CHAPTER 15

IN-VITRO SELECTION OF SALT-RESISTANT PLANTS

15.1 INTRODUCTION

The decline in plant productivity due to salinity and drought necessitates the development of stress-tolerant crops. Of the various approaches available, cell culture selection of mutants has received increasing attention in recent years (Maliga, 1984). Salt-tolerant cell lines have been isolated from several species (see review by Tal, 1990), but few plants have been recovered from NaCl- tolerant cells (Nabors *et al.,* 1980, 1982; Tyagi *et al.,* 1981; Nabors and Dykes, 1985; Freytag *et al.,* 1990). This may in large part result from a lack, or loss during selection, of regeneration capacity in cell cultures (Ranjan and Vasil, 1983; Chandler and Vasil, 1984; Bajaj and Gupta, 1987). If embryogenic cultures are established, they can be used for selection, but these are available only in a few species (Ben-Hayyim and Kochba, 1982; Ranjan and Vasil, 1983; Chandler and Vasil, 1984). An alternative, perhaps more generally applicable approach, which has not been fully explored to date, is to use easily regenerable cultures, such as morphogenic callus or various explant cultures for selection (Mathur *et al.,* 1980; Lebrun *et al.,* 1985; Jain *et al.,* 1991a).

Vigna radiata is an important salt-sensitive grain-legume of tropical and sub-tropical regions. Callus, cell-suspension and protoplast cultures of *V. radiata* have failed to produce plantlets (Mathews, 1987; Gill *et al.,* 1987; Eapen and George, 1990; Gulati and Jaiwal, 1992). However, cotyledon explants of this species possess a high morphogenic potential to regenerate shoots (Gulati and Jaiwal, 1990). In this study, the feasibility of using cotyledon explant cultures for the selection of salt-resistant variant is assessed.

15.2 MATERIALS AND METHODS

Explant culture and selection of salt-tolerant shoots

Cotyledons were excised from 2-d-old *in vitro* raised seedlings of *Vigna radiata* cv. K-851 and cultured on 'C' (MS (Murashige and Skoog, 1962) salts + B_5 (Gamborg *et al.,* 1968) vitamins basal medium supplemented with $5x10^{-6}$M BAP hereafter referred as 'CB', for shoot proliferation (Gulati and Jaiwal, 1990). The inhibitory concentration of NaCl was determined by culturing single cotyledon per test tube (25 mm x 150 mm) containing 20 ml of CB medium supplemented with 0, 25, 50, 100, 150 and 200 mol m^{-3} of NaCl. The culture tubes were incubated under 16-h photoperiod (80μ mol $m^{-2}s^{-1}$) at 25 $\pm$ 2°C. The cultures were scored for the number of explants forming shoots, the number of shoots and the mean length of shoots per explant after one month of incubation. For each treatment, 24 explants were cultured and the experiment was repeated at least twice. The NaCl concentration which completely inhibited shoot regeneration, was taken as the inhibitory concentration. Subsequently, selection for salt

tolerance was carried out in CB medium containing NaCl concentration (200 mol m^{-3}) that determined to be inhibitory and cultures were scored for shoot regeneration after one month of incubation.

Stability of salt resistance

Two methods for testing the salt resistant of the shoots selected on NaCl medium were used:

(1) Resistance stability was tested by multiplying shoots through axillary bud culture on NaCl-free 'CB' medium for two months and then transferring them back to CB medium containing 200 mol m^{-3} NaCl. Salt-tolerance of shoots selected on salt was assessed on the basis of visual observation on shoot growth on medium with 200 mol m^{-3} NaCl.

(2) Retention of resistant phenotype at the cellular level was tested by initiating callus cultures from leaf explants excised from shoots selected on salt, on modified PC-L2 medium (Phillips and Collins, 1979) with or without salt. After one subculture on the same medium, callus pieces (each 25 $\pm$ 2 mg fresh weight) were incubated in petriplates containing 20 ml of PC-L2 medium supplemented with various concentrations of NaCl (150, 200, 250 and 300 mol m^{-3}). The callus derived from leaves of control shoots was also subjected to similar treatments. The growth of callus on salt containing medium was quantified.

Rooting of shoots

Well developed normal and selected shoots ($\geq$ 3 cm) were rooted on MS + IAA ($5x10^{-6}$M) with or without NaCl (25 to 200 mol m^{-3}).

Transplantation

The plantlets were removed from the test tubes, and after washing roots in running tap water, the plants were then transferred to pots containing sterile vermiculite. A glass beaker was inverted over each of the plants to maintain high humidity during first ten days. After 15 days, plants were transferred to the soil in the pots under greenhouse conditions.

