Integrated effect of arbuscular mycorrhizal fungi and hydrogen peroxide on the root system of trifoliate orange seedlings

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ABSTRACT: The root-system architecture (RSA) is critical for plant growth and mineral absorption and is also easily affected by various abiotic and biotic factors including arbuscular mycorrhizal fungi (AMF). The present study evaluates the integrated effects of an AM fungus (*Glomus versiforme*) and exogenous hydrogen peroxide (0, 1, and 100 μM) on mycorrhizal development, growth, RSA, and carbohydrate allocation of trifoliate orange (*Poncirus trifoliata*) seedlings. Root mycorrhizal colonization and number of vesicles increased significantly on application of 1 μM H_2O_2 but was notably inhibited by 100 μM H_2O_2. AMF colonization increased shoot and root fresh weight. A significant differences was seen with 1 μM H_2O_2 but not with 0 or 100 μM H_2O_2. Inoculation with AMF notably increased RSA traits, including total length, surface area, projected area, and volume, with 0 and 1 μM H_2O_2 but not with 100 μM H_2O_2. AMF significantly decreased the concentrations of sucrose and glucose in leaves treated with 0 and 100 μM H_2O_2, but notably increased the concentrations of sucrose and glucose in roots treated with 0 and 1 μM H_2O_2. The highest root sucrose allocation of AM seedlings appeared in the treatment with 1 μM H_2O_2. The results imply that a low concentration of H_2O_2 (1 μM) acted as a signal molecule to stimulate the development of RSA and AMF, whereas a high concentration of H_2O_2 (100 μM), which is regarded as a toxic molecule producing reactive oxygen species, interfered with the formation of RSA and AMs.

KEYWORDS: *Poncirus trifoliata*, *Glomus versiforme*, citrus, symbiosis

INTRODUCTION

The root-system architecture (RSA), the spatial configuration of the root system in the soil, determines the capacity of plants to uptake water and mineral nutrients from soils. The root-system architecture also possesses a high plasticity and can be affected by various abiotic and biotic factors, such as soil water availability, nutritional status, and micro-organisms[1]. In addition, the physiological status of plants, such as the distribution of carbon source, also regulates RSA[2] causing root growth to be tightly related to the root carbon status[3]. The carbohydrate levels in the root system are therefore critical for an optimal RSA.

Arbuscular mycorrhizas (AMs) are a common symbiont formed between arbuscular mycorrhizal fungi (AMF) and the roots of terrestrial plants[4]. External hyphae of AMs can promote the ability of the host plant to absorb water and mineral nutrients from the soil, thereby regulating the metabolic activity of the host plant[5]. AMF also affects on the RSA increasing the root branching and root diameter[6]. Wu et al[7] reported that the characteristics of the RSA, such as root

projected area, surface area, volume, and total length increased significantly in red tangerine after inoculation with an arbuscular mycorrhizal fungus, *Glomus mosseae*. The branching degree of adventitious roots, primary lateral roots, and the mean diameter of roots in *Prunus cerasifera* increased after inoculation with *G. intraradices* but not with *G. mosseae*[8]. Thus certain species of AMF can have a notable impact on the RSA.

Hydrogen peroxide (H_2O_2) is a reactive oxygen species widespread in plant tissues and can damage cells or tissues. On the other hand, H_2O_2 also plays an important role as a signal molecule in the process of plant growth and development[9]. Exogenous H_2O_2 also mediates the growth of primary root[10, 11], lateral roots, and root hairs[12, 13]. The lateral-root formation of soya bean is strongly inhibited by β-hydroxyethyl hydrazine, while the inhibitory effect is reversed after exogenous H_2O_2 treatment[14]. In mung bean and cucumber, H_2O_2 could significantly promote the formation and growth of adventitious roots in vivo[15, 16]. Interestingly, the regulation of RSA caused by exogenous H_2O_2 can be counteracted by

the addition of exogenous ascorbic acid[16]. Higher concentration (> 10 μM) of exogenous H_2O_2 however significantly decrease total root length[11]. It seems that exogenous H_2O_2 could partly promote growth and development of various root structures, but the effect is dependent on the H_2O_2 concentration.

