BIOCELL
2022 46(8): 1959-1966

Tech Science Press

Mycorrhiza improves cold tolerance of Satsuma orange by inducing antioxidant enzyme gene expression

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Key words: Antioxidant defense system, Citrus, Cold stress, Mycorrhiza, Oxidative burst

Abstract: A potted experiment was carried out to study the effect of an arbuscular mycorrhizal fungus (*Diversispora versiformis*) and arbuscular mycorrhizal like fungus (*Piriformospora indica*) on antioxidant enzyme defense system of Satsuma orange (*Citrus sinensis* cv. Oita 4) grafted on *Poncirus trifoliata* under favourable temperature (25°C) and cold temperature (0°C) for 12 h. Such short-term treatment of cold temperature did not cause any significant change in root fungal colonization and spore density in soil. Under cold stress, *D. versiformis* inoculation did not change the activity of superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) in leaves and roots, whereas *P. indica* inoculation significantly increased the activity of CAT in roots and POD in leaves only. In addition, inoculation of two mycorrhizal fungi under cold stress significantly increased the relative expression levels of *PtPOD* and *PtF-SOD* in leaves, *P. indica* up-regulated the expression levels of *PtCu/Zn-SOD* in leaves, and *D. versiformis* also induced the expression levels of *PtMn-SOD* and *PtCAT1* in leaves. In addition, inoculated Oita 4 trees maintained significantly lower hydrogen peroxide levels and malondialdehyde contents in leaves and roots under cold temperature, suggesting lower oxidative damage. Therefore, we concluded that arbuscular mycorrhizal fungi (especially *P. indica*) mainly induced the expression of antioxidant enzyme genes, depending on the fungal species, and thus mitigated oxidative damage for higher cold resistance in inoculated plants.

Introduction

Cold stress is an environmental factor that inhibits plant growth and development, limits plant geographic distribution, and reduces crop yield (Liu *et al.*, 2019; Goharrizi *et al.*, 2021). Under cold stress conditions, reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2), superoxide anion radicals ($O_2^{\bullet-}$) and hydroxyl radicals, are excessively accumulated, due to the disruption of the electron transport chain (Wang *et al.*, 2020). Excessive production of ROS leads to oxidative damage in nucleic acids, proteins and lipids, disrupting cellular functions (Barrero-Sicilia *et al.*, 2017; Ding *et al.*, 2021). Plants also possess antioxidant defense systems to maintain the balance between the production and scavenging of ROS under stress conditions (Ermakov *et al.*, 2019; Araújo *et al.*, 2020). In the chloroplasts of plant cells, enzymes and non-enzymatic antioxidants are able to prevent oxidative damage. Among them, catalase (CAT), superoxide dismutase (SOD), and peroxidase (POD) can mitigate the deleterious effects of ROS on plant cells and protect them from oxidative damage (Caser *et al.*, 2016; Ghanbarzadeh *et al.*, 2019). SODs including Cu/Zn-SOD, Fe-SOD, Mn-SOD are a metalloenzyme that eliminates the conversion of $O_2^{\bullet-}$ into O_2 and H_2O_2 (Garg and Manchanda, 2009); CAT is a heme-containing tetrameric enzyme with the potential to break down H_2O_2 directly into H_2O and O_2; POD also eliminates excess H_2O_2 (He *et al.*, 2020b; Kong *et al.*, 2020). As a result, antioxidant enzymes play a crucial role in the scavenging of ROS in plants during cold stress.

Arbuscular mycorrhizal fungi (AMF) are beneficial soil microorganisms in plant rhizosphere, which can form symbiotic associations with roots of 80% of terrestrial plants (Wu *et al.*, 2013; He *et al.*, 2020a). AMF obtain carbohydrates from their hosts to sustain their growth needs, and in return, the fungi also provide host plants with a variety of benefits, including enhanced mineral nutrition and tolerance to abiotic and biotic stresses (Parniske, 2008; Cheng *et al.*, 2021a, 2021b). It has been shown that under drought conditions, AMF reduced the accumulation of ROS

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Received: 21 November 2021; Accepted: 06 January 2022

Doi: 10.32604/biocell.2022.020391

in host plants by enhancing antioxidant enzyme activities and associated gene expression (He *et al.*, 2020b; Cheng *et al.*, 2021b; Zou *et al.*, 2021). Similarly, Pasbani *et al.* (2020) inoculated eggplants (*Solanum melongena*) with AMF at three different temperatures, and found that AMF activated antioxidant defense systems, accumulated protective molecules, and reduced membrane damage. Ma *et al.* (2015) reported that AMF improved the uptake of phosphorus and alleviated low temperature stress. Chu *et al.* (2016) observed less oxidative damage in mycorrhizal *vs.* non-mycorrhizal phyllanthus (*Elymus nutans*) under cold stress conditions, which may be related to higher antioxidant enzyme activities. These studies have shown that AMF enhanced cold tolerance in plants, while the regulation of AMF on antioxidant enzyme activities and their gene expression in citrus under cold stress conditions is not clear, especially for short-term cold stress.

