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Proline accumulation and sodium sulfate tolerance in callus cultures of *Brassica napus* L. cv. Westar

Stephen F. Chandler and Trevor A. Thorpe

Plant Physiology Research Group, Department of Biology, University of Calgary, Calgary, AB T2N 1N4, Canada

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ABSTRACT

Callus cultures of Brassica napus L. cv. Westar were selected which contained 5 - 6 times more proline than unselected callus. Callus pieces from these cultures were able to survive much better after subculture to medium containing 105 mM Na_2SO_4 than unselected callus, or unselected callus cultured on exogenous proline before or during transfer to the salt. Exogenous proline was rapidly absorbed. In unselected callus there was a peak in proline accumulation ca. 2 days after transfer to Na_2SO_4, followed by a decline. In contrast proline accumulation in tolerant callus was linear with time, reaching maximum levels at 8 days. Proline levels induced by exposure to salt were maintained in the absence of stress.

ABBREVIATIONS DW = Dry weight; FW = Fresh weight

INTRODUCTION

In a number of tissue culture systems exposure to salt or water stress results in the accumulation of proline (Hasegawa et al., 1984; Chandler and Thorpe, 1986). As in the intact plant this phenomenon has been suggested to be a result of osmotic adjustment (Daines and Gould, 1985), metabolic protection or some type of cellular damage (Dix and Pearce, 1981). It is therefore possible that proline overproduction could be used as a potential marker for salt tolerance in vitro. In this report we describe the Na_2SO_4 tolerance of callus of Brassica napus L. cv. Westar selected for proline overproduction and compare proline accumulation in unselected and tolerant callus grown on the salt.

MATERIALS AND METHODS

Material and culture conditions. Unselected callus cultures of Brassica napus cv. Westar were initiated and established on Na_2SO_4-free medium, using methods previously described (Chandler et al., 1986). Tolerant callus had been selected on 140 mM Na_2SO_4, but maintained for 13 passages in the absence of salt prior to use in these experiments. Tolerance is retained in the absence of salt (Chandler and Thorpe, submitted for publication).

All media were adjusted to pH 5.7 and sterilized by autoclaving. All cultures were maintained in the light (16 hr photoperiod; photon fluence rate ca. 80 μmol m^{-2} s^{-1}; 380-800 nm) at 27°±1°C.

Selection for proline overproduction. Three unselected callus pieces were divided into ten pieces each, and subcultured onto fresh medium. After four weeks, each of the thirty individual pieces (lines) was divided in three. One third of each piece was used for proline determination and one third in assessment of tolerance to Na_2SO_4, representing the tolerance of unselected callus. Remaining thirds of four of the 30 lines were then subcultured onto fresh medium. Eight pieces were subcultured from each of these four lines, giving 32 new lines, which were grown for 4 weeks. After this, each of the new lines was cut into two and the proline content of one half of every line measured. The four lines with the highest proline concentrations were identified, and the remaining halves of these divided into 7 or 8 pieces, giving a total of 30 new lines. These were grown on fresh medium for 4 weeks, after which each line was again cut in two. One half of every line was used to determine proline concentration and one half for assessment of Na_2SO_4 tolerance.

Assessment of Na_2SO_4 tolerance. Na_2SO_4 tolerance was assessed using callus pieces (20-30 per source), of approx. 5 mg fresh weight each. Pieces were transferred to medium supplemented with 105 mM Na_2SO_4 and survival was then assessed every 3-4 days for 32 days. Survival was defined as the proliferation of any patches of green, healthy tissue. Survival was measured in unselected callus, the callus lines selected for proline overproduction and in unselected callus grown on salt-free medium containing 0, 5, 10, 20 or 50 mM L-proline for 1 or 10 days.

Growth of unselected callus in the absence and presence of L-proline and 105 mM Na_2SO_4 was also assessed. In this experiment there were 14 replicates per treatment and growth was quantified on a fresh and dry weight basis as previously described, after one month in culture (Chandler et al., 1986).

Proline uptake and accumulation. Proline uptake was measured in unselected callus (explant FW; ca. 25 mg) grown for 0.3, 1, 3 or 10 days on salt-free

Offprint requests to: T.A. Thorpe

medium containing 25 mM L-proline. Endogenous proline concentration was also measured in unselected and tolerant callus (explant FW; 40 to 50 mg) grown on 105 mM Na_2SO_4. Samples were taken for FW:DW and proline determination at 0, 2, 4, 8, 12, 25.5, 51, 77.5, 120, 174 and 240 hours.

