Variable Chlorophyll *a* Fluorescence as a Potential Indicator of Black Spruce Seedling Freezing Tolerance Under Nursery Conditions

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Autumnal developmental decline in photosynthetic efficiency as measured by chlorophyll a *variable fluorescence (Fv/Fm) was not found to correlate well with the freezing tolerance of black spruce* (Picea mariana *Mill. B.S.P.) seedlings produced in nursery greenhouses. A late-seeded (so-called extended) greenhouse crop maintained under a warmer temperature regime retained the potential for efficient photosynthesis, yet was tolerant of freezing to -30ºC. Variable chlorophyll* a *fluorescence can, however, serve as a quick and convenient measure of a degree of damage suffered by seedlings following a -30ºC freezing test. This measurement may complement the more laborious index of injury determination in current use.* Tree Planters' Notes 46(2):107-111; 1995.

Tree nursery seedlings in northwest Ontario suffered significant losses to freezing damage during outdoor overwinter storage in 1988 and 1989. Since then, storage in large-scale freezers at -2ºC has been implemented by many growers. Based on early experience with longterm storage of bareroot stock at 0 to -2ºC, no problems with this approach were anticipated. An understanding of the physiology of the seedling dormancy cycle is fundamental to any operational nursery overwintering regime. In this study, we use commercialy grown black spruce *(Picea mariana* Mill. B.S.P.) seedlings to evaluate dormancy in terms of (a) freezing tolerance (index of injury, defined below) and (b) photosynthetic efficiency measured by chlorophyll *a* variable fluorescence (Fv/Fm, defined below). In doing so, we evaluate the reliability and utility of the fluorescence method to determine the dormancy status of greenhouse-grown seedlings.

Much of our understanding of the dormancy and freezing tolerance cycles in woody plants discussed by Weiser in 1970 remains unchanged today. While the basic model described below still applies, various tree species and ecotypes differ in timing and morphological detail. The first stage of low temperature acclimation results in morphological changes (for example, bud set), depends on active photosynthesis and other metabolism, and is generally initiated by shortening daylength. The second stage, of increasing physiological dormancy measured as increasing freezing tolerance, is induced by near-freezing temperatures and is characterized by metabolic adjustments in no-longer-growing tissues. The end result is a tolerance of desiccation, as well as the ability to tolerate sub-freezing temperatures (Mazur and others 1972). Commercially grown seedlings of black spruce belong to a group of conifers capable of tolerating freezing temperatures to -70ºC (Sakai 1983). From a practical perspective, seedling physiological dormancy, that is, freezing tolerance, is generally tested, at most, down to -40ºC and the basic freezing tolerance onset characteristics have been confirmed by the extensive work of Colombo and associates (Colombo and others 1989, Colombo 1990).

One aspect of seedling physiology that changes during the onset of dormancy is the capacity for and the efficiency of photosynthesis. The latter parameter is readily measured using chlorophyll *a* variable fluorescence, that is, the ability to release excess photosynthetic energy as light. This fluorescent (longer wavelength) light arises from dark-adapted needles due to a temporary overexcitation of the photosynthetic apparatus with saturating light flash. Capacity for this fluorescence emission has been shown to correlate well with photosynthetic efficiency (the ratio between amount of light energy and the amount of photosynthetic product, usually oxygen). By coincidence, the photosynthetic efficiency values of active and therefore efficient photosynthesis (0.7 to 0.8) coincide with a commonly used measure of fluorescence, the ratio of variable to maximal fluorescence (Fv/Fm). These ratios also range around 0.7 to 0.8 for plants capable of normal photosynthesis and decrease to 0, as photosynthetic efficiency decreases. This decrease may be due to either damage to, or disassembly of, the photosynthetic apparatus as dormancy develops.

Commercially available fluorometers measure and display the Fv/Fm ratio directly and their manuals explain how the parameters are calculated (Sivak and Walker 1985, Lichtenthaler 1988). The relationship between tree seedling dormancy and chlorophyll *a* variable fluorescence (photosynthetic efficiency) was demonstrated for Douglas-fir by Hawkins and Lister (1985) and white spruce by Vidaver and others (1989). The operational use of chlorophyll *a* variable fluorescence is gaining gradual acceptance in British Columbia (Vidaver and others 1991). Some nursery applications and potential pitfalls are discussed by these authors. Fluorometer applications in seedling production are also under active investigation by Binder (British Columbia Ministry of Forests, Victoria, BC, Canada) and Mohammed (Ontario Forest Research Institute, Sault Ste. Marie, Ontario, Canada).

