

Ornithine-induced increase of proline and polyamines contents in tobacco cells under salinity conditions

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Abstract

Majority of injuries observed in plants caused by salinity are associated with the induction of oxidative stress. The accumulation of reactive oxygen species is overwhelmed by the activities of scavenging enzymes and nonenzyme agents, e.g., proline and polyamines that are commonly synthesized directly from ornithine. The present study was undertaken to evaluate the impact of ornithine, as the common source of proline and polyamines, in the biosynthesis of proline and polyamines and in the activation of antioxidant enzymes in the tobacco cells under saline conditions. Six-day-old cells were treated with 50 mM NaCl and 0.5 mM ornithine. These concentrations were selected based on the results of two sets of preliminary studies using different concentrations of ornithine and NaCl. The results demonstrated that the production of proline and putrescine/spermidine was stimulated by ornithine under normal and stress conditions. Ornithine, in the presence or absence of NaCl, significantly increased the antioxidant enzyme activity (up to 325% for catalase, 270% for peroxidase, and 374% for superoxide dismutase), compared to those of the control group. Treatment with ornithine reduced H₂O₂ to the levels observed in the control cells, while NaCl increased it to 173% of the control. In all treatments, the level of lipid peroxidation was higher than that in the control cells and suppressed the growth of the tobacco cells. On the basis of the results, the treatment of the tobacco cells with ornithine elevates the proline and polyamine contents, with a concurrent decrease in H₂O₂ content.

Keywords: ornithine; polyamines; proline; salinity; tobacco.

Abbreviations: ADC_arginine decarboxylase; CAT_catalase; MDA_malondialdehyde; NBT_nitroblue tetrazolium; ODC_ornithine decarboxylase; Orn_ornithine; PAs_polyamines; POD_peroxidase; Put_putrescine; ROS_reactive oxygen species; SOD_superoxide dismutase; Spd_spermidine; Spm_spermine; TBA_thiobarbituric acid; TCA_trichloroacetic acid.

Introduction

Salinity is one of the major abiotic factors limiting plant growth and development. Salt stress, similar to other abiotic stresses, can lead to oxidative stress caused by the increased reactive oxygen species (ROS) production (Sairam et al., 2002; Hoque et al., 2007). These species are highly reactive and toxic to the plants and can damage the proteins, lipids, DNA, and carbohydrates, resulting in cell death (Hoque et al., 2008). In response to the oxidative stress, plants either increase the activity of their antioxidant enzymes, e.g., superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), or increase the contents of nonenzymatic antioxidants e.g., polyphenols, polyamines (PAs), and proline (Wei et al., 2009). The latter are small nontoxic molecules, which regulate osmotic potential that is disturbed by salinity (Bor et al., 2003; Celik and Atak, 2012). Proline is an important osmolyte that protects cells by stabilizing proteins and cellular membranes (Celik and Atak, 2012). The salt-induced increase in PAs and proline content has been reported in several species (Azooz et al., 2004; Legocka and Kluk, 2005; Koca et al., 2007). Polyamines are small aliphatic amines that are present in all living cells. Putrescine (Put), spermidine (Spd), and spermine (Spm) are the most common PAs

expressed in plants. PAs have been extensively studied because of their participation in the reaction of plants to several environmental stress conditions and significant alterations in PA metabolism and concentration are observed in response to these conditions (Bouchereau et al., 1999). These molecules are important modulators in various biological processes, including membrane stabilization, enzyme activity, and cell division and elongation (Galston et al., 1997; Walden et al., 1997; Kuznetsov and Shevyakova, 2007). Put, the most common PA, is indirectly produced from arginine via the activity of arginine decarboxylase (ADC), or directly from ornithine (Orn) via the activity of ornithine decarboxylase (ODC) (Bagni and Tassoni, 2001). As a recommended strategy, the exogenous application of several of these small molecules has been used to induce plant cell resistance against stress conditions (Lakra et al., 2006; Quinet et al., 2010; Amri et al., 2011). In plants, Orn is a nonprotein amino acid, which plays a significant role in signal transduction, perhaps acting as both a sensory and a regulatory molecule, and is involved in the biosynthesis of proline and PAs (Mohapatra et al., 2010; Celik and Atak, 2012). Recently the role of Orn in the regulation of other

Table 1. Activity of antioxidant enzymes of suspension-cultured tobacco cells before and after treatment with NaCl and ornithine. Enzyme activities were expressed as follows: superoxide dismutase (Unit/mg protein), peroxidase (Δ Abs 470/mg protein.min), and catalase (Δ Abs 240/mg protein. min), Data are means \pm SD with n = 5. In each column, mean values with different letters were significant different at $p \leq 0.05$ according to LSD.

