Assessing frost hardiness of *Pinus bungeana* shoots and needles by electrical impedance spectroscopy with and without freezing tests

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Abstract

**Aims**

Nursery and forest operations require that frost hardiness results be produced faster than can be provided by controlled freezing tests. There is a great challenge to develop a rapid method for predicting frost hardiness that might not necessitate controlled freezing tests. The aim of this study was to examine the assessment of the frost hardiness of shoots and needles of *Pinus bungeana* by electrical impedance spectroscopy (EIS) with and without controlled exposure to freezing.

**Methods**

The frost hardiness of current-year shoots and needles of *P. bungeana* in an 8-year-old provenance field trial was measured at Shisanlin Nursery in Beijing, China, from September 2006 to January 2007 by means of EIS and conventional electrolyte leakage (EL). In the same plants, but without controlled freezing test, were monitored the EIS parameters in current-year shoots and needles.

**Important Findings**

The results showed that (i) after controlled freezing tests, the frost hardiness estimated by EIS parameters (extracellular resistance, $r_e$, and membrane time constant, $\tau_m$) was significantly correlated with the frost hardiness assessed by EL method ($r = 0.95$) and (ii) for the samples not exposed to controlled freezing treatment, the relaxation time $\tau_1$ for shoots and $\beta$ for needles had greater correlations with the frost hardiness estimated by EL after controlled freezing tests relative to the other parameters ($r = -0.90$ for shoots and $r = 0.84$ for needles, respectively). The parameters $r_e$ of shoots and needles and $\tau_m$ of needles might be applied for measuring frost hardiness of samples after exposed to controlled freezing tests. The frost hardiness results can be obtained within 48 h. The parameters $\tau_1$ of shoots and $\beta$ of needles could be used for estimating the frost hardiness of samples without using a controlled freezing test. The frost hardiness results can be obtained within 24 h.

**Keywords**: cold hardiness • electrical impedance spectroscopy • electrolyte leakage • *Pinus bungeana*

Received: 8 August 2009 Revised: 29 October 2009 Accepted: 6 November 2009
(VSD) and chlorophyll fluorescence (CF) tests are widely used to assess the extent of plant injury in relation to temperature stress after exposure to controlled freezing tests (Burr et al. 2001). The electrical impedance spectroscopy (EIS) is also applied to estimate frost hardiness of plants after artificial freezing tests in recent years (Repo et al. 1997, 2000a,b; Väinolä and Repo 2000; Zhang et al. 2003). However, all these tests require fairly expensive equipment for conducting freezing tests and may be too laborious and time-consuming for largescale monitoring. Nursery and forest operations require that frost hardiness results be produced faster than can be provided by controlled freezing tests. There is a great challenge to develop a rapid method for predicting frost hardiness that might not necessitate controlled freezing testing (Repo et al. 1997).

Recently, the EIS and CF methods exist for predicting of frost hardiness indirectly without freezing. Promising results have been obtained with EIS in willow (Salix viminalis) seedlings, Scots pine (Pinus sylvestris) seedlings and saplings, and birch (Betula pendula) seedlings (Coleman 1989; Luoranen et al. 2004; Repo 1994; Repo et al. 1997, 2000a,b) and with CF for white spruce (Picea glauca) (Binder and Fielder 1996). Both methods seem to work well for some plants or tissues, or under certain conditions, yet neither of them is proven to be a reliable measure of frost hardiness for all plants. Moreover, Linden (2002) indicates that testing without freezing does not yield numerical estimates, unless the method is properly calibrated for the species and tissues examined. EIS is useful for studying the responses of trees to climate change. The method produces information about the physicochemical properties of cells that cannot be obtained by other physiological or microscopic methods (Repo et al. 2004). In the EIS technique, provenances and tree species affect the seasonal pattern of variables used by EIS (Repo et al. 2000a; Zhang et al. 2002). Thus, more studies are needed to develop this method for practical use in tree nurseries (Luoranen et al. 2004) and in forest ecology.

The objective of this study is to examine the relationship between the frost hardiness assessments by the EL method after freezing shoots and needles of Pinus bungeana and those based on EIS parameters of the same tissues not exposed to freezing. A close match between them can potentially validate that EIS is a rapid frost hardness assessment approach applicable to P. bungeana that does not require controlled freezing test.

