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Salt-induced lipid changes in *Catharanthus roseus* cultured cell suspensions

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Abstract

Salt treatment strongly affected cell growth by decreasing dry weight. Exposure of *Catharanthus roseus* cell suspensions to increasing salinity significantly enhanced total lipid (TL) content. The observed increase is mainly due to high level of phospholipids (PL). Hundred mM NaCl treatment increased phospholipid species phosphatidylcholine (PC) and phosphatidylethanolamine (PE), whereas it reduced glycolipid ones monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) but not sulfoquinovosyldiacylglycerol (SQDG). Moreover, fatty acid composition was clearly modified when cells were cultured in the presence of 100 mM NaCl, whereas only few changes occurred at 50 mM. Salt treatment decreased palmitic acid (16:0) level and increased that of linolenic acid (18:2). Such effect was observed in phospholipid species PC and PE and in glycolipid DGDG. Double bond index (DBI) was enhanced more than 2-fold in fatty acids of either glycolipids or phospholipids from cells submitted to 100 mM NaCl. Free sterol content was also significantly enhanced, especially at 100 mM NaCl, whereas free sterols/phospholipids (St/PL) ratio was slightly decreased. All these salt-induced changes in membrane lipids suggest an increase in membrane fluidity of *C. roseus* cells.

Keywords: *Catharanthus roseus*; Salt stress; Lipids; Fatty acids; Free sterols

1. Introduction

Saline environment highly reduces crop productivity and yield. It is generally correlated with inhibition of plant growth (Mühling and Läuchli, 2002), changes in lipid metabolism (Kuiper, 1985) and also synthesis of new proteins (Majoul et al., 2000). Membrane lipids play a fundamental role in regulating permeability (Schuler et al., 1991), fluidity (Kerkeb et al., 2001), carrier-mediated transport (Deutike and Haest, 1987) and activity of membrane-bound enzymes (Cooke and Burden, 1990). In many plants, changes in lipids, i–e sterols (St), phospholipids (PL) and glycolipids were observed as a result of salt stress (Kuiper, 1985) and may contribute to the control of membrane fluidity (Shinitzky, 1984) and protein environment, changes affecting membrane functions (Schuler et al., 1991; Tester and Davenport, 2003). Most investigations dealing with the effects of environmental stress on the regulation of lipid synthesis have been carried out on whole plants (Morpurgo, 1991), plant organelles (Müller and Santarius, 1978) and on calli (Rodríguez-Rosales et al., 1999). However, the use of cell suspension cultures as an experimental system would be of great interest in the evaluation of salinity impact at the cellular level. In studies with plants, it is difficult to separate the cellular mechanisms of tolerance from those implicated in the structure, function and cell tissue organisation existing in whole plant. Besides, a cell culture model allows a long-term subculturing of cells in salinity conditions and then offer possibilities of obtaining some salt-tolerant variants (Vazquez-Flota and Loyota-Vargas, 1994).

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Catharanthus roseus cells are well characterized and continuously growing. These cells are sensitive to auxins which modulate alkaloid biosynthesis (Mérillon et al., 1986) and proteins (Chahed et al., 1999) such as peroxidases (Limam et al., 1998). Mérillon et al. (1993), studying the effect of cytokinins on the modulation of membrane lipids in *C. roseus* cells found a relation between growth, alkaloid synthesis and membrane lipid composition.

In the present study, *C. roseus* cells have been used to evaluate the impact of salinity on growth and membrane lipid changes.

2. Results

2.1. Effect of salt on C. roseus cell growth

Cell growth was determined by measuring dry weight (Fig. 1). It clearly appears that salt treatment significantly reduced cell growth by 43% at 50 mM NaCl and 67% at 100 mM compared to control. However, 50T cells which were maintained and subcultured for 8 months in the continuous presence of 50 mM NaCl, lost only 36% of their dry weight. Noteworthy that salt treatment markedly reduced cell size, especially at 100 mM NaCl (data not shown).

