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Influence of exogenous proline on embryogenic and organogenic maize callus subjected to salt stress

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Abstract

The effect of exogenous proline (6 mM) and increasing NaCl doses (from 0.4 to 1.2% w/v) on the maintenance of organogenic and embryogenic callus lines derived from the salt-sensitive maize inbred W64Ao2 were studied. To this end, total protein, free amino acid and polyamine content were analyzed. The demand of exogenous nitrogen and especially of proline, even in the presence of salt, differed in the two types of morphogenic calluses. The total protein content of embryogenic calluses was higher in the presence of proline than in its absence, in all the cases studied. An opposite effect of proline was observed in organogenic calluses: the presence of proline and salt decreased significantly their protein content. With respect to amino acid and polyamine contents, the organogenic calluses showed physiological characteristics of salt-adaptation, whereas the embryogenic calluses were more sensitive to NaCl. Although endogenous proline increased in the organogenic calluses cultured in the presence of salt, in embryogenic calluses it only rose at the lowest salt concentration. Furthermore, the endogenous arginine content under saline conditions was higher in organogenic calluses. A compensatory effect between proline and polyamine metabolism related to the endogenous arginine content in response to salt stress was also observed. This effect differed in the two types of calluses.

Abbreviations: Ala – alanine; Arg – arginine; 2,4-D – 2,4-dichlorophenoxyacetic acid; EC – embryogenic callus; GABA – γ-amino butyric acid; MC – meristematic callus; PAs – polyamines; Pro – proline; Put – putrescine; Spd – spermidine; Spm – spermine

Introduction

The effects of salt stress on growth and on the endogenous content of nitrogenous compounds are well known. Correlations between amino acid and polyamine accumulation during salt stress have been studied by a number of authors (Lucarini and Sangwan, 1987; Lovatt, 1990; Slocum and Weinstein, 1990).

In plants and tissue cultures, salt stress results in a general increase in amino acids, albeit at varying levels (Fougère et al., 1991; Watad et al., 1983). Proline accumulation has been associated with resistance to osmotic and saline stress as well as in the control of cell pH (Bellinger et al., 1991). In some species, alanine seems to play a role similar to that of proline on osmotic stress (Pulich, 1986). Furthermore, arginine accumulation is considered to be a detoxification mechanism for ammonium produced in plants under salt stress (Slocum and Weinstein, 1990).

The concentration of nitrogen and the relative amounts of ammonium and nitrate may be critical for growth and morphogenesis of plant cells (Armstrong and Green, 1985; Wilkinson and Thompson, 1987). A number of studies have demonstrated stimulation by L-proline of somatic embryogenesis from immature embryos of maize (Armstrong and Green, 1985; Duncan and Widholm, 1987; Claparols et al., 1993). Nevertheless, there is little information on the effect of this amino acid on *in vitro* organogenesis.

Differences in the endogenous amino acid pools could affect the rate of protein synthesis during morphogenetic processes such as embryogenesis (Wetherell and Dougall, 1976), seed germination (Aguilar and Sanchez, 1984) and organogenesis (Zhu et al., 1990). In previous works (Claparols et al., 1994; Santos et al., 1993), we demonstrated the effect of exogenous amino acids on the improvement of embryogenesis and on endogenous amino acid metabolism in embryogenic callus of maize.

Polyamines are considered to be regulators of plant growth and development given their effects on cell division and differentiation (Galston and Kaur-Sawhney, 1990). The diamine putrescine (Put) and the polyamines (PAs), spermidine (Spd) and spermine (Spm) are involved in important biological processes e.g. ionic balance (Lucarini and Sangwan, 1987), DNA, RNA and protein stabilization (Kaur-Sawhney et al., 1980; Bestford et al., 1991). Put accumulation is very often a characteristic of a stress response. Although this accumulation could play a protective role in the cell (Krishnamurthy, 1991), it has been reported that Put excess may have some negative effects (DiTomaso et al., 1989). Krishnamurthy and Bagwat (1989) observed an increase in Put levels associated with a low content of Spd and Spm in salt-sensitive rice cultivars. Moreover, tolerant cultivars produced small increases in Put and high increases in Spd and Spm. PAs could, therefore, play a modulating role in these salt tolerance mechanisms. It has also been reported that the endogenous polyamine content and certain amino acid levels under stress conditions are directly related to alterations in the levels of enzymes associated with PA synthesis and degradation caused by feed-back and/or repressive mechanisms (Slocum and Weinstein, 1990).

