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Research Article

Effect of Pretreatment of Putrescine and Proline on Antioxidant System in Fruits of Citrus Species

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ABSTRACT

Effect of putrescine and proline on reactive oxygen species in three citrus speices under temperatures of 1, -1 and -3°C were invistigated through analysis of the content malondialdehyde, superoxide dismutase, antioxidant activity by Reducing Power (RP) method, anthocyanine, and HPLC of phenolics. The results of comparison of mean showed that with decreasing temperature the amount malondialdehyde and other dialdehyde were increased. Althought, proline and putrescine treatment have reduced the amount of these traits. The level of anthocyanin reduced with decreasing temperature during low temperature stress. However, application of treatments with different concentrations has led to an improvement in the amount of anthocyanins compared to control fruits. Both decreasing of temperatures and using of proline and putrescine treatments increased the enzyme activity, antioxidant capacity and phenolic compounds.

Keywords: Antioxidant activity; Cold stress; HPLC

INTRODUCTION

Low temperatures lead to the production of reactive oxygen species and differential response in antioxidant defense and serious anatomical damage in plants.

Reactive Oxygen Species (ROS) attack to proteins, lipids and nucleic acids and the degree of damage depends on the balance between formation of ROS and their removal by the anti-oxidative scavenging systems and this appears to represent an important stress-tolerance trait.

Many studies have demonstrated the involvement of antioxidant defense system in response to sub-optimal temperatures, such as the system that reacts with active forms of oxygen and keeps them at a low level (e.g. **superoxide dismutase**, **catalase** and peroxidases) and the system that regenerates oxidized antioxidants (e.g. **glutathione reductase** and ascorbate peroxidase. In plant cells, ROS detoxification is controlled by ROS scavenging enzymes such as Superoxide Dismutase (SOD), Catalase (CAT), and Peroxidase (POD) and involves the oxidation and reduction of ascorbate and glutathione mainly by the activities of Ascorbate Peroxidase (APX) and Glutathione Reductase (GR). The antioxidant systems consist of three general classes: (a) lipid soluble, membrane associated antioxidants, (b) water-soluble reductants, and (c) enzymatic antioxidants such as Superoxide dismutase (SOD; EC 1.15.1.1), Catalase (CAT; EC 1.11.1.6), Ascorbate Peroxidase (APX; EC 1.11.1.11) and guaiacol peroxidase (POD; EC 1.11.1.7). SOD, the first enzyme in the detoxifying process, converts O_2 radicals to H_2O_2 and O_2. CAT, APX, and non-specific POD scavenge the accumulated H_2O_2 to nontoxic levels or form water and oxygen. Previous work has shown that low temperature-induced stress can modify the composition of phenolic compounds in different anatomical parts of plants such as pea, rapesee, maize, soybean, winter wheat, elderberry, *Ammopiptanthus mongolicus* and *Cotinus coggygria*. Anthocyanins are flavonoid compounds that protect plant tissues from many environmental stresses including high light irradiance, freezing temperatures and pathogen infection. Regulation of anthocyanin biosynthesis is intimately associated with environmental changes to enhance plant survival under stressful environmental conditions. Various factors, such as UV, visible light, cold, osmotic stress and pathogen infection, can

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induce anthocyanin biosynthesis. Anthocyanins play key roles in diverse physiological processes, such as protecting plant tissues from damage after exposure to UV, freezing temperature and pathogens; assist in the attraction of pollinators and seed dispersers, scavenge free radicals produced under stress conditions and modulate auxin transport. One of the common responses of many plant species exposed to different abiotic stresses is the accumulation of compatible organic solutes such as proline, glycine betaine, choline and O-sulfate. Proline is an amino acid that is a highly soluble, non-toxic and has a low molecular weight. Proline accumulation has been shown in different abiotic stressed plants. It has been suggested that proline protects plants by functioning as a cellular osmotic regulator between cytoplasm. Proline is a dominant organic molecule, which contributes to the maintenance of enzymes from denaturation, interacts with membrane systems, regulates cytosolic pH, and balances the ratio of NADH/NAD + functions as a source of energy and helps plants to detoxify ROS. Polyamines are small ubiquitous nitrogenous compounds. Increased polyamine levels in stressed plants are of adaptive significance because of their involvement in the regulation of cellular ionic environment, maintenance of membrane integrity, prevention of chlorophyll loss and stimulation of protein synthesis, nucleic acids and protective alkaloids. Stabilization of membranes and minimization of water stress of various kinds of cells are of the several known physiological effects of polyamines (eg. putrescine) in the plant system (Goyal and Asthir 2010). Proline is a dominant organic molecule, which contributes to the maintenance of enzymes from denaturation, interacts with membrane systems, regulates cytosolic pH, balances the ratio of NADH/NAD+ functions as a source of energy and helps plants to detoxify ROS. Polyamines can clean free radicals in plants; they also protect the membrane to some extent from oxidative damage [1]. Exogenously applied PA also increased plant tolerance under several abiotic stresses. The objective of this work was to investigate proline and putrescine in citrus fruits under low temperature [2].

