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SELECTION OF FROST-TOLERANT CELL LINES FROM CELL CULTURES OF *SOLANUM TUBEROSUM* L.

ABSTRACT

Fourteen hydroxyproline-resistant cell lines were selected by plating 7 days old cell suspensions of *Solanum tuberosum* L. cvs. Desiree and Maris Piper on a cell plating medium containing 5 or 10 mM hydroxyproline (hyp). Cell suspensions were either plated directly on selective media or after mutagenic treatment with gamma rays at a dose of 20 Gy or after freezing to –6°C. The frequency of resistant colonies varied from 0.15 to 0.35 × 10^{-6}. Almost all the selected lines possessed increased levels of frost tolerance as compared to their non-selected controls except one, indicating that hyp resistance and frost tolerance are not necessarily linked.

Key words: cell culture, frost tolerance, hydoxyproline resistance, potato, proline, *Solanum*

INTRODUCTION

Tissue culture techniques can be used as convenient aids for selection procedures. It has long been established that unorganised tissue cultures tend to be cytogenetically unstable. Changes in both number and structure of chromosomes have been observed, and these increase as the culture period is prolonged. Thus, tissue culture-induced, or somaclonal, variation can be exploited and selection for a desirable trait be made.

Some early investigators attempted to correlate the amino acid contents of plants with frost tolerance, but the results presented a confusing picture (Levitt 1980). In some plants the amino acid content increased with increase in frost hardiness, in others no relationship was found. Of the amino acids, proline has been reported by several investigators to accumulate during hardening or to increase more in hardy plants (Stefl *et al.* 1978, van Swaaij *et al.* 1985). Proline accumulation has often been shown to occur in plants as a consequence of environmental stress. A significant increase in proline level has also been reported when cell cul-

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tures are subjected to salt stress (Eberhardt and Wegmann 1989, Dridze *et al.* 1991, Sumaryati *et al.* 1992, Chauhan and Prathapasenan 1997) or physiological drought (Corcuera *et al.* 1989, Sumaryati *et al.* 1992). The physiological role of this accumulation is assumed to be associated with ability of proline to act as osmoregulator by protecting cytoplasmic enzymes and stabilising the structures of macromolecules and organelles (Chauhan and Prathapasenan 1997).

Some workers also reported significant correlation between proline concentration and frost tolerance in different species, e. g. potato (van Swaaij *et al.* 1985), winter barley (Dobslaw and Bielka 1988) and winter wheat (Dorffling *et al.* 1990). Increased levels of proline in proline analogue-resistant lines, e. g. hydroxyproline-resistant potato (van Swaaij *et al.* 1986) and wheat lines (Tantau and Dorffling 1991) may lead to plants resistant to freezing stress. Therefore, hydroxyproline resistance could be used as an indirect route to the production of frost-tolerant lines. In the present studies, attempts were made to select frost-tolerant cell lines from cell cultures of potato cvs. Desiree and Maris Piper by the use of cellular selection for hydroxyproline-resistance.

MATERIALS AND METHODS

Preparation of cell material

Callus was initiated from leaf discs obtained from *in vitro* grown *Solanum tuberosum* L. cvs. Desiree and Maris Piper plantlets and cultured on MS medium (Murashige and Skoog 1962) containing 3 mg × l^{-1} 2.4-D and 0.3 mg × l^{-1} kinetin. Cell suspension cultures were initiated and maintained as described by Lam (1977).

Treatments investigated

In one treatment cell suspensions were plated directly on Lam (1977) cell plating medium containing 5 or 10 mM hydroxyproline (hyp). In a second treatment, cell suspensions were irradiated at a dose of 20 Gy (from Co^{60} source at the rate of 10 Gy × min^{-1}) and plated on the cell plating medium also containing 5 or 10 mM hyp. In a third treatment, cell suspensions were frozen in a freezer down to –6°C at the rate of 1°C × h^{-1} and kept at this temperature for at least 1 hour, thawed at 4°C for 1 hour and plated on the cell plating medium. After 4 - 6 weeks, growing colonies were transferred to solidified MS medium containing 5 or 10 mM hyp. In all the three treatments, 7 days old cell suspensions were used and plated at a plating density of 0.5×10^6 cells × ml^{-1}. At least forty petri dishes (10 for each hyp concentration per cultivar) were prepared for each experiment.