As stated before, both AMF and H_2O_2 are involved in root development, however the interactive effects between AMF and H_2O_2 are unknown. In clover roots, *G. mosseae* colonization significantly increased H_2O_2 concentration five weeks after sowing but it decreased seven weeks after sowing[17]. The structure of AMs can limit the damage of H_2O_2 in roots of *Medicago truncatula*, *Zea mays*, and *Nicotinana tabacum* colonized by *G. intraradices*, and the accumulation of H_2O_2 in roots occurred simultaneously with the senescence of AMs[18]. The burst of H_2O_2 is highly correlated with root AM colonization and arbuscules but not with vesicles or entry points[19]. It seems that H_2O_2 and AMF can influence each other. Until now, there are no reports showing an interaction between AMF and H_2O_2 on the RSA of the host plant.

The objective of the present work was to investigate the integrated effects of both AMF and H_2O_2 on the sugar contents and other traits of RSA of trifoliate orange (*Poncirus trifoliata* L. Raf.) seedlings.

MATERIALS AND METHODS

Plant culture

Seeds of trifoliate orange (*P. trifoliata*) were surface sterilized with 70% alcohol for 5 min, rinsed five times with distilled water, and germinated at 28 °C in the dark. The germinated seeds were sown into plastic pots (18 cm upper diameter × 17 cm height × 13 cm bottom diameter) filled with autoclaved (0.11 MPa, 121 °C 2 h) Xanthiudic ferralsol soils. At sowing, AM treated seedlings received a 40 g inoculum of *G. versiforme* (P. Karst.) S.M. Berch including the infected root segments of white clover, extra-radical hyphae, spores (∼28 spores/g), and river sand. The non-AM seedlings also received the 40 g autoclaved inoculum as the control. All the AM and non-AM seedlings were placed in the centre of the plastic greenhouse under ambient temperature at the College of Horticulture and Gardening, Yangtze University, Jingzhou, China, on 13 March 2011. The locations of the pots were randomized weekly.

Experimental design

The experimental treatments consisted of 2×3 factorial design with mycorrhizal inoculations (with or without *G. versiforme*) and exogenous H_2O_2 treat-

ments (0, 1, and 100 μM) manipulated and arranged in a completely randomized design with three replicates for each treatment to give a total of 18 pots (three seedlings per pot). The AM and non-AM seedlings were watered with 0, 1, and 100 μM of H_2O_2 at an interval of seven days from June 16 to 11, August 2011, for a total of nine times.

Parameters determinations

The AM and non-AM plants were harvested and divided into shoots and roots, and the fresh weights were recorded on 18, August 2011.

The intact root systems were scanned with Epson Perfection V700 Photo (Seiko Epson Corp, Japan) and the images of the root systems were analysed using WinRHIZO professional 2007b (Regent Instruments Incorporated, Canada). The traits of RSA including total length, projected area, surface area, average diameter, and volume were automatically obtained. Part of 1-cm root segments were cleared by 10% (w/v) KOH and stained with 0.05% (w/v) trypan blue[20]. The staining root segments were observed using a Leica DME bio-microscope, and the mycorrhizal colonization and the number of vesicles and entry point were calculated using the formula previously described by Wu et al[21]. The glucose and sucrose concentrations of leaves and roots were determined by the method of Wu et al[22]. In addition, root sucrose/glucose allocation was counted as the percent of root sucrose/glucose concentration against the sum of total leaf and root sucrose/glucose concentration, respectively.

Statistical analysis

Data (means $\pm$ SE, $n = 3$) were analysed by two-way ANOVA with SAS 8.1. Percentage of mycorrhizal colonization and root sucrose/glucose allocation were arcsine-transformed prior to statistical analysis. Significant differences in means within treatments were compared with the least significant differences ($p < 0.05$).

RESULTS

No mycorrhizal colonization was recorded in the non-inoculated roots. Compared to the absence of exogenous H_2O_2 (0 μM), 1 μM concentration of H_2O_2 significantly increased root mycorrhizal colonization by 17% and vesicles by 33% but did not affect entry points. A 100 μM concentration of H_2O_2 however notably decreased root mycorrhizal colonization and vesicles, compared to 0 and 1 μM H_2O_2 (Table 1). The significant interaction between H_2O_2 and AMF occurred in the root AM colonization and entry points.

