In vitro selection and characterization of a callus line of *Vigna radiata* resistant to NaCl, KCl and Na_2SO_4

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Abstract

A salt mixture resistant (SMR) cell line of *Vigna radiata* (L.) Wilczek was isolated by selection on agar solidified PC-L2 medium supplemented with NaCl, KCl and Na_2SO_4 (8:1:1) equimolar to 300 mol m^{-3} NaCl, a concentration inhibitory to the wild-type non-selected cells (salt mixture sensitive, SMS). This line retained its resistance after subculture for 3 passages (3 months) on normal medium. The SMR line grew significantly better than SMS line at all the levels of salts, though less in saline medium than the SMR on normal medium. The growth of SMR line was significantly higher than that of SMS line under KCl stress. However, both the lines responded similarly to Na_2SO_4 at a concentration higher than 100 mol m^{-3}. The SMR line was found to be more sensitive to NaCl than SMS line. The SMR line under salt mixture stress maintained lower levels of Na^+ and higher levels of K^+ than SMS line. The SMR line failed to regenerate shoots, although rhizogenesis was observed on PC-L2 medium containing salt mixture (300 mol m^{-3}).

Introduction

Cell lines with enhanced resistance to salt have been isolated from many plant species (for review see Tal 1990). In most of the studies, NaCl was used as the selection agent. NaCl selection is likely to produce genotypes with resistance to Na^+ and Cl^- ions, but not necessarily to other toxic ions contributing to salinity in certain agricultural situations (Rains *et al.* 1986). The results obtained with single different salts might differ from those obtained when tissues are grown on salt mixtures to which plants may be exposed in nature. Therefore, the present study deals with the *in vitro* development of salt mixture resistant callus lines of *Vigna radiata* and their response to different salt stresses with respect to growth and accumulation of ions.

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Materials and methods

Callus cultures of *Vigna radiata* (L.) Wilczek cv. K-851 were initiated from leaf explants of aseptically grown 7-d-old seedlings on modified PC-L2 (Phillips and Collins 1979) medium containing 3 % saccharose, 0.7 % agar, 0.5 g m^{-3} 2,4-D, 0.5 g m^{-3} NAA and 1.0 g m^{-3} BAP. The callus cultures were grown at a 16 h photoperiod under cool-white fluorescent radiation of 80 μmol m^{-2} s^{-1} and at a temperature of 25 ± 2 °C.

Effect of different concentrations of salt mixture: After one subculture on the same medium, 250 ± 10 mg actively growing callus was divided into ten pieces (each 25 ± 2 mg) and cultured on 20 cm^3 of modified PC-L2 medium containing increasing concentraion of salt mixture in Petri dishes. Three salts (NaCl, Na_2SO_4 and KCl) were used in the ratio of 8:1:1 to prepare a salt mixture equimolar to 0 - 450 mol m^{-3} NaCl. The Petri dishes, twenty replicates for each treatment, were sealed with parafilm and incubated under the same photoperiod and temperature as callus cultures. Subsequently, at 7-d intervals, the callus from 5 Petri dishes of each treatment was removed and its fresh and dry mass (oven dried at 80 °C for 48 h) was determined. This procedure was repeated up to 28 d after inoculation and the concentration of salt mixture inhibitory to growth was determined.

Table 1. Composition of selected ions in PC-L2 basal medium or PC-L2 supplemented with salt mixture (300 mol m^{-3}).

Ions [mol m^{-3}]	PC-L2 basal medium	PC-L2 + salt mixture
Na^+	0.82	265.52
Ca^{2+}	4.08	4.08
Mg^{2+}	1.76	1.76
K^+	23.17	46.72
Cl^-	8.16	271.71
SO_4^{2-}	1.87	14.22

Selection of salt mixture resistant callus line: The selection of spontaneous mutants resistant to inhibitory concentration of salt mixture, *i.e.* equimolar to 300 mol m^{-3} NaCl (*see* Table 1 for constituents) was made by exposing 300 callus pieces (each 25 ± 2 mg fresh mass) to this concentration. No physical or chemical mutagens were employed for their isolation. One month later, most of these callus pieces exhibited browning and arrested growth except four clones that remained green. One which showed vigorous growth, was subcultured for three more subcultures (4 weeks each) on fresh medium containing the same concentration of salt mixture. During the fourth subculture, the surviving calli grew well and did not show discolouration at 300 mol m^{-3} salt mixture. These calli have been designated salt mixture resistant (SMR) to distinguish them from wild-type salt mixture sensitive (SMS) calli.

Stability of salt resistance of the selected line: To determine the stability of selected traits, SMR calli were subcultured for two passages (4 weeks each) away from the salt mixture, and thereafter were again grown on medium containing 300 mol m^{-3} of salt mixture.