Proline determination. Callus was homogenized in 12:5:1 (v/v) methanol:chloroform:water, following which the extracts were partitioned against water and chloroform. After low-speed (150 xg) centrifugation, an aliquot of the upper layer was assayed using the method of Wren and Wiggall (1965), with the omission of permutit and substitution of benzene by toluene. Absorbance was measured at 518 nm. Each extract was assayed in triplicate and within each batch of extracts proline standards were included at all stages of the procedure. Proline was calculated on a fresh weight basis and converted to mg per g dry weight from callus fresh weight:dry weight.

RESULTS

Unselected B. napus callus contained an average of 1.5-2.0 mg.g DW^{-1} proline. However, within cultures there was variability and some callus pieces contained up to 5.0 mg.g DW^{-1} (Fig. 1A). By selecting these for subculture, callus with over 25 mg.g DW^{-1} proline was ultimately retrieved (Fig. 1C). The proline overproduction phenotype was not stable and in some lines selected callus pieces produced less proline after subculture (compare Fig. 1B to 1C). Low-yielding lines continued to be low-yielding however (Fig. 1A, B, black portions).

Selected cultures, containing 5 to 6 times more proline than unselected callus, showed a much greater tolerance to Na_2SO_4 (Table 1). Twenty-one percent of selected callus pieces not only remained viable but also grew in the presence of the salt. Average final fresh weight of surviving pieces from selected lines was 92.5 mg. Four of the five selected lines which did not have any surviving callus pieces in the final test also had low proline concentrations (Fig. 1C). Three of these (in line 4, Fig. 1C) were derived from the same callus piece (Fig. 1B, 1C).

Table 1. Sodium sulfate tolerance of unselected, and proline overproducing callus of Brassica napus.

	Unselected (Fig. 1A)	Selected (Fig. 1C)
Proline; mg.g DW^{-1} (mean ± SE)	1.64 ± 0.14 (n = 30)	9.18 ± 1.05 (n = 30)
No. of lines with any surviving callus pieces	8/30	25/30
Total number of callus pieces surviving	10/938 (1%)	147/692 (21%)

There was an improvement in the Na_2SO_4 tolerance of unselected callus if it was grown on proline before transfer to salt. If grown on 0, 5, 10 or 50 mM proline for 1 or 10 days before transfer there was no subsequent survival. However, on 20 mM for 1 or 10 days 4/27 (15%) and 1/30 (3%) pieces survived on 105 mM Na_2SO_4 respectively. The proline appeared to be taken up quite rapidly (Fig. 2).

