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Calcium Ascorbate Attenuates Chilling Injury of Tomato Seedlings by Altering the Bioactivity of Phenolic Compounds

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ABSTRACT

The present investigation focused on the role of calcium ascorbate (CaAsc) on tomato seedlings under low-temperature stress. Study was conducted by spraying aqueous solution of CaAsc at 0, 50, and 100 mM, on tomato seedlings with 5-7 true leaves in order to counteract the adverse impacts of chilling stress. One week after foliar application of CaAsc, all seedlings were exposed to a chilling temperature of 3±0.5 °C for six days (six hours per day) in a growth chamber. Loss of membrane integrity due to chilling stress led to oxidation of phenolic compounds by activation of peroxides (POD) and polyphenol oxidase (PPO) enzymes. Higher phenylalanine ammonia-lyase (PAL) activity, which is responsible for phenolic compounds accumulation, due to chilling temperature, has been considered as defense mechanism of chilling stress. The results revealed that application of 50 mM CaAsc ameliorated chilling injury of tomato seedling, which was associated with lower electrolyte leakage (16.2 %) and malondialdehyde (1.54 nmol g-1 FW) accumulation. Also, CaAsc-treated seedlings with 50 and 100 mM of CaAsc exhibited higher total phenols accumulation (36.2 and 28.4 mg pyrogallol g-1 FW, Respectively) which results from higher PAL enzyme activity concurrent with lower POD and PPO enzymes activity. Enhancing chilling tolerance in tomato seedling treated with 50 mM CaAsc by triggering phenols metabolism was associated with better seedling growth rate.

Introduction

To early harvest of tomato in the field, seedlings have to be planted during early months of spring in many parts of temperate zones before temperatures reach to the optimum ranges (15–22 °C). Due to the temperature fluctuations, seedlings may be exposed to a temperature cycling between chilling and optimum ranges for few days before temperatures stabilize. Temperature fluctuations affect plant stabilization and may retard growth, delay flowering, reduce total yield and quality and even kill the plants (Baninasab, 2009; Korkmaz et al., 2010; Aghdam and Bodbodak, 2013). Also, chilling injuries may further result in membrane malfunction and reactive oxygen species (ROS) accumulation in sensitive horticultural crops with tropical and subtropical backgrounds such as cucumber (Cao et al., 2014; Nasibi et al., 2020), tomato (Ma et al., 2018) and watermelon (Jiao et al., 2020).

Phenolic compounds such as flavonoids and phenyl propanoids increase under abiotic stress conditions that may cause chilling tolerance to plants (López-Velázquez et al., 2020; Rivero et al., 2001). Although the activity of phenols oxidation enzymes such as peroxides (POD) and polyphenol oxidase (PPO) increases in response to biotic and abiotic stresses, both enzymes have been associated with the appearance of physiological injuries caused by chilling stress in plants (Martínez-Téllez and Lafuente, 1997). The hydroxylation of monophenols to o-diphenols

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and then to o-quinones is catalyzed by PPO enzyme (Barbagallo et al., 2012; Taranto et al., 2017; Thipyapong et al., 2004). Researches have also revealed the potential roles of PPO in improving plant defense against herbivorous insects and pathogen resistance (Wang and Constabel, 2004; Zhang and Sun, 2021). Nevertheless, it has been demonstrated that suppression of PPO increases stress tolerance in tomato (Thipyapong et al., 2004). Therefore, any inhibiting factor of PPO activity may help susceptible crops to withstand against stresses such as drought and chilling through maintaining phenolic compounds content.

