

## A new application of the SFDA-staining method to assessment of the freezing tolerance in leaves of alpine plants

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**Abstract:** For the first time, this study used 5- (6-) sulfofluorescein diacetate (SFDA), a fluorescent product in plant cells converted by esterase activity to fluorescein-5- (and 6-) sulfonic acid (FSA), to assess the freezing tolerance of leaf cells. We were able to readily distinguish living and dead cells, and detect differences in freezing tolerance among five alpine plants using the SFDA-staining method. We also compared this method with two conventional methods, the electrolyte leakage test and fluorescein diacetate (FDA) staining method. The electrolyte leakage test often over- or underestimated freezing injury. With the uninjured control samples, the FDA-staining method failed to stain all leaf cells, while the SFDA-staining method stained almost 100%. From these results, we concluded that SFDA-staining is a more convenient, accurate and reproducible method for analyses of freezing tolerance.

**key words:** alpine plants, FDA (fluorescein diacetate), freezing tolerance, SFDA (5- (6-) sulfofluorescein diacetate), survival ratio

### Introduction

Examining cell viability is useful in many physiological, biotechnological and ecological studies. For example, according to Steponkus and Lanphear (1967), a detailed description of freezing injuries and cold acclimation in plants is necessary for accurately determining the extent of injury. Various methods have been developed to conduct viability tests, with the most commonly used including the regrowth capacity and tissue browning test (Sakai, 1955; Neuner *et al.*, 1997; Chen *et al.*, 2000; Menges and Murray, 2004; Wang *et al.*, 2004), the electrolyte leakage test (Dexter, 1956; Hurry *et al.*, 2000; Pennycooke *et al.*, 2003; Shou *et al.*, 2004; Yamori *et al.*, 2005), the vital staining method (Widholm, 1972; Smith *et al.*, 1982; Huang *et al.*, 1986; Swain and De, 1994; Hou and Lin, 1996), the 2, 3, 5-triphenyltetrazolium chloride (TTC) reduction test (Steponkus and Lanphear, 1967; Towill and Mazur, 1974; Ishikawa *et al.*, 1995; Pihakaski-Maunsbach *et al.*, 2003; Wang *et al.*, 2004) and the fluorescein diacetate (FDA) staining

Table 1. A summary of the main viability tests currently employed, including their advantages and disadvantages comments.

| Method                   | Comments   |
|--------------------------|--|
| Regrowth capacity test   | (Advantages)   |
| Tissue browning test     | Currently, the most reliable viability tests (1, 2, 3, 4, 5).<br>Able to evaluate differences in stress tolerance among different plant tissues, including recovery following stress (3, 4).<br>(Disadvantages)<br>Must be conducted under aseptic conditions, and the incubation period is time-consuming (2, 4, 5).  |
| Electrolyte leakage test | (Advantages)<br>Able to rapidly evaluate cell injury in many samples (3, 6, 7).<br>(Disadvantages)<br>Tends to overestimate and/or underestimate the injury (3, 8, 9, 10, 11, 12).<br>Not able to estimate comprising tissues differing in their freezing tolerance capacity (13, 14, 15).   |
| Vital staining method    | Includes the staining methods of evans blue (16, 17, 18), trypan blue (19), neutral red (18, 20, 21), methylene blue (22), and phenosafranine (18, 23).<br>(Advantages)<br>Rapid and needs only a light microscope for observation (23).<br>(Disadvantages)<br>Needs laborious and inaccurate microscopic observations in the case of natural aggregation of plant cells (24).   |
| TTC reduction test       | (Advantages)<br>Does not diffuse out of cells after reduction (25).<br>Rapid and convenient (5, 25, 26, 27, 28).<br>(Disadvantages)<br>Needs laborious and inaccurate microscopic observations in the case of natural aggregation of plant cells (5, 25, 26, 29).<br>Depends not only on the cell viability but also on the metabolic state of the cells, such as the amount of reducing sugars, glutathione, ascorbic acid, and cysteine (25, 30, 31, 32).<br>Probably not considered a general test for cell survival (4). |
| FDA-staining method      | (Advantages)<br>Convenient, simple and reproducible in cultured cells and monads (5, 23, 24), roots and seeds (33) and tissue sections of higher plants (12).<br>(Disadvantages)<br>Tends to underestimate injury (12, 24, 34, 35, 36).<br>Cannot be applied to comprised cells (5, 37).   |