This report discusses research on variable chlorophyll *a* fluorescence as a suitable and quick measure of seedling freezing tolerance under nursery conditions, firstly by measuring decline in fluorescence (decline in Fv/Fm) with the onset of dormancy, and secondly by measuring damage sustained following a freezing test which exceeds the freezing tolerance of the seedlings. The damage sustained in these treatments was also measured by determining the leakage of ions from the tissue and the resulting conductivity of the solution in which the tissue is bathed. The electrolyte leakage-based index of injury test (Flint and others 1967, Colombo 1990) is in current standard use in Ontario. Dormancy-related developmental changes reflected in the fluorescence data were compared to this index of injury.

Materials and Methods

Seedling production and crop characteristics. Black spruce seeds collected from northwestern Ontario, stock # 3425003, were sown in vent block trays (Beaver Plastics, Edmonton, AB) at Hills Nursery, Murillo, and grown to shipping size in 1991 and 1992. In the fall of 1991, two crops were studied. One, the so-called current or regular crop, was sown in March and kept outside in shaded cold frames during the period of the study. Maximum and minimum temperatures are shown in figure 1 (solid triangles). The second, "extended crop," which effectively doubles the production capacity of an individual nursery, was sown in June 1991 and maintained in a heated greenhouse well into the autumn (figure 1, open triangles), with the windows opened and heating shut off the week of October 19 (figure 1, open triangles) to induce bud set and freezing tolerance (temperatures in the ventilated greenhouse were comparable to those in the cold frame during this time period, figure 1). Operational harvesting and packaging for frozen storage took place in the week of December 2, after which time a few trays from each crop were placed in a warm greenhouse at natural short photoperiod (4 to 14 ºC, figure la) to induce flushing. The stored crop was not investigated further. Similarly grown and treated seedlings were studied in 1992-1993. Measurements of fluorescence as Fv/Fm and sampling of shoots for the index of injury test were done every 1 or 2 weeks on the dates indicated in figure 2.

Chlorophyll *a* variable fluorescence. The Plant Stress Meter (BioMonitor, Sweden) was used to measure fluorescence. The fluorescence parameter Fv/Fm (10 readings were taken per treatment on randomly selected seedlings from several locations in the greenhouse) was measured weekly in 1991, at three separate times, early morning, mid morning and afternoon to determine any variability. As most stable readings were obtained at 8 to 9 am (data not shown), all readings in 1992 were taken at this time of day. All samples were dark-adapted for 15 minutes in a clamp supplied by the manufacturer and subsequently exposed to a blue excitation beam of 300 µmol/m^2/s carried by a fiber-optic cable. Red fluorescent light in turn is carried back through the cable for measurement by the photomultiplier and display of values calculated by the instrument.

Determination of shoot frost hardiness. Shoot frost hardiness was determined using the index of injury electrical conductivity technique (Flint and others 1967, Colombo and others 1984), which quantifies leakage of electrolytes (EC = electrical conductivity) known to be proportional to freezing-induced damage (and compared to total leakage from boiled samples) to seedling tissues as described in this paragraph. The terminal 3 cm of the main shoot of 16 randomly selected seedlings were used in quadruplicate. Following 16-hr control leaching (at which time "EC control" was measured), shoot tips were frozen at a rate of 2 °C/hr to -30°C. Prior to returning samples into the pre-freezing leachates, chlorophyll *a* fluorescence was measured. After further 16 hrs of incubation, the electrical conductivity of the water was measured to determine "EC frozen." Then the test tubes containing water and shoot tips were capped and placed in 90°C oven for 4 hrs to kill the tissue and allow complete leakage to occur in subsequent 16 hours ("EC heat-killed"). Index of injury (I_t) was calculated in % as:

$$I_t \text{ (in \%)} = (\text{EC frozen} - \text{EC control}) / (\text{EC heat-killed} - \text{EC control}) \times 100$$

