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RESEARCH ARTICLE

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Relationship between parameters of electrical impedance spectroscopy and frost hardiness in stems and needles of *Pinus bungeana*

Abstract We studied the frost hardiness (FH) in stems and needles of different *Pinus bungeana* provenances during frost hardening by means of electrical impedance spectroscopy (EIS) and conventional electrolytic leakage (EL) and compared the regression equations of the two methods in order to optimize the EIS method for studying FH on plants. During frost hardening, EIS and EL were applied to one-year old stems and needles of *P. bungeana* in an 8-year provenance field trial at the Thirteen Tombs Nursery in Beijing within the provenances of Mangshan of Beijing, Liangdang of Gansu and Xiaoyi of Shanxi provinces, China. A double-DCE model and Model-A were used, respectively, for the EIS analysis of stems and needles that were not exposed to a controlled freezing treatment. After controlled freezing tests, the FH of stems and needles were assessed by EIS and EL. Without controlled freezing tests, the relaxation time (τ_1) of stems and the specific intracellular resistance (r_i) of stems and needles displayed a statistically significant correlation with FH ($R^2 = 0.79 - 0.86$); after controlled freezing tests, specific extracellular resistance (r_e) of the stems and needles, the cell membrane time constant (τ_m) of needles displayed an even higher correlation with FH ($R^2 = 0.92 - 0.94$). There were significant relationship between EIS and EL in assessing the FH of stems and needles of *P. bungeana*, but EIS underestimated FH more than EL did. EIS is one of the more promising methods for assessing FH, especially without employing a controlled freezing test.

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1 Introduction

During cold acclimation, physiological and biochemical changes in plant cell tissues occur. In these processes, the changes in cell membranes, cytoplasm and vacuole play key roles. Many methods for measuring cold hardiness are time consuming and laborious and may damage plant tissues. Such conditions sometimes limit their use in practice. Thus, the development of an easy and quick method for measuring cold hardiness is urgently needed (Repo et al., 1997). Electrical impedance spectroscopy (EIS) can measure the changes in plant tissues and organs. This EIS method uses equivalent electrical circuits to express measured tissue samples and is widely applied in the fields of estimating plant vitality (MacDougall et al., 1987), damage to fruit (Cox et al., 1993), frost hardiness (Stout, 1988a, 1988b; Repo et al., 1993, 1997, 2000a), salt tolerance (Mancuso and Rinaldelli, 1993) and root growth (Ozier-Lafontaine et al., 2005; Repo et al., 2005). Among these studies, the EIS method measures intracellular resistance, extracellular resistance and membrane changes in a non-destructive way (Mancuso, 1998). The impedance spectrum is composed of a real and an imaginary part and the EIS method is used to analyze the impedance data, after which frost hardiness of plants can be assessed (Repo et al., 1990). In their study of *Pinus sylvestris* sapling shoots, Repo et al. (2000a) found that frost hardiness in the stems assessed by the EIS method (using the specific extracellular resistance r_e) correlated with frost hardiness in needles estimated by an electrolytic leakage (EL) method. So, during cold acclimation the changes of electrical properties in the stems and needles of *P. bungeana* might be obtained by means of the EIS method. For China, the EIS method for measuring frost hardiness of plants and its

application in the field of plant physiology is a new field. In our experiment, we measured the frost hardiness using the EIS and EL methods for the stems and needles in different *P. bungeana* provenances during cold acclimation and compared the relationship between these two methods in order to optimize the technology of EIS. We hypothesize that the physiological and biochemical changes of *P. bungeana* tissues occur during cold hardening and that changes also occur in impedance parameters. Therefore, the frost hardiness of stems and needles can be estimated using the EIS method.

2 Materials and methods

2.1 Plant material

One-year-old shoots of *P. bungeana* were sampled in an 8-year-old provenance field trial at the Thirteen Tombs Nursery in Beijing within the three provenances of Mangshan of Beijing (40°44′N, 116°35′E), Xiaoyi of Shanxi (37°05′N, 111°45′E) and Liangdang of Gansu (33°55′N, 106°40′E). In each provenance, ten saplings were randomly selected. The experiment was carried out from September, 2006 to January, 2007 as well as the EIS and EL measurements of the one-year-old stems and needles. Samples from ten saplings from each provenance were taken at one-month intervals. On each sampling occasion, one-year-old shoots of lateral branches were sampled. At each sampling time, six current year branches were cut from each sapling for a total of 60 branches in each of the three provenances.

