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Selection and characterization of mannitol-tolerant callus lines of *Vigna radiata* (L.) Wilczek

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Abstract

An osmotically (mannitol) tolerant callus line of *Vigna radiata* (L.) Wilczek has been isolated from callus cultures grown on modified PC-L2 medium supplemented with increasing concentrations of mannitol. The tolerance was stable and retained after growth in the absence of mannitol selection for 2 months. The growth of the tolerant line, in the presence of mannitol (540 mol m^{-3}) was comparable to that of a sensitive callus line growing in the absence of mannitol. This line not only grew well on media containing up to 720 mol m^{-3} mannitol, but also required 450 mol m^{-3} mannitol for its optimal growth. Osmotically tolerant callus also showed increased tolerance to NaCl (0–250 mol m^{-3}) stress as compared to sensitive callus. Accumulation of Na^+ was lower, and the level of K^+ was more stable in osmotically tolerant than in sensitive calli, when both were exposed to salt. The free proline content of both tolerant and sensitive calli increased on media supplemented with mannitol or NaCl. However, the proline content of sensitive callus was higher than in tolerant callus in the presence of same concentrations of mannitol or NaCl.

Abbreviations: NAA – α-naphthaleneacetic acid, 2,4-D – 2,4-dichlorophenoxyacetic acid, BAP – 6-benzylaminopurine

Introduction

Salt tolerant cells have been isolated from a wide range of plant species (Tal 1990). There are, however, only few reports on osmotically stressed cell cultures (Handa et al. 1982b) which can be used to elucidate the mechanism operating at the cellular level by which plants survive under water stress (Bressan et al. 1981). Cells resistant to PEG-induced water stress in tobacco (Heyser & Nabors 1981) and tomato (Bressan et al. 1981) and mannitol-adapted cell lines in carrot (Harms & Oertli 1985) and potato (Sabbah & Tal 1990) have been isolated. The osmotically-adapted cell lines of tomato, carrot and potato have shown an increased resistance to salt stress as compared to non-adapted cells. However, no information is available on the mechanism of osmotic-tolerance in important crop plants especially grain-legumes, which are considered to be the most sensitive to water and salt stresses.

The present paper describes the selection of an osmotically (mannitol) tolerant callus line of

Vigna radiata and its characterization with respect to growth, accumulation of ions and proline and its cross-tolerance to NaCl.

Materials and methods

The callus cultures of *Vigna radiata* (L.) Wilczek cv. K-851 were initiated from the primary leaves of aseptically grown 7 day old seedlings on modified PC-L2 (Phillips & Collins 1979) medium containing 3% sucrose, 0.7% agar, 0.5 mg l^{-1} NAA, 0.5 mg l^{-1} 2,4-D and 1 mg l^{-1} BAP. The callus cultures were grown under a 16-h photoperiod (80 μmol $m^{-2} s^{-1}$) of cool-white fluorescent light at 25 ± 2°C. After one subculture on the same medium, 250 ± 10 mg actively growing callus was divided into ten pieces (25 ± 2 mg) and inoculated into petri-dishes (100 mm × 17 mm) containing 25 ml of modified PC-L2 medium supplemented with increasing concentrations of mannitol (0, 180, 360, 449, 540, 629, 720 mol m^{-3} (iso-osmotic to 0 to 400 mol m^{-3} NaCl)) in order to determine the concentrations which inhibited the growth of sensitive (wild type) cells. The petri-dishes were sealed with parafilm and incubated under the same photoperiod and temperature as for callus cultures. After 4 weeks of culture, callus from petri-dishes was removed and its fresh and dry weights (oven dried at 80°C for 48 h) were determined for each treatment. The inhibitory concentration, thus, determined was used for the selection of variant callus lines resistant to this concentration of mannitol.

Estimation of Na^+ and K^+ ions

One hundred mg of dried well-ground callus tissue was transferred to 50 cm^3 digestion flasks to which 1 ml of 60% perchloric acid, 5 ml of conc. HNO_3 and 0.5 ml of conc. H_2SO_4 were added. The flasks were heated gently over a hot plate for 5–10 min until the solution became colourless. The digest was cooled and diluted to 100 ml by addition of double distilled water. Na^+ and K^+ in the final acid digest extract were determined using Elico flame photometer.

Free proline estimation

Each 100 mg fresh weight of frozen callus was homogenized in 10 ml of 3% sulphosalicyclic acid. Aliquots (2.0 ml) of this extract were used for proline quantification. Proline was determined by acid-ninhydrin method of Bates et al. (1973).

Chemicals

'Analar' grade chemicals, plant growth regulators (Sigma Chemical Company) and agar (Hi-media, Bombay) were used.

