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Chlorophyll fluorescence as an indicator of frost hardiness in white spruce seedlings from different latitudes

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Application. This study shows that variable chlorophyll fluorescence (F_{var}) can be used as a stock quality indicator of frost hardiness and freezing damage in white spruce (*Picea glauca* [Moench.] Voss) seedlings. F_{var} changes can indicate freezing-induced changes to the photosynthetic system when no visible needle damage, or even electrolyte leakage from needle tissue, are evident. The F_{var} curve attributes can be obtained on whole 1 + 0 seedlings in seconds and the measurement is non-destructive. In practical terms, seedling frost hardiness can be determined from the ratio of frozen to unfrozen values of F_p or F_v/F_m. If this ratio remains close to one, hardiness to that particular freezing temperature can be assumed. We believe F_{var} may have diagnostic value for genetic adaptive and screening studies with regard to frost hardiness in white spruce and other conifer species.

Abstract. This study examined the utility of variable chlorophyll fluorescence (F_{var}) to detect freezing damage in white spruce seedlings of four seedlots. Logistic regression analysis done for freezing tests in September showed that visible needle damage from freezing could be estimated by the F_{var} attributes F_o/I_{ABS}($r^2 = 0.94$), F_p($r^2 = 0.98$), F_v/F_m ($r^2 = 0.99$), and F_t($r^2 = 0.86$). The regression curves indicated that for all four fluorescence attributes, inflection points occurred between 10 and 20% visible needle damage. The lack of a relationship between fluorescence attributes and visible seedling needle damage in October through December is because the minimum temperature (−18 and −24 °C respectively) applied was insufficient to cause needle damage. Freezing-induced changes to F_{var} attributes can be detected which also result in photosynthetic rate decreases when no visible needle damage, and even electrolyte conductivity changes are evident. F_{var} attribute differences due to freezing can be resolved to the seedlot level. The Fvar curve feature manifested 5 seconds after dark-adapted seedlings have been exposed to light (F_{5s}) will estimate ($r^2 = 0.76$) photosynthetic rate after freezing.

Introduction

The determination of frost hardiness in forest trees is important to nursery operators, tree breeders, and researchers. In British Columbia, nursery seedling storability is determined by the seedlings' ability to withstand freezing to −18 °C with less than 25% needle damage when observed after seven to ten days in a heated greenhouse (Simpson 1989). From a nursery management

point of view, when lifting decisions are important to maintain stock quality, such a period is quite long. A test that would quickly, and accurately, provide information about seedling frost hardiness, for the purpose of seedling lifting and cold storage, as well as to detect suspected seedling damage from freezing, would be of considerable value. Visible needle damage, detection of ion leakage increase, differential thermal analysis, and vital staining are indicators of frost damage (see, for example, van den Driessche 1976; Binder 1981; Duryea 1985; Burr et al. 1990; Rose et al. 1990). The advantages and disadvantages of each have been discussed (Binder 1981; Calkins and Swanson 1990; Adams and Perkins 1993). Also, Senser and Beck (1977) described a 'chlorophyll method' for white spruce suggesting that irreversible injury occurs if the chlorophyll content of frozen and thawed needles decreased by more than 50%.

Frost hardiness and freezing injury have been correlated with changes in metabolic activities, level of chemical constituents, and effects on membranes (Heber et al. 1973; Levitt 1980; Duryea 1985; Rose et al. 1990). Senser and Beck (1977), for example, concluded that frost damage to spruce chloroplasts is due to an attack of toxic compounds or lytic enzymes released upon freezing from membrane compartments other than the photosynthetic ones. In addition, the involvement of toxic oxygen species (Vidaver et al. 1991), and the light and temperature-dependent inhibition of the photo- and biochemical systems (Gillies and Vidaver 1990; Demmig-Adams and Adams 1992) provide good evidence that the chloroplasts are directly involved in frost hardiness and frost protection.

Chlorophyll fluorescence has been used as a diagnostic tool (Lichtenthaler 1988; Lichtenthaler and Rinderle 1988; Vidaver et al. 1991) to study chilling injury (Smillie 1983), cold acclimation, and freezing damage in various crop plants (Sundbom et al. 1982), conifer and broad-leaf forest tree species (Brown et al. 1977; Martin et al. 1978; Sundbom and Öquist 1982; Hallgren et al. 1982; Öquist and Strand 1986; Strand and Lundmark 1987; Bolhar-Nordenkampf and Lechner 1988; Strand and Öquist 1988; Öquist and Malmberg 1989; Sundblad et al. 1990; Vidaver et al. 1991; Fisker 1992; Gillies 1993; Mohammed et al. 1995). Based on weighted criteria, this technology ranked highest among the most promising physiologically-based methods for better diagnostic testing of seedling stock quality. (Hawkins and Binder 1990; Mohammed et al. 1995).

Some theoretical aspects of Fvar in relationship to freezing injury

Complete theoretical details of *in vivo* chlorophyll fluorescence induction kinetics are complex and well documented elsewhere (Krause and Weis 1984; Krause and Somersalo 1989; Gillies and Vidaver 1990; Baker 1991; Vidaver

et al. 1991; Krause and Weis 1991). In simple terms, of the two photosystems resident in chloroplast thylakoid membranes, photosystem II is known to be one of the two sites of red light emission from chloroplasts, and subject to stress induced photoinhibition and photodamage. The other site is the antenna green pigment chlorophyll system that emits initial fluorescence (F_o). The general curve shape and the individual curve attributes identified in a typical chlorophyll *a* fluorescence induction (Kautsky) curve reflect the efficiency with which the energy from absorbed photons can reach a reaction centre of photosystem II, and how effective these reaction centres are at transferring electrons to an acceptor (Baker 1991; Vidaver et al. 1991). In practical terms, this means that if the fluorescence curve tail attributes, (i.e. those following about one second after the dark adapted seedling is exposed to the actinic light), and CO_2 fixation (photosynthesis), show a progressive decline while the fluorescence photochemical curve attributes (i.e. curve features leading to F_p) do not, the enzymes of the photosynthetic carbon reduction cycle (ATP and $NADPH^+$ production) are affected but not photosystem II photochemistry. If, however, freezing stress is intense enough to cause chloroplast thylakoid membrane structures to change (i.e. become altered or damaged) the fast fluorescence kinetics resulting in F_p (and therefore F_v/F_m) will decrease because the photochemical efficiency of photosystem II becomes impaired. Strand and Öquist (1988) interpret this as an inhibition of the electron flow from Q_A, the primary plastoquinone pool acceptor of Photosystem II. They believe that in *Pinus sylvestris L.* the irreversible freezing injury to needles is caused by damage to the Q_B protein.