(1) Sakai, 1955; (2) Sterigo and Howell, 1973; (3) Burr *et al.*, 1990; (4) James and Bert, 1990; (5) Ishikawa *et al.*, 1995; (6) Dexter *et al.*, 1930; (7) Dexter *et al.*, 1932; (8) Kettle *et al.*, 1972; (9) Van den Driessche, 1976; (10) Pellett and Carter, 1981; (11) Boorse *et al.*, 1998; (12) Yamori *et al.*, 2005; (13) Dexter, 1956; (14) Aronsson and Eliasson, 1970; (15) Iija and Jiri, 1998; (16) Gaff and Okong'Oogola, 1971; (17) Smith *et al.*, 1982; (18) Swain and De, 1994; (19) Hou and Lin, 1996; (20) Nobel *et al.*, 1995; (21) Nobel and Barrera, 2003; (22) Huang *et al.*, 1986; (23) Widholm, 1972; (24) Steward *et al.*, 1999; (25) Parker, 1953; (26) Towill and Mazur, 1974; (27) Duncan and Widholm, 1990; (28) Burke, 2001; (29) Ishikawa *et al.*, 1990; (30) Mattson *et al.*, 1947; (31) Nash and Davies, 1975; (32) Matanguihan *et al.*, 1994; (33) Noland and Mohammed, 1997; (34) Ogawa *et al.*, 1997; (35) Wang *et al.*, 2001; (36) Winkelmann *et al.*, 2004; (37) Harding and Benson, 1995.

method (Widholm, 1972; Vaňková *et al.*, 2001; Pokorna *et al.*, 2004; Winkelmann *et al.*, 2004; Yamori *et al.*, 2005) (Table 1). Each method has a number of advantages and disadvantages (Table 1), and there is no single viability test that can be used for all types of plant material or under all types of experimental conditions.

The fluorescein diacetate (FDA) staining method can be reliably used to determine plant cell survival using cultured cells and monads (Widholm, 1972; Ishikawa *et al.*, 1995; Steward *et al.*, 1999). FDA passing through the cell membrane is converted into a fluorescent substance, fluorescein, by endogenous esterase activity. In general, it is possible to distinguish living from dead cells using this method. However, FDA does not efficiently penetrate some types of membrane in certain species (Harding and Benson, 1995). Moreover, the product resulting from the conversion of FDA, fluorescein, tends to leak from the cells (Söderstöm, 1979; Thomas *et al.*, 1979). Previous studies have shown that the method fails to give 100% cell survival ratios even for uninjured control samples of cultured cells (Ogawa *et al.*, 1997; Steward *et al.*, 1999; Wang *et al.*, 2001; Winkelmann *et al.*, 2004) and leaf tissue sections (Yamori *et al.*, 2005). Ishikawa *et al.* (1995) also reported that the FDA staining method does not give an accurate estimation of cell survival in a large assembly of cells such as cross sections of intact tissues.

To solve many of these problems, the dye 5- (6-) sulfofluorescein diacetate (SFDA) appears to be effective. It was previously reported that all living cells and spores of microbial strains, including bacteria, actinomycetes, cellular slime molds and algae, are clearly stained with SFDA (Tsuji *et al.*, 1995). The basic principle is similar to that of FDA (Fig. 1). That is, non-fluorescent SFDA is converted to a fluorescent compound, fluorescein-5- (and 6-) sulfonic acid (FSA; mixed isomers), through the hydrolyzing ac-

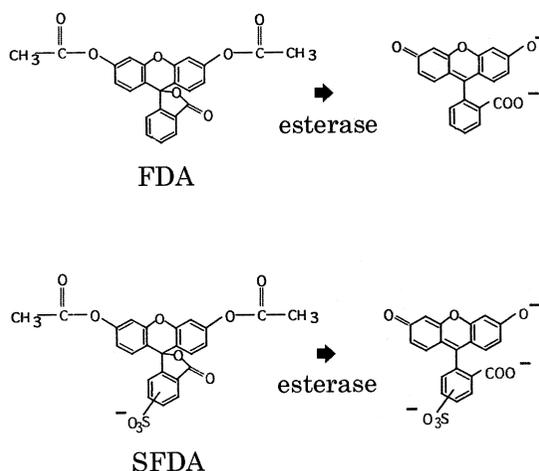


Fig. 1. Transformations of FDA and SFDA by esterase. Non-fluorescent FDA is converted to a fluorescent substance, fluorescein, by endogenous esterase activity. Non-fluorescent SFDA is also converted to a fluorescent compound, fluorescein-5- (and 6-) sulfonic acid (FSA; mixed isomers), by endogenous esterase activity.

tivity of esterase in the living cells. Ethanol (EtOH), a solvent of SFDA, penetrates cell walls and membranes, allowing SFDA to diffuse into the cytoplasm easily. Almost all living cells are stained by SFDA, and its ability to distinguish living from dead cells is superior to that of FDA (Tsuji *et al.*, 1995).