In this paper, the effect of exogenous Pro and that of increasing salt doses on the maintenance of maize meristematic and embryogenic callus were studied. To this end, total protein, amino acid and PA content of these calluses were analyzed.

Materials and methods

Plant material and callus culture

In this study, organogenic and embryogenic calluses obtained from immmature embryos of the salt-sensitive W64Ao2 maize inbred line were employed. The organogenic callus line was obtained using the method established by Torné et al. (1984). The MC calluses were subcultured at 30 day intervals in test tubes containing Murashige and Skoog (1962) medium in accordance with the modifications used in previous studies (Torné et al., 1984). The embryogenic callus line (EC) was obtained using the method of Claparols et al. (1993). The EC calluses were subcultured at 20 day intervals in test tubes containing N6 basal medium (Chu et al., 1975) supplemented by the organic components of the same medium. In both cases, the concentration of 2,4-D used was always 9μM for the maintenance of the callus. The medium was solidified with 2 gl^{-1} Gelrite and the pH was adjusted to 5.8 before autoclaving. All the cultures were maintained at 27±1°C under cool-white fluorescent lights (90–100 $\mu molm^2\ s^{-1}$) in a 16-h photoperiod (Santos et al., 1986). For these experiments, both the MC and EC calluses were cultured in their respective media in the presence or absence of 6 mM exogenous Pro. Furthermore, different NaCl concentrations (0, 0.4, 0.8 and 1.2% w/v) were applied. Fifteen replicates of one-year-old calluses with a minimum of 0.5 g fresh weight each were employed for each treatment using individual tubes.

After three subcultures, a number of MC and EC calluses corresponding to each treatment were frozen and stored at −80°C for analysis.

Protein content

The total protein content of the calluses was determined according to Bradford (1976) using γ-globulin (Sigma Chemical Co, St Louis, MO U.S.A.) as a standard.

Polyamine analysis

Polyamines were extracted, separated and detected by dansylation and thin layer chromatography following procedures of (Tiburcio et al., 1985). Briefly, samples were extracted with 5% perchloric acid and centrifuged at 27000 × g for 20 minutes after which aliquots from the supernatant and the pellet fraction were hydrolyzed with 12N HCl. The unhydrolyzed perchloric acid supernatant containing the free PAs (S fraction) as well as the hydrolyzed perchloric acid supernatant (SH fraction) and the hydrolized pellet containing PAs liberated from conjugates (PH fraction) were then dansylated, solvent purified, separated by thin layer chromatography and quantified using a Perkin Elmer spectrofluorimeter. Nine replicates of each analysis were done.

Table 1a. Free amino acid content (pmol/mg fw) of EC calluses cultured with different concentrations of NaCl in the presence (+) or absence (-) of Pro.

%NaCl/Aa	Pro	Glu	Arg	GABA	Ala	Pro	Totals
0.0	+	2548 ± 189	–	2620 ± 223	15889 ± 1345	19657 ± 1587	51217 ± 3245
	-	3474 ± 285b	–	1985 ± 175a	11436 ± 1324a	545 ± 66c	22943 ± 1897c
0.4	+	2421 ± 165	67 ± 9 c	2071 ± 198 a	15890 ± 1346	21715 ± 1689	44182 ± 2984
	-	3190 ± 212 b	141 ± 13bc	2240 ± 189	13527 ± 1426	991 ± 85cb	25442 ± 1976c
0.8	+	3181 ± 196 a	201 ± 23 c	4197 ± 413 b	26527 ± 2148 b	15640 ± 1254 a	58705 ± 3360
	-	2792 ± 198 a	50 ± 80cc	4512 ± 221 c	24214 ± 1987 c	1743 ± 123cc	45120 ± 3454bc
1.2	+	2126 ± 123 a	128 ± 12 c	2566 ± 213	14025 ± 1234	3837 ± 322 c	28770 ±1342 c
	-	2355 ± 179 b	43 ± 70cc	6528 ± 389cc	18569 ± 1745ab	1342 ± 99cc	39458 ± 2986bb

1st significance symbol: Pro effect, snd symbol: NaCl effect. (a) $p > 0.05$, (b) $p > 0.01$, (c) $p > 0.001$. Aa=amino acid

Table 1b. Free amino acid content (pmol/mg fw) of MC calluses cultured with different concentrations of NaCl in the presence (+) or absence (-) of Pro.

