone or in combination with exogenous PCIB on different classified root lengths of trifoliolate orange (*Poncirus trifoliata*) seedlings

PCIB levels (mM)	Inoculation treatments	Different classified root length (cm)					Total
		0–1 cm	1–2 cm	2–3 cm	3–4 cm	>4 cm	
0	Non-AMF	353.5±31.7b	9.9±2.0b	4.6±2.0a	2.3±0.5a	0.1±0.0a	372.4±12.9b
	AMF	428.0±22.9a	18.2±4.8a	6.5±0.6a	2.9±0.7a	0.5±0.1a	456.1±39.7a
10	Non-AMF	125.4±15.0d	5.0±0.5b	3.5±1.0a	0.3±0.1b	0.1±0.0a	134.3±19.3d
	AMF	170.1±13.6c	5.7±1.4b	4.5±1.9a	0.1±0.0b	0.0±0.0a	180.4±12.2c
<i>Significance</i>							
AMF		**	NS	NS	NS	NS	**
PCIB		**	**	NS	**	NS	**
AMF×PCIB		NS	NS	NS	NS	NS	NS

Note: Data (mean ± SE, n=4) followed by different letters within a column mean significant differences among treatments at  $P<0.05$ .

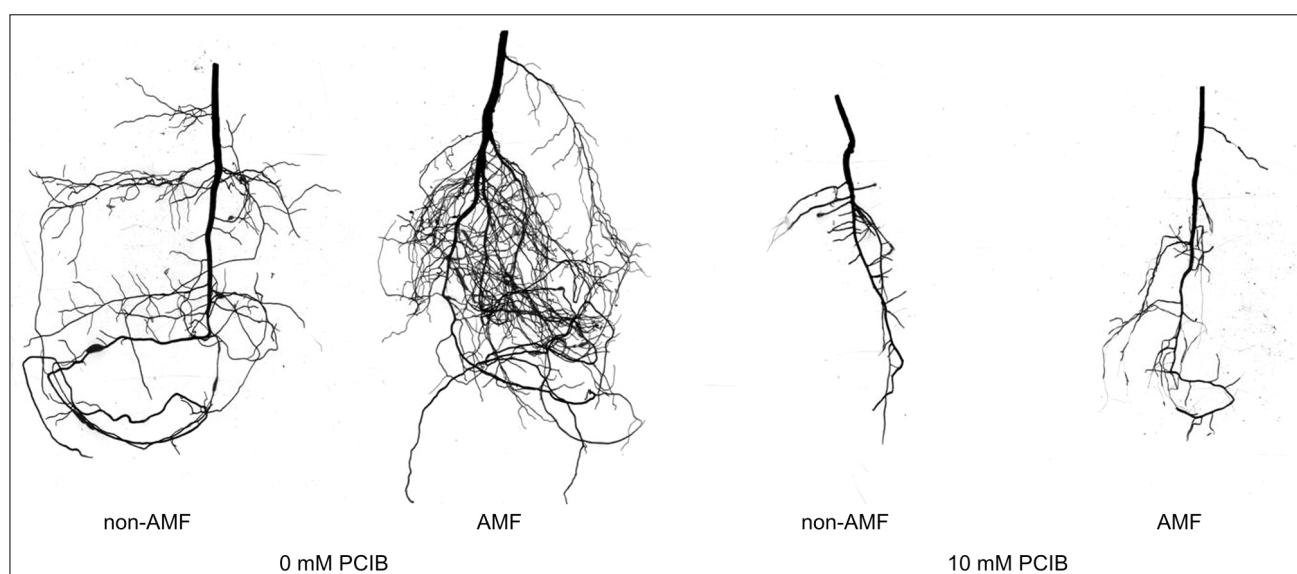


Fig. 1 Root system morphology of trifoliolate orange (*Poncirus trifoliata*) seedlings treated with or without *Glomus versiforme* and exogenous PCIB

length, whereas the AMF colonization significantly increased root length sizes under 0–1 and 1–2 cm categories and total root length, but without any difference under 1–2 cm root length category between AM and non-AM seedlings at 10 mM PCIB. Yao *et al.* (2009) reported that inoculation with AMF, especially with *G. caledonium* increased the fine root lengths of trifoliolate orange seedlings. Wu *et al.* (2012a) obtained the similar results on tangerine orange seedlings when inoculated with *G. mosseae*. These results suggested that AMF mainly induced the response under 0–1 cm under root length category to increase root total length for root modification. Such responses should be viewed from better preparedness of host plant in mining water and nutrients more rigorously.

#### CONCLUSION

Exogenous application of PCIB was observed inhibitory to root mycorrhizal colonization, the plant growth and root morphology, and the lateral root formation of trifoliolate orange seedlings. Nevertheless, inoculation with *G.*

*versiforme* overtook the negative effects of PCIB. AM seedlings showed greater growth performance, better development of root morphological traits, and more lateral root number with fine root lengths (0–1 cm class). Results, hence, revealed that AM-induced auxin production was the key for improved plant growth and root development of trifoliolate orange seedlings.

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