Evaluation of low temperature tolerance indices in seedlings of *Citrus aurantium* under potassium nitrate nutrition

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Abstract

In order to evaluate the effects of potassium nitrate nutrition on some low temperature tolerance indices in *Citrus aurantium* seedlings a factorial experiment was conducted based on a completely randomized design with four replications at the Institute of Citrus Research in Ramsar. Treatments were potassium nitrate nutrition at four levels (0, 2.5, 5, and 10 mM) and temperature at four levels (25, 0, -3, and -6 °C). Results showed that increasing the concentration of KNO_3 reduced superoxide dismutase activity and carotenoid. Concentration of 10 mM potassium nitrate also increased water content, leaf color, proline, phenol, and total chlorophyll. Maximum leaf damage, electrolyte leakage, and carotenoid were observed at -6 °C, while the highest antioxidant capacity and superoxide dismutase activity with means 37.7% and 24.4 IU. mg $^{-1}$ FW, respectively were observed in *Citrus aurantium* leaves at -3 °C. Highest electrolyte leakage was observed at concentration 0 mM potassium nitrate and temperature of -6 °C while highest superoxide dismutase was observed at -3 °C with the same concentration of potassium nitrate. Results revealed that concentration of 10 mM potassium nitrate increases *Citrus aurantium* tolerance under low temperature stress.

Key words: *Citrus aurantium* potassium nitrate; low temperature stress; proline

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Introduction

Citrus is a genus of flowering plants in the family Rutaceae and a tropical and subtropical plant which is sensitive to cold stress (Clark and Prakash, 2001). *Citrus aurantium* is one of commercial citrus varieties that is considered as a biotic and abiotic stress tolerant rootstock (Kaanane et al., 1988). Low temperature is the major environmental stress that limits plant growth, productivity, and distribution (Campos et al., 2003). Citrus crops which are among the tropical and subtropical fruits are generally classified as cold tender plants so these crops are vulnerable to freezing stress (Fotouhi et al., 2008). The first place that reacts to low temperature stress, is cell membrane (Chen et al., 2006). Overall, chilling results in loss in membrane integrity and increase in active oxygen radical productions, which leads to leaf damage and electrolyte leakage. The integrity of

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intracellular organelles is also disrupted leading to the loss of compartmentalization, reduction and impairing of photosynthesis, chlorophyll pigments, protein assembly, and general metabolic processes (Mahajan and Tuteja, 2005; Saneoka et al., 2004). Low temperature stress may lead to the formation of reactive oxygen species (ROS) such as superoxide radicals, hydrogen peroxide, hydroxyl, and single oxygen (Ruth, 2002). Hatamnia et al. (2013) reported that reactive oxygen species (ROS) production and antioxidant enzyme activity increased in abiotic stress condition. ROS, which is produced in chloroplast during metabolic processes, can destroy cellular components such as proteins, pigments, membrane, lipids, and nucleic acids (Hernandez et al., 2001). The participation of low temperature and increasing antioxidant capacity of citrus fruit have been demonstrated (Rapisarda et al., 2008). Generally, low temperature stress is considered as a damage factor particularly in Iranian north and south citrus trees. To avoid this phenomenon, in addition to mechanical methods can be used effective security counsels to increase plant resistance against cold and frost damage. One of the strategies to increase tolerance of plants under low temperature and one of the important activities of plant compatibility to cold stress, is osmotic balance reaction to maintain plant water content. These activities are affected by osmotic pressure regulatory concentration compounds such as secondary metabolites including proline and some inorganic ions such as potassium.