In this paper, we propose a novel application of SFDA for the assessment of cell survival in leaf cross-sections of alpine plants using a confocal laser scan microscope (LSM). Alpine plants generally have the ability to tolerate freezing stress (Körner and Larcher, 1988; Yamori *et al.*, 2005), and therefore, detection of differences between the results of a number of viability tests was thought to be relatively easy. This new procedure was also compared with two conventional methods, the FDA-staining method and electrolyte leakage test. In addition, we applied this method to assessment of the freezing tolerance of leaves of alpine plants.

## Materials and methods

### Plant materials

Plants of *Empetrum nigrum* L. var. *japonicum* K. Koch (Empetraceae), *Arctericia nana* (Maxim.) Makino, *Loiseleuria procumbens* L. Desvaux, *Phyllodoce nipponica* Makino, and *Vaccinium vitis-idaea* L. (Ericaceae) were dug out from the same west slope on Mt. Iwo (36°00'N, 138°22'N, 2760 m a.s.l.) in the Yatsugatake mountains, Nagano, Japan, in May 2001. The plant samples with soil were immediately wrapped with wet towels in plastic bags, kept on ice, and brought to the laboratory. The plants were then transferred to 1.3 l plastic pots and cultivated in an open place at Shizuoka University (34°57'N, 138°25'W, 55 m a.s.l.) where they were watered twice a week. The following examinations were conducted on 1 March 2002.

### Freezing treatments

Freezing treatments were conducted according to previous studies with some modifications (Pennycooke *et al.*, 2003; Shou *et al.*, 2004; Yamori *et al.*, 2005). The plants, including roots with soil, were kept in small polyethylene bags and cooled at a rate of 2.0 °C h<sup>-1</sup> from 4 to -16°C in the dark in programmed freezers. Samples were removed when the temperature reached -5, -10 and -16°C, respectively. Some of the samples cooled to -16°C were then transferred to a freezer at -30°C for 7 h. Some of these samples were then transferred to -80°C for 25 h. All plants cooled to -5, -10, -16, -30 and -80°C were subsequently thawed in the dark at 4°C.

### Fluorescence probes

SFDA (Molecular Probes, Inc., Eugene, OR, USA) was dissolved in EtOH at a final concentration of 2.0 mM. For the stock solution, FDA was dissolved in dimethyl sulfoxide (DMSO) at 10 mM. Just prior to use, the FDA in the DMSO solution was diluted to 0.23 mM with distilled water.

### Determination of freezing tolerance

#### 1. FDA- and SFDA-staining methods

Determination of freezing tolerance using the FDA-staining method was conducted

according to Yamori *et al.* (2005). The freezing tolerance of the plants was examined after 3 days of rewarming at 4°C in the dark. This duration was suggested as being the best condition for examining the freezing tolerance, because irreversible damage is clear and no significant regrowth has yet occurred (Mantyla *et al.*, 1995). Tissue sections, with a thickness of approximately three cells, were cut with a new razor blade and placed in a micro-tube (1.5 ml) then soaked in 50  $\mu$ l of 0.23 mM FDA or 2.0 mM SFDA solution for approximately 60 min at room temperature (from 20°C to 25°C). The sections were observed under a confocal laser scan microscope (LSM410, Zeiss, Oberkochen, Germany). From analyses of the concentration dependence of SFDA, we determined that 2.0 mM SFDA was optimal for evaluations of the freezing tolerance (data not shown). The excitation and emission wavelengths selected were 493 and 510 nm, respectively, and the degree of the cell survival was assessed. Only cells that exhibited a bright fluorescence from their cytosol were judged as viable.

The survival ratio (%) was calculated as the ratio of cells emitting fluorescence to total cells. The total cell number was counted in the same field of the microscope. In leaves of *V. vitis-idaea*, the total cell number counted using the microscope was approximately 200. At each freezing temperature, at least three leaf sections from different shoots were examined for each species.

## 2. Electrolyte leakage test

The electrolyte leakage test was conducted according to Yamori *et al.* (2005). Immediately after the frozen plants were thawed at 4°C, their leaves were removed and placed in screw-cap glass vials containing distilled water at a ratio of 0.1 g leaf sample (fresh weight): 10 ml of distilled water. The glass vials were kept at 4°C for 36 h in the dark. The conductivity of the supernatant was then measured with an electro-conductivity meter (Twin Compact Meter, Horiba, Kyoto, Japan). Each measurement was carried out in triplicate for each freezing temperature.

