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Improving the Antioxidant Defense System in Different Species of Citrus Fruits under Low-temperature Stress using Osmolytes

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ABSTRACT

Citrus fruits are among the most significant horticultural crops globally. Low-temperature stress, one of the most critical environmental stressors, disrupts physiological processes in plants, leading to yield reduction or plant mortality. This study examined the interaction effects of the amino acid proline at concentrations of 0, 15, and 20 mM and putrescine at concentrations of 0, 5, and 10 mM under temperatures of 1, -1, and -3 °C to evaluate changes in antioxidant systems in the fruits of three Citrus species. Antioxidant activity was assessed using the reducing power (RP) method and the ABTS free radical scavenging assay. Additionally, phenolic compounds were analyzed through high-performance liquid chromatography (HPLC). Results showed that low temperatures, as well as the application of proline and putrescine, enhanced antioxidant activity. HPLC analysis revealed that phenolic compound levels, except for naringin, increased with decreasing temperatures. Exogenous application of proline and putrescine significantly increased the contents of phenolic acids, including chlorogenic, gallic, p-coumaric, and ferulic acids, as well as flavonoids such as quercetin and rutin. However, the levels of tannic and salicylic acids were negligible. Among the studied species, the highest antioxidant capacity was observed in *C. reticulata*, while *C. paradisi* exhibited the lowest levels, suggesting that *C. reticulata* possesses greater resistance to low-temperature stress. The highest antioxidant levels were recorded in fruits treated with 20 mM proline. These findings indicate that the application of proline and putrescine under low-temperature stress enhances the resistance of citrus species by upregulating antioxidant activity and increasing the accumulation of phenolic compounds and flavonoids.

Introduction

Plant growth is adversely impacted by various abiotic environmental stresses, including cold, salinity, drought, and fluctuations in incident light. Low temperatures can cause severe injury or death in crop species, significantly affecting their productivity, survival, and ecological distribution (Rezaie et al., 2020; Adhikari et al., 2022). Citrus fruits are particularly sensitive to low-temperature damage, especially during the ripening stage, where they can only tolerate a temperature drop from 2 to −3 °C for 2–3 h. However, the extent of cold and freezing damage varies depending on species, growth conditions, and plant age (Zabihi et al., 2016).

Cold stress often increases cellular damage due to elevated production of reactive oxygen species (ROS). Therefore, resistance to cold stress is partially associated with the enhancement of

antioxidative defense systems, which include antioxidant compounds such as phenolics and flavonoids, as well as various antioxidative enzymes (Rezaie et al., 2020). Plants respond and adapt to low-temperature stress through mechanisms such as adjusting cellular metabolism and activating defensive pathways, which can serve as strategies for protection against cold or for selecting stress-tolerant cultivars (Luo et al., 2011).
Low temperatures lead to the generation of ROS, causing significant anatomical damage to plants (Garbero et al., 2012). ROS can attack proteins, lipids, and nucleic acids, with the extent of damage determined by the balance between ROS production and removal by antioxidative scavenging systems—an essential trait for stress tolerance (Scheibe and Beck, 2011). Numerous studies have highlighted the role of the antioxidant defense system in responding to suboptimal temperatures (Rahnama and Ebrahimzadeh, 2005). This system includes mechanisms that interact with active oxygen species to maintain their levels, as well as processes that regenerate oxidized antioxidants (Smirnoff, 1993).

Antioxidant systems are generally classified into three categories: (1) lipid-soluble, membrane-associated antioxidants, (2) water-soluble reductants, and (3) enzymatic antioxidants, including superoxide dismutase, catalase, ascorbate peroxidase, and guaiacol peroxidase. Research has shown that low-temperature stress can alter the composition of phenolic compounds in various plant tissues, as demonstrated in pea (Rudikovskaya et al., 2008), rapeseed (Solecka and Kacperska, 2003; Stefanowska et al., 2002), maize (Christie et al., 1994), soybean (Janas et al., 2000), winter wheat (Olenichenko et al., 2006), elderberry (Thomas et al., 2008), celeriac (*Ammopiptanthus mongolicus*) (Liu et al., 2007), and smoke tree (*Cotinus coggygria*) (Oren-Shamir and Levi-Nissin, 1997).