Potassium increases cells' tolerance against cold stress because it affects freezing point of liquid inside the vacuoles (Obreza, 2003). Potassium is one of macronutrient elements in plants which composes about 3-5% of their dry weight. Potassium can affect plant survival under different environmental stress by creating osmotic balance and protection against oxidative damage (Liu and Zhu, 1997). Potassium influences many physiological processes such as starch and sugar formation, protein synthesis, cell division, and plants growth (Obreza, 2003). Gong et al. (2011) reported that proline aggregated in corn starch under potassium deficiency and low temperature stress. Research results on ginseng plants under low temperature stress and potassium nitrate showed that potassium can increase effective genes expression on reaction of secondary metabolites and improve antioxidant reactions in decreasing reactive oxygen species (ROS) and so plant amplification survival under low temperature stress (Devi et al., 2012). In fact, the optimal concentration of potassium nitrate associated with water content maintain can affected on survival of plants under low temperature (Lindhauer et al., 1986). It is reported that potassium fertilizer increased inflationary turgor pressure and reduced the osmotic potential in cotton (Pervez et al., 2004).

The current experiment was an attempt to determine the effect of potassium nitrate on plant responses of *Citrus aurantium* in low temperature stress.

Materials and Methods

To study low temperature tolerance indices in seedlings of *Citrus aurantium* under potassium nitrate nutrition, an experiment was conducted in a factorial plan based on completely randomized design. Treatments included temperature at four levels, namely, 25, 0, -3 and-6 °C and four levels of potassium nitrate, namely, 0, 2.5, 5 and 10 mM. Seedlings were grown in soilless culture and foliar feeding with Hoagland solution (Hogland and Arnon, 1950). At the beginning of incubation, the temperature was lowered by 1 °C/day and cold acclimation was set up from 23 to 10 °C after 2 weeks. The plants were kept in each temperature for 24h (Pietrini et al., 2005). Then, leaf damage, electrolyte leakage (by fresh leaf), leaf color, leaf water content (five weeks after transfer from the incubator), total chlorophyll, carotenoid, antioxidant capacity, proline, phenol, and superoxide dismutase were assessed. Electrolyte leakage was assessed as described by Lutts et al. (1996).

Leaf discs were placed in closed vials containing 10 ml of deionized water and incubated at 25 °C on a rotary shaker for 24 h; subsequently, electrical conductivity of the solution (*Lt*) was determined. Samples were then autoclaved at 120 °C for 20 min and the last electrical conductivity (*L*0) was obtained after

equilibration at 25 °C. The electrolyte leakage was defined as follows:

Electrolyte leakage (%) = $(\frac{Lt}{L0}) \times 100$

Leaf color was determined by a colorimeter device (Minolta CR 400, Japan). Then numbers were calculated based on green leaves percentage (Sanchez et al., 2003). Also, leaf water content was calculated based on the following formula proposed by Versleous et al. (2006):

Leaf water content = $[(\frac{fresh\ weight\ of\ leaf - dry\ weight\ of\ leaf}{fresh\ weight\ of\ leaf})] \times 100$

To measure total chlorophyll and carotenoid content, one g leaf sample was ground in 90% acetone. The absorbance was measured with a UV/visible spectrophotometer (Pye Unicam SP6-550, UK) and total chlorophyll and carotenoid concentrations were calculated using the equations proposed by Strain and Svec (1966):

Total Chl = Chla + Chl b, and

Total carotenoid = (1000 A 470 -1.8 Chla -85.02 Chl b)/198

Where A470 represents absorbance values read at 470 nm wavelengths.

DPPH free-radical scavenging of the methanolic extracts was measured according to the method by Kim et al. (2003). Briefly, methanolic extract (0.1 ml) was incubated in 0.4 ml of 0.1 M HCl (pH 7.5 ± 0.1) and 0.5 ml of 0.3 mM DPPH in the dark at room temperature for 20 min. The absorbance of the solution (as sample) was measured at 517 nm and compared to the absorbance of a control (as control). The free-radical scavenging was calculated according to the following equation:

% DPPH free-radical scavenging= $(1 - \frac{Asample}{A\ control}) \times 100$

Proline was determined according to the method described by Bates et al. (1973). Approximately, 0.5 g of fresh leaf material was homogenized in 10 ml of 3% aqueous sulfosalicylic acid. Two milliliter of the filtrate was mixed with 2 ml of acid- ninhydrin and 2 ml of glacial acetic acid in a test tube. The mixture was placed in a water bath for 1 h at 100 °C. The reaction mixture was extracted with 4 ml toluene and the absorbance was measured at 520 nm with a Shimadzu UV 1601 spectrophotometer.

