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Research Report

Effects of Exogenous Salicylic Acid on Antioxidant Activity and Proline Accumulation in Apple (*Malus domestica* L.)

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Abstract. This study was conducted to determine the effects of exogenous application of different levels of salicylic acid (SA; 0 mM, 3.62 mM, and 7.24 mM) on antioxidant activity and proline accumulation in apple (*Malus domestica* Borkh cv. Red Chief Delicious) trees during late spring frost. The study was performed in Ulukısla, Nigde, Turkey from December 2012 to June 2013. We measured the levels of photosynthetic pigments, total proteins and proline in leaves, as well as superoxide dismutase and peroxidase enzymatic activities. We also performed morphological observations of the trees. The study was planned according to random experimental design. We determined that SA application increased the fruit number, shoot number, and carotenoid contents in the leaves, but this increase was not statistically significant. However, the fruit weights, superoxide dismutase and peroxidase activities, as well as chlorophyll, protein, and proline levels increased significantly in response to SA treatment compared to the control. In addition, the treated fruits were darker than the control. These results suggest that treating apple trees with exogenous SA may increase antioxidant enzyme activities as well as protein and proline levels and may alleviate the effects of late spring frost.

***Additional key words*:** peroxidase, photosynthetic pigments, superoxide dismutase, total protein

Introduction

Abiotic stress is becoming more prevalent as the intensity of agriculture and the demand for farmable land continue to increase. In addition to drought and salinity stress, chilling and freezing stress are some of the most important limiting factors of crop production throughout the world (Karlidag et al., 2009).

Plants produce several compounds to protect their cells against fatal intracellular and intercellular ice formation. Many overwintering plants accumulate sugars, amino acids and antifreeze compounds, including antifreeze proteins, in their apoplastic (extracellular) compartments (Atici and Nalbantoglu, 2003). Growth at low temperatures may increase the concentrations of reactive oxygen species (ROS) (Airaki et al., 2012; Okuda et al., 1991). To alleviate or prevent low temperature-induced oxidative injury, plants have developed mechanisms to scavenge these toxic and reactive species using antioxidant compounds and enzymatic antioxidant systems (Hasanuzzaman et al., 2013; Kang et al., 2003a).

Salicylic acid (SA) is a plant growth regulator with ubiquitous distribution among plants. SA regulates a large variety of physiological processes in plants (Klessig and Malamy, 1994). Recent reports describe the potentially valuable effects of salicylate treatment on low temperature tolerance (such as increasing protein and antioxidant levels) in crops such as barley (Mutlu et al., 2013), bean (Ding et al., 2002), cucumber (Kang et al., 2003a), eggplant (Chen et al., 2011), tomato (Orabi et al., 2015; Senaratna et al., 2000), maize (Wang et al., 2012), and wheat (Tasgin et al., 2003). Also, foliar treatments of exogenous SA increased the tolerance of apple trees to late spring frost (Turkyilmaz Unal et al., 2015). Ten countries produce 72% of the world's apple crop. Turkey is in the fourth position, with 2.9 million tons of apple production (FAOSTAT 2011). Ulukısla (Nigde), at the crossing point linking Central Anatolia to the Mediterranean regions, is a region of intensive apple cultivation. Late spring frost damage to soft-core and hard-core fruits is one of the fundamental problems in apple production in Ulukısla (Ozsoylu, 2007).

In the present study, we investigated the effects of SA soil application on the levels of biochemicals that are related to late spring frost stress (proline, soluble proteins, antioxidant enzyme activities), and on growth, leaf photosynthetic activities, and yield characteristics of apple.

Materials and Methods

Plant Material and Treatments

Apple trees were selected as the subject of this study, as apple represents an important source of income and is seriously affected by late spring frost in our region. Ten-year-old *Malus domestica* Borkh cv. Red Chief Delicious trees (a semi-dwarf variety) growing in the Nigde University Ulukısla Vocational School orchard were used in this study. From December 2012 to June 2013, the apple trees were treated with 5 L of 0, 3.62 mM or 7.24 mM SA solution via soil application once per week. In order to obtain systemic resistance, treatments were commonly applied during this six-month period, as late spring frosts are common. In June, at the end of flowering and the beginning of fruit set, leaf samples (from four randomly selected trees per group) were collected and analyzed. Measurements of shoot number, fruit number, fruit weight and fruit color were carried out on 10 trees per group in September 2013.

