BIOLOGIA PLANTARUM 46 (3): 423-427, 2003

Growth and nitrate reductase activity of *Chlorella fusca* cells as affected by long term salinity

A.A. SHAFEA

Botany Department, Faculty of Science, Assiut University, Assiut, 71516, Egypt

Abstract

Influence of various saline media on *Chlorella fusca* growth, contents of photosynthetic pigments, and activity of the nitrate reductase (NRA) was determined. KCl, $CaCl_2$, and $MgCl_2$ in concentrations relative to NaCl as 1:1, 2:1, and 3:1 promote cell number, dry mass, and contents of photosynthetic pigments. The activity of NR was enhanced by Mg^{2+} and K^+ and in some cases by Ca^{2+} at all ratios applied.

Additional key words: calcium chloride, magnesium chloride, potassium chloride, sodium chloride.

Introduction

Plant responses to salt stress are affected not only by the total salinity as measured by osmotic potential but also by composition of the saline medium (Bernstein 1975, Greenway and Munns 1980, Kingsbury and Epstein 1986, Munns and Termaat 1986). Generally, the adverse effects of salts on growth of different plant groups were recorded (*e.g.* Czernas 1978, Setter 1979, Setter and Greenway 1979, Kaiser *et al.* 1983, Seemann and Critchley 1985, Ahmed *et al.* 1985, Abdel-Basset 1986, Shafea 1987, Abdel-Fatah 1988, Cramer *et al.* 1988, Mohammed and Shafea 1992, Sweby *et al.* 1994, Kinraide 1999, Peuke and Jeschke 1999). Moreover, Weimberg (1988) in two species of wheat, *Triticum turgidum* and *Triticum aestivum* found a small additional inhibition of growth if KCl replaced NaCl as the salinizing salt. $CaCl_2$ had a little or no effect on growth inhibition beyond an osmotic effect except at the most severe stress when Ca^{2+} concentrations may be excessive.

Such adverse effects of ions on growth came from their effects on various metabolic processes. Of which, nitrogen metabolism and its further assimilation to proteins is controlled by different sequential metabolic processes. The reduction of nitrate to nitrite, catalized by nitrate reductase (NR) is considered to be the rate limiting step in nitrate assimilation (Beevers and Hageman 1969) and NR is the rate limiting enzyme in N assimilation and a key point of metabolic regulation. The activity of this enzyme is associated with protein synthesis and plant growth, both of which are affected by water stress (Sinha and Nicholas 1981). The decline in NR activity (NRA) during water stress has been attributed to a reduction in enzyme content as determined by the rate of protein synthesis and degradation (Bardzik *et al.* 1971, Sweby *et al.* 1994) as well as by inactivation of enzyme (Plaut 1974). In accordance with this, Garcia-Gonzales *et al.* (1987) found that nitrate uptake was inhibited in sodium deficient cells. However, NRA was increased by 37 % under these conditions. NRA under various saline media was not fully attended. Therefore, we studied the possible changes in growth criteria and NRA at various ratios of Na^+/Ca^{2+}, Na^+/Mg^{2+}, $Na^+/Ca^{2+} + Mg^{2+}$, Na^+/K^+, K^+/Ca^{2+}, K^+/Mg^{2+}, $K^+/Ca^{2+} + Mg^{2+}$, and K^+/Ca^+, keeping constant osmotic potential of media (-1.34 MPa).

Materials and methods

The unicellular chlorophycean alga *Chlorella fusca* was used as a test organism. Beijernck nutritive medium (Stein 1966) for chlorophyta was used for growth of this organism cultured in a growth chamber in which irradiation was performed by two fluorescent tubes (irradiance of 4.56 W m^{-2} at the surface of 500 cm^{-3} vessels)

Received 23 October 2001, *accepted* 28 August 2002.
Abbreviations: Chl - chlorophyll; NR - nitrate reductase; NRA - NR activity.
Fax: (+20) 88 342708, e-mail: shafea@acc.aun.eg

at temperature around 30 °C. Filtered dry air was bubbled through the culture vessels to provide CO_2 and to prevent settling of algal cells. After 7 d the cells were harvested.

Hemacytometer (*Boeckel*, Hamburg, Germany) 0.1 mm deep, having improved *Naubouer ruling* (*A.O. Spencer Bright line*) was used for determination of cell number. One drop of the algal suspension was pipetted on the specific slide, covered and left for 2 min for algal settling. For determination of dry mass a definite volume (100 cm^3 of algal suspension was filtered through weighed glass fiber filter (*Sartorius PF19*, Göttingen, Germany). The cells after being precipitated on filter paper, were washed twice with distilled water and dried overnight in an oven at 105 °C.

