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Induction of alternative oxidase chain under salt stress conditions

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Abstract

This paper describes the effect of NaCl on the respiration of *Citrus* cell suspensions namely on the induction of the alternative oxidase. The exposure of two *Citrus* (cvs. Carvalhal tangor and Valencia late) cell suspensions to 200 or 400 mM NaCl lead to a reduction on cell respiration rates. Under these conditions, the respiration rate decreased less in the presence of KCN indicating a stimulation of the capacity of the alternative oxidase (AOX). In addition, immunoblots showed an increase on the amount of AOX protein. Antibodies raised against the *Sauromatum guttatum* enzyme recognized the reduced form of the enzyme near the 35 kDa band. The protein accumulation was correlated with the significantly higher AOX capacity observed for cv. Carvalhal tangor.

Additional key words: cell suspension, *Citrus*, respiration rate.

Introduction

The plant mitochondrial electron transport chain includes an inner membrane protein called alternative oxidase (AOX) (Simons and Lambers 1999, Siedow and Umbach 2000, Moore *et al.* 2002). AOX catalyses the O_2-dependent oxidation of ubiquinol, producing ubiquinone and water. Electron flow from ubiquinol to AOX is not coupled to the generation of a proton motive force and hence it is a non-energy-conserving branch of the electron transport chain, bypassing the two sites of proton pumping associated with the cytochrome (cyt) pathway and decreasing energy conservation in oxidative phosphorylation. This has prompted the question of what impact AOX may have on plant growth and productivity (Amthor 2000, Moore *et al.* 2002).

Apart from a physiological role in heat production of thermogenic floral organs to volatilize compounds during pollination (Meeuse 1975), the function of the alternative pathway in plant respiration metabolism is still under debate. The non-energy-conserving nature of AOX, along with the ability of plant cells to regulate AOX activity in a sophisticated manner (Millar *et al.* 1993, Umbach *et al.* 1994) presumably provides the mitochondria with considerable metabolic flexibility. This flexibility may be particularly important during periods of abiotic or biotic stresses (Simons and Lambers 1999).

Several studies indicate that the increased contents of AOX may be a response to numerous stresses including drought (Bartoli *et al.* 2005), chilling (Fung *et al.* 2004, Ribas-Carbo *et al.* 2000), salinity (Geraldes-Laakso and Arrabaça 1997), high irradiance (Noguchi *et al.* 2005), nutrient limitation (Sieger *et al.* 2005), wounding (Hiser and McIntosh 1990), and chemical agents (Vanlerberghe and McIntosh 1994, Vandenabeele *et al.* 2003, Parani *et al.* 2004), and/or to stress-induced reactive oxygen species (ROS) (Wagner 1995, Mizuno *et al.* 2005)
Plant growth, development and productivity of citrus may be highly affected by salinity (Storey and Walker 1999). Cell cultures have been a very useful tool in trying to elucidate the mechanisms of salt tolerance operating at the cellular level. However, little is known about the effect of the excess of NaCl on the non-photosynthetic metabolism in plant cells, especially on respiration. It

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Abbreviations: AOX - alternative oxidase; R_D - dark respiration rate; ROS - reactive oxygen species; SHAM - salicyl hydroxamic acid.
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seems that under salt conditions, AOX has a protective effect, by avoiding damage to the cell due to the increase in ROS production (Maxwell *et al.* 1999, Mittler 2002).

The aim of the present work was to investigate the contribution of the alternative pathway in two different *Citrus* cell lines when subjected to salt stress. Results from this study can supply information on the possible involvement of AOX in protection under salinity conditions.

Materials and methods

Plant material and culture conditions: Cell suspensions of *Citrus* hybrid Carvalhal tangor and *Citrus sinensis* L. cv. Valencia late were maintained in a rotary shaker (140 rpm), at 24 ºC in the dark, in a liquid basal medium containing Murashige and Skoog (1962; MS) salts, supplemented with 100 mg dm^{-3} nicotinic acid, 400 mg dm^{-3} thiamine-HCl, 1 mg dm^{-3} kinetin, 0.5 g dm^{-3} malt extract and 50 g dm^{-3} sucrose, adjusted to pH 5.7. All media were autoclaved at 121 ºC at a pressure of 104 kPa for 20 min. Cultures (200 cm^3) were maintained in 500 cm^3 Erlenmeyer flasks and sub-cultivated weekly with an inoculation size 20 % (v/v).

