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Selection and breeding for salinity tolerance *in vitro**

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Key words *Citrus sinensis* *Citrus aurantium* Cloning Ovular callus Sodium chloride Stable variants

Summary Selection for tolerance to NaCl in *Citrus sinensis* and *C. aurantium* has been carried out in agar and suspension cultures. Callus was subjected to culture media containing up to 0.17*M* NaCl for ten passages. Selected cell lines were grown for three passages on media without salt before further tests on saline media. Four stable tolerant cell lines, differing in degree of tolerance, have been selected from *C. sinensis*. Four lines of similar tolerance have been selected from *C. aurantium*. The stability of most lines was very satisfactory. Most *C. sinensis* lines grew well in media containing up to 0.2*M* NaCl, and *C. aurantium* lines in media of up to 0.15*M* NaCl.

Embryos were regenerated in most selected cell lines from *C. sinensis* and, more sporadically, from *C. aurantium*. Addition of 0.5–0.6% NaCl to the media often enhanced embryogenesis. Embryos from a selected line of *C. sinensis* showed higher tolerance to NaCl in the medium than comparable embryos from an unselected line.

Single embryos derived from both selected and unselected cell lines of *C. sinensis* were successfully cloned. A limited comparison of plantlets from one tolerant line (R14) with plantlets from unselected control lines showed better adaptation of the former to salt (0.085 to 0.12*M* NaCl in the medium), and a lesser degree of leaf burn symptoms.

Introduction

A major advantage of cell culture for genetic manipulation with higher plants is the opportunity to select for new phenotypes from large cell populations cultured under defined conditions. For a trait to prove agriculturally useful, the characteristics selected from testing *in vitro* should be stable in the absence of the stress agent, and the cell lines carrying that trait should be easy to propagate, *i.e.* show satisfactory initiation and development of embryos. Moreover, one has to be aware that selection for the novel phenotype is conducted at a level of differentiation different from that at which expression of the phenotype is desired[3].

Cell lines exhibiting resistance or, rather, relative tolerance to salt stress have been reported[5,6,8,19,23]. The relative degree of salt tolerance of callus cultures derived from two barley species (*Hordeum vulgare* and *H. jubatum*) seemed to correspond to that found in the whole plants[20]. Salt-resistant plants from a cell line of tobacco have been regenerated. Progeny obtained by selfing of regenerated plants also

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proved tolerant to sodium chloride. In recent experiments a selected NaCl-tolerant tobacco line (after 50 generations in cell culture) lacked stability[8].

Fruit trees, except for the date palm and to a lesser extent also pistachio, olive and pomegranate, are very sensitive to irrigation water with a high salt content[15]. Citrus species rank among the most sensitive to saline water[4]. Thus, breeding for rootstocks or, possibly, own-rooted cultivars with a high tolerance to salt could prove very important in many arid areas where irrigation is needed. Selection for genetic variants derived from cultured material might also serve as an important step for further conventional breeding. In the present paper, isolation of several stable salt-tolerant callus lines, from both 'Shamouti' orange (*Citrus sinensis*) and 'Sour orange' (*C. aurantium*), will be described, as will the performance of embryos and plantlets derived from two of these callus lines. The ovular (nucellar) callus system seems well adapted for mutant selection because of the following characteristics: growth and maintenance of callus without exogenous growth substances (auxins and cytokinins), embryogenic capacity, stable diploid state, and ease of cloning.

Materials and methods

Callus cultures were initiated from nucelli and ovules of 'Shamouti' (*Citrus sinensis*) and 'Sour orange' (*C. aurantium*) by methods developed in our laboratory[11,14]. Further details of the technique and of the selection procedure devised have been described recently[21]. For *C. sinensis*, growth and selection for tolerance to salt were done on a solid medium (agar), while for *C. aurantium* a suspension culture was used. Selection started with the inoculation of 1000 explants (40 mg fresh weight each) on an agar medium containing 0.085 *M* NaCl. After 5 weeks of growth the best-growing cultures were selected to initiate cell lines (ten replicates each). Recurrent selection was performed over ten passages of 5 weeks each, with 0.085 *M* NaCl in the medium. The five best explants were then selected on the basis of growth or embryogenic capacity. To test stability, the selected tolerant lines were subcultured for three passages in the absence of NaCl, then recultured in the presence of salt. The lines selected were grown on various media to determine the most suitable medium for embryo formation. Embryos were counted at 10× or 40× magnification. Small globular embryos were developed into cotyledonary embryos in suspension culture containing 2% sucrose, 1 g l^{-1} malt extract, and 1 mg l^{-1} gibberellic acid (GA_3). A procedure for cloning individual embryos was also developed, to provide uniform material for comparison of salt tolerance between plants derived from selected cell lines and plants of the wild type.