The naturally occurring amino acid proline functions as a compatible osmolyte, accumulating in the cell cytoplasm to protect the structure and function of proteins and enzymes under stress conditions. Proline levels increase during stress, acting as an osmotic regulator to prevent water loss (Demiral and Turkan, 2005; Fedotova and Dmitrieva, 2016). Its accumulation under stress conditions surpasses that of other amino acids, aiding osmotic regulation, preserving cell membrane phospholipids, and neutralizing hydroxyl radicals (Gad, 2005). The application of proline contributes to the elimination of reactive oxygen species and the stabilization of cellular membranes. As a source of nitrogen and carbon, proline enhances plant growth under cold stress conditions (Posmyk and Janas, 2007). Proline functions as a cellular osmotic regulator, maintaining enzyme stability and preventing denaturation. It interacts with membrane systems, regulates cytosolic pH, balances the $NADH/NAD^+$ ratio, serves as an energy source, and aids in the detoxification of ROS (Konstantinova et al., 2002; Demiral and Turkan, 2005).
Polyamines are small, ubiquitous nitrogenous compounds that play an adaptive role in plants under stress by regulating the cellular ionic environment, maintaining membrane integrity, preventing chlorophyll loss, and stimulating the synthesis of proteins, nucleic acids, and protective alkaloids (Kusano et al., 2008). Among their physiological effects, polyamines—such as putrescine—help stabilize membranes and reduce water stress in various cell types (Goyal and Asthir, 2010). Polyamines also scavenge free radicals and offer some protection against oxidative damage to membranes (Besford et al., 1993). Exogenous application of polyamines has been shown to increase plant tolerance to several abiotic stresses (Nayyar and Chander, 2004).

The three major polyamines—putrescine, spermidine, and spermine—are essential for cell survival and growth due to their molecular interactions with nucleic acids during transcription and translation, as well as with cellular membranes. These polyamines also serve as precursors for gamma-aminobutyric acid, which plays a crucial role in various cellular functions in plants. Their interactions with polyanionic macromolecules, hydroxycinnamic acids, fatty acids, or alkaloids contribute to their roles in abiotic and biotic stress responses (Majumdar et al., 2016). This study aimed to investigate the effects of temperature, proline, and putrescine on the antioxidant activity of three citrus species: *C. reticulata*, *C. sinensis* var. *Valencia*, and *C. paradisi* var. 'Redblush'.

Material and methods

Plant materials

The samples of three speices of *Citrus* (*C. reticulata, C. sinensis* var valencia, and *C. paradisi* var. redblush) were collected from a commercial garden located in Jiroft city (Kerman province) in the geographical location of 28° 40′ 13″ North and 57° 44′ 13″ East. The tested trees, grafted on *Citrus aurantium* rootstocks, were 10 years old and distributed in identical environmental and

growth conditions. The experimental design was a completely randomized factorial with three replications. Branches containing fruits of the desired trees were treated with proline at concentrations of 0, 15, 20mM and putrescine at concentrations of 0, 5, and 10 Mm (Koc et al., 2016). The branches which were approximately identical in length and the number of fruits were selected from trees of similar age. After 24 h of spraying, the branches treated with putrescine and proline were exposed to temperatures 1, -1, and -3 °C for 3 h (Gill and Tuteja, 2010; Zabihi et al., 2016). The fruits were harvested at the stage of physiological maturity. After harvesting, the samples were transferred to the laboratory in Shahid Bahonar University of Kerman.

Antioxidant activity

Determination of antioxidant activity using the reducing power (RP) method

A method used by Amira et al. (2012) was used for estimating the capacity of sample extracts to reduce Fe^{3+}. Methanolic solutions (0.5 g of fruits was homogenized in 1 mL of methanol 80%) were mixed with 250 µL of sodium phosphate buffer (0.2 M, pH 6.6) and 250 µL of 1% K_3Fe (CN), and then incubated at 50 °C. The mixture was centrifuged after adding 250 µL of 10% trichloroacetic acid. Then, the supernatants were withdrawn and quickly mixed with 100 µL of methanol and 25 µL of 0.1% ferric chloride. After incubation for 10 min, the absorbance was determined at 700 nm. The absorbance of samples was compared to ascorbic acid as a standard and the results were represented in terms of ascorbic acid equivalents.

Determination of Antioxidant Activity Using the ABTS Free Radical Scavenging Method

The antioxidant activity of the plant extracts against ABTS ((2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)) was determined according to Stratil et al. (2006). Radical $ABTS^{\bullet+}$ was prepared through oxidation of ABTS by potassium persulfate. A mixture (1:1; v/v) of ABTS (7 mM) and potassium persulfate (4.95 mM) was prepared and kept in the dark for 16 h at room temperature. Aliquots of 0.1 mL of methanolic extract (0.5 g of fruits was homogenized in 1 mL of methanol 80%) of each sample was added to 3.9 mL of the $ABTS^{\bullet+}$ dilution. The absorbance decrease was measured at 734 nm in a UV-30 spectrophotometer. The blank was prepared with $ABTS^{\bullet+}$. The results were expressed in mg equivalents of quercetin mg^{-1} of fresh weight.

