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The Physiological Mechanism of Arbuscular Mycorrhizal in Regulating the Growth of Trifoliate Orange (*Poncirus trifoliata* L. Raf.) Under Low-Temperature Stress

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Abstract

In recent years, low temperature has seriously threatened the citrus industry. Arbuscular mycorrhizal fungi (AMF) can enhance the absorption of nutrients and water and tolerance to abiotic stresses. In this study, pot experiments were conducted to study the effects of low-temperature stress on citrus (trifoliate orange, *Poncirus trifoliata* L. Raf.) with AMF (*Diversispora epigaea D.e*). The results showed that AMF inoculation significantly increased plant growth, chlorophyll fluorescence, and photosynthetic parameters. Compared with 25 °C, −5 °C significantly increased the relative conductance rate and the contents of malondialdehyde, hydrogen peroxide, soluble sugar soluble protein, and proline, and also enhanced the activities of catalase and superoxide dismutase, but dramatically reduced photosynthetic parameters. Compared with the non-AMF group, AMF significantly increased the maximum light quantum efficiency and steady-state light quantum efficiency at 25 °C (by 16.67% and 61.54%), and increased the same parameters by 71.43% and 140% at −5 °C. AMF also significantly increased the leaf net photosynthetic rate and transpiration rate at 25 °C (by 54.76% and 29.23%), and increased the same parameters by 72.97% and 26.67% at −5 °C. Compared with the non-AMF treatment, the AMF treatment significantly reduced malondialdehyde and hydrogen peroxide content at 25 °C (by 46.55% and 41.29%), and reduced them by 28.21% and 29.29% at −5 °C. In addition, AMF significantly increased the contents of soluble sugar, soluble protein, and proline at 25 °C (by 15.22%, 34.38%, and 11.38%), but these increased by only 9.64%, 0.47%, and 6.09% at −5 °C. Furthermore, AMF increased the activities of superoxide dismutase and catalase at 25 °C (by 13.33% and 13.72%), but these increased by only 5.51% and 13.46% at −5 °C. In conclusion, AMF can promote the growth of the aboveground and underground parts of trifoliate orange seedlings and enhance their resistance to low temperature via photosynthesis, osmoregulatory substances, and their antioxidant system.

Keywords: cold stress; *Diversispora epigaea*; growth parameters; light quantum

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1. Introduction

Low-temperature stress often occurs in crop production and cultivation, which inhibits growth and development, thereby reducing the yield and quality of crops. Cotton (*Gossypium hirsutum* L.) seed suffers from cold damage during germination, showing no germination or delayed germination [1]. Maize (*Zea mays* L.) and cucumber (*Cucumis sativus* L.) wilt when subjected to cold, and necrotic spots will appear on the leaves if the cold continues for 24 h; the edges of the leaves are usually dry after warming [2].

Plants are unable to migrate to favorable environments, forcing them to develop a series of strategies to cope with low-temperature stress. This adaptation involves a range of physiological and biochemical changes [3]. For instance, low-temperature stress promotes the production of reactive oxygen species (ROS) within plants, thereby damaging the membrane system. Some enzymes, such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (ASP), can eliminate reactive oxygen free radicals, thereby preventing the toxicity of these free radicals [4]. By measuring the changes in SOD and CAT activities in *Camellia* leaves under low-temperature stress, the cold tolerance of different varieties of *Camellia* can be elucidated [5]. Under low-temperature stress, water in plant cells is passively lost, resulting in indirect water stress [6]. In order to maintain the mutual balance of water inside and outside cells, plants will increase their intracellular osmotic regulatory substances to alleviate the damage of stress to plants [6]. Osmotic regulatory substances include cerebrosides, free sterols, sterol glycosides, acylated sterols, glycosides, raffinose, glycoxylans, and other soluble sugars, in addition to aminoglutaric acid, amino acids, polyamines, betaine, etc. [7].

Low-temperature stress can also affect the photosynthesis of plants. Low-temperature stress affects the structure of photosynthetic organs and the synthesis of photosynthetic pigments [8,9]. The composition, selective permeability, and fluidity of the chloroplast thylakoid membrane change under low-temperature stress, which affects the structure of the reaction center, light-trapping antenna, electron transmitter, and other proteins on the thylakoid membrane, thus blocking the function of PSII [8]. As a result, the activities of key enzymes in the Calvin cycle, such as 1,5-diphosphate ribulose carboxylase (RuBP) and 1,6-diphosphatase (FBPase), are reduced, thus affecting the photosynthetic carbon assimilation of plants and blocking the production and transportation of photosynthetic products [10]. Under low-temperature stress, the resistance of leaf stomata to CO_2 diffusion increases, and the transport of photosynthetic products is slow, resulting in the accumulation of photosynthetic products in the leaves that cannot be effectively transported to various tissues and organs of the plant [11]. Under low-temperature stress, the water absorption and transport capacity of plant roots is weakened, resulting in water deficit. This leads to stomatal closure and reduced gas exchange between plants and the external environment, thus reducing the photosynthetic rate [11]. In summary, low-temperature stress affects the cell membrane system, antioxidant system, osmotic regulation system, photosynthesis system, etc., thus affecting the normal metabolism, growth, and development of plants.

