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BRIEF COMMUNICATION

Shoot regeneration and protein synthesis in tomato tissue cultures

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Abstract

Shoot regenerations from hypocotyls and cotyledons of tomato (*Lycopersicon esculentum* Mill.) was inhibited by NaCl (100 and 150 mM). Shoot fresh and dry masses were also reduced. Addition of proline (100 mg dm^{-3}) counteracted the inhibitory effect of NaCl. SDS-PAGE analyses of extracted proteins, revealed that in cultures grown in medium with 25 mM NaCl plus proline, extra polypeptides of M_r 190, 58, 45 and 26 kDa accumulated. As NaCl was increased in the medium a new protein of M_r 67 kDa also accumulated.

Additional key words: *Lycopersicon esculentum*, proline, protein accumulation, salinity.

Utilization of the tissue culture technique to derive cell lines tolerant to NaCl stress is one approach to the improvement of the salt tolerance of tomato (Dix and Street 1975, Mathur *et al.* 1980, Alfocea *et al.* 1993). NaCl beyond 80 mM affected fresh and dry masses and differentiation of shoots and roots in tomato cell cultures (Rahman and Kaul 1989, Tal *et al.* 1989) and induced protein changes in cultured cells (Singh *et al.* 1985, Ramagopal 1988, Ginzburg *et al.* 1990, El-Frash *et al.* 1994). Exogenously added proline was very effective in counteracting the effect of salinity (Pandey and Ganapathy 1985, Armstrong and Green 1985, Shaddad 1990).

In the present study, the shoot regeneration and protein synthesis at different NaCl levels with and without proline, was investigated.

Tomato (*Lycopersicon esculentum* Mill.) seeds were floated on distilled water for 10 min, surface-sterilized by immersion in 5 % *Chlorox* for 5 min, and washed with distilled water three times. Seeds were germinated on Murashige and Skoog (1962) medium without hormones (MS-I).

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Abbreviations: M_r - relative molecular mass, PAGE - polyacrylamide gel electrophoresis, SDS - sodium dodecyl sulphate, Tris - Tris(hydroxymethyl)aminoethane.

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The hypocotyls and cotyledons were excised from 10- to 12-d-old seedlings grown *in vitro*. The excised explants were transfered in aseptic conditions to 50 cm^3 conical flasks containing 20 cm^3 of MS-medium supplemented with [mg dm^{-3}]: 6 IAA, 5 kinetin, 40 adenine sulfate, 170 $NaH_2PO_4 . H_2O$, 100 inositol, 0.1 thiamine-HCl, 0.5 pyridoxine, 0.5 nicotinic acid, saccharose (30 g dm^{-3}) and agar (7 g dm^3) (MS-II).

Sodium chloride was incorporated into the MS-II medium at 0, 25, 50, 100 and 150 mM. Proline (100 mg dm^{-3}) was added in combination with NaCl. In controls MS-II was supplemented with proline only. For all combinations, pH was adjusted to 5.5 ± 0.2 before autoclaving. Each flask contained three cotyledons or five hypocotyl explants, in three replicates. Cultures were kept in an incubator at 27 °C and under continuous fluorescent white light (irradiance 376.8 μmol m^{-2} s^{-1}) as recommended by Lercari *et al.* (1986). After 3 weeks shoot formation, fresh and dry masses were recorded. Proline content was determined according to Bates *et al.* (1973).

For protein extraction lyophilized cultures [0.2 g(f.m.)] was ground in 0.1 cm^3 extraction buffer (50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1 % mercaptoethanol, 2 % sodium dodecyl sulfate (SDS) and 5 % glycerol), boiled for 5 min and centrifuged (10.8 *g*) in an Eppendorf microcentrifuge for 15 min. 0.05 cm^3 of extract containing about 4 mg protein was applied. SDS-PAGE was performed as described by Laemmli (1970) by means of vertical slab gel unit *SE 600* (*Hoefer Scientific Instrument*, USA). The running gel was 12 % acrylamide (0.4 % bis) at pH 8.8 and stacking was 2.5 % acrylamide (0.625 % bis) at pH 6.8. Both solutions of running gel and stacking gels were supplemented with SDS 0.4 %. The reservoir buffer was 1.5 M Tris-glycine plus SDS 2 %. Electrophoresis was performed at room temperature of 25 °C, 140 V and 60 mA with phenol red as "tracking dye". Molecular mass markers ranged from 200 - 8 kDa. Gels were stained with Coomassie brilliant blue *R-250* and destained with a 5 % MeOH/acetic acid mixture. Stained bands were scanned at 525 nm with a *Seroscan elvi 146* densitometer.