Chromatographic analysis of phenolic compounds by high-performance liquid chromatography

About 0.5 g of fruits was homogenized in 1 mL of methanol 80%. The resultant mixture was centrifuged at 12,000 g for 10 min at room temperature and the supernatant was used for high-performance liquid chromatography (HPLC) analysis (S2100/Saykam). The mobile phase contained 1% aquatic acetic acid solution (solvent A) and acetonitrile (solvent B); the flow rate was adjusted to 0.7 mL min^{-1}. The samples were eluted by the following gradient: 90% A and 10% B as initial conditions, 60% A and 40% B for 28 min, 40% A and 60% B for 32 min, finally, 10% A and 90% B for 45 min. HPLC chromatograms were detected using a UV\VIS detector at a wavelength of 272 nm according to absorption maxima of analyzed compounds. The column was a C18 column and injection volume was 20 µL (Seal et al., 2016). The stock solution of concentration 1 mg mL^{-1} was prepared by dissolving 1 mg of phenolic acids and the flavonoids in 0.5 mL HPLC grade methanol. The resulting volume was made up to 1 mL with the solvent for the mobile phase (acetonitrile and 1% aq. acetic acid). The standard and sample solutions were filtered through 0.45 µm polyvinylidene fluoride (PVDF)-syringe filter and the mobile phase was degassed before the injection of the solutions.

Statistical analysis

The experimental design was a completely randomized factorial with three replications. Data were analyzed by analysis of variance (ANOVA) and the means were compared ($P \leq .05$) by Duncan's multiple range test (DMRT). All analyses were performed using a version of the software SAS (SAS Institute, Cary, NC, USA).

Results

The results of this research demonstrated that low-temperature stress in the three citrus species led to an increase in antioxidant levels. The exogenous application of putrescine and proline further enhanced antioxidant capacity compared to the control. Among the citrus species studied, *C. reticulata* exhibited the highest antioxidant capacity, while *C. paradisi* showed the lowest, both in treated and untreated fruits (Fig. 1).

The results of the antioxidant activity analysis using the reducing power (RP) method indicated that exposure to different temperatures increased antioxidant capacity in Citrus species. Among the tested conditions, the highest

antioxidant capacity was observed in fruits exposed to -3 °C. Furthermore, the application of proline and putrescine significantly enhanced antioxidant activity (Fig. 2). Notably, the highest levels of antioxidants in citrus species were recorded in fruits treated with 20 mM proline.