Arbuscular mycorrhiza (AM) is a symbiotic system that combines Arbuscular mycorrhizal fungi (AMF) in soil with the roots of plants and can form reciprocal symbionts with more than 80% of terrestrial plants on earth [12]. Host plants provide carbon sources for AMF to survive and reproduce, and AMF acts as root hair in host plant roots, expanding the root absorption area through mycelium, transporting nutrients to the plant, and helping plant roots absorb water and inorganic nutrients such as nitrogen (N), phosphorus (P), and potassium (K) [13,14]. AMF can cope with adverse conditions by improving plant water absorption, mineral nutrients, root configuration, photosynthesis, osmotic balance, etc. [12,15,16]. Thus, AMF can improve host plant stress resistance.

Citrus is a small evergreen tree in the citrus subgenus of *Rutaceae*, belonging to tropical and subtropical evergreen fruit trees [17]. It likes light, is more resistant to shade, and prefers a warm climate; in fact, the whole growth and development process requires a temperature between 12.5 and 37 °C [17–19]. As a typical plant of the subtropical and tropical areas, citrus is especially susceptible to the adverse impact of low-temperature stress. In spring, when citrus seedlings are planted in an orchard, they frequently experience damage from cold temperatures, impairing their growth and even killing citrus seedlings in some cases, such as frost. In addition, low-temperature stress is also detrimental to the flowers and fruits of citrus; if environmental temperatures fall below −2 °C for several hours, ice crystals nucleate in the extracellular spaces of citrus fruits, which results in marked reductions in yield [3,20]. Especially in some orange gardens with poor microclimate conditions, grapefruit or late-maturing citrus varieties that are not tolerant to freezing damage are more susceptible to cold damage and freezing damage [21].

A large number of studies have shown that AMF and citrus plants can form a good symbiotic relationship. AMF inoculation can promote the elongation and growth of citrus roots, improve the photosynthetic efficiency of leaves, increase plant biomass, regulate osmosis and the antioxidant content of plants, and maintain the internal hormone balance of plants to enhance the stress resistance of citrus [16]. In recent years, more and more studies have begun to pay attention to the symbiotic relationship between citrus and AMF, which mainly includes the following three aspects: first, the effect of AMF inoculation on citrus growth; second, the effect of AMF inoculation on citrus stress resistance under abiotic stress; third, the interaction of AMF with other chemicals or microorganisms, affecting citrus growth [12,16]. In this study, trifoliate orange (*Poncirus trifoliata* L. Raf), a major citrus rootstock, was treated with low-temperature stress to study its morphological, physiological, and biochemical changes. We observed the effects of *Diversispora epigaea* on citrus cold resistance and further explored the mechanism from a physiological perspective. This study aims to enrich and develop the mechanism of AMF in enhancing citrus stress resistance and guide the application of AMF in the growth and cultivation of citrus. Furthermore, this study offers a systematic characterization of the physiological changes in trifoliate orange during cold stress and AMF treatment and thereby establishes a robust foundation for the investigation of key genes that may enhance citrus cold tolerance.

2. Materials and Methods

2.1. Experimental Design and Plant Material and Growth Conditions

The trifoliate orange (*Poncirus trifoliata* L. Raf) was used as plant material, donated by Huazhong Agricultural University in the form of seeds. The arbuscular mycorrhizal fungus used in this study was *D.e* (BGC JX08B) from the Bank of Glomeromycota in China (BGC), which was stored in a refrigerator at 4 °C and trapped with white clover for 12 weeks. The white clover root segments and growth substrates were collected and mixed well as the fungal inoculum, containing 22 spores/g.

The experiment comprised a 2 × 2 two-factor test. The first factor was temperature, including normal temperature (25 °C) and low temperature for 9 h −5 °C, 9 h). The second factor was AMF, including no inoculation (non-AMF) and inoculation with *D.e*. The experiment consisted of 4 treatments with 5 replicates per treatment. Trifoliate orange seeds were surface sterilized with 75% ethanol and germinated in autoclaved sands at 27–29 °C on 15 April 2023. After germination, seedlings with four leaves were transplanted into culture pots (height × diameter = 30 × 20 cm). Each pot was thinned to 3 seedlings. In a completely randomized setup, each treatment was replicated 5 times for a total of 5 pots and 15 seedlings. The pots were pre-filled with an autoclaved (121 °C, 0.11 MPa, 1.5 h) mixture of soil and sand at a ratio of 1:1 (*v:v*) to eliminate indigenous AMF spores. The soil

and sand were collected from the citrus orchard of Yangtze University, and the soil belongs to the Ferralsol class (FAO system). The chemical properties of the soil were as follows: a pH of 6.28, Olsen-P of 10.12 mg/kg, soil organic carbon of 9.78 mg/g, and available K of 60.63 mg/kg. AMF inoculation was carried out at a dose of 100 g (2200 spores) inoculum/pot at the time of seedling transplanting. The non-AMF treatment also involved 100 g of autoclaved inoculum. All potted seedlings were placed in a light incubator (the light flux density was 907 μmol/m^2/s) with 150 mL of purified water added every 3 days. The plants received no additional chemical fertilizers during the experiment. The temperature in the incubator was 25 °C and the air relative humidity was 70%. After 5 months of culture (10 November 2023), the potted seedlings were exposed to normal temperature and low temperature (25 °C and −5 °C) for 9 h. After treatment, the fresh weight of the aboveground part and the underground part was determined, the rhizosphere soil (50 g) was collected for subsequent determination of soil mycelia length, and root segments with a length of 1–2 cm were stored in the FAA fixing solution to determine the mycorrhiza colonization rate. Fresh leaves were collected for the measurement of chlorophyll via fluorescence imaging, the relative conductance rate (REC), and the relative water content. The remaining leaves and roots were quickly frozen in liquid nitrogen and stored at −80 °C for other physiological and biochemical measurements.