15.3 RESULTS

Response of explants on NaCl-free medium

The explants produced callus at the proximal end on NaCl-free medium within 7-d. After 10-14 d of culture, multiple shoots were differentiated in 54.5% of cultures. An average of 5 shoots per explant were produced within 4 weeks of culture (Figs. 1 and 2).

Response of explants on NaCl containing medium

NaCl not only effected the amount of callus produced at the proximal end of explants cultured on media supplemented with 25 to 200 mol m^{-3} NaCl, but also delayed the differentiation of shoots by 7-10 days. The shoot regeneration capacity in terms of explants forming shoots and the number and the mean length of shoots per explant decreased significantly ($P < 0.05$) with

increasing NaCl concentration (Figs.1 and 2). However, NaCl at 25 mol m^{-3} enhanced the shoot forming response of explants as well as the growth of the shoots compared to the control cultures. At 150 mol m^{-3} of NaCl, out of 91% explants that survived, only 16.6% regenerated an average of 2 shoots per culture. The shoots that regenerated on NaCl medium were stunted with small leathery and light-green leaves. NaCl beyond 150 mol m^{-3} caused complete inhibition of shoot organogenesis, although 33.3% explants survived at 200 mol m^{-3} NaCl (Fig.1). However, surviving explants produced only a small amount of callus at 200 mol m^{-3} NaCl (the concentration termed inhibitory). Therefore, a large number of explants were screened for shoot regeneration on medium containing 200 mol m^{-3} NaCl. Out of 850 screened cotyledons on 200 mol m^{-3} NaCl, only three produced one shoot each (Plate 12A).

Persistence of resistance in the absence of sodium chloride

(1) Through shoot multiplication

The shoots developed on 200 mol m^{-3} NaCl were stunted and therefore, were transferred to NaCl-free 'CB' medium without BAP for elongation. Within 28 days, the shoots elongated to 30 mm and provided 3 culturable nodal segments. These nodal segments, ~ 10 mm in length, were cultured on 'CB' medium (in the absence of NaCl) for one month. Each nodal segment produced four shoots, each was 20-30 mm in length. These shoots were further multiplied by axillary bud culture on NaCl-free medium. The persistence of resistance was tested by transferring these shoots back to 200 mol m^{-3} NaCl containing medium. These selected shoots grew on 200 mol m^{-3} NaCl medium (Plate 12B). In contrast, the control shoots turned brown and died in 200 mol m^{-3} NaCl. These observations indicate that resistance trait is maintained (in the absence of NaCl) upto 3 passages of one month each (tested so far).

(2) Through callus cultures

The leaf segments excised from the shoots differentiated on normal medium did not produce callus in the presence of 200 mol m^{-3} NaCl. On the other hand, such explants excised from shoots selected on NaCl (200 mol m^{-3}) produced profuse callus not only at the cut ends but also all over the surface of explants. In the latter, the callus growth was stimulated at 150 mol m^{-3} NaCl while it was not affected at 200 mol m^{-3} NaCl. On the other hand at 200 mol m^{-3} NaCl the callus derived from control shoots showed more than 65% reduction in the growth (Fig.3). Thus, salt-resistant shoots yielded salt-resistant callus without prior selection on salt containing medium.

Rooting of salt-resistant selected shoots

The rooting response of selected shoots was much better than the control shoots in the presence of NaCl. The percentage of selected shoots rooting on medium containing 25 and 50 mol m^{-3}

NaCl was greater than control shoots rooting on NaCl free rooting medium. The root growth, i.e. root number and the root length decreased with increase in salt concentration. At 200 mol m^{-3} NaCl, 30% of the selected shoots were rooted (Figs. 4 and 5; Plate 12C).

Transplantation

The rooted shoots were transferred to the pots containing sterile vermiculite. After 15 days, one hundred plantlets were transferred in the soil in pots, where 90% of them survived and resumed growth. All the regenerated plants were stunted and flowered precociously. Although, flowering was precocious in R_0 plants, normal pods and viable seeds were produced. Screening and genetic analysis of R_1 plants for salt tolerance is currently in progress.