Selection for hyp-resistant cell lines

Resistant colonies, growing on the selective media, were transferred to solidified MS medium containing the same concentration of hyp and

maintained by subculturing onto fresh medium at 4 - 6 week intervals. The frequency of hyp-resistant cell lines was calculated using the following formula:

$$\nu = \frac{n}{N}$$

where,
ν - Frequency of resistant cell lines
n - No. of hyp-resistant cell lines
N - Total number of cells plated

Determination of frost tolerance

Calluses of the selected lines were frozen as 200 mg pieces in closed tubes and care was taken to prevent damage to callus structure. To avoid supercooling, samples were cooled at -1°C for 1 h and inoculated with a small piece of crushed ice to initiate freezing. Then the temperature was decreased at the rate of 2°C h^{-1} to various sub-zero temperatures (-2 to -8°C) and samples were exposed to each test temperature for 1 h. Samples were removed at each test temperature and thawed overnight at 4°C (Lee *et al.* 1992). Freezing damage was determined using the TTC-viability assay of Towill and Mazur (1975), as follows.

Two ml of 0.8% 2.3.5-triphenyltetrazolium chloride (TTC), prepared in 0.05 M K-phosphate buffer, pH 7.5, was added to the tubes containing callus withdrawn at different freezing temperatures and incubated for 18 h in the dark at 25°C without shaking. After incubation, cells were pelleted and washed once with deionized water. The red formazan was extracted by adding 3 ml of 95% ethanol to the pelleted cells and incubating at 60°C for 1 h. Brief heating (60°C) for about 15-30 minutes was necessary to remove formazan from large cell clumps. Absorbance of the extract was recorded at 485 nm in a digital spectrophotometer (CECIL, CE 1020). Cell survival at each test temperature was calculated according to the formula:

$$S_{\%} = \frac{r_1}{r_2} \times 100$$

where,
$S_{\%}$ - Per cent survival
r_1 - TTC reductions at freeze temperature
r_2 - TTC reduction at 25°C (control)

Freezing temperature that resulted in 50% cell death was determined as the frost-killing temperature (FKT).

Assessment of frost tolerance stability in the selected lines

Two lines showing higher levels of frost tolerance (D-13 and M-12), which had been selected to grow on media containing hyp, were transferred to the normal (hyp-free) medium. After 2 subcultures on normal medium, the degree of frost tolerance of these lines was determined as described in the previous section.

Osmoticum test

In the cell plating medium 0.05 M mannitol was added as an osmoticum and cell suspensions were also plated on this medium at a plating density of 0.5 × 106 cells × ml^{-1}. After 2 subcultures on the medium, the degree of frost tolerance of the cultures was determined as described in the previous section.

RESULTS

Selection of hyp-resistant cell lines

A. Direct Selection: When leaf-derived cell suspensions of *S. tuberosum* cvs. Desiree and Maris Piper were plated on Lam (1977) cell plating medium containing 5 or 10 mM hyp, growth of cells was completely inhibited. From 40 petri dishes prepared, a few cells were able to grow to form cell colonies. After 6 weeks, these colonies were transferred onto solidified MS medium containing the same concentration of hyp. Upon subculture, some colonies failed to survive, but those which grew well, were subcultured at 4 week intervals onto fresh medium. After 4 subcultures, out of 2×10^7 cells plated, only four colonies were found to be hyp-resistant and had been selected (Table 1). Therefore the frequency of hyp-resistant cell lines was only 0.20×10^{-6}.

Table 1

Hyp-resistant cell lines selected on MS medium containing 5 or 10 mM hyp using different selection procedures.

