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PEG-tolerant cell clones of chili pepper: Growth, osmotic potentials and solute accumulation

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Abstract

Cell cultures of chili pepper (*Capsicum annuum* L.) were established from callus tissue inoculated in MS liquid medium supplemented with 6.25 μM 2,4-D and 0.44 μM BA. Cell clones were isolated by plating the cell suspension on filter paper discs supported by polyurethane foam that were bathed with culture medium containing 15% PEG. The cell clones T6 and T7 were chosen based on their characteristics of growth and friability. These cell clones were established as cell suspensions in the presence of 15% PEG and subsequently subcultured in increasing concentrations of osmoticum. By this approach the cell clones T7 and T6 were capable of growing in the presence of 20 and 25% PEG, respectively. The cell clone T7 was found to grow better in the presence of 5–10 % PEG after a period of subculturing in the absence of osmoticum indicating that the tolerance trait was stable. The tolerant cell clones exhibited a 3 to 3.5-fold decrease in the osmotic potentials in comparison with the nonselected cells suggesting that osmotic adjustment occurred. K^+ was the major contributing solute to the osmotic potential in all the cell cultures among those tested and was found to be higher in concentration in the PEG-tolerant clones (1.3–3 times higher than nonselected cells). Proline and glycine betaine levels showed a positive correlation with the degree of tolerance to water deficit in the PEG- tolerant cell clones. The levels of proline in the cell clone T7 subcultured in the absence of PEG in the culture medium decreased to values similar to those of nonselected cells, whereas the contents of glycine betaine in the same conditions were maintained at high levels.

Abbreviations: BA – benzyladenine, 2,4-D – 2,4-dichlorophenoxyacetic acid, MS – Murashige and Skoog medium, PEG – polyethylene glycol

Introduction

Plant cell cultures have been used as models for studying physiological and biochemical processes as well as those mechanisms that operate at the plant cell level (Lerner 1985).

The genetic variability observed in plant cell cultures has allowed the isolation of variant and mutant cell lines that differ in their tolerance to either biotic or abiotic factors (Larkin & Scowcroft 1981; Meredith 1983; Salgado-Garciglia et al. 1985). In vitro selection for cells exhibiting increased tolerance to water stress has been reported (Bressan et al. 1981; Harms & Oertli 1985; Sabbah & Tal 1990; Borkird et al. 1991) and the regeneration of drought- tolerant plants has been achieved recently (Sumaryati et al. 1992). PEG and mannitol are osmotic compounds used to simulate drought conditions and to isolate tolerant cells. The mechanisms by which these selected cells tolerate water stress have been investigated (Singh et al. 1985; Singh et al. 1987; Iraki et al. 1989; Singh et al. 1989; Borkird et al. 1991). Osmotic adjustment through solute accumulation has been suggested as one of the possible means for overcoming osmotic stress in PEG-adapted cell suspensions of tobacco (*Nicotiana tabacum* L.) (Heyser & Nabors 1981) and of toma-

to (*Lycopersicon esculentum* L.) (Bressan et al. 1982; Handa et al. 1982). Measurements of the intracellular solutes that contribute to osmotic adjustment in drought-tolerant cell lines have been limited to those reported by Handa et al. (1983), Sabbah & Tal (1990) and Gulati & Jaiwal (1993).

Additional research is needed to better understand the physiological and biochemical nature of cell adaptation to drought. In this study we describe the isolation and characterization of PEG-tolerant cell clones using cell suspensions of the drought-sensitive chili pepper (*Capsicum annuum* L.) as the first step for the possible drought tolerance improvement of this important plant species.

Materials and methods

Cell cultures

Establishment of callus and cell suspensions of chili pepper (*Capsicum annuum* L., cv. Tampiqueño 74) was carried as earlier described (Salgado-Garciglia & Ochoa-Alejo 1990). The cell cultures were maintained by subculture at 15 day intervals with an initial inoculum equivalent of 5 mg dry weight (determined by drying the cells in an oven at 90°C for 5 h) in Erlenmeyer flasks (125 ml) containing 50 ml of autoclaved MS liquid medium (Murashige & Skoog 1962) supplemented with 6.25 μM 2,4-D and 0.44 μM BA, pH 5.8. Cell suspensions were kept on a rotary shaker (120 rpm) at 25 ± 2°C under a 16/8 h light/dark photoperiod (cool white fluorescent lamps; 123 μmol m^{-2} s^{-1}).