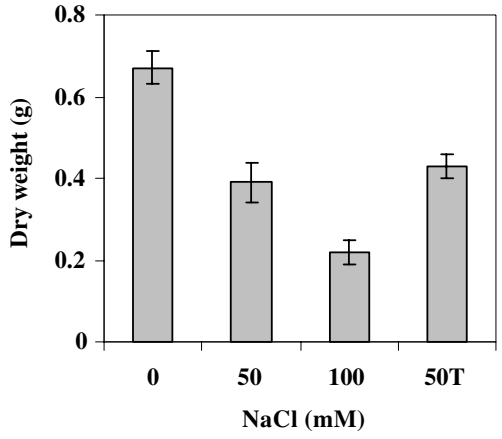

Fig. 1. Salt effect on *Catharanthus roseus* cell growth. 0, 50 and 100 cells: cells subcultured in standard medium during 31 and submitted to 0 (control) 50 or 100 mM NaCl, respectively, during 7 days (32th week). 50T: Cells subcultured and maintained during 32 weeks in the continuous presence of 50 mM NaCl. Each value is the mean ± SD of three independent experiments.

2.2. Effect of NaCl treatment on lipid amounts

Lipid content was evaluated from the amount of total fatty acids. As shown in Table 1, total lipid content increased sharply with NaCl concentrations reaching its highest value at 50 mM. Such an increase was reflected by raised amounts of neutral lipids and to a lesser extent by glycolipids and phospholipids. 50T cells showed comparable lipid amounts than cells acutely subjected to 50 mM NaCl. At 100 mM NaCl phospholipid contents increased significantly whereas glycolipid and neutral lipid levels dropped.

Table 2 showed changes in amounts of individual glycolipid and phospholipid compounds. Monogalactosyldiacylglycerol (MGDG) content dropped at 100 mM NaCl, whereas digalactosyldiacylglycerol (DGDG) content increased slightly at 50 mM and declined thereafter. In contrast to major glycolipids MGDG and DGDG, the minor SQDG increased regularly with NaCl concentrations. Once again 50T cells showed the same behaviour than 50 mM NaCl-treated cells, except for SQDG level which is higher in 50T cells. Concerning phospholipids, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) compounds increased at all salt concentrations especially at 100 mM NaCl.

Table 2 also showed that exposure of cells to 100 mM NaCl induced an increase in PL/TL ratio and a decrease in PC/PE one.

2.3. Effect of NaCl treatment on fatty acid composition

We reported in Table 3 the overall changes induced by salt treatment on fatty acid composition (molar percent) of total lipids and individual glycolipids and phospholipids. We focused mainly on the two most prominent fatty acid species found in membrane lipids of *C. roseus* i–e palmitic acid (16:0) and linolenic acid (18:2).

Table 1
Effect of salt on different lipid classes of *C. roseus* cells

NaCl (mM)	Lipids (mg g^{-1} dry wt)			
	Total lipids (TL)	Phospholipids (PL)	Glycolipids (GL)	Neutral lipids (NL)
0	17.76 ± 0.76^{c}	7.24 ± 0.16^{d}	5.83 ± 0.10^{c}	4.69 ± 0.11^{c}
50	27.21 ± 1.15^{a}	7.54 ± 0.19^{c}	$6.960.15^{b}$	12.71 ± 0.52^{a}
100	21.67 ± 0.35^{b}	13.76 ± 0.51^{a}	4.62 ± 0.16^{d}	3.29 ± 0.13^{d}
50T	26.30 ± 0.58^{a}	8.41 ± 0.19^{b}	7.34 ± 0.13^{a}	10.55 ± 0.41^{b}

Lipid content was estimated from the amounts of fatty acids. Data are expressed by mean values ± SD of three independent experiments. Values followed by superscript letters in each column differ significantly (Duncan's test, $P = 0.05$).