15.4 DISCUSSION

Salt-tolerant cell lines have been selected in several species including grain-legumes, i.e. *Cicer arietinum* (Pandey and Ganapathy, 1984); Glycine max (Jia-Ping *et al.,* 1981); *Pisum sativum* (Gosal and Bajaj, 1984); *Cajanus cajan* (TCCP, 1987) and *Vigna radiata* (Gosal and Bajaj, 1984; Kumar and Sharma, 1989a). However, plant regeneration has not been obtained from these salt selected cell lines. The inability of selected cell lines to regenerate shoots may be the result of increased somatic age (Murata and Orton, 1987) and/or accentuation of genetic abnormalities that accompanied selection in high salt (McCoy, 1987b). In the present study with *Vigna radiata*, three NaCl-resistant somaclones have been isolated on media supplemented with 200 mol m^{-3} NaCl. Resistance to NaCl within plants regenerated from *in vitro* cultured tissue may be due to mutation within the regenerative cells (Freytag *et al.,* 1990). NaCl-resistance regenerated *Vigna radiata* shoots reported here may have developed from single mutant cells. Thus, the approximate mutation frequency for salt tolerance is 3.5×10^{-3} explants.

Another interesting observation was the enhanced morphogenic response of cotyledon explants cultured on medium containing 25 mol m^{-3} NaCl compared to those cultured on salt free medium. Similar promotive morphogenic effects of Na_2SO_4 were also observed in *Nicotiana tabacum* (Pua *et al.,* 1985) and *Brassica species* (Chandler *et al.,* 1986).

In the present study, the regenerated shoots showed the persistence of resistance not only during *in vitro* multiplication in the absence of sodium chloride (upto three passage of one month each), but the callus derived from them was also salt-resistant without additional selection on NaCl containing medium. Demonstration of the persistence of NaCl-resistance trait is essential as reports exist on the loss of tolerance after one passage away from the selective agent (see Tal, 1990).

The effect of salinity on root growth *in vitro* has been extensively studied (Waisel, 1985), since roots are usually the first plant organs to be affected by salts. When subjected to moderate salinity stress, growth and mineral uptake of roots as well as their production of cytokinins, are immediately reduced and totally inhibited under severe salinity (Itai *et al.,* 1968; Weimberg *et al.,* 1984). Results of this study also showed reduced growth, with a low root number and root length in *Vigna radiata* shoots rooted in the presence of NaCl. However, knowledge on the effect of salinity on root initiation is limited (Pua and Thorpe, 1986). The shoots selected on salt showed higher percent rooting in the presence of salt than the control shoots. NaCl at 25 and 50 mol m^{-3}, stimulated the rooting response of shoots selected on salt than the control shoots on salt free medium. Thus, rooting studies indicate that selected shoots are NaCl-tolerant as they can be rooted even in the presence of 200 mol m^{-3} NaCl.

This study clearly demonstrated the importance of using cotyledon explants for *in vitro* selection experiments on *Vigna radiata* which possesses a high morphogenic potential to regenerate shoots (Gulati and Jaiwal, 1990). This system can also be used for developing genotypes resistant to other abiotic or biotic stresses, where *in vitro* selection strategies are available.

EXPLANATION OF FIGURES

Fig. 1 Survival and shoot forming response of *Vigna radiata* cotyledon explants on CB medium supplemented with NaCl (0-200 mol m^{-3}) after 4 weeks of culture. Vertical bars represent standard error of the mean.

Fig. 2 Effect of no salt and different concentrations of NaCl supplemented on average number of shoots and mean length of shoots per explant after 4 weeks of culture. Vertical bars represent standard error of the mean.

Fig. 3 Effect of different concentrations of NaCl on fresh weight of callus derived from NaCl-resistant shoots (•—•) and callus derived from control shoots (o—o) after one month of growth. Vertical bars represent standard error of the mean.

Figs.4 & 5 Influence of NaCl on percent rooting (Fig.4) and number of roots (Fig.5) of selected and control shoots of *Vigna radiata* after 4 weeks of culture. Vertical bars represent standard error of the mean.