For each treatment five replicates were taken and each experiment was repeated twice.

Results and discussion

Selection of mannitol-tolerant callus line

The fresh callus pieces of approximately 25 mg were grown on PC-L2 medium containing 540 mol m^{-3} mannitol (iso-osmotic to 300 mol m^{-3} NaCl). Within a month, most of the calli exhibited browning and arrested growth except a few clones that remained green. The one(s) which showed vigorous growth was subcultured for three more additional passages (4 week each) on fresh medium containing the same concentration of mannitol in order to remove the escapants. The selected clones were tested for the stability of the altered response by allowing them to grow away from mannitol for two transfers over a period of 60 days. Then the tolerant clones were again grown on the medium containing inhibitory concentration of mannitol (540 mol m^{-3}) where they grew satisfactorily. Such a callus line was designated as mannitol-tolerant.

Growth characteristics

The relative growth (fresh and dry weights) of sensitive and tolerant callus lines on medium containing different concentrations of mannitol is

shown in Fig. 1. The tolerant calli had a slightly higher fresh and dry weights at 0 mannitol, and at 180 and 360 mol m^{-3} both the lines had almost similar fresh and dry weights. At 450 mol m^{-3} or higher concentrations, tolerant calli had significantly higher fresh and dry weights. The growth of sensitive calli was stimulated by low concentrations (180 to 360 mol m^{-3}) of mannitol, but decreased with further increase in mannitol concentration and showed more than 50% reduction in their fresh weight at 450 or high concentration of mannitol. On the other hand, tolerant calli showed optimal growth at 450 mol m^{-3} and only a 25% reduction in their fresh weight at the highest concentration (720 mol m^{-3}) of mannitol. The stimulatory effect of lower concentrations of mannitol on the growth of cultured cells of other systems has also been reported (Hassan & Wilkins 1988; Sabbah & Tal 1990).

The growth kinetics of osmotically sensitive and tolerant callus lines was compared on normal and mannitol (540 mol m^{-3})-containing medium for over a period of 28 days (Fig. 2A,B). The growth of tolerant callus lines on mannitol medium was comparable with that of the sensitive callus lines grown on normal medium. However, under non-stress conditions, the osmotically tolerant callus lines grew faster than the sensitive callus lines. The osmotically tolerant clones exhibited the greatest increase in fresh and dry weights on normal medium except on day 28, when the dry weight accumulation of osmotically tolerant callus line grown under mannitol was greater than that of callus lines grown on normal medium. These observations indicate that growth and metabolic activity of the mannitol tolerant callus lines under stress conditions exceeded that of the control callus line and increased still further when grown on normal medium.

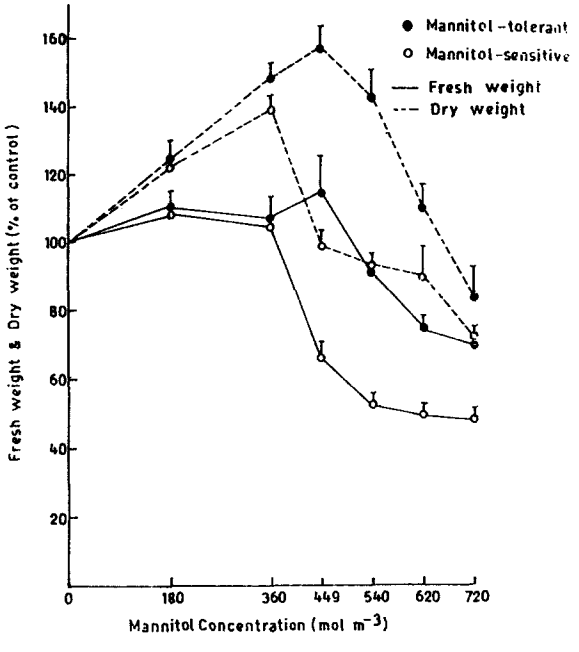

Fig. 1. Effect of increasing concentrations of mannitol on fresh and dry weights (% of control) in mannitol-tolerant and sensitive cells of *Vigna radiata* after 28 days. Vertical bars represent standard error of the mean (n = 5) of two independent experiments. FW in the absence of mannitol were 1985 ± 63.9 mg (sensitive) and 2573 ± 71.8 mg (tolerant). Average DW were 210 mg (sensitive) and 275 mg (tolerant).