Growth characteristics of the selected line: The growth (fresh and dry mass) of SMS and SMR calli grown at 300 mol m^{-3} salt mixture was measured at intervals of 7 d over a period of one month.

To compare tolerance of SMS and SMR callus lines, callus pieces of 25 ± 2 mg were inoculated on media containing different concentrations (0 - 350 mol m^{-3}) of salt mixture. Fresh and dry mass of the two lines were determined after 4 weeks of culture.

Resistance of the selected line to different salts: Resistance of SMS and SMR callus lines to different constituent salts of salt mixture was tested by inoculating callus pieces of 25 ± 2 mg on media containing NaCl (0 - 350 mol m^{-3}), KCl (equimolar to 0 - 350 mol m^{-3} NaCl) and Na_2SO_4 (equimolar to 0 - 250 mol m^{-3} NaCl). Fresh and dry masses of both the lines were determined after 4 weeks of culture.

Estimation of Na^+, K^+, Cl^- and SO_4^{2-} ions: A known amount of oven dried callus samples were digested with nitric acid and Na^+, K^+, Cl^- and SO_4^{2-} were determined as described earlier (Gulati and Jaiwal 1992).

All the experiments were repeated at least twice.

Results and discussion

Effect of salt mixture on callus growth: The fresh mass of callus decreased with increasing concentrations of salt mixture in the medium (Fig.1). However, the reduction in growth was less during the first week of culture. The maximum growth of callus tissue was observed during the third week of culture as evidenced by an increase in fresh mass for concentrations up to 200 mol m^{-3} of salt mixture. Beyond this, there was little change in fresh mass. Salt mixtures at 25 mol m^{-3} caused stimulation in callus growth over the control up to the second week of culture. Similar results with stimulatory effects of low salt concentrations have also been reported for other cultured cells (*e.g.* Gale and Boll 1979, Gosal and Bajaj 1984, Pandey and Ganapathy 1984). However, concentrations of salt mixture higher than 150 mol m^{-3} caused browning and necrosis of callus, and hence reduced callus growth, which was completely inhibited at a concentration of 300 mol m^{-3}. Such an inhibition of callus growth at higher salt levels is in accordance with the results of Chen *et al.* (1980), Pandey and Ganapathy (1984), and McCoy (1987). The decrease in dry mass was less than that of fresh mass at almost all the levels of salinity (Fig. 1).

Selection of salt mixture resistant callus line: The selected SMR calli (see Materials and methods) showed the persistence of the tolerance trait in the absence of salt mixture. Similar results were also observed in other systems (Croughan *et al.* 1978, Kochba *et al.* 1982, Rangan and Vasil 1983, Salgado-Garciglia *et al.* 1985). At present, we have no evidence whether SMR cell lines are a real mutant or only epigenetic variants. The ultimate proof of a true genetic variant lies in the regeneration of salt mixture tolerant plants and then testing the inheritance of tolerance at the whole plant level. Unfortunately, our attempts to regenerate plants from the selected cells were unsuccessful. SMR calli produced only roots on the modified PC-L2 medium with or without salts.

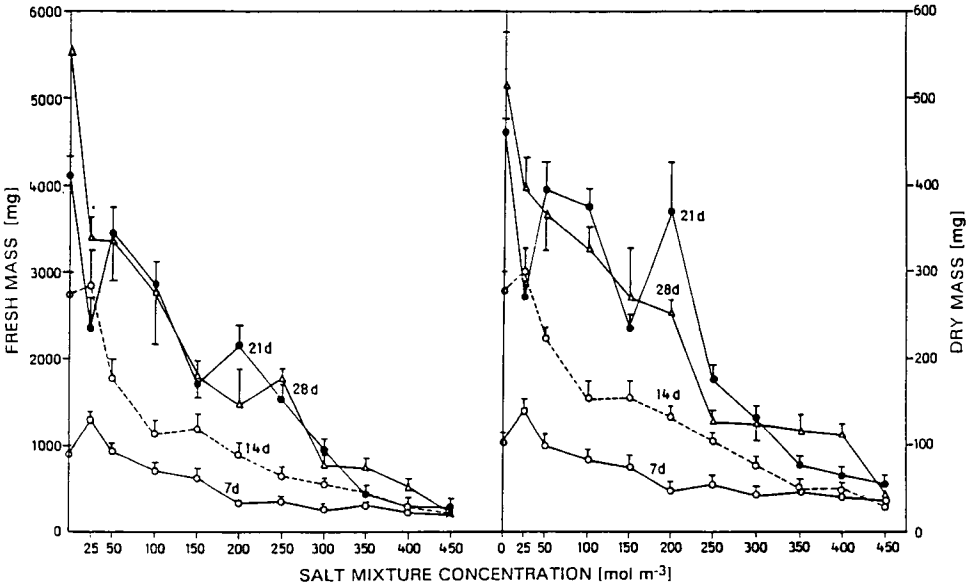

Fig. 1. Effect of salt mixture concentration on fresh and dry mass of callus cultures of *Vigna radiata* cv. K-851. Vertical bars denote standard errors.