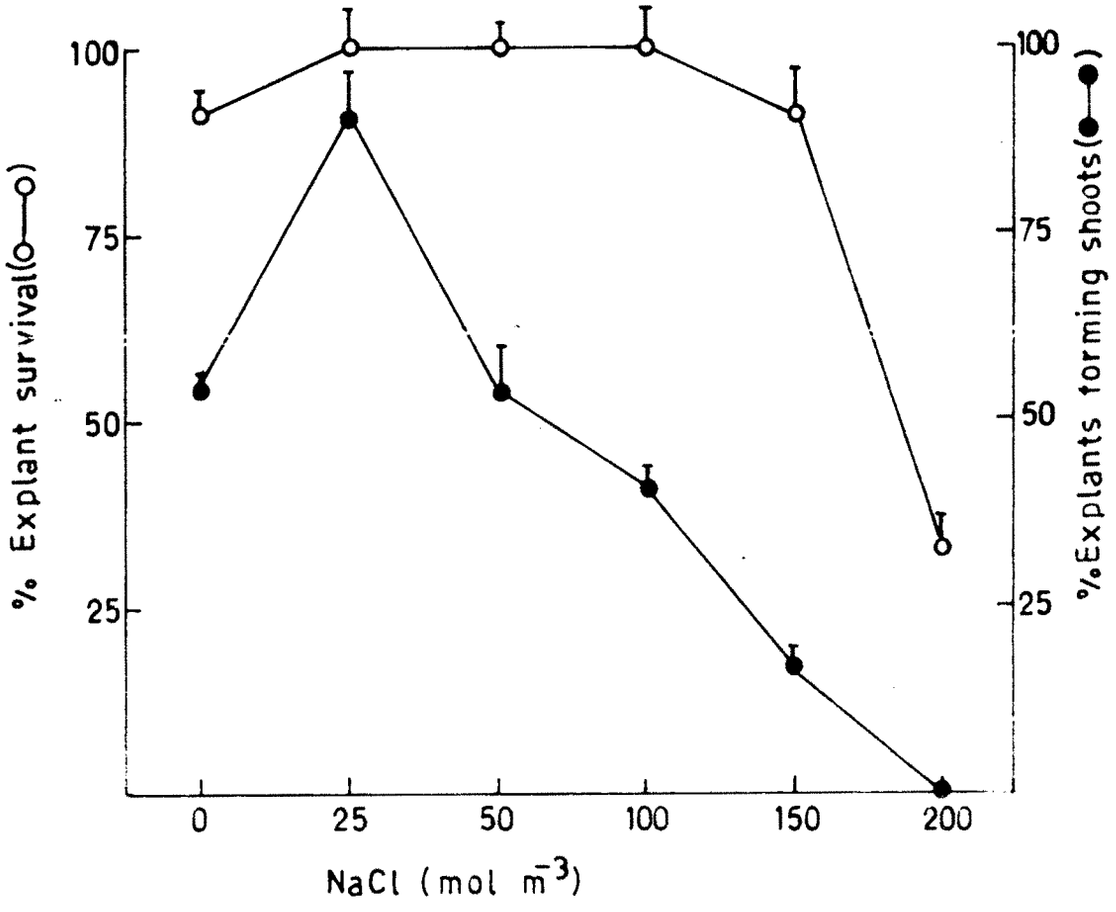
100
75
50
25
% Explant survival(o—o)
%Explants forming shoots(●—●)
0
25
50
100
150
200
NaCl (mol m⁻³)

Fig. 1.

