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Electrical conductance of red onion scale tissue during freeze–thaw injury

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SUMMARY

Changes in the electrical conductance of red onion bulb scale tissue in relation to freeze–thaw treatments were measured by implanting wire electrodes in pieces of the tissue. Continuous measurements were made during the imposition of the stress. Tissue frost-hardiness was assayed by both vital staining and the retention of anthocyanin in vacuoles of outer epidermal cells. Results showed that the tissue had an LT_{50} of -15°C. Treatments of -3 and -6°C caused only sublethal injury (no dead cells). Tissue conductance was stable during cooling cycles involving only supercooling, but all frozen–thawed samples displayed a rapid increase in conductance during thawing, followed by constant conductance within 30 min of the initiation of thawing. Estimation of the relative quantities of electrolytes released showed that apoplasmic electrolytes increased by 300–400% following sublethal injury and by 600–900% following lethal injury. Electrolyte leakage following sublethal injury was unaffected by time spent frozen, but freeze–thaw cycles produced cumulative leakage.

Key-words: Allium cepa L., apoplasmic electrolytes, freeze–thaw injury, membrane rupture, tissue conductance.

INTRODUCTION

Freeze–thaw injury leads to electrolyte leakage from plant cells (for review, see Levitt 1980). In lethal freeze–thaw injury, electrolyte leakage results from the breakdown of the plasma membrane (Sukumaran & Weiser 1972; Steponkus & Wiest 1978; Stout *et al.* 1980). In sublethal freeze–thaw injury there is also significant electrolyte leakage, but the retention of cellular behaviour characteristic of semipermeability indicates that the plasma membrane remains intact (Palta *et al.* 1977a, b, 1982; Palta & Li 1980). The nature of sublethal injury is a matter of dispute (see Palta & Li 1980; Sobczyk *et al.* 1985; Zhang & Willison 1989).

In earlier work we examined electrolyte leakage into bathing water from bromegrass tissue culture cells which had been variously frost-stressed (Zhang & Willison 1989). Unexpectedly, it was found that treatments which were apparently not lethal resulted in patterns of leakage comparable to those found after lethal freezing treatments. A possible disadvantage of the technical approach used is that electrolytes may be retained in the apoplasm and subsequently released over time. If patterns of electrolyte leakage from

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symplasm to apoplasm are to be described more accurately, particularly in the critical stages during and after thawing, some other method is required.

Here, we have implanted electrodes in plant tissue during the imposition of freeze–thaw stress and have measured the electrical conductivity of the tissue continuously at 125 Hz. We chose this frequency because it has been demonstrated that low frequency conductance (e.g. 60–200 Hz) is related to the amount of apoplasmic electrolytes, whereas high-frequency conductance (e.g. 100 kHz) is related to the total amount of electrolytes, both apoplasmic and symplasmic (Hayden *et al.* 1969; Stout *et al.* 1987).

MATERIALS AND METHODS

Estimation of survival after injury

Bulbs of red onion (*Allium cepa* L.) were obtained locally and stored at 5°C. Tissue frost hardiness was estimated on a cell-by-cell basis after frost stress treatments (see below) had been applied, using two methods of determining cell survival: (1) vital staining of peeled inner epidermis and shaved outer epidermis, using fluorescein diacetate (Widholm 1972); and (2) retention of red anthocyanin in the outer epidermal cells. Consistent results were obtained by both methods, and the simpler anthocyanin-retention method was commonly used alone in later experiments.

In-situ *electrolyte leakage*

Instrument layout. Electrode pairs were made from 0·2 mm diameter platinum wire, 5·0 mm in length and 5·0 mm apart, tightly sealed with dental wax in a glass tube. The electrodes were led to a conductance meter in shielded wire. Attached to the electrode pair was a thermocouple from a cryothermometer which recorded the temperature in the experimental tissue piece. To allow continuous monitoring, the conductance meter and the cryothermometer were connected to chart recorders with the same recording speed.

Sampling and freeze–thaw procedures. The third living scale from the outside of the onion was used for all tests. A 5-mm thick part of the scale was cut into 10 × 10 mm squares, two of which were selected and washed three times with deionized water (200 ml water/g tissue). The two tissue pieces were stacked at the bottom of a 2 × 15 cm test tube such that the outer epidermes were in contact. The electrode pair and the thermocouple were inserted in the upper piece; the lower piece was simply a convenient support. It was found experimentally that the tissue conductance of the experimental piece was the same whether onion tissue or dental wax was used for support. To prevent drying, a drop of water was added to the bottom of the tube and the test tube was sealed with Parafilm during the experiments.