%NaCl/Aa	Pro	Glu	Arg	GABA	Ala	Pro	Totals
0.0	+	1560 ± 159	321 ± 38	1670 ± 125	16860 ± 2312	1711 ± 126	26433 ± 1845
	-	2001 ± 243	512 ± 58b	2132 ± 186a	12046 ± 978a	278 ± 24c	22955 ± 1615
0.4	+	1925 ± 163b	938 ± 77 c	7960 ± 1258 c	36052 ± 2589 c	10011 ± 858 c	65317 ± 3421 c
	-	810 ± 85 b	387 ± 25ca	2703 ± 320b	10185 ± 842c	173 ± 14cb	17324 ± 1360cc
0.8	+	2426 ± 172 c	1267 ± 106 c	9637 ± 1854 b	46178 ± 3548c	10106 ± 864 c	77674 ± 3854 c
	-	2254 ± 285	1137 ± 130	5165 ± 266 c	27868 ± 1854cc	750 ± 78cc	45132 ± 3258cc
1.2	+	2915 ± 178 c	1310 ± 115 c	6261 ± 959 b	40898 ± 3211 c	9343 ± 754 c	68246 ± 3524 c
	-	2915 ± 321 a	875 ± 115b	3476 ± 458 b	22870 ± 1435cc	695 ± 63cc	37395 ± 2914cc

1st significance symbol: Pro effect, snd symbol: NaCl effect. (a) $p > 0.05$, (b) $p > 0.01$, (c) $p > 0.001$. Aa= amino acid

Amino acid analysis

In order to analyze the endogenous amino acid content, calluses were frozen in liquid nitrogen and kept at −80°C. For each treatment and analysis, 0.5 g fresh weight of callus was used. Four replicates of each treatment were done. After freezing, the material was ground in a mortar with liquid nitrogen, adding double distilled water in a 1:3 ratio (w/v) at 4°. The homogenate was sonicated and centrifuged at 10,000 rpm for 30 min. For free amino acid analysis, one fraction of the supernatant was filtered through an inverted-phase column (Sep-Pak C-18) which was subsequently derivatizated with phenylisothiocyanate (PTC) in alkaline medium. The resultant phenylisothiocyanate-amino acids were separated by HPLC using a Pico-Tag C-18 column, 300 × 3.1 mm at 46°C. The elution was performed under the following conditions: solvent (A) acetate buffer pH 6.4 0.14 M; solvent (B) acetonitrile/water (60/40); gradient from 100% (A) to 100% (B) in 21.5 min; flow rate 1 ml min^{-1}; detection UV at 254 nm. The remaining supernatant and the pellet were treated with 2N perchloric acid, sonicated and centrifuged for protein amino acid analysis. The pellet was hydrolyzed with 6N HCl and treated as in the case of free amino acids, but using a C-18 column, 150 × 3.1 mm at 38°C. In each case, the gradient from 100% (A) to 100% (B) was run in 13.0 min.

Standard amino acids (Pierce Chemical Co., Rockford, Illinois, USA) were used for calibration. An auto-amino acid-analyzer HPLC (Millipore Waters, Massachussets, USA) was employed for all the analyses.

Results and discussion

Preliminary experiments culturing the MC and EC calluses in both MS and N6 media demonstrated that the optimum growth rate was obtained when the MC calluses were cultured on MS and the EC calluses on N6 basal medium.