2.2. Determination and Methods

A tape was used to measure the plant height, a vernier caliper was used to measure the stem diameter, the number of leaves was manually calculated, and the fresh weight of the above- and belowground parts of the plant was determined by using an electronic scale. The harvested roots were scanned and analyzed for root morphological parameters using WinRHIZO (Regent Instruments Inc., Quebec, QC, Canada).

The method used to determine the mycorrhizal colonization rate followed Zhang et al. [13]: the 1 cm long root segments were treated with 10% KOH solution for 1.5 h, bleached with 10% hydrogen peroxide solution for 16 min, acidified with 0.22 mol/L hydrochloric acid solution for 12 min, and stained with 0.06% trypan blue in lactophenol for half a minute. The root segments were observed under a microscope (Olympus IX71, Beckman Coulter, Inc., Brea, CA, USA). The formula is as follows:

$$\text{Mycorrhizal colonization rate (\%)} = (\text{colonized root lengths}/\text{observed root lengths}) \times 100$$

The relative water content, chlorophyll fluorescence parameters (maximum light quantum efficiency—QY_max; steady-state light quantum efficiency—QY_Lss; and non-photochemical fluorescence quenching—NPQ_Lss), and photosynthetic parameters (net photosynthetic rate—Pn; transpiration rate—Tr; stomatal conductance—Gs; and intercellular CO_2 concentration—Ci) of trifoliate orange functional leaves (4–5) were determined according to Jian et al. [10]. The chlorophyll fluorescence parameters of the leaves were determined by using the portable PAM-2000 fluorometer (Walz, Effel-trich, Germany) on a sunny day at 9:00 am in November 2023. The photosynthetic parameters of the leaves were determined by using the Li-6400 portable photosynthetic system analyzer (Li-COR Inc., Lincoln, NE, USA) on a sunny day at 9:00 am in November 2023. The formula for calculating the relative water content of the leaves is as follows:

$$\text{Relative water content of leaves} = (\text{fresh weight of leaves} - \text{dry weight of leaves}) \times 100/(\text{saturated weight} - \text{dry weight})$$

The membrane permeability was determined by using the relative conductivity method, the malondialdehyde (MDA) content was determined by using the thiobarbituric acid method, and the hydrogen peroxide (H_2O_2) content was detected by using a H_2O_2 assay kit (Beijing Boxingong Technology Co., Ltd., Shanghai, China). The proline (Pro) content was determined by ninhydrin colorimetry. The SOD and CAT activities were measured with the Enzyme-Linked Immunosorbent assay using the corresponding kit (mll614100, ml902210, and ml201168) (Shanghai Enzyme-link Biotechnology Co., Ltd., Shanghai, China) on the basis of the user manual and according to Theocharis et al. [2]. The concentration of soluble protein and soluble sugar was measured according to the protocol of Chen et al. [8]. The 0.2 g fresh sample was homogenized with 5 mL of distilled water and centrifuged at 4000× *g* for 20 min. The 0.1 mL supernatant was reacted with 6 mL of Coomassie brilliant blue G-250 (Sigma-Aldrich Merck KGaA, Darmstadt, Germany) solution. After standing for 3 min, the absorbance was determined at 595 nm using bovine serum protein as a standard curve.

2.3. Statistical Analysis

We employed analysis of variance (ANOVA) (SAS software 8.1v) for statistical analysis of the data. Microsoft Excel 2003 and Photoshop 7.0.1 software were used for data processing and graphing, and Duncan's multirange experiment compared significant differences between treatments with $p < 0.05$.

3. Results

3.1. Effects of AMF on Growth of Trifoliate Orange Seedlings

As shown in Figure 1A, AMF colonization was evident in the root segment of the inoculated plants, and corresponding soil mycelia could also be seen during microscopic examination. The average AMF colonization rate in the AMF treatment group was 60.40% (Table 1), while no AMF colonization was observed in the non-AMF group, and no soil mycelia were detected. As shown in Figure 1B,C, the biomass of the AMF group was significantly higher than that of the uninoculated group. Compared with non-AMF, the plant height, stem diameter, number of leaves, and fresh weight of the aboveground and underground parts of trifoliate orange seedlings inoculated with AMF were significantly increased by 91.70%, 43.14%, 45.24%, 131.25%, and 50.88%, respectively (Table 1). AMF inoculation significantly increased the total root length, total surface area, and volume by 15.61%, 47.05%, and 43.90%, respectively (Table 2). Compared with the control group, the projected root area and average diameter of the AMF treatment group also increased by 4.99% and 1.72%, respectively, but the difference was not significant compared with the control group (Table 2).

Table 1. Effects of AMF inoculation on mycorrhiza development and plant growth of trifoliate orange (mean ± SD).

