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## Alleviation of drought stress by mycorrhizas is related to increased root H<sub>2</sub>O<sub>2</sub> efflux in trifoliolate orange

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Received: 12 July 2016

Accepted: 10 January 2017

Published: 08 February 2017

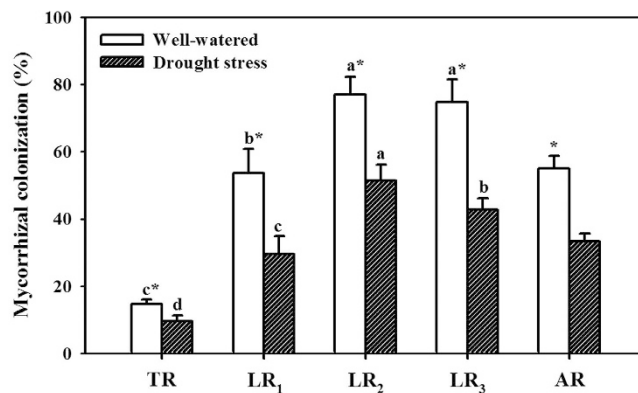
The Non-invasive Micro-test Technique (NMT) is used to measure dynamic changes of specific ions/molecules non-invasively, but information about hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) fluxes in different classes of roots by mycorrhiza is scarce in terms of NMT. Effects of *Funneliformis mosseae* on plant growth, H<sub>2</sub>O<sub>2</sub>, superoxide radical (O<sub>2</sub><sup>-</sup>), malondialdehyde (MDA) concentrations, and H<sub>2</sub>O<sub>2</sub> fluxes in the taproot (TR) and lateral roots (LRs) of trifoliolate orange seedlings under well-watered (WW) and drought stress (DS) conditions were studied. DS strongly inhibited mycorrhizal colonization in the TR and LRs, whereas mycorrhizal inoculation significantly promoted plant growth and biomass production. H<sub>2</sub>O<sub>2</sub>, O<sub>2</sub><sup>-</sup>, and MDA concentrations in leaves and roots were dramatically lower in mycorrhizal seedlings than in non-mycorrhizal seedlings under DS. Compared with non-mycorrhizal seedlings, mycorrhizal seedlings had relatively higher net root H<sub>2</sub>O<sub>2</sub> effluxes in the TR and LRs especially under WW, as well as significantly higher total root H<sub>2</sub>O<sub>2</sub> effluxes in the TR and LRs under WW and DS. Total root H<sub>2</sub>O<sub>2</sub> effluxes were significantly positively correlated with root colonization but negatively with root H<sub>2</sub>O<sub>2</sub> and MDA concentrations. It suggested that mycorrhizas induces more H<sub>2</sub>O<sub>2</sub> effluxes of the TR and LRs, thus, alleviating oxidative damage of DS in the host plant.

Drought stress (DS) is usually regarded as one of the major abiotic factors limiting crop growth and yield, including citrus. DS generally triggers oxidative stress, due to the accumulation of reactive oxygen species (ROS) in plant cells. ROS, the byproduct of aerobic metabolism *in vivo*, mainly includes hydroxyl radical (OH·), superoxide radical (O<sub>2</sub><sup>-</sup>), and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), and it can potentially damage a variety of biological molecules, such as proteins, lipids, and nucleic acids<sup>1,2</sup>. At the same time, plants also develop an elaborate antioxidant protection system to eliminate part of ROS in cells and tissues<sup>3,4</sup>.

H<sub>2</sub>O<sub>2</sub> is considered to be not only a toxic molecule inducing oxidative damage, but also an intermediary molecule participating in a variety of physio-biochemical processes in plants. The intermediary function of H<sub>2</sub>O<sub>2</sub> is closely related to its ability to travel in cellular membranes and migrate into different compartments<sup>2,5</sup>. Moreover, H<sub>2</sub>O<sub>2</sub> is a reliable indicator for the oxidative burst, because it is more stable than O<sub>2</sub><sup>-</sup>. The formation of O<sub>2</sub><sup>-</sup> in mitochondria can be measured according to the production of H<sub>2</sub>O<sub>2</sub> as determined by H<sub>2</sub>O<sub>2</sub> effluxes<sup>7,8</sup>. H<sub>2</sub>O<sub>2</sub> effluxes may be an important cellular mechanism against oxidative stress<sup>9,10</sup>. H<sub>2</sub>O<sub>2</sub> is effused from the root surface into the rhizosphere in trifoliolate orange<sup>11</sup>.

