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Freezing tolerance in alpine plants as assessed by the FDA-staining method

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Abstract: We established a method employing fluorescein diacetate (FDA)-staining with a laser scan microscope for evaluation of the freezing tolerance of leaf mesophyll cells, and applied it to assessments of freezing tolerance in leaves of nine alpine plants, *Arcterica nana*, *Cassiope lycopodioides*, *Diapensia lapponica*, *Empetrum nigrum*, *Loiseleuria procumbens*, *Phyllodoce nipponica*, *Rhododendron aureum*, *Schizocodon soldanelloides*, and *Vaccinium vitis-idaea*, which naturally occur on Mt. Iwo (36˚00´N, 138˚22´W, 2760 m a.s.l.) in 2001. The results obtained with the FDA-staining method were strongly correlated with those of the electrolyte-leakage test, suggesting that this method is highly reliable. Leaf mesophyll cells in all these plants collected in early September survived after freezing at −5˚C, but did not survive below −30˚C. The survival ratio at subzero temperatures, ranging from −5 to −16˚C, varied among the species. On the other hand, all the mesophyll cells of the plants collected in the middle of November survived freezing at −30˚C. However, the survival ratio decreased after freezing at −80˚C in *Phyllodoce nipponica* and *Empetrum nigrum*. Using the FDA-staining method, we were able to confirm the increase of the freezing tolerance of alpine plants from September to November. Advantages of the new method are discussed.

key words: alpine plants, FDA (fluorescein diacetate), freezing tolerance, seasonal change, survival ratio

Introduction

Alpine plants survive low temperature through a long winter, thanks to their freezing tolerance (Sakai and Otsuka, 1970). The freezing tolerance of alpine plants shows seasonal changes, and the degree of tolerance increases from autumn to winter (*e.g.* Sakai and Otsuka, 1970; Levitt, 1980; Sakai, 1982). Steponkus *et al*. (1993) reported that most plants growing in cold climates in winter have the ability to survive temperature even below −30˚C. On the other hand, they are subject to freezing injury at temperature just below −3˚C, when they are actively growing in summer. Studies on the freezing tolerance of and damage to alpine plants are important for understanding their distribution in natural vegetation and their adaptation to cold temperature.

As indicators of freezing damage, a tissue-discoloration method (*e.g.* Sakai, 1955; Aronsson and Eliasson, 1970) and a regrowth capacity method (*e.g.* Lapins, 1961; Sakai, 1965; Kobayashi *et al*., 1983) have been used. However, these evaluations are time-consuming and needed for many samples (Stergio and Howell, 1973). The extent of freezing damage is also estimated using stains such as neutral red (Swain and De, 1994), evans blue (Smith *et al*., 1982; Swain and De, 1994), trypan blue (Hou and Lin, 1996), methylene blue (Huang *et al*., 1986), and phenosafranine (Widholm, 1972; Swain and De, 1994). Although these tests are suitable for cultured cells and monads, it is difficult to detect surviving cells in tissue sections. The reasons are thought to be slow penetration of these stains, and invisibility of cells inside a large assembly of cells. Among evaluation methods of freezing damage, the electrolyte-leakage test has been most frequently used (Dexter *et al*., 1930, 1932; Stuart, 1939, 1941; Bigras, 1997; Hurry *et al*., 2000; Shou *et al*., 2004). However, some electrolytes can leak out of unfrozen (control) samples, especially from cut surfaces. Therefore, results of the leakage test vary depending on the species and/or tissue types, and measurements with unfrozen tissues sometimes give relatively high conductance values (Palta *et al*., 1977; James and Bert, 1990; Iija and Jiri, 1998). Thus, careful control experiments are absolutely needed in the electrolyte-leakage test.

The fluorescein diacetate (FDA) method can be applied to determination of plant cell viability. This method is convenient, simple and reproducible in cultured cells and monads (Widholm, 1972; Huang *et al*., 1986; Ishikawa *et al*., 1995; Leborgne *et al*., 1995; Steward *et al*., 1999). Esterase activity is sufficiently high in any living cell (Steward *et al*., 1999). Thus, FDA passing through cell membranes can be converted to a fluorescent material, fluorescein, by the activity of an endogenous esterase. On the other hand, dead cells cannot accumulate FDA, because the esterase activity in the dead cells is lost or their membranes are perforated (Tsuji *et al*., 1995). It is possible to distinguish living cells from dead ones in cultured cells and monads. Although the FDA-staining method has many advantages, this method has not been used for assessment of freezing tolerance with tissue cross sections. The FDA staining has been thought to not give an accurate estimation of survival ratio in a whole tissue because of the slow penetration of FDA and because of the invisibility of cells inside a large assembly of cells, such as cross sections of intact tissues (Ishikawa *et al*., 1995).

