CHAPTER 8

COMPARATIVE SALT RESPONSES OF CALLUS CULTURES

8.1 INTRODUCTION

The decline in plant productivity due to salinity and drought in saline and arid lands necessitates the development of stress tolerant crops. Of various approaches, cell culture selection for mutants has received increasing attention in recent years (Maliga, 1984). Salt-tolerant cell lines have been isolated in a number of plant species (Rains *et al.,* 1986; Chandler and Thrope, 1986) and tolerant plants have also been regenerated (Nabors *et al.,* 1980, 1982; Tyagi *et al.,* 1981; Mc Hughen and Swartz, 1984; Nabors and Dykes, 1985). Moreover, cell cultures that lack the differentiation and structural integrity of higher plants can also be an ideal system to assess the physiological effects of salt and/or water stress at the cellular level (Lerner, 1985). In most of the salinity resistance studies in tissue culture, NaCl as a single salt has been used to induce salt stress (Sabbah and Tal, 1990). NaCl selection is likely to produce genotypes with resistance to Na^+ or Cl^- ions, but not to other ions contributing to salinity in certain agricultural situations (Rains *et al.,* 1986). The results obtained with single different types of salt might differ significantly from those obtained when tissues are grown on salt mixtures to which plants may be exposed in nature. Therefore, in the present study, the response of callus cultures of *Vigna radiata* - a salt sensitive grain-legume to NaCl, Na_2SO_4, KCl or their mixture and to mannitol (osmotic stress) with respect to growth and ion accumulation has been studied to acquire knowledge of the role and contribution of ions and osmotic stress to salinity.

8.2 MATERIALS AND METHODS

The callus cultures of *Vigna radiata* (L.) Wilczek cv. K-851 were initiated from leaf explants of aseptically grown 7-day-old seedlings on modified PC-L2 (Phillips and Collins, 1979) medium containing 3% sucrose, 0.7% agar, 0.5 mg l^{-1} 2, 4-D, 0.5 mg l^{-1} NAA and 1 mg l^{-1} BAP. The callus cultures were grown under a 16-h photoperiod of cool-white fluorescent light at 25 $\pm$ 2°C. After one subculture on the same medium, 250 $\pm$ 10 mg actively growing callus was divided into ten pieces (25 $\pm$ 2 mg) and cultured on modified PC-L2 medium containing increasing concentrations of NaCl (0, 100, 200, 300 and 400 mol m^{-3}) in petridishes (100 mm x 17 mm) using five replicates. The petridishes were sealed with parafilm and incubated under the same photoperiod and temperature as for callus cultures. After 4 weeks of culture, callus growth was studied in terms of fresh and dry weights. The callus from petridishes was removed and its fresh and dry weights (oven dried at 80° C for 48 h) were determined for each treatment. The effects of Na_2SO_4 and KCl (equimolar to 100, 200, 300, and 400 mol m^{-3} NaCl) and mannitol (at concentrations iso-osmotic to 100, 200, 300 and 400 mol m^{-3} NaCl) were also investigated in a similar way. Three salts (NaCl, Na_2SO_4 and KCl) were used in the ratio of 8: 1: 1 to prepare a salt mixture equimolar to 100, 200, 300 and 400 mol m^{-3} NaCl. This salt mixture was added to modified PC-L2 medium to study its effect on callus growth.

Estimation of Na^+ and K^+ ions

One hundred mg of dried well ground callus tissues was transferred to a 50 cm^3 digestion flask to which 1 ml 60% PCA, 5 ml conc. HNO_3 and 0.5 ml 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 an Elico flame photometer.

Estimation of Cl^- and SO_4^{-2} ions

Chloride was extracted with concentrated HNO_3 and estimated by the mercuric nitrate titration method using diphenyl-carbazone and bromophenol blue mixed indicator (Krishnamurthy and Bhagwat, 1990).

Sulphate was also extracted with concentrated HNO_3 and determined by turbidimetry (Tabatabai and Bremner, 1970) using blanks and standards prepared in concentrated HNO_3. All the experiments were repeated at least twice.