Low percentage values approaching 0 indicate decreasing damage, that is, a high degree of physiological dormancy and freezing tolerance. A value of 10% or less

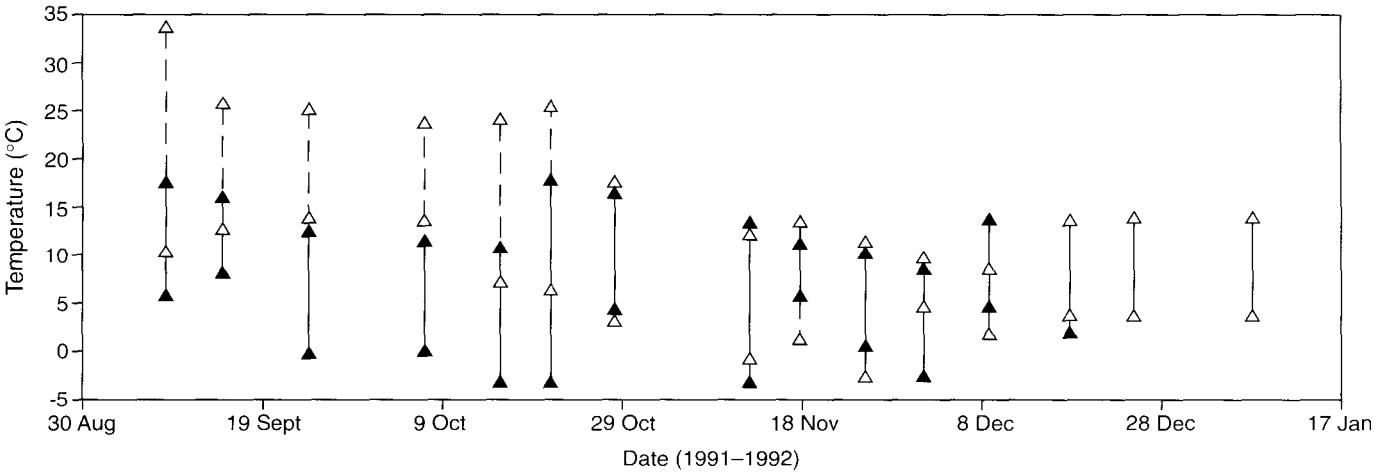

Figure 1—*Maximum and minimum air temperatures for the current crop (***solid triangles***) and extended crop (***open triangles***) in 1991–1992.*