Table 2
Salt effect on glycolipid and phospholipid species of *C. roseus* cells

NaCl (mM)	Lipids (mg g^{-1} dry wt)					PL/TL ratio	PC/PE ratio
	Glycolipids			Phospholipids			
	MGDG	DGDG	SQDG	PC	PE		
0	1.42 ± 0.11^{a}	4.06 ± 0.15^{b}	0.35 ± 0.09^{b}	2.42 ± 0.10^{c}	1.80 ± 0.18^{b}	0.41	1.34
50	1.23 ± 0.19^{ab}	5.29 ± 0.10^{a}	0.44 ± 0.10^{b}	2.52 ± 0.09^{c}	1.87 ± 0.13^{b}	0.28	1.34
100	0.84 ± 0.10^{c}	2.96 ± 0.20^{c}	0.82 ± 0.13^{a}	3.96 ± 0.11^{a}	3.57 ± 0.14^{a}	0.63	1.11
50T	1.13 ± 0.10^{b}	5.21 ± 0.13^{a}	1.00 ± 0.16^{a}	2.72 ± 0.10^{b}	1.89 ± 0.17^{b}	0.32	1.44

Data are expressed by mean values ± SD of three independent experiments. Significance was assessed using the Duncan's test.

In control cells, fatty acids were characterized by high levels of 16:0 and 18:2, both representing about 76% of total content. Others fatty acid species such as palmitoleic (16:1), stearic (18:0), oleic (18:1) and linolenic (18:3) were less abundant and accounted for about 24% of the total fatty acids. When cells were acutely treated or chronically subcultured for several months with 50 mM NaCl, only weak changes in the total fatty acid composition were detected. Exposure of cells to 100 mM NaCl markedly decreased fatty acid 16:0 level and increased that of 18:2. The other fatty acids such as 16:1, 18:0 and 18:3 were only slightly modified.

Fatty acid composition in glycolipid and phospholipid species has also been analysed. Data confirmed that fatty acids 16:0 and 18:2 are the most prominent species. Salt treatment always decreased the 16:0 level and increased that of 18:2 of DGDG and SQDG glycolipid compounds. In MGDG species the 18:2 levels did not change even in the presence of 100 mM NaCl. Concerning phospholipids PC and PE, similar changes in fatty acids levels occurred under salt stress i–e decrease in 16:0 and increase in 18:2. Data also showed that salt treatment enhanced unsaturated fatty acid levels (18:1, 18:2 and sometimes 18:3) but decreased those of satu-

Table 3
Fatty acid composition of total lipids, glycolipids and phospholipids in control and salt-treated *C. roseus* cells

NaCl (mM)	Fatty acids	Total lipids (%)	Glycolipids (%)				Phospholipids (%)		
			MGDG	DGDG	SQDG	Total GL	PC	PE	Total PL
0	16:0	44	26	43	44	39	50	50	52
	16:1	3	2	3	8	3	1	1	2
	18:0	9	14	2	19	5	2	3	5
	18:1	7	8	6	Nd	7	9	4	8
	18:2	32	40	37	22	38	37	42	32
	18:3	5	10	7	9	3	Nd	1	
	(DBI)	(1.68)	(3.00)	(2.24)	(1.16)	(2.57)	(1.79)	(1.68)	(1.35)
50	16:0	44	29	35	39		46	48	52
	16:1	2	1	1	6	1	1	1	1
	18:0	4	3	2	13	2	1	2	2
	18:1	5	4	5	12	5	6	Nd	4
	18:2	40	52	46	23	47	44	48	39
	18:3	5	11	11	7	11	3	2	1
	(DBI)	(2.13)	(4.44)	(3.41)	(1.63)	(3.14)	(2.21)	(2.06)	(1.65)
100	16:0	34	20	19	28	19	31	28	35
	16:1	1	1	1	4	1	1	1	1
	18:0	4	5	2	8	3	2	3	3
	18:1	11	15	11	19	12	11	9	11
	18:2	43	42	49	39	47	52	56	48
	18:3	7	17	19	2	19	3	2	3
	(DBI)	(3.13)	(6.04)	(7.95)	(2.97)	(5.32)	(3.79)	(4.13)	(3.08)
50T	16:0	40	23	38	43	35	46	41	46
	16:1	2	3	1	1	1	nd	1	2
	18:0	3	2	2	1	2	1	2	2
	18:1	8	5	6	5	6	8	5	6
	18:2	42	55	41	48	44	40	50	40
	18:3	5	12	12	2	13	4	2	3
	(DBI)	(2.53)	(6.16)	(3.13)	(2.45)	(3.62)	(2.00)	(2.60)	(2.02)

Data are given as % of fatty acids. nd: not detected.