Growth characteristics of SMR and SMS callus lines: The SMR calli, in the absence or presence of salt, showed rapid increases in fresh and dry mass which continued up to 28 d. The SMS calli did not show any increase in fresh mass and only a slight increase in dry mass in the presence of salts. The SMR calli had a higher fresh mass than SMS in the absence of salt. However, their fresh mass in the presence of stress was smaller than that of SMS growing on normal medium. Similar growth patterns of salt stress-selected cell lines have been reported in many species (*e.g.* Ben-Hayyim and Kochba 1982, Salgado-Garciglia *et al.* 1985). However, in some cases, the growth of the former was not less than that of the control (Pandey and Ganapathy 1984, Binzel *et al.* 1985, Kumar and Sharma 1989).

The SMR callus showed significantly higher growth at all the salinity levels as compared to SMS line (Fig. 2). Thus, the SMR cell line showed a shift towards a halophytic nature. A similar pattern of growth was exhibited by *Medicago sativa*

(Croughan *et al.* 1978), *Oryza sativa* (Rains *et al.* 1980), *Citrus sinensis* (Ben-Hayyim and Kochba 1983), *Ipomoea batatas* (Salgado-Graciglia *et al.* 1985) and *Lycopersicon peruvianum* (Hassan and Wilkins 1988).

Tolerance of SMR cells to individual salts: The growth of the SMS line was significantly higher than that of the SMR line on 50 mol m^{-3} Na_2SO_4. However, at higher Na_2SO_4 concentrations both callus lines responded similarly. Neither of the two lines could tolerate 200 mol m^{-3} or higher concentrations of Na_2SO_4 (Fig. 3). Similar results were also obtained in *Citrus sinensis* by using NaCl-resistant cell lines (Kochba *et al.* 1982).

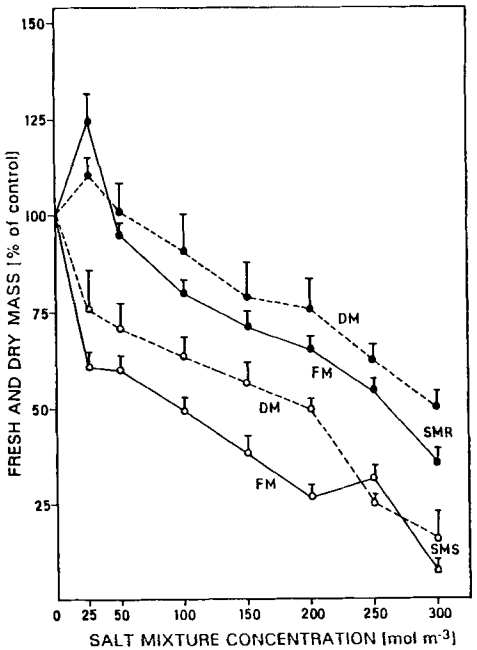

Fig. 2. Effect of salt mixture concentration on the growth of SMR and SMS calli of *Vigna radiata*. Vertical bars represent standard errors of the means (n = 5) of two independent experiments. Fresh mass (FM) in the absence of salt mixture averaged 2541 ± 115 mg and 2624 ± 624 mg in SMS and SMR calli, respectively. Average dry mass (DM) was 516 and 282 mg, respectively.

The relative growth of SMR calli was significantly higher than that of SMS calli up to 250 mol m^{-3} of KCl. But both of the lines responded similarly at higher KCl concentrations, *i.e.* 300 - 350 mol m^{-3} (Fig. 3).

The relative growth of SMR calli on NaCl containing medium was slower than that of SMS calli at all the concentrations of NaCl (Fig. 3).

Ion analysis: Na^+ and Cl^- ions accumulation in the callus increased with increasing concentrations of salt mixture in the medium. A considerable amount of Na^+ and Cl^- was accumulated by 7 d but they further increased by very small amounts with the age of culture.

K^+ content of the callus declined continuously with increasing salt mixture levels. During the first week, K^+ content remained stable at all the salt concentrations. This probably suggest that K^+ plays an important role in osmotic adjustment during the early stages of growth under salt stress (Bernstein 1977). The increase of Na^+ and Cl^- and decrease of K^+ in callus as a function of external salt mixture concentrations in the medium is in agreement with the results of Taleisnik-Gertel *et al.* (1983), Pandey and Ganapathy (1984) and Garcia-Reina *et al.* (1988). SO_4^{2-} amounts in the callus remained unchanged with increased salt mixture concentrations.

