Target Seedling Symposium

Chapter 8
State of the Art Seedling Stock Quality Tests Based on Seedling Physiology

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ABSTRACT

This chapter describes the methodology and potential application of two physiology-based seedling assessment tests under development (variable chlorophyll fluorescence and stress-induced volatile emissions); the application of two established seedling assessment tests which are in limited operational use (mitotic index and electrolyte leakage); and a short description of three other physiological assessments (tri phenyl tetrazolium chloride, days to bud break and the phytogram). The advantages and liabilities of the individual physiology-based tests are discussed.

An argument is presented for the use of an integrated battery of stock quality measures under varied environmental conditions, so that probability based predictions of future seedling performance can be generated. Finally, a variety of seedling assessment technologies are rated individually (sum and product) according to nine criteria. This procedure reinforces the hypothesis that no one test will be able to predict seedling stock quality or move forest regeneration toward the target seedling concept.

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8.1 Introduction

The 1984 Oregon State University Workshop on Evaluating Seedling Quality (Duryea 1985a) was the first major North American attempt to provide a sound basis for evaluating the various past, present, and projected methods of evaluating forest seedling stock quality. Traditionally, morphological specifications have been important grading criteria. However, we stress the theme of others (Wakely 1948, Kramer 1956, Sutton 1979, Bunting 1980, Ritchie 1984, Glerum 1985, Navratil et al. 1986, Sutton 1988, Lavender 1989, Puttonen 1989, Ritchie 1989) that, although seedling morphology is an important management standard (Sutton 1979, Puttonen 1989), it is not what the tree looks like before planting but how it performs after planting that is important to its future performance (Wakely 1948, Sutton 1979).

Morphological seedling grading for height and root collar diameter is rapid but it is unchanging, whereas stresses occurring between grading and planting significantly change physiology without altering morphological grade (Duryea 1985b). This has been aptly summed by Norris (in Duryea 1985a) that seedlings are not of equal physiological quality when planted. Physiology is critical. Its interaction with the environment and its morphological package determine the success or failure of every plantation (Wakely 1948). Seedling physiological assessments should not be used in isolation because there is no one effective method of measuring seedling vigor (Lavender 1989). Rather, they should be done in concert so that a composite of physiological evidence, as to the health of the seedling, is generated. Therefore to ensure plantation success, it is imperative that the physiological condition or vigor of forestry seedlings be monitored from sowing in the nursery through to planting at the reforestation site.

The intent of this review is to report on new and improved seedling physiological assessments with operational potential and novel methods of their integration, rather than reviewing all possible physiological tests. We include theory, methodology, and data interpretation at different levels (depending on the test' historical use in conifer regeneration) for promising operational stock quality assessments. It is hoped this information will aid operational practitioners in understanding and selecting an appropriate series of physiological-based assessments. The expansion and integration of useful stock quality assessments will promote, by definition, the management-driven target seedling stock quality concept.

8.2 Variable Chlorophyll Fluorescence (F_{VAR})

8.2.1 Background and theory

In a living plant, some of the light energy absorbed by green chloroplast pigments used to drive photosynthesis is re-emitted as long-wave infra-red light (Kautsky and Hirsch 1931a,b, Kautsky and Frank 1943). This phenomenon has been coined the Kautsky effect and abbreviated F_{VAR} (Hipkins and Baker 1986, Goedheer 1972, Bose 1982, Geacintov and Breton 1987, see Figure 8.1). The basic principles that govern the yield of fluorescence in the photosynthetic system of plants is complex and have been reviewed elsewhere (Butler 1977, Krause and Weis 1984, Briantais et al. 1986, Krause and Weis 1988). In general, the red light emitted from the plant chloroplast thylakoid membrane reflects the primary processes of photosynthesis including light absorption, excitation ener-

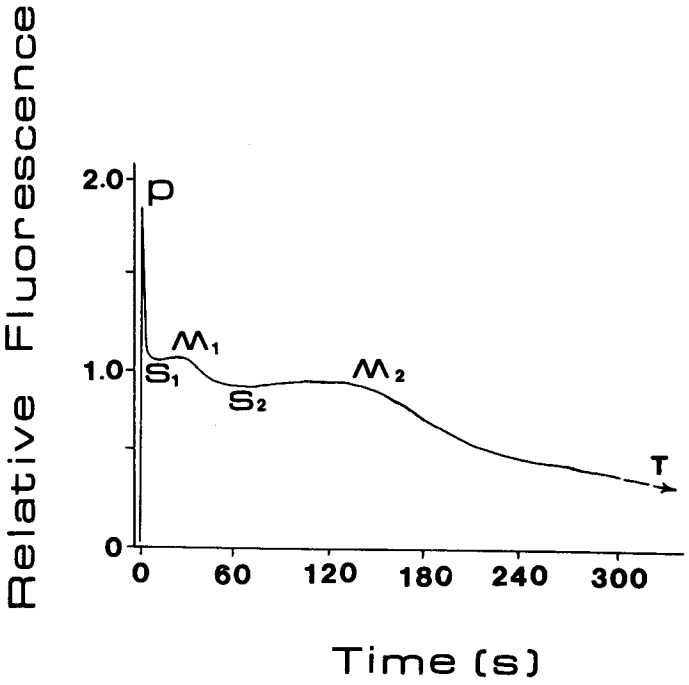

Figure 8.1—*Generalized F_{VAR} curve from 0 to 5 minutes of a typical Douglas-fir seedling. Fast-rise from 'O' to 'P': 'O' to 'I', rapid reduction of Q_A associated with water-splitting charge accumulation; 'I' to 'D', oxidation of Q_A as Q_B is reduced; 'D' to 'P', Q_A and the plastoquinone pool are highly reduced and the photochemical quenching of fluorescence approaches zero (Papegeorgiou 1975, Bothar-Nordenkampf et al. 1989). Slow change decline from 'P' through S_1: re-oxidation of Q by electron transport as CO_2 reduction to carbohydrate level activity increases. Rate through Mll interaction between build up of membrane proton potential, rate of non-cyclic electron flow ATP synthesis, and CO_2 reduction. S_2 and M_2: slow equilibrium. M_2 to T: attainment of steady state equilibrium between proton gradient, ATP synthesis and CO_2 reduction to carbohydrate. After Vidaver etal. (7990).*