Several methods have been suggested for PPO activity reduction in plants which results in resistance to cold stress. Barbagallo et al. (2012) reported that degradative enzymatic activity of PPO in eggplant cubes decreased by dipping into calcium ascorbate (CaAsc) solution. CaAsc is a strong antioxidant and commercial anti-browning agent to be used for fresh-cut apple (Aguayo et al., 2015; Fan et al., 2005). CaAsc may affects PPO activities when sprayed on seedlings. It probably increases ascorbic acid levels as a strong antioxidant and scavenge over-produced ROS under stress condition. However, no information is available about the CaAsc treatments in tomato seedlings. In this sense, the aim of this work was to study the effects of CaAsc treatment on phenolic metabolism, activity of phenols oxidizing enzymes (PPO and POD), phenolic compounds biosynthesing enzyme (phenylalanine ammonia-lyase) and chilling injuries of tomato seedlings.

Materials and Methods

Plant material and cultural practice

Tomato seeds, cv. Early Urbana, were soaked in distilled water for 24 h at room temperature. The seeds were sown immediately into 1.5 L pots (made with black polyethylene) filled with a 1:1:1 mixture of fine sand, leaf mold and clay loam soil. The pots were transferred into the greenhouse with an average temperature of 25/20 °C (day/night) and natural light. When seedlings achieved 5-7 leaves (35 days after sowing) were treated with 0 (control), 50 and 100 mM of CaAsc solution until both sides of leaves were completely wet. Irrigation was done twice a week to keep the optimum moisture level in the growth medium.

Imposition of chilling stress

One week after foliar CaAsc application, all seedlings were exposed to a chilling temperature of 3±0.5 °C for six days (six hours per day) in a growth chamber and then returned to the greenhouse. Seventy two hours after the end of chilling imposition, the following parameters were assessed to evaluate their resistance to chilling stress (Baninasab, 2009).

Injury rating scales (IRS)

IRS was visually assessed on leaves and shoots and classified using the following scales (Korkmaz et al., 2010): Normal (1), no visible symptoms; trace (2), small necrotic areas on shoots but without growth restriction (less than 5% of necrotic leaf area); slight (3), small necrotic areas on shoots (less than 15% of necrotic leaf area); moderate (4), well defined necrotic areas on shoots (less than 30% of necrotic leaf area); and severe (5), extensive necrotic areas and severe growth restriction (more than 50% of necrotic leaf area but plant still alive).

Electrolyte leakage (EL)

EL was used to assess the membrane permeability. EL was measured using an electrical conductivity meter (model 664, Met Rohm, Herisau, Switzerland) according the method described by Lutts et al. (1995). Six randomly chosen leaf discs per plant per replicate were taken from the youngest fully expanded leaf and placed into the test tubes containing 10 mL distilled water. The samples were shaken for 24 hours at room temperature. Electrical conductivity (EC) of solution following 24 h shaking (EC1) was recorded and the then same samples were placed into a boiling water bath for 20 min. The second measurement (EC2) was performed when the solution cooled to room temperature and electrolyte leakage percent calculated.

Relative water content (RWC)

RWC was determined by using 1 cm sized discs from the middle portion of the leaves which randomly taken from the seedlings of each replicate. Discs were freshly weighed (FW) and immediately floated in distilled water for five hours in the dark. Turgid weights (TW) of leaf discs were obtained after removing excess surface water with paper towels. Dry weights (DW) of discs were measured after drying at 75 °C for 48 h (Korkmaz et al., 2010). RWC was calculated by using the following equation:

$$RWC\ (\%) = (FW - DW)/(TW - DW) \times 100$$

Chlorophyll and carotenoid contents

Sample of leaves (0.25 g) was homogenized with 5 mL of acetone (80%) in a pestle and mortar and centrifuged at 3000 rpm. The absorbance of

supernatant was measured with a UV/visible spectrophotometer (Shimadzu UV-1280) at 470, 663 and 645nm and chlorophyll and carotenoid contents calculated with the following equations (Lichtenthaler and Wellburn, 1983):

Chlorophyll a (µg ml-1) =12.21(A663)-2.81(A646)

Chlorophyll b (µg ml-1) = 20.13(A646)-5.03(A663)

