CHAPTER 9

IN VITRO EVALUATION OF NaCl TOLERANCE IN WILD AND CULTIVATED SPECIES OF VIGNA

9.1 INTRODUCTION

The presence of genetic variability for salt tolerance among species of different crops have been documented (Epstein *et al.,* 1980;) and offer encouragement to develop salt-tolearnt plants. Cell culture systems provide a reliable and efficient alternative method for screening, selecting and characterizing salt tolerance at cellular level (Chandler and Thorpe, 1986; Tal, 1990). Salt-tolerant cell lines have been isolated from a number of species including a few legumes, i.e. *Medicago sativa* (Croughan *et al.,* 1978); *Glycine max* (Jia-Ping *et al.,* 1981); *Pisum sativum* (Gosal and Bajaj, 1984); *Cicer arietinum* (Pandey and Ganapathy, 1984)) and *Vigna radiata* (Gosal and Bajaj, 1984; Kumar and Sharma, 1989a). Unfortunately, there have been only few cases where *in vitro* selection resulted in heritable NaCl tolerance expressed at the whole plant level (Nabors *et al.,* 1980; Tyagi *et al.,* 1981; Nabors and Dykes, 1985; Vajrabhaya *et al.,* 1989; Kirti *et al.,* 1991; Winicov, 1991; Sumaryati *et al.,* 1992). In most of the cases above, either plants were not regenerated from NaCl-tolerant cell lines or the regenerated plants have not demonstrated increased NaCl tolerance (Smith and McComb, 1983). Since selection has been performed at the cellular level, it is important to know, whether salt tolerance in culture is reflected at the whole plant level and whether salt tolerance expressed by intact plants is seen at the level of callus culture. These questions are not adequately resolved in the literature. However, some studies have demonstrated positive correlation between whole plant and cell culture responses for salt tolerance (Tal *et al.,* 1978; Orton, 1980; Smith and McComb, 1981b; Warren *et al.,* 1985), while others have shown no correlation between *in-vivo* and *in-vitro* NaCl tolerance (Smith and McComb, 1981a; Hanson, 1984; McCoy, 1987a). The objective of the present study was to determine whether a cellular mechanism for whole plant NaCl tolerance exists in *Vigna radiata*. If such a mechanism exists, then callus culture technique could be used for screening the germplasm for salt tolerance. This report describes a cellular and whole plant response to NaCl of *Vigna radiata* and *in-vitro* screening of cultivated and wild species of *Vigna* for salt tolerance. Most of the species tested can be hybridised with *Vigna radiata* using embryo rescue technique (Gosal and Bajaj, 1983a,b; Ahn and Hartmann, 1978).

9.2 MATERIALS AND METHODS

Seeds of cultivated and wild species of *Vigna* and of 12 cultivars of *Vigna radiata* were precurred from NBPGR, New Delhi; Directorate of Pulse Research, Kalyanpur, Kanpur and Pulse Research Laboratory, Division of Genetics, IARI, New Delhi.

Whole plant study

Ten seeds of *V. radiata* cv. K-851 were germinated per plastic pot containing 5 kg of acid washed sand. One week after germination, thinning was done leaving only 4 plants per pot. They were watered with Hoagland's nutrient solution containing either 0, 25, 50, 100, 150, 200, 250, 300 mol m^{-3} NaCl with there replicates per treatment. Extreme care was taken to completely flush all pots at each watering to leach any residual NaCl. The pots were placed in the greenhouse at temperatures of 24-27°C during day and 22-24°C during the night. After 4 weeks, all plants were removed from the pots, the roots were washed thoroughly, excess water was blotted off and fresh weight of each plant was recorded. Plants were then dried at 80°C for 48 hours and dry weight was determined. Four plants from each treatment were selected at random and their shoots and roots were ground separate in pestle and morter and analysed for ions estimation. The effect of NaCl stress on whole plants of wild species could not be studied due to limited number of seeds.