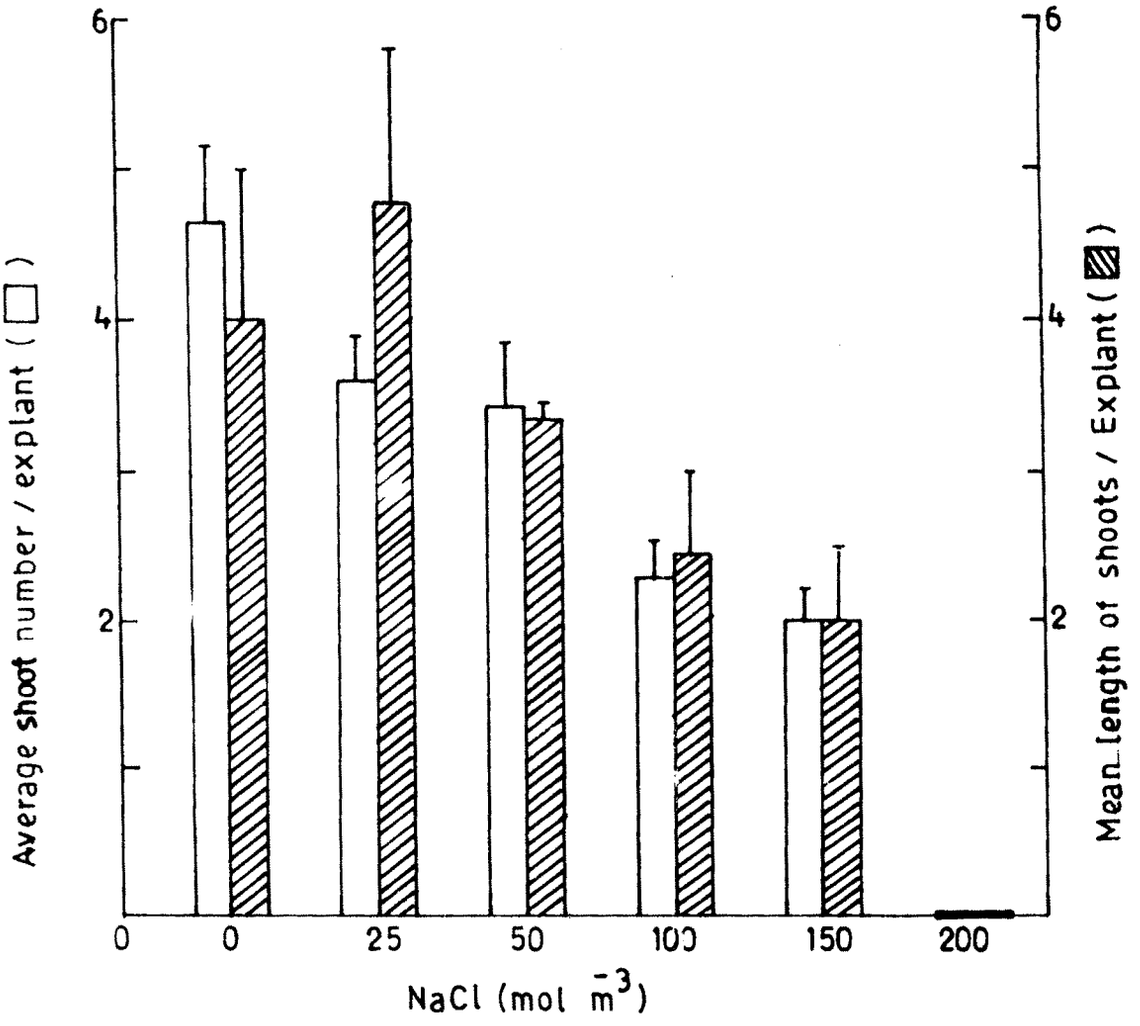
Average shoot number / explant (□)
Mean length of shoots / Explant (▨)
6
4
2
0
0
25
50
100
150
200
NaCl (mol m⁻³)

Fig. 2.

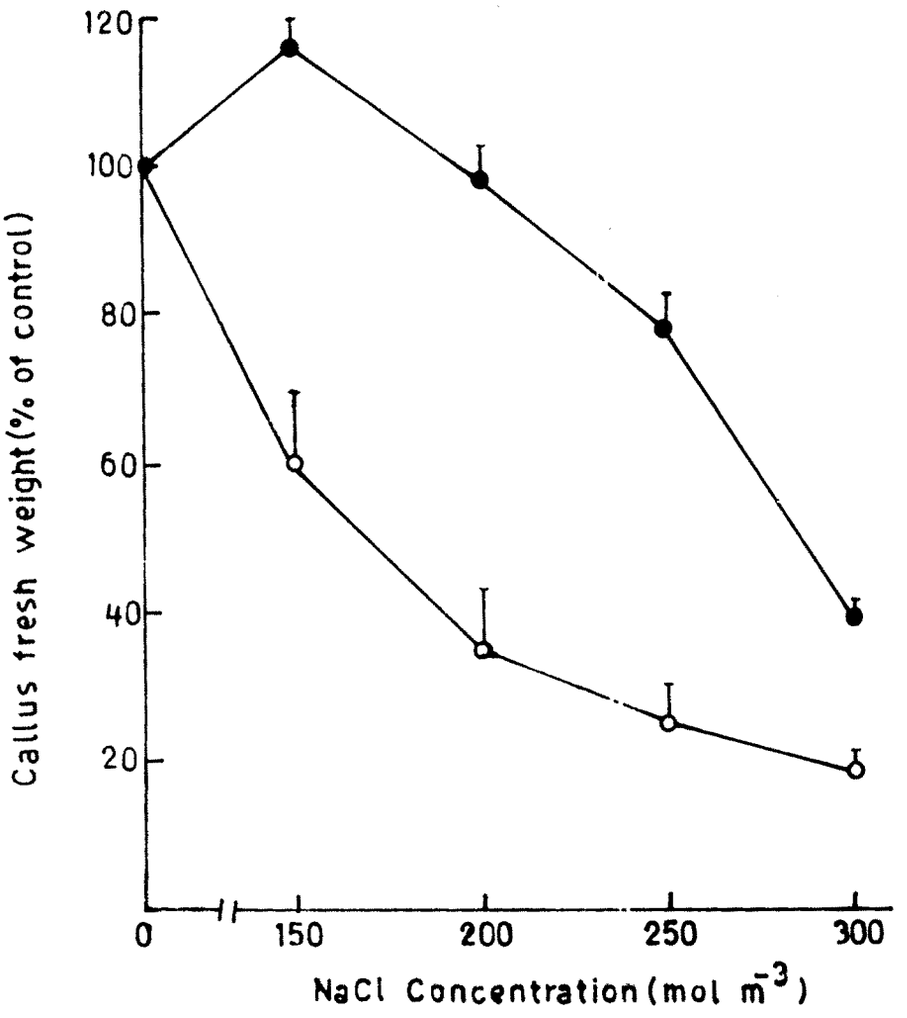
120
100
80
60
40
20
Callus fresh weight (% of control)
0
150
200
250
300
NaCl Concentration (mol m⁻³)