MATERIALS AND METHODS

Plant material

The study was based on a field provenance trial with three P. bungeana origins. The current-year shoots were sampled in an 8-year provenance field trial at Shisanlin Nursery in Beijing (N 40°13', E 116°13', elevation 79 m, China) with provenances of Mangshan of Beijing (N 40°44', E 116°35', elevation 659 m), Xiaoai of Shanxi (N 37°05', E 111°45', elevation 770 m) and Liangdang of Gansu (N 33°55', E 106°40', elevation 1500 m). In each of the three provenances, 10 saplings were selected with the plant height of 70–90 cm (around the provenance means). The measurements of the current-year shoots and needles were carried out from September 2006 to January 2007. Samples from 10 saplings of each provenance were taken at 1-month intervals. On each sampling occasion, shoots of lateral branches grown during the current year were sampled. On each sampling date, six current year branches were cut on each sapling with a total of 60 branches in each provenance.

Impedance analysis of non-frost-exposed shoots and needles

The electrical impedance spectra of the 16 shoots and needles (one stem and needle per sapling) from each provenance at each sampling date were measured in the laboratory immediately after sampling (a total of five times from September 2006 to January 2007). The total number of non-frost-exposed samples used for the impedance analysis was 480. A 15-mm section was cut from the middle of the shoot and needle samples. The impedance spectra were measured as described by Repo et al. (2000a). The sample was placed in direct contact with the electrode paste and the Ag/AgCl electrodes (RC1, WPI Ltd, Sarasota, USA) were set in contact with the paste. The impedance spectrum was measured at 42 frequencies between 80 Hz and 1 MHz (HP 4284A LCR meter, Agilent Technologies, USA). The input voltage level of the sine signal was 100 mV (r.m.s.).

The shoots were modelled by an equivalent circuit with two distributed circuit elements (DCEs) in series with a resistor (double-DCE model) (Repo et al. 1994, 2000a). The DCE element comprises a constant phase element in parallel with a resistor and two distribution coefficients of the relaxation times. The letter i refers to the imaginary unit.

The extracellular resistance ($R_e$) was obtained as:

$$R_e = R + R_1 + R_2,$$

and the intracellular resistance ($R_i$) as:

$$R_i = R \left(1 + \frac{R}{R_1 + R_2}\right).$$

The resistance parameters were normalized with respect to the cross-sectional area ($A_{shoot} = \frac{\pi d^2}{4}$, $d$ = diameter) and the length ($l = 15$ mm) of the sample in order to obtain the specific resistances (4):

$$r_s = \frac{A}{l} \cdot R_s,$$

where $r_s$ is the specific resistance and $R_s$ is the estimated resistance.
The needles were modelled by an equivalent circuit Model-A (Zhang et al. 1995):

\[ Z_{\text{Model-A}} = R_\infty + \frac{(R_0 - R_\infty) \cdot (1 + \beta)}{1 + \beta \cdot (1 + j \cdot \omega \cdot \tau_m)^{\frac{m}{\beta}}} \]  \hspace{1cm} (5)

There are four parameters in this model: \(R_0\) and \(R_\infty\) represent the low- and high-frequency resistance, respectively; \(\tau_m = R_3 C_m\) is the membrane time constant (where \(R_3\) and \(C_m\) are the resistance and the capacitance of the cell membrane, respectively), and \(\beta\) is a factor controlling spectrum skewness and impedance locus centre depression (Zhang et al. 1995). The complex number operator is \(j = \sqrt{-1}\), and \(\omega = 2\pi f\) is the angular velocity (\(f\) = alternating current frequency). The low-frequency resistance \(R_0\) corresponds extracellular resistance \(R_c\).

The intracellular resistance was calculated as:

\[ R_i = R_\infty \cdot \frac{R_0}{R_0 - R_\infty} \]  \hspace{1cm} (6)

Specific resistances were calculated as equation (4). Where the \(A\) is the cross-sectional area of the needle (assumed as a sector with 60° central angle, so \(A_{\text{needle}} = \frac{d^2 \cdot \pi}{12}\), where \(d\) is the thickness of the needle). The length of the needle (\(l\)) was 15 mm.

The parameters of the double-DCE and Model-A were estimated using an automated complex non-linear least squares (CNLS) fitting program (T. Repo), which uses LEVM v 8.06 (obtained from J. R. Macdonald, Department of Physics and Astronomy, University of North Carolina, Chapel Hill, NC, USA), which has been further developed and automated by Dr Repo and co-workers.