Materials and Methods

Thiobarbituric Acid Reactive Substance (TBARS

100 mg of the fruits tissue was homogenized in 10 ml of 0.1% TCA (W/V), and then centrifuged at 10,000 × g for 15 min. One ml of supernatant was then swirled with 4 ml of 20% TCA (W/V) containing 0.5% 2-Thiobarbituric Acid (TBA) (W/V), and the solution was heated for 30 min at 90°C. Samples were cooled on ice for 5 min and then re-centrifuged for 10 min at 10,000 × g. For Malondealdehyde (MDA) measurement, the nonspecific absorbance of supernatant at 600 nm was subtracted from the maximum absorbance at 532 nm and an extinction coefficient (ε) of 1.55 × 105 M1 cm^{-1} was used for determination of MDA concentration. For other aldehydes measurement, absorbance of 600 nm was subtracted from maximum absorbance of 455 nm and the extinction coefficient of 0.457 × 105 M^{-1} cm^{-1} was used for the calculations [5,6].

Antioxidant activity

RP assay

In this method, based on the extracts' reducing power, the yellow color of the sample changes to various shades of green and blue. The reducers (antioxidants) cause the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. Hence, by measuring the formation of Perl's Prussian blue at 700 nm, Fe^{2+} concentration can be monitored [7]. The method was used to estimate the capacity of date extracts to reduce Fe^{3+}. The samples' methanolic solutions were mixed with 250 µL of sodium phosphate buffer (0.2 M, pH 6.6) and 250 µL of 1% K_3Fe (CN) 6 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 [8]. 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 [9].

Enzyme assays

Preparing of enzyme extracts

1 g of fruit in 4 ml of 50 mM K-phosphate buffer (pH 7.0) containing 2 mM Na–EDTA and 1% (w/v) Polyvinyl–polypirrolidone (PVP). The experiment was performed on ice cold and then the homogenate was centrifuged at 10,000×g for 10 min. The supernatants were collected and stored at −20°C until using. The total protein content of samples was determined according to Bradford protein assay using Bovine Serum Albumin (BSA) as a standard. The absorbance is recorded at 595 nm.

Guaiacol peroxidase (GPX) activity (Ec 1.11.1.7)

GPX activity was measured using guaiacol as a substrate. Reaction mixture (3 ml) contained 25 µl of enzyme extract, 2.77 ml of 50 mM phosphate buffer) pH 7.0), 0.1 ml of 1% H_2O_2 (V/V), and 0.1 ml of 4% guaiacol (V/V). The increase in absorbance at 470 nm due to the guaiacol oxidation was recorded for 3 min. One unit of enzyme activity was defined as the amount that causes a change of 0.01 in the absorbance per minute [10].

Superoxide dismutase (SOD; EC 1.15.1.1)

SOD activity was determined by measuring the ability of the enzyme to inhibit the photochemical reduction of Nitro Blue Tetrazolium (NBT) in the presence of riboflavin in light. The reaction mixture (3 ml) contained 50 mM K-phosphate buffer (pH 7.8), 13 mM methionine, 75 mM NBT, 4 mM riboflavin, 0.1 mM EDTA, and 0.25 ml enzyme extract. One unit of enzyme activity was determined as the amount of the enzyme to reach an inhibition of 50% NBT reduction rate by monitoring absorbance at 560 nm with spectrophotometer [11,12]. The test tubes were shaken then placed in a light box consisting of six 15 W fluorescent lamps for 10 min. Reaction was stopped by switching off the light and placing the test tubes into dark [13].