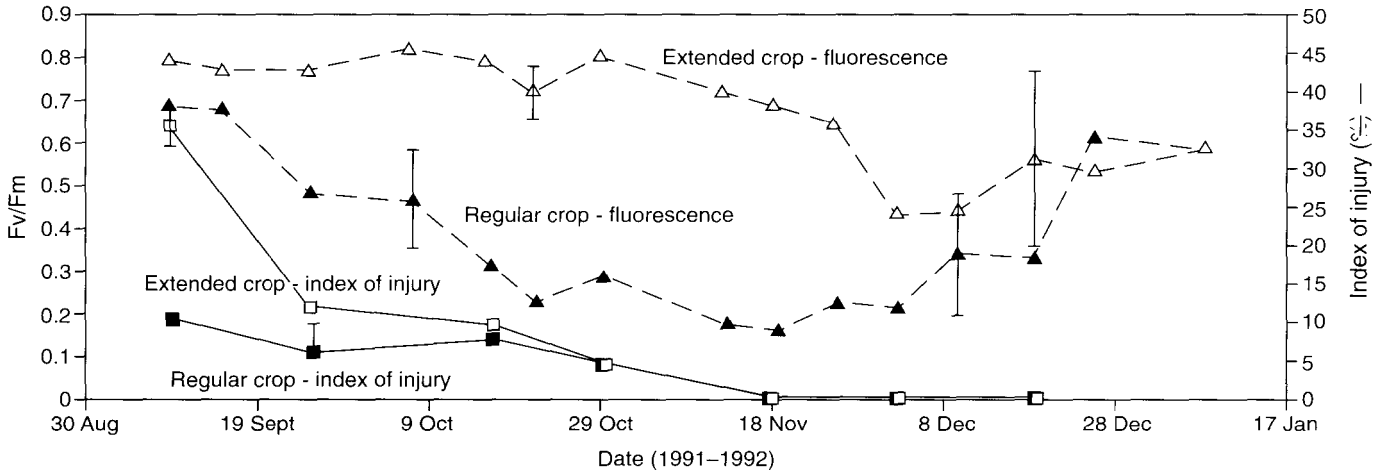

Figure 2— *Seasonal changes (1991–1992) in variable chlorophyll a fluorescence (Fv/Fm,* **dashed lines, triangles***) and freezing tolerance index of injury,* **solid lines, squares***). Current crop (***solid symbols***) and extended crop (***open symbols***). Averages of at least 10 measurements ± SE. For clarity, only the largest error bars for each treatment are presented.*

has been selected as acceptable level of freezing tolerance for seedlings overwintered out of doors.

The fluorescence data following freezing treatments (figure 3) are not presented as a simple Fv/Fm ratio (as described in the Introduction and Section 2), because this parameter changed (declined) in the course of the study with developmental time (onset of dormancy) of seedlings (see figure 2). Instead, a difference is reported (designated as Greek letter delta,)) between the final (after freezing test) Fv/Fm and initial (prior to freezing test) Fv/Fm for measurements made on a particular date. Negative values for extended crop) Fv/Fm in September indicate damage to the photosynthetic apparatus, that is, difference between readings of 0 in damaged seedlings after freezing test and 0.6 to 0.7 initially. Values for extended crop) Fv/Fm near 0 in late November result from the small difference between prefreezing values of dormant seedlings near 0 and postfreezing values in undamaged dormant seedlings still giving readings near 0.

Results

Two major findings emerged from this study. Firstly, decreasing photosynthetic efficiency (measured as declining Fv/Fm) and increasing freezing tolerance (measured as declining index of injury) changed simultaneously in the current crop (figure 2, solid symbols), although relatively high degree of freezing tolerance was measured in this crop in early September. In contrast, the extended crop that was grown in greenhouses under natural photoperiod but at elevated temperatures until late October (figure 1, open triangles), does not

show this parallel between increased freezing tolerance (rapid decrease in the index of injury during September, figure 2, open squares) and photosynthetic efficiency (Fv/Fm values remaining high at above 0.8 until late November, figure 2, open triangles). A decline in photosynthetic efficiency of the extended crop (figure 2) took place only when open greenhouse minimum temperatures started dropping below 0°C in early December, figure 1, open triangles). This was about 2 months after freezing tolerance (index of injury = 10%) was established. The experiments described above were repeated again in 1992/3 with similar results (not shown). In both years, multiple regression statistical analysis did not detect a positive correlation between the index of injury measurements and Fv/Fm measurements in the extended crop.

The second finding of this study pertains to figure 3, which describes the ability of the photosynthetic efficiency measurement (Fv/Fm) to detect freezing damage to seedlings which are not freezing tolerant, that is, the extended crop in October. The) Fv/Fm measurements for the extended crop in October detect and illustrate the damage suffered by the needles following the freezing treatment to -30 /C (figure 3, open triangles). These values (about -0.5) Fv/Fm) result from the difference between the high (0.7 to 0.8 Fv/Fm) measurements before the freezing treatment, and the low values (0 to 0.3 Fv/Fm) detected after thawing from freezing at -30 °C, that is, after damage sustained by the photosynthetic apparatus. As the extended crop increased its freezing tolerance through November, its pre-freezing test Fv/Fm values decreased gradually to about 0.4 due to dormancy onset and the post freezing test measurements gave values near 0.2, resulting in) Fv/Fm of about - 0.2 through late November and December (open triangles, figure 3). In contrast, the current crop had) Fv/Fm values near 0 throughout (dark triangles, figure 3), simply because the pre-freezing test values of 0.2 to 0.3 are subtracted from similar values after the -30 /C freezing treatment, indicating that the freezing tolerant current crop seedlings did not undergo a change in the Fv/Fm due to damage by freezing. These experiments were repeated in the 1992-1993 season with essentially the same results (data not shown).