### Frost hardiness of shoots and needles measured after controlled freezing

#### Controlled freezing tests.

During cold acclimation, five freezing tests were conducted with 1-month intervals. The shoots and needles were rinsed three times by tap water and three times by distilled water to remove surface pollutants. The samples were set into plastic bags with six branches in each and a little distilled water was sprayed into the plastic bag in order to avoid excessive supercooling. One sample bag (six branches) of each provenance was exposed in each of seven temperatures (six frost temperatures and one control +4°C; Table 1). The freezing temperatures included temperatures that killed the samples and temperatures that caused no damage. The target freezing temperatures were chosen according to the predicted hardness level except for the last three target freezing temperatures owing to the lack of proper freezing instrument. The rate of cooling was 6°C h⁻¹. The samples were kept at the target temperature for 4 h and then moved into a refrigerator at 4°C to thaw gradually for 24 h. Immediately after the freezing test, the degree of frost damage in the shoots and needles was quantified by the electrical impedance analysis and the EL method, respectively.

## Table 1: the temperatures used for determining frost hardiness in five controlled freezing tests

<table>
<thead>
<tr>
<th>Date</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>13 September</td>
<td>4, -2, -5, -8, 12, -18, -25</td>
</tr>
<tr>
<td>13 October</td>
<td>4, -3, -6, -9, 15, -25, -35</td>
</tr>
<tr>
<td>13 November</td>
<td>4, -6, -10, -15, -25, -35, -70</td>
</tr>
<tr>
<td>13 December</td>
<td>4, -8, -15, -22, -30, -40, -70</td>
</tr>
<tr>
<td>13 January</td>
<td>4, -10, -17, -25, -35, -45, -70</td>
</tr>
</tbody>
</table>

### Frost hardiness assessed by EIS and EL.

EIS: Assessment of frost hardness by the electrical impedance analysis is based on the decrease of the extracellular resistance due to damage in plasma membrane and consequent leaking of the intracellular ions into the extracellular space. Even though the method is destructive, it is an in situ measurement, and it gives a primary measure of frost damage in cells. In this method, alternating current (AC) is applied to a piece of plant tissue. The proportion of current going through the extracellular and intracellular space of a tissue is dependent on the AC frequency and the tissue properties. The tissue features (e.g. extracellular resistance) can be quantified by the equivalent circuit analysis (Repo et al. 1994, 2000a; Ryyppö et al. 1998). The impedance spectra were modelled using the double-DCE model for shoots and Model-A for needles as described previously (Repo et al. 1994; Zhang et al. 2002).

After freezing treatment, eight shoots and eight needles were used as replication in each provenance and each freezing temperature. To obtain the frost hardness, the specific extracellular resistance (\(\Omega_m\)) was modelled by a logistic sigmoid function (in equation 7) with respect to the treatment temperature:

\[ y = \frac{A}{1 + e^{B(C-x) + D}}, \] \hspace{1cm} (7)

where \(y\) is extracellular resistance \(R_c\), \(x\) is treatment temperature, \(B\) is slope at inflection point \(C\), \(C\) is frost hardness and \(A\) and \(D\) determine the asymptotes of the function (Repo et al. 1994, 1997, 2000a,b).

EL: After freezing treatment, 32 needles and 4 shoots from each freezing temperature for each provenance were selected for the EL test. The samples, 10 mm in length, were cut from the middle of the needles and shoots. The shoot samples were cut further vertically into four pieces. The samples were rinsed with distilled water and put into test tubes (eight needle samples and four shoot samples per tube) as four replicates. Thirteen millilitres of distilled water was added to each test tube for needles and shoots, which was then shaken at room temperature for 24 h before the first conductivity measurement (\(C_1\)). The samples were then heat-killed at 100°C for 20 min and shaken for another 24 h before the second conductivity measurement.
measurement \( (C_2) \). The relative electrolyte leakage \( \text{REL} \) was defined as:

\[
\text{REL} = C_1 \times \frac{C_2}{3} \times 100, \quad (8)
\]

Frost hardiness was estimated as the inflection point (parameter \( C \)) of equation (7), with \( y \) being the relative electrolyte leakage (REL).

The total numbers of shoots and needles used for frost hardiness determination by controlled freezing tests were 840 and 840 for EIS method and 420 and 3360 for EL method, respectively.

**Analysis of the data**

The relation of the frost hardiness in shoots and needles assessed by EIS and EL methods after exposure to freezing tests was studied by linear regression analysis. The relation of the frost hardiness in shoots and needles to the equivalent circuit parameters of the non-frost-exposed shoots or needles was studied by polynomial regression analysis. The original data (the means of each origin at each given time) from all the three provenances over the whole study period were pooled and the polynomial regression curve fit was applied (SPSS 12.0 for Windows, SPSS Inc., Chicago, IL, USA). Then the pooled data were split according to the assessment dates, and the correlation of frost hardness with the parameters of the non-frost-exposed samples was calculated separately for each time. For the evaluation of the reliability and accuracy of the models, the coefficient of determination was examined. The correlation coefficient of linear regression was also given (SPSS Inc.). Repeated measurement analysis was applied for analysing the differences in frost hardiness of shoots and needles between different methods, and in parameters of EIS during cold acclimation with a pooled data of three provenances.

