Scientia Horticulturae 234 (2018) 227–235

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Mycorrhiza-induced change in root hair growth is associated with IAA accumulation and expression of *EXPs* in trifoliate orange under two P levels

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ARTICLE INFO

Keywords:
AMF
EXPs
IAA synthesis
IAA transport carriers
Root hair density

ABSTRACT

Root hair growth can be regulated by substrate nutrient levels, phytohormones, and arbuscular mycorrhizal (AM) fungi. However, the information about effects of AM fungi and substrate phosphorus (P) levels on root hair features is scarce. This study evaluated the effect of an AM fungus, *Funneliformis mosseae,* on root hair morphology, root indole-3-acetic acid (IAA) level, indole-3-acetic acid oxidase (IAAO) activity, and expressions of auxin-related genes and expansins (*EXPs*) in trifoliate orange (*Poncirus trifoliata*) seedlings under 0.1 mM ($P_{0.1}$) and 1.0 mM (P_1) P levels for 16 weeks. AM seedlings showed better root colonization under $P_{0.1}$ than under P_1. Under $P_{0.1}$ and P_1, mycorrhizal seedlings recorded higher concentrations of P and IAA and lower IAAO activity in roots than non-mycorrhizal seedlings. Root hair density was significantly higher in AM seedlings under $P_{0.1}$ but dramatically lower under P_1 than non-AM seedlings. Root hair length and diameter of AM seedlings were significantly increased under P_1 only, relative to non-AM seedlings. Indole-3-pyruvate monooxygenase (*PtYUC2*) and auxin transporter-like protein 3 (*PtLAX3*) expressions in roots were significantly up-regulated by mycorrhizal treatment under both $P_{0.1}$ and P_1. The transcript levels of root auxin efflux carrier component 2 and 8 (*PtPIN2* and *PtPIN8*) were dramatically down-regulated by mycorrhizal inoculation, irrespective of P levels. Root *PtEXPB2*, *PtEXPA2,* and *PtEXPA4* expressions were notably up-regulated by mycorrhiza under $P_{0.1}$ but down-regulated under P_1. These results indicated that mycorrhiza-induced changes in root hair growth is closely related with up-regulated expressions of *EXPs* and simultaneous IAA accumulation in roots.

1. Introduction

Phosphorus (P) is one of the necessary macronutrients in plant growth and metabolism, such as photosynthesis, energy accumulation, and respiration. Approximately 80% of soil P exists in organic forms (Jungk et al., 1993), or is fixed by soil organic matters, causing P immobility and deficiency in plants and soils (White and Hammond et al., 2005; White and Brown, 2010). Plants have evolved two pathways, at least, to respond to P-deficient stress, namely, the direct uptake pathway from the rhizosphere by root epidermal cells and root hairs, and the indirect uptake pathway via AM fungi (Richardson et al., 2009).

Arbuscular mycorrhizal fungi (AMF), one of soil inhabiting microorganisms, can establish mutual symbiosis with roots of ~80% terrestrial plants. Mycorrhizal hyphae act as a living bridge between the soil and roots to absorb water and mineral nutrients from the soil to the plant partner (Achatz and Rillig, 2014; Xie et al., 2014). Studies confirmed the stimulated N, K, Cu, Zn, and P-acquisition of the host plant by mycorrhizal fungi (Smith and Read, 2008; Cozzolino et al., 2010). Hereinto, the P uptake rate by mycorrhizae is six times higher than by root hairs, and the P transfer rate is ten times faster in AMF than in root hairs (Smith et al., 1994).

Root hairs are highly specialized cells originating from the root epidermis that play a role in water and mineral nutrient absorption in plants (Janiak et al., 2012; Salazar-Henao et al., 2016). As a general rule, root hair formation can be modulated by environmental signals, especially inorganic phosphate (Pi), as well soil microorganisms (Salazar-Henao et al., 2016). Studies showed the effect of inoculation with AMF on root hair growth of host plants (Orfanoudakis et al., 2010; Brown et al., 2013). In sand culture, AMF inoculation significantly increased root hair density of trifoliate orange, while the mycorrhizal effect on root hair length was dependent on lateral root orders (Wu et al., 2016). In addition, under drought stress conditions,

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https://doi.org/10.1016/j.scienta.2018.02.052
Received 13 December 2017; Received in revised form 18 February 2018; Accepted 20 February 2018

mycorrhization induced an increase in root hair density of trifoliate orange (Zou et al., 2017).