Table 4
Effect of salt on total free sterol content and composition of *C. roseus* cells

NaCl (mM)	Free sterol composition (%)					Total free sterol contents (mg g^{-1} dry wt)	St/PL ratio
	Cholesterol	Campesterol	Stigmasterol	Sitosterol	Avenasterol		
0	nd	49.26	15.77	34.96	nd	2.40 ± 0.17^{c}	0.33
50	1.50	42.68	6.87	40.23	8.73	2.78 ± 0.17^{b}	0.37
100	1.71	41.61	3.30	38.61	14.77	3.75 ± 0.46^{a}	0.27
50T	1.48	42.68	6.90	40.23	8.71	2.61 ± 0.12^{b}	0.31

Total free sterol contents are expressed by mean values ± SD of three independent experiments. Duncan's test has been used to assess significance. St/PL: free sterols/phospholipids.

rated ones (16:0 and 18:0) in almost all lipid species of *C. roseus* cells. This salt-induced effect on the fatty acid unsaturation of all lipid species was confirmed by increased double bond index (DBI) with salt treatment.

2.4. Effect of salt on free sterol content and composition

Table 4 showed data about the effect of salt on total free sterols of *C. roseus* cells. Sterol content was enhanced with increasing salinity till 100 mM NaCl, reaching 1.5-fold the control amount. However, St/PL ratio which was not modified in 50 and 50T cells, slightly decreased in the presence of 100 mM NaCl. Analysis of free sterol composition showed that the major sterol compounds in control cells were campesterol, stigmasterol and sitosterol. Salt treatment mainly reduced the percent of campesterol and stigmasterol but increased levels of the other compounds such as sitosterol, avenasterol and cholesterol, especially at 100 mM NaCl.

3. Discussion

Various physiological and biochemical changes take place within the plant cells after a high salinity exposure which can lead to numerous damages in the structure and function of the plant cells. The present study deals with the salt-induced changes in the membrane lipid profiles of a continuously growing cell line: *C. roseus*.

Salt treatment reduced drastically cell growth especially at 100 mM. It also induced an increase in the total lipid content. In order to determine which lipid species were modified by salt treatment, we undertook the analysis of glycolipids and phospholipids. We found that glycolipid levels increased at 50 mM NaCl (and at 50T) but decreased at 100 mM as previously shown in *plantago* species (Erdei et al., 1980). Further analysis revealed that DGDG which is the most prominent specie was mainly affected by salt. The decrease in DGDG content (especially at 100 mM) might reduce the stability of the membrane bilayers (Williams, 1988), the activity of membrane bound-proteins (Cooke and Burden, 1990) and the membrane permeability (Schuler et al., 1991). MGDG content did not change at 50 mM NaCl but decreased significantly at 100 mM. Concerning the SQDG species, we always observed an increase with salt treatment at 50 and 100 mM as previously reported (Brown and DuPont, 1989); the highest increase was obtained with 50T cells i–e with cells subcultured and maintained in the continuous presence of 50 mM NaCl during several months. In halophytes high level in SQDG was often associated with tolerance to salt stress (Taran et al., 2000).