The purpose of this study was to explore the effects of AMF (*Diversispora versiformis*) and arbuscular mycorrhizal like fungus (*Piriformospora indica*) on antioxidant enzyme activities and relevant gene expression of Satsuma orange under cold stress, in order to evaluate the function of AMF on tolerating cold stress of the host. Such results will provide a theoretical basis for the application of AMF in tolerating cold stress of citrus trees.

Materials and Methods

Experimental design

The experiment was arranged in the completely randomized block design: (i) fungal treatments with *D. versiformis* (DV), *P. indica* (PI), and non-AMF; (2) temperature stress at 25°C (favourable temperature, FT) and 0°C (cold temperature, CT) for 12 h. A total of six treatments was achieved, and each treatment had four replicates, for a total of 24 pots.

Fungal inoculum

The tested strain of arbuscular mycorrhizal fungus, *D. versiformis* (P. Karst.) Oehl, G.A. Silva & Sieverd, was provided by the Institute of Root Biology, Yangtze University, and propagated by the host plant, white clover (*Trifolium repens* L.), for three months under potted conditions. The collected inoculum of *D. versiformis* included spores, hyphae, and colonized root segments. *P. indica* was provided by Prof. Zhihong Tian (Yangtze University, Jingzhou, China). The proliferation of PI was carried out by Yang *et al.* (2021). The spore suspension of PI was used as the inoculum, in which the concentration is 3.16×10^8 CFU/mL (OD_{600}).

Plant culture

The tested plant was *Citrus sinensis* cv. Oita 4, an early-ripening Satsuma mandarin selected from Oita (Japan), with trifoliate orange (*Poncirus trifoliata* L. Raf.) as the rootstock. It was budded in autumn 2018. The Satsuma mandarin trees were provided by the Institute of Fruit and Tea Research, Hubei Academy of Agricultural Sciences (Wuhan, China). Before transplanting, the mycorrhizal colonization rate of the trees was 26.7 ± 5.2%, based on the protocol of Phillips and Hayman (1970).

On May 17, 2019, we transplanted the Oita 4 trees with similar growth vigor into a 5-gallon plastic pot that was supplied with 11 kg of growth substrate. The potted soil was collected from a citrus orchard on the West Campus of Yangtze University (Jingzhou, China), removed impurities, and mixed with the substrate consisted of peat, vermiculite, and perlite (69:25:6, v/v/v) in the ratio of 2:1 (v/v) as the growth substrate. Inoculation of fungi was carried out at the time of transplantation of Oita 4. For the DV treatment, a total of 600 g mycorrhizal inoculums of *D. versiformis* was applied to a pot. The 1.0-L spore suspension of *P. indica* was applied into a pot as the PI treatment. The non-fungi treatment also received the same amount of autoclaved mycorrhizal inoculums as the control. After inoculated with the fungi, the treated Oita 4 trees were grown in a greenhouse without any heating equipment.

On 20 November 2020 at 6:00 am, inoculated and uninoculated Oita 4 trees were divided into two parts: half of the trees was exposed to 25°C for 12 h, and the other half was subjected to 0°C for 12 h, along with 900 μmol/m^2/s photo flux density and 68% relatively air humidity in an illumination incubator. Then, the experiment was ended.

Variable determinations

After 12 h of cold stress (0°C) and favourable temperature (25°C), we immediately collected some of fine roots from all the potted trees and gently shook off the soil attached to the roots as rhizosphere soil. The top 4–5 leaves of treated trees were collected, immediately frozen in liquid nitrogen, and stored at –80°C for measurement of later physiological and molecular indicators.

Root fungal colonization was stained as per the protocol described by Phillips and Hayman (1970). The degree of root fungal colonization was calculated as the percentage of fungi-colonized root segment number per observed total root segment number. Spore number in soil was assayed by Wang *et al.* (2016).

Malondialdehyde (MDA) concentration in leaves was measured according to the method described by Sudhakar *et al.* (2001), based on the reaction with thiobarbituric acid. H_2O_2 concentration was determined by the method of Velikova *et al.* (2000), accompanied with the KI reaction. SOD activity was determined by the colorimetry described by Wu (2018). POD activity was assayed by the method of He *et al.* (2020b). CAT activity was determined by the colorimetry (240 nm) of He *et al.* (2020b). Soluble protein concentration was analyzed as per the coomassie blue G-250 method outlined by Wu (2018).

qRT-PCR analysis

Frozen leaf samples were ground in liquid nitrogen. According to the manufacturer's protocol, total RNA was extracted from powdered leaf tissue (50 mg) using RN38 EasySpin plus plant RNA Kit (Aidlab, Beijing, China). RNA integrity was examined in 1% agarose gel, and quantitative analysis was performed by spectrophotometric analysis using Bio Photometer Plus 6132 (Eppendorf, Hamburg, Germany). RNA purity was estimated by calculating the A_{260}/A_{280} ratio. The RNA was reversely transcribed into the first strand cDNA using the transcript first strand cDNA synthesis kit with gDNA Eraser (PC5402, Aidlab, Beijing, China). Five antioxidant enzyme genes (*PtFe-SOD*, *PtMn-SOD*, *PtCu/Zn-SOD*,

