Fast, nondestructive measurement of frost hardiness in conifer seedlings by VIS+NIR spectroscopy

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Summary  Frost hardiness development from mid-August to mid-November was evaluated in seedlings of three provenances of Norway spruce (Picea abies (L.) Karst.) and three provenances of Scots pine (Pinus sylvestris L.) raised at nurseries in north, central and south Sweden. Measurements of the visible + near infrared (VIS+NIR) spectra of shoots were made simultaneously with estimates of frost hardiness based on electrolyte leakage following artificial freezing. Nine physiological variables known to influence frost hardiness were measured throughout the experiment.

Multivariate analysis showed that VIS+NIR spectra explained 69% and 72% of the variation in frost hardiness in Scots pine and Norway spruce, respectively. Stem lignification, dry weight fraction, and starch, glucose, fructose, galactose, sucrose, raffinose and stachyose concentrations together explained 80% and 85% of the variation in frost hardiness in Scots pine and Norway spruce, respectively when used as independent X variables in a partial least squares model. These physiological variables could be related to varying degrees with variation in the VIS+NIR spectra. We conclude that VIS+NIR spectroscopy provides a rapid nondestructive technique for measuring frost hardiness in conifer seedlings based on causal relationships between the spectra and the physiology of seedling frost hardiness.

Keywords: dry weight fraction, multivariate analysis, Picea abies, Pinus sylvestris, sugars, starch.

Introduction

The ability of plants to survive low temperatures can be estimated by evaluating damage after artificial freezing. The assessment of damage can be made in several ways. Most directly, whole seedlings are frozen, transferred to favorable conditions and examined after a few days for visible signs of frost damage (Nilsson and Andersson 1987). This procedure has the advantage that it integrates all types of freezing injury. However, the technique is slow because at least 1 week in favorable growth conditions is necessary for damage to manifest itself. Furthermore, the method requires large numbers of seedlings to achieve statistically reliable results.

Visible injury caused by controlled freezing treatments can also be assessed on needles, buds, stems or other samples from entire plants. However, visible damage caused by tissue excision may obscure the effects of freezing injury. Alternative methods of assessing freezing injury on excised plant parts that may be less ambiguous than visual assessment include those based on electrolyte leakage (Dexter et al. 1930, Aronson and Eliasson 1970), chlorophyll a fluorescence (Westin et al. 1995), triphenyl tetrazolium chloride reduction (Steponkus 1971) and ninhydrin reactions (Siminovitch et al. 1962). However, these methods are labor intensive and are fully or partially destructive, which limits their usefulness in the study of frost hardiness changes over time.

Several nondestructive methods for measuring frost hardiness in conifer seedlings have been described including changes in needle color in Scots pine (Toivonen et al. 1991), stem electrical impedance (Glerum 1973) and chlorophyll a fluorescence/ luminescence kinetics (Sundblad et al. 1990). However, measurements made with these techniques reflect changes in only some of the processes underlying frost hardiness. This means that the techniques may be valid only under special conditions or at particular stages of hardiness.

A desirable technique for estimating frost hardiness should therefore be fast, nondestructive, and based on several relevant physiological variables that underly frost hardiness. In the present study, we describe a method that fulfils these requirements. We used visible + near infrared (VIS+NIR) spectroscopy in combination with multivariate modeling of spectra to develop a method that is fast, nondestructive and based on several physiological variables important for frost hardiness.

Methods

Experimental design and plant material

Seedlings of three provenances of Norway spruce and Scots pine were grown at three nurseries in northern, central and
southern Sweden (Table 1) from seed sown in March. Seedlings were raised in containers and held in a greenhouse until mid-July, when they were moved outdoors.

Measurements started on Week 34 when one fifth of the seedlings from the northern nursery were sampled for measurements of frost hardiness, VIS+NIR spectra and nine physiological variables. Similar measurements were made the following week at the nursery in central Sweden, and, in the week following that, at the nursery in southern Sweden. This routine was repeated four times over the following 12 weeks.

