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NaCl-tolerant plants of *Poncirus trifoliata* regenerated from tolerant cell lines

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Summary. Salt-tolerant cell lines of citrus rootstock (*Poncirus trifoliata* cv Pomeroy) were selected by subculturing embryo-derived calli on media containing sublethal concentrations of NaCl (5 and 10 g/l). Selected lines showed a normal growth in the presence of salt at the concentrations used for selection, and salt tolerance persisted after a passage on a salt-free media. Their K^+ and Ca^{2+} content remained higher than in control cells for increasing NaCl concentration in the medium, suggesting a modification of cell membrane permeability as the main cause of NaCl tolerance. Shoots and plants regenerated from selected cell lines showed improved growth and salt tolerance. Calli induced from these plants tolerated a salt concentration of 10 g/l, indicating the persistance of the selected trait.

Key words: Salt tolerance – In vitro selection – *Poncirus trifoliata* – Somaclonal variation – Citrus

Introduction

Citrus plants are very sensitive to salinity and genes for salt tolerance are not known in the genus (Furr et al. 1963). Nevertheless, problems of salt toxicity are frequent in many tropical and subtropical countries where this crop is important. When classical breeding involving sexual crossing is not effective or is too time-consuming, mutation and cell selection can be used. In many plant species somaclonal variation and selection of salt-tolerant cells by culture on selective media have succeeded in isolating modified cell lines. However, many of those lines were unstable, and tolerance was lost after cultivation under unselective conditions. On the other hand, the regeneration of tolerant plants has rarely been achieved.

The genus *Citrus* represents one of the few groups of woody species where cell culture and biotechnology have been successfully applied. In vitro selection of salt-tolerant cell lines and plant regeneration have been reported for several cultivated Citrus species (Kochba et al. 1982; Ben-Hayyim and Goffer 1989).

The present paper reports the different steps of in-vitro selection for sodium chloride tolerance in a citrus rootstock and describes experiments that confirm the stability of the selected trait and provide some information on its nature.

Materials and methods

Plant material and callus culture

Seeds of *Poncirus trifoliata* cv 'Pomeroy' were received from the National Institute of Agronomic Research of Marocco (INRA). Callus development was induced from embryos cultivated on the modified medium of Murashige and Tucker (1969). The medium was supplemented with 2,4-D (2 mg/l), benzylaminopurine (BAP, 2 mg/l), casein hydrolysate (1 g/l) and sucrose (5%). For induction of somatic embryos and adventitious shoots, the best results were obtained with NAA (1 mg/l) and BAP (5 mg/l). Shoot elongation and rooting were promoted by gibberellic acid (1 mg/l) or NAA (1 mg/l) (Beloualy 1991). For the selection of tolerant cells, calli were divided into small fragments (± 50 mg fresh weight), which were cultivated in petri dishes on media containing NaCl at various concentrations.

Callus growth

Calli were transferred to fresh culture medium every 4 weeks and, at the same time, their weight was measured. The effect of the different salt concentrations was assessed by comparing growth curves and final weight of calli. In general, growth rate is expressed by the weight increase divided by the initial weight of the callus fragments.

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Determination of ion content

Calli of ±250 mg were grown for 4 weeks in the presence of different concentrations of NaCl. Samples were then calcined and acid digested according to Cotlove (1965) and Rodier (1976). Cations (Na^+, K^+ and Ca^{2+}) were analysed by emission spectrometry and Cl^- was titrated using the method of Charpentier-Volhard (Rodier 1976).

Results

Determination of selective NaCl concentrations

Lethal concentrations of NaCl were estimated after the inoculation of callus fragments (±50 mg) on culture media containing up to 15 g/l NaCl. The weight of the calli was measured after 4 weeks (Table 1). Growth was completely inhibited for the highest concentrations (12.5 and 15 g/l), and almost all calli died. Growth was reduced to 50% of that of the control at 5 g/l NaCl and to 34% at 10 g/l. These two latter concentrations are not lethal to the cells in culture, but they probably reduce the rate of cell proliferation, and they will be used in the following experiments for selecting salt-tolerant cells that could possibly be present in the calli.

Selection of NaCl-tolerant cell lines

The first step was the culture of a large number (13,984) of callus fragments (±50 mg) under selective conditions (5 or 10 g/l NaCl) for 4 weeks. Only 6 calli grew normally under these conditions remaining in good condition and reaching a weight comparable to that of the calli cultivated on a salt-free medium. Four lines (VR5-1 to VR5-4) were selected in the presence of 5 g/l NaCl: they derived from the same explant. Two other tolerant cell lines (VR10-9 and VR10-10), induced from a second original explant, were selected on the medium with 10 g/l NaCl.

