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Effects of exogenous ornithine enantiomers on tobacco cells under salinity conditions

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Abstract

Ornithine is a non-proteinogenic amino acid, which plays an essential role in the metabolism of plants. Regard to the chirality of the molecule, physiological response of the plant cells to its two enantiomers have not been widely investigated yet. In the present study, suspension-cultured tobacco cells were treated with 1 mM of D- and L-enantiomers of ornithine in normal conditions as well as under stress of 50 mM NaCl. Differential effects of L- and D-enanthiomers were observed either in normal or under salinity stress conditions. L-ornithin adversely affected the growth of tobacco cells in normal conditions and its detrimental effect was intensified by salinity. Treatment with D-ornithine however, not only did not change the growth but also alleviated it under salt stress. Physiological response of tobacco cells to D-ornithine in normal conditions was accompanied by increasing of polyamines. Under salinity stress however, D-ornithine treatment increased the activity of major antioxidant enzymes i.e., superoxide dismutase, catalase, and peroxidase and also increased proline content of the cells, all together resulted in lowering H_2O_2 and maintenance of cells membrane integrity. The results suggested that D-ornithine is a potent compound for activation of anti-oxidant system of plant cells and alleviation of stress condition.

Keywords: Antioxidant enzymes, (D/L)-Ornithine, Polyamines, Proline, Salt stress

Introduction

Application of certain amino acids is a well-known strategy to alleviate the stress effects upon plants. These amino acids function both as N and C sources in plant cells and stimulate metabolism and acclimation under stress conditions (1). Ornithine (Orn) is a non-protein amino acid which is used by plant cells to produce arginine, urea, polyamines (PAs), and proline (Pro). All of these compounds are of metabolites relevance for stress conditions (2, 3). Salinity is one of the major abiotic stresses which affect plant growth and productivity in many areas of the world. High salinity produces osmotic stress by decreasing the water chemical activity and affecting the cell turgor. It also causes a rapid and excessive accumulation of reactive oxygen species (ROS) (4, 5). Scavenging of ROS is mainly achieved by the activity of antioxidant enzymes, i.e., catalase (CAT; EC1.11.1.6), superoxide dismutase (SOD, EC 1.15.1.1), and peroxidase (POD; EC 1.11.1.7). A part of ROS is also scavenged by Pro, a small molecule which also functions as an osmolyte (6). Polyamines such as putrescine (Put), spermidine (Spd), and spermine (Spm) are another class of metabolites whose stress-induced accumulation and ROS-scavenging function have been reported widely (2). One of manifestations of the antioxidant effect of PAs is their ability to regulate an expression of genes encoding antioxidant enzymes, such as POD or SOD (7).
In the present study biosynthesis of PAs and Pro

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in combination with different elements of antioxidant system were measured in Orn-treated tobacco cells. The purpose was to explore the impact of this amino acid on physiological and biochemical responses to normal conditions and salinity stress. Like other amino acids Orn has two chiral forms: L and D, however due to the general belief that the plants have low capacity to metabolize D-enantiomer of most amino acids (8), little attention has been paid to the application of D-enantiomers so far. In this research differential impacts of D- and L-enantiomers of Orn on accumulation of PAs, Pro, and enzymatic antioxidants are discussed.

Materials and methods

Cell culture, treatments, growth, and viability

Suspension cultures were established from calli of tobacco cells (*Nicotiana tabacum* L. cv. Burley 21) that had been maintained in our laboratory for more than 110 subcultures. Both calli and subsequent suspensions were grown in a modified *Murashige and Skoog* (9) medium, without glycine and supplemented with 3 mgL^{-1} NAA, 3 mg L^{-1} IAA, and 1 mg L^{-1} kinetin, pH 5.8. (10). They were incubated in darkness, 25ºC on rotary shaker with 110 rpm. Preliminary studies showed that the suspension-cultured cells were in their logarithmic growth phase from day 5 to 13 (Fig. 1). Therefore, the cells were subcultured every 7 days. The amino acids and NaCl were filter-sterilized and added to the cells. Treatment with 1 mM of L- and D-Orn enantiomers (Sigma, St. Louis, MO, USA) to gather with or without 50 mM NaCl was achieved on day 6 and the cells were allowed to grow for additional 6 days and were harvested at day 12. The cells were frozen with liquid N_2, and then kept at -80°C until used for biochemical analysis. Viability assay was conducted using Evans blue (1% w/v aqueous solution). The cells were observed by a light microscope (BH2, Olympus, Japan) (11).

