CHAPTER 11

SCREENING FOR SALT MIXTURE RESISTANCE

11.1 INTRODUCTION

Cell lines with enhanced resistance to salt have been isolated from many plant species (see review by 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 may not to other toxic ions contributing to salinity in certain agricultural situations (Rains *et al.*, 1986). The results obtained with single different types of salt might differ from those obtained when tissues are grown on salt mixture 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.

11.2 MATERIALS AND METHODS

The 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% sucrose, 0.7% agar, 0.5 mg l^{-1} 2,4-D, 0.5 mg l^{-1} NAA and 1.0 mg l^{-1} BAP. The callus cultures were grown under 16-h photoperiod of cool-white fluorescent light of 80 μ mol m^{-2} s^{-1} at 25 $\pm$ 2°C. The callus subcultures were maintained under same conditions of light and temperature.

Effect of different concentrations of salt mixture

After one subculture on the same medium, 250 $\pm$ 10 mg actively growing callus was divided into ten pieces (each 25 $\pm$ 2 mg) and cultured on 20 ml of modified PC-L2 medium containing increasing concentration of salt mixture (0-450 mol m^{-3}) in petridishes (100 mm x 17 mm). 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. For each treatment twenty replicate dishes were used. The petridishes were sealed with parafilm and incubated under same photoperiod and temperature as for callus cultures. Subsequently, at 7-d intervals, the callus from 5 petridishes of each treatment was removed and their fresh and dry weights (oven dried at 80°C for 48-h) were determined. This procedure was repeated up to 28-d after inoculation and the concentration of salt mixture inhibitory to growth was determined.

Selection of salt mixture (SM) resistant callus line

The selection of spontaneous mutant callus line resistant to inhibitory concentration of salt mixture i.e. (equimolar to 300 mol m^{-3}) was made by exposing three hundred callus pieces (each 25 $\pm$ 2 mg fresh weight) 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 in colour. 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 fourth subculture, the surviving calli grew well and did not show discolouration at 300 mol m^{-3} salt mixture. These variant calli have been designated salt mixture-resistant (SM^r) to distinguish them from salt mixture sensitive (SM^s) calli (Plate 10).

Stability of salt resistance of the selected line

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

Growth characteristics of the selected line

The growth (fresh and dry weights) of SM^s and SM^r calli grown at 300 mol m^{-3} SM was measured at the intervals of 7-d over a period of one month.

To compare tolerance of SM^s and SM^r callus lines, callus pieces of 25 $\pm$ 2 mg were inoculated on media containing different concentrations (0-350 mol m^{-3}) of salt mixtures. Fresh and dry weights of the two lines determined after 4 weeks of culture.

Cross resistance of the selected line

Cross resistance of SM^s and SM^r callus lines to different constituent salts of salt mixture was tested by inoculating callus pieces of 25 $\pm$ 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 weights of both the lines were determined after 4 weeks of culture.

Estimation of Na^+ and K^+ ions

A known amount of oven dried callus samples were digested with nitric acid as described in section 8.2. Na^+ and K^+ in the final acid digest extract was determined using Elico flame photometer.

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

Cl^- and SO_4^{-2} were extracted with concentrated HNO_3 and estimated as described in section 8.2

All the experiments were repeated at least twice.

11.3 RESULTS AND DISCUSSION

Effect of salt mixture on callus growth

The callus growth in terms of fresh and dry weights was determined on medium containing 0-450 mol m^{-3} salt mixture at weekly intervals over a period of 28 day. (Figs. 1,2). The fresh weight of callus decreased with increase in the concentrations of salt mixture in the medium.

However, the reduction in growth was less during first week of culture. The maximum growth of callus tissue was observed during third week of culture as evidenced by an increase in fresh weight for concentrations up to 200 mol m^{-3} of salt mixture. From third week onwards, there was little change in fresh weight. Salt mixture at 25 mol m^{-3} caused stimulation in callus fresh weight over the control upto second week of culture. Similar results of simulatory effects of salt at low concentrations have also been reported from cultured cell of other systems (Gale and Boll, 1979; Gosal and Bajaj, 1984; Pandey and Ganapathy, 1984). These observations suggest that the osmotic strength of the medium was sub-optimal and that the presence of low levels of salt mixture in the medium stimulated cell growth. However, beyond 150 mol m^{-3} of salt mixture in the medium caused browning and necrosis of callus and hence, reduced callus growth which was inhibitory at 300 mol m^{-3} of salt mixture. Such inhibition of callus growth at higher levels of salt is in accordance with that of other systems (Chen *et al.*, 1980; Pandey and Ganapathy, 1984; McCoy, 1987 b). Similar trends as in fresh weight were also observed in dry weight. However, decrease in dry weight was less than fresh weight almost at all the levels of salinity.

Selection of salt mixture resistant callus line

Three hundred callus pieces (each 25 $\pm$ 2 mg fresh weight) were exposed to a concentration of 300 mol m^{-3} salt mixture which was inhibitory to the growth of callus. The four clones which remained green after 28 days of growth were picked up and grown on modified PC-L2 medium with no salt for 2 transfers over a period of 2 months. These clones were again grown on medium with 300 mol m^{-3} of SM where they grew similar to SM^r clones growing on SM. This showed the persistence of tolerance trait in the absence of SM. Similar results were also observed in other systems (Coughan *et al.*, 1978; Kochba *et al.*, 1982; Ranjan and Vasil, 1983; Salgado-Garciglia *et al.*, 1985). At present, we have no evidence whether SM^r cell lines are real mutant or only epigenetic variants. The ultimate proof of true genetic variant lies in the regeneration of salt mixture tolerant plants and then testing the inheritance of tolerance at whole plant level. Unfortunately, our attempts to regeneration plants from the selected cells failed.

Growth characteristics of SM^r and SM^s callus lines

Growth of SM^r and SM^s with or without 300 mol m^{-3} salt mixture

The growth of SM^r and SM^s callus lines was compared on normal and salt mixture (300 mol m^{-3}) containing medium for a period of 28 days (Figs. 3,4). SM^r calli in the absence or presence of salt showed a rapid increase in fresh weight which continued upto 28 days. The SM^s did not show any increase in growth in presence of salts (Plate 10). The SM^r calli had higher fresh weight than SM^s in the absence of salt. However, their fresh weight in the presence of stress was lower than that of SM^s growing on normal medium. Similar trends as in fresh weight were also reflected in dry weight. However, the dry weight of SM^s on normal medium was comparable to that of SM^r in the absence of stress upto 14 days. Thereafter, the dry weight of latter exceeded that of the former. In the present study, calli selected for salt mixture tolerance grew at a lower rate in saline medium than non-selected calli on normal medium. Similar growth pattern of salt stress - selected cell lines have been reported in many species (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).

Growth of SM^r and SM^s calli on different SM concentrations

Fig.5 shows the effect of different concentration (0-300 mol m^{-3}) of salt mixture on relative growth of SM^r and SM^s callus lines. The SM^r callus showed significantly higher growth at all the salinity levels compared to SM^s line. SM^r cultured attained half maximal fresh weight at 265 mol m^{-3} SM compared to 97.5 mol m^{-3} for sensitive cultures. Thus, the former cell line showed a shift towards the 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 batata* (Garciglia *et al.*, 1985) and *Lycopersicon peruvianum* (Hassan and Wilkins, 1988).

Cross tolerance of SM^r cells

Effect of Na_2SO_4

The increase in fresh and dry weights of SM^r and SM^s callus lines as a function of different Na_2SO_4 concentrations (0-250 mol m^{-3}) is shown in Figs. 6 A,B). The growth of SM^s line was significantly higher than SM^r 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} and high concentrations of Na_2SO_4. Similar results were also obtained in *Citrus sinensis* by using NaCl-resistant cell lines (Kochba *et al.*, 1982).

Effect of KCl

The relative growth of SM^r was significantly higher than SM^s upto 250 mol m^{-3} of KCl. But the both the lines responded similarly at higher KCl concentrations, i.e. 300-350 mol m^{-3} (Figs. 7A,B).

