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# Proline metabolic pathways in calli from *Lycopersicon esculentum* and *L. pennellii* under salt stress

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## Abstract

The enzyme activities of the proline metabolic pathways were determined in control and salt-treated (140 M NaCl) calli derived from cotyledons of the domestic salt-sensitive tomato *Lycopersicon esculentum* and the wild salt-tolerant *L. pennellii*. Glutamate, glutamine, asparagine, and aspartate levels increased in both genotypes under salt stress, while proline accumulation increased markedly only in the salt sensitive tomato. Activity of glutamine synthetase (GS) decreased in the salt-treated calli of the domestic species, whereas both NADH- and NADPH-glutamate synthase (GOGAT) activities increased; GS and NADPH-GOGAT decreased together in the salinized calli of the wild species. Decreasing ornithine levels were found due to NaCl in both tomato populations, while ornithine transaminase (OT) decreased in the wild type only. Increasing NADPH-Δ-pyrroline-5-carboxylate reductase (P5CR) and decreasing proline oxidase (Pro oxi) occurred in the salinized calli of the wild type. Conversely, Pro oxi and proline dehydrogenase (Pro dH) decreased highly in the salinized calli of the domestic population, while no significant changes in P5CR were found.

## Introduction

Proline accumulation in xerophytic and halophytic species under water and saline environments has been suggested to play several roles, namely, to act as an osmoticum, to be a protectant of organelles and cytosolic enzymes, a reservoir of C

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*Abbreviations:* DCPIP - dichlorophenol 2,6 indophenol; DTT - dithiotritol; FAD - flavin adenine dinucleotide; GOGAT - glutamate synthase; GS - glutamine synthetase; OT - ornithine transaminase; P5CR - Δ-pyrroline-5-carboxylate reductase; Pro dH - proline dehydrogenase; Pro oxi - proline oxidase; RGR - relative growth rate.

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and N sources or energy for post-stress conditions, or a redox buffer/shuttle (Stewart and Larher, 1980, Nash *et al.* 1982, Venekamp 1989). However, in some cases, proline accumulation was also found in plants or cells suffering from NaCl excess such as tomato (Tal *et al.* 1979, Bourgeais-Chaillou and Guerrier 1992). One approach to elaborate these roles in sensitive cells/plants is to attempt to study the proline metabolic pathways.

Proline accumulation in higher plants entails (*1*) the precursor-formation (glutamate, glutamine, via the chloroplastic and cytosolic glutamine synthetase/glutamate synthase (GS/GOGAT) route, (*2*) proline biosynthesis enzyme activities as chloroplastic and cytosolic Δ-pyrroline-5-carboxylate reductase (P5CR), (*3*) proline catabolism *via* mitochondrial proline oxidase (Pro oxi) and proline dehydrogenase (Pro dH), and (*4*) some amino-acid (ornithine, arginine) inter-conversions (Noguchi *et al.* 1966, Stewart and Boggess 1977, Huang and Cavalieri 1979, Treichel 1986, Rayapati *et al.* 1989, Venekamp 1989).

Up to now, the major studies on proline biosynthesis pathways have been carried out on proline accumulating species surviving under NaCl conditions (Treichel 1986, Laliberte and Hellebust 1989, La Rosa *et al.* 1991). No study on proline-accumulating species as a symptom of stress has been conducted so far. That is why the objective of the present work is to compare the pathways of proline metabolism, and more particularly the cytosolic biosynthesis, in white calli of the domestic tomato, salt-sensitive and proline accumulator, with those of a wild NaCl-tolerant species.

## Materials and methods

**Callus multiplication:** Seeds of *Lycopersicon esculentum* (L.) Mill. cv. P-73 and *L. pennellii* (Correll) D'Arcy, accession PE-47 were surface sterilized for 10 min in a 8 % calcium hypochlorite solution, then rinsed three times for 5 min with sterilized water. After 12 d of germination under controlled conditions (16 h light at 20 °C and 60 W $m^{-2}$ irradiance, 8 h night at 20 °C), calli were initiated from 5 mm segments of cotyledons and then routinely sub-cultured each 4 weeks according to Bourgeais-Chaillou and Guerrier (1992). In NaCl treatments, 140 mM concentration was used.