In this study, we first established a protocol for the FDA-staining method for evaluation of cell survival in leaf cross-sections using a confocal laser scan microscope (LSM). We also compared this method with the electrolyte-leakage test which has been most frequently used for evaluation of freezing tolerance. With the FDA-staining method, we investigated the freezing tolerance of and damage to nine alpine plants on Mt. Iwo (2760 m a.s.l.) in Japan. We sampled leaves of the alpine plants in early September and the middle of November in 2001, and examined the viability of leaf mesophyll cells, because the leaves are exposed to very low temperature during winter. We also examined changes of freezing tolerance during cold acclimation in the nine alpine plants.

Materials and methods

Plant materials

Plant species of three families, Diapensiaceae, Empetraceae and Ericaceae, were collected on Mt. Iwo, in the Yatsugatake mountains (36˚00´N, 138˚22´W, 2760 m a.s.l.),

Nagano, Japan on 1 September and 17 November 2001. As members of Diapensiaceae, *Diapensia lapponica* L. subsp. *obovata* (Fr. Schm.) Hulten and *Shortia soldanelloides* (Sieb. et Zucc.) Makino f. *alpina* (Maxim.) Makino were used. As members of Empetraceae, *Empetrum nigrum* L. var. *japonicum* K. Koch was used. As members of Ericaceae, *Arcterica nana* (Maxim.) Makino, *Cassiope lycopodioides* (Pall.) D. Don, *Loiseleuria procumbens* (L.) Desvaux, *Phyllodoce nipponica* Makino, *Rhododendron aureum* Georgi and *Vaccinium vitis-idaea* L. were used. Air temperature was continuously measured at the height of appropriately 15 cm from the ground surface in the shade, and the measurements were recorded with a data logger (Data-Mini, HIOKI, Nagano, Japan), which recorded the air temperature hourly from 1 August to 16 November 2001. All the plants including roots were sampled on the same west slope. This place was often blown by a strong wind. In the middle of November, the ground was covered with shallow snow. Just after sampling, the plant samples were wrapped with a wet towel, kept on ice, and brought to a laboratory. It took 12 hours until the freezing treatments discussed in the next section. In this study, only current-year leaves were used.

Freezing treatments

The shoots kept in small polyethylene bags were cooled at a rate of 2.0°C h^{-1} from 4°C to −16°C in programmed freezers in the dark. Parts of samples were removed when the temperature reached −5°C, −10°C and −16°C. The rest, cooled to −16°C, was transferred to a freezer at −30°C and kept for 7.0 h. A part of these shoots, cooled to −30°C, was transferred to −80°C, and kept for 25 h. The shoots cooled down to −5°C, −10°C, −16°C, −30°C or −80°C, were allowed to thaw at 4°C in the dark.

Determination of freezing tolerance

1) The FDA-staining method

Freezing tolerance of the plants was examined after 3 days of rewarming at 4°C. This duration for rewarming is suggested to be the best for examining the freezing tolerance, because irreversible damage is clear and no significant regrowth occurs (Mantyla *et al*., 1995). Tissue sections, approximately three cells thick, were cut with a new razor blade and placed in a micro-tube (1.5 m*l*). These small sections were soaked in 50 m*l* of 0.23 mM fluorescein diacetate (FDA) for 60 min at room temperature (from 20 to 25°C) to maximize formation of fluorescein. A confocal laser scan microscope (LSM410, Zeiss, Oberkochen, Germany) was used for observations. Excitation and emission wavelengths were selected at 493 nm and 510 nm, respectively, and the degree of cell survival was assessed. Only cells that exhibited bright green fluorescence from their cytosol were considered to be viable.

Survival ratio (%) was calculated as the ratio of cells emitting fluorescence to total cells. The total cell number was counted for the same field of the microscope. For each freezing temperature, at least three leaf sections from different shoots were examined for one species.