Biochemical analysis

The H_2O_2 content was assayed according to the method described by Velikova et al., (12). In brief, the cells (0.2 g) were homogenized with 5 mL of 0.1% (w/v) TCA in an ice bath. The homogenate was centrifuged at 12,000 ×*g* for 10 min, and 0.5 mL of the supernatant was mixed with 0.5 mL of 10 mM potassium phosphate buffer (pH 7) and 1 mL of 1 M KI. The absorbance of the solution was read at 390 nm using spectrophotometer (Cintra 6, GBC, Dandenong, Australia) and H_2O_2 content was calculated using a standard curve with concentrations ranging from 0.1 to 1 mM.

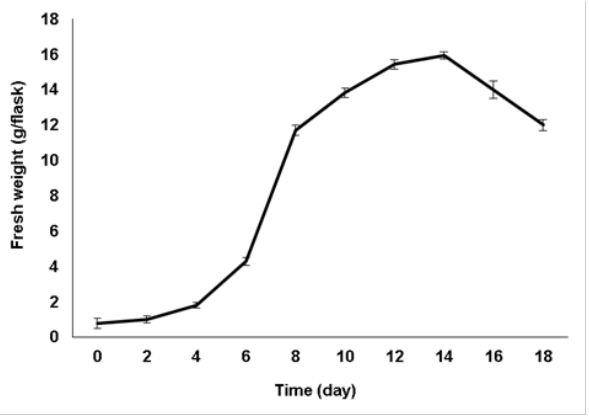

Figure 1. Growth curve of suspension-cultured tobacco cells in modified Murashige and Skoog medium. Data are presented as mean ± SD, n = 5.

The level of damage to cell membranes was determined by measuring malondialdehyde (MDA) content. Lipid peroxidation was determined by estimating MDA content as an end product of lipid peroxidation. In brief, fresh cells (0.2 g) were homogenized in 3 mL of 10% (w/v) TCA in ice bath. The homogenate was centrifuged at 10,000 ×*g* for 10 min, and the supernatant was collected. One mL of supernatant was mixed with 1 mL of 0.5% (w/v) thiobarbituric acid (TBA). The mixture was heated at 70°C for 30 min followed by rapid cooling in ice bath. Absorbance of the mixture solution was measured at 532, 600 and 440 nm by spectrophotometer. Malondealdehyde content was calculated using constant absorption coefficient of 157 mM^{-1} cm^{-1} (13).

Quantification of Pro was performed according to the method by Vendruscolo et al., (14). Briefly, 0.2 g of cell samples were homogenized in aqueous sulfosalicylic acid (3%) and centrifuged at 12,000 ×*g* for 20 min. Two milliliters of the supernatant was mixed with 2 mL of ninhydrin solution (1.25 g ninhydrin in 30 mL of glacial acetic acid and 20 mL of 6 M phosphoric acid).

Then 2 mL of acetic acid was added, and the solution was boiled at 100°C for 1 h. After cooling at room temperature, proline was extracted with 3 mL of toluene and quantified by spectrophotometer at 529 nm with proline standard solutions of 0-30 mM.

For extraction of PAs, aliquots of the cells (0.2 g) were homogenized in 1 mL of 5% (v/v) perchloric acid (PCA) and incubated at 4°C for 1 h. The homogenate was centrifuged at 12,000 ×*g* for 20 min at 4°C, and the supernatant was collected. For derivatization, an aliquot (200 µL) of each extract was added to 200 µL of saturated Na_2CO_3 and 400 µL of dansyl chloride (5 mg in 1 mL of acetone) at room temperature, mixed, and incubated at 70°C in dark for 90 min. Excess dansyl chloride was inactivated by adding 200 µL of proline (100 mg/mL) and incubating it for 30 min. Dansylated polyamines were extracted into 0.5 mL of toluene after 1 min of vortex stirring. The upper organic phase was collected and stored at -20°C for high-performance TLC (HPTLC). Thin-layer chromatography was performed using a CAMAG TLC system equipped with a Linomat 5 autosampler, TLC scanner 3, and winCATS 1.2.2 software (CAMAG, Muttens, Switzerland). Chromatography was performed on precoated silica gel 60 F254 TLC plates (10 × 20 cm, Merck, Darmstadt, Germany) using cyclohexane:ethyl acetate (5:4, v/v) as the mobile phase. Fluorescence measurement of compounds was performed via peak area after scanning with excitation and emission at λ = 250 and 366 nm, respectively. Quantification of polyamines was performed using a calibration curve obtained for each PA in the range of 0-1000 ppm (15).