Sr. No	Selection procedure	Potato cultivar	Hyp concentrations	
			5 mM	10 mM
1.	Direct selection	Desiree	-	D-11 & D-12
		Maris Piper	M-1	M-11
2.	After mutagenic treatment	Desiree	D-1 & D-2	D-13
		Maris Piper	M-2 & M-3	M-12 & M-13
3.	After freezing treatment	Desiree	D-3	D-14
		Maris Piper	-	M-14

B. Selection after mutagenic treatment: Mutagenic treatment was carried out with gamma rays using a low dose of 20 Gy that was not found to affect the cell viability or growth after plating. Irradiated cell suspensions of both cultivars were plated on the cell plating medium containing

5 or 10 mM hyp. After 6 weeks only a few colonies were found growing and were transferred onto solidified MS medium containing the same concentration of hyp. After 4 subcultures, a total of seven colonies; four on the medium containing 5 mM hyp and three on the medium containing 10 mM hyp, were found growing (Table 1) and hence the frequency of hyp-resistant colonies recorded was 0.35×10^{-6}.

C. Selection after freezing treatment: Cell suspensions were frozen to -6°C, held at this temperature for at least one hour, raised to 4°C and plated on the cell plating medium. Growth of cells was inhibited at an early stage, but later small cell colonies started to grow. Eight colonies were isolated and four of these were transferred onto solidified MS medium containing 5 mM hyp, while the other 4 were transferred onto the medium containing 10 mM hyp. Only three colonies survived (Table 1) and therefore the frequency of hyp-resistant lines was only 0.15×10^{-6}.

Frost tolerance of the selected hyp-resistant cell lines

When hyp-resistant cell lines were well established on the selective media, these were used for determination of frost tolerance by using the TTC-viability assay of Towill and Mazur (1975). FKT values (°C) of these hyp-resistant lines and their controls are given in Table 2.

Table 2

Frost-killing temperatures (FKT) of selected hyp-resistant cell lines and non-selected controls

Genotype/cell line	FKT (°C)	Genotype/cell line	FKT (°C)
Desiree (control)	-2.8 ± 0.1	M. Piper (control)	-2.9 ± 0.0
D-1	-3.2 ± 0.2	M-1	-3.3 ± 0.0
D-2	-2.8 ± 0.0	M-2	-3.5 ± 0.1
D-3	-3.7 ± 0.1	M-3	-3.1 ± 0.0
D-11	-3.9 ± 0.1	M-11	-3.7 ± 0.1
D-12	-3.3 ± 0.1	M-12	-4.2 ± 0.2
D-13	-4.1 ± 0.0	M-13	-4.0 ± 0.2
D-14	-3.5 ± 0.0	M-14	-3.4 ± 0.2

Data represent means ± SD of three independent estimations

All of the hyp-resistant lines showed higher levels of frost tolerance than non-selected controls except one (D-2) which had nearly identical FKT value to its respective control (Table 2). Among the seven lines selected from Desiree cells, six were found better, with lower FKT (°C) values than control, while among the seven lines selected from Maris Piper, all were better than control with greater frost tolerance.

Frost tolerance stability of the selected cell lines

To discover whether the hydroxyproline tolerance in the selected cell lines on hyp-containing media could be permanent or just a temporary adjustment to cellular metabolism, the two lines D-13 and M-12, which

had been selected to grow on media containing hyp, were transferred to the normal (hyp-free) medium. On the normal (hyp-free) medium, these grew better compared with those maintained throughout on hyp-containing medium. After 2 subcultures on normal medium, when their degrees of frost tolerance were determined, the FKT values obtained were -4.0°C and -4.2°C for D-13 and M-12, respectively. It appears that line M-12 had maintained its frost tolerance level, while the frost tolerance of D-13 was slightly decreased and FKT value changed from -4.1°C to -4.0°C.

Osmoticum Test

To find out whether the increase in frost tolerance is due to the osmotic effect of hyp added to the medium, or whether some sort of physiological mechanism was involved, 0.05 M mannitol was added in separate cultures. Addition of mannitol to the medium reduced the growth of cultures to a lower extent but did not result in an increase in frost tolerance.