Ovular callus from 'Sour orange' was grown in suspension on a gyratory shaker (100 cycles/min), starting with 250-mg explants. For measurement of fresh weight, cells were collected by vacuum filtration through Whatman No. 1 filter paper after 3 weeks of growth. Selection was done in a manner similar to that described for 'Shamouti.' Because of the faster rate of growth of 'Sour orange,' transfers from the liquid medium were made every 21 days. Selection was done in the presence of 0.12 *M* NaCl. Because of partial loss of embryogenic capacity in the selected 'Sour orange' cell lines, no whole-plant comparisons have yet been made with this species between plantlets derived from selected cell lines and from a cell line of the wild type. Experiments to improve embryogenesis, as well as subsequent embryo growth and development in these cell lines, are still in progress.

Results

Salt tolerance and embryogenesis in selected callus

On the basis of the selection procedure outlined, ten 'Shamouti' orange callus lines with significantly better growth on NaCl than that of the sensitive control were isolated. Of these, four have been retained for further tests. Growth of these lines in the presence of 0.1 to 0.2*M* NaCl, relative to the control, is shown in Figure 1. The four tolerant lines retained (R4, R7, R10 and R13) differ in their performance. Line R10 shows no decline in growth at 0.1*M* NaCl relative to growth in a medium with no salt. This line retains over 35% relative growth at 0.2*M* NaCl. The other three selected lines performed less satisfactorily, especially in the presence of salt concentrations exceeding 0.1*M* NaCl. Still, line R4 showed 28% relative growth at 0.2*M* NaCl. By comparison, the unselected control line achieved only 33% relative growth at 0.1*M* NaCl and very little growth at 0.2*M* NaCl. Stability tests for the four selected lines (results not shown) indicated that all lines had much better growth in the presence of 0.1*M* NaCl than did the control when the test was performed after three passages on medium without salt. However, growth on 0.2*M* NaCl after three passages without salt was decidedly better for some lines (R10) than for others (R4)[9].

Figure 2 presents the relative growth (percent of control) of the four selected cell lines of 'Sour orange' in the range of 0–0.2*M* NaCl. All four lines were distinctly superior in growth to the unselected line in the presence of salt. The unselected line ceased to grow in the presence of 0.15*M* NaCl. Differences in growth response of the selected lines were comparatively slight. One line (01-7-3) attained as much as 50% growth in the presence of 0.2*M* NaCl, compared with the control. In stability tests (Fig. 2B) performed after three passages on a medium without salt, growth of line 01-7-3 was definitely superior to that of the unselected control, but inferior to that attained by the three selected lines 01-7-2, 01-7-4, and B-5-1. These three lines performed similarly, manifesting relatively good growth (50% of control) with 0.15*M* NaCl, and very little growth with 0.2*M* NaCl.

The selection of stable tolerant cell lines resulted, however, in impairment of the embryogenic capacity in certain lines, especially in the selected lines of 'Sour orange.' In 'Shamouti' orange, with galactose as a carbon source, only the unselected line L1 and the selected lines R4 and R13 maintained a fairly high embryogenic capacity. In further experiments the addition of 0.085 or even 0.17*M* NaCl to the galactose medium significantly enhanced the capacity of lines R13 and R14 to form embryos. Line R14 at present forms embryos on both galactose

