Plant Viability as a Function of Temperature Stress

The Richards Function Applied to Data from Freezing Tests of Growing Shoots

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Abstract

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Frost resistance of growing Salix viminalis L. shoots was determined by rating mortality percentage under two commonly used freezing conditions: a condition in which plants were encased in crushed ice and another in which plants were moistened with tap water prior to freezing. The mortality-temperature data were fitted with a logistic function (having a fixed inflection point halfway between the asymptotes) and with a Richards function, which is a double asymptotic sigmoid function with a variable inflection point. Different frost resistance curves were obtained, depending on the freezing conditions used. However, conditions were inadequate for efficient ice nucleation under either condition. This implies that the applied freezing conditions are not suitable when the purpose is to induce and duplicate early ice crystal formation conditions. The Richards derivatives were negatively skewed in the one case and positively skewed in the other case, giving inflection points, as a function of the upper asymptote, situated at 0.37 when shoots were frosted in the presence of ice and at 0.81 when shoots were frozen in the presence of added moisture. These values differed significantly from 0.50, through which the logistic function would have forced the curves. Because of the significant asymmetry in these frost-resistance curves, the Richards function led to a more accurate reflection of the temperature-mortality course of growing Salix stems than the logistic function. The Richards function possesses the flexibility needed to describe plant injury response in terms of physical and plant physiological mechanisms. Therefore, the Richards function is recommended rather than the logistic function for the assessment of frost resistance.

Extreme temperatures often limit plant productivity through damage to living tissues. Not only temperature but also the rate of temperature change (Steffen et al., 1989) and duration of exposure (Rajashekar et al., 1983) determine the amount of injury. Furthermore, plant susceptibility to such damage is genetically determined and is also a function of precondition treatment and growth stage as well as physiological state (Levitt, 1980).

To quantify the plant injury response and to be able to express differences between plant material and treatments, much research has been devoted to finding absolute measures of plant injury resulting from temperature stress (Levitt, 1980). The percentage of plants surviving a standardized treatment is considered to be a reliable and accurate measure of plant injury (Stergios and Howell, 1973), but electrolyte leakage (Palta and Li, 1978; Ingram and Buchanan, 1984; Ingram, 1985; Anderson et al., 1988), impedance measurements (Glerum, 1973; Repo and Pelkonen, 1986; Repo and Lappi, 1989), and mortality percentage (Christersson, 1978; Christersson and von Fircks, 1990) have also been used.

An easily comparable measure is the temperature at which 50% of the plants are killed. This 50% level of dosage, LD50 (Proebsting et al., 1966; Sakai and Larcher, 1987), has been used to test woody species (McLeester et al., 1968; Pomeroy et al., 1970; Christersson and von Fircks, 1990). Other single-point measures, e.g. LD0, defined as the lowest temperature to which plants can be exposed without showing visual damage (Fuchigami et al., 1971; Sakai and Larcher, 1987), LD100, the temperature at which 10% of the individuals are killed (Hansen, 1992), or LD100, where no survivors are left (Howell and Weiser, 1970; Chen and Li, 1976) have also been used.

In the case of frost damage, there is a lower temperature range in which there is 100% mortality and an upper temperature range in which all plants survive. In the case of heat damage, the situation is reversed. Percentage mortality-temperature curves resulting from cold and heat treatments show that plant response to temperature stress is sigmoidal and bounded by a lower and upper asymptote of a sigmoid function (Zhu and Liu, 1987; Janacek and Prasil, 1991). Studies in which electrolyte leakage or impedance measurements are made also show good fits of sigmoid functions with fixed asymptotes to data, describing plant response to extreme temperatures (Ingram and Buchanan, 1981, 1984; Ingram, 1985; Schaff et al., 1987; Repo and Lappi, 1989).

In the studies mentioned, the logistic function was used. One of the main properties of the logistic function is its symmetry, having a point of inflection situated exactly between its asymptotes and yielding a symmetric first derivative (Hunt, 1982). Using the logistic function, Rmax, which we define as the highest rate of change in plant response, occurs at a temperature, Tmax, which coincides with LD50. In several studies using a logistic model, the conclusion has been drawn that the highest rate of plant response is situated at LD50 (Ingram and Buchanan, 1981, 1984; Su et al., 1987), which,

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Abbreviations: INA, ice nucleation active; LD, lethal dose.
by definition, does not allow for other conclusions. However, the widespread assumption that $T_{\text{max}}$ is equal to $LD_{50}$ implicitly made when using the logistic function, has never been substantiated for freezing test data.