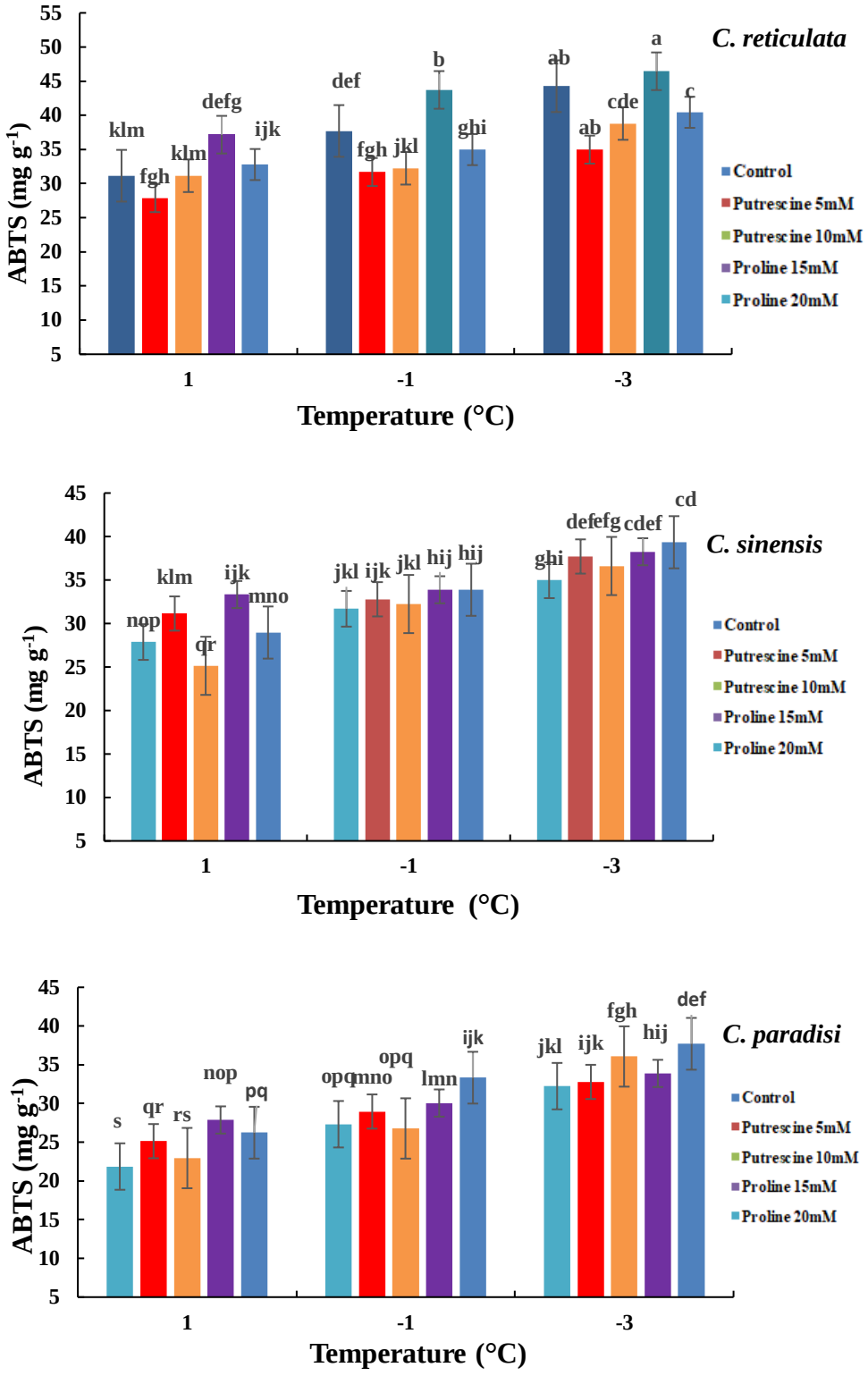

Fig. 1. Effect of different concentrations of putrescine and proline on levels of ABST in three citrus species under different temperatures. The same letters on the top of the bars indicate that there is no statistically significant difference at $P \leq 0.05$ level using Duncan's test.

