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Electrolyte release from frozen–thawed *Bromus inermis* suspension-cultured cells

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SUMMARY

By measuring the electrical conductivity of bathing water solutions, this study examines the post-thaw permeability to electrolytes of suspension-cultered cells freeze–thaw stressed to temperatures in the range from −3 to −20°C. These permeability measurements are compared to the post-thaw viability of the cells assayed cell-by-cell using vital staining. Supercooling to −5°C had no effect on permeability or viability by comparison with a +5°C control, but all freeze–thaw treatments affected permeability based on measurements of electrolyte leakage. This leakage occurred in two stages: (a) an acute stage during thawing which accounted for most of the electrolytes leached (incipient leakage), and (b) a chronic stage in which leakage was relatively slow and declined with time to become comparable to the rate in undamaged control samples. Significantly, this pattern was also found in samples frozen to −3°C and −5°C, which killed few cells according to the vital staining assay. It is proposed that the first, acute stage of leakage results from membrane rupture. On the basis of the observations that viable freeze–thawed cells also display incipient leakage, and that chronic leakage is elevated above that of the control, it is tentatively proposed that ruptured membranes subsequently reseal but are transformed with respect to their permeability.

Key-words: Bromus inermis Leyss, electrolyte leakage, freezing injury, membrane permeability.

INTRODUCTION

The plasma membrane is now generally regarded as the most sensitive part of the cell to freezing injury (for review, see Levitt 1980; Morris & Clarke 1981; Steponkus 1984), but the role it plays is as yet incompletely understood.

One method of measuring frost injury to cells and tissues is to estimate the conductivity of bathing water into which electrolytes have leaked after a freeze–thaw treatment (Dexter *et al.* 1932; Flint *et al.* 1967; Pearce 1980; Zhang & Willison 1987). Although such tests are widely used, their theoretical basis is in doubt. It is clear that electrolytes released as a result of cellular disruption during thawing contribute to the increase in conductivity, but there is also evidence that not all of this increase arises in this way, particularly because

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electrolytes may be released from frost-injured yet still-living cells (Palta *et al.* 1977a,b). Palta and colleagues (Palta & Li 1980; Palta *et al.* 1982) have suggested that this increased leakage of electrolytes from living frost-stressed cells is due to damage to active transport proteins and have proposed that such damage is critical in frost injury in general. By contrast, Carter & Lindstrom (1979) invoked responses of membrane lipids to temperature fluctuations to explain leakage phenomena. In other models, leakage from cells that survive thawing is not considered important and all leakage is attributed to catastrophic breakdown of semipermeability (Levitt 1972; Sukumaran & Weiser 1972; Singh & Miller 1985), which may include both expansion-induced plasma membrane lysis (Wiest & Steponkus 1978; Steponkus & Wiest 1979) and loss of osmotic responsiveness (Wiest & Steponkus 1979; Steponkus *et al.* 1982).

Suspension-cultured plant cells are very suitable for measuring electrolyte leakage following frost-stress because they leak individually and directly into the bathing medium, but few results have been reported (Zhang & Willison 1987). In an attempt to clarify understanding of electrolyte leakage following frost-stress treatments, we have measured the conductivity of bathing water solutions containing frost-stressed *Bromus inermis* suspension-cultured cells at various times after both lethal and non-lethal treatments.

MATERIAL AND METHODS

A bromegrass (*Bromus inermis* Leyss) cell suspension culture was maintained at 20°C with a 12:12 h (light:dark) photoperiod in 25 ml Erikssons's medium (Gamborg & Wetter 1975), held in 125-ml Erlenmeyer flasks, gently shaken at 100 r.p.m. (for details, see Zhang & Willison 1987). The culture was subcultured every 7 days and harvested on the seventh day of growth for the experiments.

Immediately before use, cells were filtered from their culture medium using 40 μm mesh nylon cloth and were washed by pouring 150 ml deionized water over them while held on the cloth. Samples, each weighing about 0·50 g and wet on the surface, were placed in test tubes and frost-stressed using a microprocessor-controlled HAAKE F3K cryostatic bath (HAAKE Mess-Technik, Karlsruhe, FRG) containing a liquid hydrocarbon (see Zhang & Willison 1987).