The initial fluorescence (Fo) is independent of photosystem II photochemical events and represents red light emission by excited antenna chlorophyll *a* molecules occurring before the electrons have migrated to the reaction centres (Hipkins and Baker 1986). F_o emission indicates constant fluorescence when all the reaction centres of photosystem II are open and Q_A is oxidized (Papageorgiou 1975). Again, in practical terms, this means that red light emission from the pigment bed will increase either because it has been damaged directly, and cannot transfer all its photon energy to the photosystem II reaction centre, or because the H_2O splitting reaction centre, Q_A or Q_B have been damaged and therefore cannot accept all the energy (Briantais et al. 1986; Krause 1988). Chlorophyll pigment structural alterations resulting from environmental stresses are known to occur (Krause and Weis 1984), and F_o has been reported to increase after freeze-stressing (Strand and Öquist 1988), as well as after other environmental stresses (Layne and Flore 1993). We calculate the ratio of F_o (red light emission from the seedling) to I_{ABS} (the amount of background light absorbed by the seedling) to produce a better estimate of true F_o corrected for seedling size (Dubé and Vidaver 1990).

In another manuscript (Binder and Fielder 1996) we describe seasonal measures of fluorescence and examine different fluorescence attributes which can be used to distinguish seedlot effects. That paper showed how instantaneous measurements of fluorescence over the season could be used to indicate when white spruce seedlings were ready for winter lifting and cold storage. We also suggested that certain fluorescence attributes were indicating physiological changes associated with changes in dormancy and cold hardiness. The present paper deals with using fluorescence to predict visible damage to seedlings as a result of freezing. Using nursery operational conditions our study examined the potential utility of variable chlorophyll fluorescence (F_{var}) technology for quick detection of suspected freezing damage in white spruce seedlings, and whether fluorescence can estimate photosynthesis if the seedling photosynthetic system has been damaged by freezing.

Materials and methods

Seedling material

Seedling origin, culture and greenhouse growth conditions were the same as described previously (Binder and Fielder 1996).

Sampling for frost hardiness testing

Frost hardiness tests were done on September 16–20, October 7–12, November 4–8, and December 5–6, 1991. In September, four freezing treatments were applied including control (+3 °C), −6, −9, and −18 °C. In October treatments were: control (+3 °C), −9, −14, and −18 °C. In November treatments were: control (+3 °C), −14, −18, and −24 °C. In December only two treatments, a control (+3 °C), and a −24 °C, were applied. For each freezing temperature forty or forty-five seedlings per seedlot were chosen randomly from a starting population of 1500 seedlings grown at the British Columbia Ministry of Forests, Glyn Road Research Station in Victoria, on Vancouver Island. The same ten seedlings were used for chlorophyll fluorescence and apparent photosynthesis (A) measurements, another 25 for visible needle damage to shoots of whole seedlings, and a third group of ten for relative conductivity (RC) of electrolytes leached from excised needles along the length of the shoot (except in September when five were used). Removing needles along the entire length of the shoot for RC approximates the visible needle damage assessment and the integrating sphere measurement of fluorescence.

Hardiness testing procedure for whole seedlings and needle segments

The protocols and equipment for hardiness testing of whole seedlings and visible needle damage assessment are described elsewhere (Binder and Fielder 1996). Equivalent freezing temperature treatments for whole seedlings and needle tissue were done on different days but the same freezing chamber was used. For freezing whole seedlings, due to size restraints, freezing temperatures were applied in random order over a maximum of three days. Seedlings for visible needle damage assessment and Fvar were treated together. Freezing treatments of excised needle tissue were run over one day; the appropriate rack of samples was removed from the freezer when the target temperature was reached. For all treatments, the freezer temperature was reduced at 6 °C/h to the target temperature and held for one hour before samples were moved to a cool room (+2 °C) in an insulated box at +2 °C for thawing overnight. The same procedure was followed for all freezing treatments except in October when, because of freezing controller failure, the cooling rate for the −14 °C test was inadvertently increased to 11 °C/h (from +3 °C down to −14 °C) for whole seedlings to be tested for fluorescence and photosynthetic rate. These data were reported because they showed seedling damage differences due to rate of freezing as well freezing intensity.

Enough needles were excised from the whole length of each seedling to provide 20 needles per temperature. Excised needles were mixed and kept temporarily in a plastic weighing boat lined with damp filter paper. After excision, each set of 20 needles was placed into a dry glass tube with a piece of moistened filter paper (about 1 ml of distilled water) stuck to the upper part of the tube. The tubes were all capped with a plastic cap and placed in 4 racks, one rack per temperature treatment, in a refrigerator at 4°C until ready to carry out the freezing treatments. Seedlots were randomized within each temperature rack and one blank tube per temperature treatment was included in each rack. After thawing (+2 °C overnight), on day two, the treated needles were cut into 3 parts so that each section was about 0.3 cm long. The cut needles were put back in the tubes and 5 ml of deionised water added. All tubes were placed in a refrigerator at 4 °C for 24 h. (We used 4 °C rather than 20 °C as an incubation temperature environment because preliminary tests showed that, for periods longer than 24 h, the electrical conductivity (EC) (μmho) of those incubated at 20 °C continued to increase in contrast with those incubated at 4 °C in which electrical conductivity remained almost constant. We concluded that a smaller error would be associated with incubation at the lower temperature over 24 h even though the total electrolytes leached was less at 4 than at 20 °C by about 10–20% of the final killed value.) On day three the tubes were allowed to equilibrate to 25 °C in a water bath and the initial EC of the water bathing the needles was measured with a Radiometer CDM83

Table 1. Scan F_{var} feature attributes, units of measurement, and definition obtained from the Fluoroview data acquisition program and from analysis of the normalized curves.