gy transfer, and the photochemical reactions in photosystern II (Schreiber and Vidaver 1976, Schreiber et al. 1976, Holzwarth 1988, Krause and Weis 1988, Walker 1988, Vanselow et al. 1989ab). The basis of the chlorophyll fluorescence assessment is that the FVAR response, which is an indicator of the plant's photochemical activity, varies with plant species, season of the year, changes in environmental conditions, previous history of the sample and other factors which may have physiological effects (Vidaver et al. 1990).

In recent years, the measurement of fluorescence has been increasingly applied to various fields in plant physiology. Pertinent reviews on the subject include that of Papageorgiou (1975), Schreiber (1983), Krause and Weis (1984), and Lichtenthaler and Rinderle (1988b). Fluorescence has been studied in relation to chilling injury (Hetherington and Öquist 1988), as a screening method for cold tolerance (Schapendink et al. 1989, Serrano et al. 1988), in relation to the effects of different water regimes (Mugnozza et al. 1988), for detection of stress conditions in plants (Lichtenthaler and Rinderle 1988b, Lichtenthaler 1988b), and in ecophysiological investigations (Lichtenthaler et al. 1986). The first monograph devoted to the applications of fluorescence was published in 1988 (Lichtenthaler 1988a).

With reference to conifers, fluorescence has been studied in relation to seasonal variations in photosynthetic activity (Lichtenthaler et al. 1988, Vidaver et al. 1988, 1989, 1990, Brooke et al. 1989), stress evaluation (Toivonen 1985, Bothar-Nordenkampf and Lechner 1988), forest decline (Lichtenthaler and Rinderle 1988a), frost hardening and dehardening (Strand and Öquist 1988a, Öquist and Malmberg 1989), water stress (Toivonen and Vidaver 1988), nutrient deficiency (Baillon et al. 1988), light stress (Öquist and Malmberg 1989), and provenance differences (Vidaver et al. 1989, 1990).

8.2.1.1 Instrumentation

A portable probe for *in vivo* detection of plant chlorophyll a fluorescence has been commercially available since 1979 (Richard Branker Research Ltd., Ottawa, Ontario). Other units using fiberoptics (Schreiber 1983) with microprocessor (Öquist and Wass 1988) or laser-equipped portable field systems (Lichtenthaler and Rinderle 1988a) have also been described. These include the PSM (BioMonitor), MFMS (Hansetech Limited) and PAM 100 (Heinz Walz). All fluorometers have their unique assets and liabilities. These systems are all useful in determining the state of the plant's photosynthetic membrane. However, they cannot assess large samples or entire seedlings and therefore have low utility in conifer applications. Toivonen and Vidaver (1984) constructed a fluorometer to make F_{VAR} measurements on whole conifer seedlings.

The system constructed by Toivonen and Vidaver (1984) uniquely incorporates an integrating sphere, light source, photographic shutter, optical fibers, and a photodetector. These components are arranged in an appropriate housing and interfaced to a microcomputer which triggers the shutter opening and acquires and stores the fluorescence emission data at onset of shutter opening (Figure 8.2).