Chromatographic analysis of phenolic compounds by high-performance liquid chromatography

About 0.5 g of fruits was homogenized in 1 ml of methanol 80%. Extraction was aided by means of vortexing for 30 min intervals. The resultant mix 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. 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 [14]. The column was a C18 column and injection volume was 20 µl. HPLC profiles were determined for samples exposed to the lowest of temperature (-3°C) [15]. The stock solution of concentration 1 mg/ml was prepared by dissolving1 mg of phenolic acids and the flavonoids in 0.5 ml HPLC grade methanol and the resulting volume was made up to 1ml 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 [16-20].

RESULT

The results of comparison of means showed that with decreasing of temperature the amount of malondialdehyde increased in fruits. In all temperatures, the highest amount is related to the control fruits [21-25]. The amount of malondialdehyde among the species indicated that the highest amount of malondialdehyde was in all three species at -3°C. However, application of putrescine and proline with different concentration in three citrus species resulted in a decrease in malondialdehyde content at different temperatures (Figure 1). The highest amount of malondialdehyde is in C. *paradisi* control fruits at -3°C. The lowest level related to C.*reticulata fruits treated with 10 mM putrescine at 1*°C *(Figure 1).*

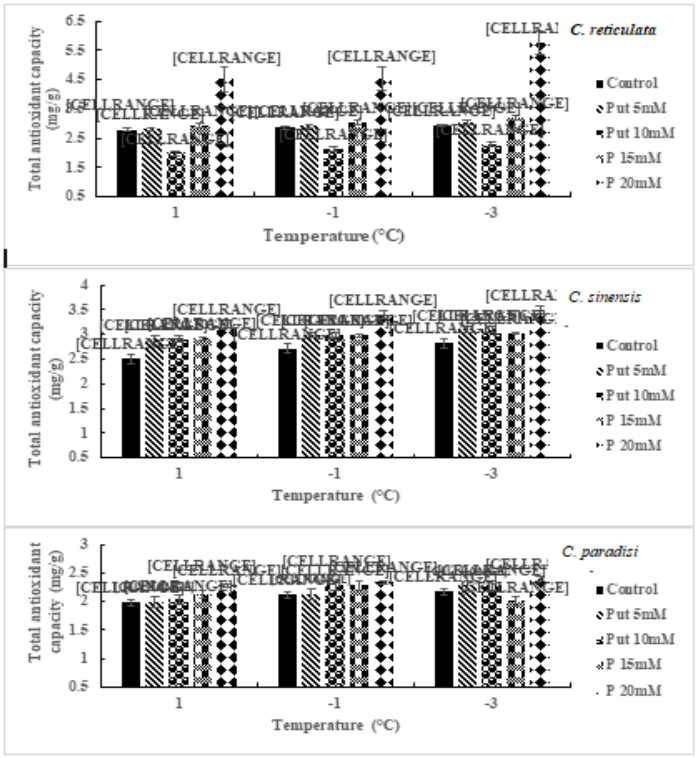

Figure 1: Effect of different concentrations of putrescine and proline on level of malondialdehyde in three citrus species under different temperature. The letters on the top of the bars, which are the same, indicate that there is no statistically significant difference at p<0.05 level based on Duncan's test.

The results showed that the amount of other aldehydes was increased with decreasing of temperature in all three species [26-28]. However, application of different concentrations of putrescine and proline resulted in a reduce in the amount of other aldehyde (Figure 2). In each species, the highest amount is in the control fruit at -3°C and the lowest related to fruits treated with proline 20 mM. Comparison between species showed that the highest amount of other aldehydes were observed in C. paradisi control fruits when exposed to the lowest temperature [29,30].