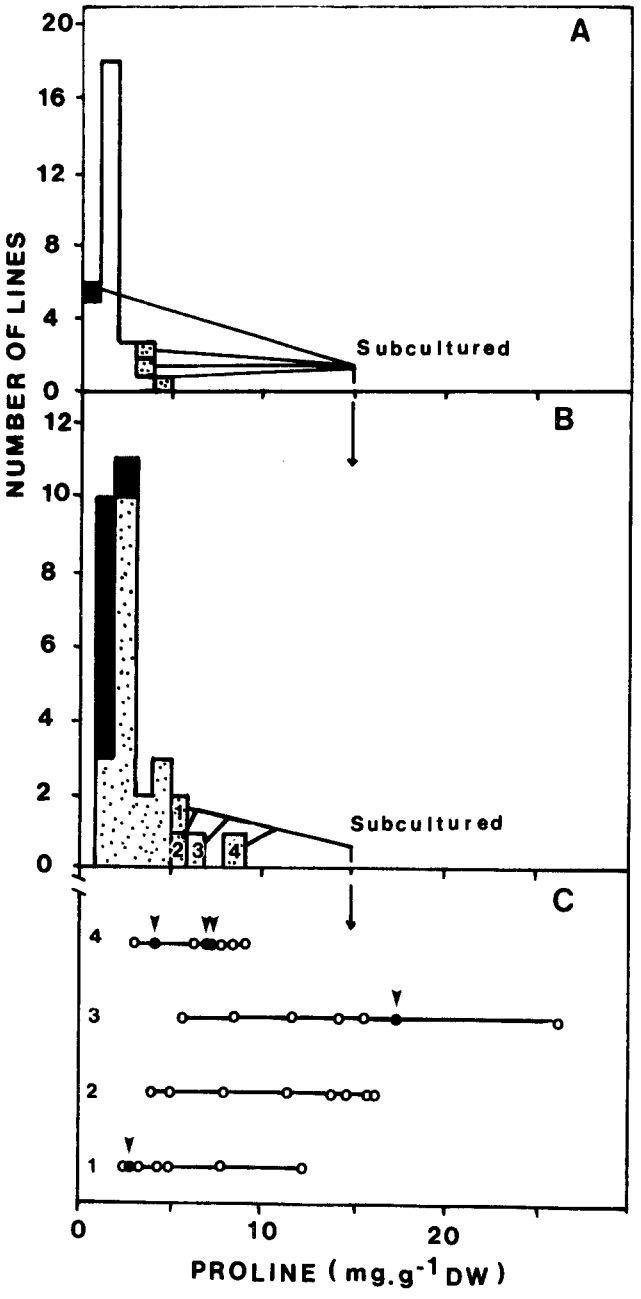

Figure 1. Proline concentration in lines of unselected (A) and selected (B,C) B. napus callus. Four callus pieces from an unselected population (A; ■, ⊡) were subcultured to give a new population of selected lines (B). The four lines with highest proline content from this new population (1-4) were then divided and subcultured, and proline content was determined after one month (C). Some lines in this final selected population showed no survival (●, arrowheads), while callus pieces from others survived (o) exposure to 105 mM Na_2SO_4. The full protocol for this selection procedure is described in Materials and Methods.

Table 2 shows the effect of continuously supplied exogenous proline on growth of unselected callus on Na_2SO_4. Addition of proline did not reduce the inhibitory effect of the salt.

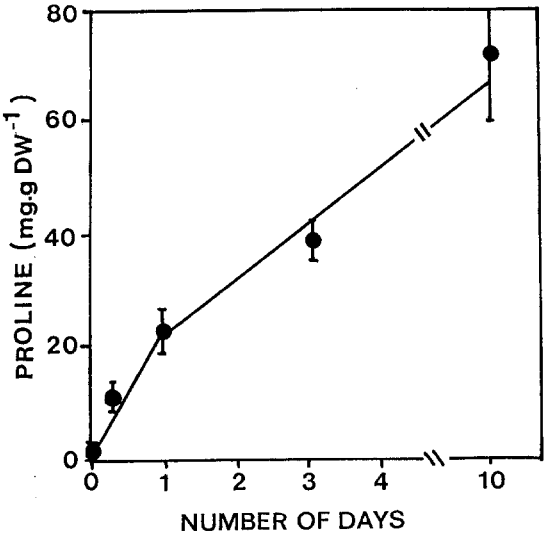

Figure 2. Proline concentration in unselected callus of B napus grown on medium containing 25 mM L-proline. Each point is the mean (± SE) of 3 replicates.

Fig. 3 shows the pattern of proline accumulation by unselected and tolerant callus on 105 mM Na_2SO_4, without any exogenous proline. Whereas there was a gradual build up of proline in the tolerant callus, proline accumulation in the unselected callus was much faster. However, in the latter case levels dropped back after peaking 2 - 3 days after transfer. Unselected callus pieces grown on Na_2SO_4 for four days survived and proliferated when transferred back to salt-free medium. Proline overproduction persisted in these cultures (Table 3).

Table 2. The effect of continuously supplied exogenous proline on growth and Na_2SO_4 tolerance in unselected callus of Brassica napus.

Media (mM)		Tissue			
Na_2SO_4	L-Proline	Survival (%)	FW (mg)	DW (mg)	Proline$_1$ (mg.g DW^{-1})
0	0	100	306±45	22.5	1.6±0.3
0	1	100	564±35	38.4	6.2±3.0
0	10	100	352±43	32.9	67.8±14.4
0	100	21	97±35	12.3	114.6±7.6
105	0	0	16±1	3.1	29.1±6.6
105	1	0	29±2	5.8	20.6±7.9
105	10	0	20±2	4.0	61.1±13.3
105	100	0	15±2	3.5	100.4±20.4

Mean (± SE) of 14 (FW) or 3 (proline) replicates.