To apply low temperature stress, ice crystals were introduced at −2°C and the samples were kept for 14 h at −3°C. Samples were subsequently subjected to further cooling to a range of ultimate subzero temperatures at a rate of $-4{\cdot}8°C\ h^{-1}$. In one set of control experiments, treatment was similar to that above except that no ice crystals were introduced and the samples consequently supercooled (i.e. did not freeze). In another control experiment, samples were prefrozen for only 3 h at −3°C before being subjected to −5°C for various time periods. In this experiment, five replicates were used.

All the frozen samples were thawed at 5°C in a cold room and the thawing was monitored visually. Thawing usually took about 30 min. In one set of experiments, 15 ml of 5°C deionized water was added to each test tube soon after thawing and a time series of conductivity readings was obtained. In a second set, samples were first washed for several minutes with 100 ml of 5°C deionized water, which was discarded before addition of the 15 ml of water from which conductivity readings were taken. After the conductivity readings had been obtained, each test tube was autoclaved to release all electrolytes remaining in the cells and the conductivity was measured again to permit estimation of the percentage leakage (see, Pearce 1980). In all experiments duplicate samples were used and results are expressed as averages. Deviation ranged from 0·11 to 2·5%. Direct cell-by-cell

Table 1. The relationship between electrolyte leakage and cell survival (vital staining assay)

Lowest temperature experienced (°C)	Percentage leakage (1 h after immersion)	Percentage survival
+5	7·5	95
−3	42	95
−3*	7·5	95
−6	60	80
−6*	7·5	95
−9	78	60
−12	82	10
−18	86	0
−26	86	0

*Supercooling.

estimation of the relative numbers of living and dead cells was determined by fluorescein diacetate vital staining, as described and discussed by Zhang & Willison (1987).

RESULTS

Freezing and leakage

Comparative data for electrolyte release (1 h after thawing) and cellular survival (by vital staining assay) after the various treatments are given in Table 1.

In samples exposed to a freeze–thaw cycle, leakage increased as the minimum freezing temperature decreased. The vital staining assay indicated progressive loss of viability of frost-stressed samples at temperatures below −3°C. In supercooled samples at −3 and −6°C, the leakage was the same as that for an unfrozen control. Similarly, there was no loss of cellular viability following supercooling according to the vital staining assay.

Leakage in relation to time

After standard freeze–thaw treatment, most leakage had occurred by the time the first measurement was made, which was a maximum of 10 min after thawing (Fig. 1). This incipient leakage increased markedly with decreasing freezing temperature. Following this rapid incipient leakage, the leakage rate declined progressively with the length of immersion of the samples in all treatments. In this second prolonged (post-incipient) leakage phase, the quantity of electrolytes released declined as the temperature experienced by the sample decreased, presumably because progressively greater quantities of leakage had occurred incipiently. Supercooling caused no increase in incipient leakage by comparison with the control (Fig. 1). This experiment was repeated three times with similar results (data not shown).

In order to test whether leakage was related either to a continuous phenomenon occurring while samples were held in the frozen state or to events occurring during thawing, samples were left for various times at −5°C, then thawed and their leakage patterns compared (Fig. 2). Only slight differences were found.

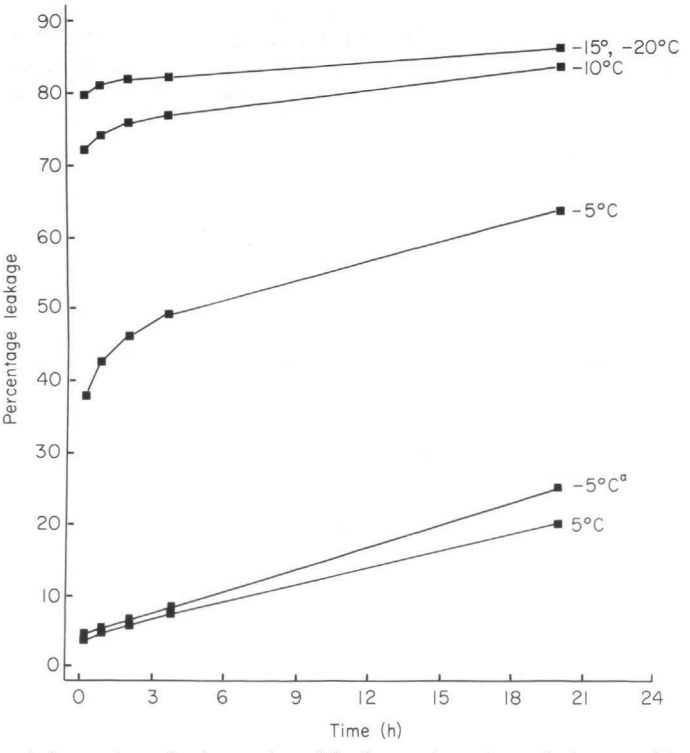

Fig. 1. Leakage in relation to time after immersion of the frozen–thawed samples in water. For each curve the lowest temperature experienced by the sample during the frost-stress treatment is indicated to the right. 'a' Indicates a supercooled sample.