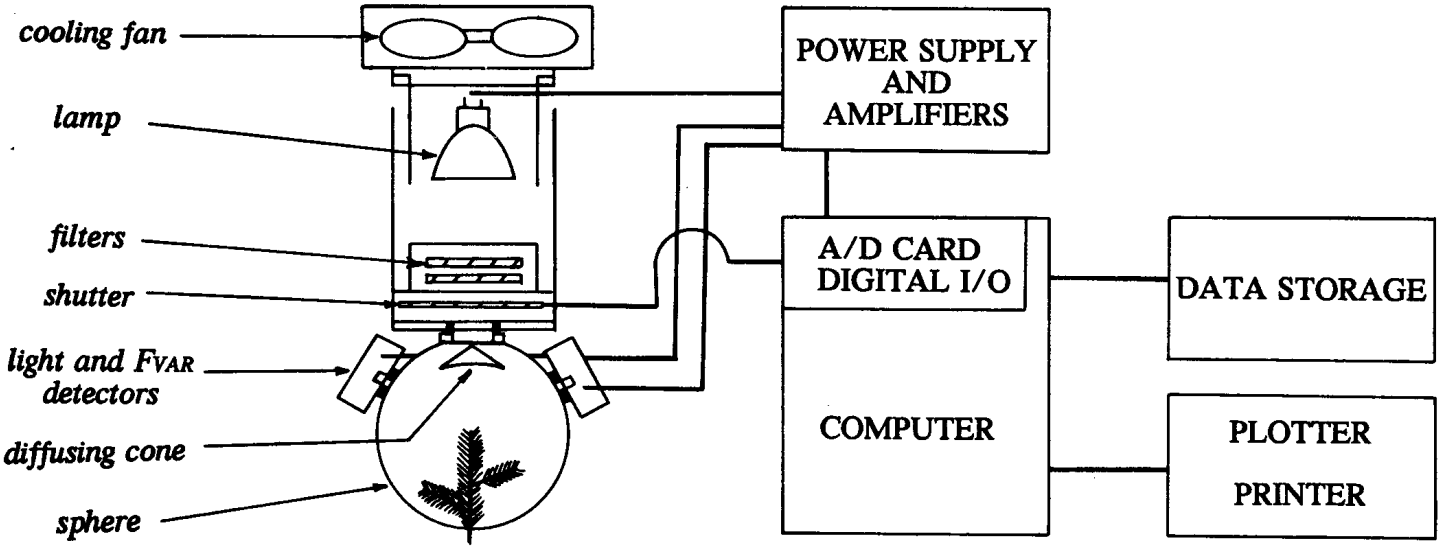

Figure 8.2—*Diagram of the integrating fluorometer showing major mechanical and electrical components for detecting, converting, and storing fluorescence events. From Vidaver et al. (1990); with permission.*

Chlorophyll fluorescence emission from the plant has a peak wavelength of approximately 680-685 nm with a secondary shoulder at 740 nm. Measurement durations can vary from milliseconds (fast change) to five minutes or longer. The data of the completed F_{VAR} time courses can be normalized. This removes the effect of sample size (fluorescence emission amplitude) when comparing data from different samples or when averaging the responses of more than one sample. In practice, any number of samples can be added. A complete description of the system and operation is given in Vidaver et al. (1990).

8.2.2 Applied data

Variable chlorophyll fluorescence induction analysis is a direct measure of the physiological status of the thylakoid membrane (photosynthesis). It can be used in conjunction with other types of physiological assessments such as electrical conductivity, root growth potential, and stress-induced volatile emissions. After placing the plant material in the dark for 20 minutes, the technique requires only a few minutes for measurement. It is also reliable, provides an immediate response, and is completely non-destructive of the sample, so the sample can be remeasured as many times as required by a trained technician or outplanted for future reference.

8.2.2.1 Stress-induced photosynthetic inactivation

Fluorescence is useful as a stress indicator (Conroy et al. 1986, Lichtenthaler 1988b). Conifers and other evergreen perennials possess an unknown mechanism which causes the reversible inactivation of photosynthesis when the needles are exposed to low temperature (Hawkins and Lister 1985) or experience water stress (Brooke et al. 1989). This mechanism apparently prevents the pigments from becoming damaged (photodamage or photoinhibition) under the above conditions, when CO_2 assimilation rates are minimal (Plaut and Bravdo 1973, Boyer 1976, Kaiser et al. 1981). Annual and deciduous plants which are easily damaged by light and chilling temperatures appear not to have this protective mechanism.

The capacity for photosynthetic inactivation enables inactive conifer needles to withstand relatively long periods of drought or subfreezing temperatures even when exposed to high light intensities. Inactivation in conifers is completed within a few hours and probably involves a change or reorganization of the chloroplast thylakoid membrane (Parker and Philpott 1963, Perry and Baldwin 1966, Kimball and Salisbury 1973, Senser et al. 1975, Senser and Beck 1977). The onset of freezing or water stress can cause damage if it occurs more rapidly than the time required for the chloroplast to become inactivated. Fluorescence time courses are distinctly different in damaged and undamaged needles. It is not difficult to distinguish between them. In inactivated but undamaged needles, F_{VAR} is absent but gradually reappears when the stress is relieved (Fink 1976, Hawkins and Lister 1985).

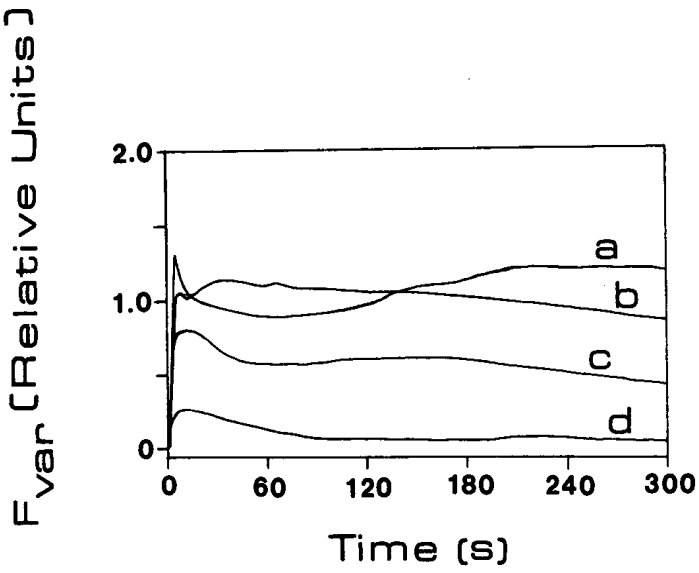

Figure 8.3—*Progression toward seasonal inactivation of F_{VAR} for 2+0 white spruce seedlings monitored during 1986 in a container nursery. Modified from Vidaver et al. (1989); with permission.*

Recovery of F_{VAR} requires adequate root function for needle rehydration, and failure of recovery can reflect root damage.

For example, data obtained on white spruce show that photosynthetic (photochemical) inactivation occurs in parallel with bud set and progression toward dormancy (Vidaver et al. 1988, 1989, 1990; Figure 8.3). This inactivation is apparently a long-term cued event induced by shortening daylength, beginning in mid-August. Daylength-dependent photochemical inactivation may therefore be an adaptation which protects against shoot photodamage from sunlight during winter (c.f. Hawkins and Lister 1985). Because of the ease of obtaining variable chlorophyll fluorescence data on whole seedlings with the integrating fluorometer and its reliability, F_{VAR} assessment could become the method of choice for determining fall nursery lifting dates for interior spruce (Vidaver et al. 1989). The sensitivity of F_{VAR} assessment is demonstrated by its ability to distinguish between provenance types. Seedlings from more northerly seedlots begin inactivation at an earlier time than more southerly seed lots even though they are grown at the same nursery under, as near as possible, identical conditions (Vidaver et al. 1989). The ability to distinguish between provenance types could be useful not only to nursery growers but could also be of great value in coniferous tree improvement and genetic studies.

