In fields, citrus plants rely strongly on soil arbuscular mycorrhizal fungi (AMF) to form arbuscular mycorrhizas (AMs) for their optimum performance through mycorrhizal extraradical hyphae in nutrient and water uptake (Srivastava et al. 2002, Ngullie et al. 2015, Zou et al. 2018). Citrus rhizosphere is reported to inhabit as many as 45 species of AMF, which colonize roots to enlarge the absorptive surface of roots (Wu et al. 2013). Earlier studies showed that AMF presence could promote nutrients and water absorption, accelerate plant growth, modify root architecture and root hairs, enhance stress tolerance, increase soil fertility, and improve soil structure (Wu et al. 2017). Hence, AMF has been accepted as a biofertilizer and used so extensively and intensively in citrus production.

Despite these developments highlighting the utility of AMF in citrus orchards, the propagation technique of AMF is a gap. In this study, indigenous AMF in rhizosphere of Citrus unshiu grafted on trifoliate orange were isolated from fresh root segments (Φ<2 mm), fresh rhizosphere soil (< 4 mm size), and air-dried rhizosphere soil (< 4 mm size) as AMF-source and propagated with white clover. Subsequently, indigenous AMF inocula were inoculated into potted trifoliate orange to assess the inoculated efficiency. Our results showed that AMF isolated from fresh root segments multiplied by 333.9% significantly higher than those isolated from fresh or air-dried rhizosphere soil. Similar results were obtained with regard to root mycorrhizal colonization (37.16–55.41%) and soil hyphal length (3.88–13.38 cm/g) in trifoliate orange after inoculated with AMF-source from root segments. Mycorrhizal trifoliate orange seedlings carrying AMF inoculum from fresh roots exhibited higher plant growth performance, root morphology, leaf P, K, Mg, Cu and Zn levels, and leaf superoxide dismutase, peroxidase, and catalase activities, compared to non-AMF treatment. Our study, hence, suggested that root segments would be a great choice to propagate indigenous AMF for later inoculating into the rhizosphere of target plants.

Key words: Antioxidant enzyme, Arbuscular mycorrhiza, Citrus, Root morphology, Spore

MATERIALS AND METHODS

Isolation of native AMF and propagation: The rhizosphere soil (< 10 cm of soil layer) and roots (Φ < 2 mm) were collected from a citrus orchard with 30-year-old Citrus unshiu cv. Guoqing 1 grafted on Poncirus trifoliate spaced
at of 3 m × 4 m in apart in campus of Yangtze University (30°36′ N, 112°14′ E, 36 m above sea level). From rhizosphere soil and root segments, a total of 4 treatments, replicated 5 times were formulated, which comprise $T_1$ as fresh roots (6 g fresh root segments having colonization of native AMF species was mixed with autoclaved (0.11 MPa, 121°C, 2 h) rhizosphere soil after removing roots/other impurities and sieving with 4 mm size); $T_2$ having air-dried rhizosphere soils (Soils were dried naturally, removed root segments/other impurities and screened by 4 mm size); $T_3$ as rhizosphere fresh soil (Collected fresh rhizosphere soil was cleaned of roots/other impurities and sieved through 4 mm to retain native AMF species in soils; and control (CK) as sterilized soils (after removing roots and impurities and sieving through 4 mm, the rhizosphere soils were autoclaved (0.11 MPa, 121°C, 2 h) to eliminate native AMF spores). In all the four treatments, sterilized (0.11 MPa, 121°C, 2 h) river sands (3:1, v/v) were evenly mixed. Seeds of white clover (Trifolium repens L.) were then sown and grown in a Walk-in Artificial Plant Growth Chamber (Zhejiang Qiushi Artificial Environment Co. Ltd. China) having air temperature 28°C/18°C (12 hours in the day / 12 hours in the night), light intensity 1200 Lux, and relative air humidity 68% for spore germination. After 3 months (February 19 to May 19, 2017) of AMF propagation with host plants in pots, the indigenous AMF was collected, and the spore density of AMF was determined by wet sieving-sucrose centrifugation using the procedure as outlined by Brundrett et al. (1994).