Effect of post-thaw washing

The effect of removing most of the incipiently released electrolytes by washing the thawed samples is shown in Fig. 3. The rate at which prolonged leakage occurred, in relation to the quantity of electrolytes still remaining in the frozen–thawed samples, increased as a result of exposure to a freeze–thaw cycle. Although lethally frozen samples exhibited this most strongly, even sublethally frozen samples (−5°C) were greatly affected by comparison with the control (+5°C).

The prolonged leakage pattern seems to consist of two components, a complex phase (before 4 h in Fig. 3) and a steady phase (after 4 h in Fig. 3). It seems possible that the steady phase represents membrane permeability (as opposed to acute injury), and it is striking that the rates in uninjured (−5°C, supercooling), slightly injured (−5°C) and severely injured (−10°C) cultures are similar to the control rate. In the complex phase, the effect is similar after all freezing treatments, but this phase is effectively absent from the supercooled and control treatments (i.e. the 'complex' and 'steady phase' rates are the same).

DISCUSSION

The results presented here show that electrolyte leakage following a freeze–thaw treatment occurs mainly during a brief period after thawing (Fig. 1). Longer immersion of thawed cells in deionized water produced little increase in conductivity in those samples

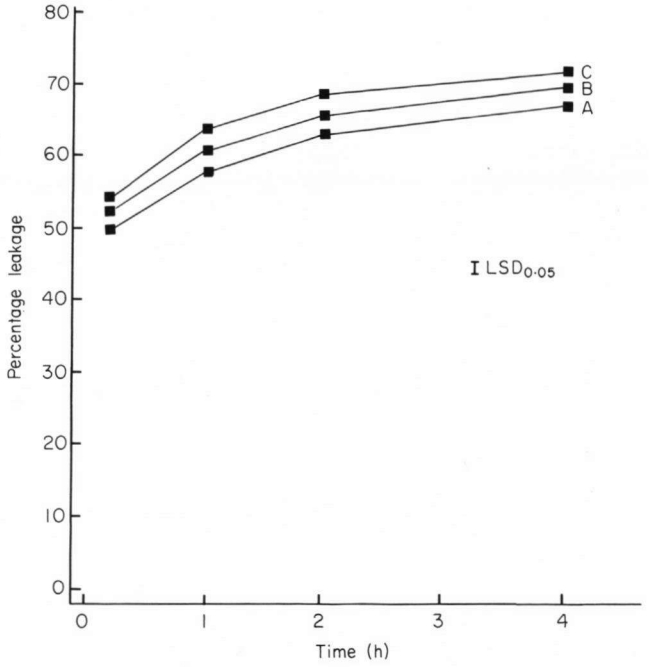

Fig. 2. Leakage in relation to length of immersion in water, after thawing, for samples kept frozen at −5°C for 0 h (A), 1 h (B) and 3 h (C).