Isolation of PEG-tolerant cell clones

Cell suspensions were adjusted to a density of 70,000 cells ml^{-1} and samples of 1 ml were plated on filter paper discs (Whatman # 1; 5 cm in diameter, placed in 135 ml glass bottles) supported by polyurethane foam (purchased from Plasticos Espumosos S. A., Mexico) bathed by liquid medium containing 0, 5, 10, 15, 20, 25 or 30% PEG (M.W. 8,000; Sigma Chemical Co.) (Conner & Meredith 1984). Each treatment was replicated 5 times. The cultures were incubated in a growing room at 25 ± 2°C and at low light intensity (4.5 μmol m^{-2} s^{-1}). Cell colonies were individually subcultured in the same conditions described above for 8–12 months (1.5–2 month intervals). By this procedure, we isolated colonies T6 and T7, which were capable of growing in the presence of 15% PEG. These cell colonies were subsequently established as suspension cultures in MS medium supplemented with 15% PEG. The cell clones were subcultured 10 times (2–3 weeks between subcultures) and were then inoculated in media with either 20, 25 or 30% PEG added. Cell clone T6 was capable of growing in 25% PEG, whereas clone T7 grew in 20% PEG. T7 was maintained in both 15 and 20% PEG and T6 in 20 and 25% PEG. The initial inoculum at subculture was equivalent of approximately 5 mg dry weight ml^{-1} (90°C; 5h).

Stability of PEG-tolerance

Cell clone T7, maintained in 15% PEG (22 subcultures, 2 to 3 week intervals), was subcultured in medium with 7.5 and 3% PEG (two times each) and then in 0% PEG (5 times, 15 day subcultures). The cell clone in these conditions was designated T7 (P 15 → 0). Cell clone T7, subcultured in medium with 20% PEG for at least 5 times (2 to 3 week intervals), was serially subcultured in 10 and 5% (2 times each) and in 0% PEG (5 times, 15 day subcultures). This cell clone was designated T7 (P20 → 0). The gradual exposure to decreasing levels of PEG was imposed because direct transfer of cells from high PEG to medium without osmoticum resulted in considerable cell damage. In order to test the stability of tolerance to PEG, cell clone T7 (P 15 → 0 and P 20 → 0) was inoculated in culture medium supplemented with PEG ranging from 0 to 30%. The initial inoculum was equivalent of 5 mg dry weight ml^{-1}. The cells were placed on a rotary shaker (120 rpm) and at 25 ± 2°C for 15 days. Cells were collected and centrifuged in conical tubes at 4,500 rpm to determine packed cell volume. Cell growth was expressed as the net increase (Δ) in packed cell volume determined by substracting the initial inoculum to the final value at the time of sampling.

Osmotic potential measurements

Cell suspension samples (10 ml) were collected by filtration and the culture medium was discarded. The cells were frozen with liquid nitrogen and stored at −70°C. Cell samples (0.5 ml packed cells) were ground in Eppendorf tubes using a glass rod and centrifuged in a microfuge at 10,000 rpm for 10 min at 4°C. The osmotic potentials were determined in a vapor pressure osmometer (Wescor model 5100 C) using 20 μl samples of supernatant. Milliosmoles were converted to MPa using the van't Hoff equation.

Determination of ions

The measurements of ions were made at 3 day intervals during the growth cycle. The culture medium was eliminated from 10 ml samples of cell suspension by filtration. The cells were frozen with liquid nitrogen, lyophilized, homogenized with 0.7–2 ml of deionized water and centrifuged at 10,000 rpm for 10 min. Na^+ and K^+ were determined with an automatized flame photometer (Instrument Laboratory 943) using 20 µl samples. Ca^{2+} levels were quantified by atomic absorption spectroscopy (Perkin Elmer atomic absorption spectrometer) according to Cali et al. (1973).

