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SELECTION AND CHARACTERIZATION OF SODIUM CHLORIDE AND MANNITOL TOLERANT CALLUS LINES OF RED PEPPER (*CAPSICUM ANNUUM* L.)

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SUMMARY

Callus cultures of *Capsicum annuum* cv. G_4 were selected on different concentrations of NaCl and mannitol containing media. Growth, viability and proline content of adapted and unadapted calluses were affected when treated with different osmotica like NaCl (ionic, penetrating) and mannitol (non-ionic, penetrating). The tissues adapted to a low concentration of NaCl (35mM) showed less growth with high proline content compared to the tissues adapted to a low concentration of mannitol (110mM). Proline content was similar in tissues adapted to high concentrations of NaCl (175mM) and mannitol (329mM) but growth in the latter case was relatively low. Growth and viability were subsequently correlated with proline content of the tissues after osmotic treatments. The loss of tissue viability of the adapted calluses was comparatively less than the unadapted callus even after osmotic treatments with 210 mM NaCl and 329 mM mannitol. The adapted calluses retained the capability of regrowth, though at a slow rate and also retained more proline. The results indicated that the effects of different osmotica on plant tissues varied depending upon the physio-chemical nature of the compounds used as stress inducing agents and that the retention and diffusion of proline was altered when the tissues were treated with high concentration of these compounds.

***Key words*: *Capsicum annuum* L., growth, osmotic stress, proline content, viability.**

INTRODUCTION

The physiological and biochemical changes in plant tissues in response to different types of osmotic stresses are not completely understood. Stress effects on plant cells and tissues have been investigated and the stress-inducing compounds used in different experiments were either ionic and penetrating (e.g. NaCl), non-ionic and penetrating (e.g. mannitol, sorbitol etc.) or non-ionic and non-penetrating (e.g. polyethylene glycol) (Gangopadhyay *et al*., 1997a). Results of such experiments, however, have shown the following general trends in plant tissues: retardation growth (Rains, 1989, Thomas *et al*. 1992), acquisition of the ability to adapt to stressful environments (Binzel *et al*. 1985, Fallon and Phillips, 1989), and increased level of proline accumulation (Rudulier *et al*. 1984, Jain *et al*. 1991, Gangopadhyaya *et al*. 1997a, b Shah *et al*. 2002).

Effects of different osmotica having different physio-chemical properties are yet to be critically compared. These compounds, at high concentrations produce shock-effects on tissues causing tissue damage, either permanent or temporary (Leone *et al*. 1994), depending on the tissue under consideration, adapted or unadapted. Damage may be due to leakage of osmotically active substances

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resulting from the loss of membrane function (Harrington and Alm 1988). Further proline, an osmotically active substance (Rains 1989) accumulates in shock-treated tissues in certain cases and improves cell viability (Fallon and Phillips 1989).

In the present study, the unadapted and adapted calluses of *Capsicum annuum* cv. G_4 were grown on media containing various concentrations of NaCl or mannitol. Growth was correlated with the proline accumulation and physiological significance of these effects of osmotic stresses were evaluated.

MATERIALS AND METHODS

Seeds of *Capsicum annum* cv. G_4 were soaked in sterile distilled water for 24 h, surface sterilized with 0.1% $HgCl_2$ solution for 3-5 min and finally rinsed thrice with sterile distilled water. Seeds were aseptically germinated on Murashige and Skoog's (1962) basal medium. Hypocotyl segments (~1 cm) of 3-week old seedlings served as explants.

Culture conditions

The hypocotyl explants were implanted on MS medium containing 2% sucrose, 100 $mg1^{-1}$ meso-inositol, 0.5 $mg1^{-1}$ kinetin and 2.0 $mg1^{-1}$ 2, 4-dichlorophenoxylacetic acid. The pH of the medium was adjusted to 5.8, the medium solidified with 8 $gm1^{-1}$ difco-bacto-agar, and dispensed into culture tubes (with each tube containing 15 ml medium and only one explant) before autoclaving at 121ºC and 103.4 kPa for 15-20 min. Calluses were later maintained on the same medium, which was used for callus induction. All cultures were maintained at 25 ± 1ºC under 16 h photoperiod at 40-50 µ mol $m^{-2}s^{-1}$ light intensity produced from cool-white fluorescent tubes.