Antioxidant enzymes activity	Control	NaCl	Ornithine	Ornithine + NaCl
SOD	10.47 \pm 1.50 ^c	25.77 \pm 1.26 ^b	27.25 \pm 3.04 ^b	39.23 \pm 2.43 ^a
POD	166.18 \pm 4.40 ^d	255.53 \pm 5.7 ^b	214.49 \pm 3.32 ^c	539.66 \pm 9.43 ^a
CAT	16.14 \pm 0.73 ^c	24.43 \pm 3.49 ^b	43.66 \pm 1.23 ^a	39.41 \pm 2.01 ^a

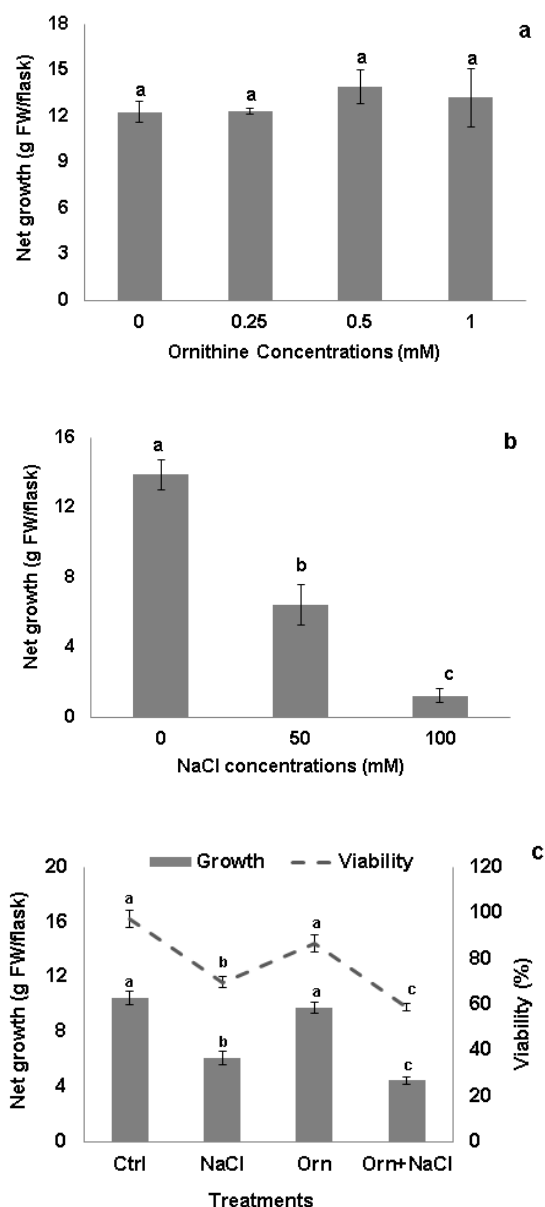


Fig 1. Effects of different concentrations of ornithine and NaCl on growth and viability of tobacco cells. a: Net growth under ornithine treatments, b: Net growth under NaCl treatments, c: Viability and growth of the cells treated with or without 50 mM NaCl and 0.5 mM ornithine. Data are presented as mean \pm SD, n = 5. Different letters show significant differences at $p \leq 0.05$ according to LSD.

amino acids and PAs metabolism have been studied by overexpression of ODC in *Arabidopsis thaliana* (Majumdar et al., 2013). However, to the best of our knowledge, only a few studies have been conducted on the role of exogenous Orn on plant cell metabolism (Gholami et al., 2013). The aim of the present study was to evaluate the effects of exogenous Orn, as a common source of PAs and proline, on the contents of proline and PAs and on the activity of certain antioxidant enzymes in the tobacco cells under salinity stress.