Treatments	Mycorrhiza Development		Plant Growth			Biomass (g FM/Plant)	
	Mycorrhizal Infection Rate AMF (%)	Hyphal Length (cm/g)	Plant Height (cm)	Stem Diameter (mm)	Leaf Number	Shoot	Root
AMF	60.40 ± 4.63 a	20.48 ± 0.60 a	25.17 ± 0.92 a	2.19 ± 0.11 a	17.53 ± 1.04 a	1.48 ± 0.12 a	0.86 ± 0.07 a
CK	0.00 ± 0.00 b	0.00 ± 0.00 b	13.13 ± 1.10 b	1.53 ± 0.09 b	12.07 ± 1.11 b	0.64 ± 0.05 b	0.57 ± 0.04 b

Note: Different letters after the data in the column indicate significant differences between treatments at the 0.05 level, $n = 5$. CK was the control, and AMF was the inoculated *D.e.*

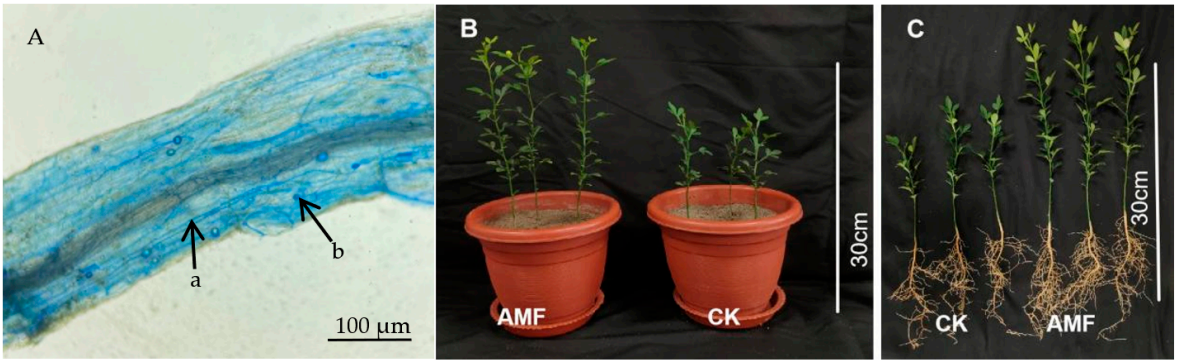


Figure 1. Effects of AMF inoculation on mycorrhiza development and plant growth of trifoliate orange. Note: (**A**)—Observation of root colonization of trifoliate orange seedlings; (**B**,**C**)—Effects of AMF inoculation and non-AMF inoculation on the growth performance of trifoliate orange. Note: CK was the non-AMF treated, AMF was the inoculated with *D.e.* a indicates the mycelium of the AMF, b indicates the spores of AMF.

Table 2. Effect of AMF inoculation on root morphology of trifoliate orange.

Treatments	Overall Length (cm)	Projected Area (cm^2)	Total Surface Area(cm^2)	Diameter (mm)	Root Volume (cm^3)
AMF	174.37 ± 6.71 a	14.10 ± 0.87 a	13.72 ± 1.17 a	0.59 ± 0.02 a	0.59 ± 0.04 a
CK	150.82 ± 9.04 b	13.43 ± 1.29 a	9.33 ± 0.64 b	0.58 ± 0.04 a	0.41 ± 0.03 b

Note: Different letters after the data in the column indicate significant differences between treatments at the 0.05 level, $n = 5$. CK was the control, and AMF was the inoculated *D.e.*

3.2. Effect of Low Temperature on the Morphology of Trifoliate Orange

Leaves are the main parts of plants for photosynthesis and transpiration. When plants are stressed, the leaves will curl, wither, and dehydrate. After 9 h of treatment at −5 °C, compared with the control group, the leaves of trifoliate orange seedlings showed water-loss phenomena such as wilting and severe curling, regardless of whether they were exposed to bacteria (Figure 2).

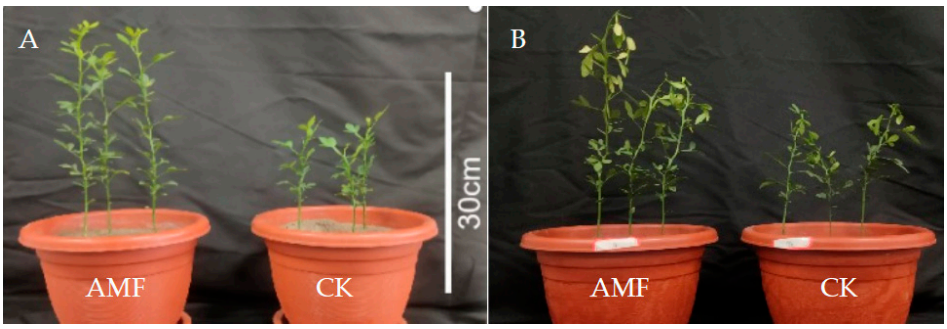


Figure 2. Effect of AMF on the morphology of trifoliate orange under low temperature. Note: (**A**)—Low temperature −5 °C treatment 0 h; (**B**)—Low temperature −5 °C treatment 9 h. As shown in the figure, the left seedlings (AMF) were treated with AMF (inoculated with *D.e*), and the right seedlings (CK) were not treated with AMF.