Effect of NaCl

The relative growth of SM^r on NaCl containing medium was poor than SM^s at all the concentrations of NaCl (Fig.8).

Ion analysis

Kinetics of Ion accumulation of SM^S cell line

Na^+, K^+, Cl^- and SO_4^2 contents of SM^s callus grown on salt mixture were estimated at 7-d intervals over a 28-d culture period (Figs. 9,10) 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. Thus, salt has penetrated the tissue within a few days and reached to a high concentration in the cells long before the growth processes have started.

K^+ content of the callus declined continuously with increasing salt mixture levels. The reduction was more pronounced during the fourth week of culture. During first week, however, K^+ content was maintained at all the salt concentrations. This probably suggest that K^+ plays an important role in osmotic adjustment during 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 (Talcinsk *et al.*, 1983; Pandey and Ganapathy, 1985; Garcia-Reina *et al.*, 1988).

SO_4^{-2} amount in the callus remained almost similar with increased in salt mixture levels.

Comparison of Na^+ and K^+ content in SM^r and SM^S callus lines

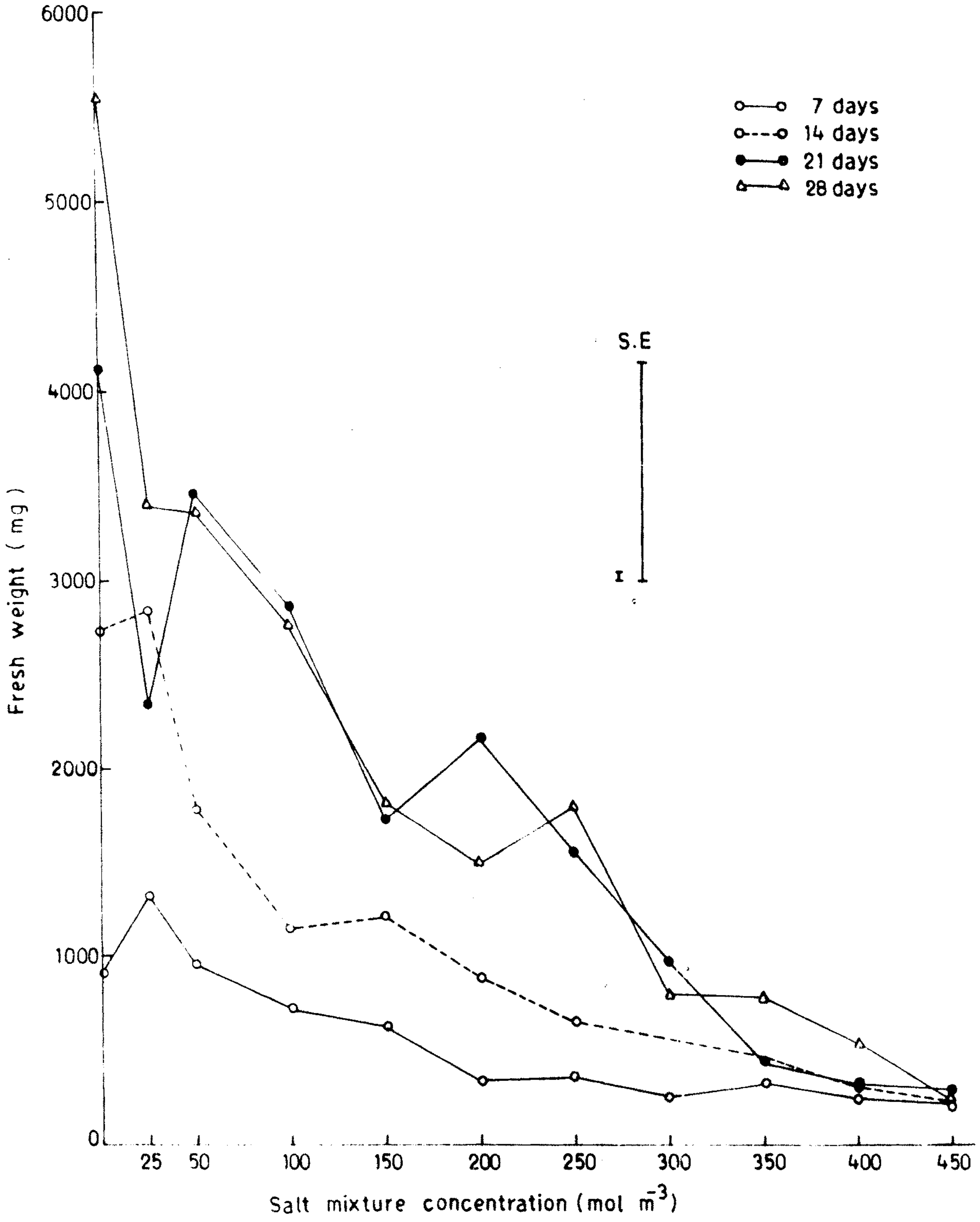
Na^+ contents in SM^r and SM^s lines are shown in Fig.11. The Na^+ content of SM^r callus was comparable to those of SM^s line, when both were grown on normal medium. Na^+ level in both the lines increased with increase in the concentration of salt in the medium. However, the sensitive callus line accumulated more Na^+ than SM^r under the same degree of stress.

K^+ content : The SM^r callus line accumulated more K^+ than SM^s line when both were grown in the absence of salt. The K^+ contents of both the lines decreased with increase in saline levels. However, this decrease was more pronounced in SM^S callus line than SM^r (Fig. 12).

Thus, SM^r has maintained low levels of Na^+ and high levels of K^+ than SM^s cell lines. Similar results with regard to K^+ and Na^+ content in the salt-resistant and sensitive callus lines have been obtained earlier in other systems (Croughan *et al.*, 1978; Watad *et al.*, 1983).