Table 1 Effects of exogenous H_2O_2 on AM development and shoot and root fresh weights of *P. trifoliata* seedlings.[†]

H_2O_2 (µM)	Inoculation	Mycorrhizal (%)	Vesicles (per cm root)	Entry points (per cm root)	Shoot (g)	Root (g)
0	Non-AMF	0 ± 0^d	0 ± 0^d	0 ± 0^b	0.888 ± 0.023^c	0.351 ± 0.018^c
	AMF	65.1 ± 2.8^b	9.0 ± 0.2^b	2.0 ± 1.0^a	0.957 ± 0.008^{bc}	0.364 ± 0.011^{bc}
1	Non-AMF	0 ± 0^d	0 ± 0^d	0 ± 0^b	0.804 ± 0.047^d	0.332 ± 0.005^d
	AMF	75.9 ± 4.7^a	12.0 ± 1.3^a	2.7 ± 0.7^a	1.033 ± 0.025^b	0.387 ± 0.008^a
100	Non-AMF	0 ± 0^d	0 ± 0^d	0 ± 0^b	1.129 ± 0.036^a	0.373 ± 0.003^{ab}
	AMF	55.3 ± 3.0^c	4.0 ± 0.5^c	2.0 ± 1.0^a	1.163 ± 0.084^a	0.380 ± 0.002^{ab}
Significance[‡]						
AMF		$< 0.001^{**}$	$< 0.001^{**}$	$< 0.001^{**}$	$< 0.001^{**}$	$< 0.001^{**}$
H_2O_2		$< 0.001^{**}$	$< 0.001^{**}$	0.579	$< 0.001^{**}$	0.010^{**}
$AMF \times H_2O_2$		$< 0.001^{**}$	$< 0.001^{**}$	0.579	0.006^{**}	0.002^{**}

[†] Data (mean $\pm$ SE, $n = 3$) followed by the different letters within a column indicate significant differences among treatments at $p < 0.05$.

[‡] ANOVA p values (p-values at level of 0.05 or 0.01 are shown with double asterisks).

Application of 1 µM H_2O_2 decreased shoot and root fresh weights of non-AM seedlings but increased that of AM seedlings compared with the treatment without H_2O_2 (Table 1). Treatment with 100 µM H_2O_2 significantly increased shoot and root fresh weights, irrespectively, of the mycorrhizal status in seedlings. Although AMF inoculation increased shoot and root fresh weights, the significant differences between AM and non-AM seedlings were only seen under 1 µM H_2O_2. Exogenous H_2O_2 and AMF presented a significant interaction effect on shoot and root fresh weights (Table 1).

Inoculation with *G. versiforme* improved status of RSA in trifoliate orange seedlings, especially under 0 and 1 µM H_2O_2 (Fig. 1). With 0 and 1 µM H_2O_2, the inoculated seedlings had significantly higher root length, projected area, surface area, and volume compared to non-AM control seedlings (Fig. 2a–e). Treatment with 100 µM H_2O_2 and inoculation with AMF only significantly increased root length, compared to the non-AM treatments (Fig. 2a). Root average diameter was significantly affected neither by both exogenous H_2O_2 nor the AMF (Fig. 2d).

The AMF in combination with exogenous H_2O_2 altered sucrose and glucose concentrations in the plant tissue (Fig. 3). Compared to non-AMF control, AMF treatment significantly increased leaf sucrose concentration by 7, 25, and 29% with 0, 1, and 100 µM H_2O_2 (Fig. 3a) and root sucrose concentrations by 44 and 16% with 0 and 1 µM H_2O_2 levels, respectively (Fig. 3b). Compared to non-AMF control, AMF treatment notably decreased leaf glucose concentration by 36 and 22% with 0 and 100 µM H_2O_2 levels (Fig. 3c) but increased root glucose concentration by 52, 73, and 15% with 0, 1, and 100 µM
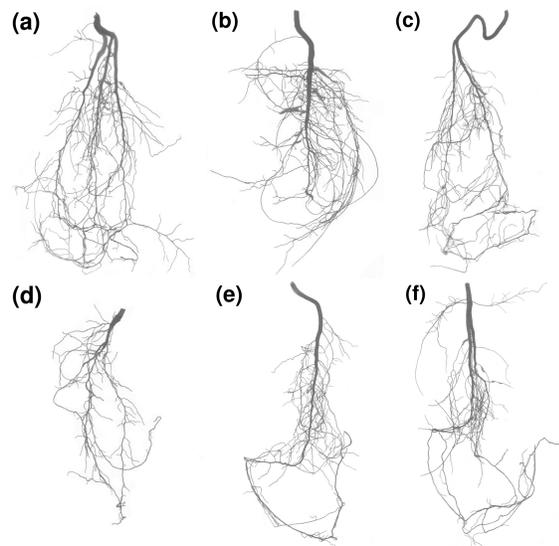