Parameter	Units	Description
F_o	mV	The estimated initial fluorescence emitted by the sample chlorophylls before the onset of measurable photochemistry. Proportional to the total number of excited chlorophyll molecules (Dubé and Vidaver 1990).
I_{ABS}	μmol m^{-2} s^{-1}	Excitation light absorbed by the seedling.
F_o/I_{ABS}	mV/μmol m^{-2} s^{-1}	Adjusts F_o for the light quanta absorbed (i.e. seedling size).
F_p	rfu.	Maximum normalized variable fluorescence within the first second of the 300 s scan.
F_v/F_m	no units	*Maximum variable* fluorescence of a 'dark-adapted' sample (i.e. the fluorescence intensity above F_o)/*Maximum* fluorescence yield of a 'dark-adapted' sample (i.e. with all the photosystem II reaction centers fully reduced). *Note*: internal actinic light level in the integrating fluorometer is 100 μmol m^{-2} s^{-1}
F_{5S}	rfu.	Normalized variable fluorescence at 5 s of the 300 s scan.
F_t	rfu.	Normalised variable fluorescence at 300 s

conductivity meter. All tubes were then heated at 90 °C for 2 h and put back in the refrigerator at 4 °C for 24 h. On day four all tubes were re-measured at 25°C for total electrolytes. Relative conductivity (RC) was calculated using the following formula [RC = (Initial EC-blank)/(Killed EC-blank)].

Fluorescence and gas exchange measurements

Variable chlorophyll fluorescence (F_{var}) and apparent photosynthesis (A) of whole seedlings (10 per temperature × seedlot × date) were measured the day after the freezing test on the same seedlings. The measurement protocol and instrument settings for the integrating sphere fluorometer and closed gas exchange system are described in Binder and Fielder (1996). Fluorescence scan attributes F_o/I_{ABS}, F_p, F_v/F_m, and F_t, shown in Table 1, were determined in the same way as previously described. The F_v/F_m attribute was calculated from F_m (maximum unnormalized fluorescence (mV) at 100μmol m^{-2} s^{-1} corrected for stray light), and F_v which is equivalent to $F_m - F_o$. The integrating sphere cannot yield the true maximum (F_m) because this can only be obtained with a saturation pulse of very high intensity actinic light.

Statistical analysis

For each date, the mean (n = 25) of visible needle damage was regressed against the mean (n = 10) of selected fluorescence scan attributes. Visible needle damage (y) and F_o/I_{ABS}, F_p, F_v/F_m (x) were fitted to a non-linear model of the form $y = a/(1+e^{c+bx})$ and F_t to a power function of the form $y = ax^b$. Parameter estimates for a, b, and c were approximated by iterating the best fit using the PROC NLIN procedures of SAS (SAS Institute Inc, 1988). Coefficients of determination (r^2) were calculated from the corrected sum of squares (CSS) and the residual sum of squares (RSS), i.e., $r^2 = 1-(RSS/CSS)$. Each regression was made on 16 points (4 seedlots and 4 temperatures). Corresponding measures of apparent photosynthesis and fluorescence attributes F_o/I_{ABS}, F_{5s}, F_p, F_v/F_m, and F_t for individual trees over all dates were regressed using linear regression (PROC GLM of SAS) where n = 552 (3 dates × 4 seedlots × 4 temperatures × 10 seedlings = 480, 1 date × 4 seedlots × 2 temperatures = 80, total = 552, due to instrument problems eight corresponding measures were lost).

Results and discussion

Effect of freezing on fluorescence, net photosynthesis and electrolyte conductivity

Non-linear regressions of visible needle damage versus fluorescence attributes were strongest for September freezing treatments when seedlings exhibited a broad range of visible needle damage response (Figure 1). On subsequent dates, freezing treatments were not severe enough to inflict greater than about 15% damage. The lowest temperature had been chosen for its relevancy to operational stock quality testing. We were not able to fit non-linear curves to visible needle damage versus fluorescence attributes in October, November, and December because of the reduced range of visible damage and because fluorescence attributes tended to decline through the fall. Consequently, useful predictive relationships could not be obtained by combining regression analyses across dates, therefore, results and discussion of relationships will focus on September data.

In September, the coefficients of determination for the fit of non-linear functions to the relationships between needle damage and F_o/I_{ABS}, F_p, F_v/F_m, F_t (n = 16) were generally high. For the logistic fit between needle damage and F_o/I_{ABS}, F_p, F_v/F_m coefficients of determination were 0.86 (MSE = 10279), 0.98 (MSE = 10544), 0.99 (MSE = 10567) respectively (df = 3). (Figure 2A, B, and C). For the power fit (n = 16, df = 2) between needle damage and F_t the