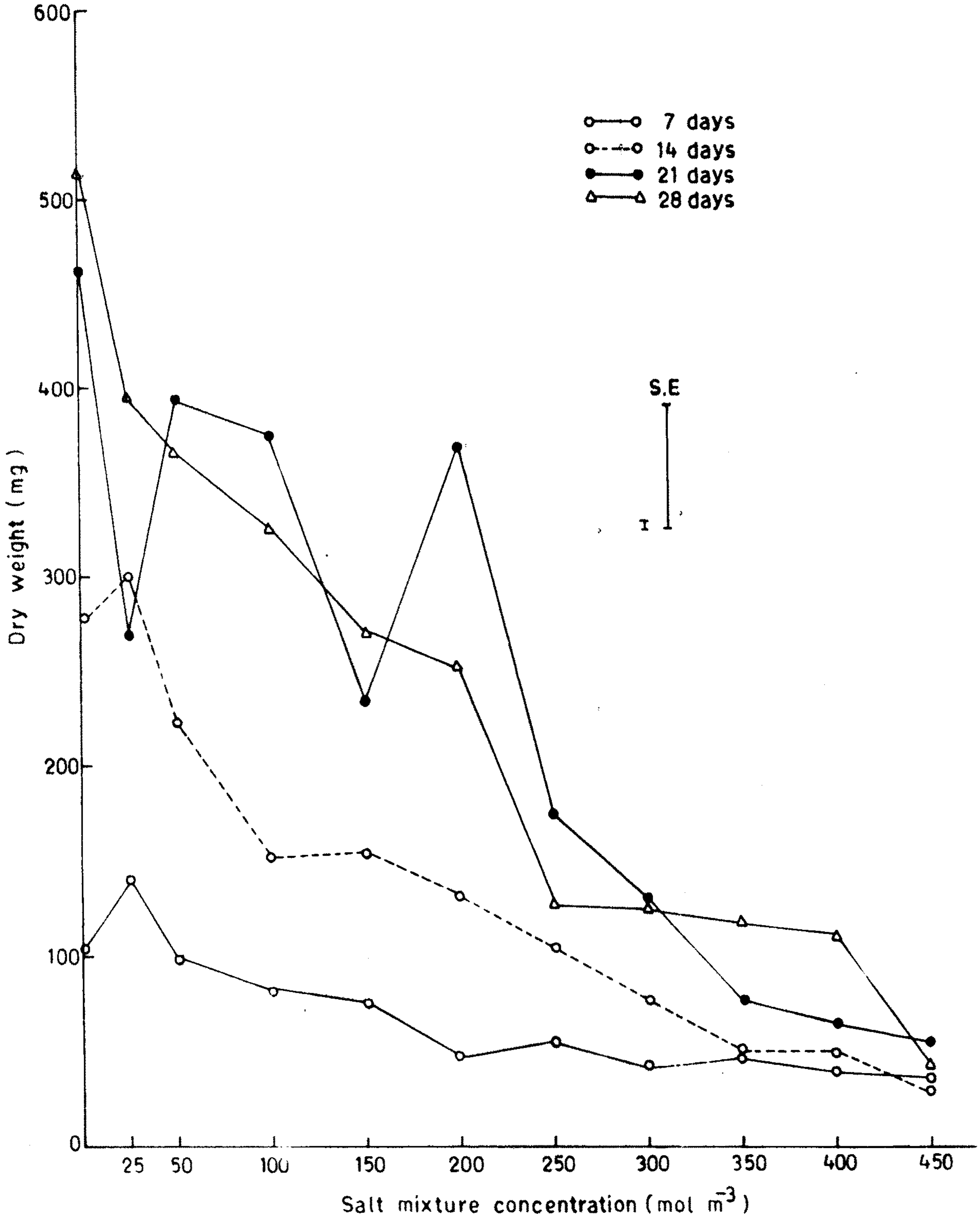
EXPLANATION OF FIGURES

Figs.1 & 2	Effect of salt mixture stress on fresh (Fig.1) and dry (Fig.2) weights of callus cultures of *Vigna radiata* cv.K-851. Vertical lines are the minimum and maximum standard error observed with the data in this figure.
Figs.3 & 4	Fresh (Fig.3) and dry (Fig.4) weights of SM^r and SM^s calli on medium with or without SM. SM^s without SM (o---o) and with 300 mol m^{-3} SM (o—o); SM^r without SM (•---•) and with 300 mol m^{-3} SM (•—•). Vertical bars represent standard error of the mean.
Fig. 5	Effect of SM on the growth of SM^r and SM^s callus of *Vigna radiata*. Vertical bars represent standard error of the mean (n = 5) of two independent experiments. FW in the absence of SM were 2541 $\pm$ 115 mg (SM^s) and 2624 $\pm$ 624 mg (SM^r). Average DW were 516 and 282 mg.
Figs.6 (A & B)	Effect of Na_2SO_4 on fresh (A) and dry (B) weights of SM^s and SM^r callus of *Vigna radiata*. Vertical bars represent standard error of the mean (n = 5) of two independent experiments. FW in the absence of Na_2SO_4 were 2502 $\pm$ 152 mg (SM^s) and 3604 $\pm$ 292 mg (SM^r). Average DW were 260 and 309 mg.
Figs.7 (A & B)	Effect of KCl on fresh (A) and dry (B) weights of SM^s and SM^r callus of *Vigna radiata*. Vertical bars represent standard error of the mean (n = 5) of two independent experiments. FW in the absence of KCl were 2502 $\pm$ 152 mg (SM^s) and 3604 $\pm$ 292 mg (SM^r). Average DW were 260 and 309 mg.
Fig. 8	Effect of NaCl on the growth of SM^r and SM^s callus of *Vigna radiata*. Vertical bars represent standard error of the mean (n = 5) of two independent experiments. FW in the absence of NaCl were 2118 $\pm$ 51 mg (SM^s) and 2624 $\pm$ 625 mg (SM^r) average DW were 254 and 283 mg.
Fig. 9	Effect of SM on Na^+ and K^+ content of *Vigna radiata* callus cultures. A,0; B,25; C,50; D,100; E,150; F,200; G,250; H,300; I,350; J,400; K, 450 mol m^{-3} SM. Vertical lines are the minimum and maximum SE observed with the data in this figure.
Fig. 10	Effect of SM on Cl^- content of *Vigna radiata* callus cultures. A,0; B,25; C,50; D,100; E,150; F,200; G,250; H,300; I,350; J,400; K, 450 mol m^{-3} SM. Vertical lines are the minimum and maximum SE observed with the data in this figure.
Fig. 11	Sodium content of *Vigna radiata* SM^s and SM^r calli as a function of salt mixture. Vertical bars represent standard error of the mean.
Fig. 12	K^+ content of *Vigna radiata* SM^s and SM^r calli as a function of salt mixture. K^+ content in the absence of SM were 68.2 $\pm$ 1.02 μ moles g^{-1} DW and 75.8 $\pm$ 1.0 μ moles g^{-1}.



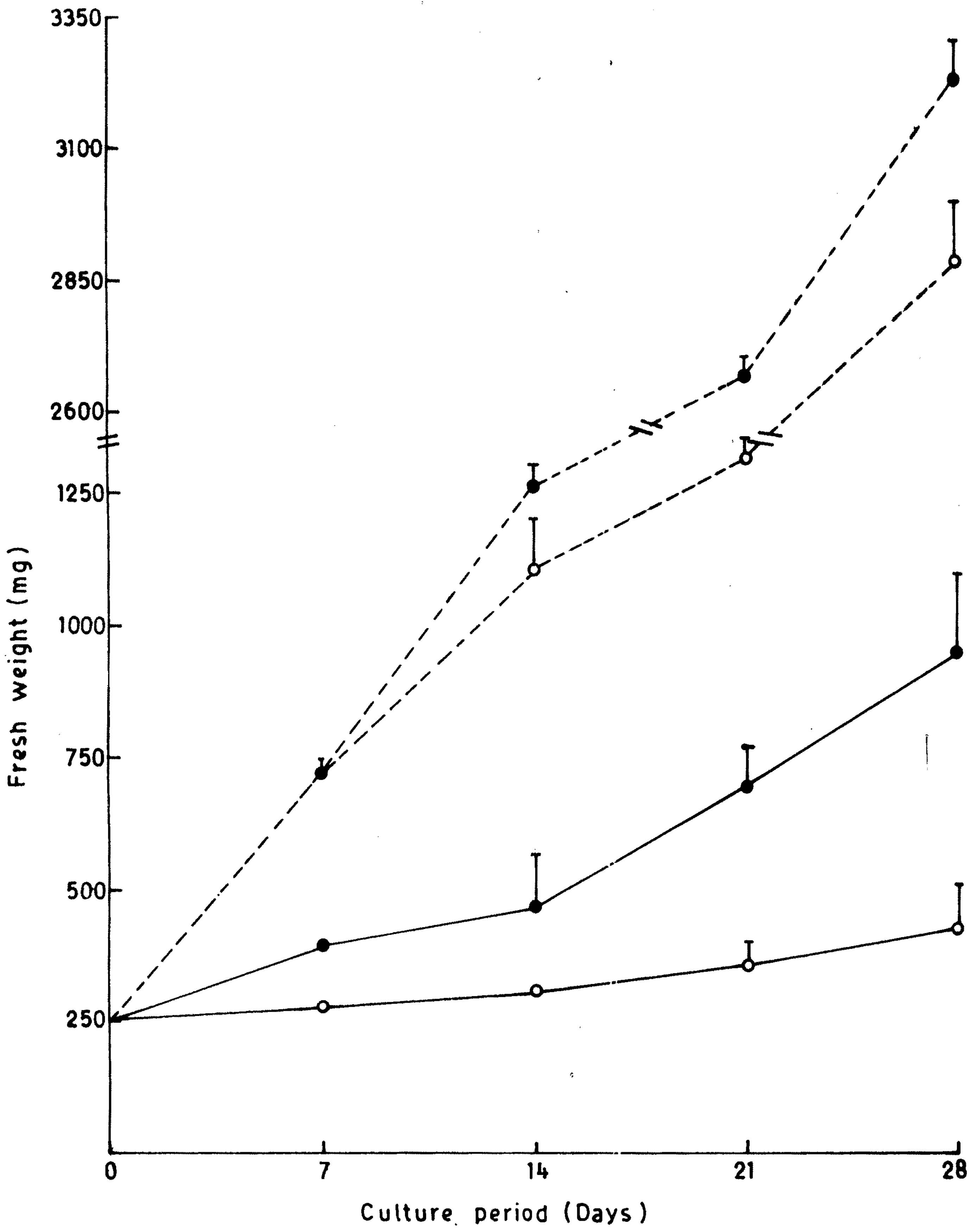
6000
5000
4000
3000
2000
1000
0
Fresh weight (mg)
25
50
100
150
200
250
300
350
400
450
Salt mixture concentration (mol m^{-3})
7 days
14 days
21 days
28 days
S.E

Fig.1.