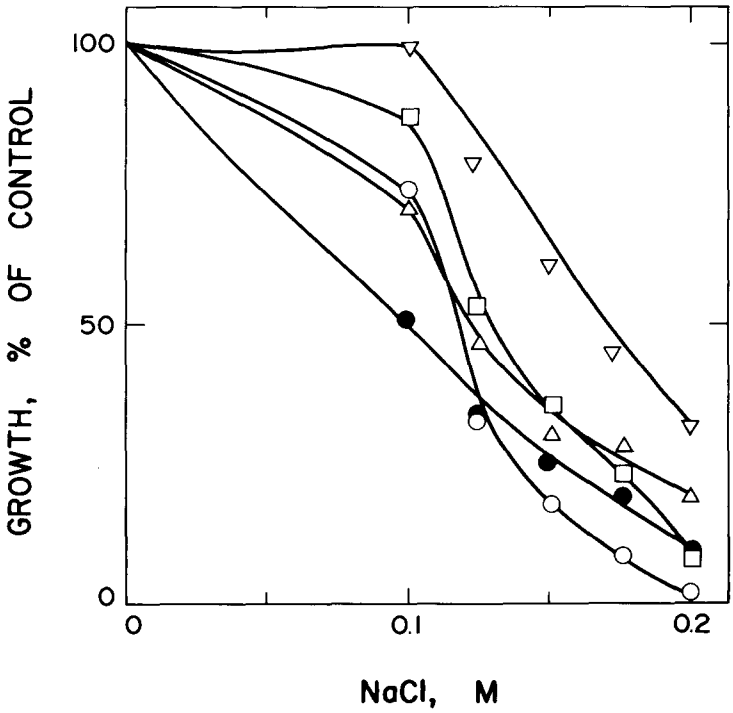

Fig. 1. Relative growth of the salt-sensitive and four salt-tolerant cell lines of 'Shamouti' orange (*Citrus sinensis*) after recurrent selection (ten passages) in the presence of 0.085 *M* NaCl. • Control line; △ R4; ○ R7; ▽ R10; □ R13. Cells were collected after one month of growth. 100% growth for control line, R4, R7, R10 and R13: 430, 565, 650, 695 and 515 mg, respectively.

and sucrose, even without the addition of NaCl. Experiments performed recently showed that the latter line forms embryos quite readily also in media with sucrose as a carbon source. In contrast, line L1 (unselected control) forms no embryos on sucrose. Enhancement of the embryogenic capacity of line R14 was achieved also by including antigibberellins such as Alar (succinic acid, 2,2-methylhydrazide) or PP333 (1-(4-chlorophenyl)4,4-dimethyl-2(1,2,3-triazole-lyl)pentan-3-ol) in the galactose medium.

A more definite loss of embryogenic capacity and a greater difficulty in regaining this capacity was evident with selected salt-tolerant cell lines of 'Sour orange.' However, the control line also shows a decrease in embryogenic capacity. With 'Sour orange,' too, galactose proved the most suitable carbon source for the manifestation of embryogenic capacity, which was enhanced by the addition of 0.1 *M* NaCl to the medium. The possible role of antigibberellins in cell lines of 'Sour orange' is still unclear. None of these lines formed embryos in the presence of sucrose.

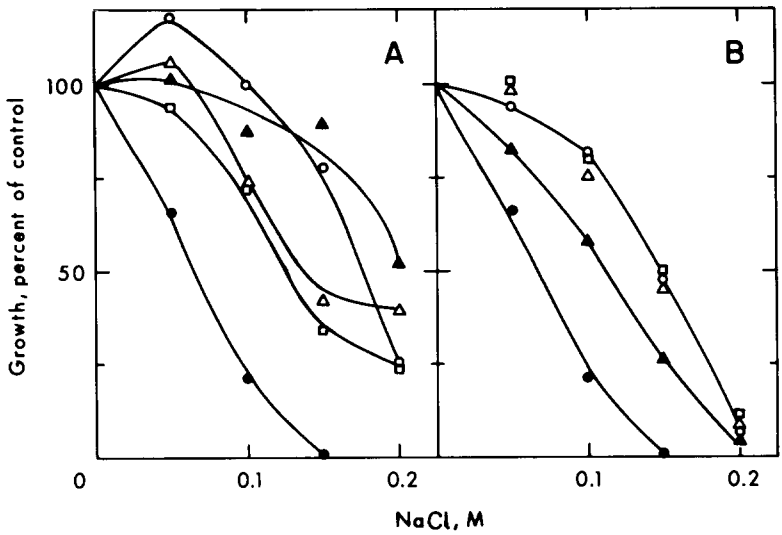

Fig. 2. Relative growth of salt-sensitive and four salt-tolerant cell lines of 'Sour orange' (*Citrus aurantium*) in the presence of NaCl. *A*: relative growth after recurrent selection (ten passages) on 0.17 m*M* NaCl. *B*: Relative growth after recurrent selection and three further passages in the absence of NaCl. • Control line; ▲ 01-7-3; ○ 01-7-2; △ 01-7-4; □ B-5-1. 100% growth for control line, 01-7-3, 01-7-2, 01-7-4 and B-5-1: 309, 453, 530, 437 and 398 mg, respectively.