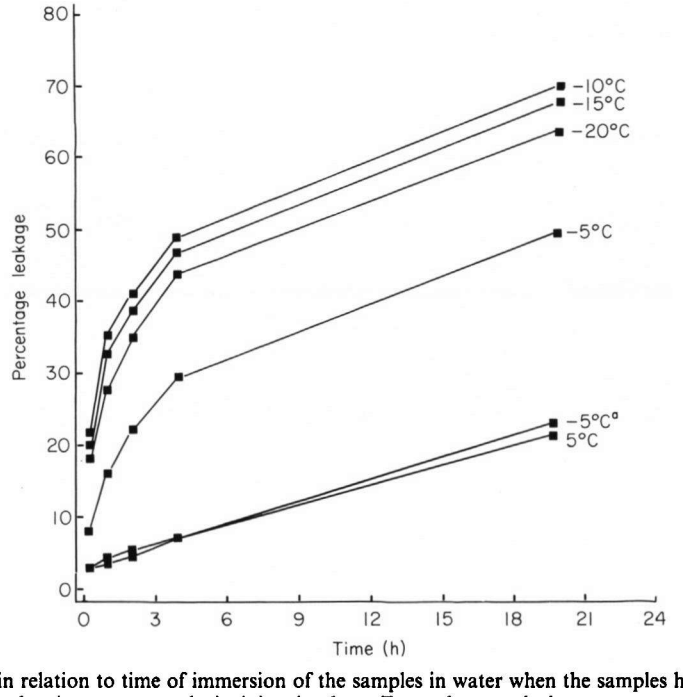

Fig. 3. Leakage in relation to time of immersion of the samples in water when the samples had been washed immediately after thawing to remove the incipient leachate. For each curve the lowest temperature experienced by the sample is indicated to the right. 'a' Indicates a supercooled sample.

that had experienced subzero temperatures sufficient to kill all or most of the cells (−15 and −20°C). In those samples that survived freezing at −5°C (or partly survived at −10°C), leakage increased with the length of immersion in deionized water. These results indicate that two components of leakage can be distinguished: (a) rapid leakage arising from freeze–thaw injury to the plasma membrane (the 'incipient leakage'); and (b) slow but prolonged leakage due to plasma membrane permeability to electrolytes in the frost-injured, but still living, cells (see also, Zhang & Willison 1987). Supercooling did not induce incipient leakage (Table 1, Figs 1 and 3), thus this phenomenon is a consequence of freeze–thaw, not of low temperature *per se*. Our data also indicate that the holding time during sublethal near-equilibrium freezing may not be critical to leakage (Fig. 2), which implies that initial freezing and/or thawing play major roles.

It is generally considered that ion transport is facilitated by carriers in the membrane (see, Harrison & Lunt 1975). Based on the observation that ion leakage is virtually unaltered by supercooling to −5°C (Figs 1 and 3), it may be concluded that ion transport-related proteins in the membrane are not affected directly by exposure to this temperature. In this context it should be noted that Lindstrom & Carter (1985) also found no effect of supercooling on electrolyte leakage from potato leaves, when supercooling (−12·5°C) was applied for 12 h. Only after 10 days of supercooling (−4°C), when metabolic effects might be expected to be expressed, did they find significantly increased electrolyte leakage.

The mechanism of massive acute electrolyte leakage following freeze–thaw exposure (incipient leakage) is not known. Palta and colleagues (Palta *et al.* 1977a,b; Palta & Li 1980; Palta *et al.* 1982) interpreted electrolyte leakage data to indicate that active transport systems responsible for pumping electrolytes into cells might be damaged by freezing, resulting in a net increase in electrolyte leakage. However, we found that leakage during the immediate post-thaw period was always much more rapid than subsequently, and in damaged samples always accounted for more than 50% of cellular electrolytes. This suggests that membrane damage is more traumatic than Palta and colleagues propose, probably involving membrane rupture during freezing and/or thawing.

Based on observations of isolated protoplasts, Wiest & Steponkus (1978; see also: Steponkus & Wiest 1979; Steponkus 1984) suggest that rapid expansion of the contracted protoplast, as water is reabsorbed during thawing, causes plasma membrane lysis in non-acclimated cells due to their insufficient membrane reserve after contraction. Expansion-induced lysis might account for the small proportion of dead cells in samples exposed to −6°C and −9°C (Table 1). However, we also found substantial incipient leakage from samples at −3°C (data not shown, pattern comparable to −5°C, Fig. 1) that showed no evidence of loss of viability by vital staining assay (see Table 1). This suggests that if membrane lysis accounts for incipient leakage, then plasma membranes which rupture following a freeze–thaw cycle may subsequently reseal. Support for this concept can be found in the work of Morris & McGrath (1981), who concluded that liposomes subjected to a freeze–thaw cycle ruptured and resealed, in view of evidence that 75% of entrapped glucose was released, yet the liposomes were intact after the freeze–thaw treatment. Similarly, Clarke *et al.* (1982) showed that in *Chlamydomonas reinhardii* freezing leads to release of enzymes, and presumably plasma membrane rupture, yet electron micrographs of lethally damaged frozen–thawed cells show intact plasma membranes.

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