7 days
14 days
21 days
28 days
S.E
Dry weight (mg)
600
500
400
300
200
100
0
25
50
100
150
200
250
300
350
400
450
Salt mixture concentration (mol m^{-3})

Fig. 2.



3350
3100
2850
2600
1250
1000
750
500
250
Fresh weight (mg)
0
7
14
21
28
Culture period (Days)

Fig. 3.

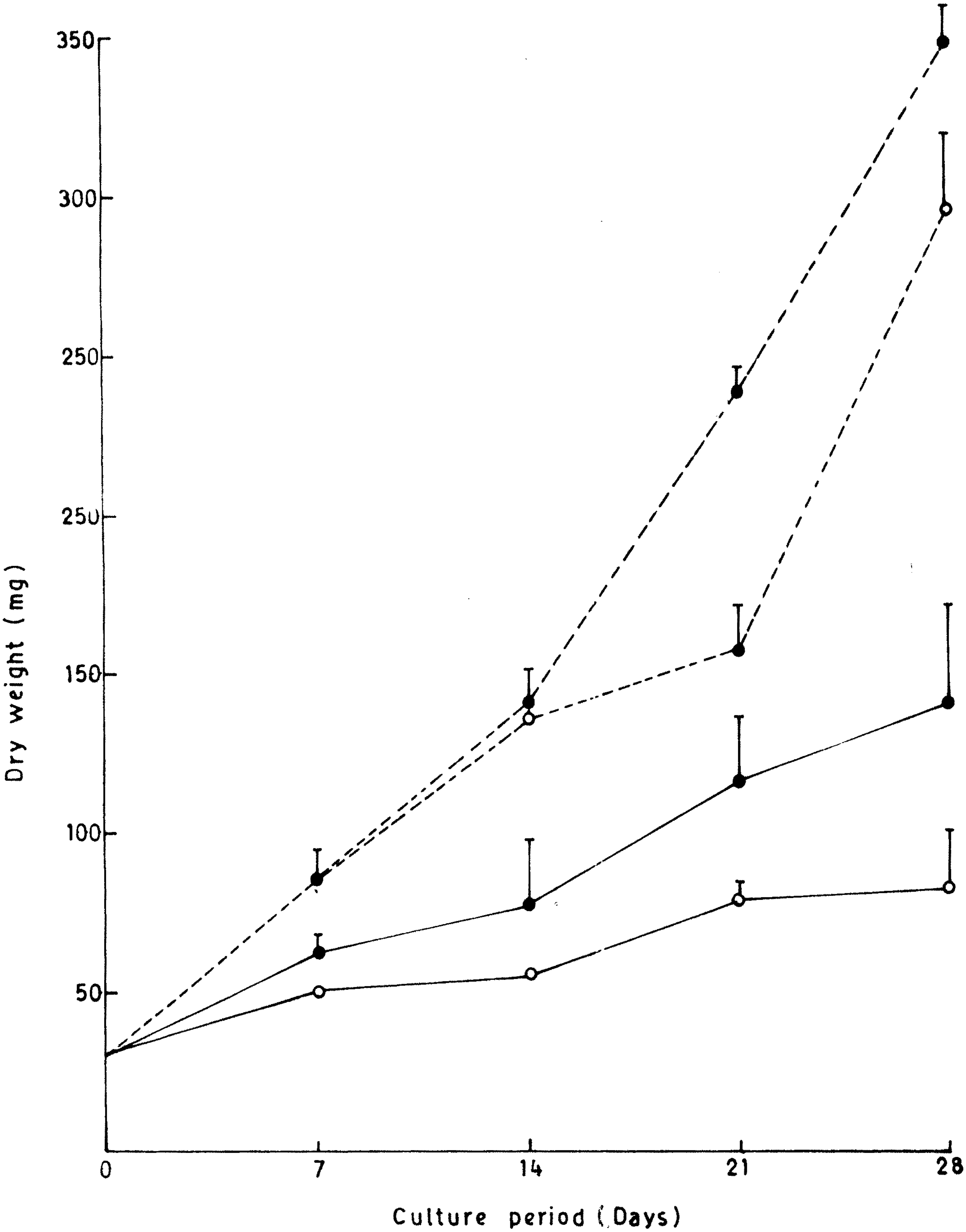
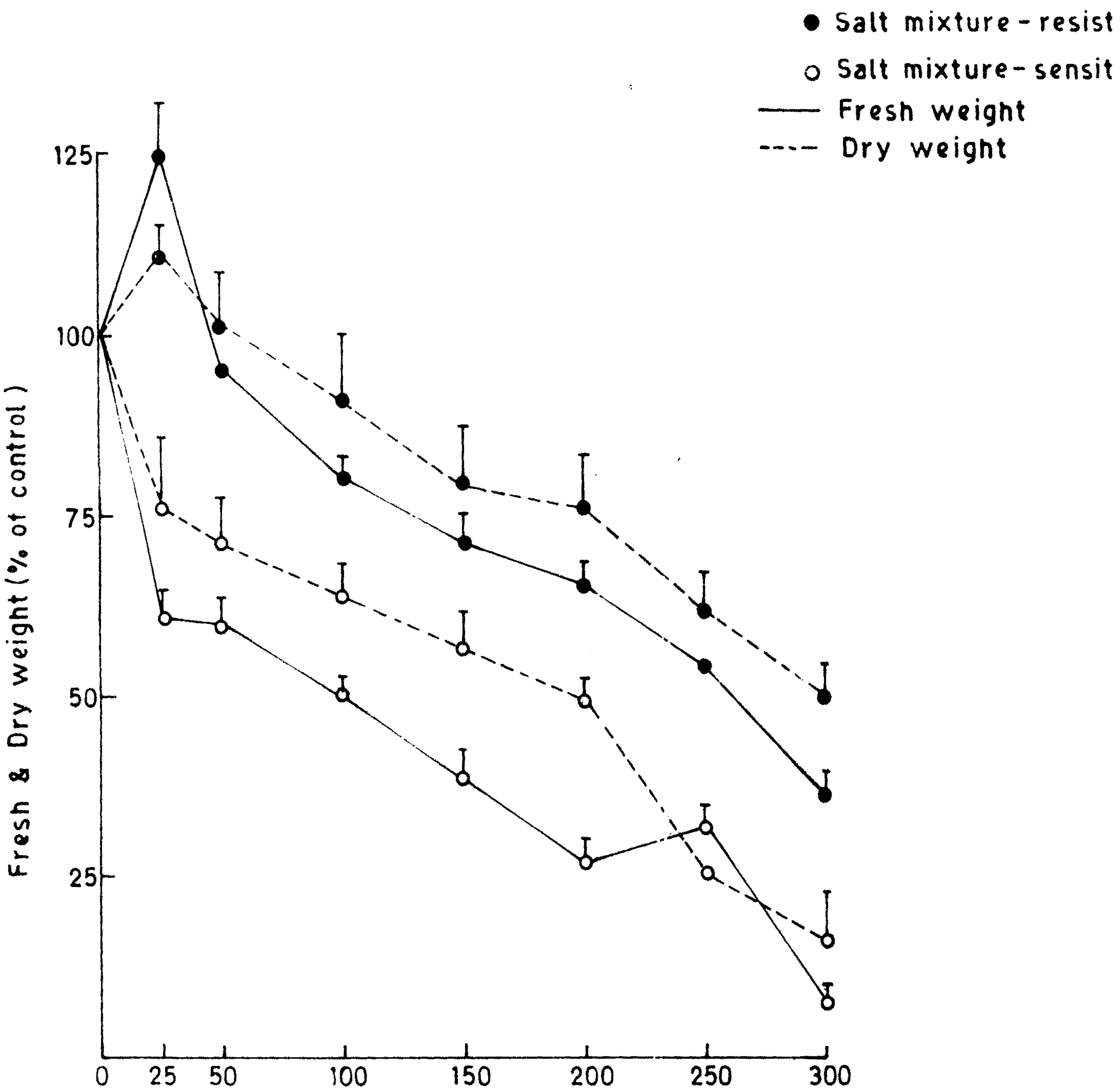

350
300
250
250
150
100
50
Dry weight (mg)
0
7
14
21
28
Culture period (Days)

Fig. 4.