Superoxide dismutase was assayed by a photochemical method based on nitroblue tetrazolium (NBT)(16). The cells (0.2 g) were homogenized in 3 mL of 50 mM HEPES-KOH buffer (pH 7.0) containing 0.1 mM EDTA. The homogenate was centrifuged at 12,000 ×*g* for 20 min at 4°C, and the supernatant was collected. Reaction mixture (3 mL) consisted of 50 mM HEPES-KOH buffer (pH 7.8), 0.1 mM EDTA, 50 mM Na_2CO_3 (pH 10.2), 12 mM L-methionine, 75 µM NBT, 300 µL enzyme extract and one µM riboflavin. One unit SOD activity was defined as the amount of enzyme required to result in a 50% inhibition of the rate of NBT reduction measured at 560 nm and was manifested against mg of protein of the extract. Protein content was determined by the method of Bradford (17), using bovine serum albumin as a standard.

Peroxidase activity was determined with guaiacol as the substrate in a total volume of 3 mL. The reaction mixture contained 60 mM sodium phosphate buffer (pH 6.1), 28 mM guaiacol, 5 mM H_2O_2 and variable amounts of the enzyme preparations. The turnover of guaiacol was monitored using spectrophotometer at 470 nm for 1 min. The activity was referred to the protein content of each enzymatic fraction (18).

For measurement of CAT activity, frozen sample (0.2 g fresh weight) were homogenized in 3 mL of sodium phosphate buffer (25 mM, pH 6.8). The homogenate was centrifuged at 12,000 ×*g* for 20 min at 4°C. Catalase activity was measured by adding H_2O_2 and decrease in the absorbance at 240 nm for 1 min. Enzyme activity was defined as the change of the absorbance against mg of protein (19).

Statistical analysis

All of the experiments were carried out with three independent repetitions each with five samples. Statistical program SPSS (version 16, Chicago, IL, USA) was used and the significance of differences between treatments was evaluated using LSD test at level of $p \leq 0.05$.

Results

Exposure of the tobacco cells to NaCl resulted in high suppression of their viability and growth (to 50% of the control group) (Table 1). While D enantiomer of Orn had no effect on cell growth and viability, L-Orn adversely affected it and this detrimental effect was intensified by salinity. D-ornithine in salinity condition however, to some extent, restored the viability and growth of the cells (Table 1). Total soluble protein content of NaCl-treated cells were significantly higher than those of the control group, while those of Orn-treated cells were identical to the control one. When salinity was accompanied by Orn treatment, protein content of the cells significantly decreased (Table 1).

The rate of membrane lipids peroxidation and accumulation of H_2O_2 are shown in Figure 2. Salinity conditions significantly increased H_2O_2

and MDA contents of the cells, compared to the control group (Fig. 2). Exposure to L-Orn promoted the rate of lipid peroxidation and accumulation of H_2O_2 and these effects were more pronounced when exposure to L-Orn was accompanied by salinity, compared to the control group (Fig. 2). Treatment of tobacco cells with D-Orn increased MDA a nd H_2O_2 contents, compared to the control group. In comparison with salinity conditions however, combination of D-Orn with salinity treatment significantly reduced both MDA and H_2O_2 of the cells (Fig. 2). Neither treatment with Orn enantiomers nor NaCl alone did not change Pro content of the cells (Fig. 3). Combined treatment of the cells with L-Orn and NaCl significantly reduced Pro content of the cells while D-Orn+ NaCl significantly increased it, in comparison with the control cells (Fig. 3).

Table 1. Growth parameters of tobacco cells treated with or without 50 mM NaCl and 1 mM (D/L)-Orn. Data are presented as mean ± SD, n = 5.