Inoculation of host plants with indigenous AMF: Plastic pots having 1.5 L capacity were filled with 1.3 kg autoclaved (0.11 MPa, 121°C, 2 h) rhizosphere soils, then inoculated with 200 g inoculum of indigenous AMF (composed of infected root segments, spores and soil hyphae). Each pot had three five-leaf-age trifoliate orange seedlings raised in non-mycorrhized autoclaved sands. The trifoliate orange seedlings were harvested after three months (May 19 to August 19, 2017) of indigenous AMF-inoculation.

Variable determinations: Plant height, stem diameter, leaf number per seedling, and fresh weight of shoots and roots in trifoliate orange were measured. The mycorrhizal colonization of roots was studied by staining and examining them with the help of optical microscope (Phillips and Hayman 1970) and calculated as the percentage of mycorrhized colonized root lengths against total observed root length. Soil spore density was determined with wet sieving-sucrose centrifugation (Brundrett et al. 1994). Spore propagation rate was calculated by the following formula: Spore propagation rate = (soil spore density after propagation – soil spore density before propagation)/spore density before propagation × 100%.

Leaf mineral nutrient (P, K, Ca, Mg, Fe, Cu, Mn, Zn, and B) levels were determined by the Plasma Emission Spectrometer (IRIS Advantage, American Thermoelctric Company, USA) (Black et al. 1982). Leaf superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) activities were assayed using nitrogen blue tetrazole method (Giannopolitis and Ries 1997), guaiacol method (Chance and Maehly 1955) and ultraviolet spectrophotometry (Wu 2018), respectively.

Statistical analysis: The data were analyzed with the ANOVA in SAS (8.1) for significance levels, and the Duncan’s Multiple Range Test (P=0.05) was used to compare significant differences amongst treatments.

RESULTS AND DISCUSSION

Propagation of indigenous AMF: The soil spore density after propagated with white clover for three months was 81, 122, and 128 spores / 10 g dry soil under T1, T2, and T3 conditions, respectively. Treatment $T_1$ induced maximum spore propagation rate of 339.44% followed by 154.35% with treatment $T_2$ and 104.89% with treatment $T_3$. These observations suggested that the fresh roots possessed the maximum spore density compared with either dried rhizosphere soils or fresh rhizosphere soil. Using the Internal Transcribed Spacer Fragment sequencing, Wu et al. (2017) reported that AMF communities in citrus roots were much higher than citrus rhizosphere soil. Such results further indicated that AMF diversity in roots was more than those in rhizosphere soil.

Mycorrhizal status in trifoliate orange as host plant: No mycorrhiza was observed in the CK-treated trifoliate orange. Root mycorrhizal colonization with treatments $T_1$, $T_2$, and $T_3$ was 55.4%, 51.1% and 37.2%, soil hyphal length was 13.38, 5.93, and 3.88 cm/g soil, respectively. The response of different treatments for magnitude of root colonization and soil hyphal length could be ranked as $T_1$> $T_2$> $T_3$ and $T_1$> $T_2$> $T_3$ respectively, in the decreasing order. The observation further indicated a higher mycorrhizal colonization and soil hyphal length in mycorrized soil treated with AMF inoculum form of fresh roots. Although spore propagation rate with $T_2$ was marginally higher than that of $T_3$, the difference was non-significant. But a significantly greater inoculation effect of AMF was observed on root mycorrhizal colonization and soil hyphal length with treatment $T_2$ than with $T_3$ suggesting that moderate soil water deficit with a short time is beneficial for multiplication of AMF spores within citrus rhizosphere, even in field conditions (Jacobson 1997, Augé 2001).

Plant growth responses with indigenous AMF inoculation: After three months of indigenous AMF inoculation, no significant difference in stem diameter and root volume of trifoliate orange seedlings was observed among all the four treatments (Fig 1 and Table 1). Compared with CK treatment, $T_1$ treatment likewise produced no significant response on different growth parameters, viz. plant height, leaf number per plant, biomass of shoot and root, total root length, and root projected area. However, both treatments $T_1$ and $T_2$ showed a significant increase in plant height, leaf number per plant, biomass of shoot and root, over treatments CK but treatments $T_1$ and $T_2$ were at par with each other. Compared with non-AMF seedlings (CK), mycorrhizal seedlings represented by $T_1$ and $T_2$ observed a significantly higher total root length, root projected area, and
Table 1 Effects of different AMF inocula on plant growth performance and root morphology in trifoliate orange seedlings