8.3 RESULTS

1. Effect of saline stress on growth

The dry weights (percent of control) of *Vigna radiata* callus cultures on modified PC-L2 medium containing increasing concentrations of NaCl, Na_2SO_4, KCl and their mixture are shown in Fig.1. The dry weight of callus decreased with increasing concentration of any of the salinizing salts of their mixture in the culture medium. A 100 mol m^{-3} salt resulted in a 62 to 94% decrease in dry weight, depending on the salt, and at 300 mol m^{-3} dry weights were only 22 to 10% of those of non-stressed callus (Fig.1). Na_2SO_4 at 50 mol m^{-3} stimulated callus growth in comparison to non-stressed callus, while both KCl and NaCl at this concentration had decreased it by 15% (Fig.1). Callus dry weight decreased sharply with further increase in concentration of any of the salts in the medium above 50 mol m^{-3}. Na_2SO_4 at 200 mol m^{-3} or higher concentrations was more inhibitory than any of the salts and their mixture. KCl was less inhibitory for callus growth at 100 mol m^{-3} than NaCl and salt mixture, while the reverse was true at higher concentrations (250-400 mol m^{-3}). Callus growth was almost completely inhibited by Na_2SO_4 and KCl at 250 mol m^{-3}, and by NaCl at 350 mol m^{-3}. Thus, the inhibitory effect of the three individual salts on callus growth was not equal but increased according to the following order: Na_2SO_4 < KCl < NaCl. Salt mixtures at 50 and 100 mol m^{-3} were more inhibitory than their constituent salts, while at higher concentrations (200-300 mol m^{-3}), they were more inhibitory than NaCl, and less inhibitory compared with Na_2SO_4 and KCl. Salt mixture at 350 mol m^{-3} had almost completely inhibited the growth of callus.

Na^+, K^+

Sodium levels in callus tissue increased with increasing concentrations of NaCl, Na_2SO_4 and salt mixture in the culture medium. Na^+ accumulation was dependent upon the accompanying ions: in Na_2SO_4 grown callus Na^+ was twice as great as in callus grown in NaCl and salt mixture (Fig.2). However, Na^+ accumulated in more or less similar amounts in callus grown on NaCl and salt mixture. In contrast, levels of K^+ in the callus declined continuously with increasing NaCl, Na_2SO_4 and salt mixture (Fig.2). On the contrary, K^+ ions increased continuously with increase in KCl levels in the culture medium. K^+ ion accumulation from KCl was greater than that of Na^+ from NaCl and salt mixture.

The Cl^- levels of callus tissue increased with increasing concentrations of NaCl, KCl and salt mixture in the medium. Cl^- accumulation was greater relative to $Na^+ + K^+$ in callus grown in salt mixture than in NaCl or KCl grown ones (Table 1).

Sulphate in callus tissue increased with increasing concentration of Na_2SO_4 in the culture medium. The concentration of SO_4^{-2} accumulation in Na_2SO_4 grown callus was 10-12 fold greater than the concentration of Cl^- in callus grown on approximately equimolar amounts of NaCl. Na_2SO_4 grown callus contained 60 to 70 times more sulphate than Na^+. The amount of SO_4^{-2} in callus grown on salinity with mixed cation salts remained almost similar to increased salinity as if the media had contained only NaCl.

II. Osmotic stress

Mannitol at 180 and 360 mol m^{-3} stimulated both the fresh and dry weights of the callus over those of the control. These parameters decreased with further increase in the concentration of mannitol (Fig.3). Callus incubated at higher concentrations of mannitol remained green and was less necrotic than callus on iso-osmotic concentrations of NaCl and salt mixture.

The reduction in dry weight was also less with mannitol than with all of the iso-osmotic concentrations of the saline media (Fig.3).

Mannitol grown callus accumulated less monovalent ions than any of the salt treatments. Accumulation of Na^+ and Cl^- remained uneffected but the K^+ content of the callus decreased with increase in the concentration of mannitol. However, K^+ content was greater in tissue grown on mannitol than NaCl, Na_2SO_4 and salt mixture containing media (Fig.2).

8.4 DISCUSSION

Growth inhibition by saline stress is commonly accepted to be due to a lowering of the water potential of growth media caused non-specifically by dissolved excess ions (Flowers *et al.*, 1977; Greenway and Munns, 1980). Most studies concerning the effects of salinity on callus growth

have dealt with the effect of one salt, NaCl. Studies restricted to NaCl stress can not describe the relationship of inorganic solute accumulation to growth. Callus did not respond equally to stress nor to the compounds used for the stressing salt. Callus growth was inhibited more severely with Na_2SO_4, followed by KCl, salt mixture and NaCl. Such severe inhibition of cell growth with Na_2SO_4 compared with NaCl has also been reported in *Beta vulgaris* (Pua and Thorpe, 1986). This is due to more uptake of Na^+ by cells growing on Na_2SO_4 medium than NaCl and salt mixture containing media. Moreover, Na_2SO_4 grown cells accumulated 60-70 times more SO_4^{-2} than Na^+. Thus, results show that the effect of Na^+ is highly dependent on the nature of the accompanying anion, for reasons yet not clearly understood. Stimulation of callus growth at low levels (50 mol m^{-3}) of Na_2SO_4 may be due to sub-optimal osmotic strength of the medium.

