

## Alleviation of Salt Stress Using Exogenous Proline on a Citrus Cell Line

M.E. Lima-Costa, S. Ferreira, A. Duarte and A.L. Ferreira  
Centro de Desenvolvimento de Ciências e Técnicas de Produção Vegetal  
Faculdade de Engenharia de Recursos Naturais  
Universidade do Algarve, Campus de Gambelas, 8005-139 Faro  
Portugal

**Keywords:** abiotic stress, cell suspension culture, osmolyte, salinity

### Abstract

Salinity constitutes an important abiotic problem since ancient times, worldwide, for it leads to a decrease in productivity of crops with agronomic value. Under salt stress conditions, plant cells develop strategies to cope with Na<sup>+</sup> and Cl<sup>-</sup>, including exclusion and compartmentalisation, induction of antioxidant enzymatic systems and compatible solutes accumulation, such as proline. The precise function of this osmolyte still remains unclear. Proline may act on osmotic adjustment, as a free radical scavenger, protecting enzymes and avoiding DNA damages. It has been also suggested the role of proline in prevention of lipid peroxidation and as a signalling/regulatory molecule. A salt-sensitive *Citrus sinensis* 'Valencia late' cell line has a smaller growth rate and accumulates proline in the presence of NaCl (>200 mM). The addition of external proline to this cell line was evaluated in terms of cell metabolism. A positive influence on the relieve of salt stress symptoms due to the presence of exogenous proline 5 mM and 100 mM NaCl was obtained, with increased growth of this salt sensitive citrus cell line.

### INTRODUCTION

Since ancient times salinity is a serious problem, that limits world plant production and decreases the crop productivity of plants with agronomic value (Flowers, 2004; Mansour and Salama, 2004). Under salt stress conditions plant cells have different strategies to survive such as Na<sup>+</sup> and Cl<sup>-</sup> exclusion/compartmentalizing (Flowers et al., 1977; Walker and Douglas, 1983; Binzel et al., 1987), activation of antioxidant enzymatic systems (Dat et al., 2000; Blokhina et al., 2003; Sairam and Tyagi, 2004) and accumulation of compatible solutes as proline (Petruša and Winicov, 1997; Ehsanpour and Fatahian, 2003; Khedr et al., 2003). The function of this osmolyte remains unclear, but several authors refer that proline may act on osmotic adjustment (Flowers et al., 1977; Flowers and Yeo, 1988; Petruša and Winicov, 1997), as a free radical scavenger or that it protects enzymes avoiding DNA damages (Matysik et al., 2002). Others investigators suggested that proline may prevent lipid peroxidation (Saradhi and Mohanty, 1993) and act as a signalling/regulatory molecule (Maggio et al., 2002). It is clear, however, that proline may alleviate salt stress injury on cell tissues. Ferreira (2005) showed that *Citrus sinensis* 'Valencia late' is sensitive to salt stress and demonstrated that, in the presence of NaCl, the growth rate decreased and proline accumulated in cells. In the present work we investigated the effect of exogenous proline addition on a *Citrus sinensis* 'Valencia late' cell line grown with or without NaCl.

### MATERIALS AND METHODS

Cell suspension cultures were obtained from induced *calli* of 'Valencia late' leaves on MTK medium (Murashige and Tucker, 1969) supplied with 1 mg ml<sup>-1</sup> of kinetin, 0.5 g/L of malt extract and 50g/L of sucrose. The cell suspensions were kept on the MTK medium in a rotary shaker (140 rpm), at 24°C in the dark, until the exponential phase of growth was attained. Cultures were maintained in Erlenmeyer flasks (500 ml), sub-cultivated weekly with a 20% (v/v) inoculum. Assays were performed under the same culture conditions and run for 20 days: A) in the absence of NaCl (control), B) in the presence of 100 mM NaCl and C) with 5 mM exogenous proline plus 100 mM NaCl.