Establishment of callus cultures

Selection of medium for optimal callus growth

Callus cultures of *Vigna radiata* cv. K-851 were initiated from leaf explants of aseptically grown 7-d-old seedlings. Leaf explants (5 mm x 5 mm) were transferred aseptically to culture tubes (150 mm x 25 mm) containing either MS (Murashige and Skoog, 1962), B_5 (Gamborg *et al.*, 1968), C(MS salts + B_5 vitamins) or PC-L2 (Phillips and Collins, 1979) basal media supplemented with NAA (0.5 mg/l), 2,4-D (0.5 mg/l) and BAP (1 mg/l). The media were adjusted to pH 5.8, solidified with 0.7% agar and autoclaved at 1.05 kg cm^{-2} for 20 min. The culture tubes were incubated under 16-h photoperiod of 80 µmol m^{-2} s^{-1} at 25 ± 2°C. After establishment, a known amount of callus was subcultured on their respective modified basal media using 5 replicates. After 4 weeks of culture the callus from all replicates was removed and its fresh weight was determined. It was then oven dried at 80°C for 48 h prior to determining its dry weight.

Effect of NaCl on callus growth

Callus cultures of different cultivated and wild species of *Vigna* and cultivars of *V. radiata* were initiated as above and maintained on modified PC-L2 medium. After one subculture, actively growing callus, 250 ± 10 mg, was divided into 10 pieces (25 ± 2 mg) and inoculated into pertidishes (100 mm x 17 mm) containing 20 ml of modified PC-L2 medium supplemented with increasing concentrations of NaCl (0, 25, 50, 100, 150, 200, 250, 300, 350 mol m^{-3}). The petridishes were sealed with parafilm and incubated under the same photoperiod and temperature as for the callus cultures. After 4 weeks of culture, callus from petridishes, was removed and its fresh and dry weight, (oven dried at 80°C for 48 hrs) were determined for each treatment.

For each treatment, five replicates were taken and each experiment was repeated twice. The relative growth rate (RGR) in terms of fresh weight of the callus and index of tolerance based on RGR (INTOL) were calculated as per the formula given by Shah *et al.*, (1990).

$$RGR = [l_n \text{ (final weight)} - l_n \text{ (initial weight)}]/4 \text{ wk.}$$

$$INTOL = RGR \text{ (treatment)}/RGR \text{ (control)}$$

The index of tolerance expresses the RGR for each treatment as a proportion of the mean RGR of the appropriate control (i.e. no NaCl). Such an index of tolerance is useful for comparing responses of different species and cultivars to stress if they have different growth rates in unstressed conditions.

Determination of Na^+ and K^+

Oven dried callus samples were digested with nitric acid described earlier in section 8.2. The concentrations of Na^+ and K^+ were determined using an ELICO flame photometer. All the experiments were repeated atleast twice.

9.3 RESULTS AND DISCUSSION

Selection of medium for optimal callus growth

The frequency of leaf explants forming callus and callus growth/explant on four different media tested are shown in Table 1. The explants showed 100% callusing at all the media tested. However, the variation of callus growth among media being significant. The callus growth showed the following gradation: PC-L2 > C > MS > B_5.

The callus growth was maximum at PC-L2 medium and minimum at B_5. Hence PC-L2 medium was used for all other experiments.

Comparison of whole plants and callus cultures

Dry Weight

The effect of various, concentrations of NaCl on dry weight of whole plants of *Vigna radiata* cvs. K-851, ML-1 and G-65, their corresponding calluses and survival of plants were compared (Fig.1). The dry weight of whole plants and callus cultures exhibited similar trends, both decreased gradually with the increase of NaCl concentration in cvs. K-851 and ML-1. In cv G-65, dry weight of whole plant and callus increased under low salt concentrations and decreased somewhat under higher concentrations. Similar trends were also observed in fresh weight of the whole plants and calluses.

Survival of plants of all the three cultivars was significantly affected by NaCl at concentrations higher than 100 mol m^{-3}. At 150 mol m^{-3} NaCl, only 40% of the plants of ML-1

and 50% of the plants of cvs. K-851 and G-65 survived. However, none of the plants survived in cvs. MI^{-1} and G-65 at 200 mol m^{-3} NaCl and in cv. K-851 at 300 mol m^{-3}. Similarly, there was almost complete inhibition of callus growth at 300 mol m^{-3} in all there cultivars.