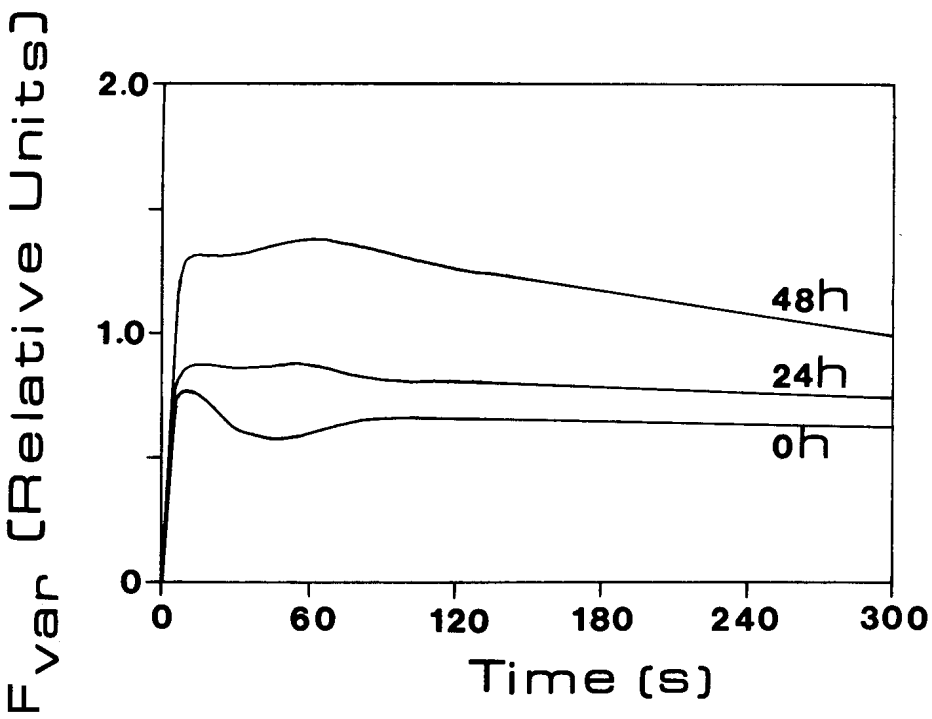

Figure *8.4*—*Recovery of F_{VAR} activity in 2+0 white spruce seedlings upon removal from cold dark storage. The F_{VAR} response at 48h is near normal and indicates efficient root function. From Vidaver et al. (1989); with permission.*

8.2.2.2 FVAR and other physiological responses

Since the high quality of seedlings is a critical factor to plantation success, reliable assessments of seedling quailty are badly needed. Present tests, such as Root Growth Potential or Capacity (RGP or RGC), tend to be time consuming and controversial (Burdett 1987, Binder et al. 1988, Landis and Skakel 1988). Fluorescence data indicate the technique has the potential to become an extremely valuable tool for determining post-storage seedling quality. For example, seedlings that have high RGP values and optimum field performance in early farmfield tests show near pre-storage levels of F_{VAR} within 24 hours of removal from cold storage and complete recovery within 48 hours (Vidaver et al. 1989, Figure 8.4). Slower or incomplete recovery appears to indicate that the seedlings are physiologically impaired. After cold storage, white spruce seedlings were assessed for photosynthetic capacity (B.C. Ministry of Forests, Research Branch EP 737, Victoria, British Columbia). There was little. Within an hour of severing the roots and providing the shoot with ample water (base of shoot cut under water and immersed in a vial of water), photosynthetic capacity increased tenfold to about 5 mg $CO_2h^{-1}g^{-1}$ (W.D. Binder and G.R. Lister, unpublished results). This is evidence that in some cases poor root function may be the cause of incomplete photosynthetic recovery.

In addition to the long-term daylength-dependent photochemical inactivation observed in spruce species, seedlings of all conifer species tested have shown that photosynthetic activity can be influenced by environmental changes (Vidaver et al. 1988, 1989, Brooke et al. 1989). Both exposure to water deficits, especially at high summer temperatures, and exposure to low temperatures will both induce inactivation. The extent of inactivation and subsequent recovery is dependent upon severity and duration of exposure (Brooke et al. 1989). In the case of spruce, the short-term inactivation was superimposable on the long-term daylength-dependent inactivation. Fluorescence monitoring of a crop could, therefore, warn when remedial measures should be taken to protect against the physiological stress which causes reduced growth potential and decreased seedling vigor.

Daylength-dependent fall inactivation has been observed in white and Engelmann spruce, but not in coastal Douglas-fir or any of the pines so far examined (Vidaver et al. 1990). In coastal Douglas-fir, in response to low temperature, provenance type elevational differences have been observed using F_{VAR} analysis (Brooke et al. 1989, Vidaver et al. 1989). Fluorescence inactivation was induced at lower temperatures on high elevation seedlings than on low elevation seedlings growing under the same conditions at the same nursery site. Such results provide evidence F_{VAR} could be useful in tree improvement as well as in nursery operations.