Salt mixture - resist
Salt mixture - sensit
Fresh weight
Dry weight
125
100
75
50
25
Fresh & Dry weight (% of control)
0
25
50
100
150
200
250
300
Salt mixture Concentration (mol m-3)

Fig. 5.

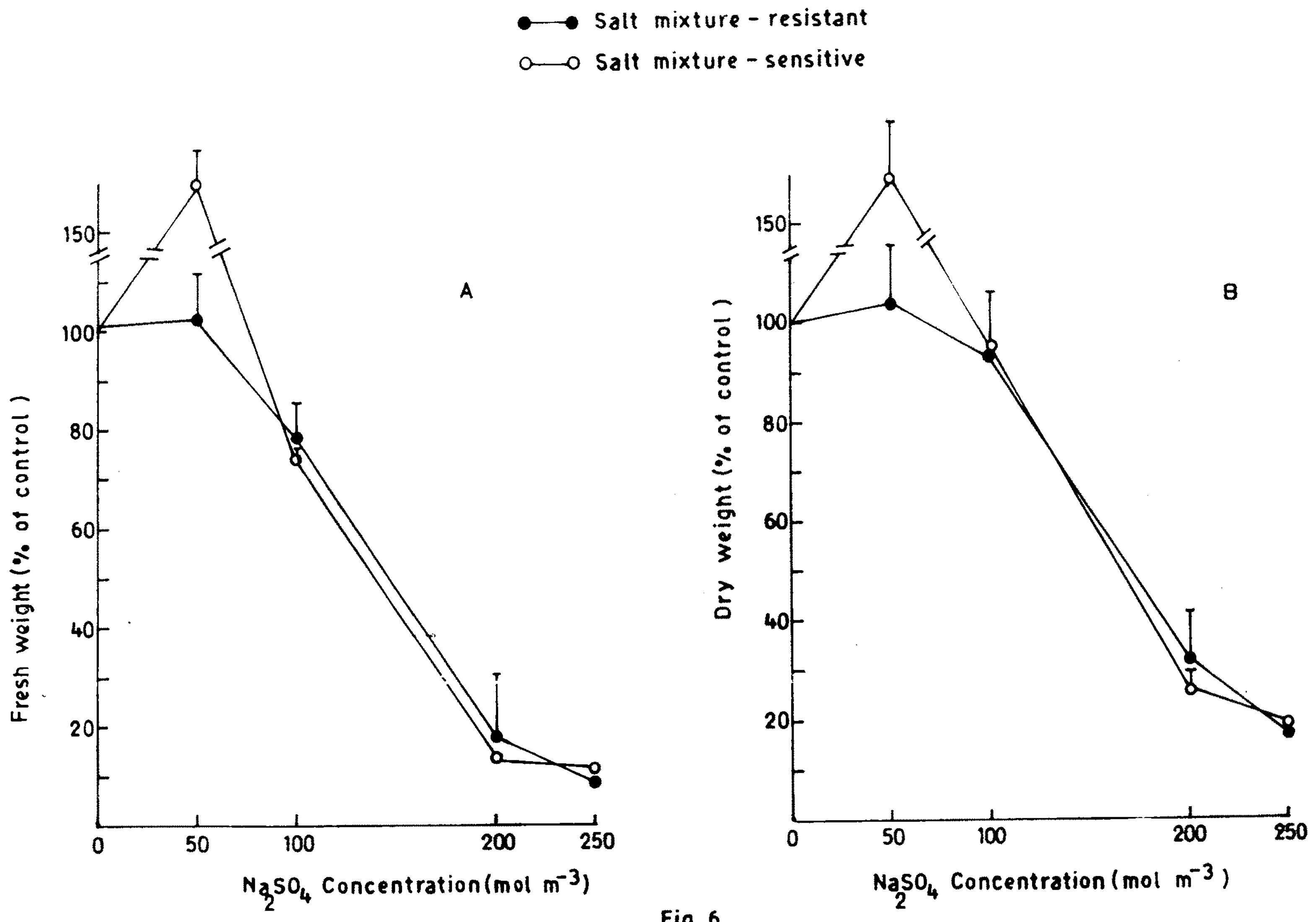
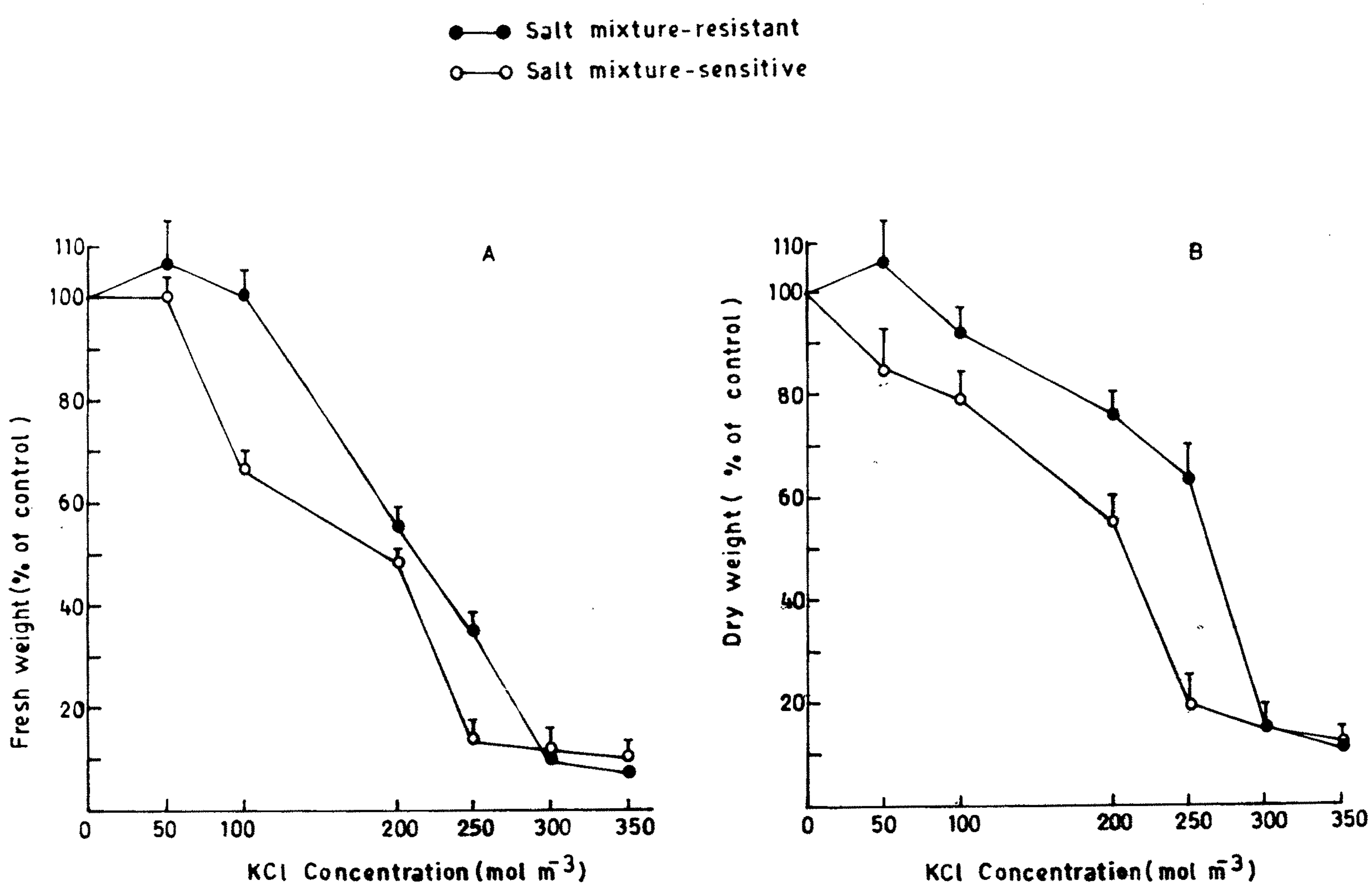
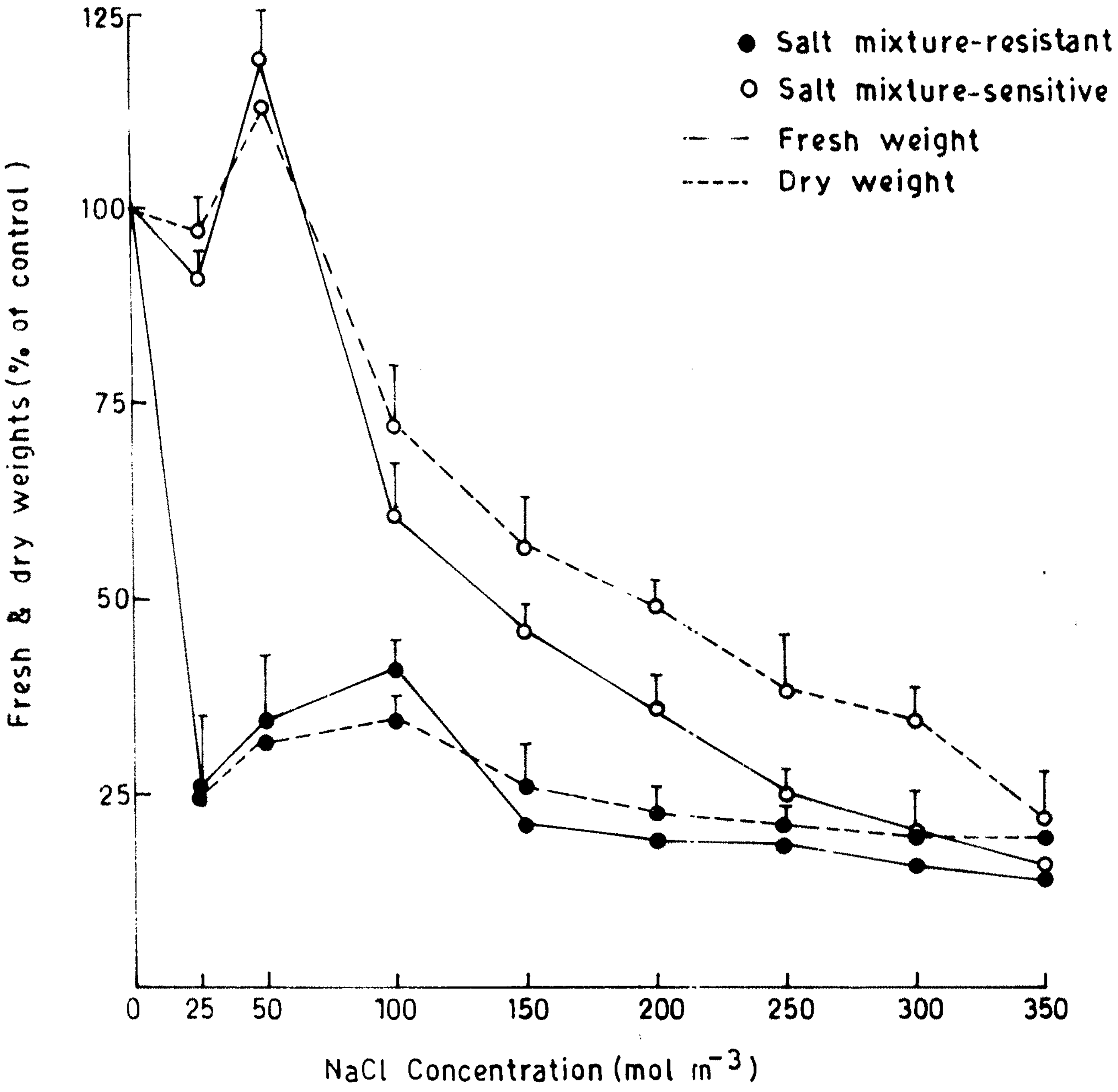