Phospholipid contents increased with salt treatment especially at 100 mM. This result could explain the increase in the total lipid content described upper. Moreover, the PL/TL ratio also increased with NaCl treatment. The salt-induced increase in phospholipid levels might stimulate membrane biosynthesis, which is essential to vesicles formation involved in compartmentalization or export of Na^{+} ions (Yoko et al., 2002). Noteworthy that the content of phospholipids did not change or increase in halophytes (Blits and Gallagher, 1990; Wu et al., 1998) while it decreased in some glycophytes (Mansour et al., 1993; Surjus and Durand, 1996). Our data also showed that in control cells, PC and PE were the predominant species representing 58% of total phospholipids. These phospholipid species were significantly enhanced with salt treatment especially at 100 mM, which is in accordance with data reported from salt-stressed jojoba calli (Chrétien et al., 1992), tomato calli (Rodriguez-Rosales et al., 1999; Kerkeb et al., 2001), and *Plantago* roots (Erdei et al., 1980). Salt induced a little change in the PC/PE ratio. This result confirms that obtained with the tolerant tomato calli (Kerkeb et al., 2001) but it is in discordance with those of (Mansour et al., 1994), who found a decrease in both PC and PC/PE ratio of wheat roots exposed to salt. The little increase in PC/PE ratio observed for cells cultured in the presence of 50 mM NaCl induced only few modifications in membrane structures. At 100 mM NaCl, a decrease in PC/PE ratio as well as reduced cell growth and size reflected a weak tolerance to high salinity. These results have led us to look at the impact of salinity on fatty acid unsaturation and sterol composition.

Indeed analysis of fatty acid composition revealed that 16:0 and 18:2 were the major fatty acids present in

C. roseus cells. This result confirmed previous data obtained in the same cellular system (Mérillon et al., 1993) and in roots of wheat (Mansour et al., 1994) or tomato seedlings (Ouariti et al., 1997). Even in MGDG, we found a low level of fatty acid 18:3, which indicated that *C. roseus* cells did not contain chloroplasts. The salt-induced changes in fatty acid composition showed roughly the same pattern i–e increase in 18:2 and decrease in 16:0. Moreover, salt always increased the unsaturation level confirmed by high DBI values of almost all lipid species. These results are in discordance with those obtained with halophytes such as *Dunaliella salina* and *Spartina patens* (Peeler et al., 1989; Wu et al., 1998). An increase in unsaturation and length of fatty acid chain enhance membrane fluidity and thickness, respectively (Mansour et al., 1994), which might affect membrane permeability to ions (Singh et al., 2002).

Salt also increased the content of the total free sterols (Erdei et al., 1980) which are known to be important in membrane rigidity. Free sterols are effective in the regulation of membrane stability and passive ionic permeability. In control cells, campesterol and sitosterol are the most prominent species as previously found (Mérillon et al., 1993). At 50 mM NaCl, only a minor increase was observed in the total free sterol content. Concerning the distribution of sterol species, we again observed only a little increase in sitosterol and avenasterol. Our data are in disagreement with those of (Mansour et al., 1994) who showed an increase in more planar sterols with salinity. However they confirmed those of (Wu et al., 1998), who found an increase in less planar sterols as sitosterol. In the presence of 50 mM NaCl the little increase in sitosterol and St/PL ratio only slightly modify membrane rigidity to avoid Na^+ and Cl^- fluxes into cells. This inability of *C. roseus* cells to maintain intracellular ion homeostasis lead to decreased cell growth. At 100 mM NaCl an increase in total free sterol content was obtained and mainly concerned sitosterol, avenasterol and cholesterol compounds. At the same NaCl concentration, phospholipids greatly increased (two times over control) while the St/PL ratio diminished. Sterol enrichment and increase in St/PL ratio are interpreted as mechanisms of salt adaptation based on sterol-induced membrane rigidity (Shinitzky, 1984). In contrast, sterol enrichment and decrease in St/PL ratio, as obtained in the present case, reflect an increase in membrane fluidity which is a characteristic of sensitive plants (Kerkeb et al., 2001), confirmed by strong inhibition of cell growth. Moreover, it is generally found that salt treatment increased the St/PL ratio much more in salt-tolerant than in salt-sensitive species (Douglas and Walker, 1984).