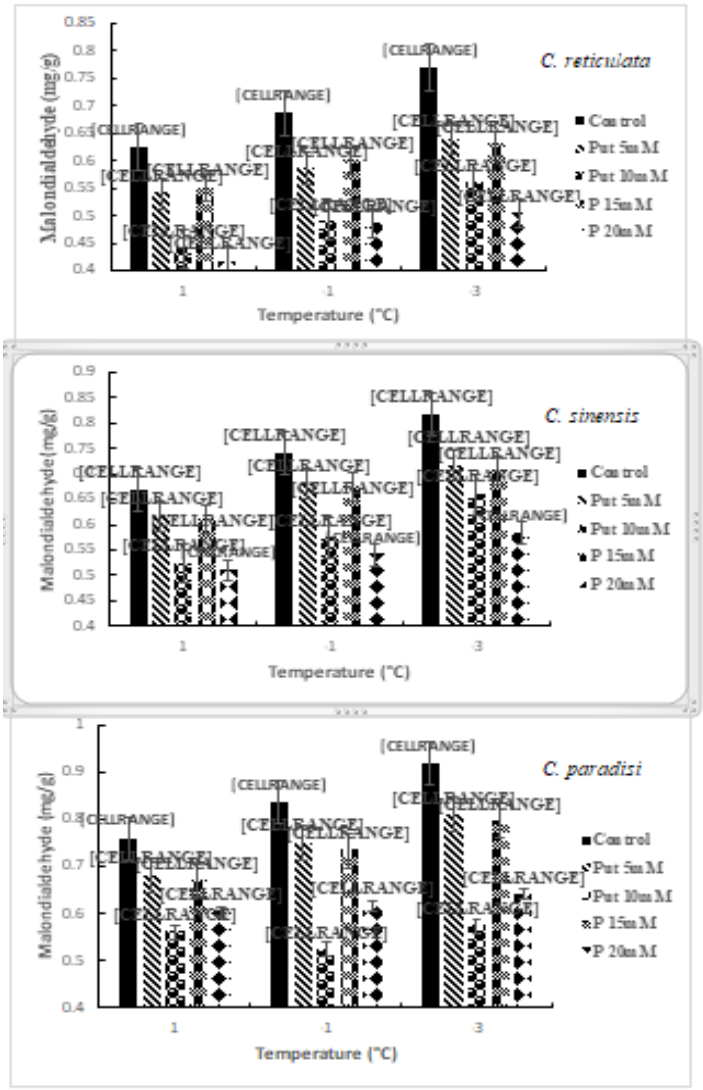

Figure 2: Effect different concentrations of putrescine and proline on level of other aldehydes in three citrus species under different temperature. The letters on the top of the bars, which are the same, indicate that there is no statistically significant difference at p<0.05 level based on Duncan's test.

Anthocyanin levels reduced with decreasing of temperature in all three citrus species. The lowest amount was observed in control fruits at -3°C. The use of proline and putrescine in all three citrus species also increased the anthocyanin content (Figure 3). The highest levels of anthocyanins are related to fruits treated with proline 20 mM and putricin 10 mM. Intraspecific comparisons show that the lowest levels of anthocyanins were observed in C. paradisi fruits and the highest levels were found in C. reticulata fruits.