PtCAT1, and *PtPOD*) were identified from the genome-wide data of *Citrus sinensis* (http://citrus.hzau.edu.cn/) and the data obtained from our early transcriptome data. The Prime Premier 5 was used to design the primer sequence (Table 1). The qRT-PCR reaction contained 10 µL 2 × AceQ qPCR SYBR Green Master Mix kit, 0.4 µL of each primer, 2 µL diluted cDNA template, and 7.2 µL ddH_2O. qRT-PCR reaction was carried out using CFX96 real-time PCR detection system (Biorad Laboratories, Hercules, CA, USA). The amplification procedure was as follows: initial denaturation at 95°C for 5 min; 40 cycles at 95°C for 10 s, 60°C for 30 s and 95°C for 15 s; 60°C for 60 s; and 95°C for 15 s. The experiment was repeated three times for each gene. Relative quantitative of genes was calculated by $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The β-actin gene was selected as an internal reference gene. Measured transcripts were normalized to the values of non-AMF plants under FT conditions.

Statistical analysis

Data were statistically analyzed by analysis of variance (ANOVA) in SAS (8.1v), and the significance between treatments was compared by the Duncan's multi-range test at the level of 0.05.

Results and Discussion

Effects of cold stress on mycorrhizal development of roots and soil

In this study, un-inoculated Oita 4 trees with AMF had a small amount of root mycorrhizal colonization and soil spores, while DV and PI inoculation significantly promoted the root fungal colonization and spore density in soil (Fig. 1; Table 2). Under FT conditions, compared with non-inoculated treatment, inoculation with DV and PI significantly increased root fungal colonization degree by 39% and 74% and spore density in soil by 319% and 2000%, respectively (Table 2). However, under CT conditions, mycorrhizal colonization distinctly increased respectively by 34% and 75% under DV and PI inoculation *versus* un-inoculated treatment, and spore density in soil considerably increased by 434% and 2503%, respectively (Table 2). This suggests that PI recorded better colonization in roots and soil than DV. Cold stress did not alter root mycorrhizal colonization degree and spore density in soil in DV- and PI-inoculated trees, which is inconsistent with the results of Pasbani *et al.* (2020) that cold temperature conditions reduced AMF colonization by the difference in the duration of cold temperature treatment. Wu (2011) also reported dramatical reduction of root mycorrhizal colonization by *Glomus mosseae* in trifoliate orange exposed to 15°C for 2 months, relative to 25°C. It concluded that short-term cold temperature treatment (only 12 h) had no significant effect on mycorrhizal formation in the rhizosphere.

Effects of fungal inoculation on H_2O_2 concentrations under cold stress

Reactive oxygen species (ROS) are by-products of metabolism, and their production is increased under stress conditions through disruption of the electron transport system and oxidative metabolic activities occurring in chloroplasts, mitochondria and microsomes (Adriano *et al.*, 2015). H_2O_2 is an important and relatively stable ROS, accumulated excessively under cold temperature (Pinheiro and Chaves, 2011; Adriano *et al.*, 2015). In the present study, cold temperature caused a rapid accumulation of H_2O_2 in leaves and roots, independent of inoculated status (Fig. 2), which is in agreement with the findings of Wang *et al.* (2020) in tomato. Compared to FT-AMF treatment, H_2O_2 content was reduced by 51.2% and 16.7% in roots of FT+DV and FT+PI, and by 19.5% and 17.1% in leaves (Fig. 2), respectively; compared to CT-AMF treatment, H_2O_2 content was decreased in roots by 38.9% and 41.7% in CT+DV and CT+PI, and by 25.1% and 31.5% in leaves, respectively. The result implies that AMF reduced the accumulation of H_2O_2 in roots under cold stress, thus alleviating the damage of cold stress on citrus roots. This may be due to the fact that AMF promoted the activity of antioxidant enzymes to scavenge more ROS, thus, reducing the oxidative damage of cold stress on plant cell membrane (Zou *et al.*, 2021). In addition, the formation of mycorrhizas promoted uptake of

TABLE 1

Gene-specific primer sequences used in the present study

Genes	Gene ID	Primer sequence (5' → 3')
PtFe-SOD	Cs7g19250	F: AGTAAGGAGCGGCGAGTA
		R: GTGGCTAATGCGGTGAAT
PtMn-SOD	Cs7g29850	F: GGCGAGCCACCACATAGT
		R: CACCCTCAGCATTCATCTTTT
PtCu/Zn-SOD	Cs3g12080	F: GGACCAGCATGGACTACAAGACC
		R: GGATGCCGGTGGAAGTGTTACC
PtPOD	Cs1g18600	F: GGCTCAACTTGTCCACCTC
		R: TATCGTCGCCCTGTCTG
PtCAT1	Cs3g27280	F: TAACAGTGGAGGAGCGAACA
		R: GGAGCCAGTGCTAAGGGT
β-Actin	Cs1g05000	F: CGACCGTATGAGCAAGGAAA
		R: TTCCTGTGGACAATGGATGGA

