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The Effect of Salinity on Light and Dark CO_2-Fixation of Salt-Adapted and Unadapted Cell Cultures of *Atriplex* and Tomato

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ABSTRACT

The effect of salinity on light and dark CO_2-fixation was determined in cells of *Atriplex portulacoides* and tomato (*Lycopersicon esculentum* Mill.) grown in culture. CO_2-fixation of tomato cells was also determined in cultures adapted to mannitol and polyethylene glycol (PEG). Salinity up to 400 mM NaCl in the case of *Atriplex* and up to 50 mM in the case of tomato enhanced the rate of light-induced CO_2-fixation in unadapted cells. Higher salt concentrations led to a marked decline in CO_2-fixation in both species. In salt-adapted *Atriplex* cells no decline in the rate of light CO_2-fixation was seen even at 500 mM NaCl. Dark CO_2-fixation was approximately 40% and 80% of the light fixation in control cell cultures of *Atriplex* and tomato, respectively. No enhancement in dark CO_2-fixation was seen as salinity was increased, but a decline was found at similar salt concentrations that decreased fixation in the light. Mannitol- and PEG-adapted tomato cells fixed CO_2 at somewhat lower rates than the control cells in the light but not in the dark.

Key words: Salinity, CO_2-fixation, cell cultures, *Atriplex*, tomato.

INTRODUCTION

Much effort has been and is being directed towards the development of genotypes which are more salt-tolerant than existing ones, in order to maintain productivity of agricultural crops under saline conditions. One method which was used in several laboratories and led to preliminary promising results was the isolation and development of cell lines that exhibit greater tolerance to salinity (Ben-Hayyim and Kochba, 1983; Binzel, Hasegawa, Havda, and Bressan, 1985; Chandler and Vasil, 1984; Croughan, Stavarek, and Rains, 1978; Heyser and Nabors, 1981; Watad, Lerner, and Reinhold, 1985). Binzel *et al.* (1985) showed, for instance, that cell lines of tobacco could grow in media containing up to 770 mM NaCl. The kinetics of dry weight formation of these cells remained relatively unchanged; any change being mainly attributed to osmotic adjustment, being a result of the uptake of inorganic solutes. Salt-tolerant lines were obtained either from relatively tolerant species (Croughan *et al.*, 1978; Dix and Street, 1975; Flowers, Lachno, Flowers, and Yeo, 1985) or by adapting salt-sensitive cell lines to grow under salinity (Ben Hayyim and Kochba, 1983; Binzel *et al.*, 1985; Lerner, 1985). Such adaptation was obtained by stepwise increases of NaCl concentration in the growth medium. Watad *et al.* (1985) showed that adaptation was obtained when 35 mM increments in NaCl were made once in approximately every ten generations. Such cells could then be transferred in one step to 500 mM NaCl and grown without a distinctive lag phase.

It was shown that cells of several species like rice and tobacco could be adapted to higher NaCl concentrations in culture than the whole plant could withstand. Such a difference in salinity tolerance between intact plants and cell cultures of whole plants (Flowers *et al.*, 1985; Watad *et al.*, 1985) might be attributed to one of two possible factors. One is related to the fact that in whole plants the energy and the carbon sources are derived from photosynthesis, in contrast to cells grown in culture in which these factors are supplied externally. The second

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the possibility of CO_2-fixation which was not completely light driven. High activity of PEP carboxylase was shown by Chunhe *et al.* (1988), such activity could also be detected in the dark and may be responsible for the determined CO_2-fixation. Rates of light and dark CO_2-fixation were, therefore, determined simultaneously (Table 1, Exps 2 and 3). It can be seen that the dark fixation rates of cells which were exposed to 50 mM NaCl during the assay as well as during growth were approximately 40% of the light fixation rates. However, while the light fixation was stimulated by NaCl up to 400 mM, the dark fixation was unaffected in this range. At higher NaCl concentrations light fixation was inhibited more strongly than dark fixation, and at 800 mM both rates became equal. This would indicate complete abolishment of photosynthesis at this salt level.

In *Atriplex* cell cultures which were gradually adapted to grow at NaCl levels of up to 200 mM, there was a gradual increase in the CO_2-fixation rates determined at a NaCl concentration identical to that in the growth media (Table 2). Moreover, in contrast to unadapted cells maintained at 50 mM, in which the rate of CO_2-fixation was considerably inhibited at 500 mM NaCl, it was not affected when cells adapted to 100 or 200 mM NaCl were transferred to 500 mM shortly before the determination of CO_2-fixation rate. This trend, which was very similar in both experiments is, in fact, similar to the increase found for cells which were transferred to the elevated but lower salinity levels shortly before assay (Table 1).