Screening and establishment of adapted callus cultures

Callus (~ 15-20 mg) was transferred to MS medium containing NaCl (35-210 mM) or mannitol (110-329 mM) for isolation and maintenance of adapted tissues. NaCl and mannitol adapted lines were isolated by repeated subculturing in stress inducing media using 15-20 mg inocula. Adapted calluses were subcultured (25 days after each treatment along with controls) in respective stress and stress-free (control) media prior to further experimentation. Five replications of each treatment were oven dried (80ºC, 16 h) to estimate the final weight of the tissues (5 mg). Per cent final weight was calculated using the formula : Final weight-Initial weight/Initial weight x 100 (each passage of 25 days).

Tissue viability

Tissue viability was assessed by the TTC test. A portion of NaCl or mannitol treated tissue (along with control ~50 mg from each replica after 25 days) was tested for recording the reduction of 2,3,5-triphenyl tetrazolium chloride (TTC) to red formazan following the method of Eberhardt and Wegmann (1989).

Proline Estimation

Proline was extracted and determined colorimetrically by the method of Bates *et al.* (1973). Callus tissue (100 mg) was homogenized with 4 ml of 50 mM phosphate buffer (pH 7.8) containing 1% w/v polyvinyl pyrrodoline (PVP) and 0.01% (w/v) Triton X-100, and centrifuged at 800 rpm for 15 min. Proline was determined in the supernatant by measuring the absorbance of the proline-ninhydrin product formed at 520 nm in a spectrophotometer using toluene as a solvent.

RESULTS AND DISCUSSION

Growth of adapted tissues in stressful media and regrowth assay

All the adapted tissues grown on NaCl or mannitol containing media showed a decreased growth rate in comparison to the unadapted one grown on stress-free media (Fig. 1). Maximum growth attained by adapted tissues was observed when tissues were grown on low mannitol (110 mM) containing media. However, the tissues grown on NaCl (35 mM) containing media of equivalent osmotic potential to the one with low mannitol concentration, showed considerably less tissue growth. Tissue growth was lowest in high mannitol (329 mM) or high NaCl (210 mM) containing media. Although the tissue growth was less in adapted tissues than the control one, the tissues survived well in their respective stressful media in consecutive passages. Moreover, the tissues

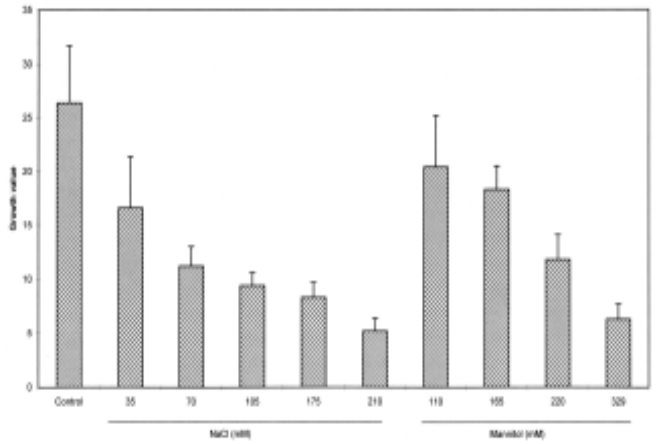

Fig. 1. Growth values (final/initial fresh weight) of unadapted and adapted calluses in media containing different conc. of NaCl and Mannitol (pooled data of five passages, each passage of 25 d). Mean ± SE (n=5)

showed a gradual increment of dry weight in the initial passages on the stressful media.

All adapted calluses (20 mg from each replica) were transferred to their respective media containing either NaCl or mannitol. Callus growth declined initially in culture media supplemented with all the concentrations of NaCl. The initially reduced growth, however, recovered and became steady after at least three passages of sub culture in the respective NaCl containing media (Fig. 2a) indicating establishment of NaCl adapted callus lines.