2.2 Impedance analysis of non-frost-exposed stems and needles

The electrical impedance spectra of 16 randomly selected needles and 16 stems from 60 shoots of each provenance at each sampling time were measured. The samples were placed in plastic bags and kept in a refrigerator at 4°C. A 15 mm section was cut from the middle of the stem and needle for measurements. The impedance spectra were measured following the protocol by Repo et al. (2000a). The samples were in direct contact with the electrode paste and the Ag/AgCl-electrodes were put into contact with the paste. The impedance spectra were measured at 42 frequencies between 80 Hz and 1 MHz (HP 4284A LCR meter, Agilent Technologies, USA). The stems were modeled by an equivalent circuit with two distributed circuit elements (DCE) (double-DCE model) (Repo et al., 2000a):

$$Z = R + \frac{R_1}{1 + (i\tau_1\omega)^{\psi_1}} + \frac{R_2}{1 + (i\tau_2\omega)^{\psi_2}} \quad (1)$$

where Z is the complex impedance, ω the angular velocity ($\omega = 2\pi f$, where f is the AC frequency). The letter i refers to the imaginary number. In the double-DCE model, there are three measures of resistance (R, R_1 and R_2), two relaxation times (τ_1 and τ_2) and two distribution coefficients (ψ_1 and ψ_2) of the relaxation times. Figure 1 shows the determination of the distributed model parameters (double-DCE) of an impedance spectrum of stem of *P. sylvestris* schematically and the mathematical explanations of the parameters (Repo et al., 2000a). R_1 and R_2 are obtained according to the intersections of the high and low frequency arcs, respectively, with the x axis and τ_1 and τ_2 are obtained from the apex of the high and low frequency arc of the impedance spectrum. The center of the circles is below the x-axis, i.e., the 'depressed center' defined by parameters ψ_1 and ψ_2. The EIS parameters of the equivalent circuit were estimated with an automated complex non-linear least squares (CNLS) fitting program (Macdonald, 1987).

Since the low frequency current may not pass the cell membranes but flows in the apoplastic space, the extracellular resistance (R_e) is obtained as:

$$R_e = R + R_1 + R_2 \quad (2)$$

At high frequencies the current may pass the cell membranes and accordingly flows both in apoplastic and symplastic space. The intracellular resistance (R_i) is obtained as:

$$R_i = R\left(1 + \frac{R}{R_1 + R_2}\right) \quad (3)$$

Since the horizontal area and length of the sample can decrease and increase the impedance proportionally, the geometric shape of the sample should be considered, i.e., the resistance parameters need to be normalized with respect to the cross-sectional area:

$$r_x = \frac{A_c}{l} R_x \quad (4)$$

where r_x is the specific resistance (Ωm) and R_x the estimated resistance (Ω) (x means the extracellular e or the intracellular i); A_c is the horizontal area of the sample (m^2) (for stems: $A_c = \pi d^2/4$, where d is diameter) and l is the length (m) (l = 0.015 m) (Repo et al., 1990, 2000a).

The needles were modeled by an equivalent circuit Model-A (Zhang et al., 1995; 2002):

$$Z_{\text{Model-A}} = R_\infty + \frac{(R_0 - R_\infty)(1+\beta)}{1 + \beta(1 + j\omega\tau_m)^{0.5}} \quad (5)$$

where R_∞ and R_0 represent the high and low frequency resistance, respectively; τ_m is the membrane time constant, $\tau_m = R_3 C_m$ where R_3 and C_m are the resistance and the capacitance of the cell membrane respectively; the coefficient β is a factor controlling the skewness of the spectrum and the impedance locus center 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) (Zhang et al., 1995, 2002).