Fig. 1 Root system architecture of *P. trifoliata* seedlings inoculated with or without *G. versiforme* and treated by exogenous H_2O_2: (a) 0 µM H_2O_2 + AMF; (b) 1 µM H_2O_2 + AMF; (c) 100 µM H_2O_2 + AMF; (d) 0 µM H_2O_2 − AMF; (e) 1 µM H_2O_2 − AMF; (f) 100 µM H_2O_2 − AMF.

H_2O_2, respectively (Fig. 3d). There was a significant interaction effect between AMF and H_2O_2 on the glucose and sucrose concentrations in the leaf and root (Table 2). Compared to non-AM control, AMF treatment significantly increased sucrose allocation to the root by 25, 22, and 16% (Fig. 4a) and glucose allocation by 44, 10, and 16% under 0, 1, and 100 µM H_2O_2, respectively (Fig. 4b). There was a significant interaction effect between AMF and H_2O_2 on root glucose allocation (Table 2).
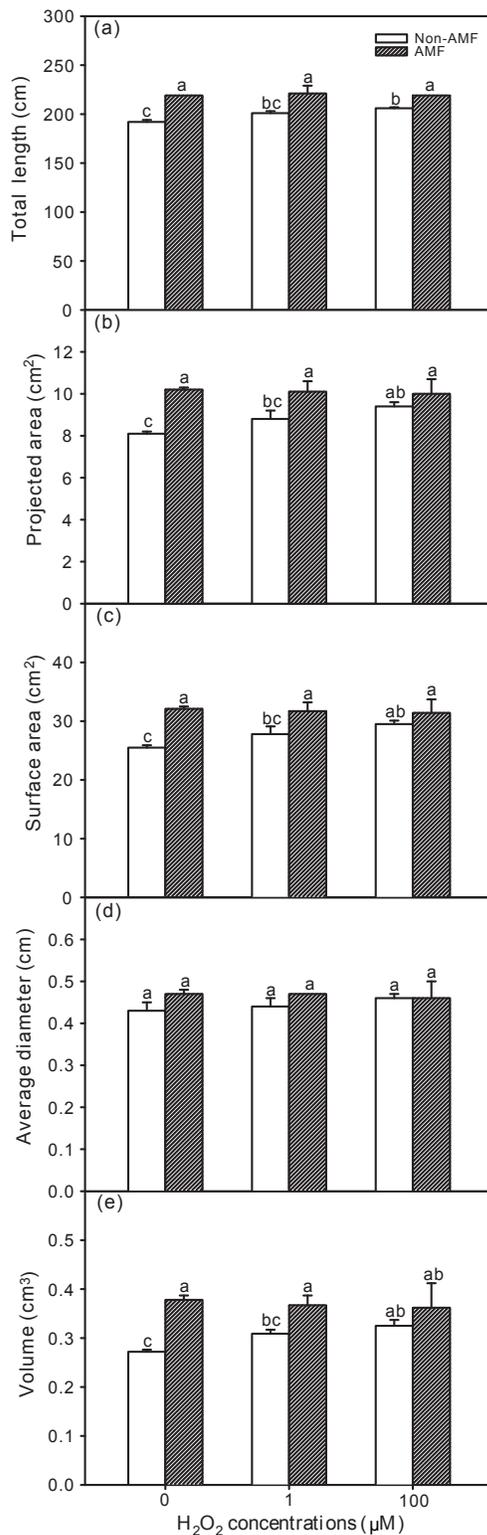
Fig. 2 Effects of exogenous H_2O_2 and arbuscular mycorrhizal fungi on traits of root system architecture of *P. trifoliata* seedlings. Data (mean $\pm$ SE, $n = 3$) marked by different letters are significantly different at $p < 0.05$.
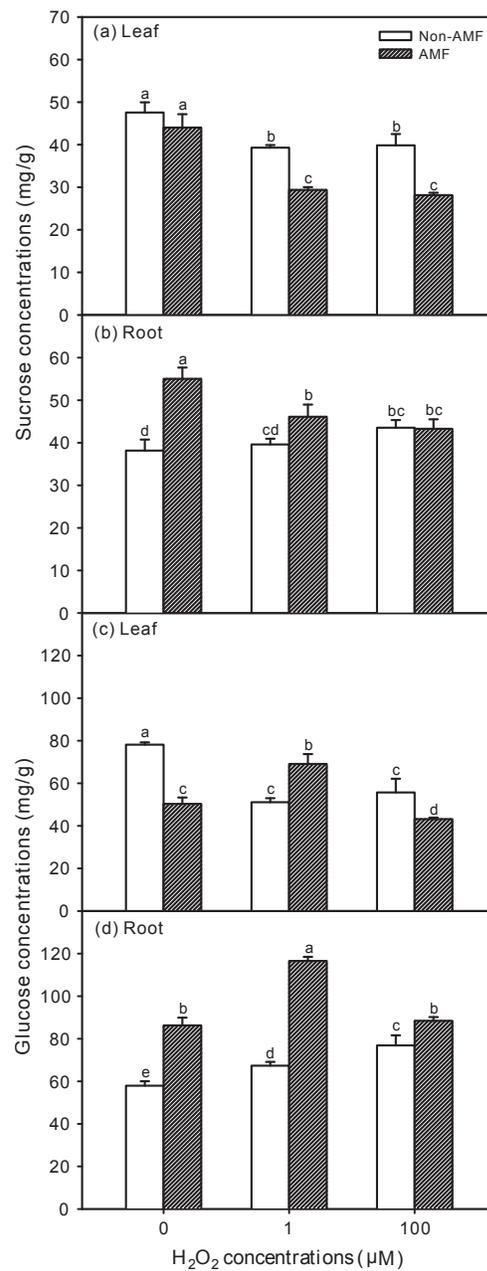
Fig. 3 Effects of exogenous H_2O_2 and arbuscular mycorrhizal fungi on glucose and sucrose concentrations of *P. trifoliata* seedlings. Data (mean $\pm$ SE, $n = 3$) marked by different letters are significantly different at $p < 0.05$.