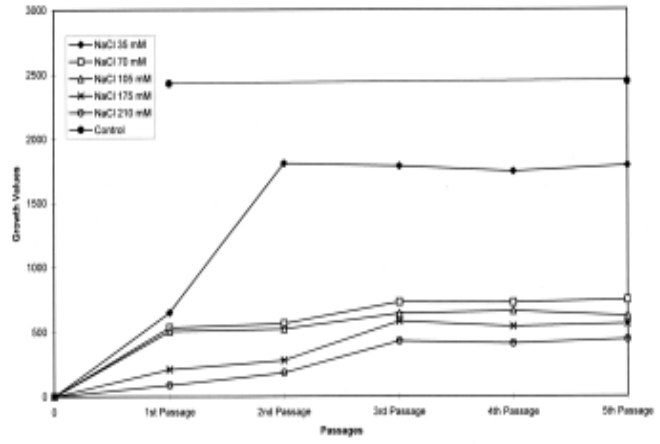

Fig. 2a. Growth values (final/initial fresh weight) of unadapted and adapted calluses on NaCl media (pooled data of five passages, each passage of 25 d). Mean + SE (n=5)

Mannitol-adapted callus-lines were established by direct adaptation procedure on media containing various concentrations of mannitol (210-329 mM). The callus lines selected, against various concentrations of mannitol, were grown on similar concentrations of mannitol (110-329 mM). Growth was reduced in the initial passages but recovered in the consecutive passages (Fig. 2b) indicating the establishment of mannitol adapted callus lines.

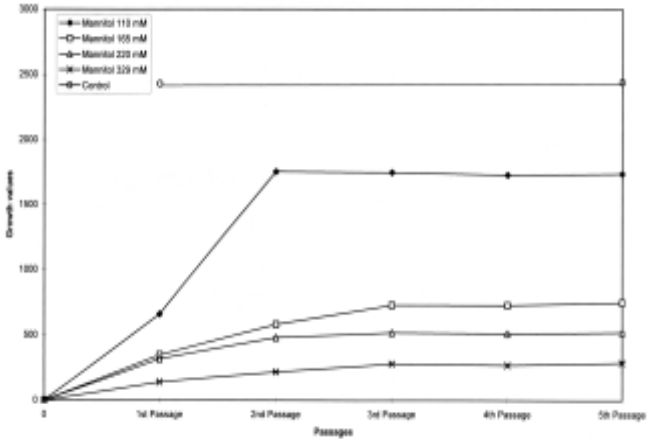

Fig. 2b. Growth values (final/initial fresh weight) of unadapted and adapted calluses on mannitol media (pooled data of five passages, each passage of 25 d). Mean + SE (n=5)

Growth rate of adapted callus lines of *Capsicum annuum* in NaCl or mannitol containing media was steady and sustainable but it was lower than the growth of the control callus grown on stress-free medium. Reduced growth of tissues in stressful medium is a usual phenomenon (Cushman *et al.* 1990, Jain *et al.* 1991a, b, Thomas *et al.* 1992) and it has been interpreted that a certain amount of the total energy available for tissue metabolism is channeled to overcome the stress (Cushman *et al.* 1990).

The process of adaptation of tissues in stressful media was gradual. This was revealed by the gradual increase of growth of calluses from the second subculture. The growth became stable after four to five passage of culture indicating the occurrence of a new homeostatic equilibrium through alteration in cellular metabolism in the adapted calluses, which was compatible with the imposed stress (Leone *et al.* 1994). The stepwise adaptation by sequential treatment of calluses from low to high concentrations of NaCl (Collin and Dix 1990; Basu *et al.*

1997, Gangopadhyay *et al.* 1997a, b) or mannitol (Gangopadhyay *et al.* 1997a, b) was of added advantage because this procedure helped in better selection of adapted lines.

Tissue viability

All the adapted calluses grown on NaCl or mannitol containing media showed a lower percent viability in comparison to the unadapted (control) calluses grown on stress-free media (Table 1). Maximum tissue viability was attained by adapted tissues on low NaCl (78.4%) or low mannitol (84.4%) containing media. Tissue viability was lowest in high mannitol (28.3%) or high NaCl (14.3%) containing media (Table 1).

Table 1. Percent viability of unadapted (control) and adapted calluses on different concentrations of NaCl and mannitol

Osmotica	Conc.(mM)	% viability
	Control	100.0 ± 0.53
NaCl	35	78.4 ± 2.32
	70	63.8 ± 2.32
	105	48.3 ± 1.49
	175	25.6 ± 2.18
	210	14.3 ± 1.86
Mannitol	110	84.4 ± 1.68
	165	78.6 ± 1.28
	220	53.5 ± 1.82
	329	28.3 ± 1.89

The viability was calculated on the basis of a TTC test considering the O.D. values (at 448 nm) of unadapted tissues as 100% (after 25 days of culture)
Values are mean ± SE of the five replicate samples.