Salt mixture - resistant
Salt mixture - sensitive
A
Fresh weight (% of control)
150
100
80
60
40
20
0
50
100
200
250
Na2SO4 Concentration (mol m-3)
B
Dry weight (% of control)
150
100
80
60
40
20
0
50
100
200
250
Na2SO4 Concentration (mol m-3)

Fig. 6.



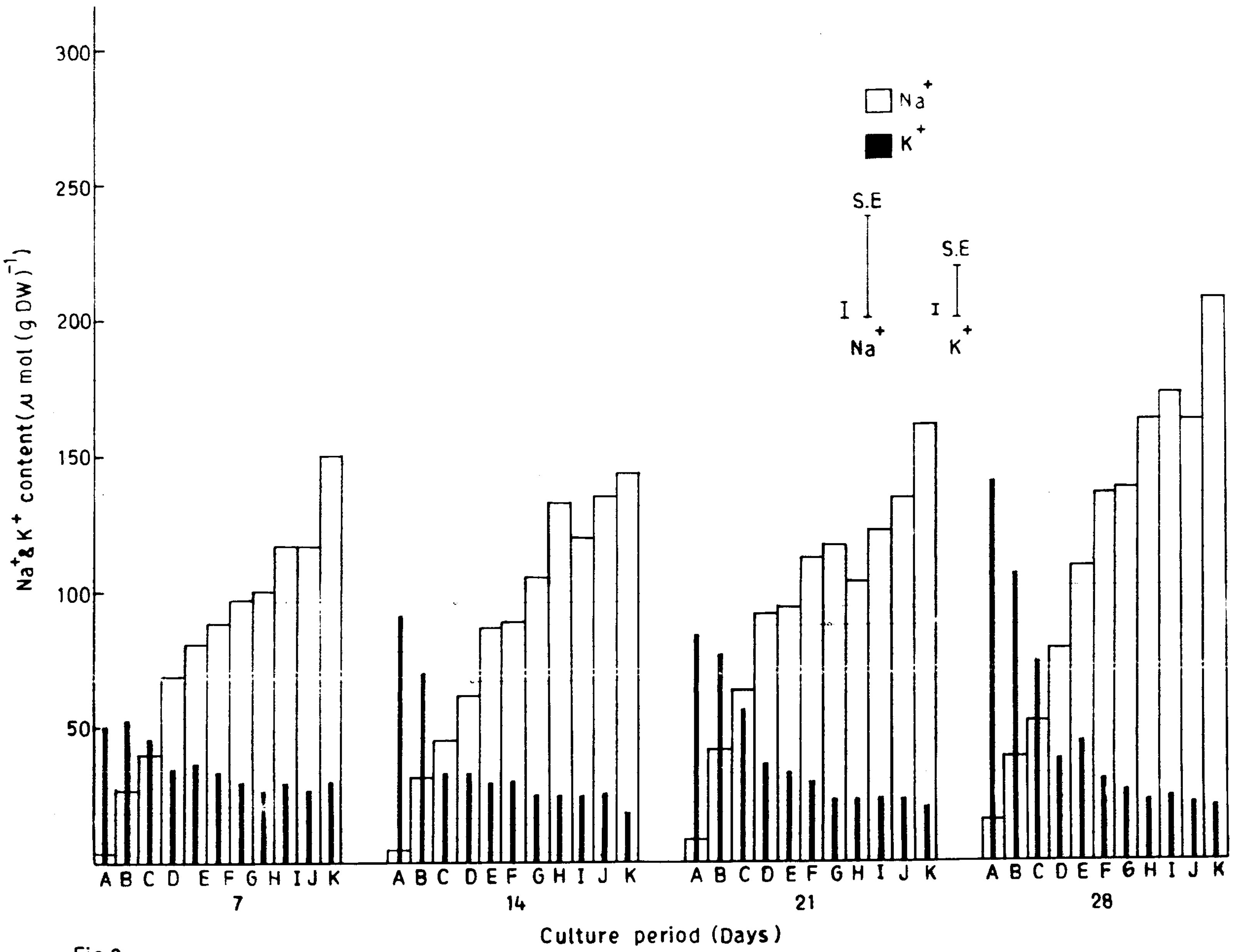
Salt mixture-resistant
Salt mixture-sensitive
A
B
Fresh weight (% of control)
Dry weight (% of control)
110
100
80
60
40
20
0
50
100
200
250
300
350
KCl Concentration (mol m^{-3})