For determination of content of chlorophylls *a* and *b* and carotenoids 10 cm^3 of algal suspension was centrifuged and the clear growth medium was decanted. The algal precipitate was dehydrated using 25 % ice acetone (10 cm^3) for a few minutes, followed by centrifugation. Care was taken that ice-cold acetone never became greenish; if so, the procedure was repeated with shortened time of exposure. The algal precipitate was then resuspended in 10 cm^3 of 100 % acetone and left for 2 to 3 h in the dark. After centrifugation, the cell debris precipitates and the clear supernatant which contains the pigments was aspirated and diluted to a definite volume. Double beam spectrophotometer (*Spectronic 2000*, *Bausch & Lamb*, USA) was used, absorbance was measured at the wavelengths of 452.5, 644, and 663 nm, and contents of pigments were calculated using equations of Metzner *et al.* (1965).

NRA (*in vivo*) was determined by the method of Jaworski (1971): aliquots 30 cm^3 of algal suspension were centrifuged and the algal precipitate was put into test tubes wrapped in aluminum foil. Five cm^3 of an assay medium containing K-phosphate buffer, *n*-proparol and chloramphenicol. After incubation in a water bath at 30 °C for 0, 15, and 30 min, the tubes were boiled in a 100 °C for 5 min. Then, the samples were filtered to remove the cell debris. One cm^3 samples were taken from each test tube and adopted for colorimetric determinations of nitrite. The sulphanilic acid is converted into the corresponding diazo compound, which couples with α-naphthylamine to form α-naphthyl-amine-P-azobenzene-P-sulphonic acid, a red azo-dye during 10 - 15 min.

NRA at time 0 represents the end products of this enzyme after 7 d of treatment. However, the values after 15 and 30 min show NRA changes after removal of the stress agent.

Results

Growth of *C. fusca* cultures at various salt ratios was mostly improved by the K^+, Ca^{2+}, and Mg^{2+} combined with Na^+ (Table 1). Cell number decreased at the osmotic potential of -1.34 MPa induced by NaCl or KCl alone and increased upon inclusion of $CaCl_2$, $MgCl_2$ or KCl to NaCl in ratios (1:1, 2:1, 3:1). Dry mass was also increased and its values were higher compared with control (Table 1) while they decreased in either NaCl or KCl salinized cultures. Accumulation of dry matter due to inclusion of Ca^{2+}, Mg^{2+}, or K^+ could be attributed to their regulative effects in the external solution as well as to their competition. This will lead to diminshing the concentration of Na^+ inside the cells. Contents of photosynthetic pigments, which are a good criterion for photosynthetic performance, were lowered under NaCl and KCl salinity. Upon isoosmotic combination with other ions they exhibited higher values, especially at the ratios of 2:1 and 3:1 (Table 1). NRA was determined at time 0 and 15 and 30 min after stress removal. In comparison with NaCl alone NRA was elevated using counteractive ions Ca^{2+}, Mg^{2+} or K^+ (Table 2). In ratio 1:1 enhancement of NRA was lesser with Mg^{2+} ions than with K^+ ions, but at the ratio 2:1, both Mg^{2+} and K^+ ions had similar promotive effect. At 3:1 ratio K^+ and Mg^{2+} showed a high effect.

Discussion

Growth of *C. fusca* cells was reduced by NaCl and KCl salinity (osmotic potential -1.34 MPa). The reduction in growth parameters (cell number, dry mass) could be attributed to a reduction in activity of some enzymes besides the delay in cell division induced by decrease in pressure potential. Such observations agree with findings of Setter and Greenway (1979) that *Chlorella emersonii* undergoes decrease in pressure potential leading to delay in cell division after transfer of cells from 1 to 335 mM NaCl. The possible causes for reduce growth of *C. fusca* under relatively high salinity (osmotic potential -1.34 MPa) was the synthesis of compounds required for osmotic adjustment. Kleinkopf and Wallace (1974) and Gale (1975) proposed that growth inhibition under salinity could be partly due to shortage of energy because processes involved in transport of salts and repair of salt damage exerted on membranes or proteins are energy-consuming. The reduction in contents of photosynthetic pigments under salinity was probably due to the inhibitory effect of accumulated ions on their

Table 1. Cell number [10^6(cell) cm^{-3}(suspension)], dry mass [g m^{-3}(suspension)] and contents of chlorophyll (Chl) *a*, Chl *b* and carotenoids (Car) [g kg^{-1}(d.m.)] of *Chlorella fusca* cultures grown for 7 d under isoosmotic combinations of NaCl with KCl, $CaCl_2$ and $MgCl_2$ (in the ratios 1:1, 2:1, and 3:1) keeping constant osmotic potential of the media -1.34 MPa. * - means significantly different from control at $P = 0.05$.