Effect of Pro on MC callus

The negative effect of exogenous Pro (Figure 1) on the total protein content of the MC calluses (organogenic) contrasts with the observed effect of this amino acid on somatic embryogenesis in maize (Armstrong and Green, 1985; Claparols et al., 1993). Moreover, this effect was more marked when salt was present in the medium. Given that MS basal medium contains 60 mM inorganic nitrogen, the addition of 6 mM Pro increases the Nred/NO_3 ratio from 1:2 to 2:2. This high ratio could impair the growth of the MC calluses, as has beeen observed by Lillo (1989). This author found that shoot formation in *Solanum tuberosum* was favored if nitrate nitrogen concentrations were about double the ammonium nitrogen concentrations. Furthermore, the total protein content of the control MC calluses cultured without Pro was not affected by the presence of salt in the medium and was always higher than that of cultured with Pro. Considering that the total protein content is indicative of the growth activity of the tissue and that these calluses were not apparently affected in the course of the experiment, these results could indicate that salt stress was not a limiting factor for the growth of MC callus.

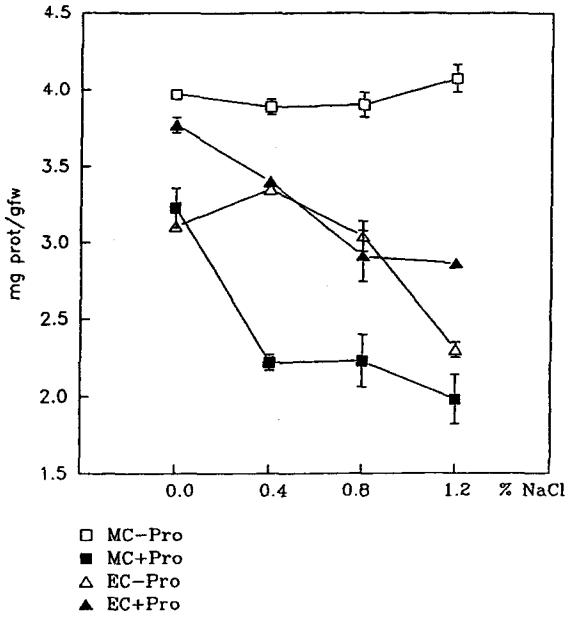

Figure 1. Total protein content of EC and MC calluses cultured with different NaCl concentrations in the presence and absence of 6 mM proline. Vertical bars indicate S.E. of the means.

Effect of Pro on EC callus

Pro addition had a positive effect on the total protein content of EC callus (Figure 1). N6 basal medium contains 35 mM inorganic nitrogen and the addition of 6 mM Pro increases the Nred/NO_3 ratio from 1:4 to 1:2, the same ratio as the MS medium without Pro addition. The influence of the nitrogenous composition on somatic embryogenesis (Wilkinson and Thompson, 1987) could account for these results. However, the presence of salt decreased the total protein content in these calluses, suggesting that they are more sensitive to salt stress than the MC calluses. The influence of Pro on osmotic regulation under stress conditions (including *in vitro* culture) acting not only as a nitrogen source but also as an organic solute of proteins has been reported (Fougère et al., 1991; Thomas et al., 1992). In our case, the different effect of exogenous Pro on the MC and EC calluses could be attributed to the varying demand of this amino acid, which might be associated with the different morphogenetic processes undergone by this callus. The presence of salt in the medium is a factor that interacts with the response of the callus to the Pro exogenous supply. This salt-response was also different in the two types of calluses.

Endogenous amino acid content

As may be seen in Figure 2, endogenous Pro content of control MC calluses cultured with Pro in the medium did not show much increase compared to those cultured without Pro. Free Ala showed a significant increase in the MC callusus cultured in the presence of Pro. Moreover, the presence of NaCl and Pro induced a significant increase in the endogenous Pro and Ala content, reaching a constant value for all the NaCl concentrations. The increase in free Pro in salt-stresssed plant tissues could be interpreted as a tolerance mechanism of osmotic regulation and/or accumulation of the excess of ammonium produced by salt stress (Bellinger et al., 1991). Furthermore, alanine could play a role similar to that of Pro on osmotic stress situations in some cases (Slocum and Weinstein, 1990).