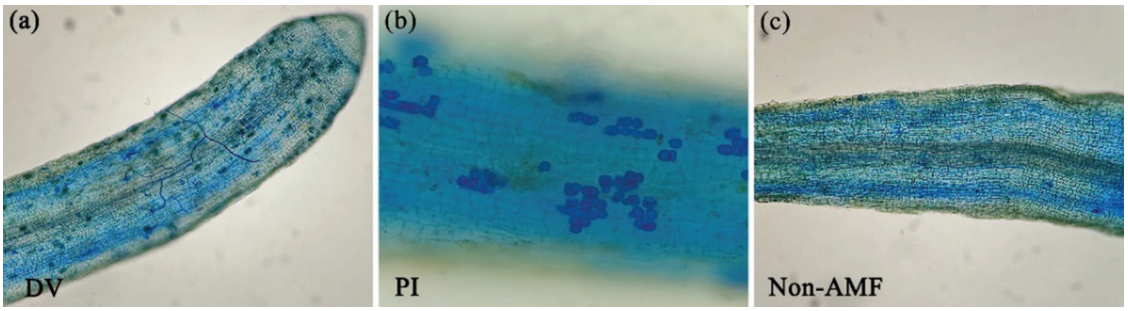

FIGURE 1. Root fungal colonization of *Citrus sinensis* cv. Oita 4 inoculated with *Diversispora versiformis* (a), *Piriformospora indica* (b), and non-AMF (c), respectively. Abbreviation: DV, *Diversispora versiformis*; non-AMF, non-arbuscular mycorrhizal fungi; PI, *Piriformospora indica*.

TABLE 2

Effects of AMF inoculation on root mycorrhizal colonization and spore density of potted Satsuma mandarin grafted on trifoliate orange under favourable temperature (25°C) and cold temperature (0°C)

Treatments	Mycorrhizal colonization (%)	Spore density (No./g soil)
CT+DV	58 ± 5b	2.83 ± 0.22b
CT+PI	100 ± 0a	13.8 ± 1.26a
CT-AMF	25 ± 3c	0.53 ± 0.10c
FT+DV	65 ± 13b	2.93 ± 0.15b
FT+PI	100 ± 0a	14.70 ± 1.16a
FT-AMF	26 ± 5c	0.70 ± 0.08c

Note: Means ± SD (n = 4) followed by different letters at the bar indicate significant differences (p < 0.05) among treatments. Abbreviation: AMF, arbuscular mycorrhizal fungi; CT, cold temperature; DV, *Diversispora versiformis*; FT, favourable temperature; PI, *Piriformospora indica*.

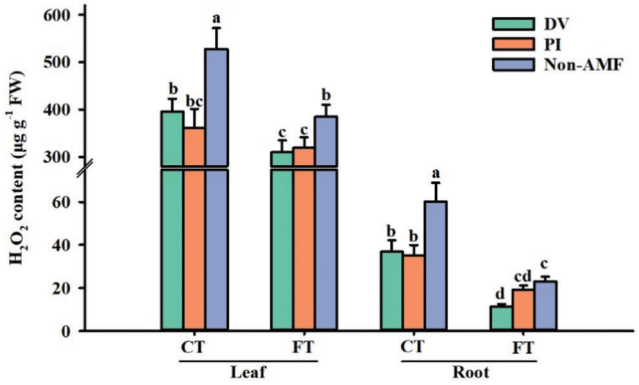

FIGURE 2. The effect of *Diversispora versiformis* and *Piriformospora indica* on H_2O_2 content of leaf and root of Satsuma mandarin under favourable temperature (25°C) and cold temperature (0°C). Data (means ± SD, n = 4) are significantly different (p < 0.05) if followed by different letters above the bars. Abbreviation: CT, cold temperature; DV, *Diversispora versiformis*; FT, favourable temperature; Non-AMF, non-arbuscular mycorrhizal fungi; PI, *Piriformospora indica*.

mineral nutrients (e.g., phosphorus) in host plants, which also facilitated resistance to cold stress (Ma *et al.*, 2015)

Effects of fungal inoculation on MDA concentrations under cold stress

Excessive production of ROS under cold stress can lead to severe oxidative damage in plants, resulting in membrane lipid peroxidation, which is manifested by elevated MDA content (Chu *et al.*, 2016; Rezaie *et al.*, 2020). In the present study, cold stress caused an increase in leaf MDA content of inoculated and uninoculated plants, implying that the 12-h cold temperature treatment had caused oxidative damage in plants with disrupted cell membranes, which is consistent with the findings of Wang *et al.* (2020). Compared to FT-AMF, the MDA content of leaves was reduced by 40.0% and 56.4% in roots of FT+DV and FT+PI, and no significant difference in leaves, respectively (Fig. 3); compared to CT-AMF, the MDA content was significantly reduced by 38.3% and 55.4% in roots of CT+DV and CT+YD, respectively, and by 42.5% and 23.3% in leaves (Fig. 3), which may be related to the reduction of H_2O_2 content and the enhancement of antioxidant enzyme activities by fungal inoculation. In addition, Wu *et al.* (2019) also reported higher unsaturation index of fatty acids of trifoliate orange seedlings after inoculated with AMF under soil water deficit conditions, which is another reason for maintaining a low membrane lipid peroxidation.