Ratio	Salts	Cell number	Dry mass	Chl *a*	Chl *b*	Car	Chl *a*/*b*	Chl/Car
Control		2305	500	21.28	7.96	6.26	2.67	4.67
1/1	NaCl	1637	400	20.47	7.96	6.03	2.67	4.66
	$NaCl/CaCl_2$	1285	780	4.12*	2.18	4.68	1.88*	1.34*
	$NaCl/MgCl_2$	1091	653	3.47*	2.14	3.13*	1.62*	2.98
	NaCl/ KCl	1510	723	14.91	5.25	4.92	2.83	4.09
	KCl	1430	372	19.13	10.10	8.01	1.89	3.65
	$KCl/CaCl_2$	1734	583	13.85	4.73	4.25	2.92	4.37
	$KCl/MgCl_2$	1530	690	14.14	5.11	4.21	2.76	4.56
	KCl/NaCl	1510	723	14.91	5.25	4.92	2.83	4.09
	$NaCl+KCl/CaCl_2$	1938	790	12.86	3.71*	3.09	2.82	4.58
	$NaCl+KCl/MgCl_2$	1856	683	10.35	3.85*	2.85*	2.68	4.97
	$NaCl+KCl/CaCl_2+MgCl_2$	2448	773	5.65*	3.18*	3.72	1.77*	2.37
2/1	NaCl	1637	400	20.47	7.65	6.03	2.67	4.66
	$NaCl/CaCl_2$	3315	666	12.52	4.03	3.42	3.10	4.83
	$NaCl/MgCl_2$	2550	683	19.01	6.58	5.57	2.88	4.59
	NaCl/ KCl	2111	696	3.66*	2.95*	4.10	1.23	1.61*
	KCl	1430	372	19.13	10.10	8.01	1.89	3.65
	$KCl/CaCl_2$	2320	956	11.85	3.92	3.46	3.02	4.55
	$KCl/MgCl_2$	3218	800	4.85*	3.18	4.35	1.52*	1.84*
	KCl/NaCl	1450	490	5.44*	4.32	5.59	1.26*	1.75*
	$NaCl+KCl/CaCl_2$	2050	1200	10.02	3.36*	2.88	2.97	4.64
	$NaCl+KCl/MgCl_2$	2693	563	12.80	4.99	4.54	2.56	3.91
	$NaCl+KCl/CaCl_2+MgCl_2$	1816	900	16.63	6.55	4.36	2.53	5.31
3/1	NaCl	1637	400	20.47	7.65	6.03	2.67	4.66
	$NaCl/CaCl_2$	1362	930	12.23	4.23	3.55	2.88	4.62
	$NaCl/MgCl_2$	1775	756	13.86	4.66	4.39	2.96	4.21
	NaCl/KCl	949	610	16.16	4.95	5.47	3.26	3.85
	KCl	1430	372	19.13	10.10	8.01	1.89	3.65
	$KCl/CaCl_2$	1530	790	14.41	4.48	4.75	3.21	3.97
	$KCl/MgCl_2$	1306	873	12.97	4.34	4.31	2.98	4.01
	KCl/NaCl	830	440	16.63	6.77	5.27	2.45	4.43
	$NaCl+KCl/CaCl_2$	1795	790	10.30	3.37	3.22	3.04	4.23
	$NaCl+KCl/MgCl_2$	1591	633	17.55	6.49	4.91	2.70	4.89
	$NaCl+KCl/CaCl_2+MgCl_2$	1785	863	15.89	5.39	4.61	2.94	4.61

biosynthesis (Strogonov 1962, Bazhanova *et al.* 1964). Salinity also effects the chloroplast structure and induce changes in pigment-proteins complexes.

Improvement of growth under various isoosmotic combination ratios 1:1, 2:1, 3:1 of Na^+/K^+, Na^+/Ca^{2+}, Na^+/Mg^{2+} or K^+/Ca^{2+} and K^+/Mg^{2+} at constant osmotic potential -1.34 MPa might be attributed to the protective effect of both Ca^{2+} and Mg^{2+} on the membranes. High Na^+ content in *Chlorella pyrenoidosa* and *Dunaliella parva* caused very high Na^+/K^+ ratio (Shieh and Barber 1971, Gimmler and Schirling 1978). Therefore addition of K^+ decreasing Na^+/K^+ ratio reduced the internal sodium concentration and ameliorates effects of salinity on metabolism and consequently on growth. Addition of Ca^{2+} affected membrane permeability (Gary-Bobo 1970, Schubert *et al.* 1993) and blocked the free penetration of Na^+ (Bange 1968). Moreover, Ca^{2+} ions prevent leakiness of organic solutes (Leopold and Willing 1984).