**VIS+NIR measurements**

The VIS+NIR spectra were measured on five seedlings of each provenance on each sampling occasion with a Foss NIRSYSTEMS 6500 spectrometer (Foss A/S, Hillerød, Denmark). Before measurement, the shoot of the intact seedling was flattened between glass plates and placed over a matt black background. Spectra were measured through a fiber-optic probe that was automatically moved along 25 mm of the middle part of the stem during measurements. The distance between the end of the fiber and the seedlings was approximately 10 mm. Fifteen complete spectra were recorded during a scan of a single seedling. From the fifteen spectra, a mean spectrum was calculated and used for multivariate modeling. The mean spectrum accordingly represented a mix of spectral information from the stem and the basal parts of associated needles. Spectral measurements took about 1 min.

Reference spectra were obtained by measuring absorbance from the box without an attached seedling. Absorbance was measured between 400 and 2300 nm with a spectral resolution of 2 nm. Spectra were thus represented by 950 absorbance values that were used as variables in multivariate modeling.

**Estimation of lignification**

Immediately after VIS+NIR measurements, digital photographs were taken of all seedlings illuminated by a metal halogen lamp. Estimation of the proportion of green versus brown/white stem was made from the photographs by measuring the relative lengths of green, brown and white stem on the digital images with a digital ruler. The ratio of brown/white stem was used as an indirect measure of stem lignification (Pulkkinen 1993).

**Measurement of dry weight fraction**

The upper 10–15 mm of the shoot tip of each seedling used for spectral measurements was excised, weighed, dried at 70 °C, and the dry weight fraction calculated.

**Measurement of frost hardiness**

Frost hardiness was measured by the electrolyte leakage method (Dexter et al. 1930, Aronsson and Eliasson 1970). A stem segment from the upper part of each seedling (below the shoot tip used for measurement of dry weight fraction) was cut into six, 4–5-mm long segments. The upper five segments were frozen in glass tubes in computer-controlled, high-capacity freezers with internal air circulation to five temperatures, the uppermost segment to −5 °C and the lower segments to −15, −25, −35 and −45 °C, respectively. Rate of freezing and thawing was 5 °C h−1. The lowest stem segment was used as an unfrozen control and stored at 4 °C in a glass tube during the freezing procedure. After freezing, the stem segments were gently shaken in 10 ml of deionized water at 4 °C for 24 h, after which the conductivity of the water was measured. Maximal leakage from dead tissue was measured after boiling the stem segments for 15 min. Freezing damage index (I) of each stem segment was calculated according to Flint et al. (1967):

\[
I = \frac{[(C_l/C_{bf}) - (C_l/C_{bc})]}{1 - (C_l/C_{bc})}, \tag{1}
\]

where \(C_l\) is conductivity after freezing, \(C_{bf}\) is conductivity after freezing and boiling, \(C_c\) is conductivity of the unfrozen control and \(C_{bc}\) is conductivity after boiling of unfrozen control.

Finally, a mean damage index (MDI), based on I at the five test temperatures, was calculated and used as a measure of the state of hardiness of each seedling. All freezing experiments were done at the Sävar nursery. Plants from other nurseries were transported to Sävar by air in cooled bags.

**Analyses of carbohydrates**

For analyses of starch, glucose, fructose, galactose, sucrose, raffinose and stachyose, approximately 10–20 needles representing a minimum of 10 mg dry weight, were randomly sampled from the seedlings. Extraction and analyses of carbohydrates were made by a two-step procedure according to Steen and Larsson (1986), but modified as described by Robenztz (1999) to allow spectrophotometric analysis of starch (U1100 spectrophotometer equipped with an AS 3000 autosampler; Hitachi, Ltd., Tokyo, Japan). Analyses of other carbohydrates

Table 1. Latitudinal origin of seedlings and nurseries.