Proline quantitation

Proline was extracted and determined colorimetrically by the method of Bates et al. (1973). Lyophilized cells (70 mg) were homogenized in 3% sulfosalicylic acid (7 ml) and filtered (Whatman # 1 filter paper) to separate cell debris. Proline was determined in filtrate samples of 50 µl to 1 ml by absorbance of the proline-ninhydrin product at 520 mm in a Pye Unicam PU 8600 spectrophotometer using toluene as the solvent.

Determination of glycine betaine

Lyophilized cells (50 mg) were extracted with 2 ml of deionized water at 25°C for 24 h and filtered as described above. Since the presence of PEG interferes with the I-KI reagent, extracts (1 ml) were treated with an equal volume of 2 N trichloroacetic acid (1 h at 4°C) in order to precipitate the osmoticum. The precipitated PEG was eliminated from the extracts by centrifugation at 4,500 rpm for 10 min. Glycine betaine was quantified by the method of Grieve & Gratan (1983).

All the experiments described above were carried out at least twice. Statistical Analysis System (SAS) was used to analyze the experimental data (t test).

Results

Stability of PEG-tolerance

Growth of nonselected cell suspensions of chili pepper was inhibited when they were exposed to 5% or higher PEG. Negative net packed cell volumes were detected at 10% PEG and higher indicating that cell death occurred (Fig. 1). Cell clone T7 (P 15 → 0 and P 20 → 0) exhibited the highest cell mass production in the presence of 5–10% PEG. Significant growth inhibition was observed only at 15% PEG or higher (Fig. 1). Higher packed cell volumes were recorded in the P 20 → 0 than in the P 15 → 0 treatment. The data of PEG-tolerance stability of the cell clone T6 (maintained in 25% PEG) was not determined in this study since cell survival was dramatically decreased in medium devoid of PEG.

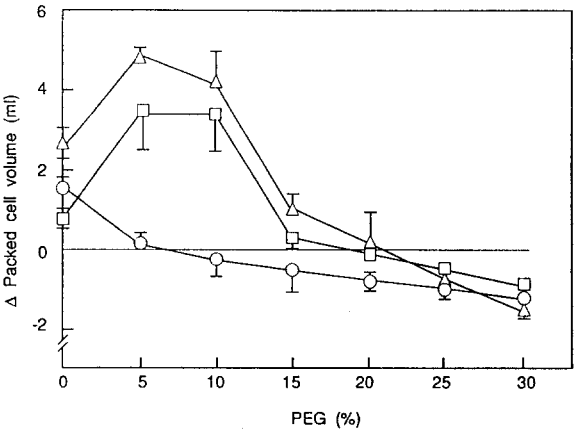

Fig. 1. Effect of increasing concentrations of PEG on the growth of cell cultures of chili pepper. (–○–○–), Nonselected cell suspension; (–□–□–) and (–△–△–), clone T7 cultured in 15 or 20% PEG, respectively, and then subcultured in the absence of PEG (P15 → 0) and P20 → 0). Inoculum: 5 mg dry weight ml^{-1}; harvesting time: 15 days after subculture. Values are the means of two independent experiments with 3 replicates each ± standard deviation (S. D.). ($p<0.05$).

Osmotic potentials

Osmotic potentials of T6 and T7 cell clones and nonselected cell cultures are shown in Fig. 2. The maximum negative osmotic potentials in the cell clones were detected during the exponential phase of growth (6–12 days), whereas in nonselected cells values remained nearly constant. Values of osmotic potential during the growth cycle of the cell clone T6 and T7, were approximately 3 to 3.5 times more negative than those of nonselected cells. Although the cell clones T6 and T7 were cultured in medium with different concentrations of PEG (20 and 25%), no significant differences in the osmotic potentials were detected.