Salt tolerance of selected embryos

Small, globular embryos from a salt-tolerant 'Shamouti' orange line (R13) and from an unselected control line (L1) were placed in Murashige and Skoog's[17] basal medium (containing 5% sugar, without phytohormones), as modified by Murashige and Tucker[18], to which 0–0.25 *M* NaCl had been added. The relative weights of the embryos after 6 weeks of growth (compared with their weight on basal medium with no added salt: 312 mg) are shown in Table 1. Differences in final weight between

Table 1. Inhibition of growth of embryos derived from a salt-tolerant 'Shamouti' orange line and a non-selected susceptible line, on media with different NaCl concentrations. Results expressed as relative weight of embryos weighed after 6 weeks of growth (100% = 312 mg)

NaCl in medium (*M*)	R13 tolerant line (% of control)	L1 susceptible line (% of control)
0	100	100
0.085	51	40
0.130	39	40
0.170	20*	10**
0.210	16	8
0.255	11	6

* 60% browning
** 100% browning

embryos of the two lines were slight. However, while globular embryos from the salt-tolerant line developed into cotyledonary embryos (3–5 mm long) and remained green on media containing up to 0.13*M* NaCl, embryos from the unselected cell line L1 turned brown in such media. On 0.17*M* NaCl, 60% of embryos from the selected cell line turned brown, as compared with 100% of those derived from the unselected line.

Salt tolerance of selected rooted shoots and plantlets

A method of cloning embryos (P. Spiegel-Roy and S. Saad, unpublished) has been developed to improve the genetic uniformity and to obviate the effects of variations in embryo size and shape. Cloning was achieved by a series of steps involving a cytokinin for shoot proliferation and an auxin-like substance for rooting. With this procedure, uniformly rooted shoots could be obtained from both selected cell lines and the unselected control. These lines were compared with respect to their performance on media or in planting mixtures containing added NaCl.

Rooted shoots of two salt-tolerant lines, R7 and R14, were tested for their response to the presence of 0.12*M* NaCl in the medium. The performance of these shoots was compared with that of shoots from an unselected line for over one month. Shoots of comparable size and vigor were selected. All shoots from plants derived from the unselected cell line showed severe stunting, leaf burn, and progressive wilting; however, 21 shoots of comparable size derived from plants obtained from the selected cell line R7 survived (out of a total of 55 rooted shoots, *i.e.* 40%). All of the latter showed no damage, while ten rooted shoots from R7 showed some leaf burn.

In another experiment, well-rooted plantlets about 3 cm tall were used. Plants from an unselected cell line, C32, served as a control; and comparable plants from a selected cell line, R14, were tested under the same conditions. The experiment was continued for 10 weeks and the results are summarized in Figure 3. Performance was rated on a scale of three: 1, no leaf burn; 2, partial leaf burn; and 3, total leaf burn. Altogether, 30 plants (15 from each line) were used. These were divided into three lots and grown in test tubes on basal medium, without NaCl, and with 0.085 and 0.12*M* NaCl. No damage to either line was evident on basal medium without the addition of NaCl. In media containing 0.085 or 0.12*M* NaCl, a larger proportion of plants from the unselected control showed leaf burn, and to a higher degree. The tolerance of plantlets derived from the selected cell line can be considered satisfactory, especially with 0.085*M* NaCl. A large-scale comparison of

plants in a potting medium of peat and perlite is now in progress in the greenhouse.