The selected calli were then subcultured for 5 months on the same culture media, where they grew as well as the unselected lines on NaCl-free medium (Fig. 1). Subcalli were then transferred to a NaCl-free medium for 3 months. Their evolution was very diverse: 61.5% of them died, 27.5% showed reduced growth and only 11% had growth similar to that of the control. Such a heterogeneity could be due to different mutations or other variations induced during in vitro culture; some lines seemed to be salt dependent. Subcalli that grew well on NaCl-free medium were used for the subsequent experiments and for plant regeneration. A new transfer to the selective media confirmed their salt tolerance: their growth rate was not affected by the presence of 10 g/l NaCl.

Growth of selected cell lines

Selected and unselected calli of ±250 mg (fresh weight) were cultivated for a month on media containing 0, 2.5,

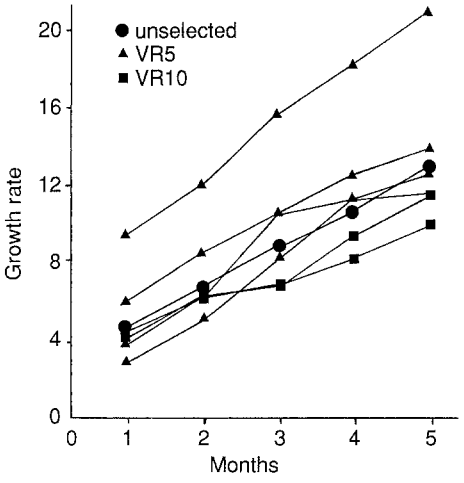

Fig. 1. Growth rate (increase of fresh weight divided by initial weight) of six cell lines (*VR5* and *VR10*) selected and cultivated in the presence of 5 and 10 g/l NaCl, and of unselected calli cultured on a salt-free medium

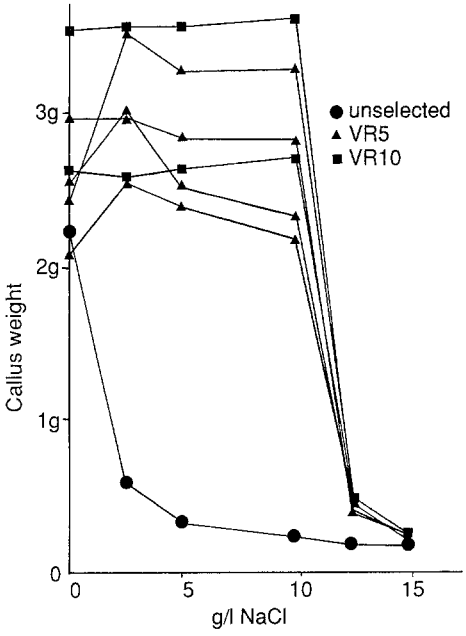

Fig. 2. Callus fresh weight of one unselected cell line and six selected cell lines cultivated for 1 month in the presence of different salt concentrations

Table 1. Weight of calli cultured on media containing 0–15 g/l NaCl

NaCl (g/l)	Weight ± SD (mg)
0	467 ± 101
5	233 ± 38
10	168 ± 13
12.5	42 ± 3
15	44 ± 3