Growth response of calluses adapted to high concentration of mannitol (329 mM) was more than that adapted to an equivalent concentration of NaCl (210 mM). Response to different osmotica (NaCl or mannitol) was not similar. The capacity of TTC reduction declined severely in callus due to treatment with either NaCl or mannitol compared to the control callus, thereby indicating considerable loss of tissue viability.

NaCl and mannitol are penetrating type of osmotica causing tissue injury by impairing cellular metabolism. However, NaCl stress had both ionic and osmotic components (Gomes-Filho and Sodek 1988) and was more detrimental to tissue than non-ionic mannitol stress. An equimolar concentration of sorbitol, a compound similar to mannitol, was reported to be less detrimental to tissue viability than NaCl (Eberhardt and Wegmann 1989).

Free proline content

Proline content in the callus lines adapted to high concentrations of NaCl (175 mM and onwards) and mannitol (329 mM) was significantly higher than the control callus (Fig. 3). The proline level in mannitol adapted calluses increased significantly in increasing concentrations of mannitol (from 165 to 329 mM) but in case of NaCl adapted callus there was not apparent increase of proline content due to increase in NaCl concentration (from 70 to 175 mM). The proline level in callus adapted to higher mannitol (329 mM) concentration showed sharp increase while the proline levels in callus adapted to NaCl showed gradual increase.

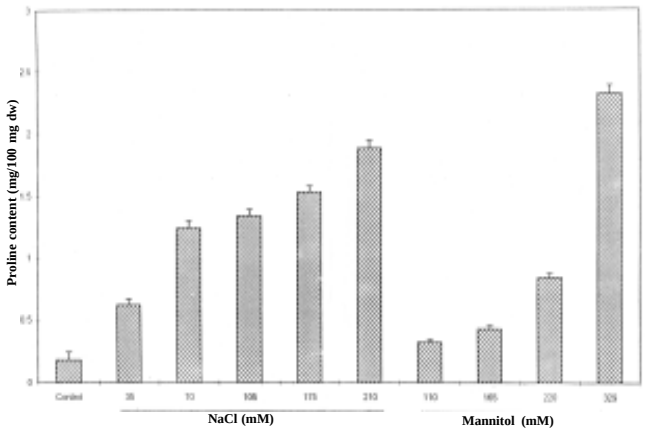

Fig. 3. Free proline content of unadapted and adapted calluses. Mean + SE (n=5)

Osmotic stress-induced accumulation of proline has been reported in many plant systems (Watad *et al.* 1983, Jain *et al.* 1991b, Gangopadhyay *et al.* 1997a, b). The increase of free proline content in NaCl/mannitol adapted calluses grown on NaCl/mannitol-containing medium compared to that of the control callus grown on stress-free medium was a general observation. However, the

level of proline accumulation was different in the callus adapted to NaCl and mannitol. At a moderate level of mannitol (110 mM) in the medium, proline content did not increase significantly compared to the control callus grown on stress-free medium. However, in the presence of an iso-osmotic concentration of NaCl (70 mM) in the medium, proline content increased sharply in the NaCl adapted callus compared to the control. This phenomenon, however, was not true in high NaCl (210 mM) and mannitol (329 mM) concentration as the level of proline accumulation was similar in the NaCl/mannitol adapted callus. The effect of an ionic and non-ionic osmoticum on proline metabolism was reported earlier in tobacco callus (Eberbardt and Wegmann 1989, Gangopadhyay *et al.* 1997a).

It can be concluded that by the use of gradual adaptation produce, NaCl and mannitol tolerant callus lines can be established. These lines show sustained growth and a high content of endogenous free proline in stressful media. This type of physiological adaptation can result in salt or drought tolerant somaclonal lines. This approach has been explored in *Brassica juncea* (Jain *et al.*, 1991a, 1991b, Gangopadhyay *et al.* 1997a), tobacco (Gangopadhyay *et al.* 1997b) and rice (Basu *et al.* 1997, Shah *et al.* 2002). Being an ionic stress, NaCl is more detrimental to tissue than non-ionic mannitol stress of equivalent osmotic potential. Although there are certain differences between the effects of different osmotica as shock-inducing agents on calluses, the adapted calluses can be differentiated from the unadapted ones by their considerable regrowth capacity and proline retention pattern after stress-shock treatments. The phenomenon of retention/leaching of proline due to shock treatments to the stressed tissues and can be used to identify adapted callus lines.

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