Accumulation of ions

Concentrations of Na^+, K^+ and Ca^{2+} in chili pepper cell cultures during the growth cycle are summarized

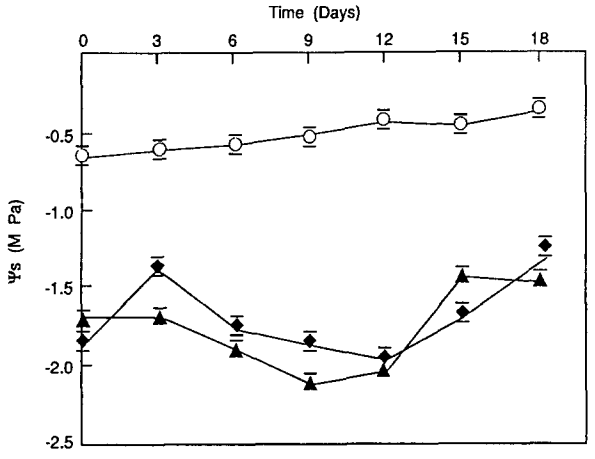

Fig. 2. Osmotic potentials during the growth cycle of nonselected cell suspension grown in medium without PEG (-○-○-), clone T6 cultured in 25% PEG (-◆-◆-), and clone T7 maintained in the presence of 20% PEG (-▲-▲-). Data represent the means of two independent experiments with 3 replicates each ± S. D. ($p<0.01$).

in Fig. 3. In general, PEG-tolerant cell clones accumulated 2 to 3 times more Na^+ than the nonselected control. Clone T7 grown in 20% PEG exhibited the highest content of Na^+, followed, in decreasing order, by cell clone T6 and the nonselected cell suspension. The contribution of Na^+ to the osmotic potential was about 0.9, 0.6 and 0.17% for cell clones T7, T6 and nonselected cells, respectively.

A positive correlation between accumulation of K^+ and degree of PEG tolerance was observed in the cell cultures. Clone T6 cultured in 25% PEG exhibited as much as 3 times more K^+ than the nonselected cell culture and approximately 1.3 times that of the cell clone T7. Levels of K^+ in the cell cultures were about two orders of magnitude higher than those of Na^+ and Ca^{2+} (Fig. 3). Potassium accumulation accounted for 68, 42 and 30% of the osmotic potentials of the cell clones T6, T7 and nonselected cells, respectively.

There was a significant difference between calcium content of nonselected and PEG-tolerant cell clones (Fig. 3). In general, nonselected cells exhibited 3–4 more Ca^{2+} than the cell clones. However, Ca^{2+} levels in clone T6 rose during the growth phase and reached a maximum value (9 days after subculture) that was similar to the maximum content found in nonselected cell suspension. The clone T7 showed a decrease in Ca^{2+} during the initial 3 day period and remained constant. The contribution of Ca^{2+} to the osmotic potential of the cells was not significant (0.5, 0.3 and 0.9% for the cell line T6, T7 and nonselected cells, respectively).