All the cultured hypocotyl and cotyledon explants produced calli at all levels of NaCl treatments, except 150 mM. The cotyledonary explants had a substantially higher rate of shoot regeneration than the hypocotyl explants. The percentage of shoot regeneration of hypocotyl and cotyledonary explants was reduced sharply as NaCl increased in the culture medium and completely inhibited at 150 mM NaCl. Exogenously supplied proline (Table 1), increased callus formation, shoot regeneration, especially at high levels of NaCl (100 and 150 mM).

The dry mass of hypocotyl explants (Fig. 1*A*) decreased as NaCl concentration increased in the culture medium. However, dry mass slightly increased at 25 and 50 mM NaCl with proline. In case of cotyledonary explants, the proline increased the dry mass at all NaCl levels (Fig. 1B). Addition of proline in the absence of NaCl improved the growth of both hypocotyl and cotyledonary explants.

Mathur *et al.* (1980) found that the capacity for regeneration of plantlets from internodal segments of *Kickxia ramosissima*, was reduced in the presence of NaCl and proline counteracted the inhibitory effect of NaCl. Proline was also shown to be the best amino acid which stimulated the somatic embryogenesis (Stuart and Strickland 1984 a,b). Glutamine and asparagine also enhanced the shoot regeneration of soybean (Green *et al.* 1990, Franklin *et al.* 1991, Shetty *et al.* 1992). They

concluded that organic nitrogen sources were important for efficient plant regeneration. Proline may serve in addition to osmoticum also as an important source

Table 1. Shoot regeneration of hypocotyl and cotyledonary explants, cultured for 3 weeks on MS-II, supplemented with different levels of NaCl, or NaCl with proline (100 mg dm^3). ERS - explants regenerated shoots.

NaCl [mM]	Proline	Hypocotyl ERS [number]	ERS [%]	shoots [explant^{-1}]	Cotyledon ERS [number]	ERS [%]	shoots [explant^{-1}]
0	0	27	100	3.77	9.9	100	6.00
0	100	27	100	9.00	9.0	100	6.45
25	0	27	100	1.22	7.0	65	4.00
25	100	27	100	2.35	9.0	100	6.02
50	0	15	55	0.88	6.0	66	4.00
50	100	18	66	3.75	7.0	77	5.78
100	0	12	44	0.55	-	-	-
100	100	15	55	1.44	5.0	55	4.34
150	0	-	-	-	-	-	-
150	100	15	55	1.00	4.0	45	4.44

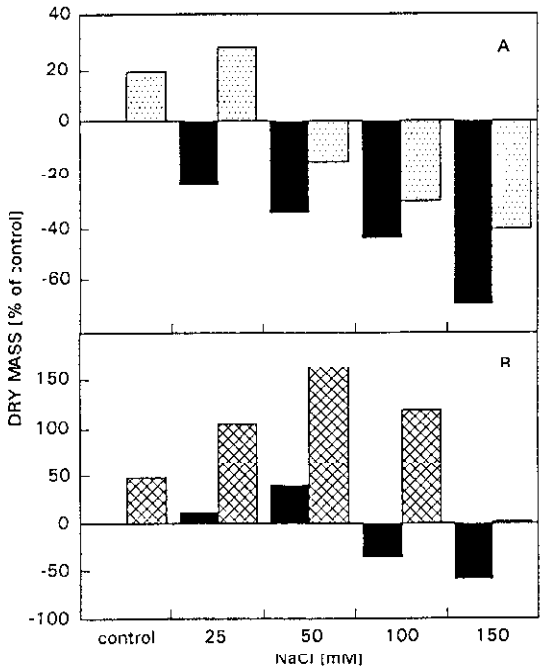

Fig. 1. Dry mass of hypocotyl (*A*) and cotyledonary (*B*) explants cultured for 3 weeks on MS-II medium supplemented with different concentration of NaCl (*black columns*) or NaCl and 100 mg dm^{-3} of proline (*dotted* or *hatch crossed columns*).

of nitrogen in plant metabolism (Britikov *et al.* 1970) and as a readily available source of energy and reducing power (Stewart *et al.* 1974).