The total phenolic concentration of each sample was determined by the modified Folin–Ciocalteu method (Singleton and Rossi, 1965). Methanolic extract (50 µl) was incubated in 450 µl of deionized water, 250 µl of the Folin–Ciocalteu phenol reagent, and 1.25 ml of 0.2 M Na_2CO_3 in the dark at room temperature for 20 min. The absorbance of the solution was measured at 735 nm and compared to a gallic acid standard curve. Superoxide dismutase (SOD) activity was measured by its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) at 560 nm. The reaction mixture (1 ml) consist of 75 µM NBT, 13 mM L-methionine, 0.1 mM EDTA, and 2 µM riboflavin in 50 mM potassium phosphate buffer (pH 7). The reaction and control mixture were incubated for 15 min in 300 $\mu mol.m^{-1}.\ s^{-1}$ irradiance at 25 °C. A non-irradiated reaction mixture was used as blank. One unit of SOD activity was expressed as the quantity of SOD required to produce a 50% inhibition of NBT photochemical reduction. The specific enzyme activity was measured as units per mg of leaf fresh wet (Sheng et al., 2006).

Statistical analysis was carried out using SAS 9.1 by analysis of variance and mean comparison was performed using Duncan's Multiple Range Test ($p \leq 0.05$).

Results

Table 1
Analysis of variance of potassium nitrate nutrition on low temperature tolerance indices in seedling of *Citrus aurantium*

		Mean of squares									
SOV	df	Leaf Damage	Electrolyte Leakage	Water Content	Leaf Color	Total Chlorophyll	Carotenoid	Antioxidant Capacity	Proline	Total Free Phenolic	Superoxide Dismutase Activity
Temperature (T)	3	9423**	6063**	4656**	14078.6**	19.9**	2.184**	2432**	0.1**	6.93**	792.1**
KNO_3 (K)	3	289^{ns}	54.27^{ns}	45.3*	283.5*	5.9**	2.749**	157.5**	0.04^{ns}	2.69*	454.4**
T×K	9	122.6^{ns}	70.2*	28.1^{ns}	77.3^{ns}	0.68^{ns}	0.44**	182.2**	0.06**	3.16**	125.3**
Error	48	182.9	28.17	16	83.2	0.77	0.102	14.14	0.01	0.71	3.16
CV (%)	-	21.72	22.45	8.13	11.9	12.14	11.03	18.99	11.01	21.83	11.18

*, **: significant at $p \leq 0.05$ and $p \leq 0.01$, respectively; ns: not significant

Table 2
Means values of low temperature stress for some plant indices of *Citrus aurantium* seedling.

Temperature (˚C)	Leaf damage (%)	electrolyte leakage (%)	water content (%)	Leaf color (%)	Total chlorophyll (mg g^{-1} FW)	Carotenoid (mg g^{-1} FW)	total free phenolic (mg g^{-1} FW)	Proline (mg g^{-1} FW)	Antioxidant capacity (%)
25	43^{c}	5.6^{d}	65.4^{a}	100^{a}	8.7^{a}	2.4^{d}	3.21^{c}	1.11^{c}	11.9^{c}
0	48.7^{c}	9.7^{c}	55.5^{b}	92.4^{b}	7.5^{b}	2.8^{c}	4.29^{c}	1.16^{bc}	11.6^{c}
-3	60.4^{b}	32.4^{b}	50.6^{c}	80.5^{c}	7^{b}	3.0^{b}	5.01^{b}	1.22^{ab}	37.7^{a}
-6	97^{a}	46.9^{a}	25.3^{d}	33.9^{d}	6^{c}	3.3^{a}	7.2^{a}	1.29^{a}	18^{b}

Means in each column followed by similar letters are not significantly different at the 5% probability Level using Duncan Test.