A positive correlation was found between the response of whole plants and calli to salt. This led to the suggestion that *V. radiata* appears to have a mechanism(s) of salt tolerance which operates at the cellular level. Similar correlation between the response of the whole plant and callus to salt has also been demonstrated in other glycophytic (Tal *et al.,* 1978; Orton, 1980; Smith and McComb, 1981 b) and halophytic (Warren *et al.,* 1985; Dains and Gould, 1985) species. In some systems, in which no such correlation was found, the organization of the cells in the whole plant has been suggested to be essential for the operation of tolerance mechanism (Smith and McComb, 1981 a; Hanson, 1984; McCoy, 1987a). Thus, the positive correlation between growth response of whole plant and callus of three cultivars of *V. radiata* is a pre-requisite for the successful application of callus tissue for screening.

Screening

The cultivated species

The effect of NaCl on the index of tolerance of callus cultures of five different cultivated species *Vigna* is shown Fig.2. Since callus morphology and growth varied among species at modified PC-L2 with O NaCl, the growth of callus cultures was expressed as an index of tolerance which thus corrects for such variation. The callus growth of all species except *V. sinensis* and *V. mungo* was depressed by the addition of 100 mol m^{-3} NaCl to medium. Callus cultures of the latter two species grew equally well at 0 (control) and 100 mol m^{-3} NaCl level. However, further increase in NaCl concentration, decreased the salt tolerance index in almost all the species of *Vigna* except *radiata* which showed higher salt tolerance upto 250 mol m^{-3} NaCl.

The wild species

The salt tolerance index of callus cultures of six wild species of *Vigna* is shown in Fig.3. A progressive reduction in tolerance was observed with subsequent addition of salt in the medium in all the wild species except in *V. setulosa* and *V. vexillata.* The salt tolerance of callus cultures of *V. setulosa* was almost equal at 0 (control) and 100 mol m^{-3} NaCl level while in the latter, the tolerance was increased at both concentrations 100 mol m^{-3} and 150 mol m^{-3} of NaCl compared to 0 (control) salinity.

The cultivars of Vigna radiata

The cultivars responsed differentially with different concentrations of NaCl. Cultivar PS-7 was comparatively more tolerant upto 200 mol m^{-3} NaCl, whereas cv. PDM-54 was more tolerant at higher salt levels (250-350 mol m^{-3}). Overall, cv. G-65 showed higher tolerance to all salt levels as compared to other cultivars (Fig 4.).

Overall the wild species *V. vexillata* and *V. setulosa* were found to be the most tolerant to salt stress compared even to the most tolerant cultivar G-65 of the most salt tolerant cultivated species, *V. radiata*.(Figs. 3 & 4).

Ion accumulation

In whole plants of cvs. K-851, ML-1 and G-65

Na^+, K^+ content and K^+/Na^+ ratio in shoot and root of plants of cvs. K-851, ML-1 and G-65 grown under NaCl stress have been shown in Table 2. K^+/Na^+ ratio in both shoot and root decreased with increase in salt levels. This decrease is due to increase in Na^+ content of both the tissues. K^+/Na^+ ratio was much higher in shoot than root suggesting greater K^+ content in shoot. However, in root, K^+/Na^+ ratio was much higher in cvs. K-851 and G-65 than ML-1, suggesting that the former two are more tolerant than the latter. Similarly, higher K^+/Na^+ ratio in roots of salt-tolerant than salt-sensitive cultures has also been found in *Zea mays* (Hajibagheri *et al.*, 1987) and *Cajanus cajan* (Subba Rao *et al.*, 1990). In the present study, cv. ML-1 exhibited greater increase in Na^+ content in shoot than G-65, at identical NaCl concentrations. Though, uptake of Na^+ was not prevented in cv. G-65, but apparently on inhibition of diffusion or transport of Na^+ into xylem sap possibility by cellular or subcellular compartmentation results into low levels of Na^+ in shoot. However, K^+ content in the shoot of G-65 was higher. These results suggest that salt tolerance is expressed at the tissue level and are compatible with the model where Na^+ is compartmentalized in G-65 root tissue, thus restricting its translocation to the shoot and interference with K^+.