DISCUSSION

In the present studies, suspension-cultured cells of *S. tuberosum* cvs. Desiree and Maris Piper were plated on Lam (1977) cell plating medium containing 5 or 10 mM hyp and several hyp-resistant colonies were isolated with a spontaneous frequency of 0.20×10^{-6}. When cells were irradiated with gamma rays at a dose of 20 Gy before plating, the frequency of resistant colonies recorded was 0.35×10^{-6} showing an improvement. Low doses of gamma radiation tend to increase the rate of mutations in plant cell cultures without discernable damage to the cell structure. Although mutations are random, sometimes mutagenic treatment may yield some desirable changes in the genome which can be selected and exploited.

Hyp-resistant lines have been reported to possess increased frost tolerance (van Swaaij *et al.* 1986, 1987, Tantau and Dorffling 1991). To test whether directly selected frost-tolerant lines are also hyp-resistant, suspension-cultured cells were exposed to a freezing temperature of –6°C and plated on the cell plating medium. Eight cell lines were established and these were transferred to hyp-containing media. Only three lines grew well, showing resistance to hyp, while the other five failed to survive on the selective medium. This suggests that frost tolerance and hyp resistance are not necessarily linked.

Addition of osmoticum (0.05 M mannitol) to the medium did not result in an increase in frost tolerance of the cultures. This indicates that increased frost tolerance was not due merely to an osmotic effect and makes an involvement of proline in the mechanism of frost tolerance more likely. This was probably due to the accumulation of proline within the cells. Over production of proline under stress is also reported in cell cultures of

various species, e. g. in rice (Chauhan and Prathapasenan 1997) and tobacco (Eberhardt and Wegmann 1989, Dridze *et al.* 1991, Sumaryati *et al.* 1992) under salt stress and in potato (Corcuera *et al.* 1989) and tobacco (Sumaryati *et al.* 1992) under water stress. The role of proline in protection against frost is well documented (van Swaaij *et al.* 1986, Dobslaw and Bielika 1988, Tantau and Dorffling 1991). It has been suggested that proline has a membrane-stabilizing effect during the stress and protects against denaturation by freeze-induced dehydration (Hellergren and Li 1981). Increase in frost tolerance in suspension-cultured cells of *S. tuberosum* has already been reported after application of 0.43 M proline to the cell suspension cultures (Hellergren and Li 1981).

Almost all the selected hyp-resistant lines of *S. tuberosum*, when evaluated for frost tolerance, showed increased degrees of frost tolerance compared with non-selected controls. Only one line which was found to be hyp-resistant did not appear to be frost-tolerant, and showed equal degree of frost tolerance to non-selected control. The increased frost tolerance in other hyp-resistant cell lines was probably due to elevated proline contents within these lines. It is hypothesised that these lines were able to convert some of the hydroxyproline into proline. Enzyme activity converting free hydroxyproline into proline has already been shown in carrot slices (Varner 1980).

In hyp-resistant lines, proline occurs in different cellular compartments: cytoplasm and/or vacuole. Proline accumulated in the cytoplasm has direct stabilising effects on cytoplasmic membranes and proteins. If proline is accumulated in the vacuole, this stabilising effect will be reduced, irrespective of the total proline concentration in the cells. Fricke and Pahlich (1990) have reported changes in the proline pools between cytoplasm and vacuole. It can therefore be hypothesised that the cell line, which did not show any increase in frost tolerance had accumulated proline in its vacuole. Alternately, in this line hyp resistance may had been due to physiological adaptation of cells to the hyp-stress by a mechanism that not yet known.

CONCLUSIONS

The results of these studies demonstrated that hyp-resistant cell lines could be selected from cell cultures of *S. tuberosum*. Selected hyp-resistant cell lines although differed in their frost killing temperatures but possessed increased levels of frost tolerance as compared to their non-selected controls. Further research is needed in this regard to determine the cause of differences in frost tolerance among hyp-resistant cell lines, including total proline contents and in compartments. However, it is of course essential that such selected lines should be able to be regenerated into plants. Further that frost tolerance should be expressed at the whole plant level and be genetically transmittable.