Carotenoids (µg ml-1) = (1000A470-3.27 [chl a]-104 [chl b])/227

Malondialdehyde content (MDA)

Levels of lipid peroxidation were expressed as the malondialdehyde (MDA) contents production and determined by useing the thiobarbituric acid method (Cao et al., 2014). The leaf samples (1 g) were homogenized in 10 mL of 0.1% trichloroacetic acid and the obtained homogenate was centrifuged at 15,000 rpm for 5 min. Four mL of 0.5% thiobarbituric acid in 20% trichloroacetic acid was added to a 1 mL aliquot of the supernatant. The mixture was heated at 95 °C for 30 min and quickly cooled in an ice bath. After centrifugation at 10000 rpm for 10 min, the absorbance was recorded at 532 nm. The value for nonspecific absorption at 600 nm was subtracted. The MDA content was calculated using its absorption coefficient of 155 mmol cm-1.

PPO activity

The PPO extract was prepared by using the method proposed by Thipyapong et al. (1995). Tomato leaves were ground to a fine powder in a pestle and extracted at a ratio of 150 mg fresh weight to 1 ml extraction buffer (100 mM Tris–HCl, pH 7.0, 100 mM KCl, 1 mM phenyl methane sulfonyl fluoride and 3% [w:v]PVP) containing SDS at 0, 0.5, 1, 2, or 4 (w:v) each. The homogenates were centrifuged at10000 ×g for 15 min, and the supernatant was used to assess the PPO activity. The PPO activity was assayed as described by Nicoli et al. (1991) with some modifications. The assay mixture consisted of 30 mM pyrogallol in 100 mM phosphate buffer, pH 7.0. Bovine liver catalase was used to prevent peroxidation of the substrate. PPO activity was determined by the change in A410 of the assay mixture (30°C) based on the measurement of the disappearance of pyrogallol by enzymatic oxidation. To investigate whether the reaction was enzymatic, the sample extract was boiled and then assayed.

POD activity

POD extraction and assay were carried out using the method described by Rivero et al. (2001). Fresh leaves was ground with 50 mM Tris–acetate buffer, pH 7.5, containing 5 mM 2-mercaptoethanol, 2 mM 1,4-dithio-DL-threitol (DTT), 2 mM ethylene diamine tetraacetic acid (EDTA), 0.5 mM PMSF, and 1% (w:v) PVP. The homogenate was centrifuged for 30 min at 13500×g and supernatant was collected for peroxidase assay. POD activity was determined following the change of A485 due to guaiacol oxidation at 30 °C. The reaction mixture contained 100 mM Tris–acetate buffer, pH 5.0, 1 mM guaiacol and 0.003 mM H_2O_2. To test whether the reaction was due to POD activity, control assays contained catalase from bovine liver (420 units in 0.1 ml H_2O). To determine whether the reaction was enzymatic, the sample extract was boiled and then assayed.

PAL activity

PAL activity was measured using the procedure of Qin et al. (2003) and the results were expressed as nmol cinnamic acid h-1 mg-1 of protein.

Total phenolics compounds (TPC)

TPC was measured by following the Tomás-Barberán et al. (2001) method. The extraction technique for each sample was performed using water: methanol (2:8) containing 2mM sodium fluoride (to inactivate polyphenol oxidase activity and prevent phenolic degradation) and quantified using the Folin–Ciocalteu reagent. Results were expressed as mg pyrogallol equivalent 100 g^{-1} FW.

After determination of chilling injury, shoots of seedlings were cut at the ground surface and the fresh weights recorded. Roots of seedlings were carefully washed under running tap water to remove growth medium and dried with paper towels to remove surface water and the fresh weights recorded. Shoots and roots were dried at 80°C for 72 h and dry weights determined.

Statistical analysis

The employed experimental design was a one-way completely randomized design and CaAsc concentration in three level was as sources of variation. All analyses were carried out in three replicates. The data were subjected to analysis of variance in SAS software (ver. 9.4, SAS, Inc. Cary, NC) and the means compared using the Duncan's Multiple Range Test.