2) Electrolyte-leakage test

The electrolyte-leakage test was conducted according to previously reported technique (Moore and Stain, 1948; Murai and Yoshida, 1998) with some modifications. Right after the frozen shoots were thawed at 4°C, leaves were cut from the shoots, and placed in screw-cap glass vials with distilled water in the ratio of 0.1 g of leaf samples (fresh weight): 10 m*l* of distilled water. The glass vials were kept at 4°C for 36 h in the dark. The conductivity of the

supernatant was measured with an electro-conductivity meter (Twin Compact Meter, Horiba, Kyoto, Japan). The unfrozen leaf samples were also kept in the screw-cap glass vials, boiled at 100˚C for 30 min, and kept at 4˚C for 36 h. The conductivities obtained for the supernatants with and without boiling were used as 100% and 0% injury, respectively. Each measurement was carried out with three replicates for each freezing temperature.

Results and discussion

FDA-staining method and electrolyte-leakage test

Figure 1B shows a result of a fluorescence image of the FDA-stained cross section in leaves of *V. vitis-idaea* without the freezing treatment. Most of the cells were stained. On the other hand, after boiling the sections at 100˚C for 30 min, fluorescence was not detected at all, and the image was black (data not shown). The same results were obtained for other species. It has been thought that FDA staining does not give an accurate estimate of cell survival in case of a large assembly of cells (Ishikawa *et al*., 1995). However, irrespective of the sample species in this study, the FDA-staining method using LSM enabled us to distinguish the living cells from the dead cells, even in cell-stacked leaf sections. This FDA-staining method is convenient, accurate and simple.

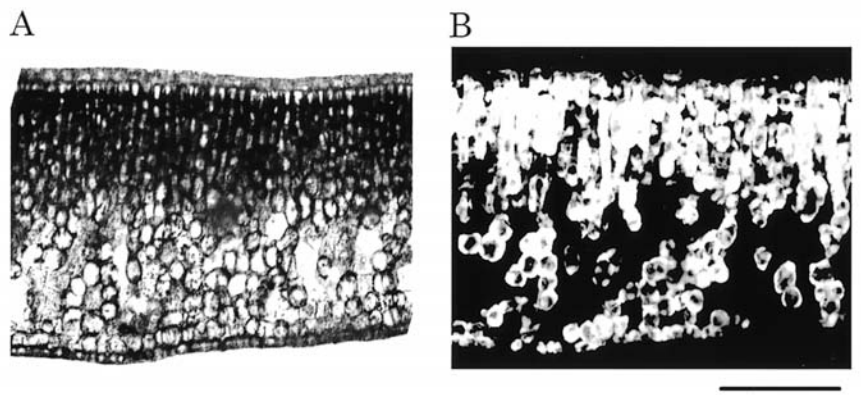

Fig. 1. Light field image (A) and fluorescence image (B) of cross-sections of unfrozen leaves of *V. vitis-ideaea*. Bar＝100 mm.

We compared the results of the FDA-staining method with those of the electrolyte-leakage test, because the electrolyte-leakage test has been widely used for assessment of the survival of plant tissues. Advantages of the electrolyte-leakage test include rapidity of injury estimation (Dexter *et al*., 1930, 1932) and necessity for only small amounts of tissues (Sakai, 1955). The results from these two methods correlated very well (Fig. 2). However, most of the points lay below the line $y=-x$. This difference can be ascribed to two things: 1) The estimation by the electrolyte-leakage test does not indicate the actual survival ratio, because injured but surviving cells can leak out of electrolytes. Therefore, the electrolyte-leakage test gives an overestimate compared to the FDA-staining method. 2) The degree of surviving cells estimated by the FDA-staining method is underestimated, because some of the surviving cells are not stained by this method. However, the discrepancy between these two methods

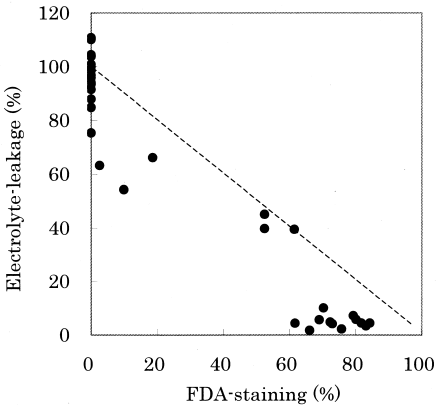

Fig. 2. Relationship between the relative conductance (%) assessed by the electrolyte-leakage test and the survival ratio (%) assessed by the FDA-staining method for leaves of *V. vitis-ideaea* sampled in September. The correlation coefficient between the two results was −0.96. The dashed line indicates $y=-x$.