Table 3
Mean values of potassium nitrate nutrition for some indices of *Citrus aurantium* seedling

KNO3(mM)	Water content (%)	Leaf color (%)	Antioxidant capacity (%)	total free phenolic (mg g^{-1} FW)	Superoxide dismutase activity (IU mg^{-1} FW)
0	47.44^{b}	71.32^{b}	16.61^{b}	2.51^{c}	10.1^{d}
2.5	48.66^{ab}	77.20^{ab}	17.7^{b}	3.47^{bc}	13.73^{c}
5	49.25^{ab}	77.61^{ab}	23.15^{a}	4.75^{ab}	22.63^{a}
10	51.46^{a}	80.54^{a}	21.73^{a}	5.09^{a}	17.13^{b}

Means in each column followed by similar letters are not significantly different at the 5% probability level using Duncan Test.

Analysis of variance revealed the significant effect of low temperature on all indices except for proline (p≤1%). Effect of potassium nitrate on characteristics of leaf water content and color was significant at 5 % probability level and on total chlorophyll, carotenoid, antioxidant capacity, proline, phenol, and superoxide dismutase this effect was significant at 1% probability level. The interaction between the two treatments on antioxidant capacity, proline, total phenol, and superoxide dismutase was also significant at p≤0.01 (Table 1). Mean comparison of the main effect of temperature on studied indices showed that leaf damage percentage, electrolyte leakage, total phenol, and carotenoid content increased significantly with reduced temperature.

Antioxidant capacity and superoxide dismutase activity increased up to -3° C and the maximum amount of these indices with mean 37.7 % and 24.4 (IU/mgr FW) were observed in this temperature, respectively. Maximum leaf water content, leaf color, and total chlorophyll were assessed in control plants (Table 2 and Fig. I).

Mean comparison of the simple effect on studied factors showed that concentration of 10 mM potassium nitrate resulted in the highest values of leaf water content, leaf color, proline, total chlorophyll, and phenol. Also in 5 mM potassium nitrate, antioxidant capacity was about 28.2 % more than control treatment. The lowest

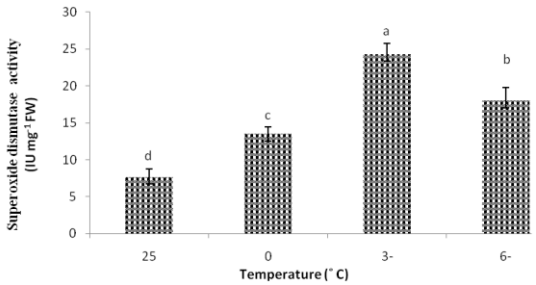

Fig. I. Effect of different temperatures on superoxide dismutase activity

superoxide dismutase activity and total chlorophyll were observed in control treatment (Table 3 and Fig. II). Results showed that

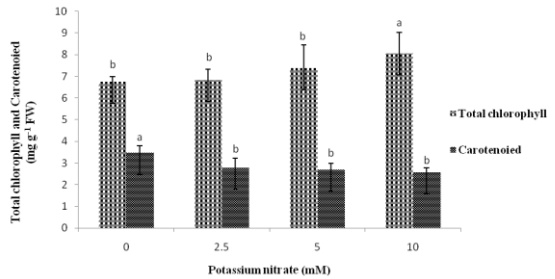

Fig. II. Effect of potassium nitrate concentrations on total chlorophyll and carotenoid content

Table 4
Means comparison of potassium nitrate × temperature effect on some indices of *Citrus aurantium* seedling in low temperature