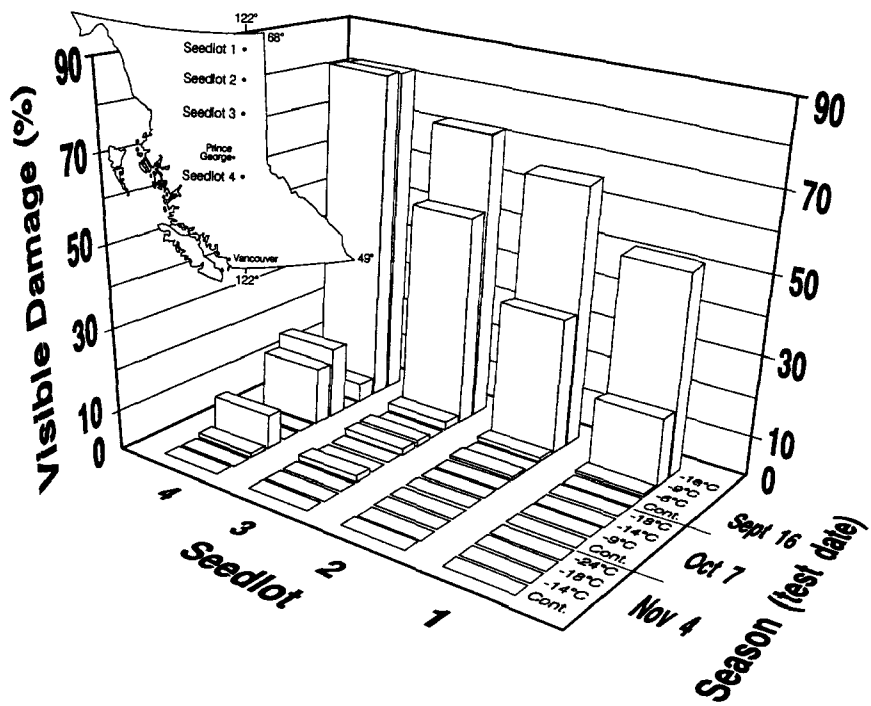

Figure 1. Mean visible damage to needles of seedlings from four seedlots frozen to different temperatures on three separate test dates. No visible damage to needles was observed for any of the four seedlots at −24 °C for the December 05 test date (not shown). Each bar is the average of 20 seedlings. The map inset of British Columbia shows the native latitudes of the seedlots.

r^2 was 0.86 (MSE = 14768) (Figure 2D). The regression curves indicated that for all four fluorescence attributes, inflection points occurred between 5 and 20% visible needle damage. These approximate threshold values were above 12.5 mV for F_o/I_{ABS}, and below 0.60, 0.36 and 0.12 (rfu) for F_p, F_v/F_m, and F_t respectively. Fluorescence attributes F_o/I_{ABS}, F_p, and F_v/F_m fit a sigmoidal response curve typical of membrane injury from freezing (Repo and Lappi 1989) or high temperature (Binder and Fielder 1995). The curve shapes are also consistent with the elastic and plastic characteristics of stress intensity as described by Levitt (1980). In contrast to other attributes, the data points for F_t versus needle damage tended to fall into two groups where damage was either present or absent. This relationship was best fit by a power curve.

The effect of freezing treatments −6, −9, and −18 °C on fluorescence in September are shown in the complete Kautsky induction curves in Figures 3A and 3B (only northern and southern-most seedlots shown). The curve attributes F_p, F_{5s}, and F_t are identified on these curves. Freezing stress clearly

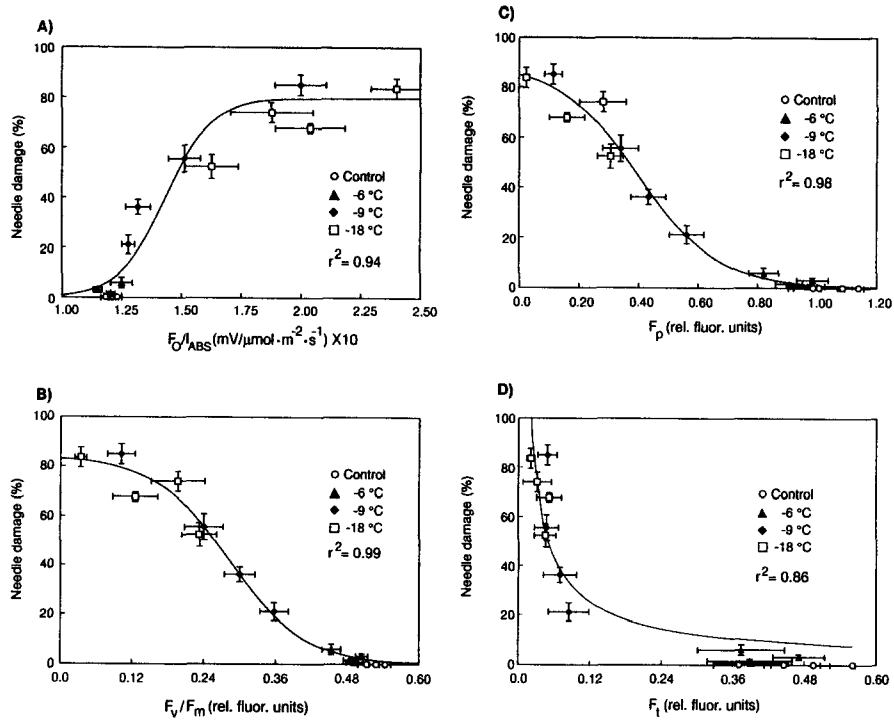

Figure 2. Regression of visible damage to needles against F_o/I_{ABS} (A), and normalized fluorescence attributes F_p (C), F_v/F_m (B), and F_t (D) of four seedlots for September 16. Each regression has 16 points (means). Each point is the mean of 10 values for fluorescence and 25 values for visible damage. Vertical and horizontal bars represent ± 1 se. Visible damage and F_o/I_{ABS}, F_p, and F_v/F_m, are fitted to a logistic function $y = a/1 + Exp^{c+bx}$ and for F_t a power function, $y = 4.615F_t^{-0.80}$ was applied. Curve parameter estimates a, b and c for the logistic curves were: F_o/I_{ABS} (75.69, −10.33, 14.81), F_p (89.34, 7.37, −2.939), and F_v/F_m (83.89, 16.11, −4.54), respectively. For the power function curve parameters were 4.62 (a), and −0.8 (b).

decreased both fluorescence emission and photosynthesis (data reported on graphs) indicating that freezing was detrimental to chloroplast function. These processes were affected to a different degree depending on the seedlot. Visible needle damage (Figure 1) and fluorescence curves (Figure 3A, B) showed the same ranking of seedlot at specific temperature treatments. The −6 °C treatment did not affect the northern seedlot compared to the control curve (Figure 3A), but the southern seedlot was affected (Figure 3B). At −9 °C both seedlots were affected but the southern-most one to a greater degree. At −18 °C both seedlots decreased maximally compared with the control. F_{var} curves for the other two seedlots (not shown) were intermediate between the two extremes.