Arbuscular mycorrhizal fungi (AMF), a kind of beneficial soil microorganism, can create a symbiotic association with plant roots forming arbuscular mycorrhizas (AMs), which play a role in the regulation of plant growth<sup>12-14</sup>. AMF can mitigate the detrimental effect of DS through restraining overproduction of ROS<sup>15,16</sup>. Less accumulation of ROS in AMF-inoculated plants is due to the enhancement of antioxidant enzyme (e.g. superoxide dismutase and catalase) activities and/or non-enzymatic antioxidants (e.g. ascorbate and glutathione) concentrations<sup>17,18</sup>. In addition, AM structures (e.g. arbuscules and hyphae) are involved in H<sub>2</sub>O<sub>2</sub> accumulation within the roots of the host plant<sup>19,20</sup>. Root H<sub>2</sub>O<sub>2</sub> effluxes are one of the important ROS-scavenging mechanisms in plants subjected to DS, which were found in citrus plants under DS by using the Non-invasive Micro-test Technique (NMT)<sup>11</sup>. NMT can non-invasively preserve the integrity of the sample and provide dynamic information of specific ions/molecules (including K<sup>+</sup>, Na<sup>+</sup>, Ca<sup>2+</sup>, H<sup>+</sup>, Cl<sup>-</sup>, Mg<sup>2+</sup>, H<sub>2</sub>O<sub>2</sub>, IAA, and glucose) near material surfaces<sup>21,22</sup>.

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**Figure 1.** Mycorrhizal colonization in the taproot (TR), first-order lateral root (LR<sub>1</sub>), second-order lateral root (LR<sub>2</sub>), and third-order lateral root (LR<sub>3</sub>) of *Funneliformis mosseae*-colonized trifoliate orange (*Poncirus trifoliata*) seedlings under well-watered (WW) and drought stress (DS) conditions. The average root mycorrhizal colonization (AR) was calculated as  $(TR+LR_1+LR_2+LR_3)/4$ . Data (means  $\pm$  SD,  $n = 4$ ) followed by different letters (a, b, c, d) above the bars represent significant differences between the taproot and lateral roots for the same soil water statue, and an asterisk (\*) above the bars represent significant differences between soil water treatments for the taproot or the same lateral roots at the 5% level.

Treatments	Biomass (g FW/plant)			Leaf relative water content (%)
	Shoot	Root	Total	
WW+AMF	4.17 $\pm$ 0.31a	2.33 $\pm$ 0.06a	6.50 $\pm$ 0.29a	89.40 $\pm$ 1.01a
WW-AMF	1.44 $\pm$ 0.58c	1.44 $\pm$ 0.27c	2.88 $\pm$ 0.85c	86.34 $\pm$ 0.87b
DS+AMF	3.00 $\pm$ 0.23b	1.98 $\pm$ 0.10b	4.98 $\pm$ 0.28b	86.60 $\pm$ 1.87b
DS-AMF	0.53 $\pm$ 0.08d	0.80 $\pm$ 0.20d	1.33 $\pm$ 0.27d	76.93 $\pm$ 2.95c

**Table 1.** Effects of *Funneliformis mosseae* (AMF) on plant biomass and leaf relative water content of trifoliate orange (*Poncirus trifoliata*) seedlings under well-watered (WW) and drought stress (DS) conditions. Note: Data (means  $\pm$  SD,  $n = 4$ ) followed by different letters among treatments represent significant differences at the 5% level.

However, the response of H<sub>2</sub>O<sub>2</sub> effluxes in different order roots and their contribution to root ROS removal are not fully known under DS conditions, as well as under mycorrhization.

In this context, the objectives of the present study were to investigate the effects of the AM fungus *Funneliformis mosseae* on plant growth, ROS (H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>-</sup>) levels, malondialdehyde (MDA) concentration, and H<sub>2</sub>O<sub>2</sub> fluxes in different classes of roots of trifoliate orange [*Poncirus trifoliata* (L.) Raf.] seedlings under WW and DS conditions, and to highlight the distinct role of AMF-alleviated oxidative damage.