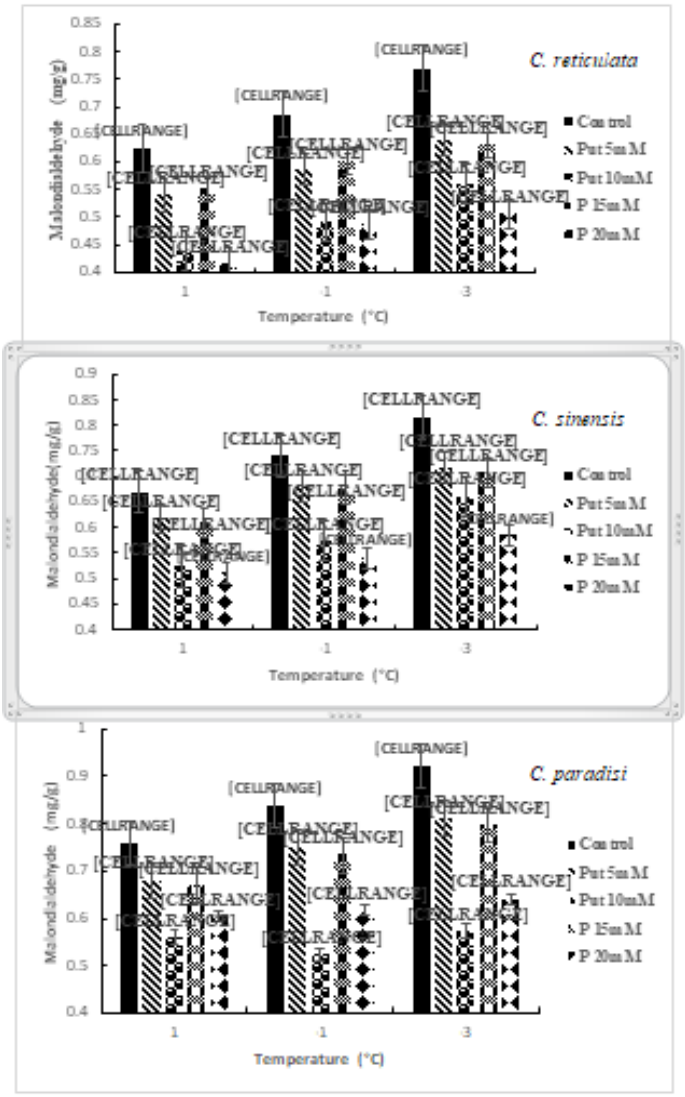

Figure 3: Effect of different concentrations of putrescine and proline on level of anthocyanin in three citrus species under different temperature. The letters on the top of the bars, which are the same, indicate that there is no statistically significant difference at p<0.05 level based on Duncan's test.

With decreasing of temperature, the amount of SOD enzyme in all three citrus species has increased [31-33]. The results of the figure 4 showed that the application of putrescine and proline leads to an increase in the activity of SOD in all three citrus species [34,35]. At each temperature, the lowest amount was related to the control fruits and the highest was related to the treated fruits with proline 20 Mm and putrescine 10 mM. The highest amount of enzyme activity was observed in C. reticulata than other species (Figure 4).

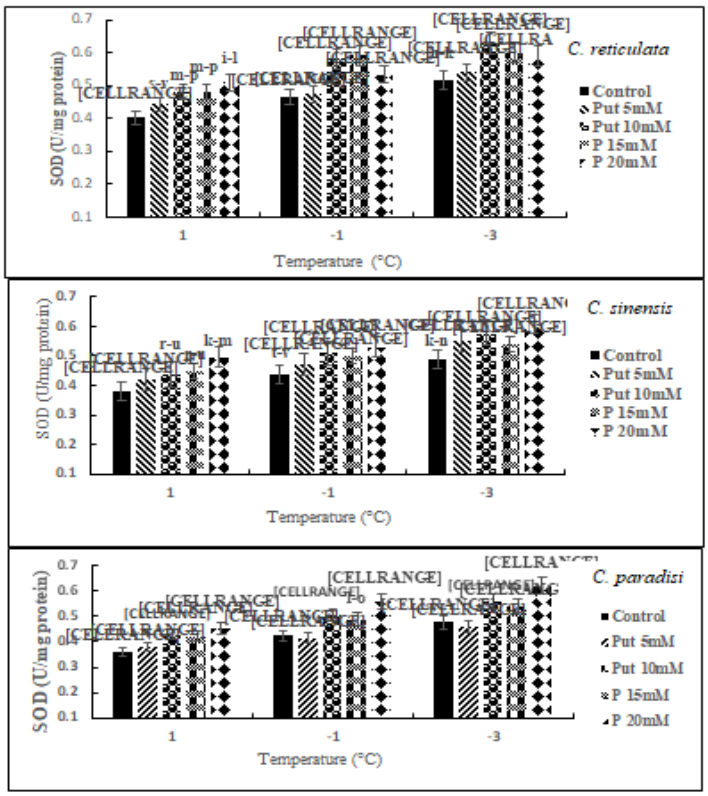

Figure 4: Effect of different concentrations of putrescine and proline on level of SOD in three citrus species under different temperature. The letters on the top of the bars, which are the same, indicate that there is no statistically significant difference at $p<0.05$ level based on Duncan's test.