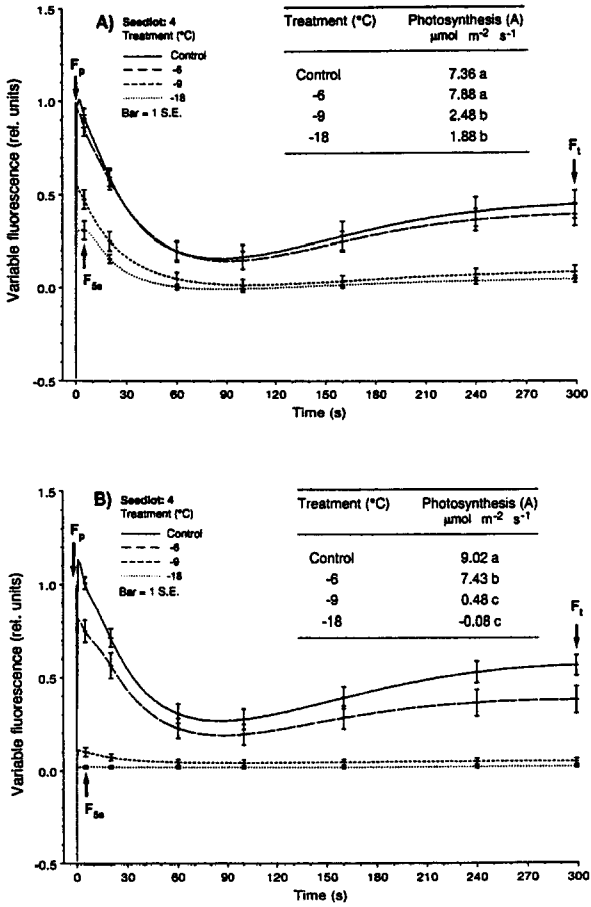

Figure 3. Mean (± 1 SE) normalized relative fluorescence curves of seedlots 1 (A), (the most northern latitude one tested) and 4 (B), (the most southern latitude one tested) over 300 seconds after freezing to −6, −9, and 18 °C on September 16. For reference the individual variable fluorescence curve feature attributes F_p, F_{5s} and F_t are shown. The definitions of these attributes are shown in Table 1. n = 20. Photosynthetic values are shown in inset.

Changes in fluorescence attributes (F_o/I_{ABS}, Fp and F_t) after freezing treatment in September are shown graphically for all four seedlots (Figure 4). Our data showed that F_o/I_{ABS} increased with increasing freezing stress. This did not agree with the results of Adams and Perkins (1993) who reported no change in F_o red light emission with increasing freezing stress, followed by a sudden decline to zero after a critical yield-point temperature is reached. The differences between the two sets of results are likely due to the different types of instruments used, test date, species, as well as the intensity of the freezing. Our treatment temperature was −18 °C, carried out in September,

while the seedlings were still hardening; their test was made in October at −40 °C using branches from mature red spruce. For all seedlots the −9 °C exposure resulted in changes in the curve attributes. Generally the attributes followed the visible needle damage trend for −9 °C on September 16 (Figure 1). All seedlot 4 (southern seedlot) curve attributes at −9 °C suggest near, or total destruction of the Photosystem II complex (Figure 4). Visible needle damage was also maximum at this temperature (Figure 1). Even at −6 °C, compared to control, this southern seedlot displayed a decrease in F_p and F_t (Figure 4) and showed about 6% visible needle damage (Figure 1). The photosynthetic rates for seedlots 1, 2, and 3 showed that the −9 °C treatment resulted in a substantial decrease in CO_2 uptake (Table 3). In the southern-most seedlot the photosynthetic rate was already reduced compared to control at −6 °C and approached zero at −9 °C (Table 3). The F_{var} attributes in all four seedlots continued to change from −9 to −18 °C although less than from −6 to −9 °C (Figure 4). Apparent photosynthesis was also further decreased between these temperature treatments for all seedlots, with seedlot 4 decreasing to a negative value (Table 3). Visible damage to needles for seedlots 1, 2, and 3 also increased depending upon their origin from north to south. The practical application of this information may be, for example, to determine which seedlot, within a seed zone, should be used for planting into an area where late and early frost are suspected, or known to occur. Fluorescence has already been suggested as measure of cold sensitivity for different provenances of Douglas-fir (Vidaver et al. 1989), lodgepole pine, and Scots pine (Lindgren and Hällgren 1993).

Trends in F_p and relative ion electrical conductivity (RC) are shown for all seedlots, freezing treatments, and all treatment dates in Table 2. Attribute F_p was chosen as a comparison because it can be determined in one second, and as a quickly derived estimate of potential damage is applicable to operational seedling quality assessment. Although of comparable predictive value, the attribute F_v/F_m, was not used because, with the integrating sphere, the F_m value for F_v/F_m, is not a true maximum value (Mohammed et al. 1995). In general, RC (Table 2) followed the trends of both F_p and corresponding photosynthetic rates (Table 3), but under certain conditions was less responsive. In September, RC of seedlots 1, 2 and 3 for treatments −9 °C and −6 °C, and RC of seedlot 4 for −6 °C, changed relatively little compared to the control values (RC/damage r^2 for September = 0.87, MSE = 14812). The lack of visible damage at −6 °C, and the abscence of an increase in RC for all seedlots in September supports the accuracy of RC as a good predictor of visible damage. Changes in F_p for the same treatments were proportionally larger suggesting that, compared to RC, fluorescence was more sensitive to stress disturbances before they cause major cellular injury. Also in December,