**RESULTS AND DISCUSSION**

**Comparison of EIS and EL methods in assessing frost hardiness of samples with artificial freezing tests**

After controlled freezing tests, the \( r_e \) of shoots and needles, the \( \tau_m \) of needles changed as a logistic sigmoid curve and could be used to measure their frost hardness (Fig. 1). During the early stages of cold acclimation, the frost hardiness estimated by EIS method was similar to that determined by EL. From October 2006 to January 2007, the level of frost hardness was lower when estimated by EIS relative to EL method (Fig. 2). The frost hardiness measured by the EIS parameters correlated linearly with that measured by the EL method \( (R^2 = 0.91; r = 0.95) \) (Fig. 3).

After controlled freezing tests, the frost hardiness calculated by parameters \( r_e \) in shoots and needles as well as \( \tau_m \) in needles correlated linearly with their frost hardiness estimated by EL method, suggesting that these parameters could be used to predict frost hardiness after artificial freezing tests. In the present study, a new finding was that the \( r_e \) and \( \tau_m \) of needles varied with a sigmoid curve after artificial freezing temperatures were used, and all these parameters could be calculated by the logistic sigmoid function. The frost hardiness of needles estimated by both \( r_e \) and \( \tau_m \) achieved a lower level of frost hardiness than that assessed by EL method in the late phase of cold acclimation. Both in this and in the former studies, the frost hardiness by \( r_e \) of shoots was typically underestimated in comparison with the frost hardness by EL method during the end of cold acclimation \( \text{(Repo et al. 1994, 2000a,b).} \) What are the reasons to make the difference in frost hardiness should be studied further.
In the present study, after artificial freezing tests the parameters $r_e$ and $s_m$ could be used to predict frost hardness with much shorter time (within 2 d) for obtaining the frost hardness results than with the other traditional methods, i.e. EL with about 5 d and VSD with 7–14 d. Suojala and Linden (1997) indicated that it is questionable to rely only on the subjective damage scoring. The VSD method requires long time to get results and is difficulty in scoring damaged and living tissues. The EL method is laborious with several steps in sample treatment and may not be suitable for hardy samples. For many plant species, curves of leakage vs. freezing temperature were flattened in the course of cold acclimation and dormancy development, rendering curve fitting more difficult (Luoranen 2000; Repo et al. 2000b; Sutinen et al. 1992; Suojala and Linden 1997). However, such situation was not found in the present study.

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The EIS method used in the present work was able to detect injury in *P. bungeana* shoots and needles after artificial freezing tests. This study was in accordance with other researches (Mancuso and Rinaldelli 1996; Repo et al. 2000a; Tsarouhas et al. 2000; Zhang and Willison 1992; Zhang et al. 1993), confirming that electrical impedance is a fast method of assessing frost hardness in plants.

**Correlation of the EIS parameters of non-frost-exposed samples with the frost hardness**

The parameters in non-frost-exposed shoots and needles changed during cold acclimation (Fig. 4). The EIS parameters
\( \tau_1, r_i \) and \( r_e \) in shoots as well as the \( \beta \), \( r_i \) and \( r_e \) in needles correlated significantly with their frost hardness assessed by EL method (\( r = 0.90–0.68 \)) (Table 2). Only parameter \( \beta \) correlated positively to the frost hardness, while the other parameters negatively correlated to the frost hardness (Table 2). Among these parameters, the \( \tau_1 \) of shoots and the \( \beta \) of needles had greater correlations with the frost hardness relative to the other parameters (\( r = -0.90 \) and \( r = 0.84 \), respectively; Table 2). The frost hardness predicted for non-frost-exposed samples by these parameters had a linear correlation with the frost hardness measured by the traditional EL method after exposure to freezing tests (\( r = 0.93–0.79 \)), in which the \( \tau_1 \) of shoots and the \( \beta \) of needles had also greater correlations with the predicted frost hardness relative to the other parameters (\( r = 0.93 \) and \( r = 0.84 \), respectively; Table 3).