KNO_3 (mM)	Temperature (˚C)	electrolyte leakage (%)	Antioxidant capacity (%)	Proline (mg g^{-1} FW)	total free phenolic (mg g^{-1} FW)	Superoxide dismutase activity (IU mg^{-1} FW)	Carotenoid (mg g^{-1} FW)
0	25	8.37^{de}	21.81^{d}	1.15^{bc}	2.47^{d}	20.13^{d}	3.11^{c}
	0	13.18 ^{d}	19.3^{def}	1.17^{bc}	3^{bcd}	23.07^{bc}	2.46^{ef}
	-3	33b^{c}	31.04^{c}	1.24^{bc}	2.51^{cd}	26.4^{a}	3.24^{bc}
	-6	49.34^{a}	20.47^{de}	1.3^{ab}	4.72^{bcd}	20.93^{cd}	3.71^{a}
2.5	25	6.12^{de}	7.88^{ij}	1.19^{bc}	4.39^{bcd}	7.2^{f}	2.21^{efg}
	0	10.75^{de}	8.9^{hi}	1.23^{bc}	4.39^{bcd}	21.87^{bcd}	3.36^{abc}
	-3	29.64^{c}	32.55^{c}	1.2^{bc}	4.29^{bcd}	24.13^{ab}	3.63^{ab}
	-6	48.96^{a}	21.48^{d}	1.26^{bc}	5.09^{b}	15.33^{e}	3.29^{abc}
5	25	4.04^{e}	2.68^{j}	1.07^{c}	2.47^{d}	1.73^{h}	2.62^{de}
	0	7.03^{de}	9.39^{ghi}	1.14^{bc}	3.47^{bcd}	4.53^{g}	2.61^{de}
	-3	26.37^{de}	49.16^{a}	1.21^{bc}	4.21	26.53^{a}	3.27^{abc}
	-6	48.62^{a}	16.11^{def}	1.21^{bc}	4.75^{bcd}	22.13^{bcd}	3.18^{bc}
10	25	3.7^{e}	14.9^{efg}	0.85^{d}	2.84^{cd}	1.73^{h}	1.79^{g}
	0	7.97^{de}	8.9h^{i}	1.2^{bc}	3.99^{bcd}	4.66^{g}	2.11f^{g}
	-3	40.45^{b}	38.26^{b}	1.25^{bc}	4.67^{bcd}	20.27^{cd}	3.03^{cd}
	-6	40.65^{b}	13.9^{fghi}	1.48^{a}	7.41^{a}	13.73^{e}	2.65^{de}

Means in each column followed by similar letters are not significantly different at 5% probability level using Duncan Test.

electrolyte leakage had a significant upward trend in all of potassium nitrate concentrations with decreasing temperature and the lowest value of electrolyte leakage observed in concentration of 10 mM potassium nitrate and control temperature (table 4). According to the results of the experiment, it was observed that the treatment with 5 mM potassium nitrate increased antioxidant capacity in -3° C temperature (Table 4). As shown in Table 4, with increasing potassium nitrate concentration an ascending trend was observed in proline content in leaves. Table 4 shows the interaction effect between potassium nitrate and temperature. According to the Table, the highest amount of proline was observed in *C. aurantium* leaves at -6° C temperature and the highest potassium nitrate concentration (10 mM).

The effect of different concentrations of potassium nitrate and temperature levels was also examined on the total phenol. The results showed that seedlings grown with 10 mM potassium nitrate and -6° C led to a higher phenol content (7.41 mg/gr FW) production. The results also showed that reducing temperature to -3° C led to a significant increase in superoxide dismutase activity in all potassium nitrate concentrations and maximum activity of this enzyme was observed at -3 °C and 0 mM potassium nitrate concentration (Table 4).

Discussion

Generally, results of the study demonstrated that among different temperature treatments, -6° C led to the highest leaf damage. In fact, low temperature affects cell membrane as it increases reactive oxygen species (ROS) production and damages lipids and membrane fatty acids causing loss of membrane integrity. It leads to activation of oxidation reactions and continuation of this process brings to complete leaf cell membrane damage (Allen, 1995). Similar increase in leaf damage percentage has been reported in coffee leaves under low temperature stress (Compose et al., 2003) and olive tree (Azzarello et al., 2009).