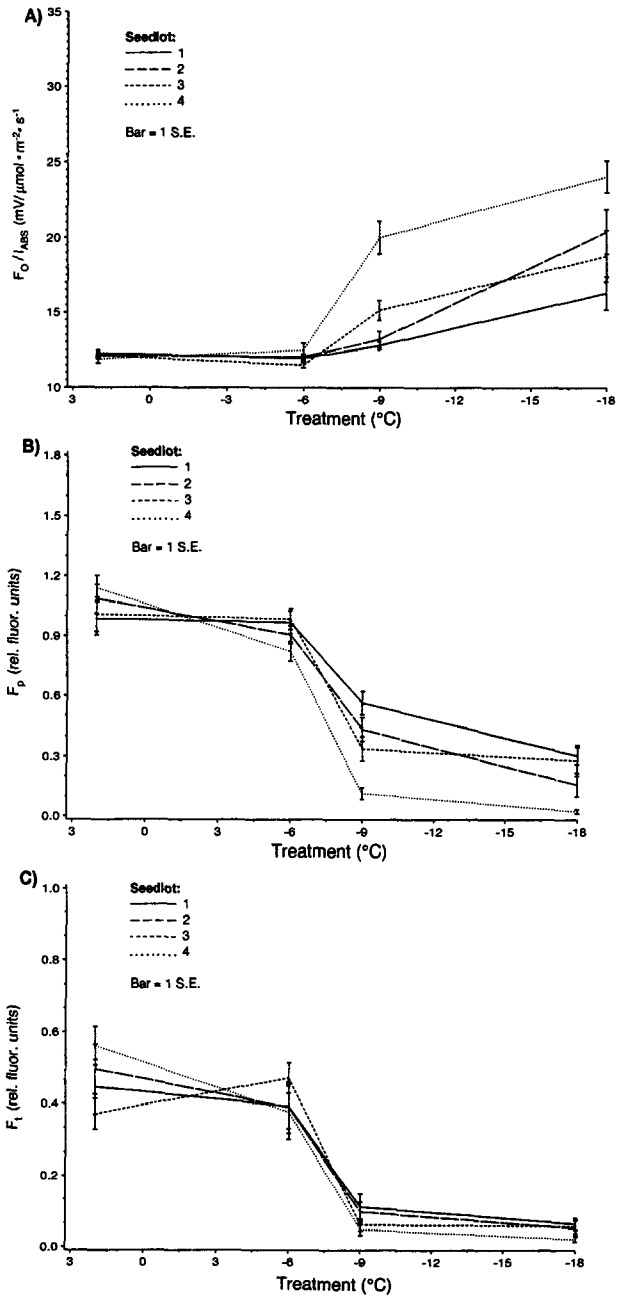

Figure 4. Mean (± 1 SE) fluorescence attribute value F_o/I_{ABS}, (A), and normalized fluorescence parameter values F_p (B), and F_t (C) of four seedlots after being frozen to −6, −9, and −18 °C on September 16. The definitions of these attributes are shown in Table 1. n = 20.

Table 2. Comparison of seedlot means of relative fluorescence attribute Fp and relative conductivity (RC) after freezing to −6, −9, and −18 °C in September, −9, −14, and −18 °C in October and −24 °C in December. Mean values are shown with one SE, n = 10 seedlings for Fp and 5 (September) or 10 (all other dates) for RC.

Sdlt	T (°C)	F_p (r.f.u)				RC (%)			
		Sept 16	Oct 7	Nov 5	Dec 6	Sept 16	Oct 7	Nov 5	Dec 6
1	Contr	0.986 ± 0.25	0.834 ± 0.18	0.525 ± 0.21	0.393 ± 0.05	13.62 ± 3.1	11.90 ± 3.7	13.48 ± 2.1	11.79 ± 2.5
	−6	0.968 ± 0.18				8.37 ± 1.5			
	−9	0.564 ± 0.19	0.852 ± 0.20			15.87 ± 1.9	13.72 ± 2.7		
	−14		*0.856 ± 0.09	0.566 ± 0.14			16.66 ± 3.9	13.76 ± 2.7	
	−18	0.305 ± 0.13	0.772 ± 0.25	0.522 ± 0.11		35.36 ± 20.7	15.85 ± 4.0	12.68 ± 2.7	
	−24			0.404 ± 0.15	0.256±0.05			14.58 ± 2.3	14.61 ± 2.3
2	Contr	1.085 ± 0.22	1.001 ± 0.25	0.483 ± 0.18	0.353 ± 0.14	9.55 ± 1.1	12.04 ± 3.1	12.05 ± 1.6	11.64 ± 1.4
	−6	0.901 ± 0.14				8.81 ± 1.5			
	−9	0.434 ± 0.18	0.970 ± 0.17			12.98 ± 2.2	12.77 ± 2.1		
	−14		*0.900 ± 0.10	0.439 ± 0.14			14.34 ± 2.0	13.33 ± 2.6	
	−18	0.156 ± 0.19	0.859 ± 0.12	0.450 ± 0.16		50.08 ± 37.4	16.60 ± 1.5	13.56 ± 3.0	
	−24			0.354 ± 0.15	0.265 ± 0.06		14.46 ± 3.4	14.94 ± 3.9	14.94 ± 3.9
3	Contr	1.01 ± 0.27	0.866 ± 0.23	0.535 ± 0.17	0.288 ± 0.08	12.62 ± 2.5	13.92 ± 2.4	12.38 ± 1.4	12.09 ± 1.7
	−6	0.989 ± 0.18				9.61 ± 2.1			
	−9	0.339 ± 0.19	0.888 ± 0.18			19.49 ± 6.9	15.94 ± 2.7		
	−14		*0.710 ± 0.16	0.505 ± 0.21			18.86 ± 2.8	12.98 ± 1.8	
	−18	0.281 ± 0.24	0.841 ± 0.18	0.508 ± 0.08		67.21 ± 23.9	22.93 ± 5.8	12.89 ± 3.1	
	−24			0.532 ± 0.26	0.253 ± 0.06			15.91 ± 2.6	6.83 ± 1.5
4	Contr	1.139 ± 0.20	1.091 ± 0.39	0.817 ± 0.14	0.530 ± 0.15	12.91 ± 1.7	14.76 ± 2.6	12.44 ± 2.3	14.61 ± 2.1
	−6	0.823 ± 0.16				10.83 ± 1.6			
	−9	0.115 ± 0.09	0.988 ± 0.12			17.95 ± 1.9	16.79 ± 2.9		
	−14		*0.674 ± 0.20	0.761 ± 0.16			28.38 ± 8.7	18.31 ± 2.8	
	−18	0.024 ± 0.03	0.823 ± 0.12	0.725 ± 0.28		71.13 ± 2.6	40.44 ± 17.1	20.71 ± 4.8	
	−24			0.678 ± 0.21	0.367 ± 0.18			21.63 ± 5.1	11.59 ± 6.1

* F_p values for the −14 °C treatment in October are based on inadvertently freezing seedlings from +3 to −14 °C in 1 h; RC values for the −14 °C treatment are shown at the normal freeze rate of 6 °C/h.