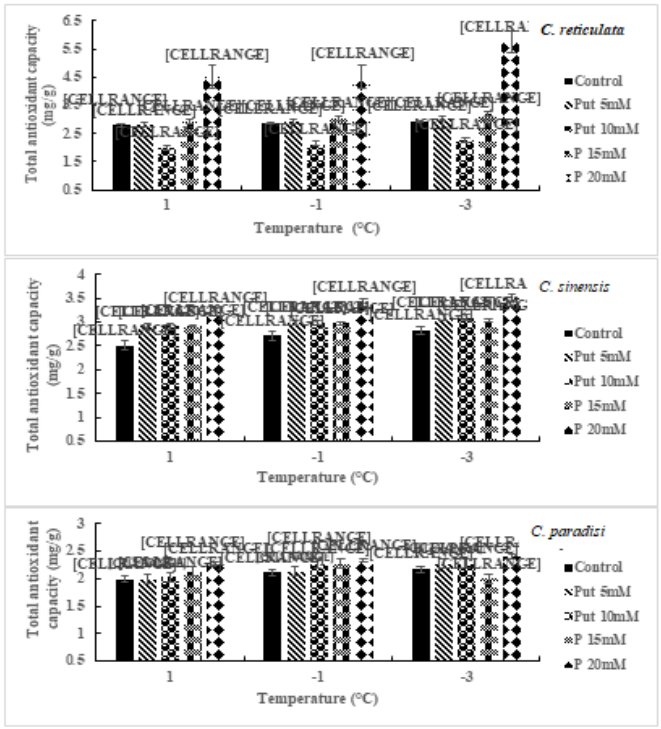

Figure 5: Effect of different concentrations of putrescine and proline on level of reducing power (RP) in three citrus species under different temperature. The letters on the top of the bars, which are the same, indicate that there is no statistically significant difference at $p < 0.05$ level based on Duncan's test.

The results of antioxidant activity by Reducing Power (RP) method showed that application of different temperatures led to an increase in antioxidant capacity in citrus species.

In three species, the highest amount is in fruits that are exposed to -3°C. Also, the use of proline and putricine have led to an increase in antioxidant activity (Figure 5).

Generally, the highest amount of antioxidants in citrus species was observed in fruits treated with 20 mm proline [36,37].

HPLC analyses of phenolics in three citrus species

HPLC chromatography was applied in order to determine phenolics compound in the extracts of three citrus fruits at different temperatures (Tables 1-3). Used standards were contained chlorogenic, gallic, tannic, p-coumaric, ferulic acid and salicylic acid from phenolic acids as well as rutin, quercetin, and naringin from flavonoids. The HPLC chromatograms of citrus species at 1, -1 and -3°C showed that with the decreasing of temperature, level of phenolic compounds except naringin have increased [38-40]. Also, exogenous plication of proline and putrescine leading to increasing phenolic compounds including chlorogenic, gallic, p-coumaric, and ferulic acid from phenolic acids and also quercetin and rutin from flavonoids (Tables 1-3). The amount of tannic and salicylic acid was negligible and was recorded as undetectable (ND). Comparison of phenolic compounds in three citrus species showed that contents of phenolics were lower in C. paradisi than two other species (Table 3).

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

	Parameters	1°C					-1°C	-3°C							
		C	Put1	Put 2	P1	P2	C	Put1	Put2	P1	P2	C	Put1	Put2	P1	P2
	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}	0.25^{de}	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}
Phenols (µg/g)	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)	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 $p<.05$ 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	Put1	Put 2	P1	P2	C	Put1	Put2	P1	P2	C	Put1	Put2	P1	P2
	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}	0.45a	0.34^{ab}	0.35^{ab}	0.4^{b}	0.45^{a}	0.48^{a}
Phenols (µg/g)	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)	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 $p<.05$ by Duncan's test. C: control, Put: 5 mM, Put:10 mM, P1: proline 15 mM, P2: proline 20 mM.

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

	Parameters	1°C					-1°C	-3°C								
		C	Put1	Put 2	P1	P2	C	Put1	Put2	P1	P2	C	Put1	Put2	P1	P2
	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}
Phenols (µg/g)	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)	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 $p<.05$ by Duncan's test. C: control, Put: 5mM, Put:10 mM, P1: proline 15 mM, P2: proline 20 mM [41,42].

DISCUSSION

Low temperature is a significant environmental element that affects plant survival, productivity, and spread. Various abiotic conditions cause plants to produce excessive amounts of Reactive Oxygen Species (ROS), which are extremely reactive and poisonous and cause damage to proteins, lipids, carbohydrates, and DNA, resulting in oxidative stress.

Pre-treatment with proline and putrescine elevated various phenolic compounds at different temperatures in the current investigation. Proline has been shown to successfully boost total phenolic content and specific phenolic metabolites, implying that phenolics are the primary molecules involved in radical-scavenging activity. Phenols protect cells from oxidative damage and improve the cell membrane's integrity. Individual polyphenols, total phenolic, and flavonoid concentration were all significantly affected by PAs. In *W. somnifera in vitro* regenerated plants, PAs and PGRs boosted the amount of bioactive substances when compared to *in vivo* plants.