In addition to AMF, substrate P level heavily affects root hair growth. Root hairs of *Arabidopsis* were overdeveloped under P deficiency (Williamson et al., 2001). Cao et al. (2013) reported that the deprivation of P highly improved initiation and elongation of root hairs. The effective use of P increased root hair density and elongation to alter the root configuration (López-Bucio et al., 2002). A study conducted by Zhang et al. (2016a) showed that low P activated auxin signals to initiate root hair formation. In auxin signaling mutants *axr1*, *axr2*, and *aux1* of *Arabidopsis*, the root hair development was defective, while P deficiency could restore the root hair defect (Schmidt and Schikora, 2001).

Auxins are an important factor affecting the occurrence and growth of root hairs (Zhang et al., 2016a). Auxins do not directly affect the fate of root epidermal cell determination but plays a role as an organizing center for environmental/hormonal signaling in root hair growth (Lee and Cho 2008, 2013). Auxins are primarily synthesized in shoots and then transported via stem vascular tissues into the root hair zone through the cortex or vascular bundle (Rigas et al., 2013). Auxin synthesis and transports are controlled by many genes. Tryptophan aminotransferase related (*TAR*) and flavin monooxygenase-like enzymes (*YUC*) are the key genes during IAA synthesis (Mano and Nemoto, 2012). Pin-formed (*PIN*) auxin exflux carriers and the AUXIN RESISTANT 1/LIKE AUX1 (*AUX1/LAX*) auxin influx carriers are indispensable for auxin transportation (Tromas and Perrot-Rechenmann, 2010). It concludes that auxin changes are highly related with root hair features.

Expansins (EXPs) are non-enzymatic cell wall proteins that mediate plant growth by catalyzing loosening of cell walls without lysing the wall polymers. They are divided into four sub-families; α-expansin, β-expansin, expansin-like A, and expansin-like B (Mohanty et al., 2018). EXPs have been widely implicated in growth and development of roots and root hairs in several plant species (Mohanty et al., 2018). Overexpression of *GmEXPB2* in soybean improves root architecture by increasing the number of cortical cells, and root hair density and size (Li et al., 2015). Balestrini et al. (2005) inoculated *Glomus versiforme* into cucumber roots and found *EXPs* involved in AMF symbiosis. Other expansins such as *Exp-like gene* and *EXPLB1* are also involved in the establishment of AMF symbiosis with *Medicago truncatula* and tomato (Dermatsev et al., 2010; Siciliano et al., 2007). However, the information regarding the effects of both P stress and mycorrhizal inoculation on root *EXPs* expression is not known.

Trifoliate orange (*Poncirus trifoliata* L. Raf) is an important citrus rootstock used in Southeast Asia. It has few root hairs and thus is highly dependent on arbuscular mycorrhizae (Wu et al., 2015). As stated above, a low substrate P level stimulates root hair initiation and growth of trifoliate orange, while the effect of both low P and mycorrhizal inoculation on root hair growth is not clear. Hence, the aim of this study was to investigate the response of mycorrhizal fungi on root-hair growth under two different P conditions. Root hair growth, auxin concentration, relative expression of root auxin synthesized, and transportation genes were determined.

2. Materials and methods

2.1. Plant culture

Seeds of trifoliate orange were surface sterilized with 70% alcohol for 10 min, rinsed three times with distilled water, and germinated in autoclaved (121 °C, 0.11 MPa, 2 h) sands at 25 °C for 30 days. Subsequently, three five-leaf-old seedlings with a height of 4 cm were transplanted into 5-L pots filled with sands. The sand collected from the Yangtze River side was sieved through 4 mm, soaked in 15% of sulphuric acid solution for one week, rinsed with distilled water, and autoclaved (121 °C, 0.11 MPa) for 2 h.