The objects of this study were (a) to evaluate data by comparing the performance of the logistic function with that of the Richards function (Richards, 1959; Causton and Venus, 1981) and (b) to show the influence of two commonly used freezing conditions (ice on shoot surfaces and shoots moistened with tap water) on the performance of the temperature versus mortality curve. Dealing with growing nonhardy shoot parts, we use the term frost resistance according to the concept given by Sakai and Larcher (1987), i.e. the ability to persist alive during exposure to subzero temperatures.

**MATERIALS AND METHODS**

**Plant Material and Growth Conditions**

*Salix viminalis* (L.) (clone No. 79046; Ager et al., 1986) plants were raised from stem cuttings in pots in a greenhouse. During the 20:4 h day:night period, the temperature was 20.15°C ± 2°C. Osram Power star bulbs (HQ I-E 400 W/D) gave a photon flux density of 210 to 250 μmol m$^{-2}$ s$^{-1}$, 400 to 700 nm, at the level of the primary leaves. The RH was kept between 40 and 60%. To maintain an adequate supply of nutrients, the plants were fertilized up to three times a week with a nutrient solution composed according to Ingestad (1979) and were well watered according to need.

**Standardized Freezing Test**

Shoots of intact plants to be exposed to freezing condition A were placed in test tubes and encased in crushed ice (crystallized deionized water), and plants to be exposed to freezing condition B were moistened with tap water (5 ± 1 mL) before being placed in test tubes. The upright test tube was positioned in a 95% ethanol bath, and before the beginning of each freezing test, plants were allowed to equilibrate to the bath temperature of 0.0°C ± 0.1°C. All plants were exposed to only one freeze-thaw cycle.

A cooling and thawing rate of 4.3°C ± 1°C/h was used in all tests. The test temperatures were maintained for 2 h and spaced at 0.5°C intervals over a temperature range of −1.5°C to −5.0°C for condition A and of −1.5°C to −6.5°C for condition B. At least four replicates were used at each temperature and each replicate contained at least 10 shoots. The temperature of the bath was regulated to ±0.1°C of the predetermined test temperature. After the freezing test, shoots were thawed at 4.0°C ± 1.0°C and then acclimated to growth temperature for 6 to 8 h in the dark before being transferred back to greenhouse conditions for assessment of viability.

**Viability Assay**

Shoots were rated for viability by regrowth of the apical meristem.

**Assessment of Ice Nucleation Activity on Shoot Parts**

To elucidate the presence of INA particles on shoots, which might interfere or trigger ice nucleation on shoot parts, we used a method based on work by Hirano et al. (1985), but modified it to avoid physical seeding of ice by handling procedures.

Excised shoots (8 cm) were left in a sterile buffer solution (100 mL KH$_2$PO$_4$ 0.01 M, pH 7.0) without immersing or wetting the cut ends. To other test tubes a volume of 50 mL of crushed (melted, deionized water) ice or 5 mL of tap water was added to the buffer solution. Test tubes with only buffer solution served as controls. Each type of sample (see Table I) consisted of more than 30 replicates. All test tubes were exposed to −2.0°C to −15.0°C, spaced at 1.0°C intervals. After 2 h of exposure at each temperature, test tubes were carefully lifted from the freezing bath and freezing of buffers in the test tubes was recorded.

**Data Analyses**

We used the Richards function (Richards, 1959; Causton and Venus, 1981) to express the plants’ response to frost as a function of freezing temperature:

$$f(M) = a(1 - e^{b(T_c)^{c/d}}),$$

where $M$ is mortality percentage, $T$ is temperature, and $a$, $b$, $c$, and $d$ are parameters. This function has its asymptotes at $f(M) = 0$ and $f(M) = a$, the latter being set to 100. The slope of the Richards function is given by:

$$\delta M/\delta T = \left(\frac{b + c}{d}\right) \frac{a}{1 + e^{b(T_c)^{c/d}}},$$

Further differentiation and equating the second derivative to zero shows that the point of inflection occurs at:

$$T_{\text{max}} = \frac{b}{\log d}/c$$

On substituting Equation 3 for $T$ in Equation 1, it was found that the point of inflection, $I$, varies with parameter $d$ and that its position as a fraction of the upper asymptote, $a$, (Causton and Venus, 1981), is given by:

$$I/a = \frac{d + 1}{e^{(1/d)}}$$

The logistic function and its slope can be obtained directly by setting $d$ to 1 in Equations 1 and 2. Solving Equation 4 for $d = 1$ shows that the logistic function has its asymptote fixed at $I/a = 0.50$.