The test tube containing the sample was immersed in a precooled (5°C) temperature-controlled bath (for description, see Zhang & Willison 1987). The bath temperature was then set to −3°C, and when the tissue temperature reached −3°C freezing was initiated by introducing a very small quantity of ice. After 10 h at −3°C, the bath temperature was decreased at a rate of 4·8°C h^{-1} to the desired temperature, whereupon the bath was warmed to 5°C at a rate of about 150°C h^{-1}.

Two standard conductances were always measured: (1) the reference value (K_r), before freezing, when the temperature first reached 5°C; and (2) the experimental value (K_5), after the freeze–thaw treatment, 20 min after the tissue temperature reached 5°C. In order to obtain an estimate of variation among samples in the calculation of K_5/K_r, seven other

identical test tubes containing tissue pieces in which electrodes had been inserted were immersed in the bath together with the one which was continuously monitored. The electrodes in these samples were connected to the conductance meter only for the purpose of measuring K_r and K_5.

In supercooling experiments, identical conditions were used except for the introduction of ice to initiate freezing.

After onion tissue has been frozen and thawed it becomes soaked with water (see Palta *et al.* 1977a). In order to study the effect of water infiltration on the electrical conductivity of tissue, independent of freeze–thaw injury, 80 standard scale tissue pieces (10 × 10 mm square) were randomly divided into two groups, one of which was vacuum infiltrated with deionized water, and the electrical conductance of both groups was measured using one of the electrode pairs described above.

Temperature correction. In order to compare *in-situ* leakage among treatments, a specific conductivity must be obtained at a standard temperature (0°C was selected for convenience), using the standard tissue piece and electrode conditions described above.

Temperature correction coefficients (a) were calculated according to a formula adapted from the YSI conductance meter instruction manual:

$$a = \frac{K_t/K_o - 1}{\text{abs(t)}} \tag{1}$$

where: K_t is the conductance value at temperature t; K_o, the conductance of the same tissue but at temperature 0°C; abs(t), the temperature difference (0°C).

In the scale tissue, the coefficient was 0·0429 for 5°C and 0·0357 for −2°C under supercooling conditions. Therefore, for a frozen and thawed sample, with its known K_t (the measured conductance at temperature t) and known coefficient, a, (calculated from equation 1 above), its specific conductivity K_o' can be calculated by:

$$K_o' = \frac{K_t}{1 + a \times \text{abs(t)}}. \tag{2}$$

RESULTS

Retention of anthocyanin as an indicator of cell survival

After a freeze–thaw cycle, faded patches appeared in the outer epidermis of samples frozen to temperatures of −9°C or below as a result of the loss of vacuolar anthocyanin (Table 1). This fading progressed as the temperature decreased, with patches increasing in number and size. At −15°C, about 50% of the area had faded, corresponding to a loss of colour from about 50% of the cells.

Faded outer epidermal cells were not stained by fluorescein diacetate and only those cells with red vacuoles could be stained. The staining of living cells was characterized by bright peripheral cytoplasm, bright nuclei and networks of fluorescent protoplasmic strands. The vacuoles themselves were not stained.

Effect of water infiltration

The conductance of tissue which had been infiltrated with distilled water using vacuum infiltration was 26·47 ± 0·53 μmho. This was significantly greater than the conductance of the uninfiltrated control, which was 19·96 ± 0·33 μmho.

Table 1. Freezing injury in relation to minimal post-freezing temperature

Minimal temperature (°C)	Percentage damage*
−3	0
−6	0
−9	10
−12	20
−15	50
−18	80
−21	95
−24	100

*Estimated visually by the amount of fading of the outer epidermal cells.