It can be concluded from these experiments that three independent phenomena occurred in these cell cultures:

(1) Photosynthesis was stimulated by NaCl up to approximately 400 mM even when added to reaction media *c.* 90 min before assay, but was inhibited at higher NaCl concentrations.

(2) In cells adapted to grow at 200 mM NaCl, photosynthesis was not inhibited up to 500 mM NaCl.

(3) Dark CO_2-fixation was not affected up to approximately 300–400 mM NaCl, but was inhibited at higher concentrations which were also inhibitory to the light fixation.

Tomato cells fixed CO_2 at a lower rate than *Atriplex* cells in the light, while their rate of dark CO_2-fixation was somewhat higher. This resulted in approximately 80% of the light fixation rate found in the dark. The activity of PEP carboxylase was probably much more dominating as compared with the activity of Rubisco in the case of the tomato cells. The lower osmotic potential

TABLE 1. *Effect of NaCl on CO_2-fixation of* Atriplex *cell cultures in light and dark*

Cells were grown in standard media containing 50 mM NaCl and transferred to different NaCl concentrations 90 min prior to assay. Values are presented for three individual experiments. Averages were not calculated as concentrations were not identical in these experiments. Assays for CO_2-fixation were conducted in the buffered cell culture medium (pH = 6·1).

Experiment No.	NaCl concentration (mM)	CO_2 fixation in light (μmol h^{-1})	
		per mg protein	per mg chl
1	50	2·31	36·9
	100	2·84	45·4
	200	3·38	51·6
	400	3·51	61·1
		$^{14}CO_2$ fixation rate (μmol mg^{-1} protein h^{-1})	
		Light	Dark
2	50	1·97	0·83
	150	2·53	0·88
	300	2·77	0·80
	500	2·68	0·75
3	50	2·85	1·11
	200	3·73	1·13
	400	4·05	1·00
	600	1·76	0·78
	800	0·60	0·67

TABLE 2. *Effect of NaCl on light CO_2-fixation by salinity-adapted* Atriplex *cell cultures*

Cells were adapted to salinity by adding NaCl to the medium for about 30 d prior to assay. The values are averages ±s.e. of four individual experiments. CO_2-fixation was conducted in a medium similar to that which served for growth.

NaCl in growth media (mM)	NaCl in assay medium (mM)	$^{14}CO_2$-fixation rate (μmol mg^{-1} chl h^{-1})
50	50	28·4±2·3
	500	16·7±1·8
100	100	37·1±3·7
	500	32·4±4·2
200	200	44·0±3·1
	500	45·2±4·2

TABLE 3. *CO_2-fixation rates in light and dark of tomato cell cultures grown in media containing different solutes*

The values are averages ±s.e. of four individual experiments. Assays for CO_2-fixation were conducted in a medium similar to that which served for growth

Experiment No.	Solute	$^{14}CO_2$ fixation rate (μmol mg^{-1} protein h^{-1})	
		Light	Dark
1	—	1·73±0·08	1·36±0·12
	PEG	1·52±0·10	1·56±0·10
	Mannitol	1·57±0·09	1·60±0·12
2	—	1·80	1·50
	PEG	1·52	1·66
	Mannitol	1·59	1·70

possibility is that the salinity tolerance of cells is not equal in different tissues and organs or is partly lost during organization of the whole plant. The first possibility can be examined by growing cell cultures under photoautotrophic conditions and determining the photosynthetic response of these cells to salinity. Photoautotrophic growth of cells in suspension cultures was previously shown for different species, such as soybean (Horn, Sherrard, and Widholm, 1983; Rogers, Ogren, and Widholm, 1987), tobacco (McHale, Zelitch, and Peterson, 1987), *Amaranthus* and cotton (Chunhe, Blair, Rogers, Govindjee, and Widholm, 1988). Such cell cultures could be adapted to grow under continuous light and in the presence of approximately 5% CO_2, in media containing some hormones and vitamins, but no sucrose or other energy or carbon source. Cells which were grown under autotrophic conditions contained chlorophyll, increased in weight and their CO_2-fixation capacity in the light was as high as 80 μmol mg^{-1} chl h^{-1} (Horn *et al.*, 1983). While fluorescence emission spectra of the cells were similar to those of chloroplasts from intact leaves (Chunhe *et al.*, 1988), the activity of ribulose 1,5-*bis*-phosphate carboxylase (Rubisco) was much lower than in mature leaves (Chunhe *et al.*, 1988; Rogers *et al.*, 1987). The potential of such cells to adapt to salinity and their photosynthetic response was never determined.