Measurement of Photosynthetic Pigment Levels

Chlorophyll and carotenoid were extracted from 0.5 g fresh leaves by homogenizing the leaves in 5 mL 80% (v/v) acetone in the dark, followed by filtration. Absorbance values were measured at 663 and 645 nm wavelengths for chlorophyll a and b, and at 450 nm for carotenoids in visible spectrophotometer. The amount of chlorophyll a and b, total chlorophyll and carotenoids were calculated according to Witham et al. (1971).

Measurement of Proline Levels

The method of Bates et al. (1973) was used to determine proline contents. Leaf samples were homogenized in 3% (w/v) sulfosalicylic acid solution and centrifuged. The supernatant was transferred to a test tube to which glacial acetic acid and acid ninhydrin solution were added. The tubes were incubated in a boiling water bath for one hour and allowed to cool to room temperature. After adding cold toluene, the mixture was vortexed and allowed to stand to enable separation of the toluene and aqueous phases. The absorbance of the toluene phase was measured at 520 nm with a spectrophotometer. The concentration of proline was calculated based on a proline standard curve and was expressed as $\mu mol \cdot g^{-1}$ FW.

Measurement of Soluble Protein Levels

Harvested fresh leaf samples were frozen in liquid nitrogen. Leaves were homogenized in 0.05 M Na-phosphate buffer (pH 7.8) containing 1 mM EDTA and 0.2 g Dowex 1 × 8 (200 × 400 mesh). The homogenates were centrifuged and the supernatants were used for enzyme activity and protein content assays.

Total soluble protein content was determined according to Bradford (1976) using bovine serum albumin as a standard. In the Bradford assay, protein concentration is determined by quantifying the binding of the dye, Coomassie Brilliant Blue G-250, to an unknown protein solution, as compared to known standards. Tubes containing 100 μL aliquots of known concentrations of bovine serum albumin (BSA; 0.156 $mg \cdot L^{-1}$ to 10 $mg \cdot L^{-1}$ in 0.15 M NaCl) were prepared. Blank tubes containing 100 μL of 0.15 M NaCl were also prepared. Then, 1 mL Coomassie Brilliant Blue solution was added to each tube and the mixtures were vortexed. The reactions were incubated at room temperature for 2 min. The absorbance at 595 nm was determined against the blank, and a standard curve of absorbance versus protein concentration was plotted.

Measurement of Antioxidant Enzyme Activities

The superoxide dismutase (SOD) activity assay was performed based on the method of Beauchamp and Fridovich (1971), which measures the inhibition of the photochemical reduction of nitroblue tetrazolium chloride (NBT) spectrophotometrically at 560 nm. One unit of enzyme activity is defined as the quantity of SOD required to produce a 50% inhibition of NBT reduction. The reaction mixture contained 50 mM Na-phosphate buffer (pH 7.8), 33 mM NBT, 10 mM L-methionine, 0.66 mM EDTA and 0.0033 mM riboflavin. Reactions were carried out at 25°C under a light intensity of approximately 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for 10 min. Peroxidase (POX) activity was determined according to Herzog and Fahimi (1973). The reaction mixture contained 3,3'-diaminobenzidinetetrahydrochloride dihydrate (DAB) solution with 0.1% (w/v) gelatin, 150 mM Na-phosphate-citrate buffer (pH 4.4) and 0.6% H_2O_2. The increase in absorbance at 465 nm was monitored for 3 min. One enzyme unit is defined as $\mu mol \cdot mL^{-1}$ H_2O_2 decomposed per min.

Statistical Analysis

Tukey's test was employed for statistical analysis using the SPSS 16.0 software package at the $p \leq 0.05$ level.

Results and Discussion

Late spring frosts occur in Ulukısla from April to July and are common in June (Fig. 1). Spring frost damages apple flower buds and reduces fruit yields and weight in this

Fig. 1. Map showing the months in which late spring frosts occur by province in Turkey (Turkish State Meteorological Service, 2014).