In calluses of V. radiata cv. G-65, V. vexillata and V. setulosa

Endogenous level of Na^+ and K^+ in callus cultures of wild species, *V. vexillata* and *V. setulosa* were compared with cultivated species *V. radiata* cv. G-65 (Table 3). Accumulation of Na^+ increased, while that of K^+ decreased in all the three species with increase in NaCl concentration. The wild species showed their high Na^+ and low K^+ content compared to cultivated species. The increase of Na^+ content and decrease in K^+ content of wild species may be due to low efficiency of Na^+ exclusion and K^+ uptake in these plants. It is suggested that a better osmotic adjustment by higher Na^+ concentration and at the same time preventing the cells

from toxic effect, is responsible atleast in part, for the superior performance of the wild species under salinity. These results are in accordance with those of *Lycopersicon* species (Tal *et al.*, 1978) and *Medicago sativa* (Croughan *et al.*, 1978).

The salt-tolerant wild species *V. vexiallata* and *V. setulosa* are, therefore, valuable source as genotypes in plant breeding for salt tolerance and as experimental plants for isolation, identification and manipulation of genes contributing to salt tolerance in *Vigna* and other crop plants.

Table 1: Callusing response of leaf explants of Vigna radiata cv. K-851 in different basal media supplemented with 0.5 mgl^{-1} 2,4-D, 0.5 mgl^{-1} NAA and 1.0 mgl^{-1} BAP*.

Medium	% callusing	Callus growth/explant	
		Fresh weight (mg)	Dry weight (mg)
MS	100	1134 ± 131	106 ± 5
B_5	100	959 ± 169	85 ± 4
'C' (MS salts + B_5 vit.)	100	1628 ± 33	154 ± 10
PC-L2	100	1806 ± 40	159 ± 8

*Data scored after 28 days of culture, Values are mean ± SE

Tabel 2: Na^+ and K^+ (μ mol g^{-1} DW) and K^+/Na^+ ratio in shoot and root of salt-treated plants of three cultivars of Vigna radiata*.

Cultivar	Salt levels (mol m^{-3})	Shoot		Root		K^+/Na^+	
		Na^+	K^+	Na^+	K^+	Shoot	Root
G-65	0	2.1	18.5	15.6	29.4	0.85	1.88
	25	3.0	20.7	19.5	27.1	6.82	1.39
	50	8.0	19.2	29.3	26.2	2.39	0.89
	100	13.4	18.2	26.0	20.1	1.35	0.78
ML-1	0	2.1	19.2	4.3	32.0	8.84	7.27
	25	4.3	21.7	19.9	27.8	5.01	1.39
	50	14.1	21.0	31.5	17.3	1.48	0.55
	100	22.6	20.5	21.7	14.1	0.90	0.47
K-851	0	2.1	14.7	22.1	33.9	6.78	1.53
	25	3.6	21.4	26.5	36.7	5.79	1.38
	50	5.4	17.3	31.5	35.8	3.18	1.14
	100	8.0	22.0	35.3	35.2	2.74	0.99

* Data scored after 28 days of culture.
5 replicates of each treatment were taken.

Tabel 3: Na^+ and K^+ (μ mol g^{-1} DW) and K^+/Na^+ ratio in NaCl treated callus of Vigna radiata cv. G-65 and of two wild (Vigna vexillata and Vigna setulosa) species.

Species	Salt levels (mol m^{-3})	μ mol g^{-1} DW.		K^+/Na^+
		Na^+	K^+	
V. radiata	0	15.2 ± 0.0	117.9 ± 0.0	7.75
cv. G-65	100	61.9 ± 5.4	52.5 ± 8.9	0.85
	200	74.9 ± 1.0	32.6 ± 5.7	0.43
	300	92.3 ± 3.2	25.6 ± 0.0	0.27
V. vexillata	0	3.10 ± 0.8	61.1 ± 0.3	19.16
	100	99.3 ± 1.5	23.7 ± 1.9	0.24
	200	126.0 ± 4.3	29.7 ± 2.5	0.24
	300	131.2 ± 4.3	19.8 ± 1.9	0.15
V. setulosa	0	3.4 ± 0.2	71.0 ± 0.0	20.46
	100	112.3 ± 8.0	51.9 ± 0.6	0.46
	200	131.5 ± 3.2	37.1 ± 0.7	0.30
	300	146.0 ± 17.8	23.1 ± 2.5	0.28

EXPLANATION OF FIGURES

Fig. 1 The effect of NaCl on the survival of plants and growth of whole plant and callus cultures of *Vigna radiata* cvs.K-851 (A), ML-1 (B) and G-65 (C).