Table 1. Correlation between relative conductance (%) assessed by the electrolyte-leakage test and the survival ratio (%) assessed by the FDA-staining method in September.

Family	Species	*r*
Diapensiaceae	*Diapensia lapponica*	−0.89
	Shortia soldanelloides	−0.94
Empetraceae	*Empetrum nigrum*	−0.85
Ericaceae	*Arcterica nana*	−0.90
	Cassiope lycopodioides	−0.80
	Loiseleuria procumbens	−0.94
	Phyllodoce nipponica	−0.93
	Rhododendron aureum	−0.96
	Vaccinium vitis-idaea	−0.96

was very small compared with the variation of freezing tolerance through the season. The second problem will hopefully be solved with improvement of penetration of FDA (under investigation). Table 1 shows correlation coefficients between the relative conductance (%) assessed by the electrolyte-leakage test and the survival ratio (%) assessed by FDA-staining for all sample species. A high correlation was observed between the two results. The maximum difference between the two methods was less than 20%. Therefore, we conclude that the FDA-staining method can be used for survival evaluation of freezing tolerance.

Freezing tolerance of alpine plants

We examined the survival ratio of mesophyll cells from leaves sampled in early September and the middle of November. Changes in air temperature on Mt. Iwo are shown in Fig. 3. The minimum temperatures on Mt. Iwo were 3˚C in early September and −10˚C in the middle of November, respectively.

From analyses of the FDA-staining method, freezing tolerances of these nine species varied considerably at the beginning of September (Fig. 4). Most mesophyll cells in all species survived after cooling to −5˚C. For all species, the survival ratio decreased with decrease in temperature between −5˚C and −16˚C, and no mesophyll cells could survive

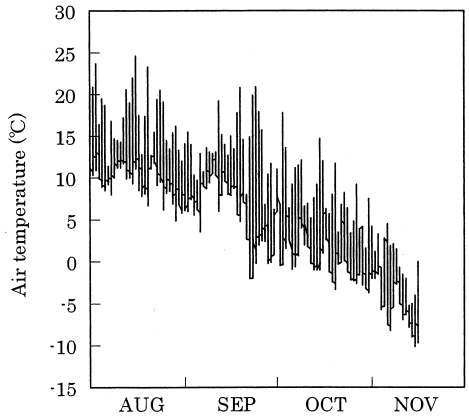

Fig. 3. Air temperature on Mt. Iwo. Air temperature was measured at the height of appropriately 15 cm from the ground surface in the shade every one hour from 1 August to 16 November 2001. In the middle of November, the ground was slightly covered with snow.