Plants need a well-coordinated and swiftly responsive antioxidant system to maintain a balance between generating and scavenging ROS under non-stress situations. Under these conditions, the antioxidant enzymes CAT, SOD, POD, and others work together to keep free radical levels low and avoid cell damage. SOD, POD, and CAT are commonly thought to be the essential components of plant antioxidant defense. MDA and other aldehydes were found to be enhanced in our experiment, which was consistent with other prior results. Fruits supplemented with exogenous Pro and Put, on the other hand, had reduced MDA and other aldehyde levels (Figures 1 and 2), owing to their stronger antioxidant defense mechanism. Exogenous Pro caused antioxidant defense up regulation and a decrease in MDA concentration in a variety of plant species, including rice. Antioxidant enzymes in three citrus species responded differentially to cold stress in our experiment. Exogenous administration of Pro and Put had a varied protective effect in those species. Exogenous polyamines boosted the level of endogenous polyamines in this study, which helped to maintain membrane integrity and permeability while also reducing lipid peroxidation. Plants have developed many mechanisms for scavenging and detoxifying ROS in order to avoid and reduce ROS buildup and oxidative stress. Although the role of Proline (Pro) in plants as an osmoprotectant is highly suggestive, proline increase in plant tissues can also contribute to the removal of hydroxyl radicals, hence improving oxidative stress tolerance. Exogenous proline has been discovered to perform functions in osmoprotection and cryoprotection. Exogenous injection of 30 mM proline counteracted the negative effects of salt on early seedling growth, according to Roy et al. Furthermore, Okuma et al. claim that exogenous proline protects enzymes and cell membranes by reducing lipid membrane oxidation under stress circumstances. Exogenous

proline, according to Hoque et al. boosts the activity of most enzymes implicated in the NaCl-induced ASC-GSH cycle and improves salt tolerance in tobacco. In axenic culture, however, Hellmann et al. found that even moderate doses of exogenous proline are hazardous to Arabidopsis. As a result, the precise roles of exogenous proline in the stress defence process of plants are yet unknown. Polyamines have been linked to a variety of environmental stresses in plants, including osmotic stress, salt stress, acid stress, heavy metal stress and UV radiation. The first line of antioxidant enzymes, superoxide dismutase, eliminates superoxide by catalysing its dismutation, with one being reduced to H_2O_2 and the other being oxidised to O_2. SOD activity is increased, which reduces abiotic oxidative stress and aids in a plant's response to stressful circumstances. Polyamines can bind to antioxidant enzymes like SOD and CAT, allowing them to reach oxidative stress sites within cells. Spm also boosted GPX activity considerably. Scavenging active oxygen and free radicals is easier in this environment. Polyamines are known to be extensively protonated at physiological pHs, implying that electrostatic binding of polyamines to negatively charged functional groups of membranes and proteins should be possible. By attaching to the negatively charged phospholipid head group, polyamines can maintain membrane integrity and permeability. Under extreme stress, antioxidant machinery may not be sufficient to mitigate the negative effects of oxidative damage. As a result, understanding how plants respond to various environmental pressures requires accumulating osmotically active chemicals in plants. Exogenous polyamine application and/or inhibitors of polyamine production enzymes have shown that these chemicals may play a role in plant adaptation/defense to a variety of environmental challenges. Polyamines (PAs) are osmotically active compounds that have been linked to drought tolerance. PAs are phytohormone-like aliphatic amine chemicals; the most common forms are Spermidine (Spd), Spermine (Spm), and their precursor diamine, Putrescine (Put). PAs are engaged in the regulation of plant development and physiological processes, as well as the modulation of plant defence responses to different environmental challenges.

CONCLUSION

Exogenous PAs, on the other hand, have been researched in several plant species to see how they affect plant tolerance to osmotic stress. Exogenous PAs cause an increase in endogenous PA content, according to Wang et al. resulting in drought resistance. Furthermore, it has been proposed that the stimulatory impact of exogenous PAs acts as an antioxidant, reducing free radicals or lipid peroxidation.

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