Fig. 2 The effect of NaCl on the index of tolerance of callus cultures of different species of genus *Vigna*.

Fig. 3 The effect of NaCl on the index of tolerance of callus cultures of different wild species of genus *Vigna*.

Fig. 4 The effect of NaCl on the index of tolerance of callus cultures of different cultivars of *Vigna radiata*.

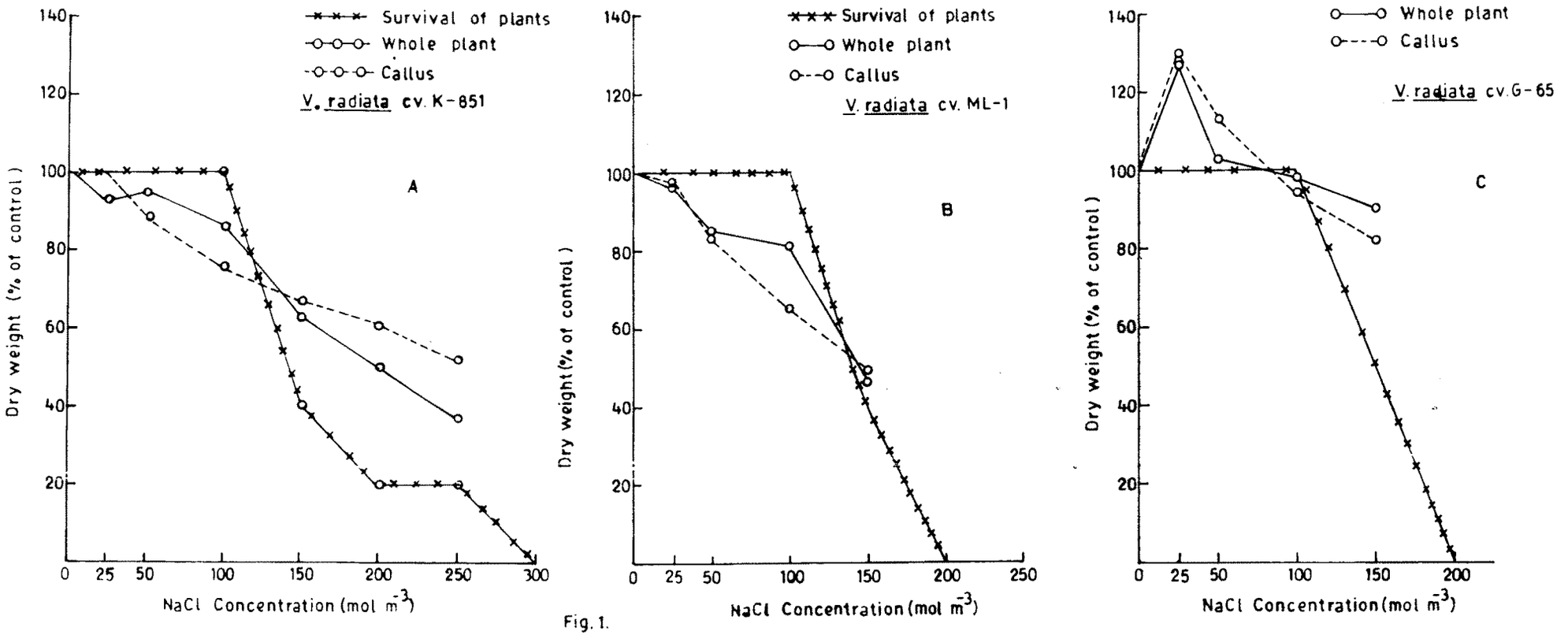
Survival of plants
Whole plant
Callus
V. radiata cv. K-851
A
Dry weight (% of control)
NaCl Concentration (mol m^{-3})
Survival of plants
Whole plant
Callus
V. radiata cv. ML-1
B
Dry weight (% of control)
NaCl Concentration (mol m^{-3})
Whole plant
Callus
V. radiata cv. G-65
C
Dry weight (% of control)
NaCl Concentration (mol m^{-3})

Fig. 1.