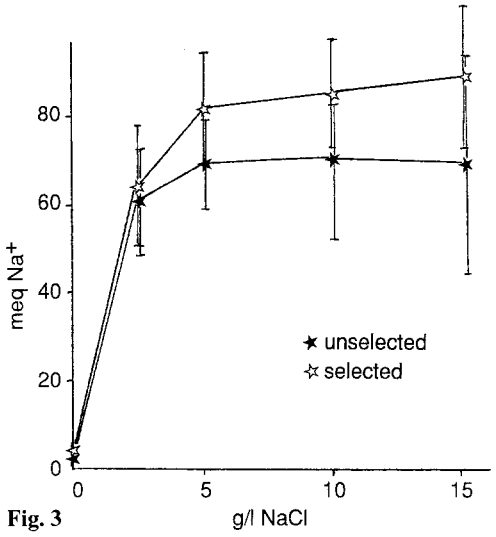

Fig. 3

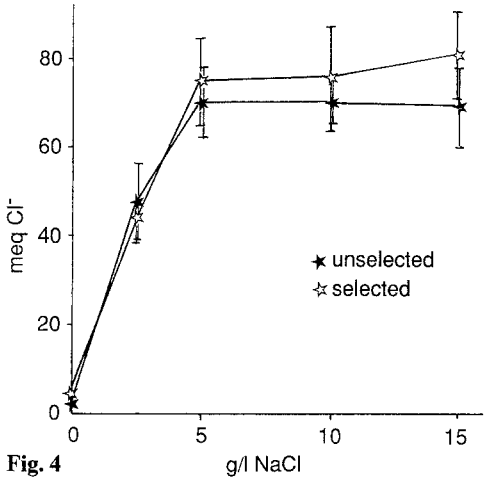

Fig. 4

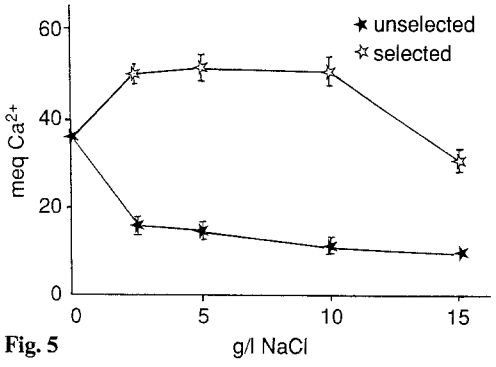

Fig. 5

Figs. 3–6. Ion content (means and standard deviations) in unselected and salt-tolerant cell lines for different NaCl concentrations in the culture medium; values are given in milliequivalents/100 g dry weight

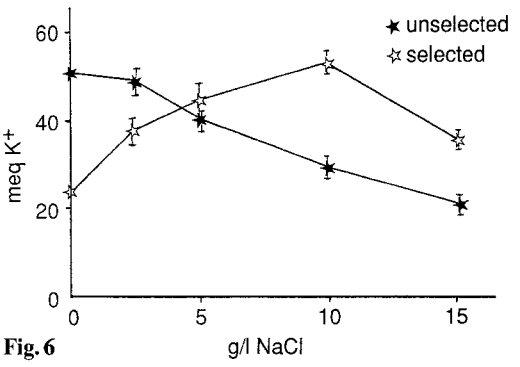

Fig. 6

5, 10, 12.5 or 15 g/l NaCl. The same experiment was repeated twice, 6 and 12 months after somaclone isolation. Figure 2 illustrates the results for an unselected cell line and for the six salt-tolerant cell lines tested 12 months after selection: the weight of 30 calli was measured for each salt concentration. It can be seen that the growth of unselected calli is significantly reduced for the lowest concentration (2.5 g/l). In comparison, cell growth of selected lines was improved for moderate salt concentrations while no strong growth reduction was observed below 12.5 g/l NaCl.

Mineral cell content

Sodium chloride can affect the cells through ion toxicity or ion unbalance by reducing the availability of other elements like potassium and calcium. When calli were cultivated on saline media, the absorption of Na^+ and Cl^- was much slower in salt-tolerant cell lines than in unselected ones. Nevertheless, after a few weeks, and for NaCl concentrations higher than 2.5 g/l, the Na^+ content (milliequivalents for 100 g dry matter) was significantly higher for tolerant cell lines (Fig. 3) than for unselected lines. The situation was similar for Cl^-, but the differences between selected and unselected cells were smaller (Fig. 4).

The calcium content was similar in the different calli cultivated on a NaCl-free medium, while the potassium level was significantly lower for salt-tolerant cells (Figs. 5, 6). With increasing concentrations of NaCl in the culture medium, the amount of K^+ and Ca^{2+} decreased in the unselected cells, but increased in the selected calli, where it remained high for salt concentrations of 2.5–10 g/l.

Under normal conditions, the K^+/Na^+ ratio was very high (189); with 2.5 g/l NaCl in the culture medium, it fell down to 0.7 after 3 days in unselected cell lines, but remained high in selected ones (3.5). The evolution of the K^+/Na^+ ratio was similar: from 105, it went down to 0.008 in the control cells and to 0.64 in selected cells. Differences between unselected and selected lines were still larger for the higher NaCl concentrations.

Plant regeneration

Five hormonal combinations were tested for shoot differentiation: culture medium number 300, containing abscisic acid and adenine, gave the best results. Unselected and selected calli (VR10) were put onto the different media, without or with NaCl (10 g/l). Table 2 gives the percentages of regenerating calli after 4 weeks. Two samples of calli were used for both lines, and the percentages were established after the elimination of infected calli. It is significant that regeneration from unselected calli only occurred in the absence of salt. Tolerant calli (VR10), on the contrary, were not affected by the presence of NaCl in the culture medium: the efficiency of shoot differentiation remained high for the different salt concentrations up to 10 g/l, the highest concentration (15 g/l) being lethal. The percentages of regenerating calli were similar in both samples.