Proline has been reported to play an important role in protecting enzymes against denaturation (Paleg *et al.* 1984 and Nikolopoulos and Manetas 1991) and in regulating the cytosolic acidity (Venekamp 1989 and Venekamp *et al.* 1989). The proline accumulation (Table 2) increased significantly as salinity level raised in the culture media and more in the cotyledonary explants compared with the hypocotyl explants. These results are in agreement with Dix and Pearce (1981), Weimberg *et al.* (1982) and Casido *et al.* (1987).

Table 2. Proline content [μg g^{-1}(f.m.)] of hypocotyl and cotyledonary explants, cultured for 3 weeks on MS-II, supplemented with different concentrations of NaCl, or NaCl and proline (100 mg dm^{-3}).

NaCl [mM]	Hypocotyl -proline	+proline	Cotyledon proline	+proline
0	25.50 ± 0.83	33.21 ± 2.64	39.03 ± 2.08	52.68 ± 1.74
25	52.71 ± 2.38**	59.06 ± 1.32**	63.75 ± 2.55**	71.52 ± 2.05**
50	76.51 ± 0.92**	83.06 ± 2.54**	80.97 ± 2.56**	96.89 ± 2.74**
100	89.54 ± 0.64**	105.48 ± 1.75**	103.29 ± 2.91**	116.97 ± 2.34**
150	105.49 ± 2.00**	114.63 ± 1.61**	127.03 ± 3.07**	133.24 ± 2.34**
L.S.D. at 5 %	2.76	3.71	4.83	4.78
L.S.D. at 1 %	3.94	5.28	6.87	6.81

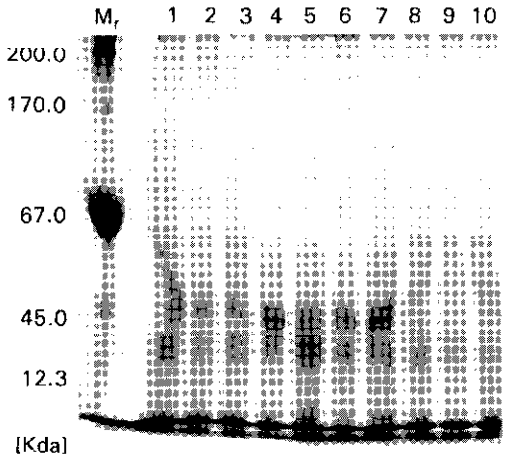

Fig. 2. SDS-PAGE of soluble protein fractions from cotyledonary explants callus cultures. Lanes 1, 3, 5, 7 and 9 correspond to different concentrations of NaCl (0, 25, 50, 100 and 150 mM, respectively). Lanes 2, 4, 6, 8 and 10 correspond to NaCl and proline [100 mg dm^{-3}]. Molecular masses [kDa] of standard proteins are shown on the left.

Proteins of M_r 190, 90 and 26 kDa were accumulated in the cultures supplemented with proline and were not detectable in untreated culture. Proteins of 170, 58, 45, 26, 12 and 8 kDa were accumulated in the cultures grown at 25 mM NaCl. Exogenous proline addition enhanced the biosynthesis of extra polypeptides of M_r 35 - 30 kDa and 67 kDa. A protein of M_r 67 kDa was apparently induced at 150 mM NaCl. In cultures supplemented with proline, newly synthesised proteins of M_r 52 and 40 kDa were recorded in addition to those produced at 150 mM NaCl (Fig. 2).

Our results indicate a major protein of 26 kDa accumulated in salt-stressed cultures and in salt-stressed cultures with proline. These results are in agreement with La Rosa *et al.* 1989, Bressan *et al.* 1988 and Ben-Hayyin *et al.* 1989. They concluded that the prologned adaptation of cells to salinity, resulted in increase of this protein ("osmotin").

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