Discussion

The lack of correlation between freezing tolerance and photosynthetic efficiency measured as Fv/Fm during the onset of dormancy in the extended crop appears to contradict suggestions in the literature, that variable chlorophyll *a* fluorescence may be a simple and fast indicator of seedling dormancy and freezing tolerance. Such a correlation clearly exists for the current crop (figure 2) and may exist for white spruce and Douglasfir seedlings produced outside, under natural conditions of a relatively mild coastal climate (Hawkins and Lister 1985, Vidaver and others 1989). The artificially extended greenhouse crop conditions in northwestern Ontario modify the onset of physiological seedling dormancy so as to make the fluorescence measurements of doubtful value as a measure of freezing tolerance. On the cellular level, the Fv/Fm parameter measures photosynthetic activity in the chloroplast and the index of injury measures the ability of the cytoplasm and the cell membrane to withstand low temperature and desiccation induced by extracellular ice. There is no known biological reason for the two cellular compartments to behave in a correlative fashion in respect to freezing tolerance. Öquist and Strand (1986) have shown no major changes in photosynthetic capacity of hardening Scots pine. However, a

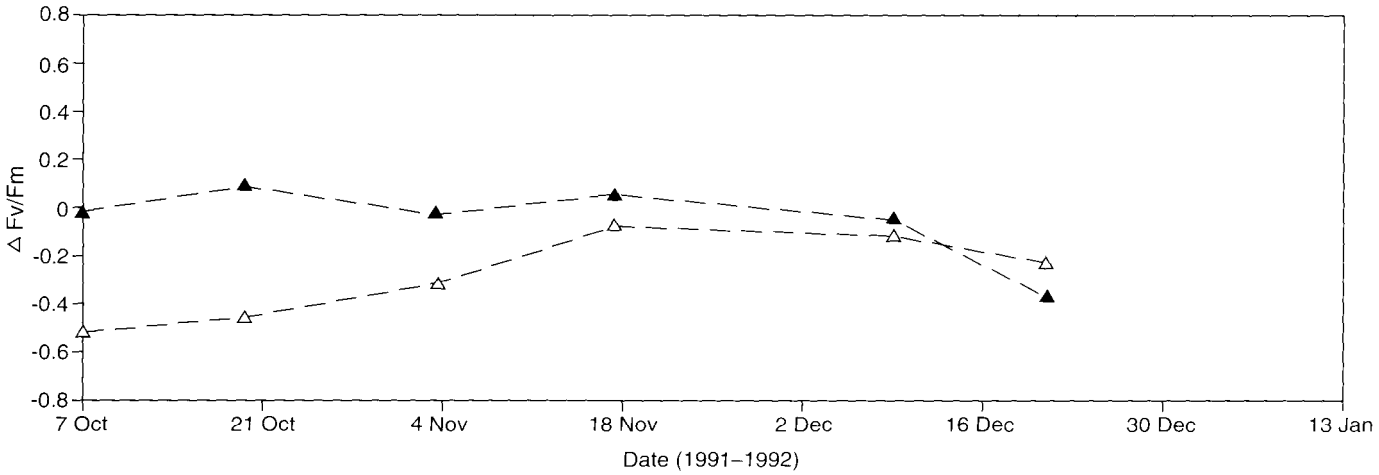

Figure 3—*Damage sustained by the 1991–1992 crops in a freezing test to –30°C as measured by the difference (Δ Fv /Fm before and after the freezing test (***dashed lines, triangles***). Current crop (***solid symbols***), extended crop (***open symbols***).*

detailed statistical analysis of chlorophyll fluorescence kinetics curves done by the same group (Sundblad and others 1990) suggested that a predictive correlation may exist between some features of the fluorescence kinetics curve and freezing tolerance in Scots pine. Our simple and readily obtained Fv/Fm measurement is not as revealing. Photosynthetic efficiency measured as Fv/Fm may be expected to decline not only due to the onset of dormancy as for the current crop in figure 2, but also due to direct damage to the photosynthetic apparatus of nondormant needles. This is the case following a damaging freezing treatment to non-hardy extended crop seedlings (figure 3). Extended crop seedlings in September had a high index of injury (20 to 40%, open squares, figure 2). Yet, these seedlings retained high photosynthetic efficiency (about 0.7, figure 2). However, following a freezing treatment at -30 °C, cell structure and photosynthetic apparatus of these seedlings was damaged, resulting in negative) Fv/Fm (as calculated above). In contrast, the photosynthetic apparatus and cell integrity of the current crop at the same time of year are already protected by physiological dormancy onset, resulting in) Fv/Fm values near 0.