**Extraction of soluble proteins:** Crude extracts were prepared by homogenizing 5 - 10 g of fresh matter in 10 $cm^3$ 0.05 M Tris-HCl buffer, pH 7.4, containing polyvinylpyrrolidone (25 % of fresh mass), 5 mM DTT, 1 mM $MgCl_2$, 1 % KCl, 3 mM EDTA and 10 % glycerol. The homogenates were centrifuged at 20 000 *g* for 15 min at 0 °C and the supernatants were directly used for the enzyme assays. Soluble proteins were measured according to Lowry *et al.* (1951) using bovine serum albumin as standard.

**Enzyme assays:** All procedures were performed in triplicate at 30 °C and were repeated in two independent extracts; the references contained buffer instead of substrates. Except for GS activity, reaction mixtures were in a final volume of 2 $cm^3$ and were started with 0.2 $cm^3$ of enzyme extracts.

P5CR (EC 1.5.1.2), GOGAT (EC 2.6.1.53) and ornithine transaminase (OT - EC 2.6.1.13) were assayed by following the NAD(P)H oxidations at 340 nm. P5CR assay buffer contained 50 mM Tris-buffer (pH 8.0), 1 mM DTT, 250 μM NADH (or NADPH) and 1 mM Δ-pyrroline-5-carboxylate (P5CT) (Rayapati *et al.* 1989). OT assay mixture contained Tris-KOH buffer, pH 8.0, 5 mM ornithine, 1.0 mM α-ketoglutarate and 250 μM NADH (Charest and Phan 1990). For GOGAT the assay mixture contained 50 mM Tris-HCl buffer, pH 7.4, 1 mM EDTA, 250 μM NADH, 1 mM glutamine and 0.5 mM α-ketoglutarate (Bourgeais-Chaillou and Guerrier 1992).

Pro dH (EC 1.5.1.2) was assayed by following the NAD reduction at 340 nm in 100 mM $Na_2CO_3$-HCl buffer (pH 10.3), containing 20 mM L-proline, and 1 mM NAD (Charest and Phan 1990). Pro oxi (EC 1.5.99.8) was assayed by following DCPIP reduction in 2 ml reaction medium containing 50 mM Tris HCl (pH 8.5), 5 mM $MgCl_2$, 0.5 mM FAD, 1 mM KCN, 1 mM phenazine methosulphate, 0.06 mM DCPIP and 100 mM proline, according to the method of Huang and Cavalieri (1979).

Reaction mixtures for GS (EC 6.3.1.2) consisted of 0.9 $cm^3$ 50 mM Tris-HCl buffer (pH 7.4), 80 mM glutamate K, 20 mM $MgSO_4$, 4 mM amonium acetate, 8 mM ATP, and 0.1 $cm^3$ of the enzyme extract. Reactions were stopped after 15 min. by the addition of 0.5 $cm^3$ 10 % trichloracetic acid. Phosphate released in the assay media was quantified colorimetrically (Bourgeais-Chaillou and Guerrier 1992).

**Amino acid determination:** 10 g of fresh calli were ground in hot water. The homogenates were centrifuged 5 000 *g* for 10 min. Free amino acid in the supernatants were determined in a *Rank-Hilger Chromaspeck Autoanalyser* (Rank Hilger Co., Westwood-Margate, UK) using a pH gradient and an ion exchange column, with ninhydrine post-column reaction and 0.1 mM norleucine as internal standard. Proline concentrations were determined according to Bates *et al.* (1973) using ninhydrin and L-proline as standard. Four replicates were performed.

## Results

**Relative growth rates (RGR) of calli:** RGR of calli were measured by the (fm-im)/im ratios, where fm was the fresh mass after 28 d of culture and im the fresh mass at the time of the transfer. Under control conditions, callus RGR was similar (mean value 5.50) for the both species. RGR of salinized *Lycopersicon esculentum* calli was only 33 % of that of the control; RGR of salt-treated *L. pennellii* reached up to 128 % of control calli.