Results

Growth and viability of the cells

Treatment of the tobacco cells with up to 1 mM Orn displayed no toxic effects. In addition, compared with the control cells, no significant difference in growth was observed between the 0, 0.25, 0.5, and 1 mM Orn-treated cells (Fig. 1a). Treatment with NaCl adversely affected the growth of the tobacco cells; the fresh weight of the cells was lowered by 50% and 90% of the weight of the control cells when treated with 50 and 100 mM NaCl, respectively (Fig. 1b). Based on these results, in the subsequent experiments, the tobacco cells were treated with 0.5 mM Orn and 50 mM NaCl, as the average of the concentrations tested. In concurrence with the effects on cell growth, the viability of the tobacco cells did not significantly change following treatment with 0.5 mM Orn alone, but it significantly decreased by 30 and 40% on treatment with 50 mM NaCl and 0.5 mM Orn + 50 mM NaCl, respectively (Fig. 1c).

Content of hydrogen peroxide and activity of antioxidants

The H_2O_2 content and the lipid peroxidation rate of the tobacco cells are presented in Fig. 2. We observed that the H_2O_2 content of the NaCl-treated cells was significantly higher than, that of the control cells (ca. 173%), whereas the H_2O_2 content of the Orn-treated cells was similar to that of the control cells. This indicates that treatment with Orn had no adverse effect on the H_2O_2 content of the cells (Fig. 2). However, the combination of Orn and NaCl restored the H_2O_2 content of the cells to the level of the control cells (Fig. 2). The extent of lipid peroxidation was higher than that of the control in all the treatments in the cells (Fig. 2). The antioxidant enzyme activity in the tobacco cells is presented in Table 1. Treatment with 50 mM NaCl significantly enhanced the activities of all antioxidant enzymes compared with those of the control cells. Similarly, treatment with Orn increased the activities of antioxidant enzymes compared with those of the control cells (Table 1). An increased antioxidant enzyme activity was also observed following the treatment with a combination of NaCl and Orn. In this context, the increase in SOD and POD activities was more prominent (Table 1).

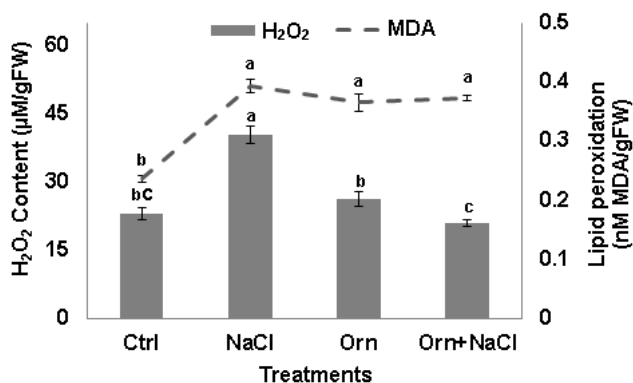


Fig 2. Hydrogen peroxide and MDA contents of tobacco cells treated with NaCl, Orn, or a combination of both. Data are presented as mean \pm SD, n = 5. Different letters show significant differences at $p \leq 0.05$ according to LSD.