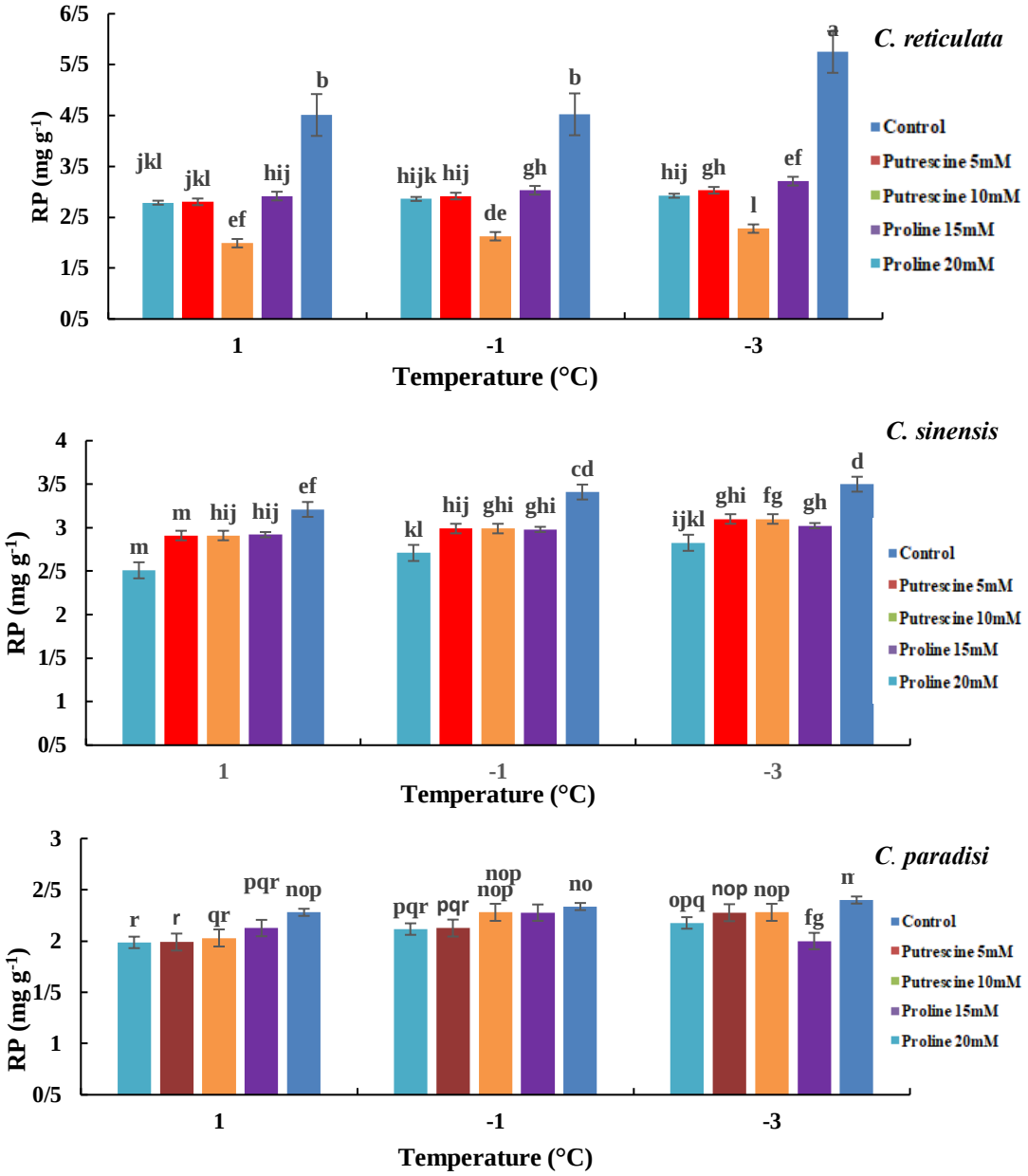

Fig. 2. Effect of different concentrations of putrescine and proline on the levels of reducing power (RP) in three citrus species under different temperatures. The same letters on the top of the bars indicate that there is no statistically significant difference at $P \leq 0.05$ level based on Duncan's test.

HPLC analyses of phenolics in three citrus species

High-performance liquid chromatography (HPLC) determined the phenolic compounds in fruit extracts of three citrus species at different temperatures (Tables 1–3). The standards used included chlorogenic, gallic, tannic, p-coumaric, ferulic, and salicylic acids from phenolic acids, as well as rutin, quercetin, and naringin from flavonoids. The HPLC chromatograms of citrus species at 1, -1, and -3 °C revealed that, with decreasing temperature, the levels of phenolic compounds increased, except for naringin, which remained unchanged. Additionally, the exogenous application of proline and putrescine significantly increased the levels of several phenolic compounds, including chlorogenic, gallic, p-coumaric, and ferulic acids from phenolic acids, as well as quercetin and rutin from flavonoids (Tables 1–3). However, the amounts of tannic and salicylic acids were negligible and recorded as undetectable (ND). A comparison of phenolic compound levels among the three citrus species showed that *C. paradisi* had lower phenolic contents compared to *C. reticulata* and *C. sinensis* (Table 3).

Table 1. Effects of exogenous putrescine and proline on total contents of phenolic acids in *C. reticulata*.

Parameters		1 °C					-1 °C					-3 °C				
		C	Put_1	Put_2	P_1	P_2	C	Put_1	Put_2	P_1	P_2	C	Put_1	Put_2	P_1	P_2
Phenols (µg g^{-1})	Chlorogenic acid	0.2^{e}	0.25^{de}	0.3^{cd}	0.3^{cd}	0.35^{d}	0.3^{cd}	0.35^{d}	0.4^{c}	0.41^{c}	0.5^{b}	0.5^{ab}	0.51^{ab}	0.55^{ab}	0.55^{ab}	0.63^{a}
	Gallic acid	0.2^{e}	0.2^{e}	0.25^{de}	0.2^{e}	$0.25d^{e}$	0.3^{cd}	0.3^{cd}	0.35^{d}	0.4^{b}	0.42^{b}	0.38^{b}	0.4^{b}	0.45^{ab}	0.45^{ab}	0.51^{a}
	P-Coumaric acid	0.1^{b}	0.14^{b}	0.18^{b}	0.16^{b}	0.18^{b}	0.19^{b}	0.19^{b}	0.2^{b}	0.2^{b}	0.25^{ab}	0.19^{b}	0.2^{b}	0.3^{a}	0.25^{ab}	0.3^{a}
	Ferulic acid	0.15^{c}	0.18^{ab}	0.2^{ab}	0.18^{ab}	0.25^{b}	0.18^{ab}	0.2^{ab}	0.3^{a}	0.25^{b}	0.35^{a}	0.18^{ab}	0.2^{ab}	0.34^{a}	0.3^{a}	0.34^{a}
	Salicylic acid	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Tannic acid	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Flavonoids (µg g^{-1})	Quercetin	0.11^{c}	0.18^{ab}	0.2^{ab}	0.18^{ab}	0.23^{ab}	0.15^{c}	0.18^{ab}	0.2^{ab}	0.25^{b}	0.3^{a}	0.15^{c}	0.23^{ab}	0.25^{ab}	0.28^{b}	0.34^{a}
	Rutin	0.14^{c}	0.15^{c}	0.18^{ab}	0.2^{ab}	0.22^{b}	0.15^{c}	0.15^{c}	0.18^{ab}	0.25^{b}	0.3^{a}	0.18^{ab}	0.25^{b}	0.25^{b}	0.26^{b}	0.3^{a}
	Naringin	0.25^{ab}	0.22^{ab}	0.2^{ab}	0.22^{ab}	0.2^{ab}	0.3^{a}	0.25^{ab}	0.25^{ab}	0.28^{b}	0.24^{ab}	0.3^{a}	0.24^{ab}	0.28^{a}	0.28^{a}	0.24^{ab}

Values in the same column with different superscript letters represent significant differences between citrus species at $P < 0.05$ by Duncan's test. C: control, Put: 5 mM, Put:10 mM, P1: proline 15 mM, P2: proline 20 mM.