Discussion

There is increasing awareness of the possibilities and limitations in the use of techniques of callus and cell suspension culture for the development of new cell lines with valuable characters for agricultural use, particularly in relation to resistance to stress and to herbicides[2,3,16]. Cell lines from crop plants showing resistance to salinity have been reported[5,6,8,23], and in a few cases plants regenerated and were tested[19]. In one case a stepwise increase in NaCl in the medium resulted in a salt-tolerant Nicotiana line stable through 24 generations in the absence of added NaCl. Gene amplification was postulated as an explanation of the phenomenon[23]. Another possibility with our citrus material is that the enhanced ability to grow in the presence of comparatively high NaCl concentrations (0.15–0.2*M*) may have originated from pre-existing resistant cells in the ovular callus[9]. We isolated four tolerant, stable cell lines from *C. sinensis*, and four from *C. aurantium*. While the isolated lines of *C. sinensis* cv. 'Shamouti' differed in their degree of tolerance, those isolated from 'Sour orange' (*C. aurantium*) were quite similar to one another. The stability of most of the lines was satisfactory. No similar degree of stability was attained in experiments on the selection of a NaCl-tolerant tobacco cell line[8].

Maintenance of tolerance in cell lines throughout all subsequent

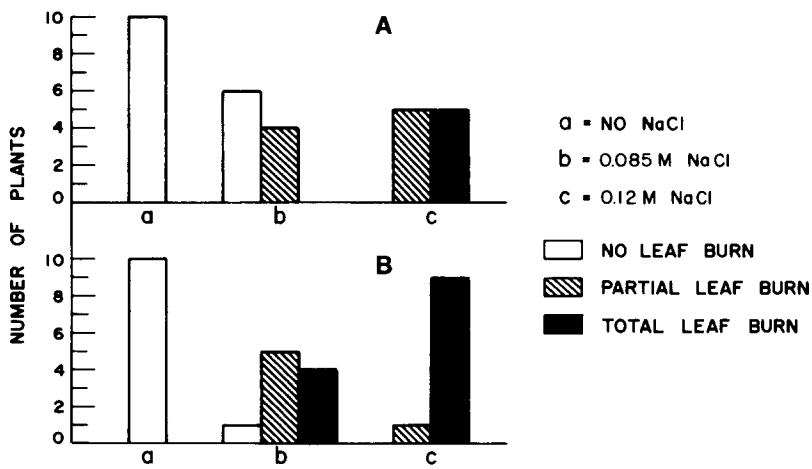

Fig. 3. Effect of sodium chloride in the medium on leaf burn in 'Shamouti' plantlets cloned from salt-tolerant (A) and salt-sensitive (B) ovular callus.

stages of plant development is considered to pose a problem. The theoretical basis involved has been discussed recently[3]. The morphogenetic characteristics of cells from NaCl-tolerant populations differ from those of cells never exposed to salt[7]. Cold-resistant carrot cell lines, although stable in resistance at the callus level, did not exhibit comparable cold resistance at the embryo stage[22]. However, in our case embryos from a selected cell line grew and developed more satisfactorily than wild-type embryos of comparable size and stage on media containing various concentrations of NaCl. Moreover, a definite enhancement in the presence of 0.085 or 0.1 *M* NaCl and, in some cases even 0.17 *M* NaCl, of embryo formation was noted in both *C. sinensis* and *C. aurantium*. In the latter, adding NaCl to the medium has proved to be the only effective means of achieving even a moderate degree of embryogenesis[1,13]. It is not yet clear whether the effect of NaCl is specific, or if NaCl enhances embryo formation in a manner similar to that attained by use of antigibberellins[12] or sucrose starvation[10].

The lack of uniformity of plant material derived from callus has been pointed out repeatedly[16]. The presence of embryos of different developmental stages creates further difficulties in comparing tolerance of selected material derived from callus or cell suspensions with the unselected control. The method of cloning individual embryos developed in our laboratory has enabled us to initiate comparisons on the basis of fairly uniform rooted shoots.

Two of these comparisons, each with a different selected salt-tolerant line of *C. sinensis*, have revealed better adaptation of plantlets derived from selected tolerant cell lines to salt in the medium than of plantlets from the unselected control. No such comparison has yet been performed with *C. aurantium*. Lengthy selection procedures and growth in the presence of high concentrations of the stress agent resulted in both a reduction in the embryogenic capacity of cell lines and a decrease in the regularity of embryo development in at least some of the lines involved. Resuppression of embryogensis in citrus by certain medium components probably involved not only sugars[13] or glycerol[1] but also NaCl[9] and possibly antigibberellins in the medium. These measures, combined with perfecting the procedure for cloning individual embryos, will enable us to adequately test the transmission of the trait of salt tolerance from cell lines to whole plants.

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