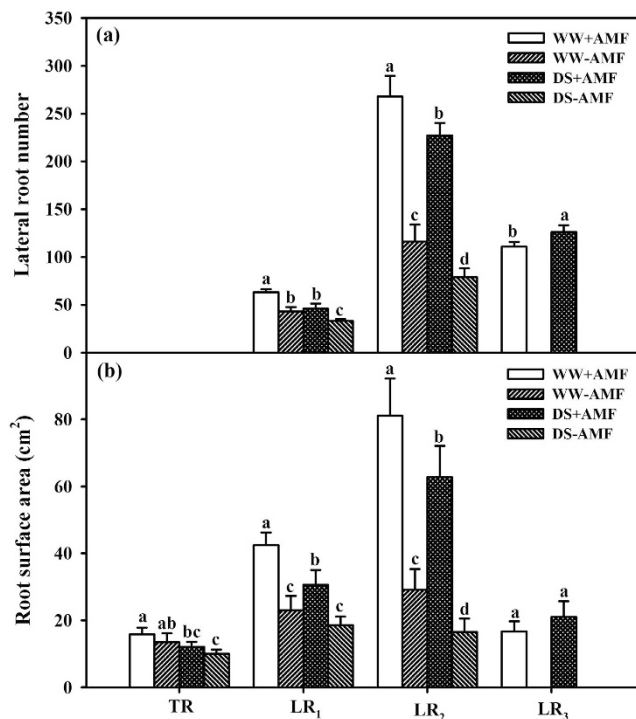
## Results

**Mycorrhizal colonization.** Root mycorrhizas were found in roots of AMF-inoculated seedlings, but not in roots of non-AMF-inoculated seedlings (Fig. 1). Mycorrhizal seedlings had 54%, 82%, 50%, 74%, and 65% significantly higher AMF colonization in the taproot (TR), first-order lateral root (LR<sub>1</sub>), second-order lateral root (LR<sub>2</sub>), third-order lateral root (LR<sub>3</sub>), and average root (AR) under WW than under DS, respectively. Moreover, AMF mainly colonized LR<sub>2</sub> and LR<sub>3</sub> in whole root systems, regardless of WW and DS.

**Plant biomass and RWC.** Mycorrhizal seedlings had significantly higher shoot, root, and total biomass than non-mycorrhizal seedlings: 190%, 62%, and 126% under WW and 466%, 148%, and 274% under DS, respectively (Table 1). Compared to non-AMF seedlings, AMF seedlings had 4% and 12% significantly higher RWC under WW and DS conditions, respectively (Table 1).

**Number and surface area of lateral roots.** Compared with non-AMF treatment, AMF inoculation significantly increased the number of LR<sub>1</sub> and LR<sub>2</sub> by 47% and 131% under WW condition and by 39% and 187% under DS condition, respectively (Fig. 2a). In addition, AMF treatment markedly stimulated LR<sub>3</sub> formation under WW and DS conditions, but LR<sub>3</sub> was not found in non-mycorrhizal seedlings. AMF inoculation notably increased the surface area of LR<sub>1</sub> and LR<sub>2</sub> by 85% and 182% under WW and by 65% and 280% under DS than non-AMF inoculation, while it did not significantly alter the surface area of the TR (Fig. 2b).

**Concentrations of O<sub>2</sub><sup>-</sup>, H<sub>2</sub>O<sub>2</sub> and MDA.** AMF seedlings exhibited significantly lower O<sub>2</sub><sup>-</sup> concentrations than non-AMF seedlings regardless of water treatments: 30% and 36% lower in leaves and 18% and 28% lower in roots under WW and DS conditions, respectively (Fig. 3a). Similarly, compared with non-AMF seedlings, AMF



**Figure 2.** Effect of *Funnelformis mosseae* (AMF) on the number of lateral roots (a) and root surface area (b) of trifoliate orange (*Poncirus trifoliata*) seedlings under well-watered (WW) and drought stress (DS) conditions. Data (means  $\pm$  SD,  $n = 4$ ) followed by different letters (a,b,c,d) above the bars among treatments represent significant differences at the 5% level.

seedlings had 14% and 22% significantly lower leaf  $H_2O_2$  concentrations and 27% and 28% lower root  $H_2O_2$  concentrations under WW and DS conditions, respectively (Fig. 3b). AMF inoculation notably decreased MDA concentrations by 13% and 15% in leaves and 17% and 20% in roots under WW and DS conditions, respectively (Fig. 3c).

**Root  $H_2O_2$  effluxes.** Net root  $H_2O_2$  effluxes (real-time effluxes of  $H_2O_2$  in the 0.5 cm length from the tip in each order root) were the highest in the TR among different classes of roots, regardless of AMF or soil water status (Fig. 4a). Compared to non-AMF seedlings, AMF seedlings showed significantly higher net root  $H_2O_2$  effluxes in the TR, LR<sub>1</sub>, and LR<sub>2</sub> by 21%, 59%, and 24% under WW condition, respectively. Under DS, AMF inoculation significantly increased net root  $H_2O_2$  effluxes by 91% in the TR, but they were not notably altered in LR<sub>1</sub> and LR<sub>2</sub>, compared with non-AMF inoculation. In addition, net root  $H_2O_2$  effluxes in LR<sub>3</sub> were decreased by 40% in mycorrhizal seedlings under DS conditions than under WW conditions. Average net root  $H_2O_2$  effluxes were not significantly different between mycorrhizal and non-mycorrhizal seedlings under WW and DS conditions.