Callus growth at low concentration of KCl was higher compared with NaCl and salt mixture. Subsequently, growth at higher concentrations of KCl decreased and became less rapid than on NaCl and salt mixture. One difference between callus grown on KCl and NaCl was greater K^+ and Cl^- contents of the KCl callus than Na^+ and Cl^- accumulation in NaCl grown callus. It is suggested that the results can be explained by the different degree of solute accumulation, which at lower levels can be beneficial to callus, allowing osmotic adjustment to occur, and at higher levels can become toxic. The difference in ionic uptake from KCl and NaCl media implies greater permeability of the cells in KCl medium.

A comparison between salt mixture and NaCl systems shows that at the same salinity level the Cl^- accumulation in callus grown on salt mixture was higher than on NaCl, resulting in a greater reduction in growth. SO_4^{-2} ions of salt mixture seem to be inhibitory to K^+ uptake as reported earlier by Rains (1972).

Plant cells show variable response in their performance under salt and water stresses. Several halophytic plants responded similarly when subjected to sea water or PEG, indicating that most of the results can be ascribed to low osmotic potential and not salinity *per se* (Jefferies *et al.,* 1979). In the present study, callus growth was less severely inhibited by mannitol than by any of the salt treatments, suggesting that in the latter cases it was affected by ion toxicity. Similar results were also obtained by Tal and Katz (1980). They reported that tomato cell cultures showed no correlation between salt stress and PEG induced water stress. The results that we obtained using mannitol were similar to those obtained by other workers with PEG (Handa *et al.,* 1982a; Sabbah and Tal, 1990).

The dry weight of cells growing in mannitol was much higher than in cells growing in the presence of iso-osmotic concentrations of any of the salts and their mixture. Similar results were also reported by Heyser and Nabors (1981b), Binzel *et al.* (1985) and Ben-Hayyim

(1987). The capability of the cells for good growth on mannitol rather than on any salt/salt mixture may be due to osmotic adjustment of the tissue by accumulation of more K^+ ions under mannitol induced osmotic stress than at iso-osmotic concentrations of any of the salts and their mixture.

The present study shows that the response of callus varies with stress and salt types. SO_4^{-2} and K^+ are highly toxic to *Vigna radiata* leaf callus while Na^+ and Cl^- are less toxic. The results also suggest that single salts (Na_2SO_4 and KCl) are more toxic to the tissue than a salt mixture.

Table 1: Effect of different salts and their mixture on Cl^- content (mmol kg^{-1} dry weight) of Vigna radiata callus.

Concentration of salt (mol m^{-3})	Salt			
	Na_2SO_4	KCl	NaCl	Salt mixture
0	225 ± 14.1	225 ± 14.1	225 ± 14.1	225 ± 14.1
50	479 ± 28.2	564 ± 27.8	564 ± 29.0	592 ± 28.2
100	535 ± 28.2	564 ± 28.6	592 ± 84.6	620 ± 29.6
200	564 ± 40.0	535 ± 84.6	592 ± 84.6	648 ± 28.2
400	620 ± 16.0	733 ± 21.0	620 ± 20.0	789 ± 56.4

Values are the mean of five replicates ± standard error.

EXPLANATION OF FIGURES

Fig. 1 Effect of NaCl, Na_2SO_4, KCl and their mixture on dry weight (% of control) of callus cultures of *Vigna radiata* cv.K-851. DW in the absence of NaCl, Na_2SO_4, KCl and their mixture were 394.6 $\pm$ 15.0 mg, 260.0 $\pm$ 16 mg, 260 $\pm$ 16 mg and 516.3 $\pm$ 53.7 mg, respectively.