freezing to −24 °C resulted in a decrease in F_p and A (Tables 2 and 3) whereas there was no corresponding increase in electrolyte leakage (Table 2) or visible needle damage (Figure 1). The difference might, in part, be explained by the fact that fluorescence reflects the state of photosystem II photochemistry directly (Vidaver et al. 1991), and electrolyte leakage the general integrity of the outer, comparatively resilient, membranes of cells (Levitt 1980). Also, the difference may be partly due to the fact that the two tests used different tissue sizes and test protocols, (i.e., detached needles in test tubes for EC versus whole plants in air for F_{var}). Such differences could contribute to variation in the amount of damage to the individual leaf cells in comparison to visual signs of damage in whole needles. We must caution, however, that an alterantive conclusion could be that the response of Fp (and A) to freezing, with no corresponding response in visible damage, could suggest that these measurement techniques are more subject to a Type I error than RC.

In September, for all seedlots, and to a lesser degree in December for seedlots 3 and 4, RC decreased after mild freezing (Table 2). Colombo et al. (1984) previously reported this phenomenon as a negative index of injury after a mild freezing treatment in shoot-tips of black spruce. It has also been reported in other types of measurements after sublethal freezing by others (Öquist 1983; Powles 1984; Steffen and Palta 1986). Interestingly, mild stress has been suggested to cause enhancement of membrane stability after a period of destabilisation (Larcher 1987). We suggest that perhaps a practical application of this phenomenon may be to facilitate frost acclimation. The idea may warrant further study.

The sensitivity of F_{var} to detect freezing stress is further demonstrated by the −14 °C data for October (Table 2). In this case seedlings were inadvertently cooled at 14 °C/h, as opposed to 6 °C/h in the rest of the study. Depending on the seedlot origin, the higher rate of freezing resulted in a lower F_p value and photosynthetic rate, compared to the −18 °C treatment cooled at the 6 °C/h rate (Tables 2 and 3), (the RC values for the −14 °C/h freezing treatment are shown at the normal 6 °C/h cooling rate (Table 2)). Therefore, not only could the fast fluorescence attribute Fp detect damage due to freezing intensity, but also could distinguish between the rates at which that freezing took place.

Practical application of fluorescence curve attributes to freezing damage detection

We believe that the F_{var} and photosynthesis data we collected here, in combination with that reported by others (Strand and Öquist 1988), under similar conditions, supports the suggestion that the primary target of low (and high) temperature stresses in plants is the thylakoid membrane (Yordanov 1992).

Table 3. Seedlot means of net photosynthesis (A) after freezing to −6, −9, and −18 °C in September, −9, −14, and −18 °C in October, 14, −18 and −24 °C in November and −24 °C in December. Mean values are shown ± SE, n = 10 seedlings.

Sdlt	T (°C)	A (μmol CO_2 m^{-2} s^{-1})			
		Sept 16	Oct 7	Nov 5	Dec 6
1	Contr	7.701 ± 1.6	6.019 ± 1.1	3.289 ± 0.9	3.221 ± 0.9
	−6	7.875 ± 1.6	–		
	−9	2.481 ± 1.2	5.968 ± 0.6		
	−14	–	*5.658 ± 1.1	3.173 ± 1.0	
	−18	1.878 ± 1.5	4.682 ± 1.4	2.580 ± 1.3	
	−24			2.103 ± 0.5	2.066 ± 0.9
2	Contr	7.896 ± 1.5	6.733 ± 1.3	2.945 ± 0.8	3.242 ± 1.3
	−6	8.347 ± 0.9			
	−9	1.752 ± 1.3	6.826 ± 0.8		
	−14	–	*6.105 ± 1.1	2.827 ± 1.0	
	−18	0.341 ± 0.7	5.519 ± 1.4	2.357 ± 0.9	
	−24			1.681 ± 0.4	2.304 ± 0.9
3	Contr	8.340 ± 1.5	5.508 ± 1.2	3.435 ± 1.4	3.197 ± 0.4
	−6	8.093 ± 1.2	–		
	−9	0.586 ± 0.5	6.227 ± 0.8		
	−14	–	*4.105 ± 1.5	2.709 ± 1.2	
	−18	0.198 ± 0.6	4.866 ± 1.1	2.413 ± 1.0	
	−24			2.052 ± 1.0	1.726 ± 0.2
4	Contr	9.016 ± 1.4	6.260 ± 1.3	5.417 ± 1.4	5.496 ± 1.6
	−6	7.434 ± 2.3	–		
	−9	0.479 ± 0.7	7.596 ± 1.5		
	−14	–	*2.605 ± 1.9	3.865 ± 2.0	
	−18	−0.079 ± 0.1	4.820 ± 1.3	2.607 ± 1.7	
	−24			2.095 ± 0.9	1.923 ± 0.7

* A values for the −14 °C treatment in October are based on inadvertently freezing seedlings from +3 to −14 °C in 1 h; RC values for the −14 °C treatment are shown at the normal freezing rate of 6 °C/h in Table 2.

Fluorescence therefore is an ideal way to assess potential damage in green plants subjected to temperature stress.

For operational freeze testing of white spruce in nurseries, damage resulting from freezing could be determined in seconds from typical regression curves using the curve attributes F_o/I_{ABS}, F_p, or F_v/F_m (Figure 2), provided such curves have been generated for that approximate time of year. In a similar way potential damage due to natural freezing events could be evaluated quickly by operational nurseries. In such cases, control curves could be generated shortly before the freezing event, and the stress exposed trees

compared to the control curve after the event. If the ratio of the Fvar attribute after freezing to before freezing remains close to one, frost hardiness to that temperature can be assumed. This means that if safe lifting for cold storage needs to be verified by the −18 °C test (Simpson 1989), for example, the longest part of the assessment then becomes the freezing procedure. From the data collected, we suggest attribute F_t would not be very useful as a predictor of freezing damage as it appears to lack sensitivity for moderate freezing stresses. Also, it requires at least 5 min to measure.