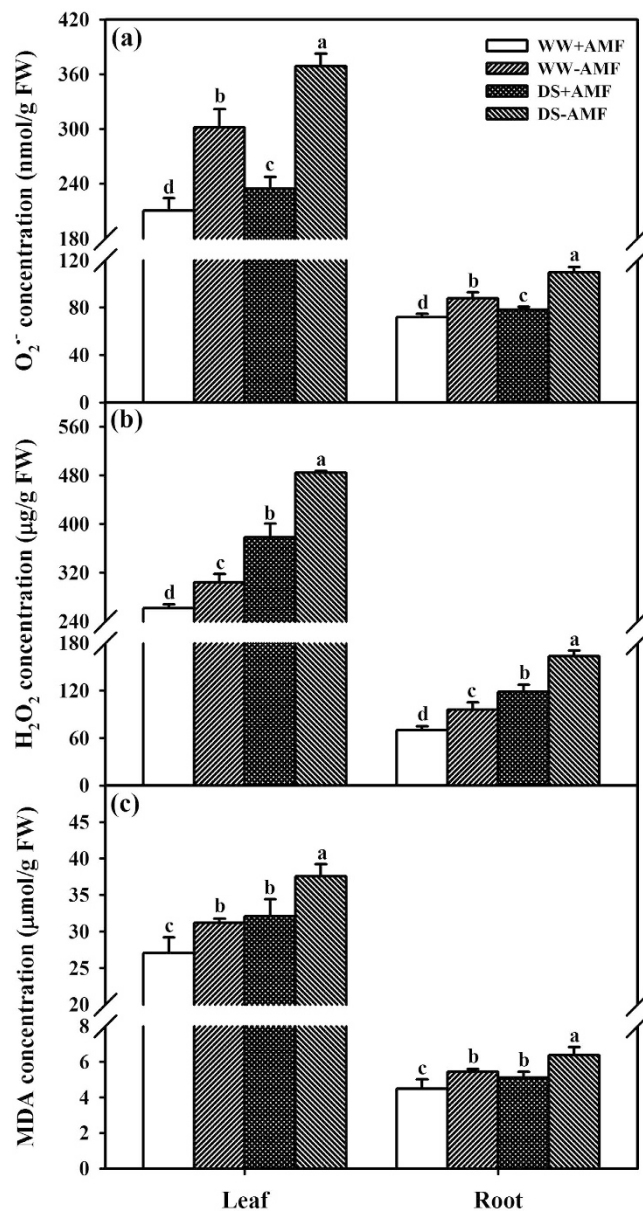
Mycorrhizal seedlings showed 43%, 194%, and 249% higher total root  $H_2O_2$  effluxes (real-time effluxes of  $H_2O_2$  in whole root systems) in the TR, LR<sub>1</sub>, and LR<sub>2</sub> under WW and 130%, 99%, and 300% higher under DS condition, respectively, as compared with non-mycorrhizal seedlings (Fig. 4b). The AMF treatment significantly increased average root  $H_2O_2$  effluxes by 113% under WW conditions and by 118% under DS conditions, compared to the non-AMF treatment.

**Correlation studies.** Total root  $H_2O_2$  effluxes were significantly positively correlated with root mycorrhizal colonization (Fig. 5a), but negatively correlated with root  $H_2O_2$  concentrations (Fig. 5b) and root MDA concentrations (Fig. 5c).

## Discussion

AMF colonization was considerably higher in LRs than in the TR and higher under WW than under DS. This is in agreement with the findings of Ding *et al.*<sup>23</sup>, who reported that *F. mosseae* colonization in LRs of soybean were higher than those in the TR at 56 days after sowing. The decrease of root mycorrhizal colonization under DS compared to under WW ascribed to the reduction of spore germination, root exudates, and root carbohydrate supply<sup>24</sup>. Possibly, greater LR formation in AMF plants, especially LR<sub>2</sub>, could provide more chances to be colonized by mycorrhizal hyphae, resulting in higher mycorrhizal colonization in LRs than in the TR<sup>25</sup>. On the other hand, mycorrhizal plants may tend to have more fine roots and less coarse roots<sup>26,27</sup>, which can be successfully colonized.

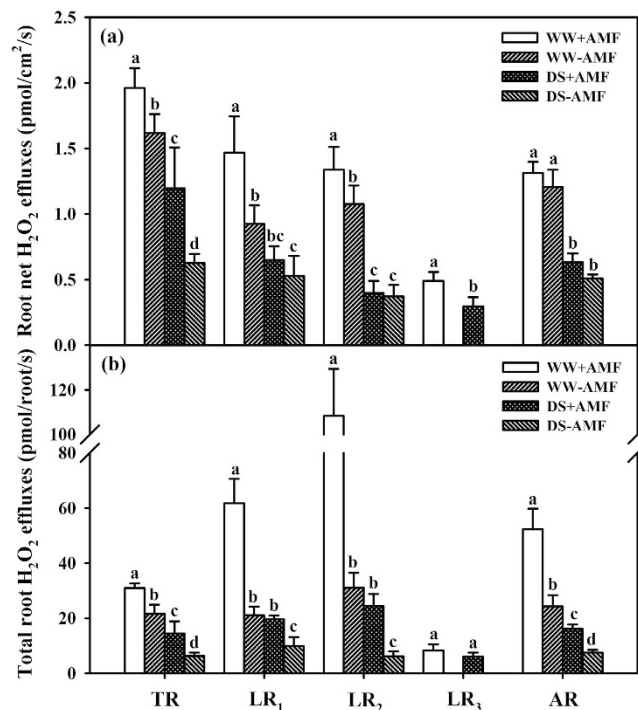
AMF inoculation with *F. mosseae* alleviated the negative effect of DS on LRs development, indicating the significantly higher number and surface area of LR<sub>1</sub> and LR<sub>2</sub> in AMF plants than in non-AMF plants. This is consistent with previous reports<sup>28,29</sup>. In general, taproots have a large diameter and play a role in fixing plants and guiding the direction of root growth, while LRs carry out absorption<sup>30</sup>. The LR formation, especially higher