NR is the enzyme limiting N assimilation and *C. fusca* cultures grown for 7 d under salinity exhibited low NRA. Upon withdrawal of salts, NR restored its activity 15 and 30 min later nearly in all cultures. The NRA was affected by individual treatments of both Na^+ and K^+. However, when K^+ was combined with Na^+ at the three, ratios used, it was the promotive ion for NRA. Mg^{2+} generally enhanced NRA, but Ca^{2+} had a little role.

The decline in NRA due to water stress was also observed, *e.g.*, by Hewitt (1975), Pate (1980), Sinha and Nicholas (1981). Such decline has been attributed to reduction in NR content as determined by the rate of

Table 2. The activity of nitrate reductase (NRA) [g(nitrite) kg^{-1}(protein)] in *Chlorella fusca* cultures grown for 7 d under isoosmotic combinations of NaCl with KCl, $CaCl_2$ and $MgCl_2$ (in the ratios 1:1, 2:1 and 3:1) keeping constant osmotic potential of the media -1.34 MPa.

Ratio	Salts	At time 0	After 15 min	After 30 min
Control		1.86	3.08	4.31
1/1	NaCl	0.24	0.40	0.48
	$NaCl/CaCl_2$	2.50	5.35	8.20
	$NaCl/MgCl_2$	1.24	5.83	9.17
	NaCl/KCl	2.38	6.36	10.34
	KCl	0.26	0.86	1.56
	$KCl/CaCl_2$	2.20	3.53	4.88
	$KCl/MgCl_2$	37.68	32.60	27.53
	KCl/NaCl	2.38	6.36	10.34
	$NaCl+KCl/CaCl_2$	117.76	82.24	46.73
	$NaCl+KCl/MgCl_2$	2.38	5.26	8.14
	$NaCl+KCl/CaCl_2+MgCl_2$	10.96	8.81	6.66
2/1	NaCl	0.24	0.40	0.48
	$NaCl/CaCl_2$	2.10	3.82	5.54
	$NaCl/MgCl_2$	1.08	2.63	4.18
	NaCl/KCl	1.83	1.77	1.71
	KCl	0.26	0.86	1.56
	$KCl/CaCl_2$	2.96	5.63	8.32
	$KCl/MgCl_2$	19.41	15.12	10.84
	KCl/NaCl	2.42	3.82	5.48
	$NaCl+KCl/CaCl_2$	186.91	116.64	46.39
	$NaCl+KCl/MgCl_2$	14.49	14.00	13.52
	$NaCl+KCl/CaCl_2+MgCl_2$	19.57	13.54	7.56
3/1	NaCl	0.24	0.40	0.48
	$NaCl/CaCl_2$	1.31	2.51	3.72
	$NaCl/MgCl_2$	1.35	2.90	4.46
	NaCl/KCl	1.90	6.39	8.97
	KCl	0.26	0.86	1.56
	$KCl/CaCl_2$	2.15	2.82	7.49
	$KCl/MgCl_2$	43.46	26.79	10.13
	KCl/NaCl	2.65	6.18	8.72
	$NaCl+KCl/CaCl_2$	2.65	5.30	7.94
	$NaCl+KCl/MgCl_2$	2.43	3.34	4.25
	$NaCl+KCl/CaCl_2+MgCl_2$	1.16	2.86	4.56

protein synthesis and degradation (Bardzik *et al.* 1971) as well as by inactivation of the enzyme (Plaut 1974). The NRA also depends on nitrate uptake (Garcia-Gonzales *et al.* 1987, Sweby *et al.* 1994, Anil *et al.* 1997). In this respect, Passera and Albuzio (1978) reported that the increase in nitrate content would lead to increase in NRA in salt tolerant plants. Changes in NRA after growth at salinity stress could also be attributed to alterations in proportions of isoenzymes (Flowers *et al.* 1976) and K^+ and Mg^+ ions affects formation of different isoezymes.

The reduction in *C. fusca* growth by salt stress was plausibly due to osmotic stress lowering the external water potential and effects of specific ions on metabolic processes ranging from absorption of nutrients to enzyme activation and inhibition.

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