8.2.3 Test potential (pros and cons)

Variable chlorophyll fluorescence data, obtained to date, strongly indicate that such analyses could be highly useful to the conifer seedling industry. There is strong evidence in the literature suggesting that F_{VAR} measurements serve to indicate the physiological condition of conifer seedling shoots and roots, the status of chlorophyll, and other parts

of the photosynthetic apparatus necessary for carbon assimilation. In the shoot, such information is useful to growth and dry matter production. In the roots, it reflects the ability to provide water and nutrients to the needles, thereby optimizing conditions for photosynthetic processes.

Potential uses of F_{VAR} include:

1) determination of winter lifting window for white spruce and probably Engelmann spruce;
2) assessing post-cold storage seedling vigor in white spruce and probably other conifer species;
3) monitoring effects of environmental factors on photochemical activities (i.e., stress) in most, if not all, conifer species; and
4) in all conifer species, detection of provenance photochemical differences.

To date, the most reliable F_{VAR} results have been observed in species which are strongly cued by photopenod, such as the white and Engelmann spruces. Typically, "P" values greater than 1 .0 indicate high photosynthetic capacity while "P" values less than 0.25 indicate photosynthetic inactivation. The seasonal data interpretations are not as clear cut for temperature-cued species, such as Douglas-fir, western redcedar and hemlock. Their "P," values in the fall fluctuate, using the apparatus described by Toivonen and Vidaver (1984), between 0.5 and 1 .0 depending on the environment. The paucity of F_{VAR} data on these species must be overcome prior to F_{VAR} becoming a high utility, stock quality assessment tool. It should be noted that, while understanding of much of the physiological basis and scientific principles which underlie the changes in F_{VAR} are becoming clearer, more work is required before the technique can be readily extended to any wide variety of applications in operational forestry situations, either alone or in conjunction with other assessments.

8.3 Stress-Induced Volatile Emissions (SIVE)

8.3.1 Background and theory

Most reforestation workers are familiar with the sweet odor emitted when a box of "not so good" seedlings is opened. The stress-induced volatile emissions (SIVE) test takes the "smell" several steps further and quantifies the odor. The test is based on low molecular weight hydrocarbons given off in response to stress events by conifer seedlings (Drakeford and Hawkins 1989, Hawkins and DeYoe 1990). The SIVE work is based, in part, on recent stress physiology research on *Pinus resinosa* and *Betula papyifera* (Kimmerer and Kozlowski 1982). Woody plants produce the gases ethylene, ethane, acetaldehyde and ethanol, among others, in response to stresses such as air pollutants (NO_X, 0_3, SO_2), water deficits, and freezing (Kimmerer and Kozlowski 1982). The amplitude of gas production is a function of the stress intensity.

The idea of using volatile gas production to determine levels of seedling stress and injury is not original. Ethylene, a gaseous plant growth regulator, is an integral part of the seedling's stress response mechanism (Abeles and Abeles 1972, Jaffe and Telewiski 1984). The gas ethane is a sensitive indicator of cell injury, and more specifically membrane breakdown (Riely and Cohen 1974, Chia et al. 1984, Johnson and Gagnon 1988). The two gases have also been used in concert to describe stress and injury (Elstner and Konze 1976, Konze and Elstner 1978, Kobayashi et al. 1981). Ethanol and its biochemical precursor, acetaldehyde, have also been investigated as a stress response pair (Kimmerer and MacDonald 1987). All four gases have been used simultaneously to assess stress events in pine and birch (Kimmerer and Kozlowski 1982).

Of the four documented stress response gases, it appears that ethanol and acetaldehyde are the best for rapid screening of stress resistance or quality of plant tissue (Kimmerer 1987, Kimmerer and MacDonald 1987, Hawkins and DeYoe 1990). If a test is developed using a single gas, ethanol would be the gas of choice because it is produced continually in woody plants (Kimmerer and Stringer 1988) under both aerobic and anaerobic conditions (Kimmerer and MacDonald 1987. MacDonald et al. 1989).

SIVE testing may have two major advantages over most of the presently used tests: speed and preventive maintenance. After the incubation time of one to two hours, the results are available immediately after the gas chromatograph (GC) run—minutes rather than hours, days or weeks after testing. SIVE distinguishes between stress and injury. This would allow remedial cultural corrections to be made prior to a crop stress becoming a crop injury. Crop injuries decrease the value and performance potential of nursery seedlings.

8.3.1.1 SIVE and other stock quality assessments

Stock quality or seedling assessment is important at all phases of the regeneration continuum (Sutton 1988, Puttonen 1989, Ritchie 1989). High levels of seedling stress resistance correlate well to seedling survival and growth (Ritchie 1984ab, 1986, 1989, Glerum 1985) and can also be used to gauge when to lift and plant stock (Burdett and Simpson 1984). Functionally, cold hardiness induction or frost resistance, mechanisms separate but parallel to dormancy induction, prepares tissues to withstand stresses inherent to winter (Weiser et al. 1979, Levitt 1980). This intensifies the resistance of the seedling to a number of stresses (Levitt 1980, Lavender 1984, Ritchie 1984b, 1989, Glerum 1985), but does not preclude it from being stressed. Clearly, knowledge of the level of seedling stress resistance and a rapid means of determining it would aid in operational decision making.

Operationally, two techniques are used for assessing frost resistance (even though many techniques are available, Keates 1990): one which is qualitative and the other which is quantitative. Visual evaluation (browning) is the qualitative assessment. This assessment takes from 5 to 10 days to complete depending on the time of year. The quantitative assessment is electrical conductivity (leakage), see Section 8.5. Conductivity is either expressed as the ratio of fresh to killed or as an index of injury (Flint et al. 1967, Section 8.5.2). This assessment takes at least three days. Correlation of SIVE data to these assessments would promote its utility.