Table 2. Effects of exogenous putrescine and proline on total contents of phenolic acids in *C. sinensis*.

Parameters		1 °C					-1 °C					-3 °C				
		C	Put_1	Put_2	P_1	P_2	C	Put_1	Put_2	P_1	P_2	C	Put1	Put2	P1	P2
Phenols (µg g^{-1})	Chlorogenic acid	0.35^{c}	0.38^{c}	0.4^{ab}	0.35^{c}	0.45^{ab}	0.4^{ab}	0.4^{ab}	0.45^{ab}	0.4^{ab}	0.5^{b}	0.48^{ab}	0.5^{b}	0.55^{b}	0.54^{b}	0.63^{a}
	Gallic acid	0.3^{c}	0.33^{c}	0.4^{b}	0.35^{c}	0.4^{b}	0.35^{c}	0.34^{c}	0.45^{a}	0.34^{c}	0.45^{a}	0.34^{ab}	0.35^{ab}	0.4^{b}	0.45^{a}	0.48^{a}
	P-Coumaric acid	0.14^{c}	0.14^{c}	0.18^{c}	0.2^{ab}	0.22^{ab}	0.17^{c}	0.2^{ab}	0.25^{b}	0.22^{ab}	0.3^{a}	0.17^{c}	0.2^{ab}	0.25^{b}	0.22^{ab}	0.3^{a}
	Ferulic acid	0.15^{c}	0.15^{c}	0.18^{c}	0.15^{c}	0.2^{ab}	0.16^{c}	0.18^{c}	0.2^{ab}	0.22^{ab}	0.3^{a}	0.16^{c}	0.2^{ab}	0.25^{b}	0.27^{a}	0.32^{a}
	Salicylic acid	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Tannic acid	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Flavonoids (µg g^{-1})	Quercetin	0.13^{c}	0.14^{c}	0.15^{c}	0.16^{c}	0.2^{ab}	0.18^{c}	0.2^{ab}	0.25^{b}	0.25^{b}	0.3^{a}	0.18^{b}	0.2^{ab}	0.25^{b}	0.3^{a}	0.34^{a}
	Rutin	0.14^{c}	0.14^{c}	0.18^{c}	0.18^{c}	0.2^{ab}	0.15^{c}	0.18^{c}	0.2^{ab}	0.24^{b}	0.25^{b}	0.15^{c}	0.2^{ab}	0.2^{ab}	0.24^{b}	0.27^{a}
	Naringin	0.34^{a}	0.3^{a}	0.25^{b}	0.28^{a}	0.25^{b}	0.3^{a}	0.25^{b}	0.2^{ab}	0.25^{b}	0.2^{ab}	0.3^{a}	0.22^{ab}	0.2^{ab}	0.25^{b}	0.2^{ab}

Values in the same column with different superscript letters represent significant differences between Citrus species at $P \leq .05$ by Duncan's test. C: control, Put: 5 mM, Put:10 mM, P1: proline 15 mM, P2: proline 20 mM.

Discussion

Citrus is among the most important tropical and subtropical fruit trees but is notably sensitive to low temperatures. Plant growth and development are influenced by ambient temperature, with each species exhibiting a specific temperature range defined by minimum, maximum, and optimum thresholds. Various studies have demonstrated significant differences in how species and cultivars respond to cold stress (Sanghera et al.,

2011). This experiment revealed that *Citrus reticulata* exhibited higher levels of phenolic compounds and antioxidants compared to two other species. These levels increased as temperatures declined and with the application of different concentrations of exogenous proline and putrescine. Phenolic compounds play a crucial role in mitigating environmental stresses such as low temperatures (Robles et al., 2003). Their antioxidant properties inhibit the production of reactive oxygen species (ROS) (Jaakola and Hohtola, 2010). Research indicates that proline effectively stimulates the total phenolic content and specific phenolic metabolites, highlighting phenolics as key contributors to radical-scavenging activity (Shen et al., 2009). Phenolics protect cells from oxidative damage and enhance cell membrane stability (Burguieres et al., 2006).