Growth Parameters	Treatments					
	Ctrl	**NaCl**	**L-Orn**	**D-Orn**	**L-Orn + NaCl**	**D-Orn + NaCl**
Net growth (g FW/flask)	15.88± 0.88^{a*}	6.95± 0.15^{d}	13.27± 0.36^{b}	14.97± 0.47^{a}	4.44± 0.46^{e}	11.75± 0.43^{c}
Viability (%)	97.2± 1.5^{a}	48.2± 0.9^{d}	86.4± 3.31.2^{b}	93.6± 9.41^{a}	39.8± 2.1^{e}	83.7± 0.8^{c}
Total protein (mg/gFW)	62.98± 2.78^{b}	69.92± 3.24^{a}	60.01± 3.01^{b}	61.82± 2.97^{b}	50.04± 0.97^{c}	40.85± 1.55^{c}

*Different letters show significant differences at p≤0.05 according to LSD.

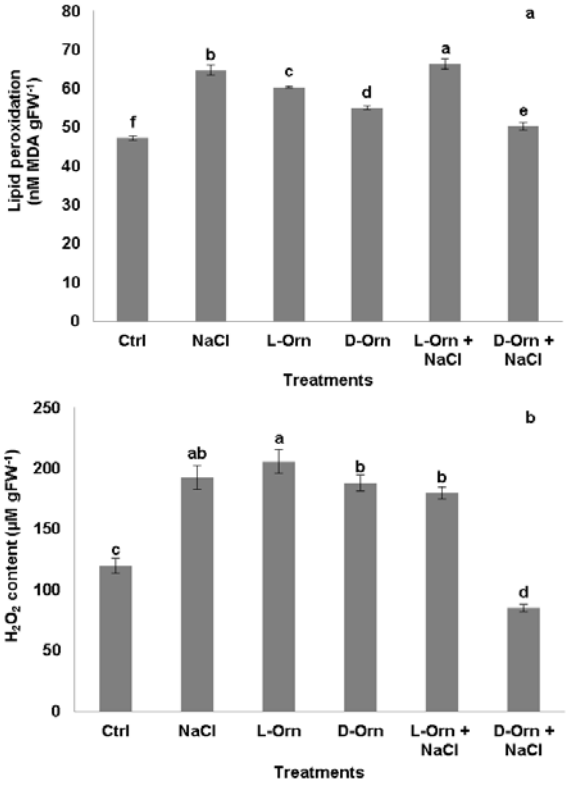

Figure 2. Lipid peroxidation rate **(a)** and hydrogen peroxide **(b)** of tobacco cells treated with NaCl, (D/L)-Orn, or a combination of both. Data are presented as mean ± SD, n = 5. Different letters show significant differences at $p \leq 0.05$ according to LSD.

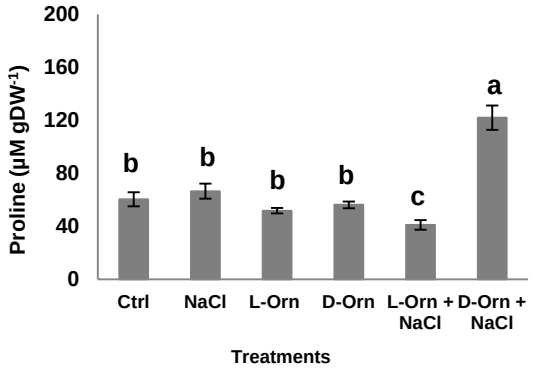

Figure 3. Proline contents of tobacco cells treated with NaCl, (D/L)-Orn, or a combination of both. Data are presented as mean ± SD, n = 5. Different letters show significant differences at $p \leq 0.05$ according to LSD.

Quantification of PAs showed that salinity had no significant effect on Put and Spd levels (Fig. 4a, b). In comparison with the control conditions treatment with Orn resulted in higher levels of PAs which was more pronounced in treatments with D enantiomer (Fig. 4a, b). In salinity condition however the effects of the two enantiomers were different, D-Orn+ NaCl resulted in significant increase of Put (Fig. 4a) whereas L-Orn+ NaCl significantly increased

Spd content (Fig. 4b). The activity of SOD of tobacco cells decreased with 50 mM NaCl but increased with Orn enantiomers, compared to the control group (Fig. 5a). Under salinity condition, the activity of SOD decreased by L-Orn but was identical to the control in those cells which were treated with D-Orn (Fig. 5a). The activity of CAT increased in NaCl, D-Orn, and D-Orn+ NaCl treatments, compared to the control (Fig. 5b). Treatment of the cells with L-Orn significantly decreased CAT activity but in combined treatment with NaCl did not significantly alter CAT activity, compared to the control cells (Fig. 5b). The activity of POD increased by NaCl and NaCl plus Orn enantiomers, however application of Orn enantiomers without NaCl significantly reduced it, in comparison with the control group (Fig. 5c).