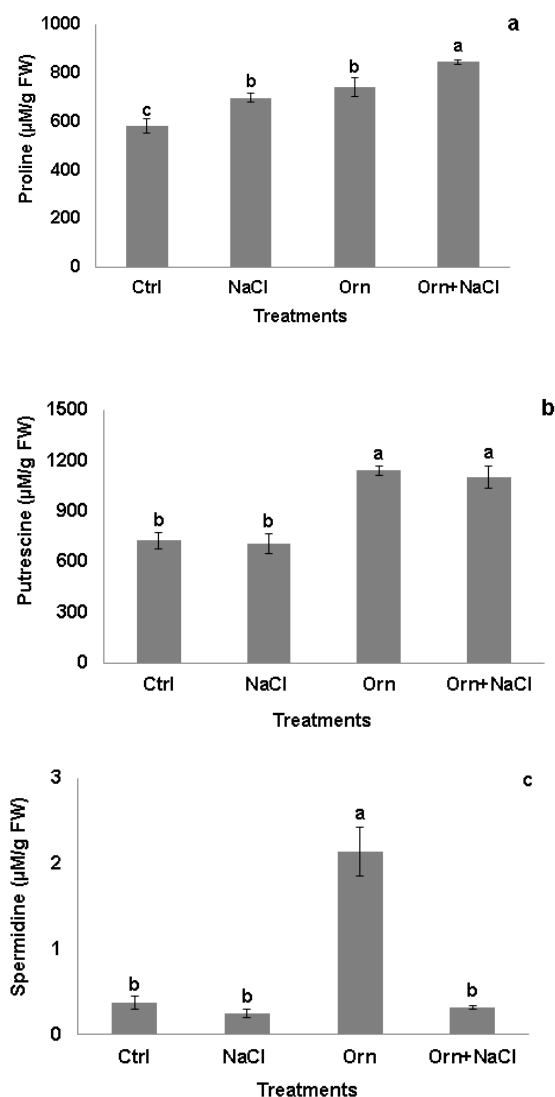


Fig 3. Proline (a), Put (b), and Spd (c) contents of tobacco cells treated with NaCl, Orn, or a combination of both. Data are presented as mean \pm SD, n = 5. Different letters show significant differences at $p \leq 0.05$ according to LSD.

The proline content of the cells increased in response to treatment with 50 mM NaCl (Fig. 3a), and the highest content of proline was observed in cells that were treated with a combination of Orn and NaCl.

Polyamine contents

Compared with the control cells, treatment with NaCl did not change the Put content of the cells, whereas treatment with Orn and Orn + NaCl significantly increased it. The content of Put in these treatments were 140% of the control group (Fig. 3b). Treatment with Orn also caused a significant increase in Spd (more than 550%) compared to that in control cells as well as other treatments (Fig. 3c).

Discussion

The results of the present study demonstrated that the treatment of the tobacco cells with Orn stimulated the antioxidant enzyme activity in the cells. Plants express several antioxidant enzymes, among which SOD, catalyzing the dismutation of $O_2^{\cdot-}$ to H_2O_2 , CAT that catalyzes the removal of H_2O_2 and PODs, involved in the removal of H_2O_2 , are quite important. High concentrations of ROS can lead to phytotoxicity, whereas relatively low levels can be used for acclimation signaling. This novel finding implies that ROS are not simply toxic by-products of metabolism, but also function as signaling molecules (Dat et al., 2000). The H_2O_2 content of the Orn-treated tobacco cells was similar to that of the control cells, although the possibility of changes in other ROS exists, but was not measured in the current study. Therefore, there is a possibility that the treatment with Orn can provide some information regarding the signal transduction sequences and molecular mechanisms responsible for the induction of antioxidant enzymes. It has previously been demonstrated that salinity induces oxidative stress caused by increased ROS generation in plant cells (Bor et al., 2003; Azevedo Neto et al., 2006; Hernandez and Almansa, 2002). Therefore, salt stress resistance may depend, at least in part, on the induction of the antioxidant defense system, which includes several antioxidant enzymes and some specific molecules. Among antioxidant enzymes, SOD is a key enzyme that promotes dismutation of $O_2^{\cdot-}$ to H_2O_2 , thus modulating the relative amounts of $O_2^{\cdot-}$ and H_2O_2 , and decreasing, in part, the risk of OH^{\cdot} radical formation via the Fenton reaction (Cabiscol et al., 1999). Hydroxyl radicals are highly reactive and may cause severe damage to membranes, proteins and DNA (Bowler et al., 1992). They initiate lipid peroxidation by removing a hydrogen atom from the unsaturated fatty acids of membrane phospholipids, a process that generates a free lipid radical. The destruction of the unsaturated fatty acids of phospholipids results in a loss of membrane integrity. Hydroxyl radicals can also damage the membrane by cross-linking of membrane proteins through the formation of disulfide bonds (Catala, 2011). However, it should be noted that OH^{\cdot} production is not confined to the Fenton reaction and other enzymes, e.g., NADPH oxidase and xanthine oxidase, also produce OH^{\cdot} . Therefore, OH^{\cdot} production and peroxidation of membrane lipids are not always regulated by the activity of SOD, CAT and POD alone. Although the activities of OH^{\cdot} -producing enzymes were not analyzed in this study, there is a possibility that the inadequacy of the NaCl- and Orn-treated tobacco cells to decrease MDA content and prevent membrane damage, in spite of increased SOD, CAT, and POD activities, may be related to the activity of the OH^{\cdot} -producing enzymes e.g., NADPH oxidase and xanthine oxidase (Sato et al., 2011).