Table 1. Effects of SA on shoots number, fruit number, and fruit weight of apple (*Malus domestica* Borkh cv. Red Chief Delicious) plants. Values are shown as mean ± SE (n = 10)

SA concentration (mM)	Shoots number	Fruit number	Fruit weight (g)
0 (Control)	10.000±2.098	37.833±6.432	131.500±8.346 a[z]
3.62	11.600±3.435	40.500±6.191	170.500±7.778 b
7.24	11.833±1.941	41.333±6.828	170.667±8.505 b

[z]Means with same letter are not significantly different at $p \leq 0.05$.

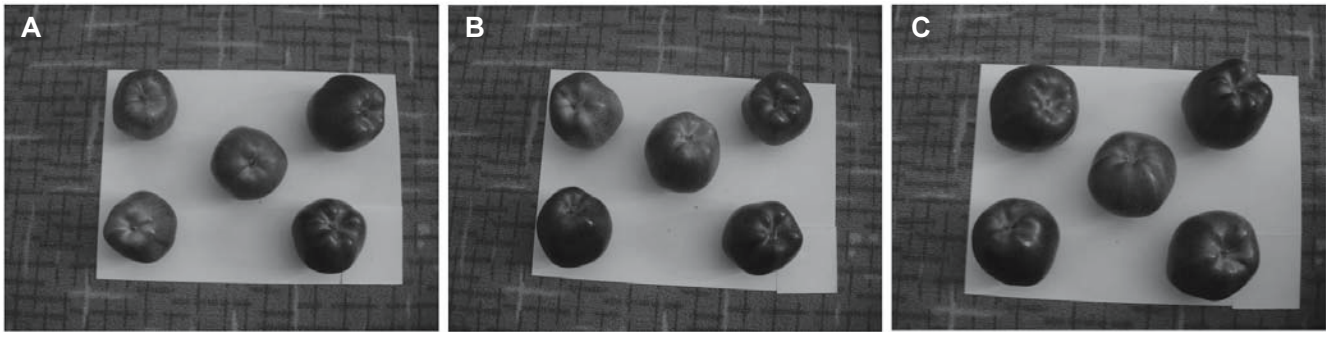

Fig. 2. Apple (*Malus domestica* Borkh cv. Red Chief Delicious) fruits [A, Control (0 mM SA); B, 3.62 mM SA; C, 7.24 mM SA].

region (Aygun and San, 2005).

The number of shoots, number of fruits and fruit weight increased after SA treatment. However, only the fruit weight was significantly higher than that of the control, without significant differences between the 3.62 mM and 7.24 mM treatments (Table 1). These results suggest that higher concentrations of SA do not strongly affect the change in fruit weight. Our results agreed with the ones of Larque-Saavedra and Martin-Mex (2007) who showed that lower concentrations of SA increase fruit weight and yield in cucumber and tomato. Moreover, SA application has a positive effect on characteristics such as fruit color, plant growth, chlorophyll content in leaves, early yield and total yield in both tomato (Yildirim and Dursun, 2008) and strawberry (Karlidag et al., 2009).

In the current study, the fruit color darkened after SA treatment (Fig. 2). This finding is in agreement with Babalar et al. (2007), who showed that SA treatment positively affects the overall quality of Selva strawberry fruit, including the production of strong red coloring. The characteristic red, blue and purple coloration observed in various tissues of a diverse assortment of plants is due to the presence of anthocyanins. The induction of anthocyanin production by chilling

Table 2. Effects of SA on chlorophyll and carotenoid contents in leaves of apple (*Malus domestica* Borkh cv. Red Chief Delicious) plants. Values are shown as mean ± SE (n = 4)

SA concentration (mM)	Chlorophyll a ($mg \cdot mL^{-1}$)	Chlorophyll b ($mg \cdot Ml^{-1}$)	Total Chlorophyll ($mg \cdot mL^{-1}$)	Carotenoid ($mg \cdot mL^{-1}$)
0 (Control)	0.631±0.088 a[z]	0.262±0.026 a	0.893±0.114 a	3.782±0.549
3.62	0.871±0.011 b	0.371±0.009 b	1.242±0.021 b	3.985±0.092
7.24	0.898±0.052 b	0.369±0.019 b	1.266±0.071 b	4.079±0.278

[z]Means with same letters are not significantly different at $p \leq 0.05$.