The response of osmotically sensitive and tolerant callus lines on medium supplemented with different concentrations of NaCl (iso-osmotic to mannitol) is shown in Fig. 3. Osmotically tolerant cells grew significantly better than sensitive cells in NaCl up to 250 mol m^{-3}. Osmotic selection over 3 months in mannitol media had enabled tolerant cultures to grow in NaCl at higher rates than sensitive cultures. Sodium chloride at 100 mol m^{-3} caused a 40% reduction in fresh and dry weights of sensitive cultures, but there was no reduction at all of the selected cultures at this concentration. Osmotically tolerant callus cultures showed a 30% reduction in both fresh and dry weights at 200 mol m^{-3} NaCl as compared to 65% (of fresh weight) and 50% (of dry weight) for sensitive cultures. Osmotically tolerant cultures attained half maximal growth at 225 mol m^{-3} NaCl for fresh weight and at 275 mol m^{-3} for dry weight as compared to 137 and 165 mol m^{-3} for sensitive cultures. Osmotically tolerant cells also showed significantly higher dry weight than sensitive cells when both were exposed to NaCl (Fig. 3).

Thus, the present study showed that osmotically tolerant cells were tolerant to both osmotic and salt stresses as compared with sensitive cultures. These results are in accordance with those obtained with carrot (Harms & Oertli 1985) and potato (Sabbah & Tal 1990).

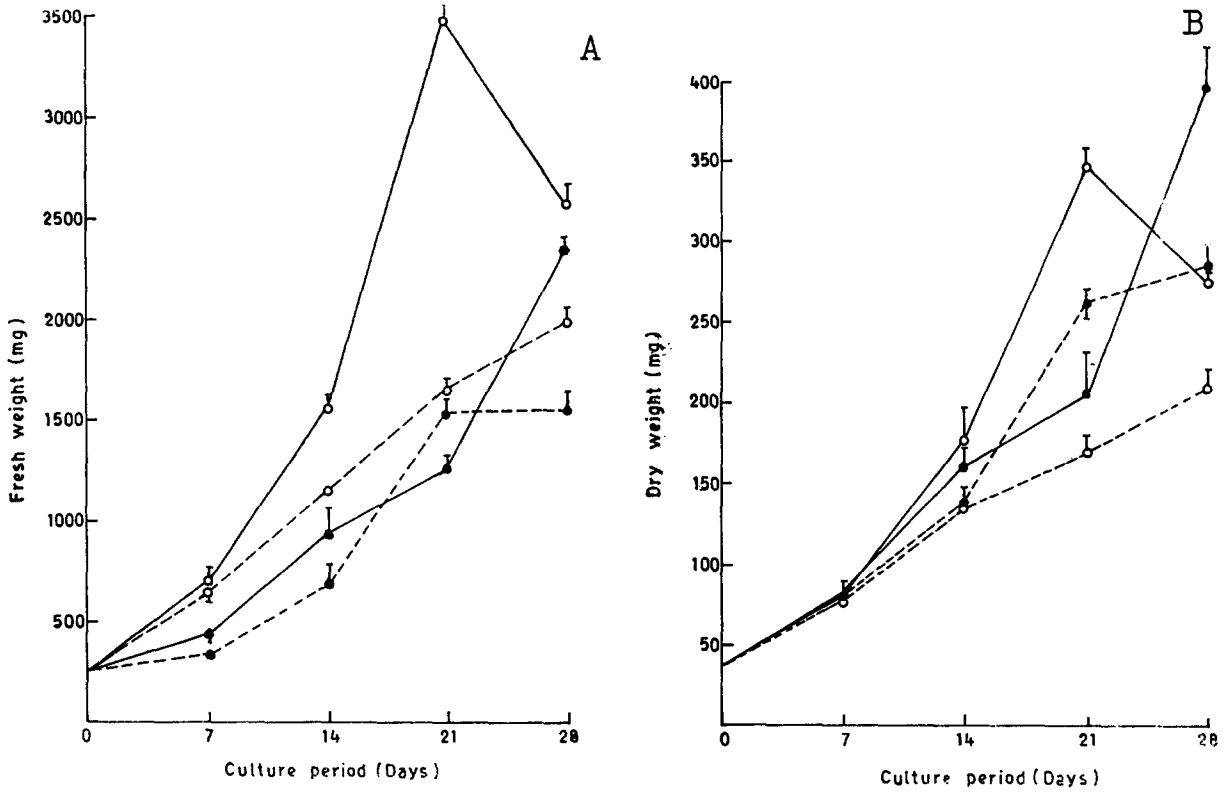

Fig. 2. Effect of osmotic (mannitol) stress on the fresh (*A*) and dry (*B*) weights of sensitive and mannitol-tolerant callus lines of *Vigna radiata*. (O---O)-sensitive callus and (O——O)-mannitol-tolerant callus grown on normal medium; (●---●)-sensitive callus and (●——●)-mannitol-tolerant callus grown on mannitol (540 mol m^{-3}) supplemented medium. Vertical bars represent standard error of the mean.