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Plant height (cm)</th>
<th>Stem diameter (cm)</th>
<th>Leaf number (#/plant)</th>
<th>Shoot (g FW/plant)</th>
<th>Root (g FW/plant)</th>
<th>Total length (cm)</th>
<th>Projected area (cm²)</th>
<th>Surface area (cm²)</th>
<th>Volume (cm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T₁</td>
<td>24.8±2.7a</td>
<td>0.25±0.02a</td>
<td>25±2a</td>
<td>1.5±0.2a</td>
<td>1.0±0.04a</td>
<td>236±19a</td>
<td>14.0±0.2a</td>
<td>19.2±0.5a</td>
<td>0.62±0.11a</td>
</tr>
<tr>
<td>T₂</td>
<td>24.3±3.5a</td>
<td>0.24±0.03a</td>
<td>23±1a</td>
<td>1.4±0.4a</td>
<td>1.0±0.06a</td>
<td>207±11b</td>
<td>13.2±0.2b</td>
<td>16.9±0.8b</td>
<td>0.74±0.13a</td>
</tr>
<tr>
<td>T₃</td>
<td>16.6±0.7b</td>
<td>0.22±0.02a</td>
<td>18±2b</td>
<td>0.8±0.1b</td>
<td>0.9±0.08b</td>
<td>198±9bc</td>
<td>12.7±0.7bc</td>
<td>15.3±0.9c</td>
<td>0.72±0.22a</td>
</tr>
<tr>
<td>CK</td>
<td>15.9±1.8b</td>
<td>0.23±0.04a</td>
<td>18±1b</td>
<td>0.9±0.2b</td>
<td>0.9±0.07b</td>
<td>188±6c</td>
<td>12.2±0.4c</td>
<td>13.6±0.3d</td>
<td>0.65±0.10a</td>
</tr>
</tbody>
</table>

Data (means±SD, n=5) followed by different letters among treatments indicate significant differences at the 5% level between treatments. T₁, the inoculums from fresh roots as indigenous AMF propagated source; T₂, the inoculums from air-dried soils as indigenous AMF propagated source; T₃, the inoculums from rhizosphere fresh soils as indigenous AMF propagated source; CK, the inoculums from sterilized soils as indigenous AMF propagated source.


root surface area. Amongst different treatments, treatment T₁ re-showed the response of highest magnitude on plant growth performance and root morphology. Hence, treatments T₁ and T₂, especially T₁, heavily significantly promoted the growth and root morphology, whereas treatment T₃ remained ineffective with respect to these attributes. These results could be effectively linked to spore propagation rate, subsequently root mycorrhizal colonization and mycorrhizal length in mycorrhizosphere (Zou et al. 2017, Liu et al. 2018). The effect of treatment T₃ on total root length, root projected area and root surface area of trifoliate orange as host plant was significantly higher than treatment T₂. It was further confirmed that the spore density and mycorrhizal development were higher in position of treatment T₁ than treatment T₂. Mycorrhizal plants develop better root architecture, conducive to plant colonization, growth and stress tolerance (Wu and Zou 2017).

Leaf nutrient composition responses with indigenous AMF inoculation: In the present study, significant difference in leaf Ca content was observed while comparing the effectiveness of all the four treatments (Table 2). Compared with CK, treatment T₁ and T₂ were associated with a significant increase in leaf P, K, Mg, Cu and Zn levels, but leaf Mn, Fe and B concentrations substantially decreased. The stimulatory effect of AMF inoculation was of higher magnitude with treatment T₁ than either T₂ or T₃. The treatment T₂ also significantly increased leaf P, K, Mg and Zn concentrations, but decreased leaf Mn, Fe and B concentrations, as compared with CK. On the whole, leaf P, K, Mg, Cu and Zn levels were significant higher in T₁ and T₂ treatments than in T₃ treatment (Table 2). Better mineral levels in mycorrhizal trifoliate orange seedlings could be attributed to effective intervention of mycorrhizal extra radical hyphae in mineral absorption from the mycorrhized soil (Leake et al. 2004), as a function of improved root morphology in AMF seedlings (Wu et al. 2017), which in turn aided in accumulating mineral nutrients, thereby, promoting the development of AMF-mycelium to add the better nutrient foraging ability of host plants (Bonfante and Genre 2010).