Fig. 3.

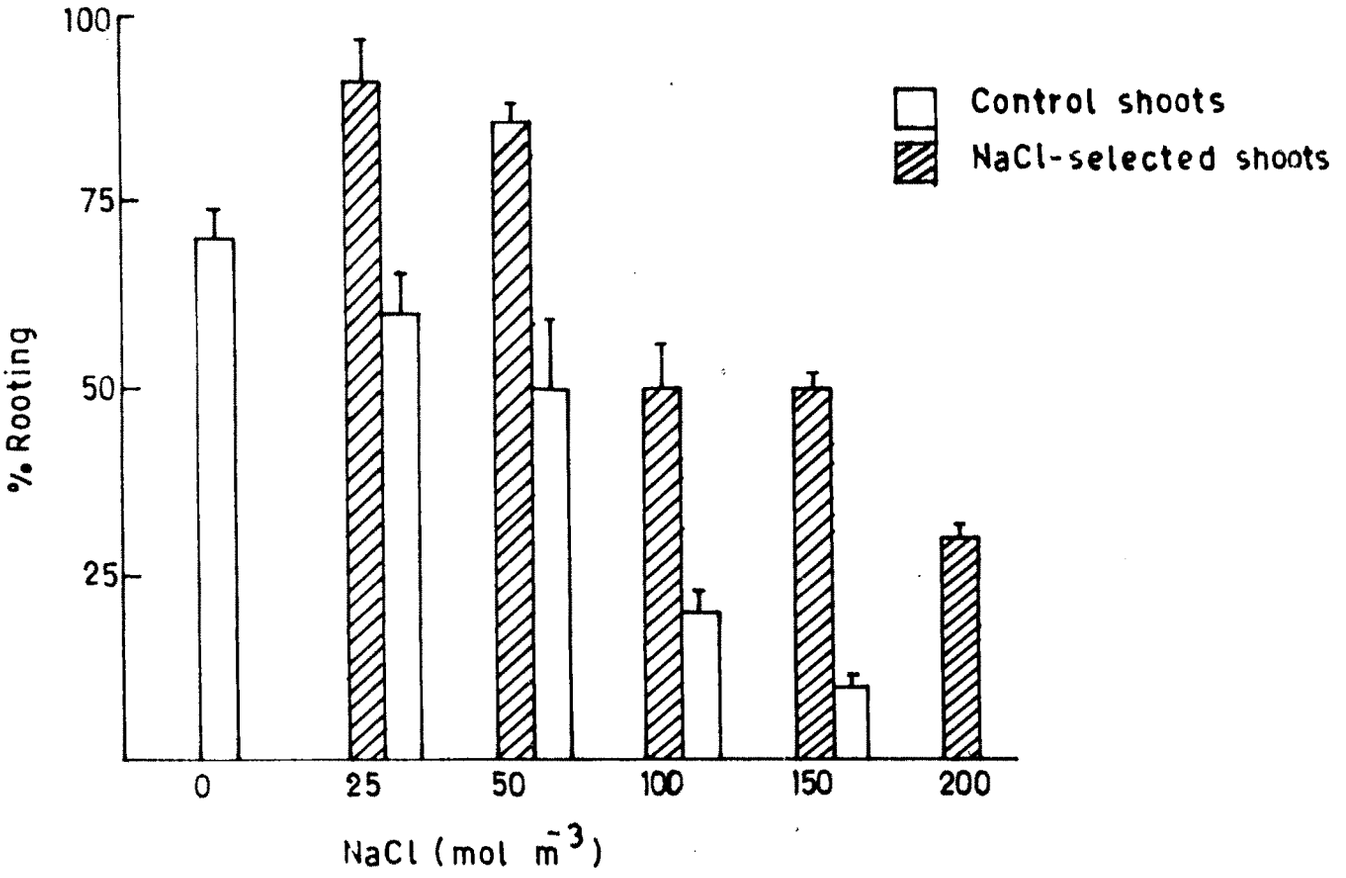
100
75
50
25
% Rooting
0
25
50
100
150
200
NaCl (mol m⁻³)
Control shoots
NaCl-selected shoots

Fig. 4.