**Amino acid and protein contents:** Under salt conditions, the levels of free amino acids and proteins increased in *L. esculentum* calli but remained constant in *L. pennellii* ones (Table 1). Proline contents increased 2- and 6-fold in the salt-treated calli from the wild and the domestic species, respectively. Increasing glutamate, glutamine, asparagine and aspartate levels were found practically in both populations under salt-stress; the arginine pool remained equal to zero in

*L. esculentum* calli under salt and control conditions, while it increased in *L. pennellii*. Ornithine levels declined in salinized calli, regardless of the populations.

Table 1. The amino acid pools [μmol $g^{-1}$(d.m.)] ± SE and protein content [mg $g^{-1}$(d.m.)] ± SE of calli from *L. esculentum* and *L. pennellii* cultured 4 weeks on medium without (control) or with 140 mM NaCl.

| | Control | | 140 mM NaCl | |
|---|---|---|---|---|
| | *L. esculentum* | *L. pennellii* | *L. esculentum* | *L. pennellii* |
| Arginine | 0 | 1.60 ± 0.213 | 0 | 3.52 ± 2.24 |
| Asparagine | 0.324 ± 0.071 | 4.40 ± 0.376 | 0.496 ± 0.012 | 10.2 ± 1.32 |
| Aspartate | 1.12 ± 0.141 | 6.48 ± 0.539 | 1.49 ± 0.113 | 10.7 ± 1.21 |
| Ornithine | 0.193 ± 0.021 | 0.728± 0.063 | 0 | 0 |
| Glutamate | 0.153 ± 0.011 | 9.02 ± 1.12 | 0.234 ± 0.028 | 11.9 ± 1.05 |
| Glutamine | 0.471 ± 0.020 | 26.6 ± 2.12 | 1.33 ± 0.112 | 24.5 ± 2.34 |
| Proline | 22.2 ± 2.14 | 8.90 ± 0.714 | 129 ± 10.4 | 17.8 ± 1.54 |
| Total amino acids | 117 ± 10.3 | 174 ± 14.5 | 260 ± 23.4 | 193 ± 21.4 |
| Total proteins | 93.2 ± 11.2 | 62.2 ± 7.31 | 124 ± 13.5 | 58.3 ± 6.12 |

**Enzymes activities:** The GS activity (Fig. 1) decreased more in NaCl-treated calli from the sensitive species than in those of the wild species. Compared with GS activity, opposite evolutions of GOGAT activities (Fig. 2) were found as a function of NaCl-treatment: indeed, both NADH and NADPH-dependent GOGAT increased 2-fold in NaCl-treated calli from the sensitive and the wild species.

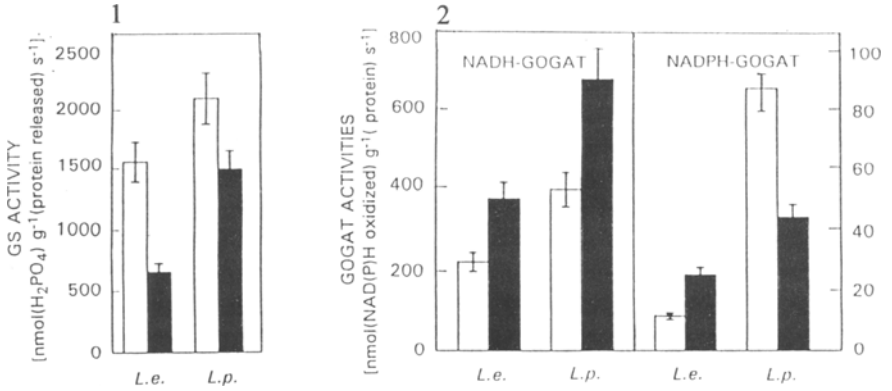


Fig. 1. GS activities [nmol($H_2PO_4$) $g^{-1}$(protein released) $s^{-1}$] activities of calli from *L. esculentum* (*L.e.*) and *L. pennellii* (*L.p.*) under control (*open columns*) and 140 mM NaCl (*closed columns*) conditions. Vertical bars represent SE.

Fig. 2. NADH and NADPH-dependent GOGAT [nmol(NADPH oxidized) $g^{-1}$( protein) $s^{-1}$] of calli from *L. esculentum* (*L.e.*) and *L. pennellii* (*L.p.*) under control (*open columns*) and 140 mM NaCl (*closed columns*) conditions. Vertical bars represent SE.