The low frequency resistance R_0 corresponds to the extracellular resistance R_e. The intracellular resistance is calculated as:

$$R_i = R_\infty \frac{R_0}{R_0 - R_\infty} \quad (6)$$

Specific resistances were calculated according to Eq. (4), where the A_c is the cross-sectional area of the needle (assumed as a sector with 60° central angle, so $A_{needle} = \pi d^2/3$, where d is the thickness of the needle) and l is the length of the needle (l = 15 mm).

The parameters of the double-DCE and Model-A were estimated using an automated complex nonlinear 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 (Table 1 shows the symbols and abbreviations of the EIS parameters).

Table 1 List of symbols and abbreviations of EIS parameters

symbol	definition	unit
ω	angular velocity	$rad \cdot s^{-1}$
β	a factor in Model-A controlling spectrum skewness and impedance locus centre depression	
τ_1, τ_2	relaxation time	s
ψ_1, ψ_2	distribution coefficient of relaxation time τ_1 and τ_2	
$\tau_m = R_3 C_m$	membrane time constant in Model-A	s
A_c	cross-sectional area	m^2
C_m	specific membrane capacitance	$\mu F \cdot cm^{-2}$
CNLS	complex non-linear least squares	
DCE	distributed circuit element	
F	frequency	Hz
i	imaginary unit	
J	complex number operator	
R, R_1, R_2	3 resistances in Double–DCE Model	Ω
R^2	coefficient of determination	
R_3	specific membrane resistance	Ωcm^2
R_∞, R_0	resistances in high frequency and low frequency in Model-A	Ω
r_e	specific extracellular resistance	Ωm
R_e	extracellular resistance	Ω
r_i	specific intracellular resistance	Ωm
R_i	intracellular resistance	Ω
r_x	specific resistance	Ωm
Z	total impedance	Ω

2.3 Frost hardiness of stems and needles measured after controlled freezing test

2.3.1 Controlled freezing tests

The stems and needles were washed three times with tap water to rid the sample surfaces of pollutants and again three times with distilled water. The washed samples were placed into plastic bags with six shoots in each. Seven plastic bags were used for each provenance. A small amount of distilled water was sprayed into the plastic bag in order to avoid excessive super cooling of the samples. For each controlled freezing test, freezing temperatures and a control temperature of 4°C were applied to determine the critical temperature range for frost hardiness. The freezing temperatures included temperatures that will kill the samples and temperatures that allow the samples survive (Table 2). The rate of cooling was 6°C/h and the samples were kept at the target temperature for 4 h. In the end the samples were put into a refrigerator at 4°C to all gradual thawing for 24 h.

2.3.2 Frost hardiness assessed by electrical impedance spectroscopy (EIS) and electrolytic leakage (EL)

The frost hardiness of stems and needles measured by the EIS method, after exposure to freezing test. Eight replications were used in each provenance and each test temperature (see 1.2 for details).

Electrolytic leakage was used for measuring frost hardiness (Repo et al., 2000a; Zhang et al., 2003; Zhang, 2005). After the freezing treatment, each 10 mm long, needle was cut at the middle of the needle and then rinsed with distilled water and put into test tubes (eight needle samples per tube). The stems, 10 mm long, were cut vertically from the middle of the 10 mm stem samples and divided into four pieces. The stem samples were rinsed with distilled water and put into test tubes (four stem samples per tube). Each test temperature used four replicates. We added 13 mm of distilled water to each test tube for needles and stems, which was then shaken at room temperature for 24 h. The first conductivity (C_1) was measured by DDSL-308 conductivity meter (Shanghai, China). The samples were then heat killed at 100°C for 20 min and shaken for another 24 h before the second conductivity measurement (C_2). The relative electrolytic leakage (REL) is defined as:

$$REL = \frac{C_1}{C_2} \times 100 \quad (7)$$

The REL and EIS parameter r_e with the frost hardiness was fitted with a logistic sigmoid model as a function of treatment temperature (Repo and Lappi, 1989):