Salinity induced proline production in the tobacco cells and treatment with Orn also resulted in the increased proline content of the cells. In plants, proline can be synthesized from Orn via the activity of Ornithine δ -aminotransferase. The enzyme has been implicated in the accumulation of proline (via pyrroline-5-carboxylate), which represents a way to regulate cellular osmolarity in response to osmotic stress (Stranska et al., 2008). Thus, as expected, the proline content of the tobacco cells was significantly higher following the combined treatment with NaCl and Orn than that of the salinity and Orn alone. Proline is a major component of structural proteins and a known osmoprotectant, capable of mitigating the impacts of drought, salt, and temperature stress in plants. It has also been accepted that proline can be considered as a nonenzymatic antioxidant in plants, essential for mitigating the impact of ROS (Chen and Dickman, 2005). The participation of PAs and their accumulation in plants under salinity stress has been frequently reported (Legocka and Kluk, 2005; Kuznetsov and Shevyakova, 2007; Liu et al., 2011; Tassoni et al., 2008). On investigating the effect of salt stress on the PA expression in maize, Ingold and his coworkers (2009) found that PAs production varies among different genotypes as well. Moreover, while the polyamine concentrations in shoots did not change, the plant roots showed significantly lower Spd and higher Spm concentrations under salt stress. However, treatment of the tobacco cells with 50 mM NaCl did not produce significant changes in the Put or Spd contents of these cells. Furthermore, treatment with Orn and Orn + NaCl significantly increased the Put content of the cells, compared to the control conditions. These results are reasonable when considering the fact that Orn is the most common precursor for Put biosynthesis (Bagni and Tassoni, 2001). Activity of spermidine synthase catalyzes the biosynthesis of the longer PA, Spd, from Put. An increase in the expression of Spd synthase and Spm synthase genes have been reported in grape plants under salt stress. However, the authors indicated that the expression of these genes was observed at the late stages of the stress, compared with that of ADC and S-adenosylmethionine decarboxylase (Liu et al., 2011), implying that the sensitivity to salt stress among enzymes involved in PA biosynthesis varies. Treatment with Orn induced the production of both Put and Spd. Majumdar et al. (2013) believe that Orn itself is a part of the sensing as well as the signal transduction mechanism to regulate its own cellular content and also plays a role in regulating Pro and PAs production. Coincidentally, Orn supply significantly increased production of both Put and Pro in tobacco cells in the present study. Mohapatra et al. (2010) found that Put overproduction in poplar cells included changes in the oxidative state of transgenic cells, accompanied by accumulation of H_2O_2 and lower activity of certain antioxidant enzymes. Therefore, it is plausible that adverse effects of higher production of Put in tobacco cells in the present study is ameliorated by increase of proline. Although the gene expression or activity of ODC/ADC and Spd synthase was not monitored in the current experiments, based on the results presented here, it can be concluded that Spd synthase was more sensitive to salinity than ODC/ADC in the tobacco cells, such that the treatment with 0.5 mM Orn + 50 mM NaCl results in a significant increase in Put expression alone. The particular structure of Spd facilitates its interaction with anions and binding to membrane phospholipids; therefore, Spd plays an important role in the maintenance/enhancement of plasma membrane integrity, subsequently resulting in a better growth of plant cells (Duan et al., 2008). Thus, based on the data presented here, it can be

concluded that the treatment of the tobacco cells with Orn under saline conditions can significantly improve the antioxidant capacity of the cell and induce proline and Put contents, but it cannot increase the Spd content, protect the cells against membrane lipid peroxidation or restore cell growth.