Fig. 7.



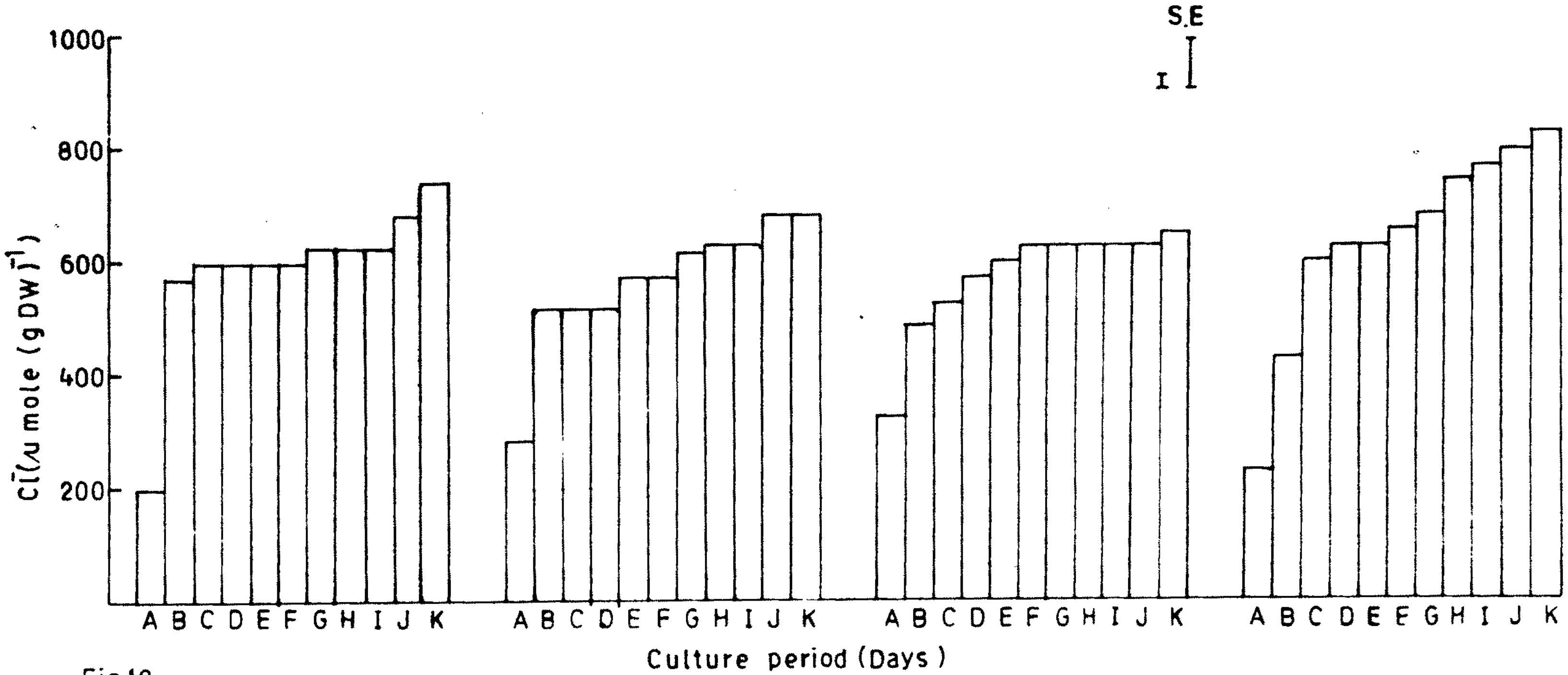
Salt mixture-resistant
Salt mixture-sensitive
Fresh weight
Dry weight
125
100
75
50
25
Fresh & dry weights (% of control)
0
25
50
100
150
200
250
300
350
NaCl Concentration (mol m^{-3})

Fig. 8.



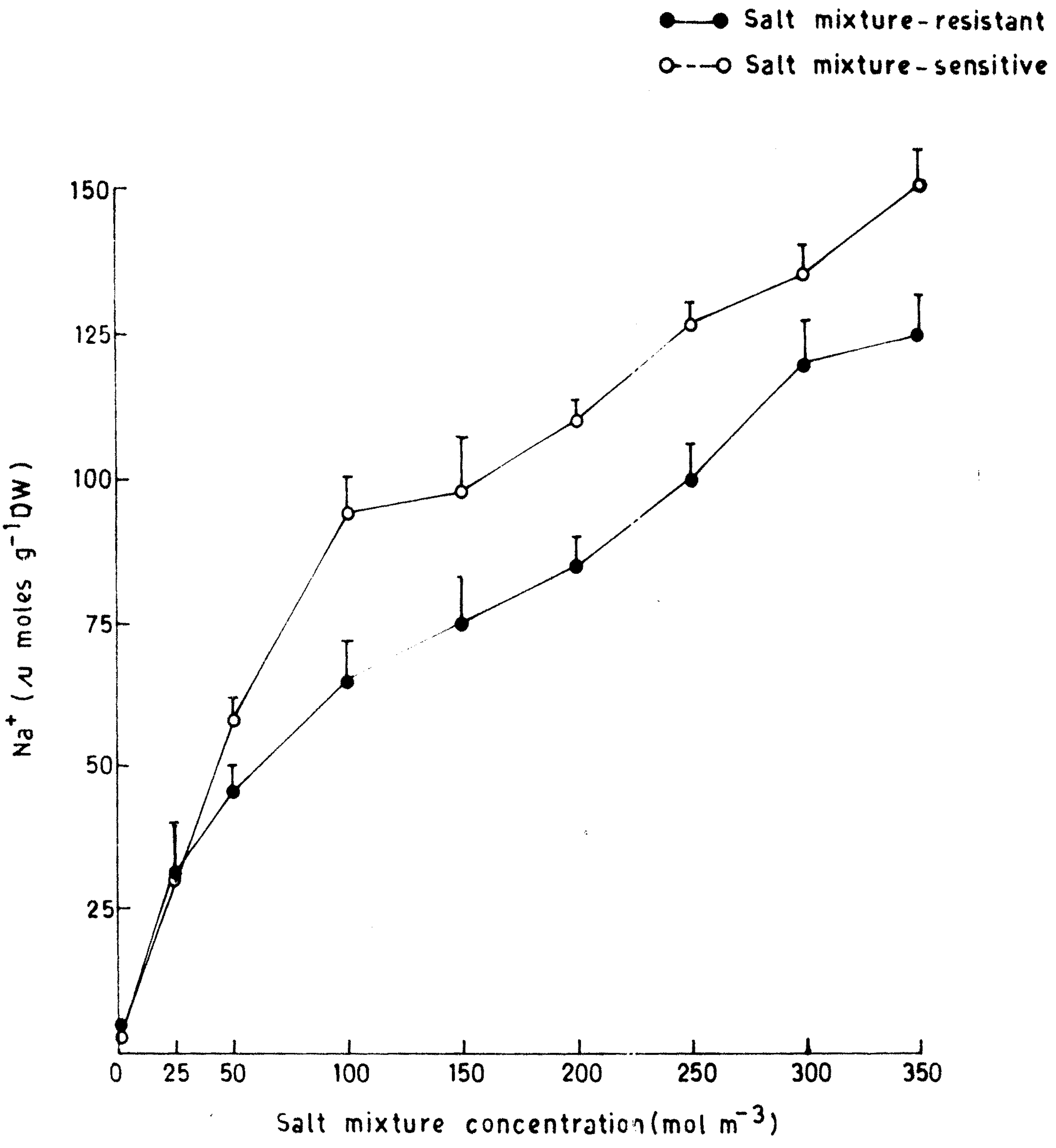
Na+ & K+ content (μ mol (g DW)-1)
300
250
200
150
100
50
Na+
K+
S.E
Na+
S.E
K+
A B C D E F G H I J K
7
14
21
28
Culture period (Days)