3.3. Effect of AMF on Chlorophyll Fluorescence Parameters of Trifoliate Orange Leaves at Low Temperature

As shown in Table 3, regardless of AMF inoculation, low-temperature stress significantly decreased QY_max and QY_Lss and increased NPQ_Lss. Compared with the non-AMF treatment, inoculation with AMF significantly increased QY_max and QY_Lss by 16.67% and 61.54% at room temperature and by 71.43% and 140% at low temperature,

respectively, after 9 h. Compared with the non-AMF treatment, AMF inoculation had no significant effect on NPQ_Lss regardless of low-temperature treatment for 0 h or 9 h (Table 3).

Table 3. Effect of AMF inoculation on chlorophyll fluorescence parameters of trifoliate orange under low-temperature stress.

Treatments	QY_max	QY_Lss	NPQ_Lss
Non-AMF and −5 °C 0 h	0.30 ± 0.03Ab	0.13 ± 0.01Ab	0.20 ± 0.02Ba
Non-AMF and −5 °C 9 h	0.14 ± 0.01Bb	0.05 ± 0.00Bb	0.26 ± 0.02Aa
AMF and −5 °C 0 h	0.35 ± 0.02Aa	0.21 ± 0.02Aa	0.17 ± 0.01Ba
AMF and −5 °C 9 h	0.24 ± 0.01Ba	0.12 ± 0.01Ba	0.28 ± 0.02Aa

Note: Different capital letters after the data in the column indicate significant differences between −5 °C treated 0 h and 9 h at the 0.05 level, n = 5; Different lowercase letters after the data in the column indicate significant differences between non-AMF and AMF at the 0.05 level, n = 5. Non-AMF was not inoculated with *D.e*, AMF was inoculated with *D.e*.

3.4. Effect of AMF on Photosynthesis of Trifoliate Orange Leaves at Low Temperature

As shown in Table 4, low-temperature stress significantly reduced leaf Pn, Gs, Ci, and Tr among trifoliate orange seedlings, regardless of AMF inoculation. Compared with the non-AMF treatment, AMF inoculation significantly increased the Pn and Tr of trifoliate orange leaves by 54.76% and 29.23% at room temperature and by 72.97% and 26.67% at low temperature, respectively, after 9 h (Table 4). Compared with the non-AMF treatment, AMF inoculation had no significant effect on Gs and Ci regardless of low-temperature treatment for 0 h or 9 h (Table 4).

Table 4. Effects of AMF inoculation on photosynthetic parameters of trifoliate orange seedlings under low-temperature stress.

Treatments	Pn (μmol/m^2·s)	Gs (μmol/m^2·s)	Ci (μmol/mol)	Tr (mmol/m^2·s)
Non-AMF and −5 °C 0 h	4.31 ± 0.37 Ab	0.14 ± 0.01 Aa	330.65 ± 14.61 Aab	3.25 ± 0.12 Ab
Non-AMF and −5 °C 9 h	0.74 ± 0.06 Bb	0.02 ± 0.00 Ba	243.22 ± 22.86 Bb	0.15 ± 0.01 Bb
AMF and −5 °C 0 h	6.67 ± 0.57 Aa	0.16 ± 0.01 Aa	355.35 ± 32.14 Aa	4.20 ± 0.39 Aa
AMF and −5 °C 9 h	1.28 ± 0.11 Ba	0.04 ± 0.00 Ba	302.67 ± 29.33 Ba	0.19 ± 0.02 Ba

Note: Different capital letters after the data in the column indicate significant differences between −5 °C treated 0 h and 9 h at the 0.05 level, n = 5; Different lowercase letters after the data in the column indicate significant differences between non-AMF and AMF at the 0.05 level, n = 5. Non-AMF was not inoculated with *D.e*, AMF was inoculated with *D.e*.

3.5. Effects of AMF on Relative Water Content and REC of Trifoliate Orange Leaf at Low Temperature

As can be seen from Table 5, regardless of whether AMF was inoculated or not, low-temperature stress significantly decreased the relative water content and increased the REC of the leaves of trifoliate orange seedlings. Compared with the non-AMF treatment, inoculation with AMF significantly increased the relative water content of leaves (13.75%) at room temperature but had no significant effect on the REC of leaves (Table 5). Compared with the non-AMF treatment, under low-temperature stress, AMF inoculation had no significant effect on the relative water content of leaves, but significantly reduced the REC of leaves (33.26%, Table 5). It can be seen that low-temperature stress increases membrane permeability, leads to electrolyte leakage, reduces the relative water content in leaves, and increases the relative conductivity. AMF can alleviate this change, thereby reducing the damage of low temperature to the leaf cell membrane and increasing the cold resistance of plants.

Table 5. Effects of low-temperature stress on relative water content, REC, the contents of MDA and H_2O_2 of leaves of trifoliate orange seedlings.