We devised a procedure to test whether the Richards function that we obtained gave an S-shaped curve with a significant asymmetry (thereby differing from a logistic function). Even if a fitted Richards function has an $LD_{50}$ value that differs from its $T_{\text{max}}$ value ($LD_{50} - T_{\text{max}} \neq 0$), the variance structure of the data may hamper the detection of significant differences. Therefore, we applied a repeated sampling procedure (Efron, 1982), taking 100 simple random samplings with replacement for each data set, and fitted the Richards function for each replicate. This procedure gave 100 pairs of $LD_{50}$ and $T_{\text{max}}$ values and, consequently, 100 values of the difference ($LD_{50} - T_{\text{max}}$ Table I). If the point of inflection, as a proportion of the upper asymptote, in the original data is located at 0.5, i.e. if the function is symmetric, the repeated
sampling procedure would have produced a frequency distribution of differences centered around zero and with a spread that depends on the variance structure of the original data. A significant asymmetry ($P < 0.05$) of the function is detected when less than 5% or more than 95% of the replicates have positive values.

A regression method that employed an iterative fitting procedure (Hartley, 1961) was used to fit the logistic and Richards equations. The initial starting values for all parameters were set to 0.1 and the tolerance criterion for convergence was $10^{-5}$ units of magnitude of each parameter. Comparison of the models, which differed in their number of parameters to estimate, was made on the basis of the adjusted $r^2$ defined as: \[ \text{adj}-r^2 = 1 - \left(1 - A/B\right)(N - 1)/(N - P), \]
where $A$ is the sum of squares explained by the model, $B$ is the total sum of squares, $N$ is the sample size, and $P$ is the number of parameters, excluding the fixed asymptote. Being sensitive to the number of parameters used in the equation, the adjusted $r^2$ measures the trade-off between a good fit and a complex model, in contrast to the basic $r^2$, which measures only the goodness of fit.

**RESULTS**

**Frost Resistance Data of Growing Stems**

The logistic function showed an $LD_{50}$ value of $-2.6\degree C$ for data obtained from frosted shoots in the presence of ice (condition A) and an $LD_{50}$ of $-4.4\degree C$ for data obtained from frosted shoots in the presence of surface moisture (condition B) (Fig. 1). The steepest slope, $R_{\max}$, by definition occurring at $T_{\max} = LD_{50}$, is for condition A, whereas condition B has a lower $R_{\max}$ ($\Delta M/\Delta T = 85.8$ and 67.5, respectively).

When we use the Richards function, we apply a double asymptotic function with a variable point of inflection. The $T_{\max}$ values thus obtained differed from the $LD_{50}$ values (Fig. 2): in the presence of ice (condition A), $T_{\max}$ occurred at a temperature warmer than $LD_{50}$ ($-2.4\degree C$ and $-2.5\degree C$, respectively), whereas when the plant surfaces were moistened with water, condition B, $T_{\max}$ occurred at a temperature colder than $LD_{50}$ ($-4.7\degree C$ and $-4.4\degree C$, respectively) (Figs. 1 and 2). Furthermore, the associated $R_{\max}$ values differed from the $R_{\max}$ values that resulted from the logistic curve-fitting procedure: the value for condition B ($\Delta M/\Delta T = 103.5$) was higher than that for condition A ($\Delta M/\Delta T = 90.5$) (Fig. 2). The point of inflection, as a fraction of the upper asymptote (I/a), was at 0.37 for condition A and at 0.81 for condition B (Fig. 3).

The use of the logistic function would have placed the point of inflection at a mortality proportion of 0.50 in both cases (Fig. 3). Considering curve fitting, the Richards function gave a slightly better fit to the data, with adjusted $r^2$ values of 0.83 and 0.91 for conditions A and B, respectively, than the logistic function, with adjusted $r^2$ values of 0.81 and 0.89.