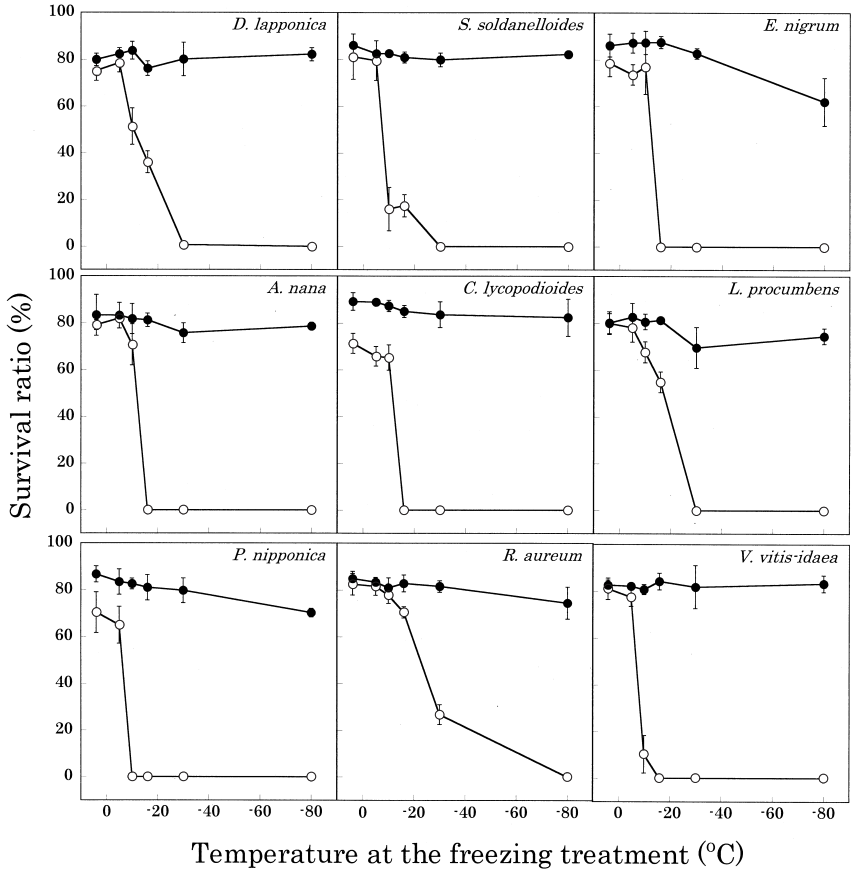

Fig. 4. Effects of freezing temperatures on the survival ratio (%) assessed by the FDA-staining method. Values are means ± SD (n = 3). Open circles: plants sampled on 1 September, closed circles: plants sampled on 17 November 2001.

below −30˚C. The survival ratios in the −5˚C freezing test were 65.0, 79.6 and 77.5% in *P. nipponica*, *S. soldanelloides*, and *V. vitis-idaea*, respectively, but in the −10˚C freezing test, the survival ratios of these three species decreased to 0.0, 16.1 and 10.3% in *P. nipponica*, *S. soldanelloides*, and *V. vitis-idaea*, respectively. These alpine plants showed more susceptibility to low temperature than other species. These results indicate that mesophyll cells of all species were damaged by low temperature below −5˚C. On the other hand, the survival ratios at the freezing test of −5˚C were 78.2 and 81.6% in *L. procumbens* and *R. aureum*, respectively. The survival ratios in the −16˚C freezing test were 55.0 and 70.5% in *L. procumbens* and *R. aureum*, respectively. These alpine plants survived better in low temperature than the others in early September.

However, these freezing tolerances changed after cold acclimation. The majority of the mesophyll cells survived even after cooling to −30˚C for all the plant species that were sampled in the middle of November. The survival ratios of mesophyll cells treated even at −80˚C did not decrease so much. The ratios at −80˚C were slightly lower in *E. nigrum* and *P. nipponica* than in other species. Survival at such an extreme temperature has also been reported in other studies. It has been reported that cacti including *Opuntia humidusa* can tolerate subzero temperatures of −24˚C, and *Opuntia fragilis* can survive a 1 h-treatment at −40˚C (Nobel and Loik, 1990; Loik and Nobel, 1993). Moreover, some alpine plants on Mt. Kurodake (1984 m) in Hokkaido were able to survive freezing to −70˚C in winter (Sakai and Otsuka, 1970). In the present study, many alpine plants on Yatsugatake in November were able to survive −80˚C. These studies at extreme low temperature are thought to be important for understanding not only their distribution and strategies in natural vegetation but also physiological mechanisms for freezing tolerance. Since freezing tolerances were analyzed by a regrowth capacity method and a tissue-discoloration method in past studies, the evaluations were time-consuming and needed many samples (Stergio and Howell, 1973). On the other hand, the FDA-staining method with LSM is reliable compared to the analyses in past studies, and also has some advantages including rapidity of injury estimation and necessity for only small amounts of tissues. Therefore, this FDA-staining method is thought to be best for analyses of the variation of plant species in freezing tolerance and understanding of their distribution in natural vegetation.

In this study, we established a method employing FDA-staining by using LSM for evaluation of freezing tolerance of leaf mesophyll cells, and applied it to the assessment of freezing tolerance of alpine plants. We were able to confirm that the freezing tolerance of alpine plants considerably vary among species in early September, and also largely increase from September to November. The FDA-staining method employed in this study has many advantages: for example, 1) we can conduct fluorescence and immunofluorescence analyses at the same time; 2) we can analyze differences in degree of survival among cell types. We did not detect differences in freezing tolerance between palisade and spongy tissue cells in the present study. However, differences in freezing tolerance among cell types can be detected; for example, a primary damage site can be identified by detailed studies using this FDA-staining method. The FDA-staining method for the evaluation of freezing tolerance can provide us with morphological information about the freezing tolerance of leaves.

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