Effects of fungal inoculation on SOD activity under cold stress

SOD can catalyze $O_2^{\bullet-}$ into H_2O_2 (Garg and Manchanda, 2009). Chu *et al.* (2016) treated *Elymus nutans* with 5°C for 5 days and found that low temperature enhanced SOD activity, but there was no significant difference between AMF-inoculated and uninoculated plants. Nevertheless, Pasbani *et al.* (2020) reported the increase of SOD activity in *Solanum melongena* at 5°C for 1 h after AMF inoculation.

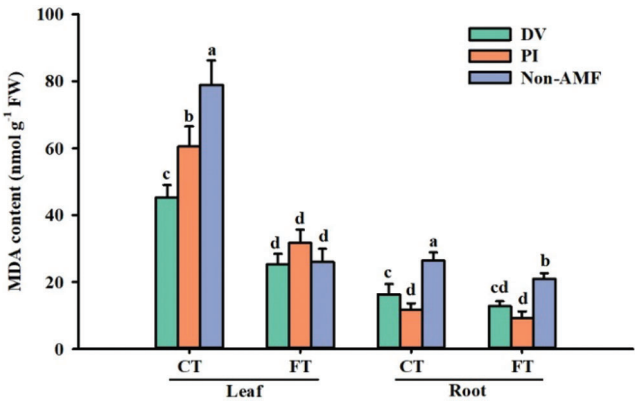

FIGURE 3. The effect of *Diversispora versiformis* and *Piriformospora indica* on MDA content of leaf and root of Satsuma mandarin under favourable temperature (25°C) and cold temperature (0°C). Data (means ± SD, n = 4) are significantly different (p < 0.05) if followed by different letters above the bars. Abbreviation: CT, cold temperature; DV, *Diversispora versiformis*; FT, favourable temperature; Non-AMF, non-arbuscular mycorrhizal fungi; PI, *Piriformospora indica*.

This implies that SOD activity may be influenced by the duration of stress as well as the host plant and mycorrhizal species. In our study, cold stress only significantly induced the increase of SOD activity in leaves and roots of PI-inoculated plants, but had no effect on SOD activity in uninoculated and DV-inoculated plants. Under FT conditions, only DV inoculation significantly increased SOD activity in leaves; under CT conditions, both mycorrhizal fungi treatments did not significantly change SOD activity in leaves and roots. It suggested that the induction of SOD activity by mycorrhizas depended on the fungal species. It seems that SODs were hardly induced by mycorrhizal fungi under cold stress. However, the expression of *SODs* isoenzyme gene revealed that the expression of these enzyme genes preceded the enzyme activity in response to temperature treatment (Fig. 8), suggesting that the expression of antioxidant enzyme genes could be used as a good indicator of resistance to cold stress.

Effects of fungal inoculation on CAT activity under cold stress

CAT is a tetrametric heme enzyme that catalyzes the breakdown of H_2O_2 to H_2O and O_2, which is required for ROS detoxification during stress (Willekens *et al.*, 1997). Our results showed that cold stress inhibited CAT activity in roots and leaves of Oita 4, which is consistent with the findings of Chen *et al.* (2014). Both DV and PI inoculations induced an increase in CAT activity in leaves and roots under FT conditions, whereas cold treatment only significantly induced an increase in CAT activity in leaves of PI-inoculated plants and a decrease in CAT activity in leaves and roots of uninoculated and DV-inoculated plants, as well as in roots of PI-inoculated plants. Compared to FT-AMF treatment, leaf CAT activity increased by 29.6% and 10.2% for FT+DV and FT+PI, respectively, and root CAT activity increased by 27.1% and 102.4% for FT+DV and FT+PI, respectively (Fig. 5). Zhu *et al.* (2010) found that cold treatment at 5°C induced a decrease in CAT activity in maize roots and leaves, which is consistent with the results of our study, while they also found that the inoculation treatment was effective in promoting CAT activity in roots and leaves compared to the non-inoculation treatment. Higher CAT activity of mycorrhizal plants exposed to cold stress would eliminate more H_2O_2, which thus causes low oxidative damage.