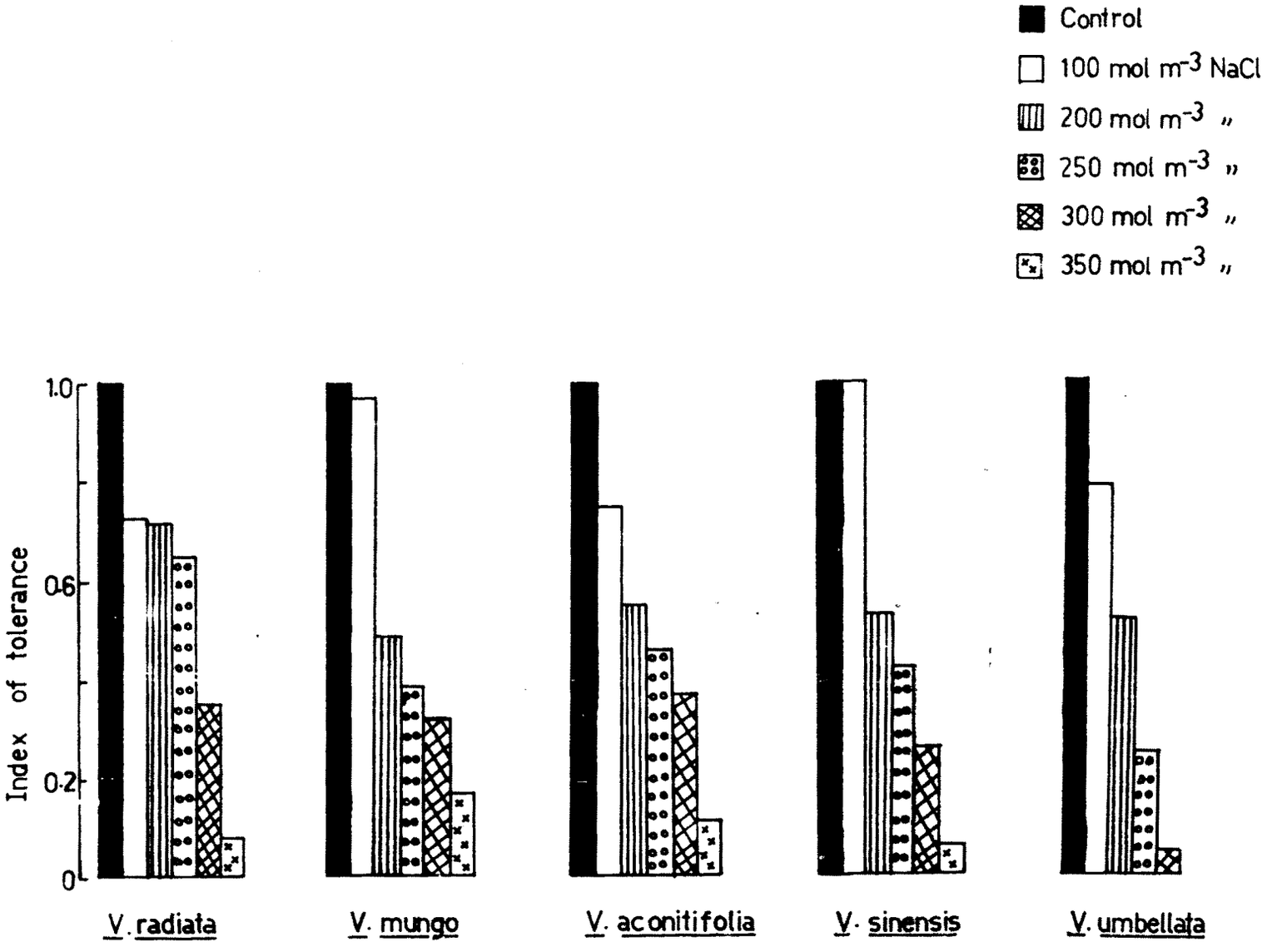
Control
100 mol m-3 NaCl
200 mol m-3 "
250 mol m-3 "
300 mol m-3 "
350 mol m-3 "
Index of tolerance
1.0
0.6
0.2
0
V. radiata
V. mungo
V. aconitifolia
V. sinensis
V. umbellata

Fig.2.