## Results and discussion

We observed fluorescence images of cross-sections of unfrozen leaves of *V. vitis-idaea* using both the FDA- and SFDA-staining methods (Fig. 2). After freezing treatment at -80°C, no fluorescence was detected; the image was black (data not shown). In the unfrozen leaves, the FDA-staining method failed to stain all the cells (Fig. 2A), while the SFDA-staining method stained almost 100% (Fig. 2B). The same results were obtained with the other species examined (data not shown). The FDA-staining method, in particular, failed to stain the spongy tissue cells rather than the palisade tissue cells (Fig. 2A). Cell characteristics are known to differ between spongy and palisade parenchyma cells even within the same leaf (for a review, see Terashima, 1989; Terashima and Hikosaka, 1995), and since FDA does not efficiently penetrate some types of membrane (for a review, see Harding and Benson, 1995), low penetration of FDA might fail to stain the spongy tissue cells in particular.

After freezing treatment at -30°C and -80°C, the survival ratios resulting from both FDA- and SFDA-staining were almost 0% for leaves of *A. nana*, *E. nigrum*, *P. nipponica* and *V. vitis-idaea* (Fig. 3). Moreover, after freezing treatment at -80°C, the survival ratios

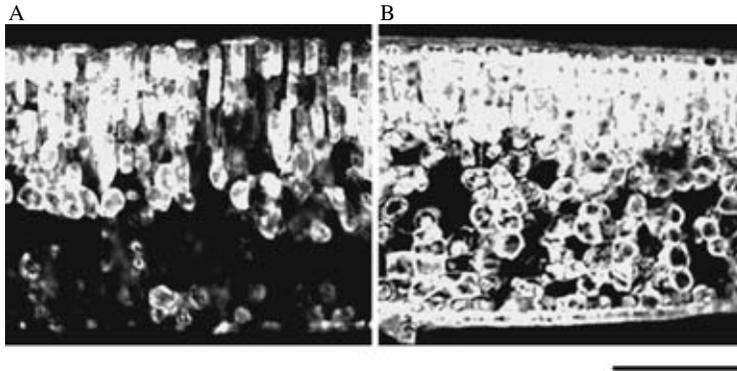


Fig. 2. Fluorescent images of cross-sections of unfrozen leaves of *V. vitis-idaea* with the FDA-(A) and SFDA-staining methods (B). Bar=100  $\mu$ m.

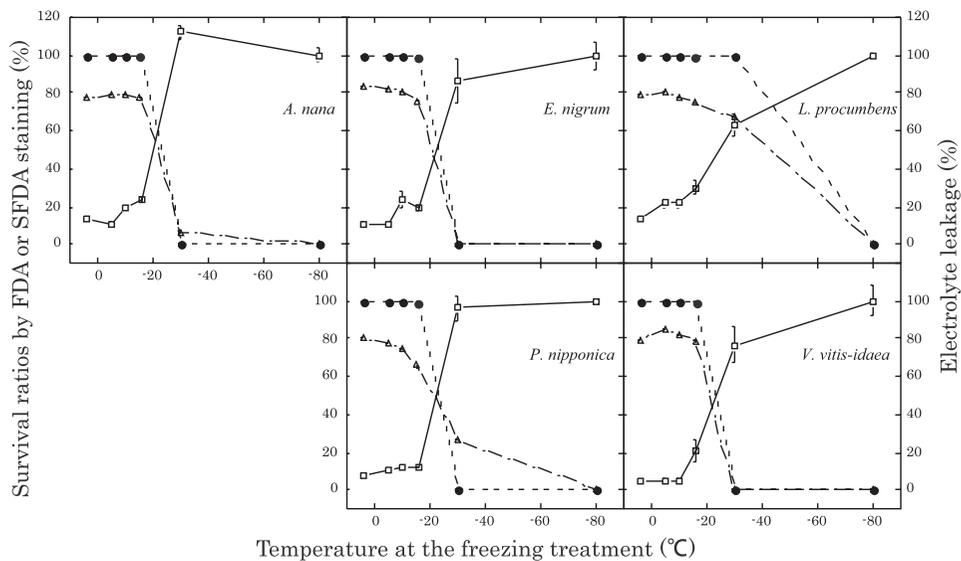


Fig. 3. Effects of freezing on the cell survival ratios (%) determined with the FDA- and SFDA-staining methods, and electrolyte leakage test (%) in five alpine plants. Values are means $\pm$ SD ( $n=3$ ). Open triangles: FDA-staining, closed circles: SFDA-staining, open squares: electrolyte leakage.