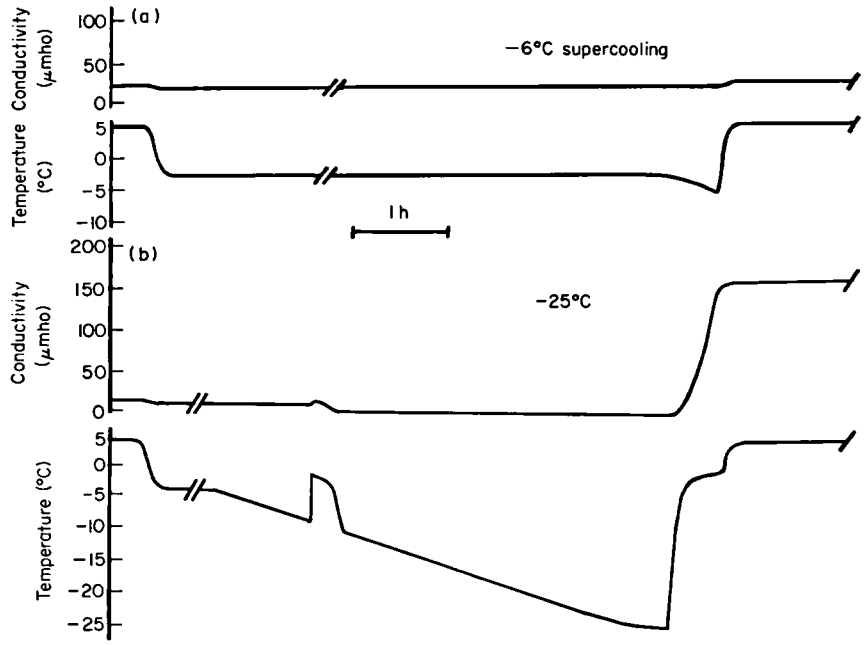

Fig. 1. (a) Tissue conductance during a cycle involving supercooling of the tissue to −6°C. The lower curve shows temperature. (b) Tissue conductance during a cycle involving freezing without ice inoculation, showing supercooling to −8°C and an exotherm at that temperature. The tissue was subsequently cooled to −25°C, which killed all cells.

Tissue conductance in relation to cooling cycles

Supercooling. It was possible to supercool the scale tissue to at least −7·5°C (Fig. 1a and b). Tissue conductance decreased only slightly during supercooling, and when the