Proline content was determined using the method of Magné and Larher (1992). Fresh cells were extracted with 80% (v/v) ethanol at 80°C for 30 min. One hundred µl of the cell extract was mixed with 400 µl of the ninhydrin acid reagent. After incubation for 1 h at 100°C, the tubes were cooled and 1 ml toluene was added per tube. The absorbance of the upper phase was determined at 520 nm and compared to a standard curve obtained using different concentrations of proline.

## RESULTS AND DISCUSSION

Fresh weight accumulation was impaired when *Citrus sinensis* 'Valencia late' cell suspension cultures were exposed to 100 mM NaCl compared with control with no NaCl (Fig. 1, 2). Cell cultures with 100 mM NaCl plus proline (5 mM), in the same conditions, had a greater fresh weight, suggesting that saline stress was smaller. Proline accumulation (Fig. 1) slightly increased in cell suspensions with 100 mM NaCl compared to control. Cell cultures with 5 mM of exogenous proline plus 100 mM NaCl had a greater accumulation of proline on day 10 (Fig. 1). This fact may be attributed to concomitant intracellular proline production and transport of exogenous proline to the cytoplasm. It is well known that water salinity reduces the productivity of many crops over the world. To avoid this problem, many crops have the ability to induce metabolic mechanisms that help overcome cellular salt stress. One of these cell strategies is the production and accumulation of compatible osmolytes such as the amino acid proline (Flowers and Yeo, 1988; Maggio et al., 2002; Matysik et al., 2002). This fact was verified by many authors and confirmed with our results – in the presence of high levels of salt, cytoplasmic accumulation of proline takes place. Arbona et al. (2003) reported accumulation of proline in Carrizo citrange (*Poncirus trifoliata* L. Raf × *Citrus sinensis* L. Osb.), a salt sensitive rootstock, with increased NaCl concentration in the growth medium. An increased accumulation of proline in 'Carvalho' cells adapted to high NaCl was also reported (Ferreira and Lima-Costa, 2006). Bajji et al. (1998) showed accumulation of proline with increase of NaCl concentration in *Atriplex halimus* L. callus, but with a concomitant decrease in growth rate. Others authors, such Lutts et al. (1996), suggested that accumulation of proline in *Oryza sativa* L. was a symptom of damage, rather than an adaptative response as an intracellular osmotic adjustment.

In this work, we showed that exogenous proline can protect cells from injury. Ehsanpour and Fatahian (2003) also reported an increase in the growth rate of *Medicago sativa* Hamedani, a salt-sensitive cultivar, when exposed to extracellular proline. However, large amounts of proline can inhibit the growth of some species (Ehsanpour and Fatahian, 2003; Heuer, 2003). Our results showed intracellular accumulation of proline under salt exposure in the presence of exogenous proline throughout the growth period, as referred by others authors (Okuma et al., 2000; Öztürk and Demir, 2002; Khedr et al., 2003). It was also reported (Ferreira and Lima-Costa, 2006) that proline accumulation may be essential to maintain turgor and cell division, and consequently greater cell viability.

## CONCLUSIONS

In conclusion, it was observed that the presence of exogenous proline favoured growth conditions when salt stress occurred, suggesting that proline can alleviate salt stress injury, helping plant cells to resist to NaCl. Proline may act as an osmoprotectant of key enzymes and membranes, maintaining cell turgor and scavenging free radicals.

## Literature Cited

- Arbona, V., Flors, V., Jacas, J., Garcia-Agustin, P. and Gomez-Cadenas, A. 2003. Enzymatic and non-enzymatic antioxidant responses of Carrizo citrange, a salt-sensitive *Citrus* rootstock, to different levels of salinity. *Plant Cell Physiol.* 44:388-394.
- Bajji, M., Kinet, J.M. and Lutts, S. 1998. Salt stress effects on roots and leaves of *Atriplex halimus* L. and their corresponding callus cultures. *Plant Sci.* 137:131-142.