Treatments	Relative Water Content (%)	REC (%)	MDA(nmol/g)	H_2O_2(umol/g)
Non-AMF and −5 °C 0 h	0.80 ± 0.04 Ab	10.21 ± 0.81 Ba	0.58 ± 0.04 Ba	55.26 ± 4.89 Ba
Non-AMF and −5 °C 9 h	0.63 ± 0.05 Ba	48.01 ± 2.25 Aa	0.78 ± 0.04 Aa	68.21 ± 5.21 Aa
AMF and −5 °C 0 h	0.91 ± 0.08 Aa	10.22 ± 0.72 Ba	0.31 ± 0.02 Bb	32.44 ± 2.78 Bb
AMF and −5 °C 9 h	0.64 ± 0.04Ba	32.04 ± 2.01Ab	0.56 ± 0.03Ab	48.23 ± 2.45Ab

Note: Different capital letters after the data in the column indicate significant differences between −5 °C treated 0 h and 9 h at the 0.05 level, n = 5; Different lowercase letters after the data in the column indicate significant differences between non-AMF and AMF at the 0.05 level, n = 5. Non-AMF was not inoculated with *D.e*, AMF was inoculated with *D.e*.

3.6. The Effect of AMF on the Content of MDA and H_2O_2 in Trifoliate Orange Leaves at Low Temperature

The effects of AMF on MDA and H_2O_2 contents in leaves under low-temperature conditions are shown in Table 5. Regardless of AMF inoculation, low-temperature stress significantly increased MDA and H_2O_2 contents in the leaves of trifoliate orange seedlings. Compared with the non-AMF treatment, the contents of MDA and H_2O_2 were significantly reduced by AMF inoculation, decreasing by 46.55% and 41.29% at room temperature and by 28.21% and 29.29% at low temperature, respectively, after 9 h (Table 5). In conclusion, low-temperature stress treatment caused damage to plants and significantly increased the MDA and H_2O_2 contents in leaves. However, AMF alleviated the damage of low temperature to the leaf cell membrane and thus increased the cold resistance of plants.

3.7. Effects of AMF on Contents of Soluble Sugar, Soluble Protein, and Pro in Trifoliate Orange Leaf at Low Temperature

Table 6 shows the changes in the content of osmotic regulatory substances in trifoliate orange leaves. As can be seen from Table 6, regardless of whether AMF was inoculated or not, low-temperature stress significantly increased the contents of soluble sugar, soluble protein, and Pro in the trifoliate orange leaf. Compared with the non-AMF treatment, AMF inoculation increased the contents of soluble sugar, soluble protein, and Pro by 15.22%, 34.38%, and 11.38% at normal temperature and by 9.64%, 0.47%, and 6.09% at low temperature, respectively, after 9 h (Table 6). In conclusion, low-temperature stress treatment caused damage to plants, and plants were protected by increasing the content of soluble sugar, soluble protein, and Pro in leaves. AMF could further increase the contents of these three osmoregulatory substances to cope with low-temperature stress.

Table 6. Effects of AMF on the contents of soluble sugar, soluble protein, and Pro, and antioxidant oxidase activity in leaves of trifoliate orange seedlings under low-temperature stress.

Treatments	The Soluble Sugar (g/L)	The Soluble Protein (mg/g)	Pro (ug/L)	SOD (U/g)	CAT (U/g)
Non-AMF and −5 °C 0 h	8.08 ± 0.34 Bb	2.56 ± 0.19 Bb	310.54 ± 21.12 Bb	3008.22 ± 198.31 Ba	152.51 ± 10.12 Bb
Non-AMF and −5 °C 9 h	15.98 ± 1.22 Ab	4.21 ± 0.21 Aa	394.26 ± 22.78 Ab	4755.92 ± 354.24 Aa	215.28 ± 13.26 Ab
AMF and −5 °C 0 h	9.31 ± 0.62 Ba	3.44 ± 0.26 Ba	345.89 ± 19.52 Ba	3409.32 ± 192.64 Ba	173.44 ± 12.51 Ba
AMF and −5 °C 9 h	17.52 ± 1.43 Aa	4.23 ± 0.35 Aa	418.28 ± 32.37 Aa	5017.52 ± 389.48 Aa	244.26 ± 19.31 Aa

Note: Different capital letters after the data in the column indicate significant differences between −5 °C treated 0 h and 9 h at the 0.05 level, n = 5; Different lowercase letters after the data in the column indicate significant differences between non-AMF and AMF at the 0.05 level, n = 5. Non-AMF was not inoculated with *D.e*, AMF was inoculated with *D.e*.

3.8. The Effects of AMF on the Activities of Superoxide Dismutase and Catalase in Trifoliate Orange at Low Temperature

Table 6 shows the changes in SOD and CAT activities in leaves. Low-temperature stress significantly increased the activities of antioxidant enzymes (SOD and CAT) in the leaves of trifoliate orange seedlings regardless of AMF inoculation. Compared with the

non-AMF treatment, the SOD and CAT activities increased by 13.33% and 13.72% at room temperature and by 5.51% and 13.46% at low temperature, respectively, after 9 h (Table 6).

4. Discussion

Whether the mycorrhizal effect can play a role depends on the affinity between AMF and the host, and the colonization rate can indicate the colonization of fungi and biomass in the root tissue, which is required for AMF to be effective [16]. Studies have shown that the colonization rate of AMF (such as *D.e*) on citrus roots is generally between 17% and 48% [22]. In this experiment, the colonization rate and promoting effect of AMF on the roots of trifoliate orange were studied. The results showed that the AMF colonization rate of trifoliate orange reached 60.40%, and a clear mycelium and arbuscular structure could be observed during microscopic examination, indicating that *D.e* could better infect the roots of trifoliate orange seedlings and had good affinity. This is roughly similar to the results of previous studies [22].