Seedlings are exposed to handling (mechanical and physical) stresses between the nursery and the planting site which can have deleterious effects on seedling performance (Tabbush 1986). An immediate means of determining the severity of such stresses has yet to be established. if mechanical and physical stresses alter volatile gas production, SIVE has the potential to be used for such assessments.

8.3.2 Applied data

Tests and their application described to this point, for the most part, have been done individually under standard defined conditions. The results from such tests provide the present health of the seedling but to date correlations allowing predictions have not been forthcoming. To move from tests which assess the past and present, to forward projections, requires that the batteries of tests be done under a range of environmental conditions. A stress test will allow the generation of a response surface for a variety of performance attributes, and from this a probability-based projection of seedling performance could be made.

This section will present individual assessment results and the results of a stress test.

8.3.2.1 SIVE and freezing stress

Starting in early August, Douglas-fir seedlings, half of which had been subjected to four weeks of nursery drought stress (Hawkins and DeYoe 1990), were transported from the nursery to the laboratory. They were subjected to five levels of temperature stress (Hawkins and DeYoe 1990).

The season can be divided into four phases in terms of frost tolerance as indicated by ethanol and acetaldehyde production and visual damage assessments (Hawkins and DeYoe 1990). The phases are late summer, early fall, late fall, and winter.

In late summer (August, September), during initiation of bud scales and filling of buds, exposure to lower temperatures resulted in unchanged or decreased ethanol production and increased acetaldehyde production (Figure 8.5). Ethanol production peaked around the LT_{25} (temperature

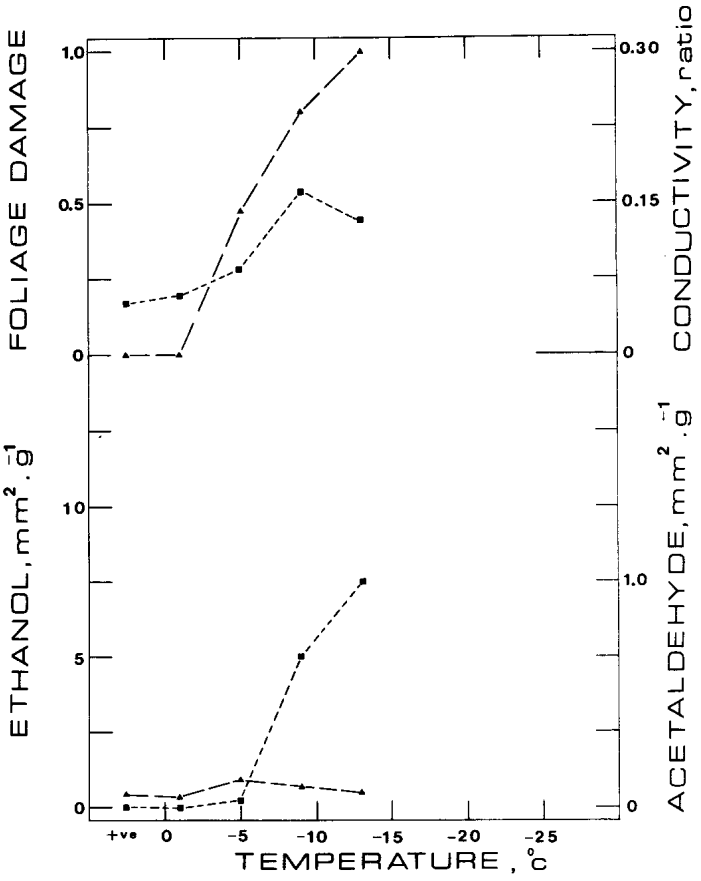

Figure 8.5—*Typical August, September pattern of foliage damage as a proportion of total foliage* (▲—▲), *conductivity damage ratio* (■—■), *ethanol* (▲–▲) *and acetaldehyde* (■—■) *production in response to decreasing temperature. Control and drought-treated stock were not separated during this time period. Modified from Hawkins and DeYoe (1990).*

resulting in damage to 25 percent of the sample) and acetaldehyde production peaked at the lowest temperature. Visual foliage damage and the electrical conductivity damage ratio both increased with decreased temperature (Figure 8.5). During this period, the LT_{25} of foliage damage ranged from about -3 to -6.5°C.

During late September and October (early fall), both ethanol and acetaldehyde production increased with decreased temperature (Figure 8.6), though ethanol production tended to plateau. The conductivity damage ratio increased with decreased temperature and the LT_{25} of foliage ranged from -4 to -6°C. Generally, nursery drought-treated stock had lower levels of damage, regardless of the variable being examined.