Fig. 2 Effect of NaCl, Na_2SO_4, KCl and their mixture and mannitol on Na^+ and K^+ contents of *Vigna radiata* callus cultures. A, 0; B, 50; C, 100; D, 200; E, 250; F, 300; G, 350; H, 400; I, 450 mol m^{-3} salt and a, 0; b, 180; c, 360; d, 450; e, 540; f, 630; g, 720 mol m^{-3} mannitol. Vertical lines are the minimum and maximum standard error observed with the data in this figure.

Fig. 3 Effect of NaCl, salt mixture and mannitol on fresh and dry weights (% of control) on callus cultures of *Vigna radiata*.

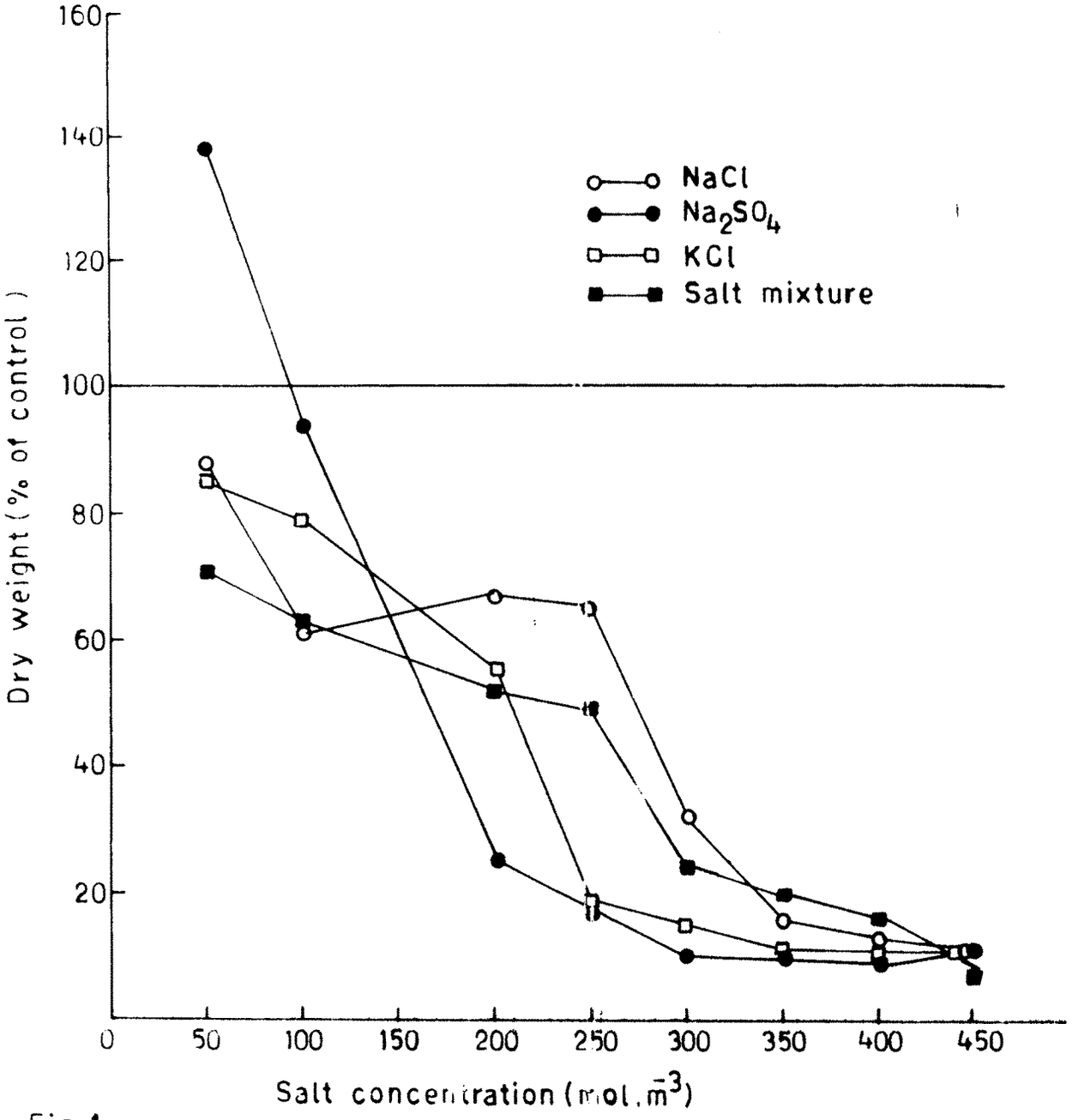
160
140
120
100
80
60
40
20
0
50
100
150
200
250
300
350
400
450
Dry weight (% of control)
Salt concentration (mol.m⁻³)
NaCl
Na2SO4
KCl
Salt mixture

Fig. 1.