Effects of fungal inoculation on POD activity under cold stress

POD is an oxidoreductase enzyme commonly found in most plant tissues that can catalyze oxidative reactions using H_2O_2 as an electron acceptor (Garg and Manchanda, 2009). The results of this study showed that cold stress significantly promoted root POD activity, but inhibited leaf POD activity. In addition, compared to FT-AMF treatment, root POD activity of plants with FT+DV and FT+PI increased by 12.5% and 33%; under CT conditions, root POD activity increased by 18.2% and 52.3% in DV and PI inoculation, respectively, compared to non-AMF treatment (Fig. 6),

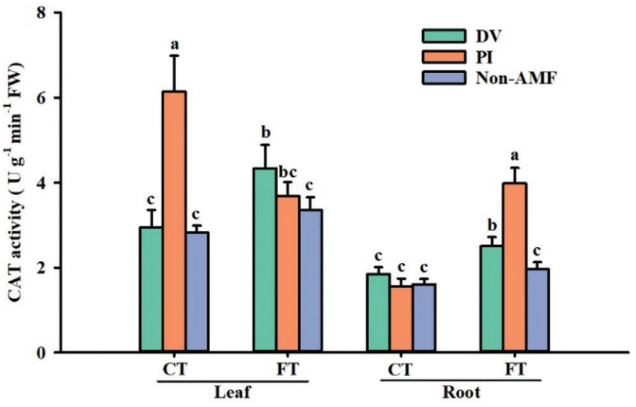

FIGURE 5. The effect of inoculation with *Diversispora versiformis* and *Piriformospora indica* on CAT activity of leaf and root of Satsuma mandarin under favourable temperature (25°C) and cold temperature (0°C). Data (means ± SD, n = 4) are significantly different ($p < 0.05$) if followed by different letters above the bars. Abbreviation: CT, cold temperature; DV, *Diversispora versiformis*; FT, favourable temperature; Non-AMF, non-arbuscular mycorrhizal fungi; PI, *Piriformospora indica*.

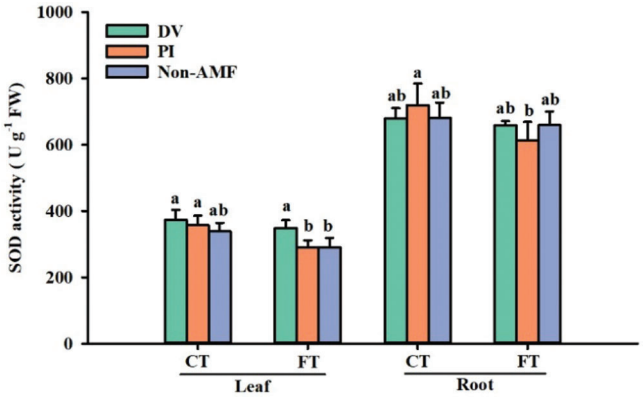

FIGURE 4. The effect of inoculation with *Diversispora versiformis* and *Piriformospora indica* on SOD activity of leaf and root of Satsuma mandarin under favourable temperature (25°C) and cold temperature (0°C). Data (means ± SD, n = 4) are significantly different ($p < 0.05$) if followed by different letters above the bars. Abbreviation: CT, cold temperature; DV, *Diversispora versiformis*; FT, favourable temperature; Non-AMF, non-arbuscular mycorrhizal fungi; PI, *Piriformospora indica*.

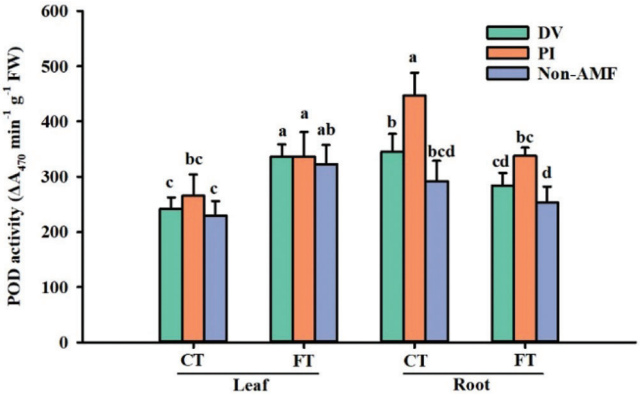

FIGURE 6. The effect of inoculation with *Diversispora versiformis* and *Piriformospora indica* on POD activity of leaf and root of Satsuma mandarin under favourable temperature (25°C) and cold temperature (0°C). Data (means ± SD, n = 4) are significantly different ($p < 0.05$) if followed by different letters above the bars. Abbreviation: CT, cold temperature; DV, *Diversispora versiformis*; FT, favourable temperature; Non-AMF, non-arbuscular mycorrhizal fungi; PI, *Piriformospora indica*.