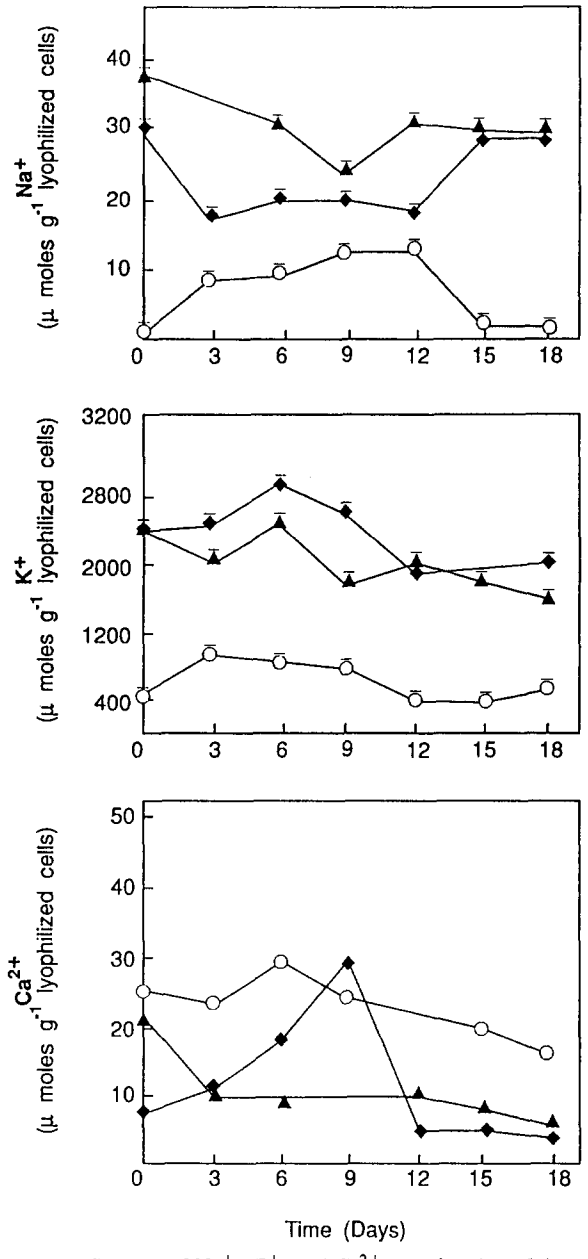

Fig. 3. Contents of Na^+, K^+, and Ca^{2+} as a function of time of culture in nonselected cells of chili pepper (-○-○-), clone T6 (-◆-◆-) and clone T7 (-▲-▲-) grown in 0, 25 and 20% PEG, respectively. Each value of Na^+ and K^+ is the mean of two independent experiments with 3 replicates each ± S.D. ($p<0.05$). No S. D. was included for Ca^{2+} analysis since the apparatus gave the means automatically).

Proline and glycine betaine accumulation

Notable differences in accumulation of proline in PEG-tolerant cell clones and nonselected cell cultures were observed (Fig. 4). Cells not exposed to PEG showed

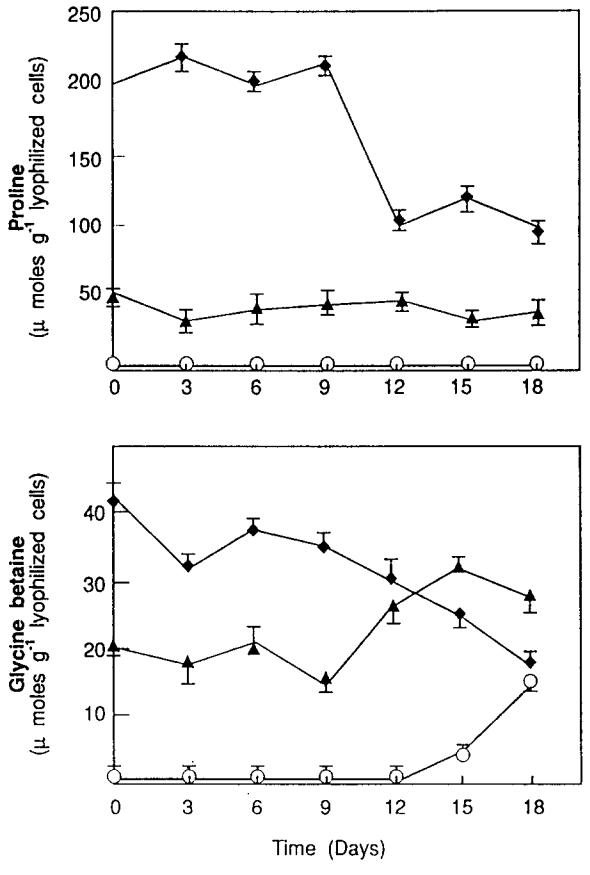

Fig. 4. Proline and glycine betaine levels during the growth cycle of nonselected and cells clones of chili pepper. (–○–○–), Nonselected cells in 0% PEG; (–◆–◆–) clone T6 grown in 25% PEG; (–▲–▲–), clone T7 cultured in 20% PEG. Points represent the mean values of two independent experiments with 3 replicates each ± S.D. ($p<0.05$).

low and constant levels of proline during the growth cycle. Cell clone T7 contained about 40–50 times more proline than nonselected cells. The highest level of proline was found in clone T6 (a 200-fold increase over the nonselected cells) where accumulation occurred during the active phase of growth and then decreased during the stationary phase. Proline accumulation accounted for 6.8, 1.4 and 0.03% of the total osmotic potential of clones T6, T7 and nonselected cells, respectively.