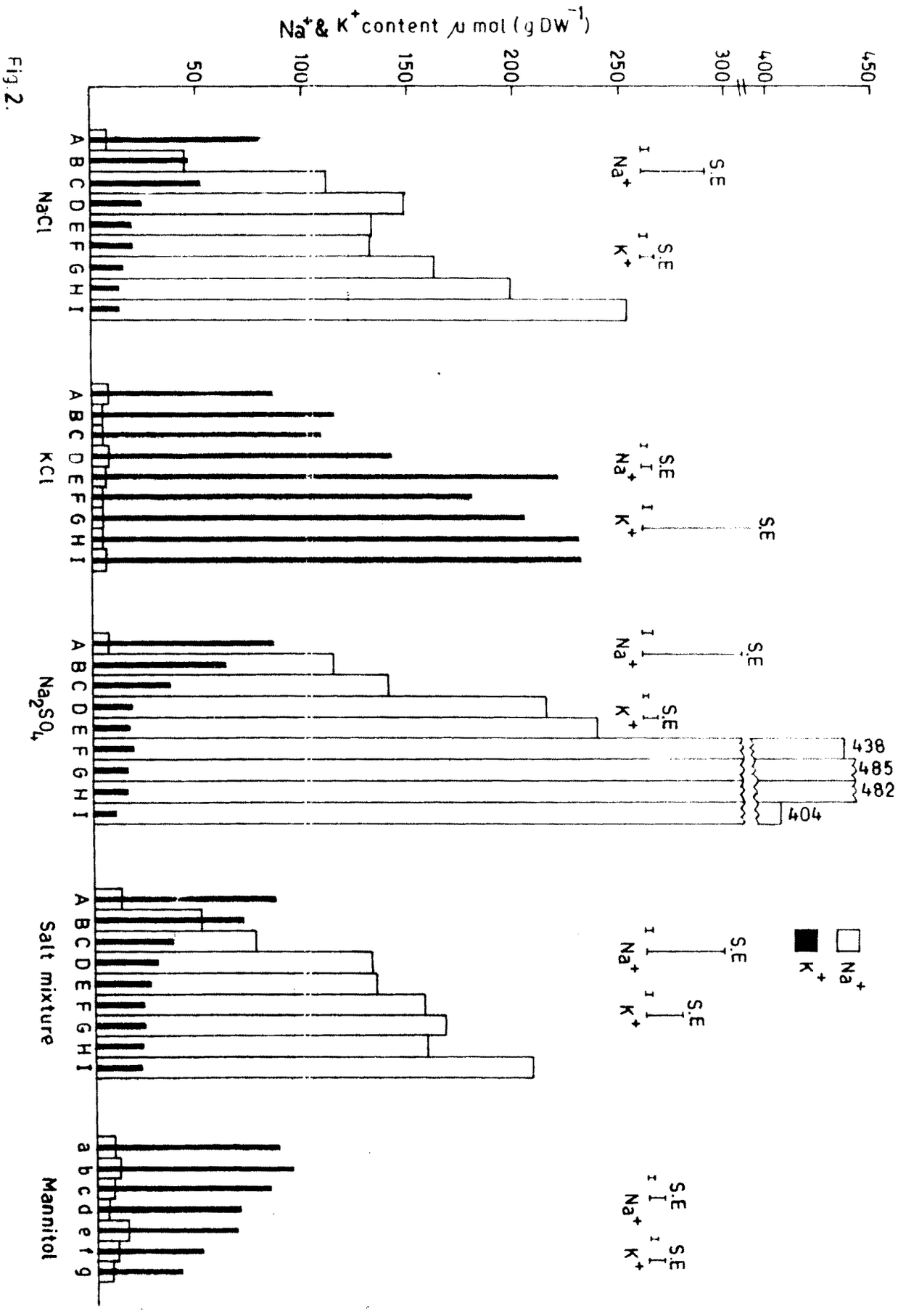
Na+ & K+ content μ mol (g DW-1)
50
100
150
200
250
300
400
450
NaCl
KCl
Na2SO4
Salt mixture
Mannitol
A B C D E F G H I
a b c d e f g
S.E
Na+
K+
438
485
482
404

Fig. 2.