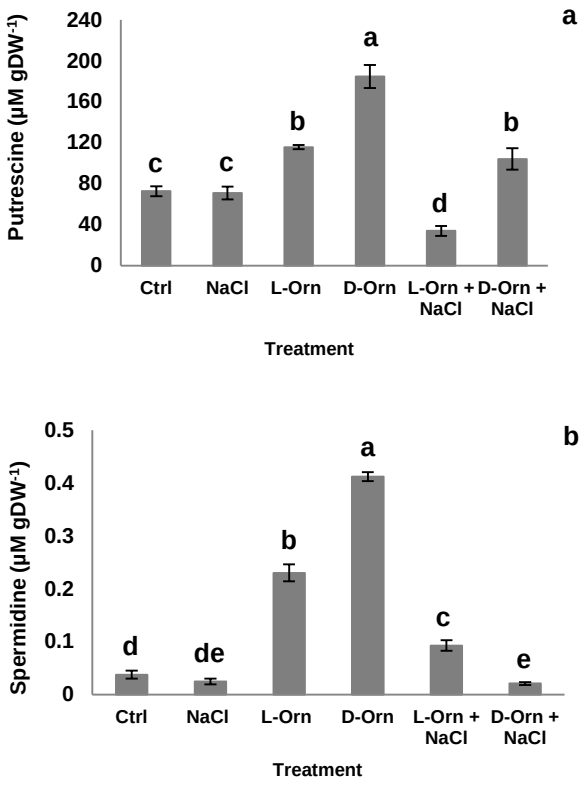

Figure 4. Put **(a)** and Spd **(b)** contents of tobacco cells treated with NaCl, (D/L)-Orn, or a combination of both. Data are presented as mean ± SD, n = 5. Different letters show significant differences at $p \leq 0.05$ according to LSD.

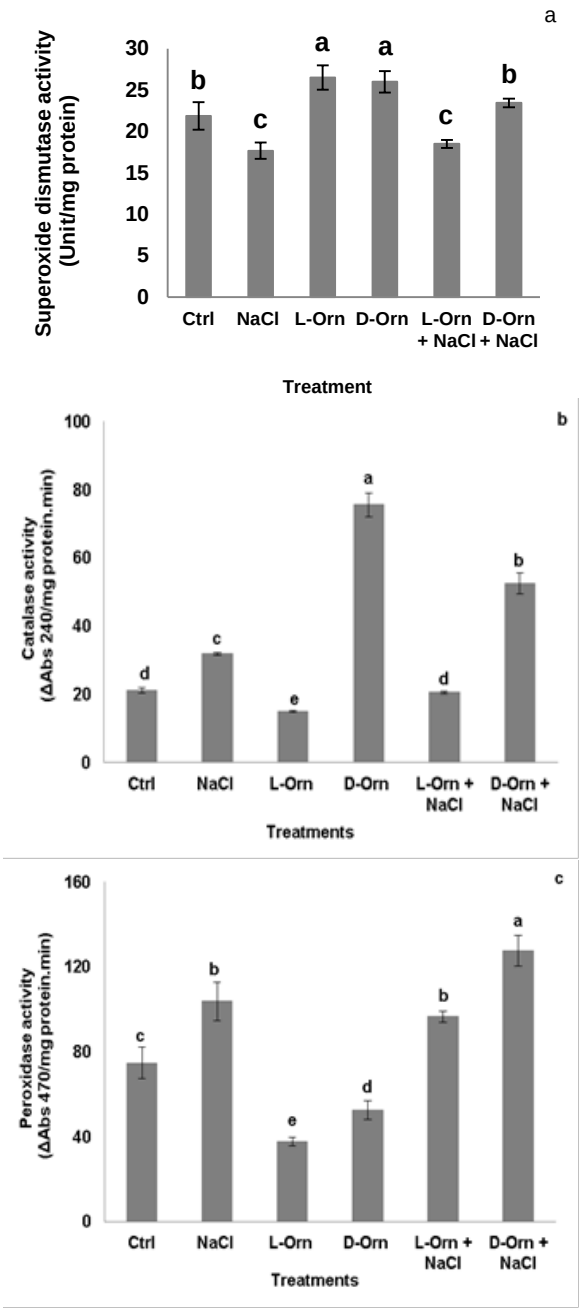

Figure 5. Activity of SOD **(a)** CAT **(b)** and POD **(c)** of suspension-cultured tobacco cells before and after treatment with NaCl and (D/L)-Orn. Data are means ± SD with n = 5. In each column, mean values with different letters are significantly different at $p \leq 0.05$ according to LSD.