$$y = \frac{A}{1 + e^{B(C-x)}} + D \quad (8)$$

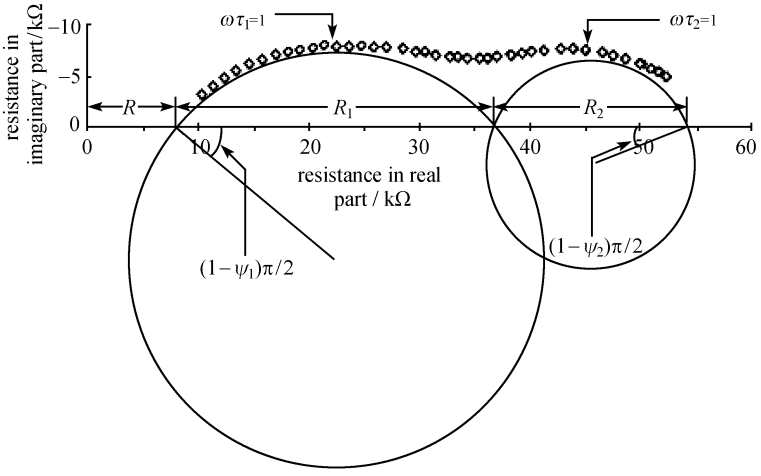

Fig. 1 Schematic drawing of an impedance spectrum of stem of *P. sylvestris* (Repo et al., 2000a)
Note: The mathematical interpretations of the distributed model parameters (double-DCE) see Repo et al., (2000a) in Fig. 1 and Appendix 1. The spectrum is composed of 42 different frequencies ranging from 80 Hz to 1 MHz from right to left, respectively.

Table 2 Temperatures used for determining frost hardiness in five controlled freezing tests

date	temperature/°C						
13 Sept.	4	−2	−5	−8	−12	−18	−25
13 Oct.	4	−3	−6	−9	−15	−25	−35
13 Nov.	4	−6	−10	−15	−25	−35	−70
13 Dec.	4	−8	−15	−22	−30	−40	−70
13 Jan.	4	−10	−17	−25	−35	−45	−70

where x (treatment temperature) and y (REL or extracellular resistance r_e) are determined by A (differences between the highest and the lowest REL or r_e results), B (slope at inflection point), C (temperature at inflection point) and D (the lowest result of REL or r_e). We used SPSS 13.0 to calculate the temperature at the inflection point expressed as the half-lethal temperature (LT_{50}) of tissues, which assesses frost hardiness.

3 Results and analysis

3.1 Electrical impedance of non-frost-exposed stems and needles

The shape and parameters of EIS in non-frost-exposed stems and needles of *P. bungeana* varied during cold acclimation. The electrical impedance spectra of stems appeared as two overlapping arcs with almost the same radius in September; from October on the radii of the two arcs differed and became almost one arc (Fig. 2(a)). The electrical impedance spectra of needles had one arc throughout the cold acclimation, at low frequencies the spectra were unstable (Fig. 2(b)). At high frequencies, the spectra of both stems and needles had the same resistance (real part) and reactance (imaginary part) at different measurement dates.

The specific extracellular resistance r_e of stems

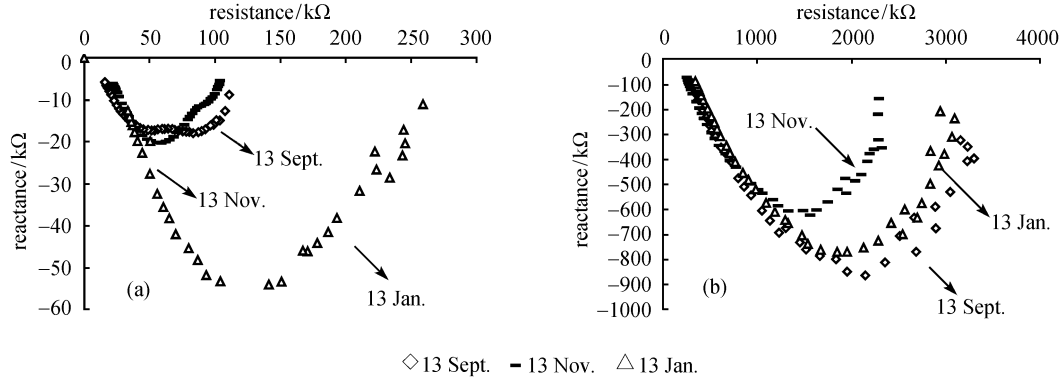

Fig. 2 Impedance spectra of non-frost-exposed stems and needles
Note: The spectra are the pooled data of three provenances and composed of 42 different frequencies ranging from 80 Hz to 1 MHz (from right to left, respectively).