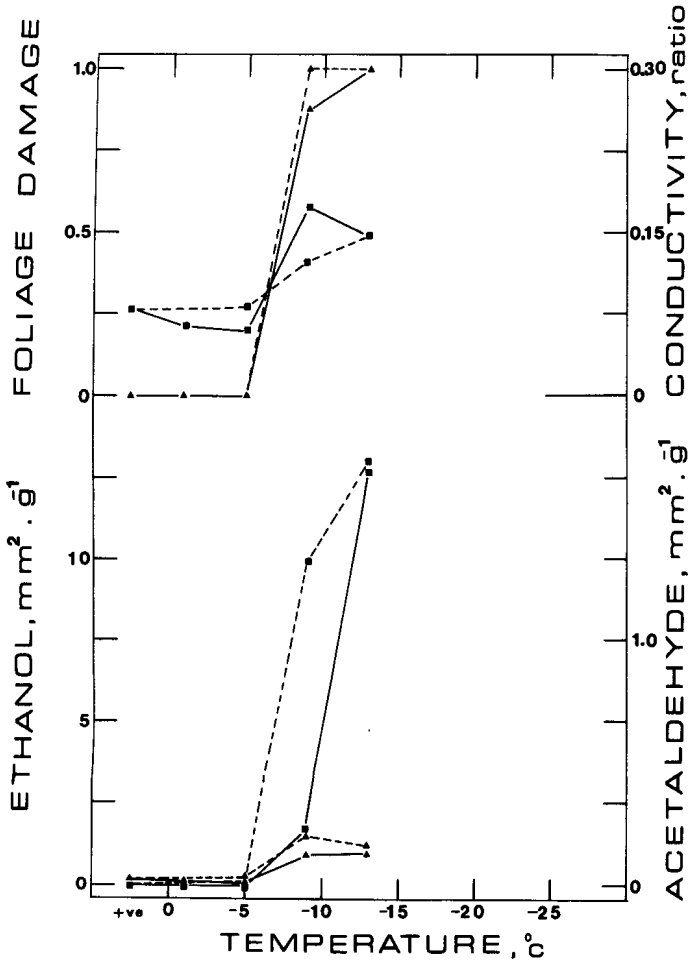

Figure 8.6--*Typical September, October pattern of foliage damage* (▲), *conductivity damage ratio* (■), *ethanol* (▲) *and acetaldehyde* (■) *production in control* (▲—▲ *or* ■—■) *and drought-* (▲—▲ *or* ■—■) *treated Douglas-fir seedlings in response to decreasing temperature. Modified from Hawkins and DeYoe (1990).*

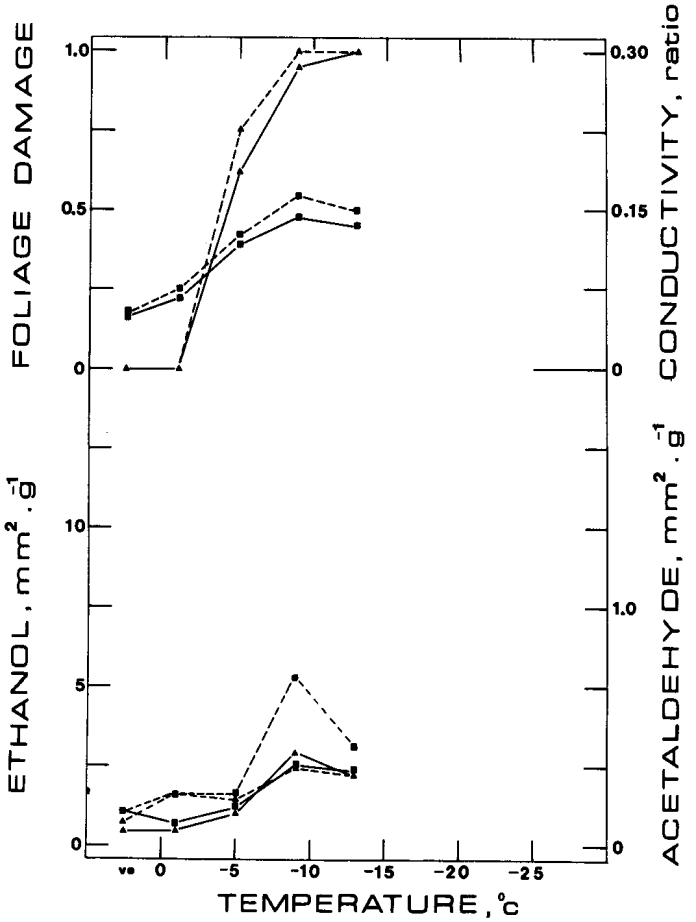

Figure *8.7—Typical October and early November pattern of foliage damage* (▲), *conductivity damage ratio* (■), *ethanol* (▲) *and acetaldehyde* (■) *production in control* (▲—▲ *or* ■—■) *and drought* (▲—▲ *or* ■—■) *treated Douglas-fir seedlings in response to decreasing temperature. Modified from Hawkins and DeYoe (1990).*

Ethanol production increased and acetaldehyde production decreased during late fall (October-early November) compared with the preceding period (Figure 8.7). Drought-treated stock generally had greater levels of damage. Interestingly, the foliage LT_{25} increased from that previously observed, and ranged from -2.5 to -4.7°C during the period. Again, the conductivity damage ratio increased with decreased temperature.

From late November through early February, maximum winter frost hardiness was achieved. Gas production and conductivity damage ratio were greatest in control stock while there was little difference between nursery treatments for foliage damage (Figure 8.8). Ethanol and acetaldehyde production increased throughout this period. The foliage LT_{25} ranged from -9 to -18°C.

Measurements were terminated when the stock was placed in storage.

Mathematical correlations have been done between gas production, visual damage and electrical conductivity damage ratio (Hawkins and DeYoe 1990). Correlations of ethanol and acetaldehyde production to the other variables were significant during the winter period. These data suggest that SIVE can be used in conjunction with and instead of slower, more established tests for the assessment of frost injury/stress resistance and for the prediction of optimum lifting windows.