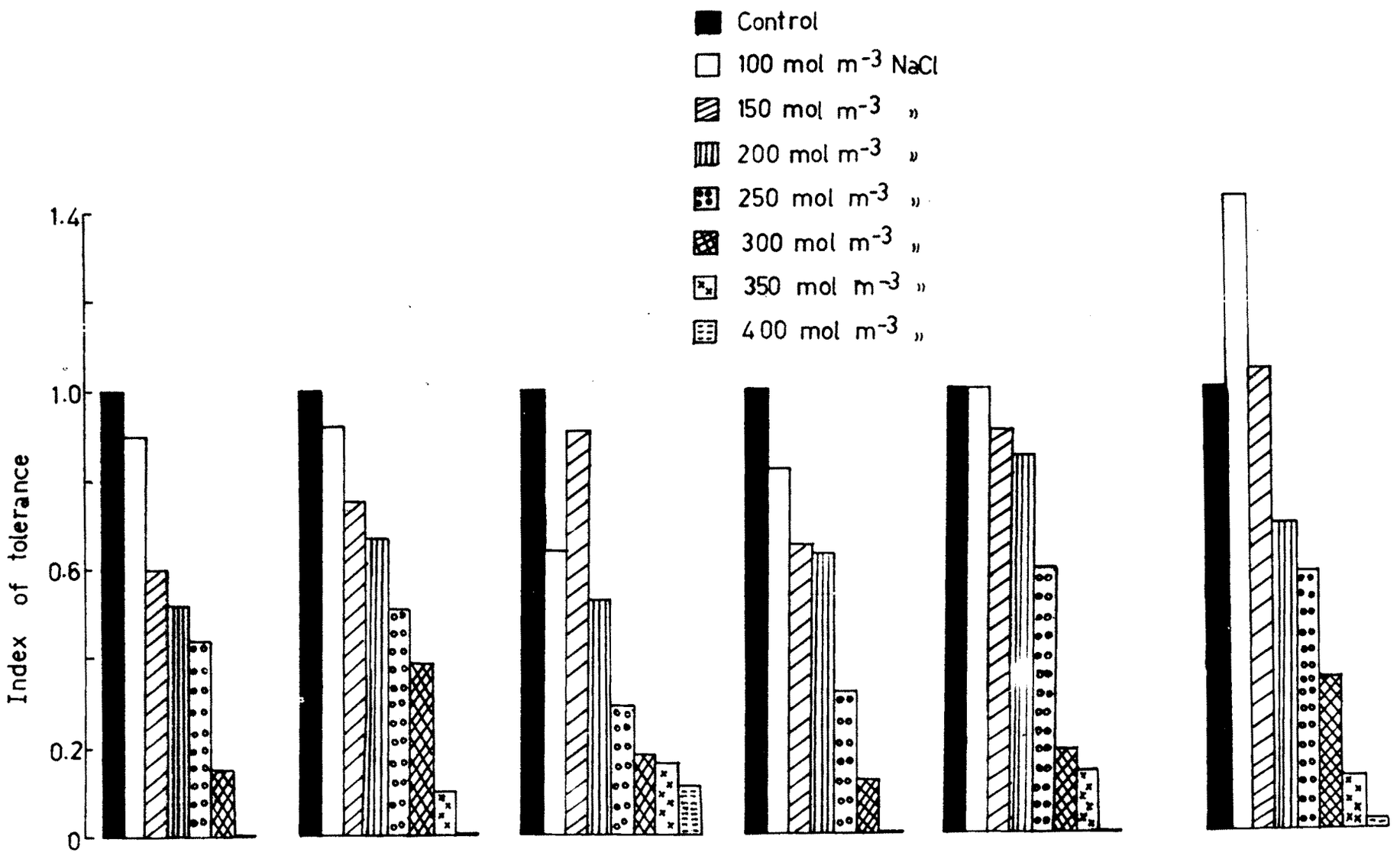
Control
100 mol m-3 NaCl
150 mol m-3 »
200 mol m-3 »
250 mol m-3 »
300 mol m-3 »
350 mol m-3 »
400 mol m-3 »
Index of tolerance
1.4
1.0
0.6
0.2
0
V. trilobata
V. sublobata Plx 270
V. sublobata Plx 478
V. sylvestris
V. setulosa
V. vexillata

Fig. 3.