Table 3. Proline concentration in unselected callus of Brassica napus grown in the absence and presence of 105 mM Na_2SO_4 for four days, followed by one month in the absence of stress.

	Proline Concentration (mg.g DW^{-1})	
Treatment	Range	mean (± SE)
Na_2SO_4-free	1.1 - 2.7 (n = 6)	2.0 ± 0.2
Plus Na_2SO_4	5.9 - 21.2 (n = 5)	15.1 ± 2.7

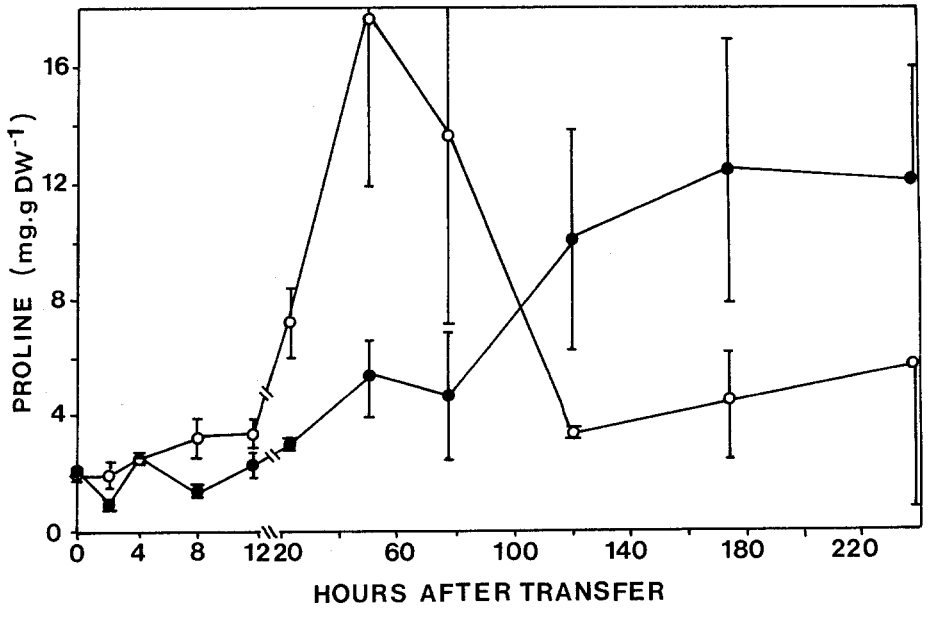

Figure 3. Proline concentrations in unselected (o) and tolerant (•) callus of B. napus grown on medium containing 105 mM Na_2SO_4 for a total of 240 hours. Each point is the mean (±SE) of three replicates.

DISCUSSION

Selection for proline overproduction in cell cultures of B. napus resulted in enhanced salt tolerance. This beneficial effect might be expected from observations in other species where exogenous application to tissue cultures improves salt tolerance (Katz and Tal, 1980; Rosen and Tal, 1981; Pandey and Ganapathy, 1985). However, in our cultures this approach was less effective than selection, even though the exogenous proline added was rapidly taken up and cultures were loaded with proline before transfer to salt. This implies that metabolites or processes associated with proline production are also important in salt tolerance and/or that exogenous proline is not located intercellularly correctly. In B. napus callus it is possible increased proline levels may both enhance salt tolerance and be a symptom of cellular damage during salt/water stress. The latter conclusion may be drawn because we have found that final concentrations of proline in unselected callus are always greater than that in tolerant callus at the same stress level, and because proline concentration is related to inhibition of growth, regardless of medium water potential (Chandler and Thorpe, 1987).

The patterns of proline accumulation were certainly different in tolerant and unselected callus when followed during the first 10 days exposure to Na_2SO_4. In unselected callus the first signs of damage are at 5 days, followed by complete necrosis after 10 (Chandler and Thorpe, submitted for publication). This is after the peak of proline production. A further indication that the proline produced by unselected callus on salt is not omoregulatory was that the high level of production induced by the salt persisted during growth on salt-free medium.

Selection for proline overproduction appears a promising approach for increasing salt tolerance. Efficiency of selection for proline over-production could be enhanced using single cells to reduce any effects of sectoring and specific metabolic blocks (Dix, 1984; Van Swaaij et al., 1986), to remove the necessity for proline determination at each stage of selection.

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