Lipid peroxidation and decrease in membrane fluidity indicators representing cell membrane injury and electrolyte leakage increased by freezing stress (Azzarello et al., 2009). Electrolyte leakage is an effective parameter to assess membrane permeability and therefore it is used as an indicator of membrane

integrity (Marangoni et al., 1996). Potassium causes leakage and maintenance of water in plant tissues with increasing osmotic pressure and cell membrane fluidity and thus it prevents cell membrane rupture, plant tissue damage, and increases electrolyte leakage (Wagner, 1967). Increase in electrolyte leakage under low temperature stress in wheat (Zhang et al., 2008), coffee seedlings (Azzarello et al., 2009), and tomato (Ghiasi and Razavi, 2013) confirm the results of this study. Webster and Ebdon (2005) found that electrolyte leakage decreased in darnel leaves (*Lolium temulentum*) under potassium nitrate nutrition and low temperature stress.

Potassium has substantial effects on stomata movement and water-relation (turgor regulation and osmotic adjustment) in plants under cold stress condition (Marschner, 1995). Numerous research studies indicated that application of high concentrations of potassium nitrate may increase leaf water content under low temperature stress (Pervez et al., 2004). The present results are in agreement with those reported on valencia leaves (Yelenosky and Guy, 1989) and walnut shoots (Poirier et al., 2010) who found that potassium deficiency reduced water content in low temperature stress. Reactive oxygen species (ROS) interferes with the balance of photosynthesis reaction and destroys cell tissues and chlorophyll pigment in chloroplast membrane and thylakoids. Therefore, leaf color reduces while carotenoid increases (Pietrini et al*.,* 2005). Potassium affects transport of sucrose from the leaves, carbon dioxide fixation, and chlorophyll production, eliminating negative effects of low temperature (Cackmak, 2005). There is a bulk of studies on some indices in *C. aurantium* including maize and rice for leaf color (Kaya et al., 2013; Yu-Chuan et al., 2008), valencia and wheat for chlorophyll (Ribeiro et al., 2009; Berova et al., 2002) and *Ilex paraguariensis* for carotenoid (Varone and Gratani, 2007).

Results of the study showed that optimum potassium concentration affected osmotic balance through accumulation of metabolites such as proline that influence osmotic pressure so it due to increase proline production that is one of secondary metabolites (Morgan, 1984). Proline, as a multifunctional amino acid, plays key roles in the osmotic regulation between cytoplasm and vacuole, the redox regulation of the NAD+/NADH ratio, membrane stabilization, and finally promoting ROS scavenging systems (Bohnert and Jensen, 1996). Studies conducted on alfalfa (Antolin and Sunchez, 1993), maize (Gong et al., 2011), and melon (Kaya et al., 2007) showed that proline was increased under potassium nutrition.

Phenolic compound is an antioxidant metabolite that impacts on neutralization of reactive oxygen species increasing plants adaptability to low temperature and increases its amount in cold stress condition. When plant growth increases and there is more photosynthesis at higher potassium rates, increased phenolic concentrations may correspondingly occur due to allocation of excess fixed carbon to the shikimic pathway (Crozier et al., 2006; Shaw et al., 1998). Low temperature stress and potassium nutrition led to an increase in total phenol as reported by Sweet Thai basil (Nguyen et al., 2010), maize (Rengel and Damon, 2008), and *Pinus banksiana* (Teklemariam and Blake, 2004). Potassium deficiency reduces photosynthetic activity because carbohydrates accumulate in leaves and absorption process of carbon dioxide is conducted incompletely. Indeed, electrons which are produced by light reactions of photosynthesis tend toward superoxide radical production increasing superoxide dismutase activity under cold stress because they do not consume carbon dioxide. There is also evidence for increased superoxide dismutase activity and antioxidant capacity in peach sapling (Leng and Qi, 2003), maize (Farooq et al., 2008; Gong et al., 2011), and *Crataegus aronia* (Kirakosiyan et al., 2003).

Conclusion

As a conclusion, this study demonstrated the positive effects of potassium nitrate on some low temperature tolerance indices. It seems that 10 mM potassium nitrate caused maintaining membrane integrity by increasing leaf water content, proline, and phenol and decreasing superoxide dismutase activity and so it due to tolerance to -6 ˚C in this experiment.

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