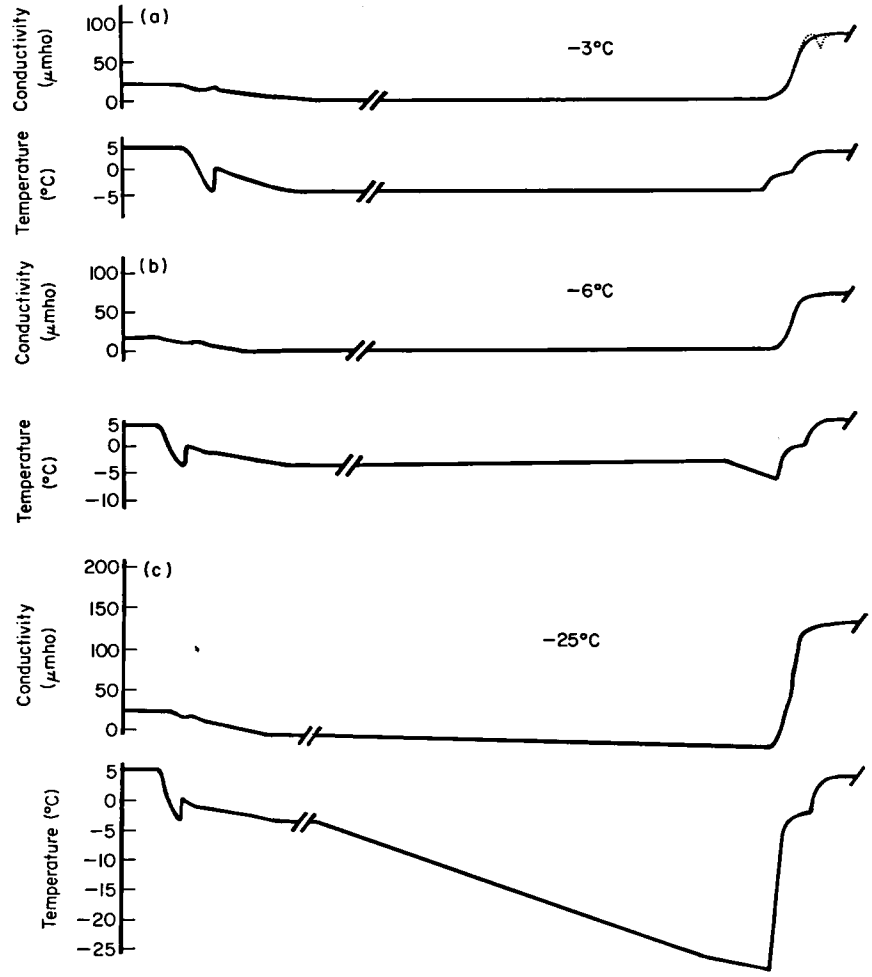

Fig. 2. Three typical curves showing tissue conductance changes during freeze–thaw cycles. Time scale the same as Fig. 1a. (a) Frozen to and thawed from −3°C; (b) frozen to and thawed from −6°C; (c) frozen to and thawed from −25°C. (In (a) the dotted line shows variation in which a peak and valley are present.)

temperature of the specimen was returned to its initial value, the tissue conductance also returned to the precooling level (Fig. 1a).

Freeze–thaw cycles. Tissue conductance curves had similar general patterns during freeze–thaw cycles, regardless of the lowest temperature experienced (Fig. 2a–c). A typical curve had the following characteristics.

(a) *Freezing.* At 5°C, the conductance remained steady with time, giving a constant reference conductance (K_r) for any tissue piece. When freezing was initiated at −3°C, tissue temperature rose to −0·5°C and the conductance increased slightly as a result of this rise in temperature. Although the bath was set at −3°C, the tissue remained above −2°C for about 1 h, and during this period the tissue conductance decreased slowly. After 2 h at a

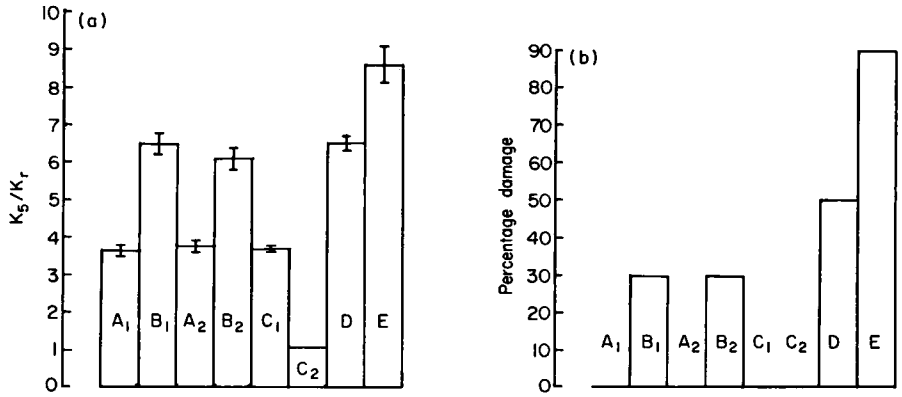

Fig. 3. (a) K_5/K_r (ratio of conductivity at 5°C after a freeze–thaw cycle to that in the same tissue piece at 5°C before the cycle had begun) in relation to minimal freezing temperature and freeze–thaw cycle frequency. The standard deviation in each treatment is indicated ($n=8$). A_1—freezing to and thawing from −3°C, freezing at −3°C for 10 h; B_1—same sample as A_1 but with an additional −3°C freeze–thaw cycle (i.e. two −3°C cycles, each of 10 h); A_2—freezing to and thawing from −3°C, freezing at −3°C for 1 h; B_2—same sample as A_2 but with an additional −3°C freeze–thaw cycle (i.e. two −3°C cycles, each of 1 h); C_1—freezing to and thawing from −6°C, 10 h; C_2—supercooling to −6°C; D—freezing to and thawing from −15°C, 10 h; E—freezing to and thawing from −25°C, 10 h. (b) Percentage damage as measured by the percentage of epidermal cells which have faded due to loss of anthocyanin. The labels A_1–E have the same meaning as in (a). A_1, A_2, C_1 and C_2 have no damage.

bath temperature of −3°C, the tissue reached −3°C and the corresponding tissue conductance became constant and close to zero. Lower temperatures gave lower conductances, but the differences were small (Fig. 2a–c).

(b) *Thawing*. When tissues were thawed using a bath temperature of 5°C, the temperature of the tissue increased quickly initially to −2°C. Subsequently, the temperature increased at a slower rate, taking about 30 min for thawing to be completed.