- Binzel, M.L., Hasegawa, P.M., Rhodes, D., Handa, S., Handa, A.K. and Bressan, R.A. 1987. Solute accumulation in tobacco cells adapted to NaCl. *Plant Physiol.* 84:1408-1415.
- Blokhina, O., Virolainen, E. and Fagerstedt, K. 2003. Antioxidants, oxidative damage and oxygen deprivation stress: a review. *Annals of Botany* 91:79-194.
- Dat, J., Vandenberghe, S., Vranova, E., van Montagu, M., Inze, D. and van Breusegem, F. 2000. Dual action of the active oxygen species during plant stress responses. *Cell. Mol. Life Sci.* 57:779-195.
- Ehsanpour, A.A. and Fatahian, I. 2003. Effects of exogenous salt and proline on *Medicago callus* in vitro culture. *Plant Cell Tis. Org. Cult.* 73:53-56.
- Ferreira, A.L. 2005. Caracterização Cinética da Cultura de Células em Suspensão de *Citrus* sp. L. Resposta Bioquímica ao Stresse Salino, PhD Thesis. University of Algarve, Faro, Portugal.
- Ferreira, A.L. and Lima-Costa M.E. 2006. Metabolic responses to salt stress in suspension cultures of sensitive and resistant *Citrus*. *J. Hort. Sci. Biotech.* 81:983-988.
- Flowers, T.J. 2004. Improving crop salt tolerance. *J. Exp. Bot.* 55: 07-319.
- Flowers, T.J., Troke, P.F. and Yeo, A.R. 1977. The mechanism of salt tolerance in halophytes. *Ann. Rev. Plant Physiol.* 28: 9-121.
- Flowers, T.J. and Yeo, A.R. 1988. p.392-413. In: D.A. Baker and J.L. Hall (eds.), *Solute transport in plant cells and tissues*. Longman Scientific and Technical, Harlow.
- Heuer, B. 2003. Influence of exogenous application of proline and glycinebetaine on growth of salt stressed tomato plants. *Plant Sci.* 165:693-699.
- Khedr, A.H., Abbas, M.A., Wahid, A.A.A., Quick, W.P. and Abogadallah, G.M. 2003. Proline induces the expression of salt-stress-responsive proteins and may improve the adaptation of *Pancreaticum maritimum* L. to salt-stress. *J. Exp. Bot.* 54:2553-2562.
- Lutts, S., Kinet, J.M. and Bouharmont, J. 1996. Effects of various salts and mannitol on ion and proline accumulation in relation to osmotic adjustment in rice (*Oryza sativa* L.) *callus* cultures. *J. Plant Physiol.* 149:186-195.
- Maggio, A., Miyazaki, S., Veronese, P., Fujita, T., Ibeas, J.I., Damsz, B., Narasimhan, M.L., Hasegawa, P.M., Joly, R.J. and Bressan, R.A. 2002. Does proline accumulation play an active role in stress-induced growth reduction. *Plant J.* 31:699-712.
- Magné, C. and Larher, F. 1992. High sugar content of extracts interferes with colorimetric determination of amino acids and free proline. *Anal. Biochem.* 200:115-118.
- Mansour, M.M.M. and Salama, K.H.A. 2004. Cellular basis of salinity tolerance in plants. *Env. Exp. Bot.* 52:113-122.
- Matysik, J., Alia, Bhalu, B. and Mohanty, P. 2002. Molecular mechanisms of quenching of reactive oxygen species by proline under stress in plants. *Current Science* 82:525-532.
- Murashige, T. and Toker, D.H.P. 1969. Growth factor requirements of *Citrus* tissue culture. p. 1151-1161. In: H. Chapman (ed.), *Proceedings of the First International Citrus Symposium*, University of California, Riverside, Vol 3.
- Okuma, E., Kenji, S., Fukuda, M., Tada, M. and Murata, Y. 2002. Negative correlation between the ratio of K<sup>+</sup> to Na<sup>+</sup> and proline accumulation in tobacco suspension cells. *Soil Sci. Plant Nutr.* 48:753-757.
- Öztürk, L. and Demir, Y. 2002. In vivo and in vitro protective role of proline. *Plant Growth Regulation* 38:259-264.
- Petrusa, L.M. and Winicov, I. 1997. Proline status in salt-tolerant and salt-sensitive alfalfa cell lines and plants in response to NaCl. *Plant Physiol. Biochem.* 35:303-310.
- Saïram, R.K. and Tyagi, A. 2004. Physiology and molecular biology of salinity stress tolerance in plants. *Current Science* 86:407-421.
- Saradhi, A.P.P. and Mohanty, P. 1993. Proline in relation to free radical production in seedlings of *Brassica juncea* raised under sodium chloride stress. *Plant Soil* 156:497-500.
- Walker, R.R. and Douglas, T.J. 1983. Effect of salinity level on uptake and distribution of chloride, sodium and potassium ions in citrus plants. *Aust. J. Agric. Res.* 34:145-153.