Results

The outcome of studies proposed a strong protection of CaAsc against chilling injury in tomato seedlings. The highest and lowest injury rating scale (IRS) referred to controls and in the

seedlings treated with 50 mM of CaAsc, respectively (Table 1). Because of the death of the seedlings, many parameters were not detectable in non-treated control plants, therefore in most cases; comparison was performed between the seedlings treated with 50 and 100 mM CaAsc only.

Application of 50 mM CaAsc provided more protection of seedlings against chilling stress, so that the treated plants showed significantly less IRS, EL and MDA when compared to the other treatments (Table 1). Also, 50 mM CaAsc treated seedlings had the higher chlorophyll a and b content, however, total chlorophyll content was not affected by CaAsc treatment at different concentrations. In addition, high stem diameter and plant height were observed in CaAsc-treated seedlings.

Table 1. Some parameters of tomato seedlings in response to calcium ascorbate (CaAsc) treatments under chilling stress condition

Calcium ascorbate (mM)	Height (cm)	Stem diameter (mm)	Chilling index (Scale)	Electrolyte leakage (%)	Malondealdehyde content (nmol g-1 FW)	Relative water content (%)
0	19.12±0.55	9.32± 0.52	4.75± 0.25	-	-	-
50	24.75± 1.39	11.04±0.16	1.16±0.09	16.26± 1.11	1.54± 0.04	70.9± 0.54
100	21.61± 1.25	11.28±0.12	1.66± 0.36	25.38±1.23	2.55± 0.13	72.44± 0.89
Significance	* a	**	***	***	**	ns

Data are means ± SE (n=12).
a Levels of significance represented by: *P<0.05, **P<0.01 and ***P<0.001.
ns: not significant

PAL activity varied significantly in response to the CaAsc concentration. The highest PAL activity was occurred in the seedlings treated with 50 mM CaAsc. The total phenolic compounds (TPC) showed significant differences in response to CaAsc application, the lower concentration of CaAsc responding to the highest phenolic accumulation, which had significant differences with the high concentration of CaAsc (Table 2). Oxidative activities of PPO and POD decreased with the application of CaAsc being quite significant in showing an inverse relationship associated with the PAL activity and TPC accumulation. TPC were positively correlated with the PAL activity (Fig. 1), but negatively with PPO and POD activities (Fig. 2).

Table 2. Chlorophyll content and phenolic metabolism of tomato seedlings in response to calcium ascorbate (CaAsc) treatments under chilling stress condition

Calcium ascorbate (mM)	Chlorophyll a	Chlorophyll b	Total chlorophyll	TPC	PPO activity	POD activity	PAL activity
0	-	-	-	-	-	-	-
50	20.87±0.18	7.3± 0.05	28.21± 0.24	36.26±0.73	18.56±1.4	10.1±0.4	50.95±0.85
100	19.98± 0.14	8.56±0.08	28.54±0.21	28.4±0.92	24.88±1.27	13.9±1.11	41.92±1.05
Significance	**b	***	ns	*	*	*	**

a Chlorophyll: mg g-1 FW; Total phenolic compounds (TPC): mg pyrogallol g-1 FW; PPO activity: mmol pyrogallol mg^{-1} protein min^{-1}; POD activity: mmol oxidized guaiacol mg^{-1} protein min^{-1}; PAL activity: mmol cinnamic acid mg^{-1} protein h^{-1}
Data are means ± SE (n=12).
b Levels of significance represented by: *P<0.05, **P<0.01 and ***P<0.001.
ns: not significant