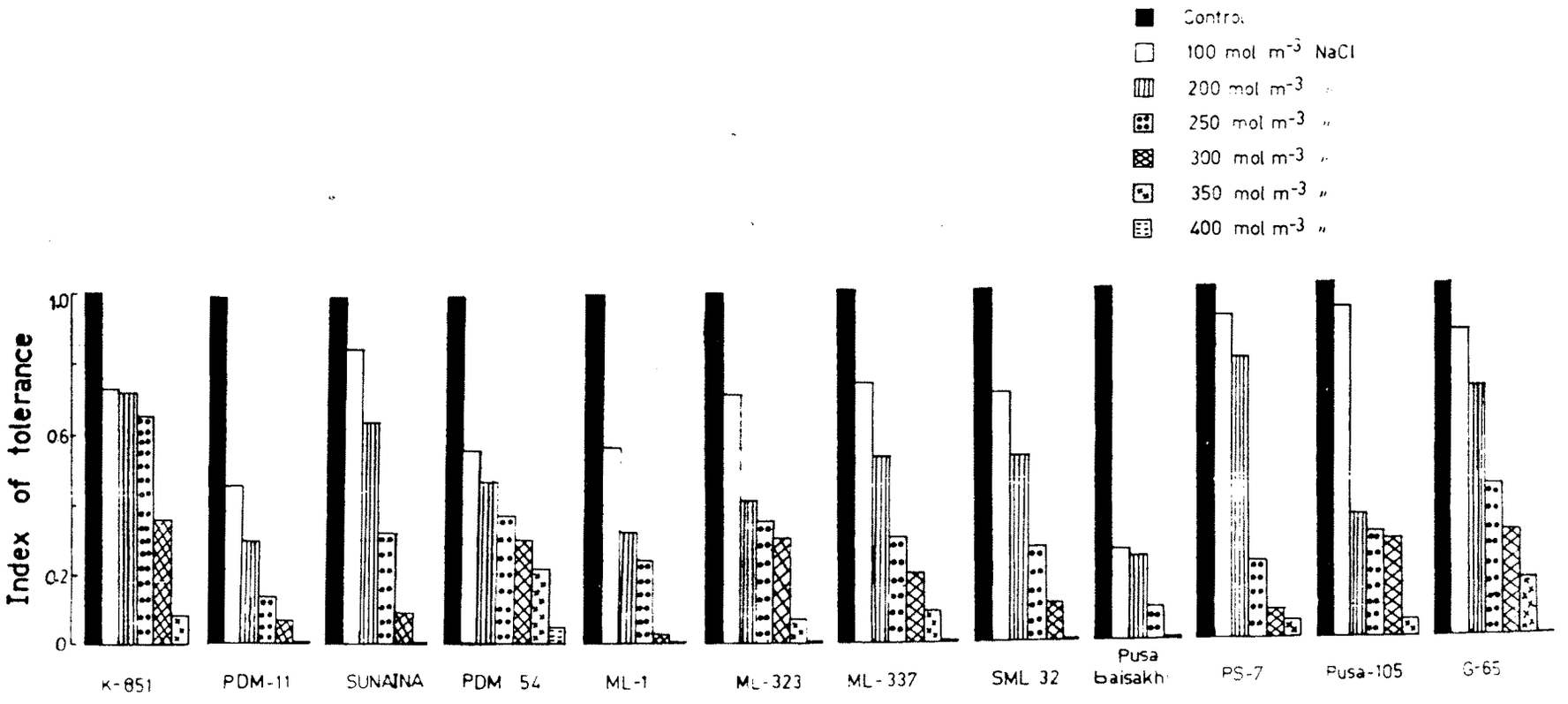
Control
100 mol m-3 NaCl
200 mol m-3 "
250 mol m-3 "
300 mol m-3 "
350 mol m-3 "
400 mol m-3 "
Index of tolerance
1.0
0.6
0.2
0
K-851
PDM-11
SUNAINA
PDM 54
ML-1
ML-323
ML-337
SML 32
Pusa Baisakhi
PS-7
Pusa-105
G-65

Fig -