**Figure 3.** Effects of *Funneliformis mosseae* (AMF) on superoxide radical ( $O_2^-$ ) (a), hydrogen peroxide ( $H_2O_2$ ) (b), and malondialdehyde (MDA) (c) concentrations of trifoliate orange (*Poncirus trifoliata*) seedlings under well-watered (WW) and drought stress (DS) conditions. Data (means  $\pm$  SD,  $n = 4$ ) followed by different letters (a,b,c,d) above the bars among treatments represent significant differences at the 5% level.

order LR (e.g., 3<sup>rd</sup> order LR formation in this study), was stimulated by AMF, which helped the host plant to absorb more water and nutrients, thus potentially enhancing drought tolerance in AMF seedlings. Maillet *et al.*<sup>31</sup> also reported that root branching of the legume *Medicago truncatula* was stimulated by *Rhizoglyphus intraradices*. Greater root branching in AMF seedling may be due to the fact that AMF can produce indole-3-acetic acid and polyamines and also further stimulate these phytohormone synthesis in roots<sup>32,33</sup>.

Our study showed an efflux behavior of root  $H_2O_2$  from roots into the rhizosphere. Compared with WW, DS resulted in lower net root  $H_2O_2$  effluxes and total root  $H_2O_2$  effluxes, regardless of the TR and LRs. Earlier studies confirmed that  $H_2O_2$  effluxes are closely related with cell membrane permeability<sup>34</sup>. As a result, DS strongly destroyed cell membranes and resulted in less effluxes of  $H_2O_2$ , thereby, inducing the oxidative burst, compared with WW. Root  $H_2O_2$  effluxes are a way of physiological metabolism of intracellular  $H_2O_2$  overproduction and the response of plant self protection to DS<sup>11</sup>. Bienert *et al.*<sup>2</sup> first provided molecular genetic evidence that indicated diffusivity of  $H_2O_2$  through specific members of the aquaporin family. The decrease in root  $H_2O_2$  effluxes by DS would induce more  $H_2O_2$  accumulation in roots, thereby resulting in a stronger oxidative burst in plants exposed to DS (e.g., a higher MDA was found under DS than under WW). Generally, net root  $H_2O_2$  effluxes gradually decreased with an increase of root order, from the TR to LRs.  $H_2O_2$  can be transported long distances across cell membranes from its generation site<sup>35</sup>. Possibly, the taproot as the first arising root in the whole root system has



**Figure 4.** Effects of *Funneliformis mosseae* (AMF) on net root hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) effluxes (a) and total root H<sub>2</sub>O<sub>2</sub> effluxes (b) in the taproot (TR), first-order lateral root (LR<sub>1</sub>), second-order lateral root (LR<sub>2</sub>), and third-order lateral root (LR<sub>3</sub>) of trifoliolate orange (*Poncirus trifoliata*) seedlings under well-watered (WW) and drought stress (DS) conditions. Average root H<sub>2</sub>O<sub>2</sub> effluxes (AR) were calculated as (TR+LR<sub>1</sub>+LR<sub>2</sub>+LR<sub>3</sub>)/4. Total root H<sub>2</sub>O<sub>2</sub> fluxes of the taproot and lateral roots were calculated as (root surface area × root net H<sub>2</sub>O<sub>2</sub> effluxes in the corresponding root classes). Data (means ± SD, n = 4) followed by different letters (a, b, c, d) above the bars represent significant differences between soil water treatments for the taproot or the same lateral roots at the 5% level.

greater capacity to transfer H<sub>2</sub>O<sub>2</sub> from the extensive original sites (the TR, as well LRs) to the epidermis of the TR, further effluxing into the rhizosphere, as compared with LRs. On the other hand, mycorrhizal structures, including intercellular hyphae and arbuscules, could accumulate H<sub>2</sub>O<sub>2</sub><sup>19,20</sup>, thereby decreasing H<sub>2</sub>O<sub>2</sub> diffusion in cell membranes. Since root mycorrhizal colonization increased with the increase of root order, from the TP to LRs, a comparatively greater mycorrhizal status in LRs would accumulate more H<sub>2</sub>O<sub>2</sub> in mycorrhizas, resulting in a decrease of root H<sub>2</sub>O<sub>2</sub> effluxes, compared with the TR.