<table>
<thead>
<tr>
<th>Seedling material/nursery</th>
<th>Origin/location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Northern nursery Sävar</td>
<td>64°00’</td>
</tr>
<tr>
<td>Northern Scots pine used at Sävar</td>
<td>67°00’</td>
</tr>
<tr>
<td>Local Scots pine used at Sävar</td>
<td>65°12’</td>
</tr>
<tr>
<td>Southern Scots pine used at Sävar</td>
<td>63°00’</td>
</tr>
<tr>
<td>Northern Norway spruce used at Sävar</td>
<td>66°12’</td>
</tr>
<tr>
<td>Local Norway spruce used at Sävar</td>
<td>64°06’</td>
</tr>
<tr>
<td>Southern Norway spruce used at Sävar</td>
<td>60°00’</td>
</tr>
<tr>
<td>Central nursery Brunnsberg</td>
<td>59°40’</td>
</tr>
<tr>
<td>Northern Scots pine used at Brunnsberg</td>
<td>61°35’</td>
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<tr>
<td>Local Scots pine used at Brunnsberg</td>
<td>59°40’</td>
</tr>
<tr>
<td>Southern Scots pine used at Brunnsberg</td>
<td>57°30’</td>
</tr>
<tr>
<td>Northern Norway spruce used at Brunnsberg</td>
<td>61°25’</td>
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<tr>
<td>Local Norway spruce used at Brunnsberg</td>
<td>59°50’</td>
</tr>
<tr>
<td>Southern Norway spruce used at Brunnsberg</td>
<td>57°30’</td>
</tr>
<tr>
<td>Southern nursery Ekebo</td>
<td>55°55’</td>
</tr>
<tr>
<td>Northern Scots pine used at Ekebo</td>
<td>57°30’</td>
</tr>
<tr>
<td>Local Scots pine used at Ekebo</td>
<td>56°00’</td>
</tr>
<tr>
<td>Southern Scots pine used at Ekebo</td>
<td>51°00’</td>
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<tr>
<td>Northern Norway spruce used at Ekebo</td>
<td>57°30’</td>
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<tr>
<td>Local Norway spruce used at Ekebo</td>
<td>55°30’</td>
</tr>
<tr>
<td>Southern Norway spruce used at Ekebo</td>
<td>49°30’</td>
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</tbody>
</table>

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were made with a Dionex ion exchange chromatograph with a CarboPac column PA1 and a pulsed electrochemical detector (Dionex, Sunnyvale, CA).

Omission of data before modeling
From the initial data set consisting of measurements on 450 seedlings, we excluded measurements on 15 pine seedlings and 15 spruce seedlings from the southern nursery that were mishandled during transportation, four spruce seedlings and two pine seedlings with abnormal spectra having negative absorbance values, nine seedlings with abnormal electrolyte leakage values because of contamination of glass tubes and 15 pine seedlings from the central nursery with abnormally yellow needles.

Data analysis and multivariate modeling
To remove irrelevant systematic variation resulting from potential instability of the spectrometer, the initial absorbance values in the VIS+NIR spectra were subjected to multiplicative signal correction (MSC). The MSC uses a mean spectrum as a reference for the corrections (Geladi et al. 1985).

Because of skew distributions of values for the variables starch, galactose, raffinose and stachyose concentrations and lignification, these values were log transformed before modeling (Brereton 1992).

All multivariate data analysis was performed with the SIMCA 7.0 program (Umetrics AB, Umeå, Sweden). For the principal component analysis (PCA), spectral data were analyzed according to;

\[ X = TP' + E, \]  
(4)

where \( q \) is a vector of regression coefficients, to give PLS the same diagnostic capabilities as PCA. For a more comprehensive presentation of PLS, see Martens and Naes (1989).

Before building the calibration models, one fifth of the observations were removed and used to make up the prediction data set. Seedlings for the prediction set were randomly sampled and chosen to represent one observation per provenance, nursery and week. For predictions of frost hardiness based on the nine physiological variables, partial least square (PLS) models according to Equation 3 were used together with regression models according to Equation 5, but with the nine variables instead of the spectra as \( X \)-variables. Throughout the multivariate analysis, separate models were used for Scots pine and Norway spruce.

Results and discussion

Frost hardness
Frost hardness increased in all seedlings from August to November (Figures 1 and 2). Among provenances and nurseries, frost hardening occurred earlier in northern provenances and at the northern nurseries (Figures 1 and 2). The effect of nursery location was partially confounded with seed origin because the provenances grown at the three nurseries were not the same. However, the northern provenances grown at the southern nursery were also tested at the central Sweden nursery. Because these provenances showed different hardening patterns at the two locations, with earlier hardening at the more northerly nursery, it is likely that the different hardening patterns observed in Figure 1 and 2 resulted from a combination of genetic and environmental factors.

Predictions of frost hardness based on spectra
Mean VIS+NIR spectra were similar for Scots pine and Norway spruce (Figure 3). Multiple scatter filtering (MSC-filtering) did not alter the general profile of the spectra (Figure 4). The jump in the spectra at around 1100 nm was caused by a change in detector from silicon to PbS. This difference between detectors did not affect the results of PLS or PCA analysis, because only relative information, i.e., deviations from means, and not absolute values were modeled.