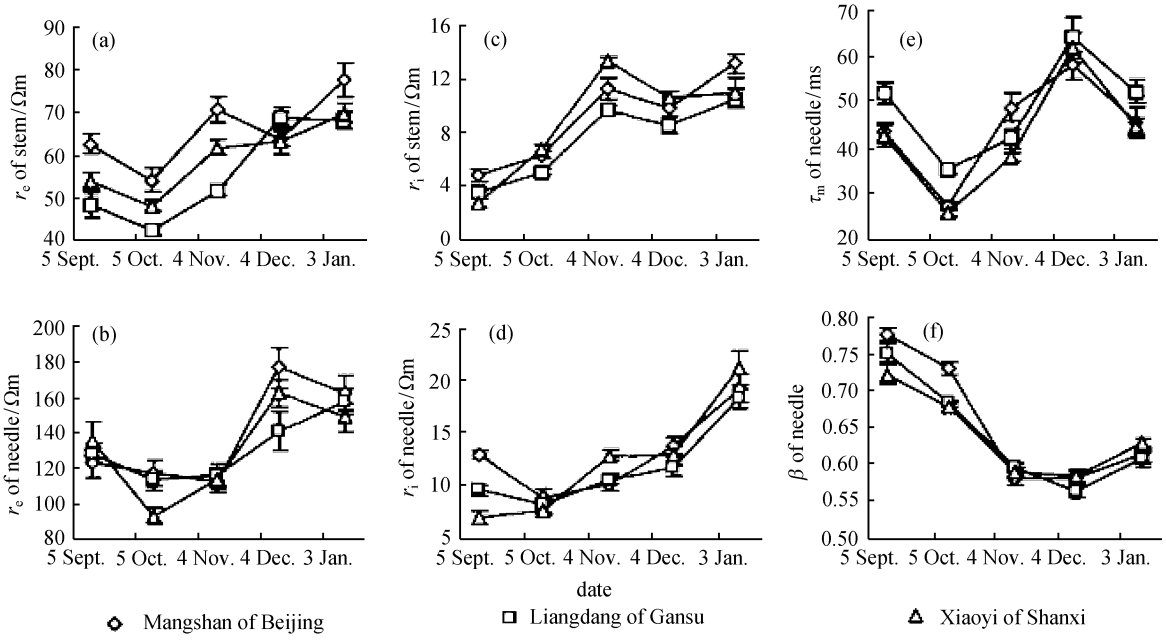

Fig. 3 Variation of impedance parameters of non-frost-exposed stems and needles during frost hardening

decreased from September to October and increased slowly from October until January (Fig. 3(a)). The specific extracellular resistance r_e of needles declined from September to October, but increased from October to December, while from December to January it remained unchanged (Fig. 3(b)). The specific intracellular resistance r_i of stems increased from September to November and then kept the same level until the end of our experiment (Fig. 3(c)). The specific intracellular resistance r_i of needles rose during the cold acclimation (Fig. 3(d)). The membrane time constant τ_m of needles changed similarly with r_e of needles. It decreased at first, then increased and for a second time decreased again (Fig. 3(e)). The coefficient β of needles changed significantly during early hardening but these differences tended to reduce from September to November. The differences disappeared entirely between November and January and the coefficient remained stable (Fig. 3(f)).

The relaxation time τ_1 of stems and the r_i of stems and needles were correlated with the frost hardiness of the same samples treated by exposure to freezing temperatures (Table 3).