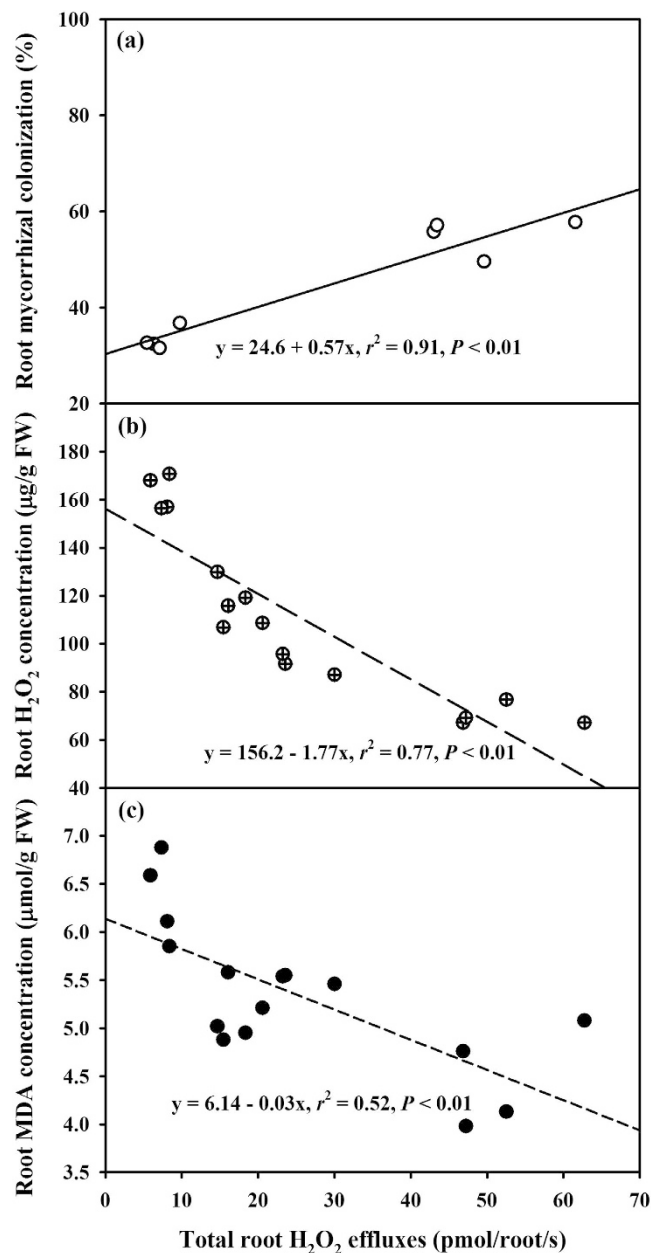
AMF colonization by *F. mosseae* significantly increased total root H<sub>2</sub>O<sub>2</sub> effluxes under WW and DS and net root H<sub>2</sub>O<sub>2</sub> effluxes in the TR, LR<sub>1</sub> and LR<sub>2</sub> under WW and in the TR under DS. Ding *et al.*<sup>23</sup> reported that H<sup>+</sup> effluxes in the TR of soybean seedlings were higher than that in LRs, and in extraradical hyphae of the TR than in extraradical hyphae of LRs. Li *et al.*<sup>36</sup> recently cloned two functional aquaporin genes from *G. intraradices*, which can express in extraradical mycelium under DS conditions. Since H<sub>2</sub>O<sub>2</sub> diffusion can be through aquaporins<sup>2</sup>, higher root H<sub>2</sub>O<sub>2</sub> effluxes in AMF seedlings than in non-AMF seedlings might be involved in the H<sub>2</sub>O<sub>2</sub> release of aquaporins by extraradical hyphae. Total root H<sub>2</sub>O<sub>2</sub> effluxes were significantly positively correlated with root AMF colonization, strengthening the functioning of AMs on H<sub>2</sub>O<sub>2</sub> effluxes. Further studies are needed to demonstrate whether AM extraradical hyphae release H<sub>2</sub>O<sub>2</sub>, and if H<sub>2</sub>O<sub>2</sub> in root cells is transported from arbuscules to extraradical hyphae and then completely effluxed through special aquaporins. In whole root systems, LR<sub>2</sub> had the highest total root H<sub>2</sub>O<sub>2</sub> effluxes, which is due to the fact that LR<sub>2</sub> had a distinctly higher root mycorrhizal colonization, root surface area and number. In the present study, the significantly negative correlation of total root H<sub>2</sub>O<sub>2</sub> effluxes with root H<sub>2</sub>O<sub>2</sub> or MDA concentrations indicated that root H<sub>2</sub>O<sub>2</sub> effluxes result in less accumulation of root H<sub>2</sub>O<sub>2</sub>, thus mitigating damage of membrane lipid peroxidation.