Recent studies have shown that AMF can promote the growth of most plants. In this study, AMF-inoculated potted seedlings were tested, and it was observed that AMF had a significant promoting effect. The plant height, stem diameter, leaf number, and other biomass indexes of mycorrhized trifoliate orange seedlings were significantly higher than those of non-mycorrhized seedlings. Plant phenotypic characteristics are the most intuitive manifestation of plant growth and development, among which the root system is the main organ of plant nutrient cycling. Therefore, maintaining the health and function of the root system is crucial to the growth and development of the aboveground plant parts [23–26]. The results of this study showed that AMF significantly increased the root volume, total root length, and root surface area, and also increased the root projection area and average root diameter, which may be related to the expansion of the root absorption area, stimulating the secretion of endogenous hormones, increasing the photosynthetic area of leaves, and accelerating the absorption of nutrients (N, P, K, etc.) by AMF [27–29]. The above results show that AMF inoculation changed the plant root morphology, increased the contact area between the roots and soil, and promoted the absorption of nutrients and water by plants.

Plants are often subjected to various abiotic stresses during their growth and development, and low-temperature stress is one of the common abiotic stresses. Plant leaves are the most sensitive parts to low-temperature stress. Under mild low-temperature stress, leaves will lose water, slowly curl, reduce photosynthesis, and dry and wither in severe cases [30]. The results of this study showed similar results: compared with the control group, the leaves of trifoliate orange seedlings treated at −5 °C for 9 h showed water loss and wilting with more severe curling.

As a method of analyzing plant photosynthesis, chlorophyll fluorescence technology can reflect the chemical properties of plant photosynthetic reaction centers and is a powerful tool for studying plant stress phenotypes [31]. This technique has been applied to analyze the abiotic stress of barley, tomato, *Arabidopsis*, tea, and other crops. The results of this study showed that low-temperature treatment significantly decreased the QY_max and QY_Lss of non-mycorrhized trifoliate orange seedlings and increased the NPQ_Lss of the leaves of trifoliate orange seedlings. This phenomenon was similar to the results of Fazal [28], indicating that low temperature damaged the PSII reaction center of the leaves of trifoliate orange. As a sensitive index of photosynthetic performance, QY_max can reflect the maximum light energy conversion efficiency of the PSII reaction center. The results of this study also showed that, compared with the absence of AMF, inoculation with AMF significantly increased the QY_max and QY_Lss of trifoliate orange seedlings, while decreasing NPQ_Lss, which is consistent with the results of Ren et al. [32], indicating

that inoculation with AMF increased the activity of the PSII reaction center in plant leaves and improved the light energy conversion efficiency and electron transfer ability of leaves. The photosynthetic function of PSII was promoted, and the ability of plants to resist low temperature was improved [33].

Low-temperature stress can affect many indexes of plant photosynthesis, such as Pn, Gs, Ci, Tr, etc., reflecting the damage of low-temperature stress on plant photosynthesis [33]. In this study, Pn, Tr, Gs, and Ci were significantly reduced by low-temperature treatment, regardless of whether plants were treated with or without fungi, which is in line with the results of Setua et al.'s [34] study on *Morus alba* under low-temperature stress: stomatal inhibition occurred during plant photosynthesis under low-temperature stress. Stomatal inhibition further affected plant gas exchange and CO_2 absorption, reduced CO_2 supply, and thus affected plant photosynthetic rate. In addition, under the same temperature condition, the Pn of mycorrhized trifoliate orange seedlings was significantly higher than that of non-mycorrhizal seedlings, which is similar to the results of Ullah et al. [28], indicating that AMF inoculation could improve the photosynthetic capacity of trifoliate orange seedlings by alleviating the effects of low-temperature stress on leaf stomatal inhibition and other reactions, thus improving their cold tolerance.

The relative water content in plant cells was closely related to the metabolic intensity, growth rate, and resistance of plants. Usually, under low-temperature stress, plants will resist the effect of low temperature by reducing the water content in their leaves, thereby increasing the concentration of cell fluid to reduce the freezing point. In this study, after 9 h of low-temperature treatment, the relative water content of leaves decreased significantly, and at this time, plants resisted the influence of low temperature by increasing the concentration of cell fluid, which is similar to the results of Ye et al. [35]. In addition, under low-temperature stress, AMF inoculation significantly increased the relative water content of the leaves of trifoliate orange seedlings, thus alleviating the damage to the seedlings caused by low-temperature stress, which is similar to the results of Zhou et al. [36].