For shoot elongation, concentrations of adenine and abscisic acid were reduced to 4 and 0.1 mg/l respectively; shoot development was not affected by the presence of 5 or 10 g/l NaCl. Table 3 reports the numbers of shoots derived from unselected calli that elongated and rooted when cultivated on a salt-free medium and in the presence of 2.5 g/l NaCl, compared to the evolution of shoots of the VR10 selected calli induced and maintained on culture media (with 0, 5 and 10 g/l NaCl). The percentages are calculated from two samples of 30 shoots. Rooting as well as elongation were not affected by NaCl in tolerant shoots, and the lines appeared similar: thus, salt-tolerance selected at the cell level was expressed in the regenerated shoots. On the contrary, shoots derived from unselected calli did not tolerate 2.5 g/l NaCl.

Rooted plantlets were transferred first to sterile vermiculite and later to soil. After 4 weeks, some plantlets developed new leaves, but recovery was slow and irregular.

Growth of selected plants

For a more precise comparison, cuttings of ± 1 cm were taken from plants derived from unselected cell lines and from plants regenerated from tolerant calli after shoot differentiation on three different media: salt free (TO) or containing 5 or 10 g/l NaCl (T5 and T10). Thirty-two cuttings were used from each group, and their size was checked 7 weeks after inoculation (Table 4). On NaCl-free medium, salt-tolerant plantlets were taller than unselected ones. On the other hand, their growth remained satisfactory on media containing up to 10 g/l salt. For higher concentrations (12.5 and 15 g/l), the plantlets grew similarly to the unselected ones when cultivated in the presence of 2.5 g/l NaCl.

In a second assay, unselected and tolerant rooted plantlets were placed on three culture media (0, 2.5 and 10 g/l NaCl) and their size compared after 4 weeks. The initial size of plantlets was ± 1.5 cm; means and standard deviations were calculated for 60 plants (Table 5). These observations confirmed the better growth of tolerant plants under the different conditions.

Calli derived from tolerant plants

The stability of the selected trait was tested by a comparison of calli induced from unselected and tolerant plants regenerated in the absence of NaCl (TO) or on a medium containing 10 g/l NaCl (T10); 50 calli from each of the different groups, each weighing about 250 mg, were culti-

Table 2. Percentages of shoot differentiation, in relation to salt content for one unselected and two selected calli (two replications)

NaCl concentration (g/l)	Unselected		VR10-9		VR10-10	
	A	B	A	B	A	B
0	46	61	54	45	41	44
2.5	0	0	59	52	49	37
5	0	0	59	63	44	55
10	0	0	61	50	38	57
15	0	0	0	0	0	0

Table 3. Percentages of elongated and rooted shoots from unselected and selected plants for different media (means and standard deviations)

Cell lines	NaCl (g/l)	Elongated shoots	Rooted shoots
Unselected	0	26 ± 4.1	30 ± 1.5
	2.5	0	0
VR10-9	0	31 ± 3.3	23 ± 2.3
	5	35 ± 1.5	32 ± 5.1
	10	42 ± 2.2	27 ± 4.2
VR10-10	0	48 ± 5.2	27 ± 3.2
	5	42 ± 3.6	20 ± 0.6
	10	53 ± 1.8	20 ± 1.8

Table 4. Influence of salt concentration on the size (cm) of unselected shoots and of salt-tolerant shoots regenerated on salt-free medium (T0) or in the presence of 5 or 10 g/l NaCl (T5 and T10)

NaCl (g/l)	Unselected	T0	T5	T10
0	4.49 ± 4.1	7.88 ± 3.3	6.94 ± 3.1	7.52 ± 2.2
2.5	3.61 ± 1.7	8.03 ± 1.2	8.28 ± 1.4	7.16 ± 1.4
5	2.77 ± 1.0	7.81 ± 1.2	7.28 ± 2.8	8.01 ± 1.2
10	–	6.79 ± 1.1	7.65 ± 1.1	7.74 ± 1.7
12.5	–	3.49 ± 0.9	3.45 ± 2.6	3.64 ± 0.5
15	–	3.49 ± 0.7	3.08 ± 1.2	3.19 ± 0.7