In conclusion, inoculation with *F. mosseae* induced considerably higher root H<sub>2</sub>O<sub>2</sub> effluxes in LRs and the TP, which might result in a lower oxidative damage in roots under DS.

## Methods

**Experimental design.** The experiment was a completely randomized block design with two factors: (1) two mycorrhizal treatments, inoculation with *Funneliformis mosseae* (+AMF) and without *F. mosseae* (−AMF), and (2) two soil water levels, well-watered (WW, 75% of maximum water holding capacity of soil) and drought stress (DS, 55% of maximum water holding capacity of soil). Each of the four treatments had four replicates, with a total of 16 pots.

**Mycorrhizal inoculum.** The AM fungus strain, *Funneliformis mosseae* (Nicol. & Gerd.) Schüßler & Walker, was isolated from the rhizosphere of *Incarvillea younghusbandii* in Dangxiang (90°45'E and 29°31'N, 4,300 m above the sea level), Tibet, and further propagated using white clover (*Trifolium repens*) as a host plant for 16



**Figure 5.** Linear regression between total root hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) effluxes and root mycorrhizal colonization (**a**,  $n = 8$ ), root  $\text{H}_2\text{O}_2$  concentration (**b**,  $n = 16$ ), or root MDA concentration (**c**,  $n = 16$ ) of trifoliolate orange (*Poncirus trifoliata*) seedlings colonized by *Funneliformis mosseae* under well-watered (WW) and drought stress (DS) conditions.

weeks under potted conditions. For the AMF treatment (+AMF), 100 g inoculum (15 spores/g), including spores, infected root segments of white clover plants, and growth substrates was mixed with the 1.2 kg soil at transplanting. The non-AMF-inoculated treatment (-AMF) received the same amount of sterilized inoculums together with 2 mL inoculums filtrate (25  $\mu\text{m}$  filter) to maintain a similar microbial community except for *F. mosseae*.

**Plant material.** Seeds of trifoliolate orange were surface-sterilized with 70% alcohol for 10 min, rinsed five times with distilled water, and placed in autoclaved (0.11 Mpa, 121  $^\circ\text{C}$ , 2 h) sands at 26  $^\circ\text{C}$  for germination. Subsequently, four-leaf-old seedlings of the same size were transferred into a 1.1 L plastic pot containing 1.2 kg autoclaved (0.11 Mpa, 121  $^\circ\text{C}$ , 2 h) soil from a citrus orchard of the Yangtze University campus. The soil collected here had a pH of 6.0, 12.1 mg/kg  $\text{KMnO}_4\text{-N}$ , 15.7 mg/kg Bray-P, 22.3 mg/kg neutral  $\text{NH}_4\text{OAc-K}$ , and 38% maximum water holding capacity. All the seedlings were placed in a glasshouse (photosynthetic photon flux density was 982  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , day/night temperature 27/20  $^\circ\text{C}$ , and relative humidity 80%) from March 24 to August 19, 2014.

**Soil water treatment.** According to Zou *et al.*<sup>11</sup>, WW treatment was selected as 75% of maximum water holding capacity, and DS was considered as 55% of maximum water holding capacity of soil. Before DS was begun, soil water of these pots was kept in WW. After 99 days, half of the seedlings were kept in DS status for 50 days and the other seedlings were still kept in WW status for 50 days. The soil water levels in the pots were determined daily through weighing, and the amount of water lost was supplied to each pot to reach the designed soil water levels. During the experiment, a 50 mL of Hoagland solution with half of P strength was biweekly added into each pot for normal growth of seedlings.

**Variable determinations.** After 50 days of DS treatment, all the plants were harvested, and plant height, stem diameter and leaf number per plant were recorded. Subsequently, the plants were divided into shoots and roots, whose fresh weights were measured. According to Hackket<sup>37</sup>, root systems were divided into the TP and LR<sub>s</sub>. The number of LR<sub>1</sub>, LR<sub>2</sub>, and LR<sub>3</sub> was carefully counted. Ten 1 cm long root segments per pot of the TR, LR<sub>1</sub>, LR<sub>2</sub>, and LR<sub>3</sub> were stained with 0.05% (w/v) trypan blue for 5 min<sup>38</sup> and observed by the LEICA DME bio-microscope for AM structures. Root AMF colonization was calculated as the percentage of colonized root lengths by AMF against observed total root length.

Different classes of roots per plant were scanned with an Epson Perfection V700 Photo (Seiko Epson Corp, Japan), and then the scanned root images were analyzed to obtain the root surface area using a WinRHIZO professional 2007b (Regent Instruments Incorporated, Canada).

The fourth fully expanded top leaf was used to determine leaf RWC by the method outlined by Bajji *et al.*<sup>39</sup>. The leaf RWC was calculated by the following formula:  $RWC (\%) = 100 \times (FW - DW) / (SW - DW)$ , where FW refers to fresh weight, DW to dry weight (oven-dried, at 75 °C for 48 h), and SW to saturated weight after leaf rehydration for 24 h.