In conclusion, 50 mM NaCl induced in *C. roseus* cells only few modifications of PC/PE and St/PL ratios, and a weak increase in DBI. All these changes slightly modify the membrane fluidity. Moreover, the salt-induced inhibition of growth of 50 and 50T cells reaches the same level versus control. At 50 mM NaCl, salt did not affect severely cell growth which reflects a rather weak tolerance of these cells towards low concentration of salt. In the same way the long-term subculturing of these cells in the presence of 50 mM NaCl seems to indicate a small adaptation. At 100 mM NaCl, salt induced important changes, i.e. increase in membrane phospholipids and in fatty acid unsaturation as well as decrease in St/PL ratio. All these changes which increase membrane fluidity rather than rigidity, suggest that *C. roseus* cells which have been obtained from glycophytes plants, are much less tolerant to high saline environment. This high concentration provoked deleterious effects leading to drastic inhibition of cell growth.

Further studies are needed to assess the exact role of membrane-bound proteins such as ATPase and Na^+/H^+-antiports, which are known to be modulated by salt stress (Niu et al., 1995; Tester and Davenport, 2003) and involved in ion homeostasis (Zhu, 2003).

4. Experimental

4.1. Cell culture

Cell suspensions of *C. roseus* (L.) G. Don (albino cell line C_{20}), were propagated on a rotary shaker (100 rpm), at 24 °C in the dark, in B5 medium supplemented with 58 mM sucrose and 4.5 μM 2,4-D and adjusted to pH 5.5 just before sterilization (Mérillon et al., 1989). The media were sterilized by autoclaving but vitamins were sterilized by filtration (across discs 0.22 μm, Gilman) before being added to the media. They were subcultured weekly at dilution 1:10 in 250 ml Erlenmeyer flasks containing 50 ml suspension. For experimental purposes, cells were regularly subcultured during 31 weeks in standard medium. 0 (control), 50 and 100 cells were subacutely submitted to 0, 50 or 100 mM NaCl, respectively, during 7 days (32th week). 50T cells were subcultured and chronically maintained during 32 weeks in the continuous presence of 50 mM NaCl.

4.2. Analytical methods

At the end of the experiments cells were filtered on nylon cloth (30 μm) under partial vacuum, rapidly washed with cold distilled water, freezed in liquid N_2 and stored at −80 °C until analysis.

4.3. Lipid analysis

Total lipids were extracted from *C. roseus* cells using $CHCl_3$–MeOH–H_2O (1:1:1) (Bligh and Dyer, 1959). Polar lipids were separated by TLC on silica gel plates 60 (Merck) in $CHCl_3$–Me_2CO–MeOH–HOAc–H_2O

(10:4:2:2:1) (Trémolières and Lepage, 1971). Lipid spots were detected by brief exposure to I_2 vapour. Identifications were carried out by comparison with reference lipids and by specific staining for phospholipids and galactolipids. Fatty acids from total lipid classes were transmethylated with MeOH–BF_3 (Metcalfe et al., 1966). Fatty acid Meester were analysed by FID-GC on a metal column (1.8 m × 3 mm i.d.) packed with GP 3% SP-2310/2% SP-2300 with N_2 as carrier gas at a flow rate of 20 ml min^{-1}. The column was maintained isothermally at 200 °C. Injector and detector tempers were held, respectively, at 210 and 240 °C. For measuring the amount of fatty acids, 17:0 was added as internal standard before methylation. Calculation of fatty acid contents was obtained using an integrator. The DBI was calculated according to (Quartacci et al., 2001) as follows: $DBI = [(1 \times \%\ \text{monoenes}) + (2 \times \%\ \text{dienes}) + (3 \times \%\ \text{trienes})]/\sum \%$ saturated fatty acids.

Free sterols were measured colorimetrically according to (Huang et al., 1961). Lipid extract was dissolved in concentrate HOAc (1:1) to which two volumes of Ac_2O–H_2SO_4 (30:1) were added. OD was measured after 30 min at 525 nm and sterol content was determined using cholesterol as standard.

The main sterol compounds were extracted, separated by GC–MS and identified through mass spectroscopy according to (Dupéron et al., 1992).

4.4. Statiscal analysis

All experiments were carried out using three cultures per treatment. Data were compared on the basis of standard error and standard deviation of the mean values. In addition, Duncan's multiple range tests were used to determine significant differences among data.

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