DISCUSSION

Symbioses between plants and AMF are mediated by signal exchanges, such as flavonoid compounds, polyamines, Nod factor, and phenolic compounds[23]. Our results show that exogenous H_2O_2 altered the root mycorrhizal colonization and the number of vesi-

Table 2 Significance of the integrated effects of AMF and exogenous H_2O_2 on RSA traits and carbohydrate concentrations of *P. trifoliata* seedlings.[†]

Indexes	AMF	H_2O_2	AMF $\times$ H_2O_2
Root length	$< 0.001^{**}$	0.160	0.256
Root projected area	0.001^{**}	0.348	0.137
Root surface area	0.001^{**}	0.353	0.140
Average root diameter	0.086	0.661	0.343
Root volume	$< 0.001^{**}$	0.528	0.135
Leaf sucrose	$< 0.001^{**}$	$< 0.001^{**}$	0.009^{**}
Root sucrose	$< 0.001^{**}$	0.033^{**}	$< 0.001^{**}$
Leaf glucose	0.001^{**}	$< 0.001^{**}$	$< 0.001^{**}$
Root glucose	$< 0.001^{**}$	$< 0.001^{**}$	$< 0.001^{**}$
Root sucrose allocation	$< 0.001^{**}$	$< 0.001^{**}$	0.500
Root glucose allocation	$< 0.001^{**}$	$< 0.001^{**}$	$< 0.001^{**}$