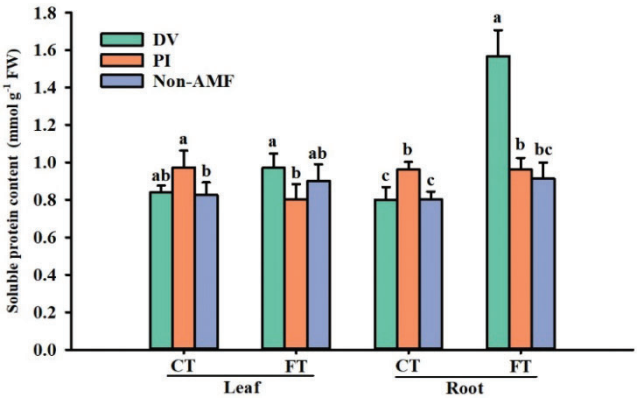

FIGURE 7. The effect of inoculation with *Diversispora versiformis* and *Piriformospora indica* on soluble protein content of leaf and root of Satsuma mandarin under favourable temperature (25°C) and cold temperature (0°C). Data (means ± SD, n = 4) are significantly different (p < 0.05) if followed by different letters above the bars. Abbreviation: CT, cold temperature; DV, *Diversispora versiformis*; FT, favourable temperature; Non-AMF, non-arbuscular mycorrhizal fungi; PI, *Piriformospora indica*.

implying that PI enhanced root POD activity under cold stress. This is in agreement with the results of Zhu *et al.* (2010), who reported that leaf POD activity was similar between inoculated and non-inoculated leaves under all temperature treatments, while root POD activity of mycorrhizal plants was higher than that of non-mycorrhizal plants. As a result, PI inoculation caused the increase of POD activity in roots under FT and CT conditions, indicating superior functioning of PI in eliminating ROS (e.g., H_2O_2) than DV.

Effects of fungal inoculation on total soluble protein concentration under cold stress

Genes induced under stress conditions are thought to protect cells from cold stress by producing several gene products, such as aquaporin proteins, protective proteins, and detoxification proteins (Shinozaki and Yamaguchishinozaki, 1996). The present study showed that cold stress induced an increase in total soluble protein in leaves and roots of PI-inoculated and uninoculated plants (Fig. 7). Compared with LT-AMF treatment, LT+PI treatment significantly increased total soluble protein in leaves and roots of citrus by 17.8% and 20.2%, respectively. In addition, DV inoculation significantly increased root soluble protein content under FT conditions by 71.6%, compared with non-AMF. Hence, DV accelerated soluble protein production in leaves and roots of citrus under cold stress conditions, indicating important roles in ROS detoxification.

Effects of fungal inoculation on relative expression levels of antioxidant enzyme genes in leaves under cold stress

Ji *et al.* (2017) found that genes involved in biosynthesis of unsaturated fatty acids, pathogen defense and phenylalanine metabolism were up-regulated at cold temperature, while genes encoding antioxidant enzymes were down-regulated for enhancing the transcription level of functional genes in resistance to cold damage. In the present study, we analyzed the relative expression level of five antioxidant enzyme genes in response to fungal inoculation and cold stress (Fig. 8). These antioxidant enzyme gene expression was down-regulated by cold stress, which is in agreement with Li *et al.* (2018). Compared with CT-AMF treatment, *PtCu/Zn-SOD* gene expression in leaves was up-regulated by 1.46 times and 7.90 times in CT+DV and CT+PI, respectively. The expression level of *PtMn-SOD* gene in leaves was up-regulated by 1.40 times and 1.05 times in CT+DV and CT+PI, respectively, and the expression level of *PtFe-SOD* gene in leaves was up-regulated by 5.88 times and 3.99 times in CT+DV and CT+PI, respectively, *PtPOD* gene expression was up-regulated by 8.67 times and 9.83 times respectively in CT+DV and CT+PI, and *PtCAT1* gene expression was up-regulated by 3.33 and 2.05 times in CT+DV and CT+PI, respectively (Fig. 8). Nevertheless, under CT conditions, SOD and POD activity was not significantly influenced by DV and PI inoculation, compared with non-AMF inoculation. mRNA and protein expression of antioxidant enzymes were not directly related to antioxidant enzyme activity (He *et al.*, 2020b). The