Fig.9.



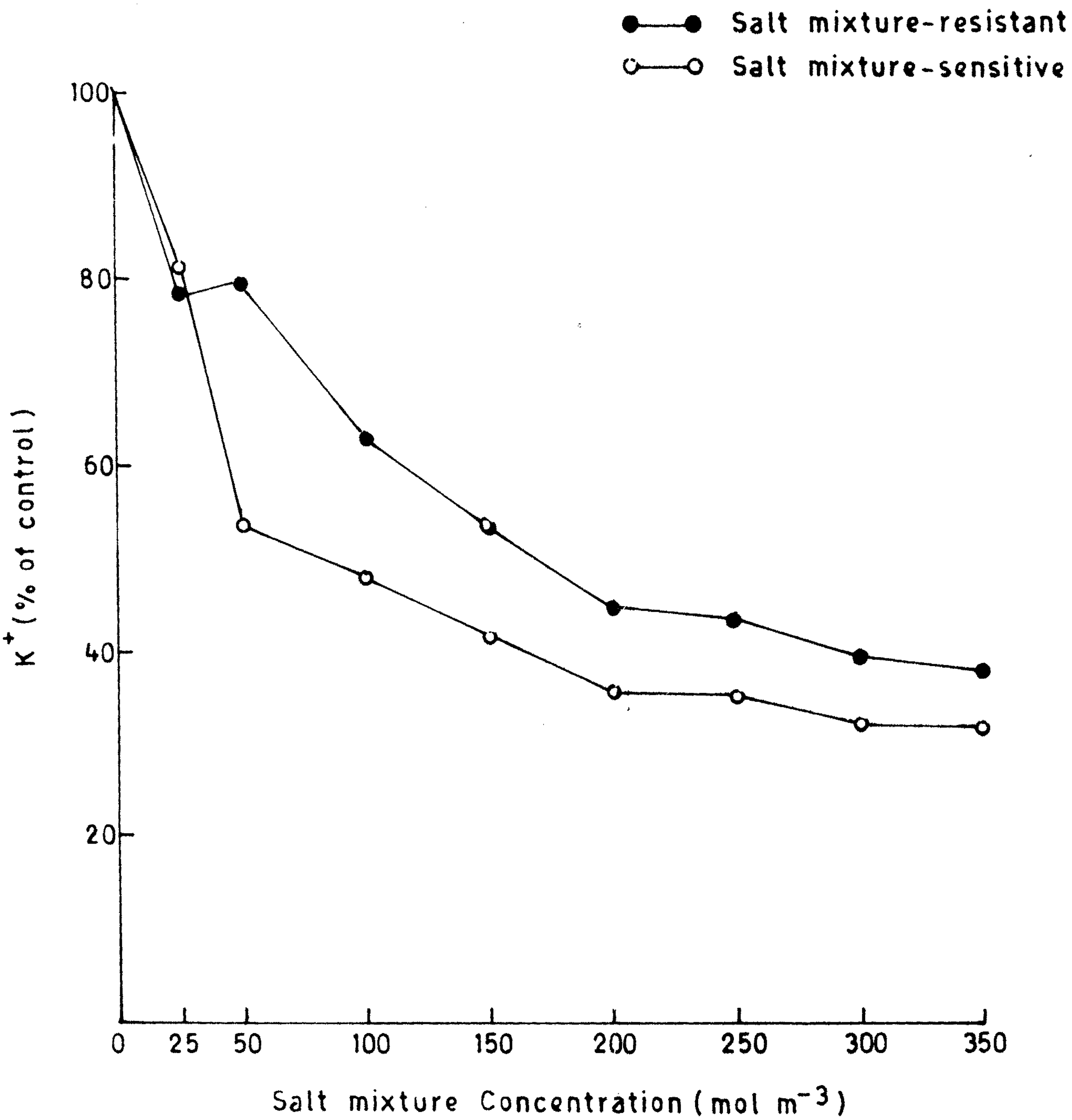
S.E
1000
800
600
400
200
Cl⁻ (μ mole (g DW)⁻¹)
A B C D E F G H I J K
A B C D E F G H I J K
A B C D E F G H I J K
A B C D E F G H I J K
Culture period (Days)

Fig.1a



Salt mixture-resistant
Salt mixture-sensitive
150
125
100
75
50
25
0
25
50
100
150
200
250
300
350
Na+ (µ moles g-1 DW)
Salt mixture concentration (mol m-3)

Fig.11.



Salt mixture-resistant
Salt mixture-sensitive
100
80
60
40
20
K⁺ (% of control)
0
25
50
100
150
200
250
300
350
Salt mixture Concentration (mol m⁻³)

Fig.12.

PLATE 10 Salt mixture sensitive and resistant calli growing in a petridish containing 300 mol m^{-3} salt mixture in PC-L2 medium after 21-d of growth.

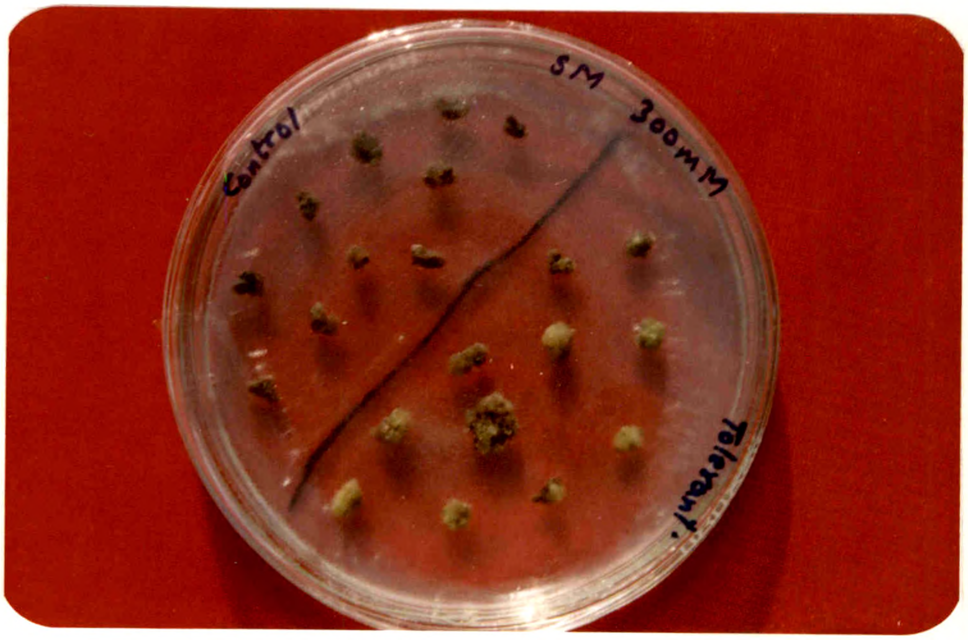


PLATE 10