Tissue conductance increased fairly slowly with the rise in temperature to −2°C, but then there was a rapid increase in conductance while the temperature rose from −2 to 0°C. This phase of rapid increase in tissue conductance was of about the same duration (not more than 30 min) in all samples and was completed when the tissue temperature reached about 0°C.

(c) *Post-thaw*. Tissue conductance increased only slightly while the temperature rose from 0 to 5°C, and once the tissue temperature had stabilized at 5°C, the conductance remained steady (Fig. 2a–c). Within the short post-thaw period when tissue temperature was rising, some variation in the pattern of change in conductance was observed among samples, as shown in Fig. 2a. A smooth curve was most usual, but in some samples there was a small peak and valley before constancy was obtained (Fig. 2a).

Freeze–thaw injury index. The ratio of conductance after a freeze–thaw cycle to that before freezing (K_5/K_r), for any tissue sample, is an index of the quantity of electrolytes released as a result of the freeze–thaw stress. It can be considered to be an index of freeze–thaw injury. As the lowest temperature experienced by any tissue sample in a freeze–thaw cycle decreased, the injury index increased (Fig. 3). There was no change in conductance after supercooling, however, indicating no injury and giving an injury index of 1·0. The freeze–thaw injury index after prolonged freezing (10 h) was almost the same as after short-term

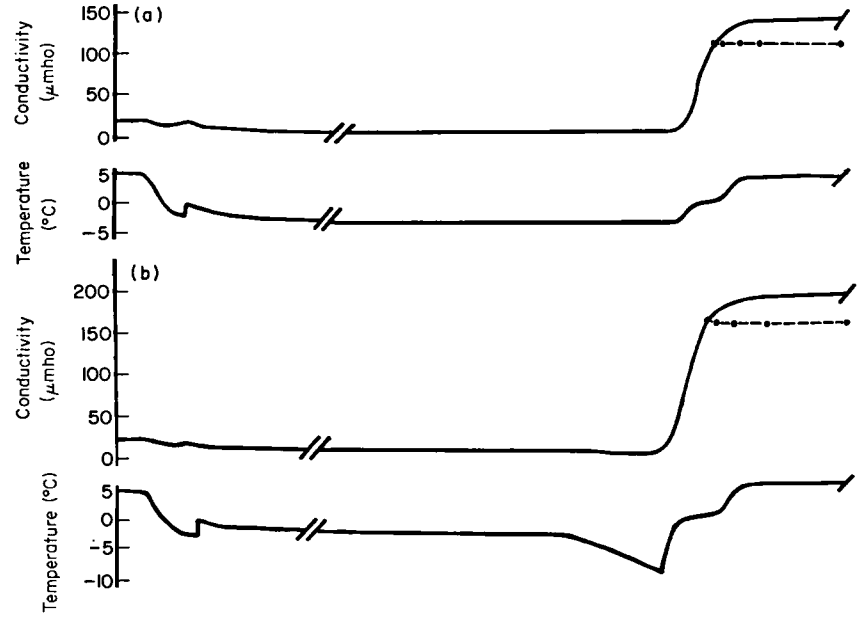

Fig. 4. Examples of temperature correction of conductance curves. The solid lines are the originally recorded conductances and the broken lines are corrected conductances. (a) Sample frozen to and thawed from −3°C; (b) sample frozen to and thawed from −9°C. Time scale the same as in Fig. 1a.

freezing (1 h) at the same temperature (−3°C). By contrast, two freeze–thaw cycles at −3°C, each of 1 h duration, resulted in a substantial increase in the injury index (Fig. 3).

Temperature correction

Tissue conductance measured at different temperatures during any freeze–thaw cycle can be temperature corrected to obtain a specific conductivity at 0°C. A common feature of temperature-corrected conductance curves was that the conductance changed more acutely around 0°C than in uncorrected curves (see, for example, Fig. 4).

DISCUSSION

Our results show that both vital staining and the presence of vacuolar red anthocyanin are reliable indicators of frost survival. The red onion scale tissue was frost resistant, with an LT_{50} around −15°C. Freezing to and thawing from −3°C and −6°C appears to have caused no damage.