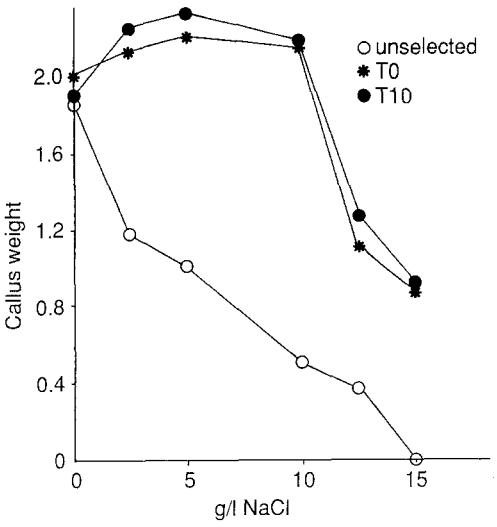

Fig. 7. Weight (g) of calli induced from unselected plants and from salt-tolerant plants regenerated in the absence of salt (*T0*) and in the presence of 10 g/l NaCl (*T10*). The calli were cultivated on different saline media

Table 5. Shoot and root lengths, and numbers of leaves of unselected and salt-tolerant plantlets at three NaCl concentrations (means and standard deviations)

NaCl (g/l)	Origin	Shoot length (cm)	Root length (cm)	Number of leaves/ plants
0	Unselected	3.95±1.6	8.87±3.6	4.5±1.8
	Tolerant	6.17±3.3	8.69±5.6	8.0±2.1
2.5	Unselected	3.62±1.2	6.63±2.5	3.4±0.9
	Tolerant	5.82±3.5	8.74±5.7	8.2±1.4
10	Unselected	1.60±0.0	1.66±0.0	–
	Tolerant	5.74±3.1	7.67±6.1	8.4±1.1

vated for 4 weeks on different media (Fig. 7). Growth was similar for calli induced from plants regenerated under both conditions. These results shows the persistence of salt-tolerance in these cells, but final evidence of the stability of the selected trait depends on the production of a seed progeny.

Discussion

Many attempts have been made previously to use somaclonal variation and in vitro selection for isolating salt-tolerant cell lines in several plant species. Unfortunately, regeneration is often impossible after the long periods of in vitro culture required for cell selection. In addition, the selected trait is frequently lost during the subcultures or disappears in the regenerated plants. *Citrus* species are among the very rare woody crops where some authors have obtained NaCl-tolerant cell lines and plants (Ben-Hayyim et al. 1985).

The procedure used here for *Poncirus trifoliata* was designed in order to maximise the homogeneity of recovered cell lines and involved four successive steps: (1) sort out lines tolerating NaCl concentrations sublethal for normal cells, (2) subculture 5 times (1 month each) in the presence of selective salt concentrations, (3) grow on a NaCl-free medium and eliminate subcultures unable to sustain a normal growth and (4) confirm the selected tolerance by returning to lethal salt concentrations. Cell lines selected in that manner appeared to be very stable in subsequent subcultures and tests and differed steadily from the control. The same differences persisted in the regenerated plantlets and in the calli induced from those. Both selected cells and plantlets tolerated a NaCl concentration of 10 g/l. In tolerant lines, growth reduction and necrosis due to salt concentrations of 12.5 and 15 g/l were comparable to the damage induced by 2.5 g/l NaCl on unselected cells.

Somaclonal variation can result from modifications present in the explant or it can occur during the subcultures. In the present case, the six calli sorted out in the first selection step derived from two embryos. They reached the same weight and their subclones remained similar during the following years. Thus, it seems that the selected modifications were either present in the explants or induced in the two primary calli before subculture.

Ben-Hayyim and Goffer (1989) reported the regeneration of plantlets from tolerant cell lines of *Citrus sinensis* selected and cultured on a salt-free regeneration medium, but they did not succeed in plant regeneration in the presence of NaCl. Under the conditions described here, shoot differentiation was equally successful on both media, and the plants from both origins were similar in terms of growth and salt tolerance.

Various modifications of cell characters can account for the salt tolerance of calli and plants. In flax, McHughen (1987) ascribed the salt tolerance of a selected mutant to a general improvement of vigour. Such a modification is also probably involved in *Poncirus trifoliata*. Although callus growth on salt-free medium was not significantly improved, the selected plantlets grew faster in the presence and absence of salt. Nevertheless, the most important trait of the selected lines probably lies in a modification of the cell membrane's permeability, which is expressed by a slower penetration of Na^+ and Cl^- ions, the final concentration of salt remaining similar, and by a more efficient penetration of K^+ and Ca^{2+}. The sensitivity to high salinity shown by *Poncirus* does not appear to be related to the toxicity of an ion nor due to a higher osmotic pressure, but to depends at first an a ion competition, which leads to a deficiency in essential elements like potassium and calcium. Selected cells toler-

ate higher NaCl concentrations thanks to an enhanced uptake and retention of these elements.

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