REFERENCES

Chauhan, V. A., Prathapasenan, G. 1997. Response of rice callus to hydroxyproline and NaCl. In: Ravishankar, G. A. and Venkataraman, L. V. (eds.). Biological Applications of Plant Tissue and Cell Culture. Oxford & IBH Publishing Co. Pvt. Ltd., New Delhi. pp. 403-409.

Corcuera, L. J., Hintz, M., Pahlich, E. 1989. Proline metabolism in *Solanum tuberosum* cell suspension cultures under water stress. J. Plant Physiol. 134: 290-293.

Dobslaw, S., Bielka, S. 1988. Studies to establish frost tolerance ranking in winter barley by means of proline accumulation. 1. Tests on indicator varieties. Arch. Zuchtungsforsch. 18: 235-240.

Dorffling, K., Schulenburg, S., Lesselich, G., Dorffling, H. 1990. Abscisic acid and proline levels in cold hardened winter wheat leaves in relation to variety-specific differences in freezing resistance. J. Agron. Crop Sci. 165: 230-239.

Dridze, I. L., Khadeeva, N. V., Maisuryan, A. N. 1991. Production of tobacco cell lines resistant to certain stress factors. Soviet Plant Physiol. 38:121-125.

Eberhardt, H. J., Wegmann, K. 1989. Effect of abscisic acid and proline on adaptation of tobacco callus cultures to salinity and osmotic shock. Physiol Plant. 76: 283-288.

Fricke, W., Pahlich, E. 1990. The effect of water stress on the vacuole-extravacuole compartmentation of proline in potato cell suspension cultures. Physiol. Plant. 78: 374-378.

Hellergren, J., Li, P. H. 1981. Survival of *Solanum tuberosum* suspension cultures to -14°C: the mode of action of proline. *Physiol. Plant.* 52: 449-453.

Lam, S. L. 1977. Regeneration of plantlets from single cells in potatoes. Amer. Potato J. 54: 575-580.

Lee, S. P., Zhu, B., Chen, T. H. H., Li, P. H. 1992. Induction of freezing tolerance in potato (*Solanum commersonii*) suspension cultured cells. Physiol. Plant. 84: 41-48.

Levitt, J. 1980. Response of Plants to Environmental Stress. Vol. 1 (2nd ed.). Academic Press, Inc., London. pp.67-344.

Murashige, T., Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-479.

Stefl, M., Trcka, I., Vratny, P. 1978. Proline biosynthesis in winter plants due to exposure to low temperatures. Biol. Plant. 20: 119-128.

Sumaryati, S. Negrutiu, I., Jacobs, M. 1992. Characterization and regeneration of salt and water stress mutants from protoplast culture of *Nicotiana plumbaginifolia* (Viriani). Theor. Appl. Genet. 83: 613-619.

Swaaij van A. C., Jacobsen E., Feenstra W. J. 1985. Effect of cold hardening, wilting and exogenously applied proline on leaf proline content and frost tolerance of several genotypes of *Solanum.* Physiol. Plant. 64: 230-236.

Swaaij van, A. C., Jacobsen, E., Kiel, J. A. K. W., Feenstra, W. J. 1986. Selection, characterization and regeneration of hydroxyproline-resistant cell lines of *Solanum tuberosum*: tolerance to NaCl and freezing stress. Physiol. Plant. 68: 359-366.

Swaaij van A. C., Talsma K., Krijgsheld H., Jacobsen E., Feenstra W. J. 1987. Frost tolerance in cell cultures of potato. Physiol. Plant. 69: 602-608.

Tantau, H., Dorffling, K. 1991. *In vitro* selection of hydroxyproline-resistant cell lines of wheat (*Triticum aestivum*): accumulation of proline, decrease in osmotic potential and increase in frost tolerance. Physiol. Plant. 82: 243-248.

Towill, L. E., Mazur, P. 1975. Studies on the reduction of 2.3.5-tiphenyl-tetrazolium chloride as a viability assay for plant tissue cultures. *Can. J. Bot.* 53: 1097-1102.

Varner, J. E. 1980. The direct conversion of free hydroxyproline to proline. Biochem. Biophys. Res. Commun. 96: 692-695.