## Figures

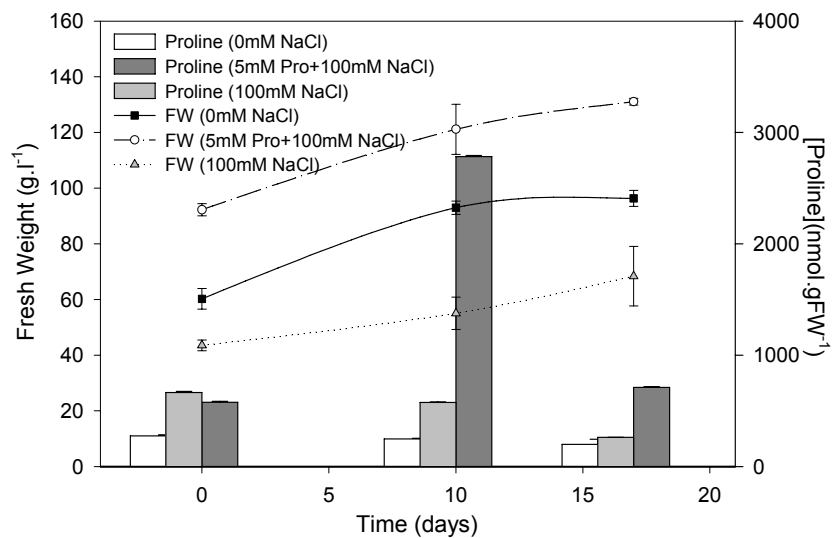


Fig. 1. Relationship between fresh weight (FW) and proline accumulation in cells suspensions of *Citrus sinsensis* 'Valencia late', without NaCl treatment, with 100 mM NaCl and with 5 mM exogenous proline plus 100 mM NaCl. Values are means  $\pm$  s.e. (n=3).

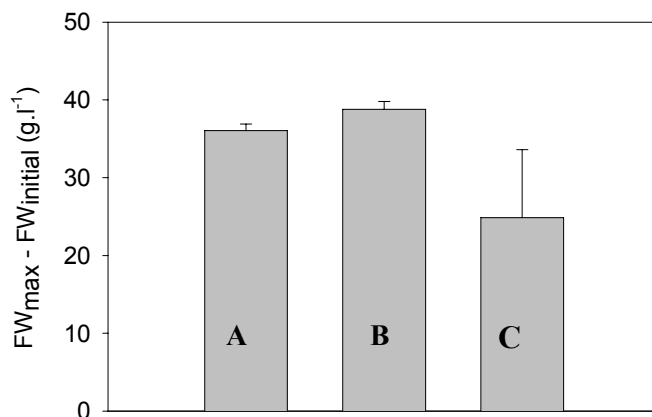


Fig. 2. Fresh weight variation between the beginning of the experiment ( $FW_{initial}$ ) and the day of maximum yield ( $FW_{max}$ ) of cell suspensions of *Citrus sinsensis* 'Valencia late', without NaCl treatment (A), with 5 mM exogenous proline plus 100 mM NaCl (B) and with 100 mM NaCl (C). Values are means  $\pm$  s.e. (n=3).