Leaf antioxidant enzyme profile responses with indigenous AMF inoculation: AMF inoculation is reported to impart better biochemical preparedness to the host plant (Liu et al. 2018). The activities of SOD, POD and CAT in leaves with treatment T₁ were, respectively observed 307.71%, 74.48% and 75.38% higher than with CK (data not shown). The activities of SOD, POD and CAT observed a significant increase by 120.96%, 18.29% and 70.07%, respectively, with treatment T₂ over CK. Compared with CK, SOD activity in leaves under treatment T₂ increased by 94.07%, but without any significant response on activities of POD and CAT. Compared with treatments T₃ the activities of SOD, POD and CAT in leaves were significantly increased with treatment T₁. Mycorrhizal plants, therefore, showed higher activity of antioxidant enzymes in the host plants to develop a greater resilience against biotic/abiotic stresses. Our observations are consistent with the studies of Huang et al. (2014) highlighting the response of inoculating AMF on trifoliate orange under drought stress. The changes in antioxidant enzymes were also reported to be closely related with AMF colonization, number of arbuscules, and amount of mycorrhiza-induced calmodulin (Huang et al. 2014).
In short, the combination of fresh root segment and white clover served as an effective indigenous spore propagation method of AMF, the role of which was duly verified by the magnitude of response observed in mycorrhized trifoliate orange. Such an attempt could be effectively developed in citrus culture. As a result, the present study clearly confers a feasible protocol for rapid proliferation of indigenous AMF to citrus orchard (Fig 2). The full potential of AMF as a biofertilizer is to be exploited in future.

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Table 2  Effects of different AMF inocula on leaf mineral levels in trifoliate orange seedlings

<table>
<thead>
<tr>
<th>Treatment</th>
<th>P/g/kg</th>
<th>K/g/kg</th>
<th>Ca/g/kg</th>
<th>Mg/g/kg</th>
<th>Cu/mg/kg</th>
<th>Zn/mg/kg</th>
<th>Mn/mg/kg</th>
<th>Fe/mg/kg</th>
<th>B/mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>2.81±0.09</td>
<td>8.95±0.54</td>
<td>26.97±1.78</td>
<td>2.51±0.08</td>
<td>12.36±0.96</td>
<td>27.96±1.69</td>
<td>34.07±2.21</td>
<td>238±10b</td>
<td>69.5±3.6b</td>
</tr>
<tr>
<td>T2</td>
<td>2.31±0.13</td>
<td>8.75±0.31</td>
<td>26.18±0.94</td>
<td>2.60±0.17</td>
<td>10.80±0.61</td>
<td>28.89±1.20</td>
<td>29.25±1.51</td>
<td>159±13c</td>
<td>47.9±2.9c</td>
</tr>
<tr>
<td>T3</td>
<td>2.17±0.08</td>
<td>7.64±0.65</td>
<td>25.34±0.96</td>
<td>2.21±0.06</td>
<td>8.55±0.56</td>
<td>23.48±1.09</td>
<td>35.28±1.85</td>
<td>240±12b</td>
<td>67.8±3.0b</td>
</tr>
<tr>
<td>CK</td>
<td>1.38±0.10</td>
<td>6.75±0.26</td>
<td>26.91±1.28</td>
<td>1.95±0.05</td>
<td>7.90±0.48</td>
<td>17.18±1.20</td>
<td>43.08±1.49</td>
<td>292±14a</td>
<td>92.9±2.3a</td>
</tr>
</tbody>
</table>

Data (means±SD, n = 5) followed by different letters among treatments indicate significant differences at the 5% level between treatments. T1, the inoculums from fresh roots as indigenous AMF propagated source; T2, the inoculums from air-dried soils as indigenous AMF propagated source; T3, the inoculums from rhizosphere fresh soils as indigenous AMF propagated source; CK, the inoculums from sterilized soils as indigenous AMF propagated source.

REFERENCES


Bonfante P and Genre A. 2010. Mechanisms underlying beneficial