Before interpreting the Richards functions that we obtained, a test of significance for the basic finding that $T_{\max}$ may differ from $LD_{50}$ was applied. For each condition, we evaluated 100 bootstrap replicates of $LD_{50}$, $T_{\max}$, and the differences between $LD_{50}$ and $T_{\max}$. The $LD_{50}$ values obtained with the Richards function did not differ from the $LD_{50}$ values obtained with the logistic function, but the $LD_{50}$ values differ from the corresponding $R_{\max}$ values (Table I) obtained with the Richards function. Consequently, all replicate values for the difference between $LD_{50}$ and $T_{\max}$ in condition A were negative, whereas the replicates from condition B all had positive values (Fig. 4). This implies that the Richards curves under both conditions have a highly significant asymmetry.

**Ice Nucleation Activity on Shoot Parts**

Buffer solution with submerged shoots froze after 2 h of exposure to temperatures between $-4\degree C$ and $-7\degree C$ (Table II). Buffer solutions to which tap water was added started to freeze below $-8\degree C$ to $-10\degree C$. The use of deionized water for making ice made it possible to supercool melted ice in the buffer solution to at least $-10\degree C$. All controls crystallized between $-9\degree C$ and $-13\degree C$.

**DISCUSSION**

In the search for plant material with superior properties of resistance to temperature stress, small differences in frost resistance performance between species have often been detected (Warnes and Johnson, 1972; Glerum, 1973; von Fircks, 1985), showing that evaluation of dose-response curves is superior to single-point comparisons. By determining the significant differences between $LD_{50}$ and $T_{\max}$, we
Figure 2. Richards functions (solid lines) and their first derivatives (dashed lines) fitted to mortality percentages obtained by exposing plants to low temperatures. A, Cooling in the presence of ice; B, cooling with water. The size of the data points is proportional to the number of coinciding replicates, ranging from 1 (smallest) to 7 (largest). $T_{\text{max}}$ is indicated with a triangle, and $LD_{50}$ is indicated with a star. The dotted lines correspond to 5\% ($LD_5$) and 95\% ($LD_{95}$) mortality.

Figure 3. The fourth parameter ($d$) of the Richards function determines where the point of inflection, as a fraction of the upper asymptote ($I/A$), is located. When $d = 1$ (location indicated with a star), the Richards function converts into the logistic function, having an inflection point fixed at $I/A = 0.5$. $I/A$ is situated at 0.37 for condition A (●) and at 0.81 for condition B (○).

Figure 4. The difference between the temperature at which a mortality of 50\% is obtained and the temperature at which the inflection point is located ($LD_{50} - T_{\text{max}}$) for 100 bootstrap replicates under cooling conditions with ice (A) and with water (B).

Table 1. Frost hardness data obtained by use of the Richards function

<table>
<thead>
<tr>
<th>Freezing Condition</th>
<th>Parameter</th>
<th>A Mean</th>
<th>A SE</th>
<th>B Mean</th>
<th>B SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$LD_{50}$</td>
<td>-2.5</td>
<td>0.02</td>
<td>-4.4</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>$T_{\text{max}}$</td>
<td>-2.4</td>
<td>0.01</td>
<td>-4.7</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>$LD_{50} - T_{\text{max}}$</td>
<td>-0.1</td>
<td>0.01</td>
<td>0.3</td>
<td>0.02</td>
</tr>
</tbody>
</table>

proved that the Richards functions were significantly asymmetric. The degree of asymmetry is determined by parameter $d$ in Equation 1, which positions the $T_{\text{max}}$ value according to Equation 3. This finding suggests that to trace and understand the physical and physiological mechanisms that lead to damage, it is of great advantage to assess the plant response over the entire range between $LD_5$ and $LD_{100}$. Furthermore, because the value of parameter $d$ varies with freezing conditions (Fig. 3), this parameter seems to be specific for each type of applied freezing condition. This must be considered when comparing test results from different laboratories.

Under the freezing conditions employed, the Richards function and the logistic function gave about the same $LD_{50}$ values, but different $T_{\text{max}}$ values at which the injury rate, $R_{\text{max}}$, was highest. Although these differences were relatively small compared with the differences between $LD_{50}$ and $T_{\text{max}}$, for the freezing conditions used, they revealed important differences between the freezing conditions that could not have been detected with the logistic function. Comparison between the logistic and Richard functions shows that, in particular, the tails of the Richards functions nearest to the...
points of inflection (the right tail for condition A and the left tail for condition B) have quite different positions from those obtained with the logistic function. In other words, point estimates such as $LD_{50}$, $LD_{50}$, or $LD_5$ (Figs. 1 and 2) would differ more between the logistic and Richards functions than their derived $LD_0$ values. Forcing the point of inflection halfway between the asymptotes with a logistic fit not only prevents us from distinguishing between $LD_0$ and $T_{max}$ but will also bias the estimates of $LD_{50}$, $LD_{90}$, $R_{max}$, or any single point one wishes to assess from a fitted function.