Cell growth evaluation: To evaluate growth, assays were performed in the same culture conditions and run for 22 d in the absence of NaCl (control assay) and in the presence of 42.7, 50, 100, 200, and 400 mM NaCl. Growth measurements in the absence of NaCl, were performed in a bioreactor (*Setric Genie Industriel 210*, Toulouse, France), with an oxygen electrode (Ingold electrode), at 60 rpm speed and with an initial aeration of 1.67 cm^3 s^{-1}. Carvalhal tangor cell line was acclimated to 100 mM NaCl, using as the starting material friable calli grown for six months in the culture medium with that salt concentration. Fresh mass (FM) was determined after the filtration of 10 cm^3 samples using *Whatman* filter paper.

Extracellular sucrose was quantified by HPLC separation (*Beckman System Golds* with *Jasco RI-1530* detector) after a supernatant filtration (*Sep-Pak*, *WAT051910*, *Waters*, Milford, USA) and suspended on 50 % (v/v) acetonitrile solution. Sucrose detection was obtained after 14 min and calculated against sucrose standards, using an aminopropile column (250 mm × 5 μm, *Merck*, Darmstadt, Germany).

O_2 uptake by cell suspensions was monitored polarographically with a Clark-type electrode system (*Hansatech Ltd.*, King´s Lynn, Norfolk, UK) at 20 ºC. *Citrus* cell suspension respiration was measured in the culture medium at pH 5.7. KCN and SHAM were both used at 1 mM, concentrations which fully inhibit *Citrus* cell respiration.

SDS-PAGE and immunoblotting: 100 μg of total protein was solubilized in 0.05 cm^3 of SDS sample buffer [(2 % (m/v) SDS, 5 % (v/v) β-mercaptoethanol, 10 % (v/v) glycerol, 62.5 mM Tris-HCl, pH 6.8] and boiled for 5 min prior the addition of 0.08 % (m/v) bromophenol blue tracking dye. SDS-PAGE separation of the proteins was carried out with the buffer system of Laemmli (1970) using a 4.5 % (m/v) stacking and a 12 % (m/v) polyacrylamide resolving gel. The resolved proteins were then blotted to a immunoblot *PVDF* membrane (*Bio-Rad*, Hercules, USA) in a transfer buffer containing 25 mM Tris (pH 8.3), 192 mM glycine (0.1 % SDS) and 20 % methanol, followed by Western blotting.
Antibodies (generously supplied by Dr. T. Elthon) recognize all three AOX proteins of *Sauromatum guttatum* as well as putative AOX proteins from other higher plants. They were used at dilutions of 1/100, for 1.5 h at room temperature. The AOX proteins were detected using an alkaline phosphatase conjugate substrate kit (*Bio-Rad*).

Statistics: The data were subjected to analysis of variance (*ANOVA*) and mean values were compared using *SPSS for Windows* (statistical program). Duncan Post-hoc tests were performed when significant differences occurred at 5 % level.

Results

Influence of NaCl on cell growth: The *Citrus* cell lines, Carvalhal tangor and Valencia late, grown in suspension cultures with sucrose as sole carbon and energy source showed that biomass increase was maintained in controls until sucrose exhaustion (Fig. 1). The presence of oxygen in the growth medium was parallel to the sucrose concentration, indicating that cell respiration must be the main metabolic mechanism of energy mobilization.

The presence of NaCl in the medium induced distinct growth patterns in both cell lines with different sensitivity to salt (Fig. 2, Table 1). The measurement of growth parameters, namely specific growth rates, biomass production and of the yield factors confirmed that Valencia late is much more sensitive towards salt than Carvalhal tangor (Table 1).

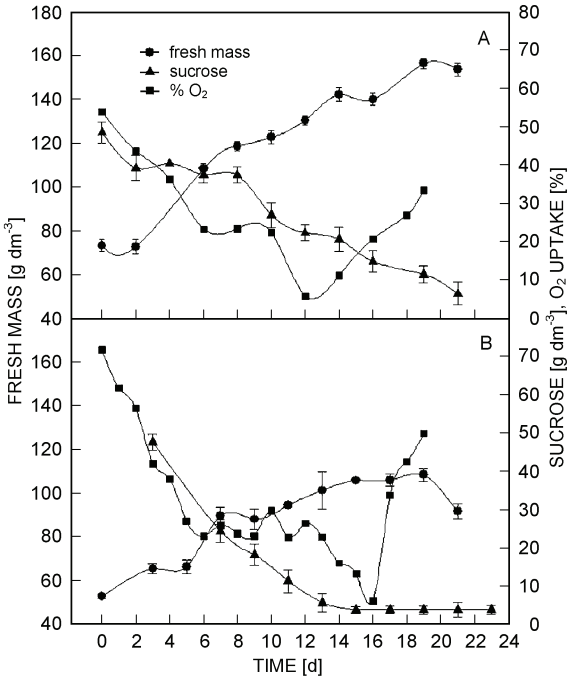

Fig. 1. Biomass production, O_2 and sucrose consumption in Carvalhal tangor (*A*) and Valencia late (*B*) cell lines in a bioreactor, in the absence of NaCl. O_2 uptake is expressed as % of saturation (dissolved O_2). Means of three replicates, *bars* represent standard errors.