Thus, the present study was aimed at determining the response of photosynthesis to salinity in adapted and unadapted cell cultures and so help to clarify the source of different tolerance levels of cell cultures and intact plants to salinity. The study was conducted on cells of two species *Atriplex*—a halophyte, and tomato—a glycophyte.

MATERIALS AND METHODS

A suspension of the NaCl-tolerant *Atriplex portulacoides* was produced. The callus was obtained from leaves cultivated on a medium developed by Murashige and Skoog (1962) (MS-medium), containing 9·05 μmol dm^{-3} 2,4-dichlorophenoxyacetic acid (2,4-D) and 9·29 μmol dm^{-3} 6-furfurylaminopurine (KIN). The callus was solidified on 0·8% agar and was transferred to liquid MS-medium containing 22·62 μmol dm^{-3} 2,4-D and 4·65 μmol dm^{-3} KIN. The cells were adapted gradually to increasing levels of NaCl at increments of 25 mM up to 500 mM. Cells were always maintained at the new step for 1–2 weeks and the salinity was increased only when new cell growth could be detected. Growth was determined on the basis of increase in fresh weight of the suspension. The MS-medium was replaced once every 15 d. The cells were at the final salinity levels for approximately 30 d prior to the CO_2-fixation measurements.

Tomato plants (*Lycopersicon esculentum* Mill.) cv. Rodeo were also used to obtain cell suspension cultures. The cell suspension was cultivated in a modified medium according to Tewes, Glund, Walther, and Reinbothe (1984), containing 0·452 μmol dm^{-3} 2,4-D and 0·093 μmol dm^{-3} KIN. The cells were adapted gradually to increasing levels of NaCl at increments of 25 mM up to 200 mM, in the same manner as for the *Atriplex* cells. The MS-medium was replaced once every 7 d. The cells were adapted to this medium for over 1 year. The tomato cell culture was also transferred to iso-osmolar media containing mannitol (400 mmol dm^{-3}) or to PEG (polyethylene glycol) 8000 (160 g dm^{-3}) and grown under these conditions for approximately 30 d.

The cultures were incubated on a rotary shaker at 130 rev. min^{-1}, 24$\pm$1 °C, at a photoperiod of 16 h with fluorescent light (300 μE m^{-2} s^{-1}). The fresh weight of the *Atriplex* culture media (at 100 mM NaCl) was 14 mg cm^{-3} containing approximately $2{\cdot}6 \times 10^4$ cells. The content of the tomato cultures was 24 mg cm^{-3} containing approximately $1{\cdot}6 \times 10^6$ cells.

Fixation rates of CO_2 were determined on 1·0 cm^3 samples taken from cell-containing media and transferred to glass scintillation vials (20 mm diameter). Sodium chloride, mannitol or PEG were added to the desired final concentration, the pH was adjusted to 6·1 by MES (2-[*N*-morpholino] ethanesulphonic acid) (50 mM) and the volume was brought to 1·45 cm^3. Small stirring rods were placed in the vials which were then closed with serum stoppers. One row of vials was mounted in a transparent water bath illuminated from both sides by fluorescent lights and placed on to magnetic stirrers. Photon flux density in the centre of the vials was approximately 250 μE m^{-2} s^{-1} and the temperature was maintained at 25 °C. The final assay mixture (not including $NaH^{14}CO_3$) was always pre-incubated at 25 °C for 1 h in the dark followed by 30 min in the light or dark according to the experiment plan. The assays were initiated by the injection of 50 mm^3 $NaH^{14}CO_3$ to a final concentration of 5·0 mol m^{-3} (1·0 μCi μmole^{-1}) and terminated after 10 min by adding 400 mm^3 of 10% HCl. Vials were then flushed with air-streams and the residual $^{14}CO_2$ was liberated and absorbed in soda lime traps. This was followed by boiling for 10 min and drying under vacuum. The dried solutes were redissolved in 1·0 cm^3 H_2O and counted by liquid scintillation. Since the number of vials to be exposed to light simultaneously was limited, we preferred to include all variables without replications in a given experiment, rather than replicate some of the variables. Each experiment was, however, repeated three or four times; in most cases the averages and s.e. of replicate experiments are presented, while others are the results of individual experiments.

The chlorophyll and protein contents were determined on separate aliquots of cells taken from the same media (Arnon, 1949; Lowry, Rosebrough, Farr, and Randall, 1951, respectively).