obtained by both staining methods were 0% for leaves of *L. procumbens*. On the other hand, after freezing treatments between  $-5^{\circ}\text{C}$  and  $-16^{\circ}\text{C}$ , leaves of *A. nana*, *E. nigrum*, *P. nipponica* and *V. vitis-idaea* were stained almost 100% by SFDA, but only 80% of the cells were stained by FDA (Fig. 3). The same tendencies were observed in leaves of *L. procumbens* after freezing treatments between  $-5^{\circ}\text{C}$  and  $-30^{\circ}\text{C}$ . The survival ratios in control samples of all species showed only 80% staining with the FDA-staining method,

as reported previously (Ogawa *et al.*, 1997; Steward *et al.*, 1999; Wang *et al.*, 2001; Winkelmann *et al.*, 2004; Yamori *et al.*, 2005). The low survival ratios with the FDA-staining method are thought to be due to the low penetration of FDA and its easy leakage from cells (Söderstöm, 1979; Thomas *et al.*, 1979; Harding and Benson, 1995). On the other hand, the SFDA-staining method was able to stain nearly all mesophyll cells in the leaves (Fig. 2). The combination of SFDA and EtOH is considered the most effective method of detecting living cells for the following reasons (Tsuji *et al.*, 1995): first, EtOH increases penetration of the cell membrane, greatly facilitating the penetration of SFDA into the cells. Second, the sulfonic group of fluorescein produced from SFDA may bind more strongly to positively charged cellular proteins compared to the fluorescein produced from FDA (Fig. 1).

The survival ratios (%) of leaf cells with the SFDA-staining method and electrolyte leakage (%), shown as relative electrolyte conductivity, are compared in Fig. 3. In this study, samples treated at  $-80^{\circ}\text{C}$  were used to represent 100% injury for the electrolyte leakage test. Both methods indicated that most leaf cells in all samples survived freezing treatment at  $-16^{\circ}\text{C}$ . For *V. vitis-idaea* treated at  $-16^{\circ}\text{C}$ , the survival ratio with the SFDA-staining method was 100%, while with electrolyte leakage was 20.8%. For *L. procumbens* treated at  $-30^{\circ}\text{C}$ , the SFDA-staining method indicated high survival ratios (100%), while the electrolyte leakage test showed substantial injuries (63.3%). These findings indicate that the electrolyte leakage test overestimates the freezing injuries. After freezing treatment at  $-30^{\circ}\text{C}$ , the survival ratios of *A. nana*, *E. nigrum*, *P. nipponica*, and *V. vitis-idaea* decreased to almost 0% when assessed with the SFDA-staining method. It is clear that the results of the SFDA-staining method and electrolyte leakage test were similar in *A. nana* and *P. nipponica*, as electrolyte leakage in *A. nana* and *P. nipponica* increased to 112 and 96.0%, respectively, after freezing treatment at  $-30^{\circ}\text{C}$ . However, electrolyte leakage in *E. nigrum* and *V. vitis-idaea* increased to only 86.7 and 76.5% after freezing at  $-30^{\circ}\text{C}$ . These results indicate that the electrolyte leakage test underestimates freezing injury. Thus, there was considerable inconsistency between the SFDA-staining method and electrolyte leakage test. Yamori *et al.* (2005) observed that the values obtained with the electrolyte leakage test were generally correlated with the survival ratios obtained with the FDA-staining method in nine alpine plants. However, the electrolyte leakage test greatly overestimated the freezing injuries in three species. The same disadvantage has been frequently pointed out in other previous studies (Pellett and Carter, 1981; Burr *et al.*, 1990; James and Bert, 1990).

The electrolyte leakage test has been widely used for determining the freezing injury in viability tests. The test provides a simple, rapid and qualitative technique, and moreover, is generally correlated with the FDA- and SFDA-staining methods, although it sometimes overestimates or underestimates the survival ratios (Fig. 3). Therefore, we conclude that the electrolyte leakage test can be used for rough estimations of freezing tolerance. However, we recommend the use of the SFDA-staining method rather than the electrolyte leakage test when a more accurate and reliable analysis is needed; for example, in comparisons of differences in freezing tolerance between palisade and spongy tissue cells. The SFDA-staining method also offers the following advantages compared with the FDA-staining method: 1) it overcomes low penetration of the dye by using EtOH as a solvent and 2) its reaction with cellular proteins is higher as SFDA has a sulfonic group. These

findings show that the SFDA-staining method is more convenient, accurate and reproducible.

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