A decline in Fv/Fm following damaging stress is well documented and has been used, among others, to assess frost sensitivity of wild and cultivated potato (Greaves and Wilson 1987); resistance of poplar clones to low and high temperature, as well as drought (Havaux and others 1988) and chilling tolerance of tomato hybrids (Walker and others 1990). **In this respect, variable chlorophyll *a* fluorescence will likely prove a simple, rapid method for the quantification of stress-induced damage to tree seedlings.** In the case of freezing tolerance studies, this approach does not appear to eliminate the use of expensive controlled freezing rate freezers. On the other hand, the chlorophyll *a* variable fluorescence measurements taken before a freezing test do not appear to be a reliable indication of the degree of physiological dormancy and freezing tolerance in seedlings grown under extended crop greenhouse conditions.

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References

Colombo SJ, Glerum G, Webb DP 1989. Winter hardening in first-year black spruce seedlings. Physiologia Plantarum 76: 1-9.

Colombo SJ. 1990. Bud dormancy status, frost hardiness, shoot moisture content anti readiness of black spruce container seedlings for frozen storage. Journal of the American Society of Horticultural Science 11;(2):302-307.

Flint HL, Boyce BR, Beanie DJ. 1967. Index of injury: a useful expression of freezing injury to plant tissues as determined by the electrolvtic method. Canadian Journal of Plant Science 47:229-230.

Greaves JA, Wilson JM. 1987. Assessment of the frost sensitivity of wild and cultivated potato species by chlorophyll fluorescence analysis. Potato Research 30:381-393.

Havaux M, Ernez M, Lannoye R. 1988. Tolerance of poplar (*Populus* sp.) to environmental stresses: L Comparative study of poplar clones using the in v.ivo chlorophyll fluorescence method. Acta Oecologica Oecologia Plantartnn 9:161-172.

Hawkins CDB, Lister GR. 1985. *In vivo* chlorophyll fluorescence as a possible indicator of the dormancy stage in Douglas fir seedlings. Canadian Journal of Forest Research 15:607-612.

Lichtenth aler HK. 1988. Applications of chlorophyll fluorescence. Amsterdam: Kluwer Academic Publishers: 287-297.

Mazur P, I eibo SP, Chu EHY. 1972. A two factor hypothesis of freezing injury. Experimental Cell Research 71:345-355.

Oquist C, Strand M. 1986. Effects of frost hardening on photosynthetic quantum yield, chlorophyll organization, and energy distribution between the two photosvstems in Scots pine. Canadian Journal of Forest Research 64:748-753.

Sakai A. 1983. Comparative study on freezing resistance of conifers with specialreference to cold adaptation and its evolutive aspects. Canadian Journal of Botany 61:2323-2332.

Sivak MN, Walker DA. 1985. Chlorophyll a fluorescence: can it shed light on fundamental questions in photosynthetic carbon dioxide fixation? Plant Cell Environment 8:439-448.

Sundblad LG, Sjöstrorn M, Malmberg G, Öquist G. 7990. Prediction of frost hardiness in seedlings of Scots pine *(Pinus sylvestris* using multivariate analysis of chlorophyll a fluorescence and luminescence kinetics. Canadian Journal of Forest Reserach 20:592-597.

Vidaver WE, Binder W, Brooke RC, Lister GR, Toivonen PMA. 1989. Assessment of photosynthetic activity of nursery-grown Picea glauca seedlings using an integrating fluorometer to monitor variable chlorophyll fluorescence. Canadian Journal of Forest Research 19:1478-1482.

Vidaver WE, Lister GR, Brooke RC, Binder WD. 1991. A manual fm the use of variable chlorophyll fluorescence in the assessment of the ecophysiology of conifer seedlings. For Res Dev Agreemt Rep 163. Victoria, BC: Forestry Canada and British Columbia Ministry of Forests.

Walker MA, Smith DM, Pauls KP, McKersie BD. 1990. A chlorophyll fluorescence screening test to evaluate chilling tolerance in tomato. HortScience 25(3):334339.

Weiser CJ. 1970. Cold resistance and injury in woody plants. Science 160:1269-1277.