An arbuscular mycorrhizal (AM) fungus, *Funneliformis mosseae* (Nicol. & Gerd.) Schüßler & Walker [BGC GZ03C], was used in this experiment. The fungal strain was provided by the Bank of Glomeromycota in China (BGC) and propagated through the identified spores with *Trifolium repens* for 16 weeks under potted conditions. Mycorrhizal inoculums consisted of spores, root segments, and sands. A 50 g (corresponding to 1500 spores) inoculum of the fungal strain was inoculated into pots as the AMF treatment when seedlings were transplanted. The non-AMF-inoculated treatment was supplied with the same amount of sterilized inoculum plus 2 mL filtrate (25 μm filter) of mycorrhizal inoculum to minimize differences in other microbial communities. All the AM and non-AM seedlings were grown in a controlled greenhouse (photosynthetic photon flux density was 982 μmol/m^2/s, day/night temperature 27/20 °C, and relative humidity 80%) from March 22 to July 26, 2016. Pots were relocated weekly to eliminate environmental effects.

Within one week of seedling transplanting, 100 mL ddH_2O per pot was supplied daily. Subsequently, P treatments were begun. The concentration of 0.1 mM and 1.0 mM P used was selected according to our previous results of Liu and Wu (2017). Each pot was irrigated with 100 mL Hoagland solutions with 0.1 mM and 1.0 mM P strengths at an interval of 3 days. At the same time, the P concentration was regulated by KH_2PO_4, and KNO_3 was added into the low P treatment to maintain a uniform K concentration. To avoid P accumulation of the sand, 100 mL ddH_2O per pot every two days was used to remove the residual P.

2.2. Experimental design

The experiment was arranged in a completely randomized blocked design with two factors: inoculation with or without *F. mosseae* and different P treatments of 0.1 mM ($P_{0.1}$) and 1.0 mM (P_1). Each treatment had six replicates, and each replicate had three seedlings, for a total of 24 pots (72 seedlings).

2.3. Variable determinations

After 16 weeks of P treatments, the AM and non-AM seedlings were harvested and divided into leaves, stems, and roots, whose fresh weights were determined. Nine integral lateral roots of a replicate in the first-, second-, and third-order (a total of 54 integral lateral roots per treatment) were stained with 0.05% trypan blue for 5 min (Phillips and Hayman, 1970), and the AM structures were microscopically observed. Root AMF colonization (m%) was calculated as the percentage of colonized root lengths by AMF against observed total root lengths. Mycorrhizal dependency was calculated by the protocol described by Yao et al. (2005).

To observe root hairs, sixty one-cm-long fresh root segments (ten taproots, twenty 1st-order lateral roots, twenty 2nd-order lateral roots and ten 3rd-order lateral roots) per treatment were pre-fixed for 24 h in 2.5% glutaraldehyde, eluted with increasing alcohol concentrations (from 30%–100%), and dried with critical-point drying (CPD) (Bray et al., 1993; Wu et al., 2016). Root hair images were obtained by a Scanning Electron Microscope (SEM, model JSM-6390LV, JEOL Co., Japan) at ×200 and ×400 magnification. Root hair density, length, and diameter were determined using the Image J software (National Institutes of Health, MD, USA).

The concentration of root P (0.25 g dry sample) was determined using the inductively compled plasma-atomic emission spectrometry (ICP-AES, IRIS Advantage, Thermo Electron Corporation, USA).

Root endogenous indole-3-acetic acid (IAA) was extracted according to the protocol of Dobrev and Kaminek (2002) and determined by High Performance Liquid Chromatography (HPLC), in which the mobile phase was a mixture with methanol and ddH_2O (2/3, v:v), 10 μL of the extracted solution was detected for 1 h under 254 nm at a 0.8 mL/min flow rate, and the column temperature was 30 °C.

Root indole-3-acetic acid oxidase (IAAO) activity was determined according to Beffa et al. (1990) with a minor modification. A 0.2 g root sample was homogenized with phosphate buffer (pH 6.0), and the absorbance was detected under 530 nm after being colored by sulfuric acid-$FeCl_3$ mixed solutions.