A positive correlation between PEG tolerance and glycine betaine accumulation in the cell suspensions was observed (Fig. 4). Glycine betaine contents in clone T6 cultured in 25% PEG were about 2 and 40 times higher than those of clone T7 grown in 20% PEG and nonselected cells maintained in 0% PEG, respectively. In general, the glycine betaine level of clone T6 was higher during the active phase of growth and decreased at the stationary phase of the cycle; however, glycine betaine levels in clone T7 and nonselected cells remained constant during the 0–9 day period after subculture and increased at the stationary phase. The contribution of glycine betaine to the total osmotic potential of clones T6 and T7 and nonselected cells was 1.3, 0.8 and 0.3%, respectively.

Differences in proline and glycine betaine levels were observed between clone T7 that was maintained in the presence of 20% PEG and the same clone that was subcultured 5 times in culture medium without PEG (Fig. 5). Proline contents in clone T7 grown in PEG were 12–17 times higher than those in the absence of PEG. Values of the latter did not differ from those of PEG-untreated nonselected cells. On the other hand, glycine betaine levels in clone T7 subcultured in medium with or without PEG were higher than those of nonselected cells. Interestingly, clone T7 subcultured in the absence of PEG exhibited a 3-fold increase in glycine betaine 9 to 12 days after subculture in comparison with that maintained in PEG.

Discussion

Several strategies have been used to isolate drought-tolerant plant cells. A single-step exposure of a tomato cell suspension to 15% PEG was reported by Bressan et al. (1981). Cell lines with increased tolerance were further selected by increasing the PEG concentration from 15% to 20, 25 and 30% (Bressan et al. 1982). However, tolerance was rapidly lost when the PEG-adapted cells were subcultured in medium without osmoticum. In another approach, tomato cells differing in water stress tolerance were isolated by cloning cells in the absence of PEG (Handa et al. 1983). A step-wise selection using mannitol in the culture medium was reported for carrot (*Daucus carota* L.) (Harms & Oertli 1985), potato (*Solanum tuberosum* L.) (Sabbah & Tal 1990) and *Vigna radiata* L. cultures (Gulati & Jaiwal 1993).

In the present work we combined a cloning step by plating cells directly in culture medium with 15% PEG and a subsequent exposure to 20 and 25% PEG. This strategy made possible the isolation of cell clones that may represent specific genetic alteration events during the in vitro culture.

The data in Fig. 1 show that clone T7 cultured in 15 or 20% PEG was capable of growing better than the control cells when the former was re-exposed to PEG after a gradual subculture in decreasing concentrations of PEG and a period of culture in its absence (40–60 days). This cell clone was found to require about 5–

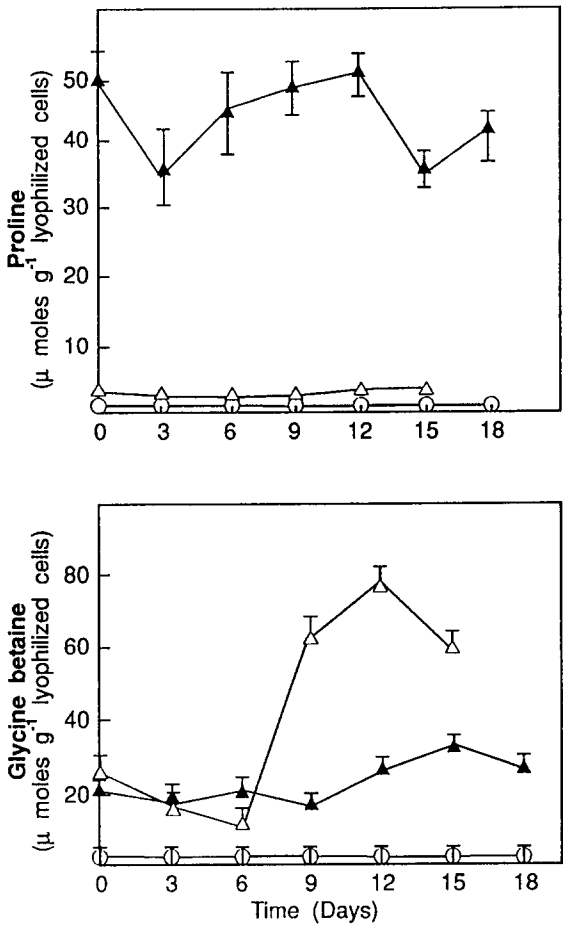

Fig. 5. Changes in proline and glycine betaine as a function of the growth cycle stage of chili pepper cell cultures. (-○-○-), Nonselected cells growing in 0% PEG; (-▲-▲-), clone T7 in 20% PEG; (-△-△-), clone T7 cultured in 20% PEG and then transferred to medium without it. All data points represent the means of two independent experiments with 3 replicates each ± S. D. ($p<0.01$).