Table 3. Effects of exogenous putrescine and proline on total contents of phenolic acids in *C. paradisi*.

Parameters		1 °C					-1 °C					-3 °C				
		C	Put_1	Put_2	P_1	P_2	C	Put_1	Put_2	P_1	P_2	C	Put1	Put2	P1	P2
Phenols (µg g^{-1})	Chlorogenic acid	0.3^{c}	0.34^{c}	0.35^{c}	0.35^{c}	0.4^{b}	0.35^{c}	0.35^{c}	0.45^{b}	0.4^{b}	0.5^{a}	0.4^{b}	0.4^{b}	0.45^{b}	0.5^{a}	0.55^{a}
	Gallic acid	0.2^{ab}	0.25^{ab}	0.25^{ab}	0.2^{ab}	0.3^{b}	0.3^{ab}	0.3^{b}	0.35^{a}	0.3^{b}	0.38^{a}	0.3^{b}	0.35^{a}	0.35^{a}	0.35^{a}	0.38^{a}
	P-Coumaric acid	0.13^{c}	0.15^{c}	0.18^{c}	0.15^{c}	0.2^{ab}	0.15^{c}	0.18^{c}	0.2^{ab}	0.2^{ab}	0.25^{a}	0.15^{c}	0.18^{c}	0.2^{ab}	0.2^{ab}	0.25^{a}
	Ferulic acid	0.13^{ab}	0.13^{ab}	0.15^{ab}	0.13^{ab}	0.18^{b}	0.15^{ab}	0.17^{b}	0.18^{b}	0.15^{ab}	0.2^{a}	0.15^{ab}	0.15^{ab}	0.2^{a}	0.18^{b}	0.2^{a}
	Salicylic acid	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Tannic acid	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Flavonoids(µg g^{-1})	Quercetin	0.1^{ab}	0.12^{ab}	0.15^{b}	0.14^{b}	0.15^{b}	0.12^{ab}	0.14^{b}	0.15^{b}	0.14^{b}	0.15^{b}	0.12^{ab}	0.15^{b}	0.2^{a}	0.18^{b}	0.2^{a}
	Rutin	0.1^{c}	0.12^{c}	0.14^{ab}	0.15^{ab}	0.15^{ab}	0.12^{c}	0.12^{c}	0.15^{ab}	0.15^{ab}	0.2^{a}	0.13^{c}	0.15^{ab}	0.18^{b}	0.18^{b}	0.2^{a}
	Naringin	0.3^{a}	0.25^{b}	0.25^{b}	0.28^{a}	0.22^{ab}	0.25^{b}	0.25^{b}	0.24^{b}	0.25^{b}	0.2^{ab}	0.25^{b}	0.24^{b}	0.2^{ab}	0.24^{b}	0.2^{ab}

Values in the same column with different superscript letters represent significant differences between Citrus species at $P \leq .05$ by Duncan's test. C: control, Put: 5 mM, Put:10 mM, P1: proline 15 mM, P2: proline 20 mM.

Polyamines (PAs) have also been shown to significantly influence individual polyphenols, total phenolics, and flavonoid content. Low temperature is a critical environmental factor limiting plant survival, productivity, and geographic distribution. Abiotic stresses, including cold, induce the overproduction of ROS in plants, which are highly reactive and toxic, leading to oxidative stress. ROS can cause damage to DNA, lipids, and proteins, resulting in DNA base oxidation, lipid peroxidation, and protein carbonylation, respectively (Gill and Tuteja, 2010). Secondary metabolites, particularly phenolic compounds and flavonoids, are crucial in the plant response to various stresses (Egert and Tevini, 2002). The accumulation of low molecular weight, water-soluble molecules—known as compatible solutes or osmolytes—is an essential strategy for plants to resist abiotic stresses (Chen and Murata, 2002). Among these, proline, polyamines, and soluble sugars are particularly significant. Their beneficial effects in modulating osmotic stress and maintaining plasma membrane integrity have been documented (Rai, 2002). Several studies indicate that exogenous application of PAs can enhance low-temperature tolerance. For instance, *Anthurium andraeanum* exposed to chilling stress and treated with exogenous putrescine exhibited reduced membrane injury and malondialdehyde (MDA) content, along with increased antioxidant and proline levels, demonstrating the effectiveness of putrescine in mitigating cold-induced damage (Shao et al., 2022). In response to ROS production, plants increase their antioxidant capacity and activate antioxidant enzymes. Studies on orange fruits of the Thomson Novel variety and five genotypes of *Citrus sinensis* have shown enhanced antioxidant

capacity under low temperatures (Rapisarda et al., 2008; Tajvar et al., 2011).