Ion accumulation

Sodium content of osmotically-tolerant and sensitive callus increased when both of them were grown on NaCl containing medium, but Na^+ accumulation was much lower in the former than the latter. The Na^+ content of sensitive cells increased with increase in NaCl concentration. However, in tolerant cells the Na^+ level did not change much with increasing NaCl levels (Table 1).

The K^+ level of sensitive and tolerant cells decreased with increasing NaCl concentrations in the medium, but this decrease was much higher in the former than the latter (Table 1).

The ratio of Na^+/K^+ in the cells increased with increasing NaCl concentration in the medium in both tolerant and sensitive cells. The increase however was higher in the latter than the former at salt concentration higher than 150 mol m^{-3} (Fig. 4).

Free-proline content

On normal medium sensitive callus had higher levels of proline than tolerant callus (Table 2 and 3). In both lines, proline accumulation increased in media supplemented either with mannitol or NaCl. However, sensitive callus accumulated more proline in the presence of mannitol or NaCl than tolerant callus grown on the same concentrations of mannitol or NaCl. Tolerant callus which was selected on medium containing 540 mol m^{-3} mannitol (iso-osmotic to 300 mol m^{-3} NaCl) contained approximately equal amounts of proline when grown on medium containing iso-osmotic concentrations of salt or mannitol.

Proline has been shown to accumulate in plant cells exposed to salt or water stress (Chandler & Thorpe 1986) and it has been implicated in osmoregulation of cytoplasmic enzymes (Pollard & Wyn Jones 1979), stabilization of proteins

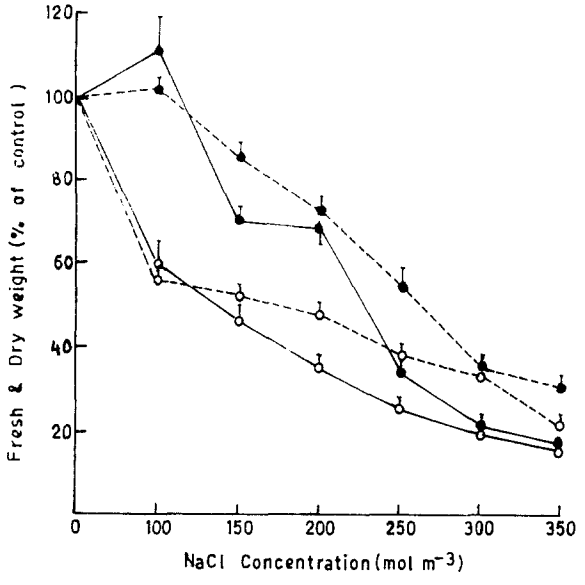

Fig. 3. Effect of NaCl stress on fresh weight (sensitive callus (○——○); mannitol-tolerant callus (●——●)) and dry weight (sensitive callus (○----○); mannitol-tolerant callus (●----●)) of sensitive and mannitol-tolerant callus lines of *Vigna radiata*. Vertical bars represent standard error of the mean.

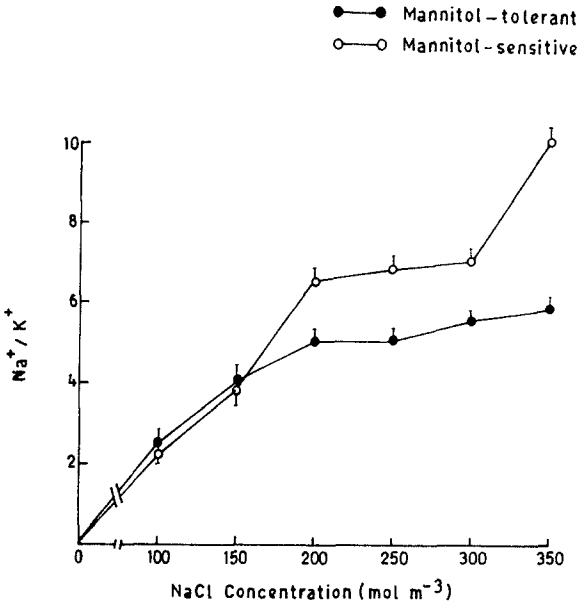

Fig. 4. Na^+/K^+ ratio in sensitive (○——○) and mannitol-tolerant (●——●) callus line grown on modified PC-L2 medium supplemented with different concentrations of NaCl. Vertical bars represent standard error of the mean.