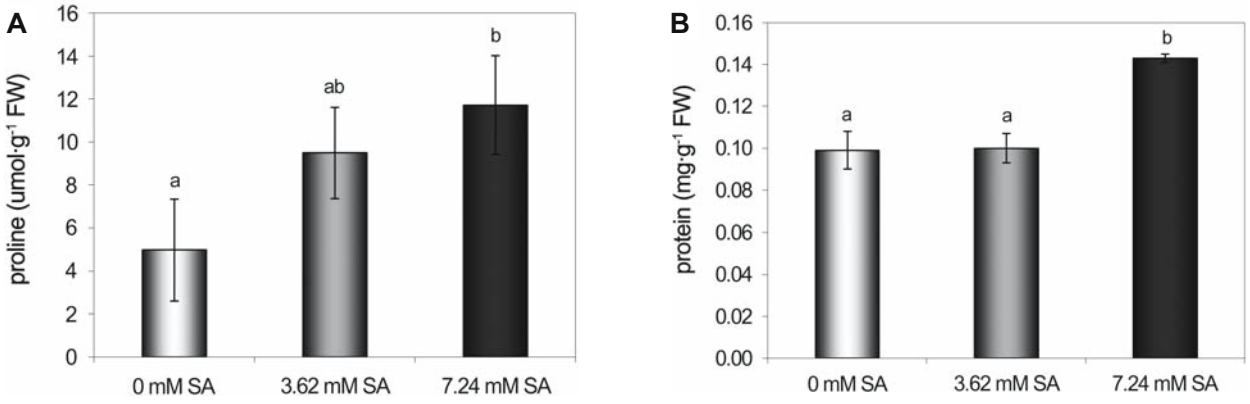

Fig. 3. Effects of SA on proline (A) and protein (B) levels in leaves of apple (*Malus domestica* Borkh cv. Red Chief Delicious) plants. Vertical bars represent SE (n = 4).

temperatures suggests that these pigments have a protective function (Chalker-Scott, 1999). McKown et al. (1996) suggested that there is an association between anthocyanin biosynthesis and freezing tolerance. Therefore, we need to measure antochyanin amounts in another study.

Recent evidence suggests that SA is an important regulator of photosynthesis as it affects leaf and chloroplast structure (Uzunova and Popova, 2000), stomatal closure (Melotto et al., 2006), chlorophyll and carotenoid contents (Fariduddin et al., 2003; Rao et al., 1997) and the activities of enzymes such as RuBisCO (ribulose-1,5-bisphosphate carboxylase/oxygenase) and carbonic anhydrase (Slaymaker et al., 2002). In the current study, chlorophyll contents significantly increased in all treatment groups compared to the control. However, there was no significant difference between the 3.62 mM and 7.24 mM SA treatments. The highest increases were observed under 7.24 mM SA treatment, with a 42.31% increase in Chla contents and 41.77% increase in total Chl contents. In addition, under 3.62 mM SA treatment, we observed a 41.60% increase in Chlb contents (Table 2). Indeed, SA treatment of jasmine under cold stress alleviates the decrease in net photosynthetic rate, stomatal conductance and primary conversion of light energy by photosystem II and it reduces the chlorophyll and starch contents compared with the control (Han et al., 2007).

The proline contents of apple trees were also affected by SA treatment; SA-treated trees (7.24 mM) had higher levels of proline than control trees ($p \leq 0.05$; Fig. 3A). Solomon et al. (1994) reported a positive correlation between proline accumulation and adaptation to stress conditions. Proline is thought to protect plants by functioning as a cellular osmotic regulator between the cytoplasm and vacuole and by detoxifying ROS, thus protecting membrane integrity and stabilizing antioxidant enzymes (Bandurska, 1993). Positive correlations between the accumulation of endogenous proline and improved cold tolerance are mainly found in chilling-sensitive plants (Korkmaz et al., 2009). Our findings are in agreement with previous reports revealing a correlation between increased proline contents and reduced freezing-chilling injury (Agham et al., 2012; Zhang et al., 2010).

Low temperatures induce the expression of a diverse array of plant genes. The products of these genes help plants adapt to subsequent freezing stress (Thomashow, 2001). In the present study, total protein levels increased after soil SA treatment, but significant increases were only detected in the 7.24 mM SA treatment group (0.143 $mg \cdot mL^{-1}$) (Fig. 3B).