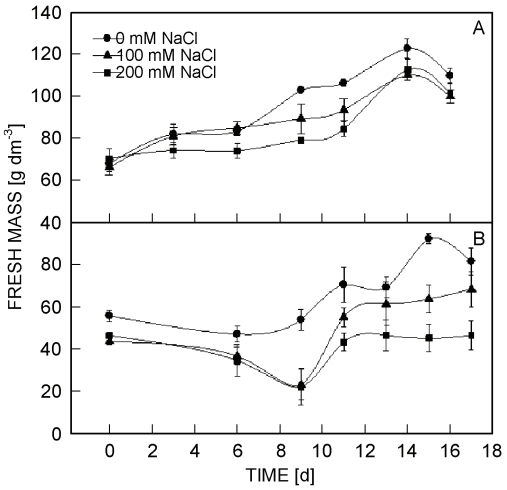

Fig. 2. Influence of NaCl concentration on the biomass production by Carvalhal tangor (*A*) and Valencia late (*B*) cell lines. Means of three replicates, *bars* represent standard errors.

Influence of NaCl on respiration rates of cells: Non-acclimated cell suspensions of Carvalhal tangor and Valencia late were grown under different concentrations of NaCl (100, 200 and 400 mM), while the 100 mM NaCl acclimated Carvalhal tangor was grown at 100 mM NaCl for 2 d and the respective respiration rates (R_D) were measured (Fig. 3). Non-acclimated Carvalhal tangor

Table 1. Influence of NaCl concentration on growth parameters: specific growth rate [d^{-1}], biomass productivity [g(d.m.) dm^{-3} d^{-1}] and biomass yield [g(f.m.) g^{-1}(initial suspension)] for Carvalhal tangor (CT) and Valencia late (VL) cell lines grown in Erlenmeyer flasks (n.d. - not determined).

NaCl [mM]	Specific growth rate		Biomass productivity		Biomass yield	
	CT	VL	CT	VL	CT	VL
0	0.127	0.110	1.13	1.55	0.26	0.21
100	0.089	0.069	1.16	0.89	0.29	0.16
200	n.d.	0.019	1.26	0.54	0.34	≅0

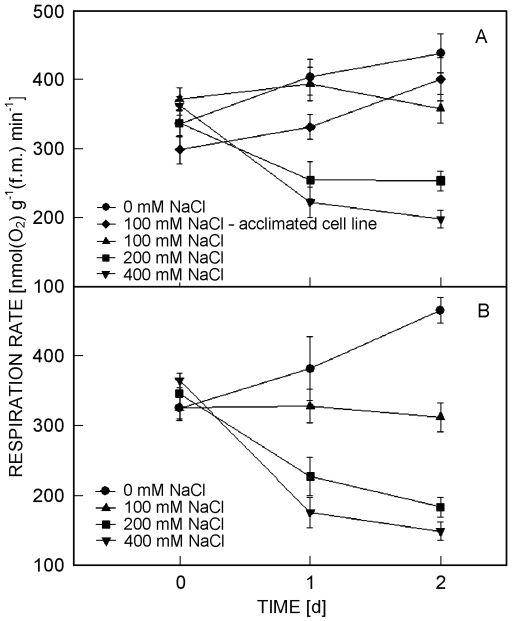

Fig. 3. Influence of NaCl concentration on the respiration rate in the absence of inhibitors, in normal and acclimated Carvalhal tangor (*A*) and Valencia late (*B*) cell lines. Means of three replicates, *bars* represent standard errors.

suspension growing in the absence of NaCl, and the acclimated line, growing at 100 mM NaCl showed a similar increase in R_D along the time. The transient exposure of the normal cell line to 100 mM NaCl resulted in a non-significant reduction of R_D. A significant decrease of R_D was visible only at 200 mM NaCl or above. On the other hand, Valencia late cell cultures submitted to NaCl concentrations above control (100, 200 and 400 mM) showed a significant decrease in R_D.