Discussion

Ornithin is an amino acid which is not used in the protein structure but is the main precursor of certain compounds such as PAs and Pro whose important role in plant cell physiology, in particular under abiotic stress conditions has been widely discussed (4, 20). The effects of mild cases of salt stress are primarily limited to plant growth and development but in extreme cases, salt stress can cause a water deficit then leads to secondary oxidative stress. During the onset and development of salt stress, all major processes are affected, including photosynthesis, protein synthesis, energy production, and lipid metabolism (21). Investigating on the relationship between salt stress, antioxidative enzymes and proline content of two varieties of tobacco, Celik and Atak (22) found that soluble protein concentration was increased in plants up to 150 mM NaCl concentrations, due to the synthesis of proteins required to protect the plant against salt stress, e.g., antioxidant enzymes, or those whose accumulation results in decrease of the growth potential e.g., hydroxyprolin-rich proteins (23).

Adverse effect of 50 mM NaCl on the growth and viability of tobacco cells was similar to those reported so far (24, 25). As expected, inhibition of the cell growth by NaCl was accompanied by increase of membrane damage and decrease of SOD activity. In order to quench these ROS, elevation was observed in the activities of antioxidant enzymes peroxidase and catalase as compared to the control. Evidently, this increase in antioxidant enzyme activity, however, did not contribute towards the total soluble protein contents of the suspension-cultured tobacco cells and a decline was hence observed in the soluble protein contents of the cells after NaCl treatment. Accumulation of H_2O_2 under salinity conditions was so severe that increased CAT and POD activities were not sufficient to detoxify it.

No significant change was observed in PA content of tobacco cells under salinity conditions. It has been usually stated that PAs are highly beneficial compounds and can be applied to protect membranes. Salt tolerance of seedlings of certain rice cultivars has been attributed to their ability to maintain leaf polyamine levels under salt-stress conditions (26). Hussain and his coworkers (27), however, argued that PAs should not be taken only as a protective molecule but rather like a double-faced molecule with key functions in signaling networks in plants subjected to abiotic stresses, although the exact molecular mechanism remains enigmatic. Measurement of ADC2 activity (key enzyme of stress condition) that converts Arginine to putrescine and also Urea as a byproduct of conversion of Arginine to Orn, that influences on antioxidants enzymes activities, may provide us more details about the mechanism of the effect of ornithine enantiomers on metabolism of tobacco cells under salinity conditions.

Proline is one of the most accumulated osmolytes in salinity and other osmotic stresses in plants. Better growth performance and lower extent of lipid peroxidation have been reported under salt stress in transgenic rice plants in which Δ^1-pyrroline-5-carboxylate synthetase (P5CS; a key enzyme for biosynthesis of proline) was over expressed (28). Other researchers also observed an evident correlation between the transcript up-regulation and the Pro accumulation in *Opuntia streptacantha* under salt stress although, it was not parallel with the changes in P5CS enzymatic activity (29). Such an accumulation of Pro however, was not observed in tobacco cells neither under salinity nor under treatment with Orn. Ornithine is a major precursor for Pro biosynthesis. As a stress signaling molecule, Orn has been shown to be accumulated under stress conditions (30). L-amino acids are sometimes toxic since they are widely being catabolized to produce other amino acids resulting in high accumulation of H_2O_2 as a by-product (8). This may explain the reason of increased H_2O_2, and membrane damages by L-Orn and L-Orn+ NaCl treatments. D- enantiomer of amino acids affect plants to a diverse extent depending on their structure and sometimes have no negative effect on growth (31). Interestingly, treatment of tobacco cells with D-Orn under salinity conditions remarkably reduced H_2O_2 and MDA but increased Pro and Put and activated CAT and POD, resulted in amelioration of salinity effects on the cells.

From the results presented here, it can be deduced that D-Orn has a potential advantage to retain cells growth and development under low salinity stress conditions.

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