RESULTS AND DISCUSSION

The *Atriplex* cells which were grown autotrophically in media containing 50 mM NaCl produced approximately 12 μg chlorophyll per mg protein, which is considerably less than the amount found in intact leaves. Since it was not clear whether CO_2-fixation was rate-limited by the low amounts of chlorophyll, or by suppressed activity of photosynthetic enzymes, the rate was expressed on both a chlorophyll and a protein basis (Table 1). The fixation rate of CO_2 in light was stimulated by increased NaCl concentration up to 400 mM NaCl when expressed in terms of both chlorophyll and protein content (Table 1, Exp. 1). The greatest stimulation per increase in NaCl concentration was found between 50 and 100 mM and was reduced at higher salinity levels. The high protein-to-chlorophyll ratio, which was about 16, as compared with about 12 in many intact leaves (Zelitch, 1971) raised

in the growth and assay media due to PEG or mannitol reduced the fixation rate but only in the light. A marked stimulation in photosynthetic CO_2-fixation was found when 50 mM NaCl was added 90 min before the assay, but at higher salt levels the rate was decreased (Fig. 1). Dark fixation was not significantly affected by NaCl. In tomato cells this stimulation took place at 50 mM, much lower than in *Atriplex*. A rise in salinity to 100 mM NaCl led to a decrease of 240% in photosynthesis, while further rises in salinity to 200 and 400 mM NaCl resulted in a decrease of approximately 60% and 30%, respectively.

The findings of the present study rule out the possibility that the higher salinity tolerance of cell cultures as compared with intact plants is related to photoautotrophic growing conditions. This can be concluded from the stimulated photosynthesis of tomato cells at 50 mol m^{-3} NaCl and the lack of inhibition at 100 mol m^{-3} found in cells which were unadapted to salinity. Moreover, when cells were pre-adapted to salinity the rate of CO_2-fixation was markedly stimulated by salt as shown for *Atriplex portulacoides* cells. A stimulation of photosynthesis under mild salinity condition was also shown for non-halophytic, and even salt-sensitive cowpeas. In freshly isolated mesophyll cells from intact plants which were grown under different salinity levels and were thus adapted to salinity, an increase in the rate of CO_2-fixation was observed up to approximately 90 mol m^{-3} NaCl (Table II in Plaut, Grieve, and Federman, 1989). In intact non-halophytic plants CO_2-assimilation was mostly found to decrease even under mild salinity levels (Gale, Kohl, and Hagan, 1967; Walker, Torkfalvy, and Downton, 1982; West, Hoffman, and Fisher, 1986; Plaut, Grieve, and Maas, 1990). This was mainly attributed to a decrease in stomatal conductance. The decline in stomatal conductance under low salinity levels exceeded the decrease in CO_2-assimilation and resulted in a decrease of intercellular $p(CO_2)$, which is interpreted as a stimulation in the rate of CO_2-fixation (Plaut *et al.*, 1990). In *Lycopersicon esculentum* an increase in net assimilation was even reported for intact leaves (Taleisnik, 1987). This stimulation in CO_2-fixation could not be due to a change in the protein-to-chlorophyll ratio, as similar rates were found on both a chlorophyll and a protein basis. The present results would thus suggest that this was either due to enhanced enzyme activity or to its improved preservation.

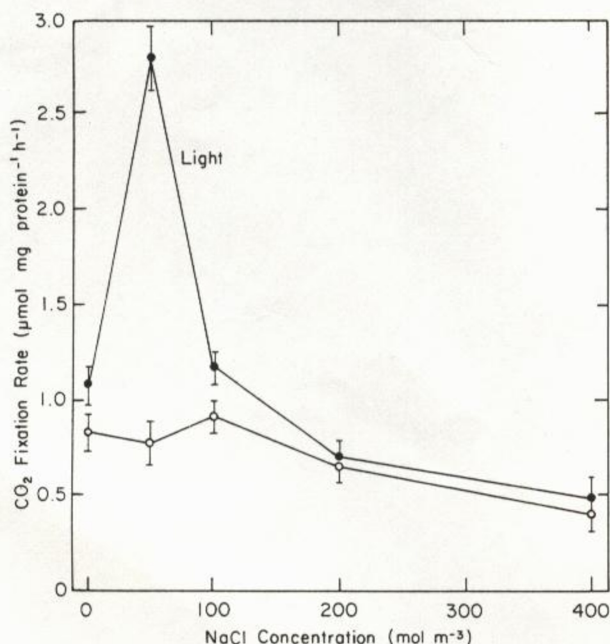

FIG. 1. The rate of light and dark CO_2-fixation by tomato cells grown in standard media and transferred to different NaCl concentrations 90 min prior to assay.

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