The molecular events involved in SA signaling are not yet fully understood. SA treatment may directly or indirectly alter freezing tolerance and cellular antioxidant enzyme activities during chilling or freezing stress, increasing the plant's ability to withstand chilling-induced injury (Kang et al., 2003a; Tasgin et al., 2003). Compared with the control, the activities of two protective enzymes, i.e., peroxidase (POX) and superoxide dismutase (SOD), increased in the

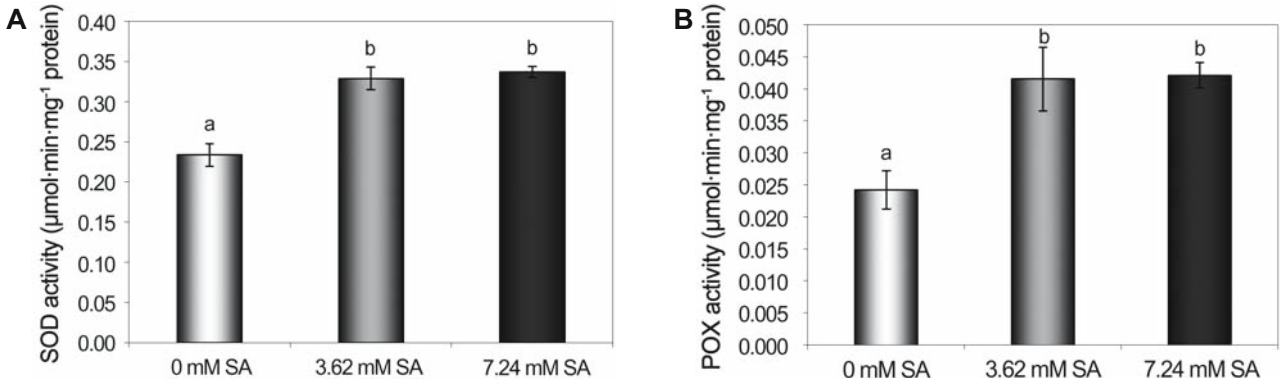

Fig. 4. Effects of SA on SOD (A) and POX (B) activity in leaves of apple (*Malus domestica* Borkh cv. Red Chief Delicious) plants. Vertical bars represent SE (n = 4).

leaves of SA-treated trees. The highest increase was observed under 7.24 mM SA treatment, with POX activity increasing by 73.64% and SOD activity increasing by 44% ($p \leq 0.05$; Fig. 4). Treatment with suitable concentrations of SA improves chilling tolerance in banana (Kang et al., 2003b), hot pepper (Zhang et al., 2008), grape (Wang and Li, 2006), hybrid maize (Farooq et al., 2008) and watermelon (Lu and Yu, 2004), mainly by activating antioxidant enzymes (including catalase, glutathione reductase, peroxidase and superoxide dismutase).

During cold acclimation in plants, a complex response takes place at the cellular, physiological and molecular levels, resulting in the enhancement of chilling and freezing tolerance. The effect of cold acclimation on chilling and freezing tolerance may be mediated by various plant growth regulators (Kocsy et al. 2001). The current results suggest that exogenously applying SA to apple trees via soil treatment may increase antioxidant enzyme activities as well as protein and proline levels, alleviating late spring frost effects. The apoplastic regions of leaves contain antioxidant enzymes and antifreeze proteins (Livingston and Henson 1998). Cellular antioxidant enzyme activity (Janda et al. 2003) and the levels of apoplastic antifreeze proteins (Antikainen and Griffith 1997) are highly correlated with freezing tolerance in plants. Therefore, SA may increase freezing tolerance in apple plants by affecting antioxidant enzyme activity and the levels of antifreeze proteins.

Conclusion,

Plants are sessile organisms and thus have to endure environmental challenges such as drought and cold temperatures. The present study suggests that SA treatment can ameliorate the deleterious effects of low temperatures on apple plants. Further research is required to determine the effects of different concentrations of SA or SA combined with other plant growth regulators. Inducing multiple stress tolerance in plants by exogenous application of SA and its derivatives may have significant practical, economic and environmental applications to agriculture, horticulture, forestry and landscaping.

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