Under absence of NaCl for Carvalhal tangor and Valencia late lines and 100 mM NaCl for the acclimated cell line, the addition of KCN resulted in decreased R_D up to 10 % (Fig. 4), maintaining the sensitivity towards the inhibitor for 2 d. However, the exposure to higher NaCl concentrations (200 and 400 mM) resulted in an increase on cyanide-insensitive respiration. The alternative oxidase capacity was much higher in Carvalhal tangor,

with values up to 39.1 % of the control at 200 mM and 54.5 % at 400 mM NaCl (Fig. 4). Valencia late presented a similar trend with respect to cyanide-insensitive respiration. However, the cyanide-insensitive R_D, reflecting the capacity of the alternative oxidase was lower (Fig. 4).

AOX- protein detection: The monoclonal antibody from *S. guttatum* recognized AOX protein with molecular mass of about 35 kDa. In both cell lines, the amount of AOX protein in control cells and when subjected to 100 mM NaCl was undetectable, consistent with the low rates of cyanide-insensitive R_D (Fig. 5).

Discussion

When living organisms are subjected to abiotic stress, the formation of ROS is generally part of the response. ROS may also be involved in ageing and on the onset of several damages. Plants may be considered well adapted to resist to oxidative stress, possessing several efficient

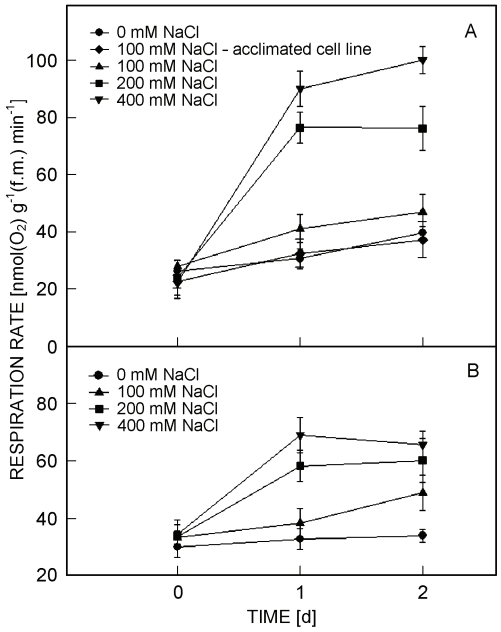

Fig. 4. Influence of NaCl concentration on the respiration rate in the presence of 1 mM KCN, in normal and acclimated Carvalhal tangor (*A*) and Valencia late (*B*) cell lines. Means of three replicates, *bars* represent standard errors.

mechanisms of radical scavenging. On the other hand, ROS may be involved in stress signalling, *e.g.*, for the synthesis of AOX in the mitochondrial respiration chain.

We used two *Citrus* cell lines with different sensitivity to salt stress, Carvalhal tangor (non-acclimated and acclimated to 100 mM NaCl) and Valencia late, to study the response and possible adaptation of these cells to NaCl stress. Both cell lines, when exposed to relatively high NaCl concentrations, showed decreased growth and parallel reduction in R_D. The decrease was more visible in Valencia late at 100 and 200 mM NaCl (Table 1, Fig. 3), suggesting the onset of salt stress in this line. For Carvalhal tangor, a restriction in the uptake of Na^+ and Cl^-, and the maintenance of a higher K^+/Na^+ ratio seem to be important mechanisms to cope with salt stress, which appear not to be as efficient in Valencia late (Ferreira and Lima-Costa 2006). A lower presence of both constitutive and inducible antioxidant enzyme activities in Valencia late may also be implied in the lower NaCl resistance (Ferreira and Lima-Costa 2006).

The similar behaviour of both the control and the acclimated cell lines of Carvalhal tangor suggested that these cultures have the capacity to adapt to the presence of NaCl, independently of eventual alterations in the electron transport chain.

As concern other stresses, reduction of R_D has been shown in *Acer pseudoplatanus* exposed to excess copper (Pádua *et al.* 1999) and in *Phaseolus vulgaris* under NaCl treatment (Jebara *et al.* 2006). Also, the addition of different herbicides resulted in a decreased R_D (Aubert *et al.* 1997). It has also been shown that AOX may play a role on stress defense of plants (Purvis and Shewfelt 1993, Wagner and Krab 1995, Wagner and Moore 1997, Mittler 2002).