(Schobert & Tschesche 1978) and membrane (Jolivet et al. 1982) and also to provide a store of nitrogen or respiratory substrate to facilitate post stress recovery (Aspinall & Paleg 1981). However, it has also been suggested that proline has no adaptive role and is merely a symptom of stress (Hanson & Hitz 1982). The proline content of tolerant callus increased with decrease in medium water potential and there were similar amounts of proline in tissue grown on iso-osmotic concentration of mannitol or salt. However, as in *Lycopersicon* species (Tal et al. 1979) and *Nicotiana sylvestris* (Dix & Pearse 1981), the unselected callus produced higher amounts of proline than tolerant callus. One explanation for this could be that even though growth was inhibited in sensitive callus, production of osmotica still proceeded and that proline was far more important here than it was in tolerant callus. In tolerant callus, other solutes or ions might be utilized in addition to proline (Handa et al. 1983). An alternative explanation for enhanced proline level in unselected callus could be that excess production was a symptom of stress damage (Hanson et al. 1979). Evidence for this was not only the non-linear increase in proline levels in unselected callus but also the

Table 1. Sodium and potassium contents (μ moles g^{-1} callus dry weight) in sensitive and mannitol-tolerant callus lines of *Vigna radiata* grown on modified PC-L2 medium supplemented with NaCl after 28 days. Values are the average of five replicates ±standard error.

NaCl (mol m^{-3})	Sensitive callus		Mannitol-tolerant callus	
	Na^+	K^+	Na^+	K^+
0	7.6 ± 0.3	86.5 ± 5.7	5.2 ± 0.2	26.9 ± 5.1
100	110.6 ± 1.0	51.7 ± 1.2	48.7 ± 4.8	19.8 ± 1.9
200	133.6 ± 5.4	24.3 ± 2.5	51.5 ± 0.6	11.8 ± 1.0
250	132.6 ± 2.1	20.1 ± 0.4	53.2 ± 1.0	10.7 ± 0.0
300	147.8 ± 8.6	19.5 ± 0.2	57.3 ± 0.0	10.5 ± 0.2
350	162.3 ± 16.7	16.0 ± 0.6	58.6 ± 2.1	9.2 ± 0.2

Table 2. Proline content (μ mol (g FW)$^{-1}$) of sensitive and mannitol-tolerant callus lines grown on modified PC-L2 medium supplemented with different concentrations of mannitol after 28 days. Values are the average of five replicates ±standard error.

Mannitol mol m^{-3}	Tolerant callus line	Sensitive callus line
0	57.7 ± 1.2	72.0 ± 4.2
180	151.6 ± 2.1	193.0 ± 15.5
360	154.5 ± 2.8	258.6 ± 3.5
449	166.2 ± 3.5	216.2 ± 18.1
540	208.0 ± 8.7	274.0 ± 8.5
629	178.4 ± 9.6	213.9 ± 3.5

Table 3. Proline content (μ mol (g FW)$^{-1}$) of sensitive and mannitol-tolerant callus lines grown on modified PC-L2 medium supplemented with different concentrations of NaCl after 28 days. Values are the average of five replicates ±standard error.

NaCl (mol m^{-3})	Tolerant callus line	Sensitive callus line
Control	57.7 ± 1.2	72.0 ± 4.2
100 (180)	149.1 ± 5.4	148.5 ± 4.9
200 (360)	158.6 ± 2.1	185.6 ± 7.4
250 (449)	190.1 ± 7.3	272.2 ± 9.6
300 (540)	206.9 ± 5.2	235.9 ± 8.2
350 (629)	99.3 ± 2.4	238.0 ± 9.5

Value in parentheses are mannitol concentrations iso-osmotic to NaCl.

relationship between the inhibition of growth and accumulation of proline. However, in sensitive callus, proline levels on NaCl were low in comparison to iso-osmotic level of mannitol. Whatever the cause of the enhance levels of proline in the unselected callus, it is clear that proline accumulation was not confined to tolerant callus, therefore unlike the situation in salt tolerant callus (Watad et al. 1983; Pandey & Ganapathy 1985; Spiegel-Roy & Ben-Hayyim 1985) it cannot be the sole mechanism of osmotic-stress tolerance.

In the present study, tolerant callus showed greater resistance to osmotic and salt stress. This may be due to the cellular accumulation of metabolic products of mannitol which contribute to osmotic adjustment (Thompson et al. 1986). Moreover, tolerant callus also maintained a higher level of K^+ ions than sensitive callus. K^+ ions, apart from involvement in various biochemical aspects, may contribute to osmotic adjustment of tolerant cells exposed to osmotic and salt stresses.

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