Plants change their membrane systems when exposed to low temperatures. In the natural environment, the ROS concentration in leaves is usually low, and the ROS concentration will increase under low-temperature stress [37]. The accumulation of ROS will not only destroy the membrane structure and affect the normal metabolism of plants, but also destroy the selectivity of biofilms, resulting in increased membrane permeability and membrane lipid peroxidation, which may eventually cause different degrees of injury or even plant death [38]. H_2O_2, a kind of ROS, is an important product of peroxide reactions, and its content reflects the degree of damage to the plant cell membrane in low-temperature environments [39]. MDA is the product of peroxidation, and an increase in its content will cause obvious damage to the cell membrane, while the selectivity and permeability of the cell membrane will be reduced, resulting in exosmosis of the electrolyte in the cell [40]. The REC indicates the ion exosmosis rate of the plant cell membrane. The degree of electrolyte exosmosis is not only related to temperature but also has a great relationship with the duration of stress [41]. Therefore, H_2O_2 content, MDA content, and the REC can be used to evaluate the index of low-temperature injury in plants. The REC and MDA content in leaves increased with the decrease in treatment temperature [42]. The REC and MDA content showed an increasing trend with the extension of low-temperature stress duration [43]. In this study, the contents of MDA and H_2O_2 and the REC of leaves of trifoliate orange seedlings increased under low-temperature stress, and the cell membrane system was seriously damaged. This is consistent with the results of previous studies [37–43]. This study also found that under low-temperature stress, AMF inoculation could reduce the accumulation of H_2O_2 and MDA and the REC, so as to alleviate the damage caused by low temperature on plants. This is similar to the research conclusion of Chen et al. [44]: the

MDA content increased significantly under low-temperature stress, while AMF treatment inhibited this effect. Liu et al. [45] also found that AMF (*D.e*) alleviates chilling stress by boosting redox poise and antioxidant potential in tomato.

Soluble sugar, soluble protein, and Pro, as osmoregulatory substances, maintain cell osmotic pressure and can help plants resist osmotic stress caused by various abiotic stresses [46,47]. Under low-temperature stress, plants can resist the damage caused by synthesizing osmotic regulatory substances to maintain their physiological state [48]. Pro is the main organic osmoregulatory substance of plants, and plants will actively synthesize and accumulate Pro to regulate osmotic potential and protect cell membrane homeostasis under low-temperature stress [49]. Soluble sugar is an important osmoregulatory substance in plant cells, which can increase the concentration of intracellular solute, reduce the freezing point of the cell solution, buffer the excessive dehydration of the cytoplasm, and protect the cytoplasmic colloid from freezing, thus reducing the damage to cells caused by low temperature [50]. Soluble protein is another important osmoregulatory substance in plant cells, which can increase the water retention capacity of cells and enhance the cold resistance of plants. Under the same low-temperature stress condition, the higher the content of soluble protein, the higher the cold resistance of plants [51]. In this study, it was found that low-temperature stress resulted in an increase in the contents of soluble sugar, soluble protein, and Pro in poncirus trifolata leaves, which echoes numerous research results [46–51]. The increase in the contents of soluble sugar and soluble protein may be due to the low-temperature stress, which leads to a reduction in cell respiration and consumption, and at the same time forces the hydrolysis of starch in leaves to increase the cytoplasmic concentration, promoting resistance to cold injury [50,51]. During this period, cells mainly enhance cold resistance by adjusting the contents of soluble sugar and soluble protein. In addition, in this study, under the same temperature condition, AMF significantly increased the contents of soluble sugar, soluble protein, and Pro in leaves of trifoliate orange seedlings compared with the non-AMF group. These results indicate that AMF inoculation can improve the cold resistance of plants by increasing the contents of soluble sugar, soluble protein, and Pro in plants.

ROS is commonly found in plants. Exposure to low-temperature stress for a long time will lead to the accumulation of excess ROS, and plants need some antioxidant enzymes to remove excess ROS [52]. Peroxidase, superoxide dismutase, catalase, and ascorbate catalase can help plants remove the increased ROS caused by abiotic stress [52]. Popov and Naraikina [53] showed that SOD and CAT are defensive enzymes related to plant cold resistance, and they can reduce the high concentration of ROS caused by low temperature, thus minimizing the damage to plants caused by excessive ROS content. This study found that SOD and CAT activities in leaves of trifoliate orange seedlings were enhanced under low-temperature stress. Under low-temperature stress, the increased activity of antioxidant enzymes helps to eliminate intracellular ROS accumulation caused by low temperature in trifoliate orange seedlings, thus protecting the cell membrane system of trifoliate orange seedlings. This is similar to Wang et al.'s [52] findings: the SOD and CAT activities of tobacco were significantly increased under different low-temperature stress conditions. However, Li et al. [54] found that SOD and CAT activities were gradually decreased in red pine seedlings under low-temperature stress. The different responses of antioxidant enzymes in different plant species to low-temperature stress may be due to their different tolerance mechanisms to stress, and may also be related to plant species and root environment. In this experiment, regardless of the temperature, the SOD and CAT enzyme activities in the leaves of trifoliate orange seedlings were increased by AMF inoculation compared with those without AMF inoculation. This is similar to the results of previous studies on cucumber [44] and maize [55]. Therefore, AMF can enhance plant

cold tolerance by increasing the activity of antioxidant enzymes and enhancing the level of antioxidant metabolism in plants.

5. Conclusions

In conclusion, AMF (*D.e*) inoculation can promote the growth of the aboveground and underground parts of trifoliate orange seedlings. Under low-temperature stress, AMF (*D.e*) can enhance plant cold resistance by improving root configuration and leaf nutrient status, enhancing photosynthesis, increasing osmoregulatory substance content, improving its antioxidant capacity, and reducing ROS levels. In the future, the key genes and metabolites that enable AMF to enhance the cold tolerance of citrus could be elucidated by transcriptomics and metabolomics. Thus, the mechanism by which AMF regulates the cold tolerance of citrus can be more completely explained.

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