Materials and Methods

Cell culture, growth, and treatments

Suspension-cultured tobacco (*Nicotiana tabacum* L. cv. Burley 21) were grown in a modified Murashige and Skoog medium (Murashige and Skoog, 1962) without glycine and supplemented with 3 mgL^{-1} NAA, 3 mgL^{-1} IAA, and 1 mgL^{-1} kinetin, pH 5.8. The cells were sub-cultured every 7 days and incubated in the dark at $25\text{ }^\circ\text{C}$ on a rotary shaker at 110 rpm. To determine the appropriate concentrations of Orn and NaCl, two series of preliminary studies were conducted using Orn at final concentrations of 0, 0.25, 0.5, and 1 mM or NaCl at final concentrations of 0, 50, and 100 mM. The treatment of the tobacco cells with Orn or NaCl was performed on day 6 of the subculture (when the cells were in their logarithmic growth phase), and the effect of Orn or NaCl was monitored by changes in the cell weight on day 12. The cell viability was assessed with Evans Blue (aqueous, 0.05% w/v) (Smith et al., 1984). Based on the results of these experiments, doses of 0.5 mM Orn and 50 mM NaCl were selected for subsequent experiments. Further analysis of growth, polyamines, proline, and H_2O_2 content, activities of antioxidant enzymes, and peroxidation rate of membrane lipids were performed with those cells that were treated with 50 mM NaCl and 0.5 mM Orn. The cells were treated on day 6 and harvested on day 12 of the subculture, washed thoroughly and dried under reduced pressure, frozen immediately in liquid nitrogen, and stored at $-80\text{ }^\circ\text{C}$, prior to being analyzed.

Evaluation of redox status and antioxidant system of tobacco cells

The hydrogen peroxide content of the tobacco cells was assayed at 390 nm based on the reaction between iodide and hydrogen peroxide in an acidic environment, as described by Velikova et al., (2000). The extent of lipid peroxidation and damage to the membranes was estimated by determining the amount of the red complex formed between MDA (the end product of peroxidation of membrane lipids) and thiobarbituric acid (TBA) at low pH and elevated temperature. Absorbance was measured at 532 and 600 nm and the MDA content was calculated using an absorption coefficient of $155\text{ mM}^{-1}\text{ cm}^{-1}$ (De Vos et al., 1991). The activity of superoxide dismutase (EC 1.15.1.1) was evaluated using nitroblue tetrazolium (NBT), which produces a water-soluble formazan dye upon reduction with a superoxide anion. One unit of 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 (Giannopolitis and Ries, 1977). The activity of POD (EC 1.11.1.7) was determined by monitoring the turnover of guaiacol at 470 nm as described by Pandolfini et al. (1992). The activity of CAT was measured at 240 nm using H_2O_2 as the precursor (Safari et al., 2012). Protein content was measured according to the Bradford method (1976), using bovine serum albumin as a standard.

Extraction and determination of proline and Polyamines

The cells were homogenized in 50% MeOH using ceramic beads in a cryo-mill (Precellys 24, Bertin Technologies, Montigny-le-Bretonneux, France) for 3 × 30-s sessions, with a 45-s delay, at a maximum temperature of 0 °C. Derivatization of proline in the supernatant was accomplished using 6-aminoquinolyl-N-hydroxysuccinimidylcarbamate (Aqc) (Boughton et al., 2011). Polyamines were extracted as previously described (Hassannejad et al., 2011). Both PAs and proline were quantified by liquid chromatography. An Agilent Zorbax Eclipse XDB-C18 Rapid Resolution HT column (2.1 × 50 mm, 1.8 µm) (Crawford Scientific™ Ltd, Lanarkshire, Scotland, UK) was used, with a flow rate of 300 µL.min⁻¹, maintained at 30°C, resulting in operating pressures below 400 bar, with a 19-min runtime. An aqueous mobile phase of 0.1% (v/v) formic acid in water (solvent A) and an organic phase of 0.1% (v/v) formic acid in acetonitrile (solvent B) were used. A gradient was run from 0 to 2 min using 1% solvent B, then it was linearly increased to 15% solvent B over 7 min, then increased to 30% solvent B over 5 min, followed by re-equilibration at 1% solvent B for 5 min.

Statistical analyses

The experiment was structured following a completely randomized design arranged in a 2 × 4 factorial with three replications. For all variables, analysis of variance (ANOVA) was performed to test for differences between NaCl and Orn treatments and their interactions (SPSS version 16, Chicago, IL, USA). The primary and interaction effects of the various experimental factors were determined. Where interactions between two factors were significant, we presented the results in the form of a combination of treatments and not separately or individually. LSD at the 5% probability level was used to compare the significance of differences among all the treatments.

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