[†] ANOVA p values (p-values at level of 0.05 or 0.01 are shown with double asterisks).
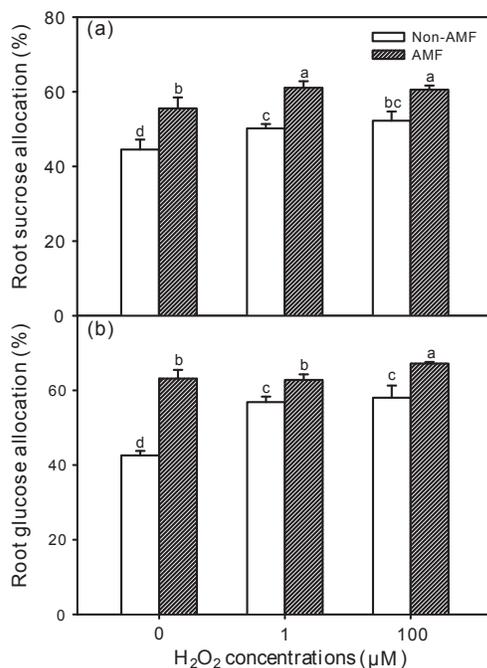
Fig. 4 Effects of exogenous H_2O_2 and arbuscular mycorrhizal fungi on (a) root sucrose and (b) root glucose allocation of *P. trifoliata* seedlings. Data (mean $\pm$ SE, $n = 3$) marked by different letters are significantly different at $p < 0.05$.

cles of the *G. versiforme*-inoculated trifoliate orange seedlings. This effect was dependent on H_2O_2 levels with 1 μM H_2O_2 increasing colonization and the number of vesicles and with 100 μM H_2O_2 decreasing colonization compared to the 0 μM H_2O_2 treatment. It seems that low concentration of H_2O_2 might act as a signal molecule in mycorrhizal development while

high concentration of H_2O_2 is a toxic reactive oxygen species that damages mycorrhizal development.

In this study, inoculation with *G. versiforme* improved RSA traits (root length, projected area, surface area, and volume, except average diameter) of trifoliate orange seedlings under 0 μM H_2O_2 conditions, which is in agreement with the report on trifoliate orange by Wu et al[24]. The RSA improvement due to mycorrhization was linked to the improvement of nutrition (e.g., phosphorus) uptake, the regulation of endogenous polyamines[7,25], and endogenous hormone synthesis, transport, or sensitivity[26]. Compared to treatment of 0 μM H_2O_2, 1 μM concentration of H_2O_2 notably altered RSA, as low concentrations of H_2O_2 are postulated to modulate abscisic acid signalling during root growth and development[27]. On the other hand, H_2O_2 itself might promote cell division, thus inducing cell elongation process[28]. In the present work, non-mycorrhizal seedlings presented a significantly higher total root length, projected area, surface area, and volume when treated with 100 μM H_2O_2 level compared to 0 μM H_2O_2 level. However, there were no significant differences in RSA traits between AM seedlings treated with 0 and 100 μM H_2O_2. This suggests that the presence of AMs reduced the adverse effects of high levels of H_2O_2 on RSA. AMF however were not able to improve RSA under 100 μM H_2O_2 conditions, the toxic effects of H_2O_2 on cell growth may also hinder the mycorrhizal effect.

The present study showed that the AMF inoculation decreased leaf sucrose concentration with 1 and 100 μM H_2O_2 but increased root sucrose concentration with 0 and 1 μM H_2O_2, thereby increasing sucrose allocation to the root in AM seedlings. The result agrees with a previous study[29] reporting an increment of the root sucrose allocation in the *Prunus persica* plants colonized by *G. versiforme*. The alteration of carbohydrates might be related to the activities of sucrose-cleaving enzymes induced by mycorrhization[30]. It is well known that carbohydrates, particularly sucrose are substrates for metabolism that strongly impact root development[31]. AMs would generally consume 10–20% of net carbon from photosynthesis of host plants and are prioritized to absorb small molecule carbohydrates, such as glucose[32,33]. As a result, higher root sucrose content and higher root sucrose allocation in AM seedlings under low concentration H_2O_2 conditions would have a potential role in root morphogenesis. In the present study, the mycorrhizal trifoliate orange seedlings also recorded significant lower leaf glucose concentration and higher root glucose concentration, regardless of exogenous H_2O_2 levels. Since AMs prefer to absorb

glucose, a higher root glucose concentration under mycorrhization could provide a better sugar supply for the AMF development.

Thus AMs in combination with application of exogenous 1 µM H_2O_2 could stimulate the root sucrose and glucose allocation, to maintain the development of AMs and the root systems.

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