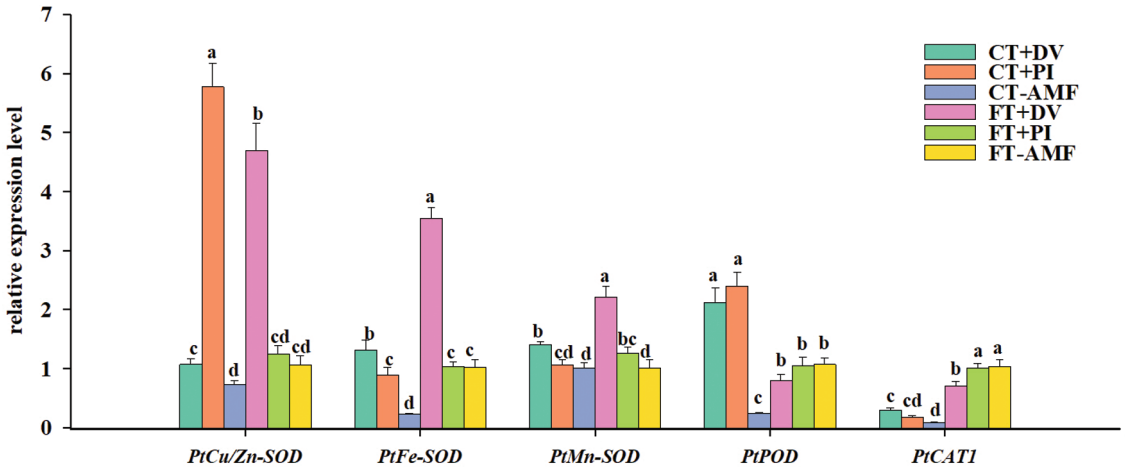

FIGURE 8. Effect of inoculation with *Diversispora versiformis* and *Piriformospora indica* on the relative expression level of leaf *PtSODs*, *PtPOD*, and *PtCAT1* genes in Satsuma mandarin under favourable temperature (25°C) and cold temperature (0°C). Data (means ± SD, n = 3) are significantly different (p < 0.05) if followed by different letters above the bars. Abbreviation: AMF, arbuscular mycorrhizal fungi; CT, cold temperature; DV, *Diversispora versiformis*; FT, favourable temperature; PI, *Piriformospora indica*.

transformation of mRNA into protein is regulated by microRNA, which is affected by ubiquitination and phosphorylation. Therefore, further research on the phosphorylation of antioxidant enzyme protein genes will be conducted. In addition, DV application also significantly up-regulated *PtCu/Zn-SOD*, *PtFe-SOD* and *PtMn-SOD* gene expressions in leaves under FT conditions, with 4.41-fold, 3,45-fold and 2.19-fold, respectively, compared with non-AMF treatment, suggesting that DV promotes the expression of *SOD* genes under FT conditions. Such induced expression of *SODs* in inoculated Oita 4 trees would remove more $O_2^{\bullet-}$, thus maintaining relatively low ROS levels in inoculated plants.

Our results suggested that AMF induced the expression of antioxidase enzyme genes in citrus leaves at cold temperature, thus, mitigating oxidative burst of ROS. This means that AMF improves the cold tolerance of citrus by regulating the gene expression of *PtCu/Zn-SOD*, *PtMn-SOD*, *PtFe-SOD*, *PtPOD* and *PtCAT1*. ROS in root cells of the host plant is suddenly and temporarily increased during the initial stage of AMF colonization, and is reduced by increased antioxidant enzyme activity and carotenoid content (Zou *et al*., 2021), which may cause reprogramming involving transcriptomes, proteomes, and metabolites to establish a new metabolic homeostasis (Fürtauer *et al*., 2019; He *et al*., 2020b; Liang *et al*., 2021), thus improving the tolerance of abiotic stresses in plants.

Conclusions

In our study, short-term cold treatment (e.g., at 0°C for 12 h) did not cause any changes in mycorrhizal colonization and spore density in soil. However, mycorrhizal fungi inoculation significantly reduced the content of H_2O_2 and MDA in leaves and roots under CT conditions, and also significantly promoted the relative expression of *PtCu/Zn-SOD*, *PtFe-SOD* and *PtMn-SOD* in leaves under FT and CT conditions, coupled with the induced expression of *PtPOD* and *PtCAT1* in leaves under CT conditions. Mycorrhizal fungal colonization almostly did not influence antioxiadnt enzyme activity in leaves and roots under cold stress, with the exception of increased CAT activity in roots and POD activity in leaves by PI inoculation, suggesting that antioxidant enzyme genes early responded to cold stress under inoculated *versus* un-inoculated conditions. This response pattern resulted in less oxidative damage to the inoculated Oita 4 trees, and thus showed higher resistance to cold stress. Among them, PI had stronger cold tolerance than DV, which is expected to be used in field, because PI can be cultured *in vitro*.

Acknowledgement: The authors would like to extend their sincere appreciation to the Researchers Supporting Project No. (RSP-2021/134), King Saud University, Riyadh, Saudi Arabia.

Availability of Data and Material: All data generated or analyzed in this study are included in this published article.

Authors' Contribution: The authors confirm contribution of the paper as follows: study conception and design: Qiang-Sheng Wu; data collection: Ming-Ao Cao and Fei Zhang; analysis and interpretation of results: Ming-Ao Cao and Qiang-Sheng Wu; draft manuscript: Ming-Ao Cao, Elsayed Fathi Abd_Allah and Qiang-Sheng Wu. All authors reviewed the results and approved the final version of manuscript.

Ethics Approval: Not applicable.

Funding Statement: This study was supported by the Plan in Scientific and Technological Innovation Team of Outstanding Young Scientists, Hubei Provincial Department of Education (T201604) and the Hubei Agricultural Science and Technology Innovation Action Project (Hubei Nongfa [2018] No. 1). The authors would like to extend their sincere appreciation to the Researchers Supporting Project Number (RSP-2021/134), King Saud University, Riyadh, Saudi Arabia.

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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