Table 3 Regression equations of relaxation time (τ_1) of stems and specific intracellular resistance (r_i) of stems and needles for non-frost-exposed samples as a function of frost hardiness (x)

EIS parameters	regression equation	R^2
τ_1 of stem /ms	$y = 0.0055x^2 + 0.0948x + 8.4339$	0.86
r_i of stem /Ωm	$y = -0.0062x^2 - 0.5627x - 1.6864$	0.80
r_i of needle /Ωm	$y = 0.0144x^2 + 0.5786x + 14.31$	0.79

3.2 Correlations between the parameters of EIS of non-frost-exposed stems as well as needles and the frost hardiness assessed by EL method

After the freezing temperature treatment, the r_e of stems and needles and the τ_m of needles decreased with an increase in freezing temperatures. Thus, the frost hardiness of stems and needles can be estimated by these parameters. Comparisons of frost hardiness between these parameters of EIS and EL for stems and needles are presented in Table 4. At the lower level of frost hardiness, the frost hardiness, assessed by the EIS method, produced results similar to those of the EL method; while at the higher level of frost hardiness the EIS method estimated lower frost hardiness than the EL method (Table 4). The frost hardiness of stems and needles assessed by EIS and EL methods formed a straight line relationship with $R^2 = 0.91$ (frost hardiness of stems and needles assessed between EL and r_e) and $R^2 = 0.93$ (frost hardiness of needles assessed between EL and τ_m). Furthermore, the parameters of EIS after exposure in the freezing tests correlated significantly with the frost hardiness calculated by EL method (Table 5).

4 Discussion and conclusions

4.1 Correlation of EIS parameters to frost hardiness with non-frost-exposed freezing tests

The electrical impedance spectra of stems had two overlapping arcs with almost similar radii in September and from October these two arcs radius differed and

Table 4 Comparison of frost hardiness (FH) in stems and needles measured by the electrolytic leakage (EL) and electrical impedance spectroscopy (EIS) methods (unit: °C)

item	date	Mangshan of Beijing		Liangdang of Gansu		Xiaoyi of Shanxi	
		EL	EIS	EL	EIS	EL	EIS
r_e of stem	13 Sept.	−11.83a	−9.40b	−9.15a	−7.91a	−14.56a	−11.15b
	13 Oct.	−15.15a	−11.29a	−14.90a	−12.12a	−20.43a	−15.83a
	13 Nov.	−35.27a	−30.80a	−30.51a	−22.76b	−32.00a	−25.56b
	13 Dec.	−42.72a	−32.17a	−40.1a	−33.23a	−41.92a	−37.63a
	13 Jan.	−47.77a	−37.93b	−45.89a	−32.18b	−53.03a	−30.30b
r_e of needle	13 Sept.	−11.15a	−10.96a	−8.72a	−8.86a	−15.62a	−11.78a
	13 Oct.	−16.86a	−9.29b	−13.63a	−12.05a	−16.63a	−14.95a
	13 Nov.	−35.10a	−28.42b	−25.66a	−17.48a	−30.79a	−22.00b
	13 Dec.	−39.42a	−30.07a	−40.53a	−30.94b	−40.30a	−34.68a
	13 Jan.	−48.91a	−33.57b	−45.85a	−26.71b	−44.62a	−30.18b
τ_m of needle	13 Sept.	−10.96a	−11.45a	−8.86a	−8.06a	−11.78a	−11.80a
	13 Oct.	−9.29a	−8.86b	−12.05a	−8.24b	−14.95a	−14.89a
	13 Nov.	−28.42a	−21.66b	−17.48a	−15.78a	−22.00a	−16.89b
	13 Dec.	−30.07a	−29.98a	−30.94a	−30.93b	−34.68a	−30.19a
	13 Jan.	−33.57a	−32.52b	−26.71a	−27.13b	−30.18a	−32.52b

Note: Within the columns of Mangshan of Beijing, Liangdang of Gansu and Xiaoyi of Shanxi, the left results were FH measured by EL method and the right results were FH measured by EIS method. At each measuring date within each provenance column, the differences between these two methods are taken as significant and marked with different letters ($p < 0.05$) (the Wald 95% confidence intervals do not overlap).