MDA, a by-product of oxidative damage to lipids in tissues, was measured following the method described by Sudhakar *et al.*<sup>40</sup>. Fresh samples (0.1 g) of leaves and roots were homogenized with 5 mL 0.1% (w/v) trichloroacetic acid and centrifuged at  $3,000 \times g$  for 10 min. The mixture including 0.5 mL supernatant and 1.5 mL 0.67% thiobarbituric acid was incubated at 100 °C for 0.5 h and centrifuged at  $3,000 \times g$  for 10 min. The absorbance at 450, 532, and 600 nm was recorded, and the MDA concentration ( $\mu\text{mol/L}$ ) was calculated by the formula:  $6.45 (A_{532} - A_{600}) - 0.56A_{450}$ .

The  $\text{H}_2\text{O}_2$  concentration in leaves and roots was determined according to Velikova *et al.*<sup>41</sup>. Fresh samples (0.1 g) were homogenized with 5 mL 0.1% (w/v) trichloroacetic acid and centrifuged at  $12,000 \times g$  for 15 min. A 4 mL mixture was comprised of 1 mL 10 mM potassium phosphate buffers (pH 7.0), 2 mL 1 M KI, and 1 mL supernatant. The absorbance at 390 nm was recorded.

The levels of  $\text{O}_2^-$  in tissues were measured using the protocol described by Wang and Luo<sup>42</sup>. Fresh leaf or root samples (0.15 g) were homogenized in 5 mL 0.1 M phosphate buffer (pH 7.8) and centrifuged at  $4,000 \times g$  for 10 min at 4 °C. The 0.5 mL supernatant was mixed with 0.5 mL 50 mM phosphate buffers (pH 7.8) and 0.1 mL 10 mM hydroxylamine chloride for reaction for 1 h at 25 °C. Subsequently, 1 mL 17 mM sulfanilamide and 1 mL 7 mM  $\alpha$ -naphthylamine were added to the mixture at 25 °C for 20 min, and the absorbance was recorded at 530 nm.

Net root  $\text{H}_2\text{O}_2$  fluxes in a 0.5 cm length from the tip of the taproot and different order lateral roots were measured using the Scanning Ion-Selective Electrode Technique (SIET) system (BIO-001A, Younger USA Sci. & Tech. Corp., Amherst, MA, USA) in the Xuyue Beijing NMT Service Center (Xuyue Beijing Sci. and Tech. Co., Ltd., Beijing, China). An  $\text{H}_2\text{O}_2$ -sensitive microsensor was polarized at +600 mV against an Ag/AgCl reference electrode before use<sup>6</sup>. Root systems *in vivo* were placed in a Petri dish and immersed in an assay solution, comprised of 0.1 mM  $\text{CaCl}_2$ , 0.1 mM KCl, and 0.3 mM MES (pH 6.0). The  $\text{H}_2\text{O}_2$  microsensor was placed 30  $\mu\text{m}$  from the root surface. Net root  $\text{H}_2\text{O}_2$  fluxes were calculated by Fick's law of diffusion:  $J (\text{pmol}/\text{cm}^2/\text{s}) = -D_0 \times (dc/dx)$ , where  $D_0$  indicates the diffusion constant,  $dc$  the concentration gradient, and  $dx$  the distance (30  $\mu\text{m}$ )<sup>43</sup>. Total root  $\text{H}_2\text{O}_2$  fluxes were counted according to  $J \times$  surface area of the lateral root.

**Statistical analysis.** Data (means  $\pm$  SD,  $n = 4$ ) were statistically analyzed by the variance (ANOVA) with SAS 8.1 software (SAS Institute Inc., Cary, NC, USA), and the significant differences between the treatments were tested by Duncan's Multiple Range Tests at  $P < 0.05$ .

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## Acknowledgements

This study was supported by the Plan in Scientific and Technological Innovation Team of Outstanding Young, Hubei Provincial Department of Education (T201604), the National Natural Science Foundation of China (31101513), and the Open Fund of the Institute of Root Biology, Yangtze University (R201401). Thanks to Dr. Edward C. Mignot, Shandong University, for linguistic advice.

## Author Contributions

Y.M.H., Y.N.Z., and Q.S.W. conceived the experiment, Y.M.H. conducted the experiment, and Y.M.H., Y.N.Z. and Q.S.W. analyzed the results. All authors reviewed the manuscript.

## Additional Information

**Competing financial interests:** The authors declare no competing financial interests.



**How to cite this article:** Huang, Y.-M. *et al.* Alleviation of drought stress by mycorrhizas is related to increased root H<sub>2</sub>O<sub>2</sub> efflux in trifoliolate orange. *Sci. Rep.* 7, 42335; doi: 10.1038/srep42335 (2017).

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