Under control conditions, high levels in NADPH-P5CR were found in the calli from the wild populations (Fig. 3). Both P5CR activities decreased due to salinity in

the domestic species whereas the NADPH-P5CR increased highly in the wild species. Thus, under saline conditions, P5CR levels were 4-fold higher in the wild species than in the domestic one.

OT activity (Fig. 3) decreased in the salt-treated calli from the wild species and was 6-fold lower than in the domestic ones.

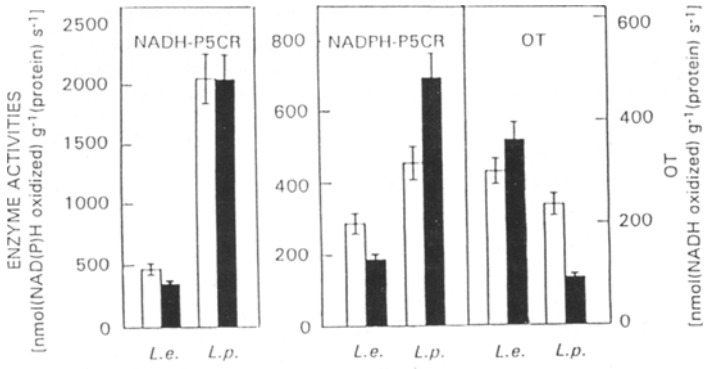


Fig. 3. NADH- and NADPH-dependent P5CR [nmol(NADPH oxidized) $g^{-1}$ $s^{-1}$] and OT [nmol(NADH oxidized) $g^{-1}$(protein) $s^{-1}$] of calli from *L. esculentum* (*L.e.*) and *L. pennellii* (*L.p.*) under control (*open columns*) and 140 mM NaCl (*closed columns*) conditions. Vertical bars represent SE.

Under control condition, NAD-Pro dH activity was practically 3-fold higher than NADP-Pro dH one, irrespective of the populations (Fig. 4). Contrary to the P5CR reverse reaction (since P5CR and Pro dH functioned with the same reactants), the NADP-Pro dH activity was the highest in the domestic species and the lowest in the wild population. Both Pro dH decreased under saline conditions in the domestic species; conversely, only NAD-pro dH activity increased in the salinized calli from the wild species.

Pro-oxi activity decreased 3- and 10-fold in salinized calli from the wild and the domestic species, respectively (Fig. 5).

## Discussion

As in *Mesembryanthemum, Opophytum* and *Acer* (Treichel 1986), the increasing proline content in both populations did not result from an enhanced proteolysis or a decreased protein synthesis. This suggests that the contribution of the protein hydrolysis during the stress was not a proline-generating mechanism in tomato populations.

The GS/GOGAT pathway, which catalyzes the major part of $NH_3$-assimilation was not linked to the evolution of proline-precursor (glutamate and glutamine) or proline levels: under saline conditions, the GS activity decreased more in the domestic species where proline and glutamine levels increased more markedly. On the other hand, compared with the domestic species, high levels of GOGAT and

glutamate were found in the wild species where proline levels were the lowest. The decreasing ornithine levels and OT activities in the salt-treated wild species are unclear; moreover, the decreasing ornithine level in the salinized calli of the domestic species was not significantly coupled with the slight stimulation of OT activity which catalyzes pyrroline-5-carboxylate or glutamate γ-semi-aldehyde conversions from ornithine. So, the formation of potential proline precursors (glutamine, glutamate, arginine and ornithine), taking into account amino-acid contents and maximum activities of GS, GOGAT and OT, did not seem to be linked to the proline levels.