Table 5 Regression equations of specific extracellular resistance (r_e) of stems and needles and membrane time constant (τ_m) of needles after freezing treatments as a function of frost hardiness

EIS parameters	regression equation	R^2
r_e of stem /Ωm	$y = 0.0135x^2 + 1.5093x + 7.3011$	0.94
r_e of needle /Ωm	$y = 0.0044x^2 + 0.9006x + 0.1049$	0.92
τ_m of needle /ms	$y = -0.0052x^2 + 0.3523x - 4.5426$	0.92

became nearly one arc. This result is different to that of stems in *P. sylvestri*s studied by Repo et al. (2000a); in their investigation the stems of *P. sylvestris* kept two arcs through the cold acclimation. However, our result was in accordance with that of Zhang et al. (2002) who studied the needles of *P. sylvestris*, i.e., where one arc was found during the cold acclimation. In the present study, whether for stems or for needles, the resistance (real part) and the reactance (imaginary part) were equal at the highest frequencies, which again differed from the results found by Repo et al. (2000b) and Zhang et al. (2002). These differences with the previous studies might be due to different tree species.

From early September to early October, i.e. during the stages of preparation and beginning of cold acclimation, r_e of stems, as well as r_e and τ_m of needles decreased. It was expected that when these parameters would reach a certain low point, i.e., the threshold point, where *P. bungeana* would achieve its hardening conditions and would prepare for overwintering. At the moment of an extensive increase in frost hardiness, i.e., the period of early October to early December, r_e of stems, as well as r_e and τ_m of needles increased too, probably because the cells contain some proteins which increased the frost hardiness and helped plants to resist low temperatures.

During the cold hardening stage, the τ_1 of stems, as well as the r_i of stems and needles, increased gradually with a similar tendency in response to frost hardiness and correlated with the frost hardiness. During cold acclimation, the soluble sugar content increases and since sugar consists of low mobile electrolytes, the increase in sugar content raised the concentration of cell sap. This may result in an increase in cytoplasm resistance and perhaps explain the trend of EIS parameters during cold hardening. In the early studies of *P. sylvestris* (Repo et al., 2000a; Zhang et al., 2003), *Medicago stativa*, *Lotus corniculatus* (Stout, 1988a; 1988b) and *Salix viminalis* (Repo et al., 1997) it was found that the r_i also increases with the process of cold acclimation. Changes in the parameters of the τ_1 of stems probably correlated with its lignification of cells and the cell types (Repo et al., 1997). It was suggested that parameters τ_1 and r_i could be used to predict frost hardiness without using controlled freezing tests.

4.2 Correlation of EIS parameters to frost hardiness with freezing tests

In the study of the relationship between parameters of EIS and frost hardiness in *P. bungeana* stems and needles, we observed that r_e and τ_m were correlated significantly with

frost hardiness. According to the trend of EIS parameters, r_e is the most suitable parameter to assess the frost hardiness of stems after exposure to freezing. After freezing, the plasma membrane is damaged and ions in cells move outside the spaces between the cells, causing a decrease in extracellular resistance, thus, the extracellular resistance decreased with an increase in frost damage (Repo et al., 1994; 1997; 2000a; 2000b). Mancuso et al. (2004) measured the frost hardiness of four *Callistemon* and two *Grevillea* plants using EIS and EL methods and they obtained very similar results between these two methods. However, in the present study when frost hardiness reached its highest level the EIS method underestimated the frost hardiness more than the EL method did, which agrees with the results of Repo et al. (1994; 2000a). This is probably due to the poor suitability by the EL method when the samples have a high frost hardiness level. Furthermore, the ion leakage changes with the season (Zhang, 2005). Our research found that the membrane time constant τ_m could be used to assess the frost hardiness of needles. Compared it to r_e, the τ_m predicted more similar frost hardiness results with the EL method.

We draw the following conclusions from our investigation: 1) EIS of stems and needles changed with frost hardiness; 2) relaxation time τ_1 of stems, specific intracellular resistance r_i of stems and needles might be used to estimate their frost hardiness without using freezing tests; 3) specific extracellular resistance r_e of stems and needles and membrane time constant τ_m of needles could be applied to assess their frost hardiness after exposure to freezing tests. In practice, it is essential to develop a fast and easy method for measuring frost hardiness for plants and the EIS is such a method. However, in China this method is rather new and is at the early stages of application. Thus, further studies are needed for different tree species and different provenances in order to understand and more widely use the EIS method and to provide a theoretical basis and technical method for measuring frost hardiness of plants.

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