The calibration models used to predict frost hardness explained 69 and 72% of the variation in frost hardness in Scots pine and Norway spruce, respectively. Predictions were made on new independent seedling samples not previously included in the calibration models (Figure 5). These seedlings represented the full range of frost hardiness states observed in the experiment, including the low values found mainly in the southern provenances grown at the southern location and measured in August, to the high values found mostly in northern
provenances grown at the northern nursery and measured during late fall.

The VIS+NIR spectra also discriminated among more subtle differences in frost hardiness as illustrated by the clustering of seedlings according to provenance in the PCA plot shown in Figure 6. The data in Figure 6 are based on observations on Norway spruce seedlings grown at the northern nursery and measured at Week 37. Southern seedlings are mainly in the upper right of the plot, northern seedlings in the center to lower left and local seedlings in the upper left, although there is some overlap. Overlapping was expected, because large within-provenance variation has been demonstrated in Norway spruce (Skröppa 1991).

The ~30% unexplained variation in frost hardiness as determined by VIS+NIR spectroscopy could be related to limitations in either the spectroscopic method or the reference method, i.e., electrolyte leakage for frost hardiness measurement. Practical limitations, i.e., small seedlings sectioned into many small tissue samples, necessary to obtain individual measures of frost hardiness and the hardness-determining variables from each seedling, probably limited the precision of the reference method. Furthermore, the electrolyte leakage method is only an indirect measure of frost hardiness. Better predictions might, therefore, have been obtained with a better reference method for frost hardness determination.

**Predictions of frost hardness based on physiological variables**

Frost hardness depends on several physiological variables, including water content (Sakai and Larcher 1987), carbohydrate composition (Ögren 1997) and stem lignification (Pulkkinen 1993). During autumn, frost hardening changes in these variables occur at different times and rates, giving rise in some cases to distinct hardening stages (Tumanov 1967, Weiser...
1970). As PLS models based on VIS+NIR spectra were able to predict hardiness over a broad range of values, they may reflect information about several underlying physiological variables.

To test this hypothesis, nine variables possibly related to frost hardiness were measured in parallel with frost hardiness throughout the experiment. In separate PLS models these variables predicted frost hardiness slightly more accurately than VIS+NIR spectra (80% for Scots pine and 85% for Norway spruce). The relative importance of the different variables in predicting frost hardiness varied between species (Figure 7). In Scots pine, carbohydrate composition, in particular starch, glucose, galactose and raffinose concentrations was the most useful predictor (Figure 7). In Norway spruce, dry weight fraction, lignification and raffinose concentration were the most useful predictors.

**Relative importance of spectral windows for prediction of frost hardiness and their relationship to chemical composition**

In the PLS models, the second component contained most of the variation in VIS+NIR spectra related to frost hardiness (data not shown). The loadings plot for this component (Figure 9) show that important information for prediction of frost hardiness was obtained in several spectral windows, especially around 540, 676, 748 and 1400–1540 nm. It is generally not possible to interpret NIR spectra in the same way as IR...
Nevertheless, some deductions can be drawn. The window around 676 nm represents information related to chlorophyll. Because spectral measurements were centred over the stem, a large proportion of the information around 676 nm represents a measure of the chlorophyll content of the stem. This parameter is negatively correlated with lignification of the stem according to Pulkkinen (1993). The peak in loadings around 676 nm is, therefore, indicative of the degree of lignification. The window between 1400 and 1540 nm represents integrated information from water, lignin, cellulose and carbohydrates (Osborne and Fearn 1986, Curran 1989). The loadings plot shown in Figure 9 seems, therefore, to confirm the importance of carbohydrates, water content and lignification for frost hardness. Loadings for wavelengths around 400 nm, which were especially prominent for Scots pine, could not be related to any of the measured physiological hardness determining variables. Information related to hardiness in this spectral region might be associated with a color transition of needles from green to purple caused by increased concentrations of anthocyanins, which have been related to autumn frost hardening in Scots pine seedlings (Toivonen et al. 1991).

We conclude that VIS+NIR spectroscopy, in combination with multivariate modeling, is a fast and nondestructive method for measuring frost hardiness in Scots pine and Norway spruce seedlings. The method is based on relevant physiological information and is not a result of coincidental correlation between spectra and frost hardiness.

References