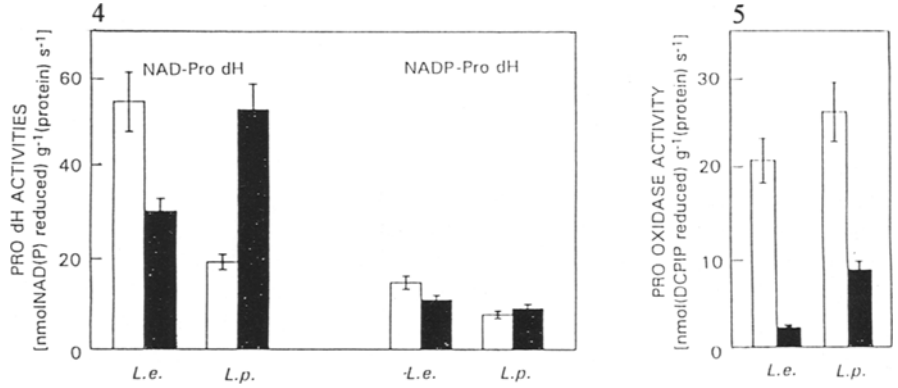


Fig. 4. NAD- and NADP-dependent Pro dH [nmol]NADP reduced) $g^{-1}$(protein) $s^{-1}$] of calli from *L. esculentum* (*L.e.*) and *L. pennellii* (*L.p.*) under control (*open columns*) and 140 mM NaCl (*closed columns*) conditions. Vertical bars represent SE.

Fig. 5. Pro oxi (nmol(DCPIP reduced) $g^{-1}$(protein) $s^{-1}$] of calli from *L. esculentum* (*L.e.*) and *L. pennellii* (*L.p.*) under control (*open columns*) and 140 mM NaCl (*closed columns*) conditions. Vertical bars represent SE.

In accordance with the results obtained with NaCl-tolerant plants (Boggess *et al.* 1976b, Rhodes *et al.* 1986), the salinized calli of the most NaCl-tolerant species were found to convert more glutamate into free proline, due to their high P5CR activity. Treichel (1986), Laliberte and Hellebust (1989) or DeLauney and Verna (1990) reported that P5CR would be involved in the proline biosynthesis regulation. However, under control and saline conditions, the proline levels of the domestic species were higher than those of the wild species, while the maximum velocities of both P5CR of the former were lower than those of the latter. Similarly, La Rosa *et al.* (1991) suggested that changes in P5CR of NaCl-adapted and unadapted cells of *Nicotiana* were not high enough to convincingly account for differences in proline pool sizes. Thus, P5CR would not be a limiting step in the proline biosynthesis pathway of tomato.

Under salt stress, high levels of proline in the domestic species resulted from high inhibitory rates of both Pro dH and Pro oxi activities, as it was reported previously in other species (Boggess *et al.* 1976a, Stewart *et al.* 1977, Charest and Phan 1990). This was consistent with the idea that proline accumulation in the sensitive tomato was a symptom of metabolic disfunction and resulted from inactivation by water stress of normal control mechanism (Stewart *et al.* 1977): (*1*) inhibition of proline

hydroxylation into hydroxyproline (Golan-Goldhirsch *et al.* 1990), since proline exerted a specific key-role in the formation of cell walls in the domestic species (Tal and Katz 1980); (*2*) impaired respiration (Bellinger and Larher 1987) as it was suggested by the accumulation of sugars or succinate (Bourgeais-Chaillou and Guerrier 1992).

The specific enhancement of NADPH-P5CR in the salinized wild species would contribute to ensure a NADP recycling for the transfer of redox potential: indeed, proline biosynthesis consumes ATP, reductants and carbon skeletons, allowing to lower over-reduction of the NADPH pool and to increase ADP-turnover, a factor limiting respiration and the whole energetic metabolism (Bellinger and Larher 1987, Venekamp 1989). Therefore, the increasing P5CR under salt stress, and the involvement of proline as a cell protector, as stated by Nash *et al.* (1982), would prove that *L. pennellii* is well adapted to NaCl. Conversely, the uncontrolled proline biosynthesis (since it was independent of P5CR) in the domestic species would result in a diversion of reduced N and C to the detriment of growth.

Salt-tolerance results from cell-mechanisms and from their regulation derived from the integrated functioning of the whole plant (Munns and Termaat 1986, Bourgeais-Chaillou and Guerrier 1992). Comparing the magnitude of proline biosynthesis enzymes in calli, it can be assumed that the increasing proline level in the salt-treated calli from the domestic species resulted from a decrease in proline catabolism rather than from an activation of its biosynthesis. However, a precise determination of the proline metabolic pathways in cells and further enzyme studies on both their substrate-specificity and the appearance of new isoenzymatic forms are clearly required for establishing the role of proline when accumulated as a symptom of stress injury.

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