Within the period tested, supercooling produced no noticeable *in-situ* leakage (Fig. 1a). Using a floating leaf method, Lindstrom & Carter (1985) also observed no effect of supercooling on electrolyte leakage from potato leaves (−12·5°C for 45 min or −4°C for up to 10 days).

On the basis of previous studies (Hudson & Idle 1962; Graham & Mullin 1976), it can be suggested that the exotherms we observed during ice inoculation of sublethally injured tissue (see Fig. 2a and b) resulted from extracellular freezing. Changes in tissue conductance during an extracellular freeze-thaw cycle might be attributed to one or more of the three episodes: freezing, thawing and post-thawing.

We found that during freezing the formation of ice proceeded slowly, as shown by the thermo-analysis curve (Fig. 2a–c). The gradual decrease in tissue conductance during

freezing probably resulted from (a) insulation due to ice encasement of electrodes, and (b) a reduction in channel continuity and an increase in electrolyte concentration in the tissue (see Steponkus *et al.* 1985). Therefore, conductivity measurement in frozen tissue does not describe its electrolyte status.

Once the tissue temperature was at 5°C, tissue conductance was constant (Fig. 4), indicating no significant change in the quantity of apoplasmic electrolytes with time in the post-thaw period.

In all experiments, tissue conductivity was greater after thawing than before freezing. The tissue infiltration experiment showed that a small part of this increase in tissue conductance is due to freeze–thaw-induced tissue infiltration. The experimental infiltration-induced increase in conductance was only about 33%, however, whereas even after non-lethal freezing to −3°C, which induced the smallest amount of post–thaw leakage, an increase in conductance of 250% was found (Fig. 3). Thus, most of the post–thaw increase in conductivity is probably due to factors other than freezing-induced infiltration. Possible causes of this increase are: (1) electrolytes leak from the symplasm while the tissue is frozen but are detected only during thawing; (2) electrolytes leak during thawing as a direct consequence of thawing; and (3) a combination of the above.

In previous studies, examples of all three of the possibilities outlined above have been found. Possibility 1 is illustrated by lethally frozen cells which lose osmotic responsiveness immediately after freezing (see Dowgert & Steponkus 1984), and possibility 2 by death due to thawing-induced membrane expansion lysis when cells are not killed during freezing (see Wiest & Steponkus 1978; Dowgert & Steponkus 1984; Gordon-Kamm & Steponkus 1984). Possibility 3 is illustrated by the example of lethally frozen serviceberry, which releases HCN both while frozen and after thawing (Stout *et al.* 1980, 1981; Stout 1983).

For sublethal injury at −3°C, the amount of leakage detected after thawing was independent of time spent frozen (Fig. 3), which suggests that a significant proportion of the leakage did not occur as a continuous process while the tissue was frozen. The fact that two freeze–thaw cycles, each of 1 h, resulted in much more leakage than one 10 h cycle at the same temperature (−3°C), indicates that in onion, leakage following sublethal injury is either a brief event associated with the initial ice-growth phase of freezing, or is a direct consequence of thawing, or is a combination of both of these.

In several previous studies, leaching of electrolytes following freeze–thaw stress has been detected in onion tissues (Palta *et al.* 1977a, b; Palta & Li 1980) and other comparable materials (Sobczyk *et al.* 1985; Pukacki & Pukacka 1987). This leakage was found to progress relatively slowly with time of submersion of the injured tissue in bathing water, and has been explained by assuming that freezing damages the ion pumps responsible for pumping back electrolytes previously released passively (Palta & Li 1980). Our relatively direct method of detecting leakage indicates that leakage (whatever its origin) is acutely detectable during thawing and is not continued into the post-thaw period (see Fig. 2a–c). This suggests that the release of electrolytes in the conventional conductivity method of assaying freeze–thaw injury (Dexter *et al.* 1932) is greatly affected by the rate of diffusion of electrolytes from apoplasm to the bathing water. It does not really assess leakage from symplasm to apoplasm (membrane permeability) as generally assumed (Palta *et al.* 1977a; Palta & Li 1980; Sobczyk *et al.* 1985; Pukacki & Pukacka 1987), but may measure the combined effect of electrolyte efflux from cells to apoplasm and from apoplasm to bathing water. A good example of this is the recent demonstration (Murray *et al.* 1989) that it takes about 3 h for most electrolytes to diffuse from a liquid-nitrogen killed red spruce twig to the bathing water.

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