The present work is the first focused on the actual induction of AOX by salt stress, although it is known that salt stress results in higher capacities of AOX and consequent heat production (Geraldes-Laakso and Arrabaça 1997). High concentrations of NaCl induced the synthesis of AOX-protein, more visible in Carvalhal tangor, suggesting that NaCl may be involved in regulation of the synthesis of this enzyme. Similar results had been obtained by Rychter and Mikulska (1990) in bean roots and González-Meler *et al.* (2001) in mung bean and soybean exposed to a low phosphate supply. Also Pádua *et al.* (1999) found a significant increase in the capacity of the alternative respiration pathway in sycamore cells, subjected to toxic levels of copper. Already Kirimura *et al.* (1996) observed an increase in the KCN-resistant respiration in *Aspergillus niger* mycelium when incubated it with antimycin.

Consistently, the stress induced AOX-protein has a molecular mass of about 35 kDa, as in *Nicotiana tabacum* (Vanlerberghe and McIntosh 1992a,b), *Glycine max* (Kearns *et al.* 1992), *Solanum tuberosum* (Hiser and McIntosh 1990), *Phaseolus vulgaris* (Lennon *et al.* 1997), *Acer pseudoplatanus* (Aubert *et al.* 1997) and *Malus domestica* (Duque and Arrabaça 1999).

In *Citrus*, KCN-insensitive respiration was very low in control cells, but it increased after incubation with NaCl. Our results indicate a correlation between the capacity of the alternative respiration in the cells

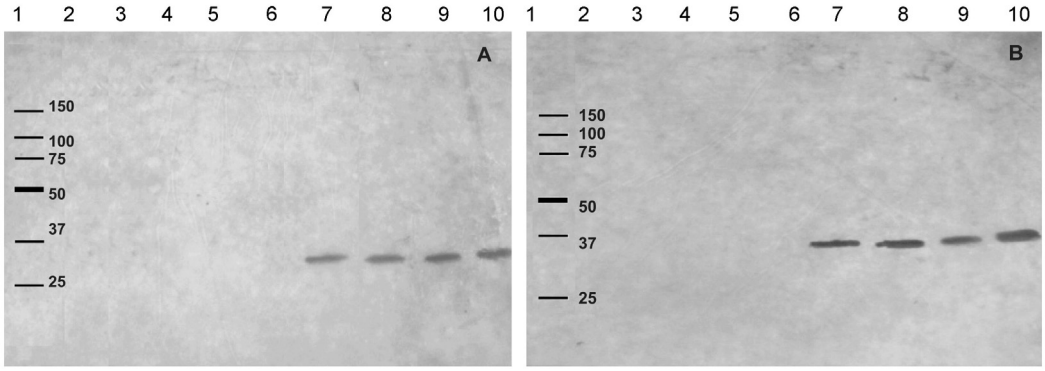

Fig. 5. Influence of NaCl concentration on alternative oxidase protein. Western blot of protein probed with the anti-AOX monoclonal antibody in Carvalhal tangor (*A*) and Valencia late (*B*) cell lines at different times. *Lanes*: *1* - molecular mass markers, *2* - control (0 mM) at time zero, *3* - control after 24 h, *4* - control after 48 hours, *5* - 100 mM NaCl at time zero, *6* - 100 mM NaCl after 48 h, *7* - 200 mM NaCl after 24 h, *8* - 200 mM NaCl after 48 h, *9* - 400 mM NaCl after 24 h, *10* - 400 mM NaCl after 48 h.

submitted to 200 or 400 mM NaCl and the appearance of the 35 kDa protein detected by Western blotting. Similar correlations were also observed in *S. guttatum*, during induction by salicylic acid (Rhoads and McIntosh 1992), and in *Nicotiana tabacum* cell suspensions in the presence of antimycin (Vanlerberghe and McIntosh 1992b).

Experiments with plant cells have suggested that the reactive oxygen species may play a role in the stimulation of the transcription of AOX (Wagner 1995, Moore *et al.* 2002, Mittler 2002). A model has been proposed (Wagner 1995) for the regulation of the synthesis of AOX-protein in which ROS, namely H_2O_2 were crucial in the signal transmission between mitochondria and the nucleus. On the other hand, the reduction of AOX levels may increase the sensitivity of plants to oxidative stress (Maxwell *et al.* 1999). Similarly, the high AOX-protein content detected at 200 and 400 mM NaCl, could be explained by an increase in ROS not efficiently eliminated by the antioxidant systems of the cells, which are depressed under our stress conditions (Ferreira and Lima-Costa 2006).

These results show that *Citrus* cell suspension cultures may constitute a useful experimental model for the study of the induction the alternative, cyanide-resistant terminal oxidase, its regulation and its functions.

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