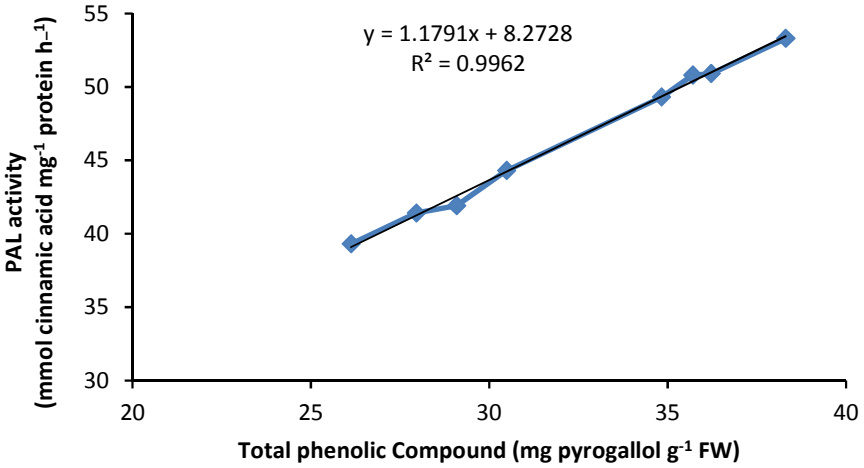

Fig. 1. Relationship between the total phenolic compounds and PAL activity in tomato seedlings under chilling condition

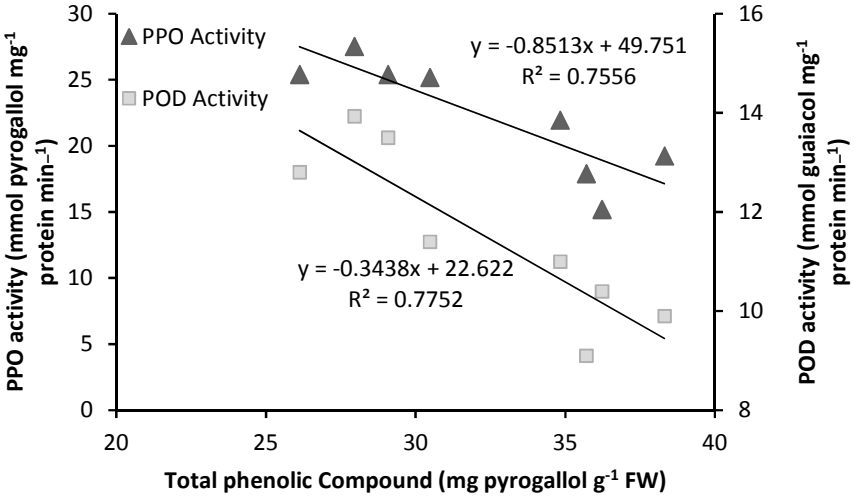

Fig. 2. Relationship between the total phenolic compounds and PPO and POD activity in tomato seedlings under chilling condition

Discussion

The control seedlings were completely injured due to imposition of chilling stress at 3 °C and ultimately showed the highest IRS in which all seedlings were killed by imposing chilling stress. Generally, the chilling symptoms such as wilting and necrosis of leaves primarily occur in chilled-seedlings and peroxidation of unsaturated fatty acids in cell membrane led to increase of MDA content. Membrane damage initiates a cascade of secondary reactions resulting loss of cellular integrity and consequential tissue death (Sayyari et al., 2011; Boari et al., 2014; Ma et al., 2018). The damages could be estimated through the measurement of electrolyte leakage (EL), which was lower in the leaves of the CaAsc treated seedlings (Table 1). According to the results, CaAsc treatments showed a concentration-dependent role in maintaining membrane permeability, in such a way that over 50 mM applications of CaAsc were not only less effective to increase the plants tolerance to low temperature stress, but also had adverse effects such as necrosis and desiccation of the leaves. Loss of selective permeability of the cell membrane occurs during low temperature exposure, leading to increase leakage of ions into the cell wall (Ni et al., 2005; Rico et al., 2007). This allowed us to hypothesize that the application of CaAsc could develop chilling resistance and membrane integrity attributable to membrane stabilization, accumulation of cell wall calcium pectate and eventually improvement of turgor pressure and cell

functions (Mignani et al., 1995). Similar findings have also been reported by Aguayo et al. (2010) when they treated apple slices with CaAsc which led to a reduction in the firmness of slices loss during storage probably due to the role of calcium in cell membranes and the antioxidant activity of ascorbate.