Because the presence of heterogeneous ice nuclei is necessary to avoid excessive supercooling, inoculation of ice crystal formation has been achieved by misting the surfaces of samples with water (Maronek and Flint, 1974; Ashworth et al., 1985; Hansen, 1992) or by addition of ice (Christersson, 1978; Harrison et al., 1978; Steffen et al., 1989). The scatter of data points in the right parts (between $LD_0$ and $LD_90$) of the dose-response curves presented in Figures 1 and 2 implies the occurrence of differences between test material or freezing conditions leading to a response variation of up to 80% units (Fig. 2B). However, growth conditions both before and after freezing were standardized, suggesting that addition of water alone still allowed a large number of plants to be subjected to random and substantial supercooling (−4.5°C, Fig. 2B). This large scatter might be due to the use of inefficient conditions for seeding of ice, shifting the mortality versus temperature curves to temperature regions that might cause interaction between the water outside the shoot parts and the random presence of shoot-derived INA particles.

Although addition of moisture in the form of water is necessary when applying the Richards function. Therefore, it would be worthwhile to search for other freezing conditions that would reduce supercooling to a smaller extent.

A significant higher $LD_{50}$ value was determined when inoculating with ice (condition A) compared with condition B, where water was used. In this respect, the fitted logistic functions led to the same interpretation as the fitted Richards functions.

Because plants do not have effective ice nucleants above −8°C to −10°C (Lindow et al., 1982), freezing might have a biogenic (INA bacteria, Vali et al., 1976) or abiotic (ice and water) origin. Particular ice nucleants active above −4°C to −5°C have a bacterial origin (Amy et al., 1976; Lindow et al., 1978, 1982). Since no such efficient ice nucleators appear to be present on shoot surfaces (Table II), the particular ice-nucleating ability of ice and water and its interaction with other shoot-derived INA particles might be the main reason for the different positions of the mortality curve along the temperature scale.

When ice nucleation was promoted by the presence of ice, there was a high injury rate at a temperature close to the temperature at which plant injury commenced (Fig. 2). This was not the case when shoots were frosted in the presence of water (condition B): the injury rate was very low initially, but kept increasing exponentially and reached a higher maximum value than in condition A. This might be due to the absence of aqueous ice nucleators effective above −8°C (Table II). Shoot-derived INA particles will then act as ice nuclei. Shoot-derived ice nuclei that were active between −4.0°C and −7.0°C (Table II) were infrequently and unevenly distributed. The resulting randomized and uncontrolled freezing of the shoots will probably affect the form of the temperature versus mortality response curve (Fig. 2B) and thereby also the parameters $d$ and $R_{max}$. This mortality-temperature curve (Fig. 2B) implies a temperature-dependent relationship, which is similar to the findings by Vali and Stansbury (1966), who showed that the probability of ice nucleation in supercooled distilled water increased exponentially with steadily decreasing temperatures. However, these processes are time dependent (Ashworth et al., 1985), so both temperature and time must be considered as the main factors that affect the different parts of the mortality-temperature curve (Fig. 2) and thereby the position of $T_{max}$.

We conclude that plant response to freezing stress follows a sigmoidal asymmetric model, irrespective of the freezing conditions applied. Under both conditions, using either ice or water as the nucleant, significant differences between $LD_{50}$ and $T_{max}$ values were obtained. Furthermore, when comparing results of frost-resistance evaluations in the literature, it should be borne in mind that reported differences between $LD_{50}$ values may be caused by the use and presence of different sources of ice nucleators to “diminish or reduce” supercooling effects and not by the inherent frost resistance of the tested plant material. The use solely of moisture or ice has to be rejected as a reliable and reproducible method of inducing early ice crystal formation.

The persistent use of logistic functions in temperature stress research may lead to erroneous interpretation of the data and may prevent researchers from explaining plant injury responses in terms of plant physiological mechanisms. Since frost-resistance curves may be inherently asymmetric, fitting
a symmetric model such as the logistic function may lead to biased estimates. Therefore, we suggest that researchers use the Richards function for the analyses of future temperature-stress data.

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LITERATURE CITED