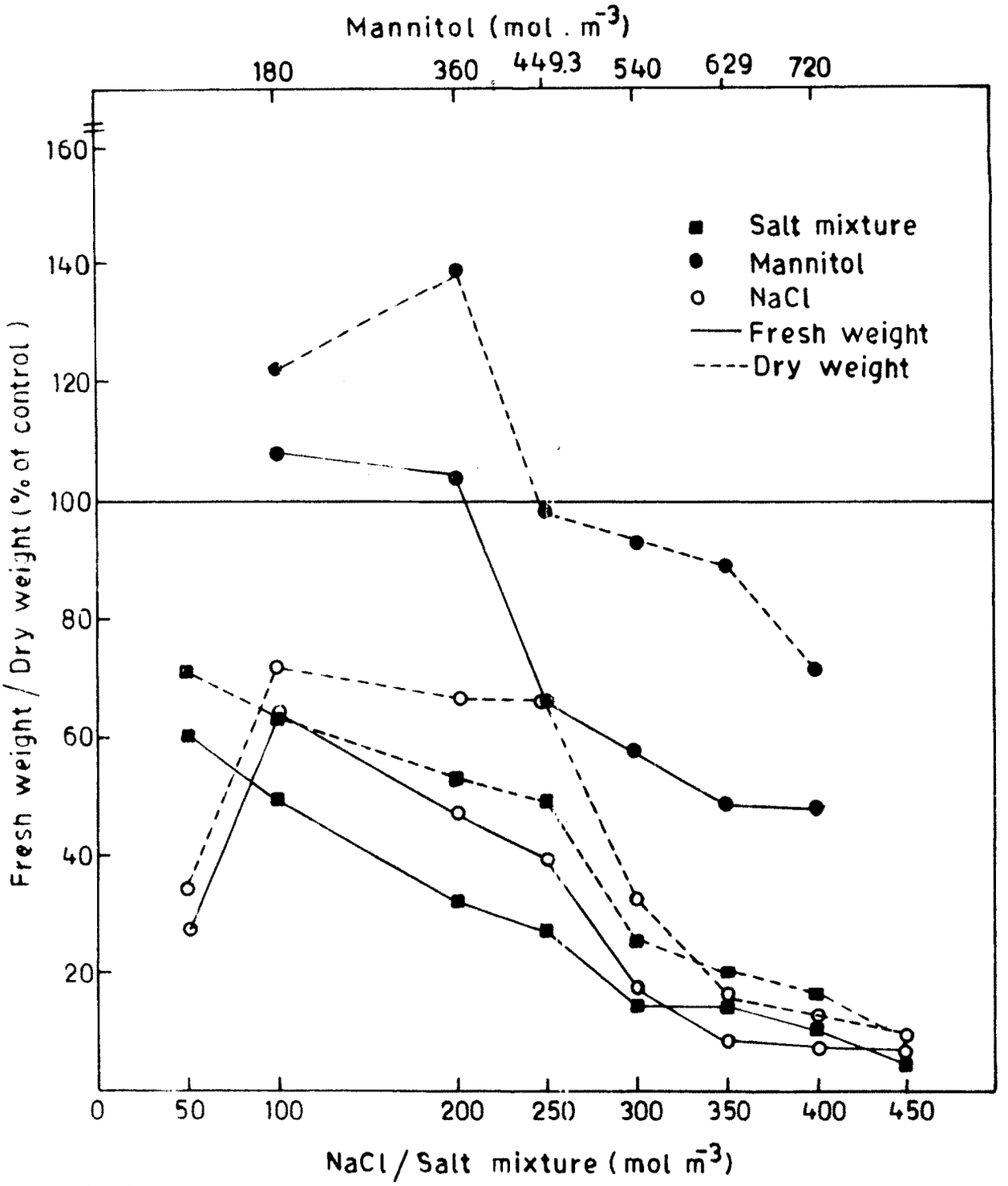
Mannitol (mol . m^{-3})
180
360
449.3
540
629
720
160
140
120
100
80
60
40
20
0
50
100
200
250
300
350
400
450
Fresh weight / Dry weight (% of control)
NaCl / Salt mixture (mol m^{-3})
Salt mixture
Mannitol
NaCl
Fresh weight
Dry weight

Fig. 3.