In the case of the EC calluses, Pro addition induced a marked increase in the endogenous Pro content of control callus (Figure 2). These results agree with the findings obtained by our group previously (Claparols et al., 1993). The presence of 0.4% NaCl induced a rise in free Pro of the EC calluses, although it decreased at 0.8 and 1.2%, suggesting perhaps that is response mechanism to salt stress attributed to Pro was not func-

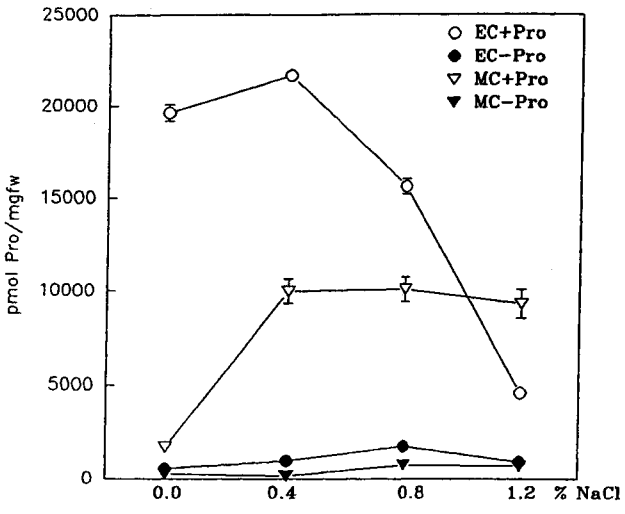

Figure 2. Effect of exogenous proline (6 mM) on the endogenous proline content of EC and MC calluses cultured with different NaCl concentrations. Vertical bars indicate S.E. of the means.

tional at the higher salt concentrations in this type of callus.

The total free amino acid content of the control EC calluses cultured with exogenous Pro was twice as high as that of the control MC calluses (Table 1). However, the total amino acid increase was considerably higher in the MC than in the EC calluses when salt was present in the medium. The relationship between the increase in the amino acid pool and the metabolic adaptation to osmotic stress has been described (Zhu et al., 1990; Fougere et al., 1991). In our case, these symptoms of adaptation are present in the MC calluses and are reinforced by the marked increment in Arg in the MC calluses under saline conditions. It is well known that free Arg can be useful to reduce the ammonium excess produced under salt stress (Slocum and Weinstein, 1990; Lovatt, 1990). This Arg increase was not as marked in the EC calluses.

Polyamine content

In general, the total PA and Put evolution in the different treatments was similar in the EC and MC calluses (Figure 3). At high salt levels, the total PA and Put contents were higher than those of the control and 0.4% NaCl treated calluses. This Put increase under osmotic and saline stress has been observed in other cases (Erdei, 1990; Basu and Gosh, 1991). However, the total PA content was considerably higher in the EC than in the MC calluses.

It is noteworthy that in the EC calluses cultured in the presence of Pro, the total PA content decreased at 0.4% NaCl (where endogeneous Pro increased) and it increased at 0.8 and 1.2% (where endogenous Pro decreased). Endogenous PAs rose in the calli cultured with 0.4% and 0.8% NaCl, when Pro was absent. Total Spd increased at 0.8 and 1.2% NaCl regardless of exogenous Pro. In the MC calluses, the presence of Pro and salt induced an increase in the total Put, which was similar to that observed in the absence of Pro. However, the Spd content was higher in the case of the MC calli cultured under saline conditions in the absence of Pro. One characteristic described in the salt-adaptation of cultivars of other cereals such as rice is their capacity to synthesize Spd (a protector of cell walls, nucleic acids, etc.) from Put by means of Spd synthase. This enzyme would be totally or partially inactivated in sensitive cultivars and as a result Put is accumulated. (Krishnamurty and Bagwat, 1989). Moreover, the significant increase in the GABA content (Table 1) of the MC calluses cultured with Pro and salt was not detected in the EC calluses cultured under the same conditions, suggesting that Put degradation is more marked in the MC calluses.

Although Arg (polyamine precursor) and Pro are synthesized from Glu independently, there is an important interconversion between them in the case of catabolic processes or stress conditions (Thompson, 1980). Our findings suggest that a compensatory effect of Pro and Arg metabolism in response to salt stress could have taken place. This effect will be different in calluses with different morphogenic pattern.