10% PEG for maximal growth during the challenging experiments suggesting not only that tolerance to PEG was stable but also that dependence for osmoticum was established after selection. These results also suggest that tolerance is not the consequence of an adaptation process as has been reported for tomato cells (Bressan et al. 1981; Bressan et al. 1982). Stimulation by PEG in the selected cell clones resembles variant cells selected for NaCl (Salgado-Garciglia et al. 1985) or p-fluorophenylalanine tolerance (Salgado-Garciglia & Ochoa-Alejo 1990) since the presence of these compounds also stimulated cell growth.

Attempts to demonstrate the stability of drought tolerance of cell clone T6 (subcultured in 25 % PEG) were unsuccessful because of the difficulty to maintain cell viability after transfer to media with lower levels of osmoticum. It is possible that a hypoosmotic shock was the cause of cell death as has been observed in tomato cells subjected to prolonged PEG-exposure (Handa et al. 1982).

Positive correlations have been demonstrated between the osmotic adjustment capacity and the tolerance to drought in different crops (Parsons & Howe 1984; Premachandra et al. 1989) and in PEG-adapted cells of tomato (Bressan et al. 1982). Significantly more negative osmotic potentials were recorded in PEG-tolerant clones of chili pepper in comparison with nonselected cells. These results suggest that osmotic adjustment is involved in the tolerance to PEG as has been observed for tomato PEG-adapted cell lines (Handa et al. 1982).

Ions, soluble sugars, polyalcohols, organic acids, free amino acids and quaternary ammonium compounds have been proposed as osmolytes in both microbial and plant cells (Yancey et al. 1982). Increases in K^+, sugars, proline and glycine betaine have been demonstrated in different plant species under water stress (Ladyman et al. 1980; Hanson & Hitz 1982; Corchete & Guerra 1986; Schwab & Gaff 1986; Premachandra et al. 1989; Irigoyen et al. 1992). Reducing sugars, K^+ and free amino acids have been demonstrated to be the major contributors to osmotic potentials in PEG-adapted cells of tomato (Handa et al. 1983). K^+ has also been shown to accumulate in cells of sour orange treated with PEG (Ben-Hayyim 1987), but the opposite has been observed in mannitol-treated wheat callus (Trivedi et al. 1991). In the present study, a positive correlation between PEG-tolerance and the K^+ levels in chili pepper cell cultures was observed. This ion was the major contributor to osmotic potentials in the PEG-tolerant clones suggesting an important role in drought tolerance. Since this ion accounts for about 40–70% of the osmotic potential in the cell clones and the other quantified solutes had only a marginal contribution, it is possible that some undetermined compounds might also have an important participation in osmotic adjustment (Hanson & Hitz 1982). It would be interesting to expand the studies to some other solutes like sugars in order to investigate their contribution to osmotic adjustment in the PEG-tolerant cell clones of chili pepper. Water stress often causes an increase in the accumulation of compatible solutes such as proline and glycine betaine. These substances have been proposed as nontoxic osmotic solutes, which act as protein protectants and stabilizers of organelles (Schobert & Tschesche 1978; Pollard & Wyn Jones

1979; Pahlich et al. 1983; Paleg et al. 1984, 1985; Schwab & Gaff 1990). Chili pepper clones T6 and T7 exhibited considerable accumulation of both proline and glycine betaine in comparison with nonselected cell suspensions. However, contribution of these compounds to osmotic potentials were not significant and therefore their role as osmolytes might be excluded.

The fact that proline was not maintained at high levels in cell clone T7 after subculture in medium without PEG, suggests that a physiological adaptation occurred. However, glycine betaine was found to accumulate even in the absence of the selection pressure indicating that the increased levels resulted from a stable alteration of the metabolism of this compound. Further studies are necessary to establish the nature of this change.

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