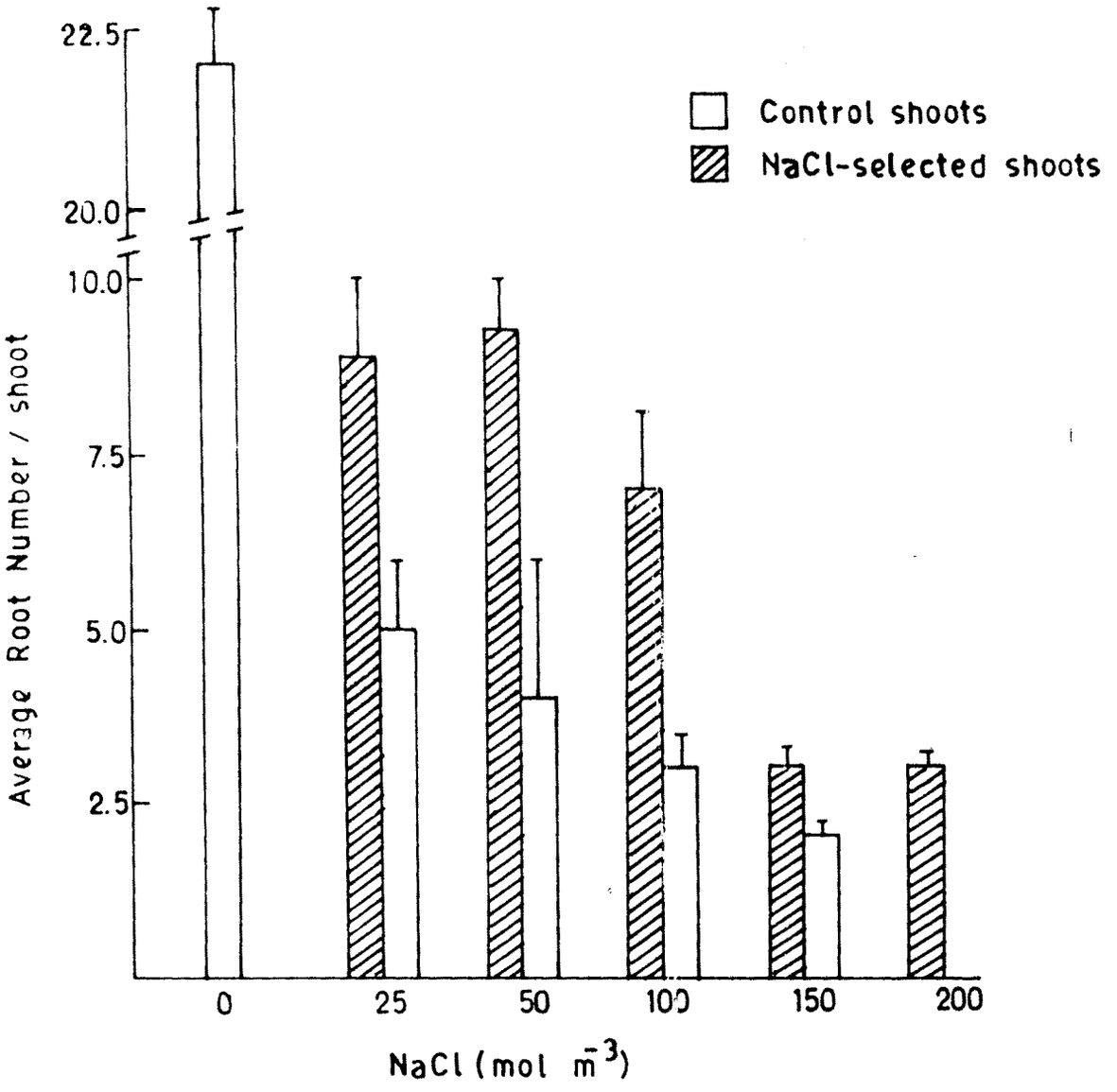
22.5
20.0
10.0
7.5
5.0
2.5
Average Root Number / shoot
Control shoots
NaCl-selected shoots
0
25
50
100
150
200
NaCl (mol m⁻³)

Fig. 5.