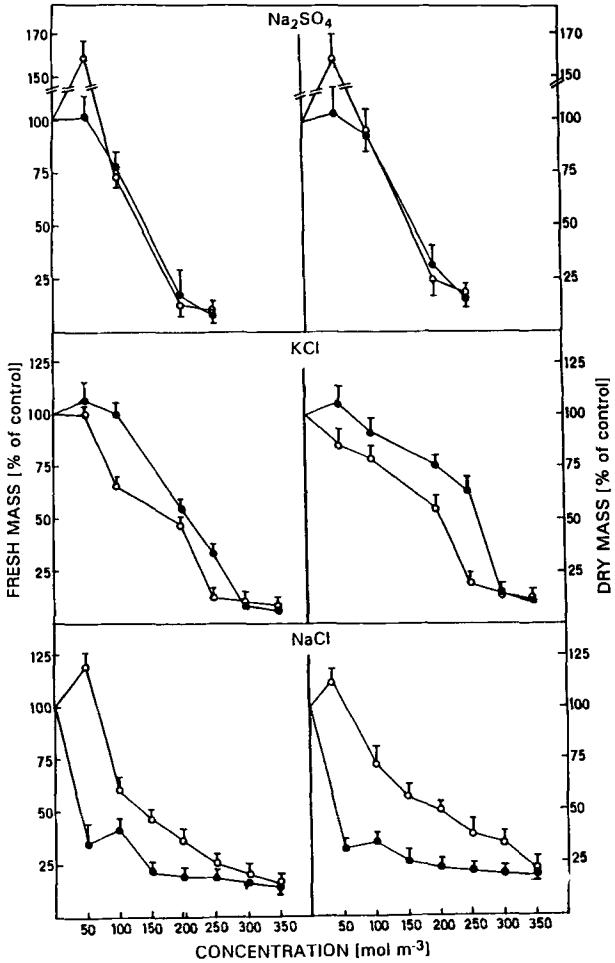

Fig. 3. Effect of Na_2SO_4, KCl and NaCl on fresh and dry masses of SMS (*open circles*) and SMR (*closed circles*) calli of *Vigna radiata*. Vertical bars represent standard errors of the means (n = 5) of two independent experiments. Fresh mass in the absence of salts averaged 2502 ± 152 mg and 3604 ± 292 mg in SMS and SMR calli, respectively. Average dry mass was 260 and 309 mg, respectively.

The Na^+ content of SMR callus was comparable to that of SMS callus, when both were grown on normal medium. The Na^+ level in both the lines increased with

increase in the concentration of salts in the medium. However, the SMS callus line accumulated more Na^+ than the SMR line under the same degree of stress (Fig. 4).

The SMR line contained slightly higher K^+ than SMS line when both were grown in the absence of salts. The K^+ contents of both the lines decreased with increase in saline levels. However, this decrease was more pronounced in the SMS callus line than the SMR line (Fig. 4). Similar results with regard to K^+ and Na^+ content in salt-resistant and sensitive callus lines have been obtained in other species (Croughan *et al.* 1978, Watad *et al.* 1983).

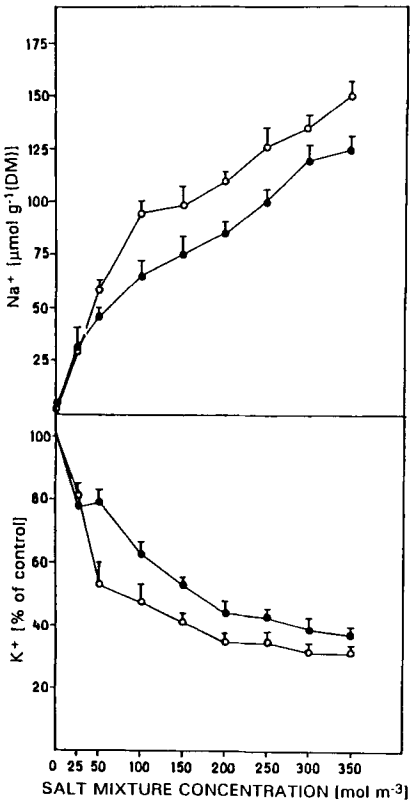

Fig. 4. Na^+ and K^+ content of *Vigna radiata* SMS (*open circles*) and SMR (*closed circles*) calli as a function of salt mixture concentration. Vertical bars represent standard errors of the means. K^+ contents in the absence of salt mixture were 68.2 ± 1.02 µmol g^{-1}(DW) and 75.8 ± 1.0 µmol g^{-1}(DW) in SMS and SMR calli, respectively.

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