The EIS parameters \( \tau_1 \) in shoots and the \( \beta \) in needles of non-frost-exposed samples could be used to estimate frost hardness without the need of freezing because they correlated significantly to their frost hardness assessment by EL method after freezing (Tables 2 and 3). The frost hardness assessed by \( \tau_1 \) of shoots without freezing showed a close match with the assessment after freezing by EL method. For example, the differences between frost hardness assessments by EIS parameter \( \tau_1 \) and that by EL method were <5.7°C, with the minimum difference of 0.1°C in shoots during cold acclimation. However, the differences between the measured and predicted frost hardness were sometimes >10°C, with the estimated frost hardness temperature range covering from –5°C to –50°C. In an early study of \( P. \) sylvestris saplings, the EIS parameter \( \tau_1 \) in shoots predicted frost hardness with an accuracy of ±2.0°C for needles in the early stage of cold acclimation (Repo et al. 2000a). The estimation of \( \tau_1 \) and \( r_i \) in shoots without freezing has also been proved by \( Rho\text{dodendron} \) (Väinölä and Repo 2000) and \( B. \) pendula (Luoranen et al. 2004). But in their studies, at the end of...
The results also indicated that needles decreased, whereas the needle frost hardiness increased.

Correlation coefficient (controlling spectrum skewness and impedance locus centre depression intracellular and extracellular resistance, respectively, and results were consistent with those found in *Pinus sylvestris* (Repo et al. 2000a)). Both correlation with frost hardiness assessed by artificial controlled freezing tests for the whole cold acclimation (Zhang et al. 2002). These results indicate that the responses of trees to the environment conditions differ among tree species. During the frost hardening, the $\tau_1$ increased (Fig. 4A) and it is caused by the changes in the ion mobility in a cellular compartment (Repo et al. 2000a). Mancuso and Rinaldelli (1996) have proposed that altered cell membrane properties affect the relaxation time of the leaves and stems of *Olea europaea*, and the water content may partially explain the behaviour of $\tau_1$ (Repo et al. 2000a). Both $\tau_1$ and $\beta$ are not affected by the size of the plant samples and they are the most reliable parameters.

In summary, we have used the EIS method to predict frost hardiness with and without freezing and compared the frost hardness assessments with those by the traditional EL method after artificial freezing tests. Results indicated that the parameters $r_1$ for shoots, $r_0$ and $\tau_m$ for needles could be used to assess frost hardness of *P. bungeana* after freezing tests. The parameter $\tau_1$ in shoots and the $\beta$ in needles could be used to assess frost hardiness without freezing tests. The EIS method saves time considerably with and without artificial freezing tests and can produce frost hardness results within 48 and 24 h, respectively.

## FUNDING

National Natural Science Foundation of China (grant number 30640035) and the Scientific Research Foundation for the Returned Overseas Scholars, Agricultural University of Hebei (grant number 200406).

## ACKNOWLEDGEMENTS

We thank The Nursery in Beijing Thirteen Tombs Forestry Centre for providing us with the opportunity to make use of the provenance field trial. Dr Tapani Repo also deserves to be thanked for his comments on the manuscript. Mrs Haisu Chen is also thanked for her technical assistance.

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### Appendix  List of symbols and abbreviations

<table>
<thead>
<tr>
<th>Symbol</th>
<th>English name</th>
<th>Unit</th>
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<tbody>
<tr>
<td>$\omega$</td>
<td>Angular velocity</td>
<td>rad s$^{-1}$</td>
</tr>
<tr>
<td>$\beta$</td>
<td>A factor in Model-A controlling spectrum skewness and impedance locus centre depression</td>
<td></td>
</tr>
<tr>
<td>$\tau_1, \tau_2$</td>
<td>Relaxation time</td>
<td>s</td>
</tr>
<tr>
<td>$\psi_1, \psi_2$</td>
<td>Distribution coefficient of relaxation time $\tau_1$ and $\tau_2$</td>
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</tr>
<tr>
<td>$\tau_m = R_3 C_m$</td>
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<td>$A$</td>
<td>Cross-sectional area</td>
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<td>$c_m$</td>
<td>Specific membrane capacitance</td>
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<tr>
<td>CF</td>
<td>Chlorophyll fluorescence</td>
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<tr>
<td>CNLS</td>
<td>Complex non-linear least squares</td>
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<tr>
<td>DCE</td>
<td>Distributed circuit element</td>
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<tr>
<td>EIS</td>
<td>Electrical impedance spectroscopy</td>
<td></td>
</tr>
<tr>
<td>EL</td>
<td>Electrolyte leakage</td>
<td></td>
</tr>
<tr>
<td>$f$</td>
<td>Frequency</td>
<td>Hz</td>
</tr>
<tr>
<td>$i$</td>
<td>Imaginary unit for shoot</td>
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</tr>
<tr>
<td>$i$</td>
<td>Complex number operator for needle</td>
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<td>$R^2$</td>
<td>Coefficient of determination</td>
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<td>Specific membrane resistance</td>
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