Under non-stress conditions, plants maintain a dynamic balance between the production and scavenging of reactive oxygen species (ROS) through a highly coordinated and rapidly responsive antioxidant system. The exogenous application of proline (Pro) and putrescine (Put) has demonstrated protective effects against oxidative damage and other stress-related injuries. Plants have developed various mechanisms to scavenge and detoxify ROS. While proline is widely recognized for its role as an osmoprotectant (Wang et al., 2015), its accumulation in plant tissues has also been linked to the scavenging of hydroxyl radicals, contributing to improved oxidative stress tolerance (Sharma and Dietz, 2006). Exogenous proline has been observed to function as both an osmoprotectant and a cryoprotectant under specific conditions (Songstad et al., 1990). For example, Roy et al. (1993) reported that applying 20 mM proline effectively counteracted the adverse effects of salinity on early seedling growth. However, contrasting evidence from Hellmann et al. (2000) indicated that even moderate concentrations of exogenous proline could be toxic to *Arabidopsis* in axenic culture. These findings highlight the complexity of proline's role in plant stress responses, necessitating further research to fully understand its mechanisms and effects.

Polyamines (PAs) also play a crucial role in plant responses to environmental stresses, including osmotic stress, salinity, acid stress, heavy metal stress, and UV radiation (Legocka and Kluk, 2005). PAs are highly protonated at physiological pH, which facilitates their electrostatic interaction with negatively charged functional groups on membranes and proteins. This interaction stabilizes membrane structure and maintains permeability by binding to the negatively charged phospholipid head groups. Under severe stress conditions, the antioxidant machinery of plants may not be sufficient to minimize oxidative damage. Consequently, the accumulation of osmotically active substances, such as polyamines, becomes a critical adaptive strategy. These molecules not only serve to counter osmotic stress but also play a role in scavenging ROS. Research on the exogenous application of polyamines, as well as inhibitors of polyamine biosynthetic enzymes, has highlighted their potential role in plant adaptation and defense against various stresses (Groppa and Benavides, 2008).

Polyamines are a class of phytohormone-like aliphatic amine compounds, including the triamine spermidine (Spd), the tetramine spermine (Spm), and their precursor, the diamine putrescine (Put). These compounds are involved in regulating plant growth and physiological processes (Kusano et al., 2007, 2008) and are key modulators of plant defense responses under environmental stress (Bouchereau et al., 1999). The effects of exogenous polyamines on plant tolerance to osmotic stress have been studied in various plant species (Liu et al., 2004). Wang et al. (2006) demonstrated that exogenous polyamines increase endogenous PA levels, contributing to enhanced drought tolerance. Moreover, polyamines have been proposed to act as antioxidants, reducing free radicals and mitigating lipid peroxidation, thereby bolstering plant resilience under stress conditions (Velikova et al., 2000). These findings collectively underscore the multifaceted roles of both proline and polyamines in plant stress tolerance. Their dual functions as osmoprotectants and antioxidants, along with their interactions with cellular structures, position them as crucial players in the broader context of plant adaptation to environmental challenges.

Conclusions

Low-temperature stress significantly impacts plant survivability, geographical distribution, and yield stability. This type of stress disrupts cellular integrity, primarily by altering membrane fluidity, reducing water potential, modifying lipid composition, diminishing ATP supply, and causing the accumulation of toxic compounds. Additionally, low-temperature stress leads to imbalances in ion supply and solute leakage, further compromising cellular functions. A key consequence of such stress is the excessive production of reactive oxygen species, an unavoidable byproduct of aerobic metabolism under adverse conditions. These highly reactive and toxic molecules interfere with various cellular processes, ultimately resulting in oxidative damage and, in severe cases, cell death. Effective management strategies and agronomic practices are essential for mitigating the damages associated with low-temperature stress. Among these strategies, proline has been identified as a critical molecule in enhancing plant tolerance to stress. The exogenous application of proline as a stress mitigator has shown great potential in strengthening plant resilience. Proline not only functions as an osmoprotectant but also plays a significant role in alleviating oxidative damage by interacting with cellular components and stabilizing cellular structures. Polyamines, such as putrescine, further contribute to stress

tolerance by interacting with polyanionic macromolecules, cellular membranes, hydroxycinnamic acids, fatty acids, and alkaloids. These interactions are fundamental to their role in both abiotic and biotic stress responses. In this study, proline and putrescine were employed to mitigate oxidative damage in the fruits of three citrus species during cold stress. Their application resulted in an increased content of non-enzymatic antioxidants, including enhanced antioxidant activity and higher phenolic compound levels. These changes effectively countered the negative effects of low-temperature stress, thereby improving the cold tolerance of citrus species.

Conflict of Interest

The authors indicate no conflict of interest in this work.

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