Changes in phenolics compounds metabolism were another important physiological effect of the CaAsc treatment. Activity of various enzymes regulate metabolism of soluble phenolics compounds in plants (López-Velázquez et al., 2020). Deamination of L-phenylalanine followed by converting to cinnamic acid and ammonium is the first necessary step for the biosynthesis of phenylpropanoid skeleton in higher plants. The PAL enzyme is commonly assumed to be the principal enzyme involved in the biosynthesis of phenolic that catalyzes the mentioned reaction. PAL activity is affected by a great number of biotic and abiotic factors and considered by most authors to have an important role in the maintenance of cell tolerance against abiotic stresses in plants (Levine et al., 1994; Leyva et al., 1995). Our research revealed that CaAsc treatment increased PAL activity follow by accumulation of phenolics compounds. The relationship between PAL activity and soluble phenolics compounds concentration in tomato seedlings (r^2=0.99, Fig. 1) indicated that the accumulation of phenolics compounds in response to CaAsc treatment is caused by the activation of PAL enzyme. Accumulated phenolics compounds through the CaAsc treatments induced chilling tolerance in tomato seedlings. Phenolics compounds accumulation can scavenge ROS, chemically reactive molecules that overproduce during imposition of environmental stress, thereby resulting in significant damage to the cell membranes, nucleic acids and proteins (Aroca et al., 2003; Król et al., 2014; Murata et al., 2012; Shi et al., 2019).

The metabolism of phenolic compounds includes the action of PPO and POD enzymes that inhibited by 50 mM CaAsc-treated seedlings. These enzymes catalyze the oxidation of phenols to quinones (Rivero et al., 2001; Thipyapong et al., 1995). The relationship between POD and PPO activity and soluble phenolic compounds suggests that the application of CaAsc can induce chilling tolerance in plants by lowering the activity of these enzymes and inhibiting oxidation of the phenolic compounds. These bioactive compounds may form complexes with some metals such as Cu and Fe and eliminate the activity of catalyzing enzymes with chelating prosthetic group of enzymes such as PPO and POD (Król et al., 2014). Both enzymes have been related to the appearance of physiological injuries caused in plants by chilling and thermal stresses (Ruiz et al., 1999; Rivero et al., 2001). Similarly, the activities of SOD, GPX and APX were increased in chickpea seedlings under chilling condition and associated chilling injury symptoms (Genisel et al., 2013). Therefore, any factor that inhibits PPO or POD activity may enable susceptible crops to biotic and abiotic stresses such as chilling injures.

PPO activity and PPO-mediated phenolic oxidation leads to the production of quinones and ROS. Once the cell compartmentalization is disrupted as a result of chilling-induced damages to the cell organelles membranes, PPO is released from the chloroplasts to react with its phenolic substrates from the vacuole. Consequently, CaAsc-induced accumulation of phenolic compounds is activated, perhaps as a result of the acclimation to overcome chilling stress in tomato seedlings.

Conclusion

Application of 50 mM CaAsc provided more protection of seedlings against chilling stress, so that the treated plants showed significantly less IRS, EL and MDA when compared to the other treatments. In addition, high stem diameter and plant height were observed in CaAsc-treated seedlings. Oxidative activities of PPO and POD decreased with the application of CaAsc being quite significant in showing an inverse relationship associated with the PAL activity and TPC accumulation. Also, 50 mM CaAsc treated seedlings had the higher total phenolic compounds and it was positively correlated with the PAL activity, but negatively with PPO and POD activities.

Conflicts of Interest

The authors indicate no conflict of interest for this work.

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