Conclusion

It may be concluded that: a) Diverse morphogenetic processes such as embryogenesis and organogenesis vary in their demand of exogenous nitrogen (especially of Pro) even in stress situations, where this amino acid could play a role as an osmorregulator. b) As for the parameters analyzed, the meristematic callus seems to be more tolerant to the presence of NaCl than the embryogenic callus, although both are derived from the same sensitive cultivar. These findings could be attributed to the different structure and cellular characteristics of these calluses.

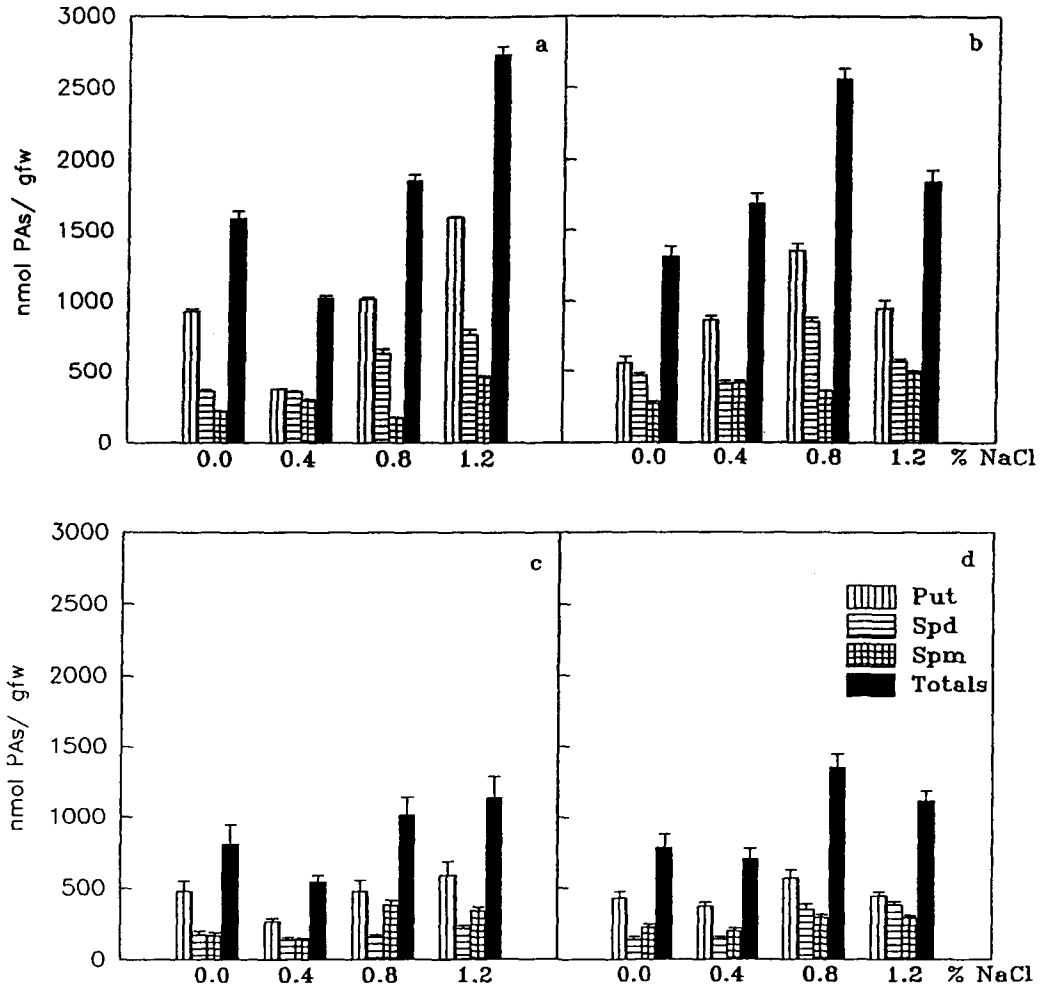

Figure 3. Polyamine content (nmol Pas g^{-1} fw) of EC (*a* and *b*) and MC (*c* and *d*) calluses cultured in the presence of different NaCl concentrations. a and c= 6 mM exogenous Pro.b and d= no exogenous Pro. Vertical bars indicate S.E. of the means.

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