PLATE 12 A. Regeneration of shoots from cotyledon explants cultured on C (MS salts + B_5 vitamins) basal medium supplemented with BAP ($5x10^{-6}M$) and 200 mol m^{-3} NaCl.

B. Salt selected shoot growing on 200 mol m^{-3} NaCl containing medium.

C. Rooting of salt selected shoots on MS + IAA ($5x10^{-6}M$) and 200 mol m^{-3} NaCl.

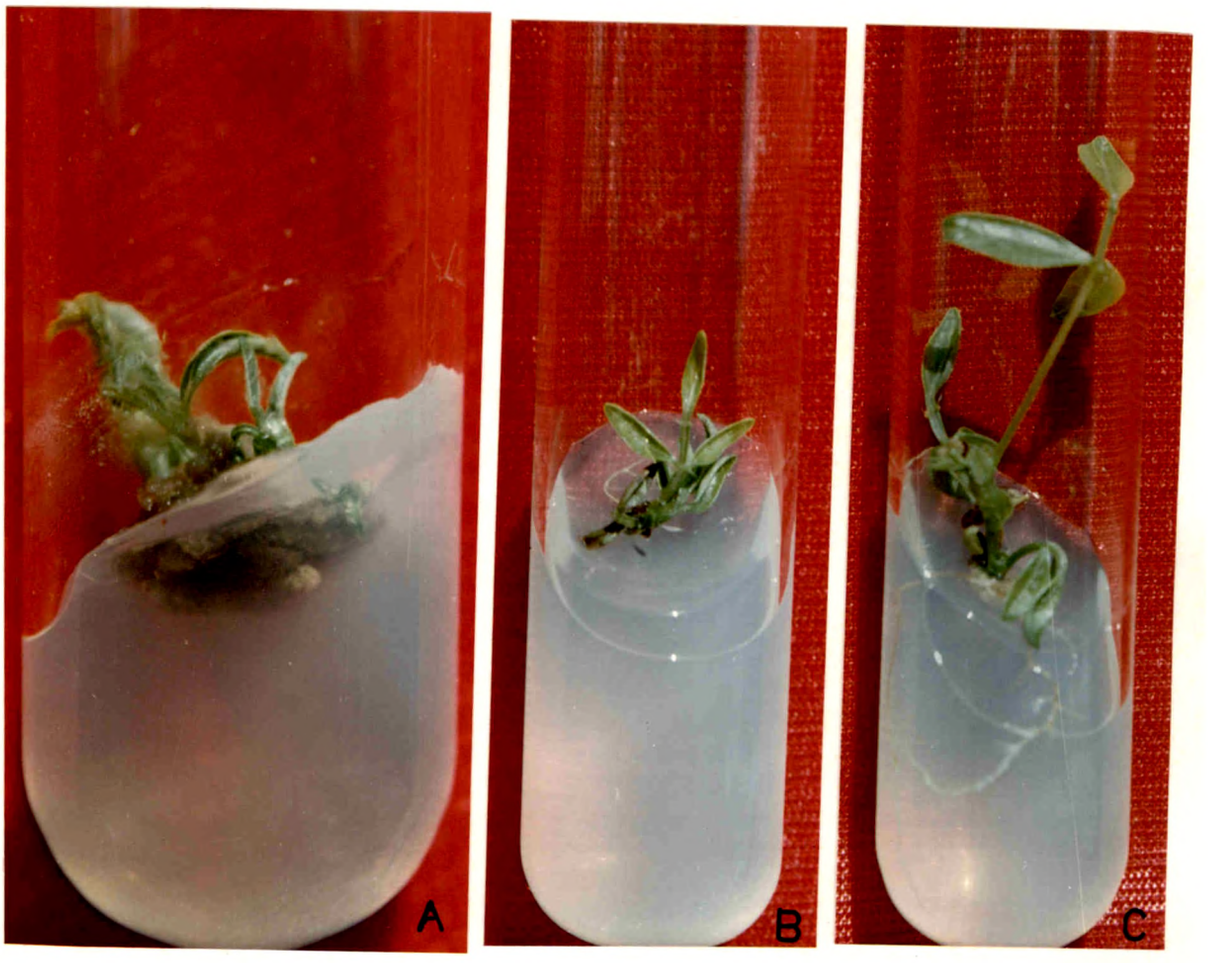

PLATE 12