We must, however, caution that the relationship of any F_{var} attribute to visible needle damage after freezing may vary with the species, cultural conditions, and the time of year. For example, the relationship for spruce becomes poor as frost hardiness to that specific temperature progresses because no visible needle injury is observed but should hold if more intense freezing is applied (see also Lindgren and Hällgren 1993).

Fluorescence parameter F_{5s} as an estimator of photosynthesis in freezing-stressed seedlings

Linear regression analyses of corresponding measurements of photosynthesis versus fluorescence attributes over all dates, seedlots and freezing treatments indicated fairly strong linear trends for most of the attributes. Selected regressions included F_{5s}, Fp, F_v/F_m, and F_t, with coefficients of determination (r^2) of 0.76 (MSE = 2967), 0.65 (MSE = 2556), 0.66 (MSE = 2582), and 0.56 (MSE = 2214), respectively (n = 552). Attribute F_o/I_{ABS} had a low r^2 of 0.07 (MSE = 265) most likely because this attribute has no direct involvement in PSII photochemistry. We suggest that the fluorescence attribute F_{5s} may be utilized as a fast estimator of apparent photosynthesis (A) after seedling freeze-stressing (Figure 5). The attribute F_{5s} is manifested close to the fluorescence curve features S_1 and M_1 which are known to be associated with the induction of net photosynthetic CO_2 assimilation (Hipkins and Baker 1986; Vidaver et al. 1991). We believe the F_{5s} fit with photosynthesis is quite good considering that the photosynthetic carbon reduction cycle (rubisco) and photophosphorylation (ATP synthesis) enzymes are temperature-sensitive and are likely to show functional decline before energy transport systems. The equation and r^2 for the correlation of fluorescence and photosynthesis differ somewhat from those reported in another paper (Binder and Fielder 1995) because those were seasonal, unstressed readings and in the present paper freezing damaged seedling photosynthetic rates are included. The practical application of this information for nursery operators and researchers is the ability to estimate apparent photosynthesis, non-destructively in seconds, after freeze-stressing. This can be done without expensive gas exchange

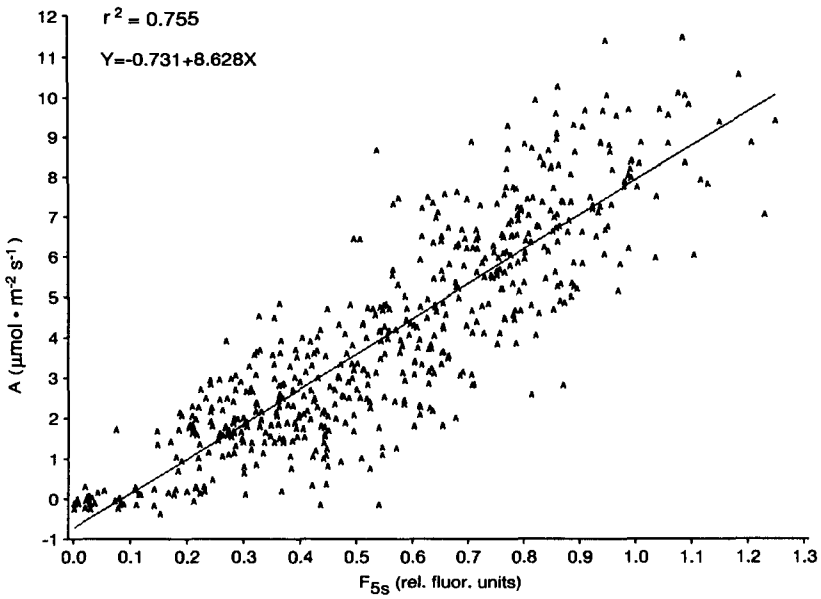

Figure 5. Regression relationship between the normalized variable chlorophyll fluorescence attribute F_{5s} and net photosynthesis (A) for control or freezing to −6, −9, −14, −18 or −24 °C in September, October, November, or December. Each point represents a seedling to seedling match of independent F_{5s} value and the dependent net photosynthesis (A) value. n = 552.

instruments and growth chambers, and without the need to estimate leaf area, which can be time-consuming for conifers.

Conclusions

This study shows that F_{var} curve attributes can be used to determine seasonal cold hardiness and freezing injury in white spruce seedlings. On an operational basis, this technology could also be used to distinguish between genetically different seedlings with regard to their latitude of origin and thus has application in genetic screening for cold hardiness and freezing stress resistance (see also Vidaver et al. 1989; Lindgren and Hällgren 1993; Devisscher and Malek 1993). Changes in F_{var} curve attributes after freezing stress were related to changes in photosynthesis, electrical conductivity, and visible needle damage. Visible needle damage due to freezing was best predicted in September by F_{var} variables F_o/I_{ABS}, F_p, and F_v/F_m. The regression fit between visible needle damage and these curve attributes and was high using a logistic fit (r^2 = 0.94, 0.98 and 0.99 respectively), and to a lesser extent F_t, using a power regression (r^2 = 0.86). The logistic curve did not fit the F_t data. Because −24 °C was the minimum freezing temperature used, we could not

fit a logistic, or power function between the fluorescence attributes and visible needle damage in October, November and December because there was little or no visible damage to seedling needles. The fluorescence attribute F_{5s} could be used to estimate ($r^2 = 0.76$) photosynthesis potential of individual white spruce seedlings after freezing damage.

We believe that variable chlorophyll fluorescence is a more sensitive measure to detect freezing stress than either visible needle damage or ion conductivity. We suggest, also, that F_{var} has greater practical application to detect freezing damage than ion conductivity because F_{var} measurements can be made non-destructively immediately after thawing seedlings, and require less time and less labour. The F_{var} attributes suggested for use here can be obtained in approximately 1.5 minutes so thirty seedlings could easily be measured in one hour.

We suggest that F_{var} curve feature characteristics can be used to determine provenance, family, and perhaps even clonal differences, as well as nursery culturally-induced changes in frost tolerance and other imposed stresses in white spruce, and probably other conifers as well. Further, F_{var} could have application in physiological stock quality assessment, for example, in the assessment of nursery culturally-induced changes in cold stress resistance, or determining recovery potential after a freezing damage event.

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