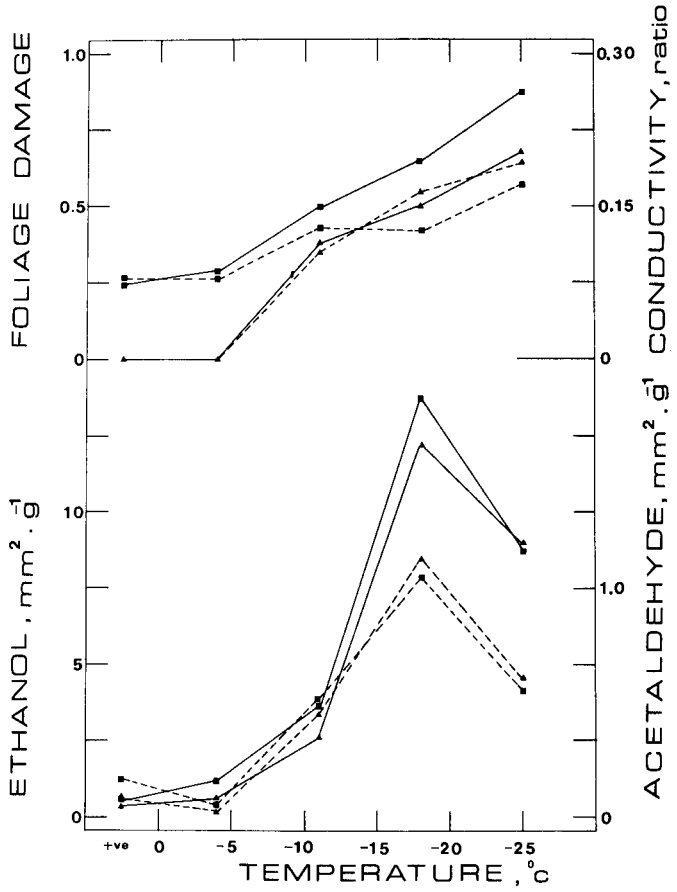

Figure 8.8--*Typical late November through early February pattern of foliage damage (▲), conductivity damage ratio (■), ethanol (▲) and acetaldehyde (■) production in control (▲—▲ or ■—■) and drought- (▲—▲ or ■—■) treated Douglas-fir seedlings in response to decreasing temperature. Modified from Hawkins and DeYoe (1990).*

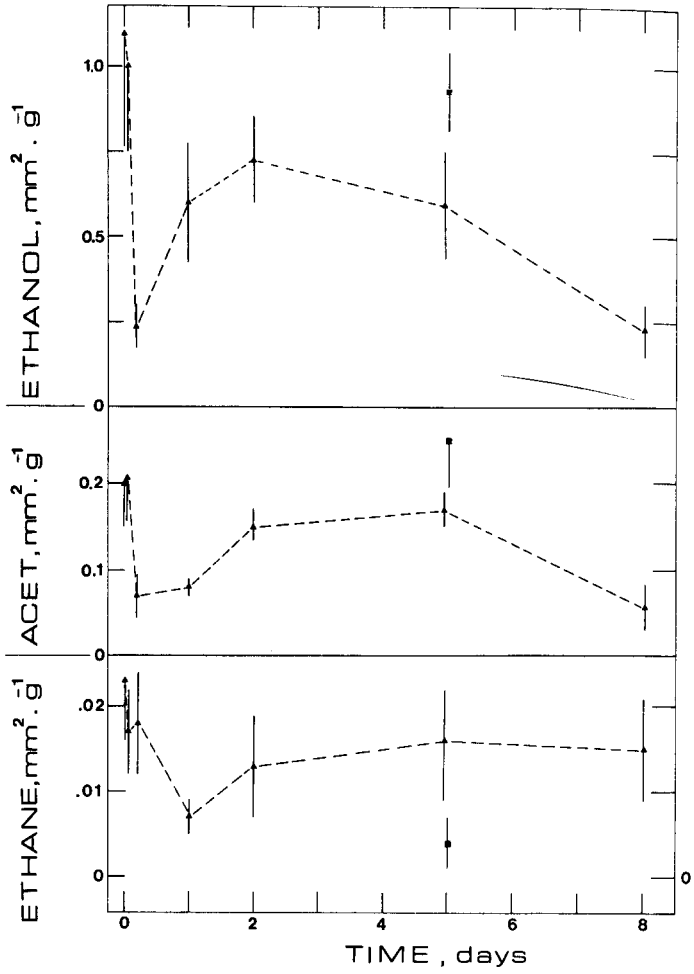

Figure 8.9—*Mean ± SE Douglas-fir seedling ethanol, acetaldehyde (ACET) and ethane production with time from sampling in the nursery through eight days in the laboratory (▲—▲). Mean ± SE gas production of seedlings subjected to physical-mechanical stress (■) on day 5 (the outlier). Modified from Hawkins and DeYoe (1990).*

8.3.2.2 SIVE and handling stress

Douglas-fir seedlings were sampled in the nursery as well as upon return of the seedlings to the laboratory for baseline ethanol production. Laboratory baseline samples were taken for several days. Seedlings were then moved within a styroblock (simulate a handling event) and left to stand for one hour prior to sampling and incubation.

Initially upon sampling and returning the seedlings from the nursery to the laboratory (Hawkins, unpublished results), gas emissions were high (Figure 8.9). A stable but gradually declining level of gas production was reached after 24 to 48 hours in the laboratory. After five days in the laboratory, a sub-sample was moved within the styroblock and this resulted in increased ethanol and acetaldehyde and decreased ethane production (Figure 8.9).

The effect of movement of the seedlings on day five was detected by SIVE. Based on this, it is hypothesized that the rapid decline in gas production observed during the first few hours in the laboratory is the recovery from the stress of sampling and transporting the seedlings to the laboratory. The observed stress response could have a short- and a long-term component. Within hours, levels of gas production have returned to what they were in the nursery. However, due to the warm, long day, low humidity laboratory conditions (as opposed to greenhouse conditions in December and January), there is a slow, one week acclimation to the laboratory conditions until low levels of gas production are again observed.

These data, though preliminary, suggest that SIVE has the sensitivity to detect low levels of physical-mechanical stress. SIVE could prove a valuable tool in screening for

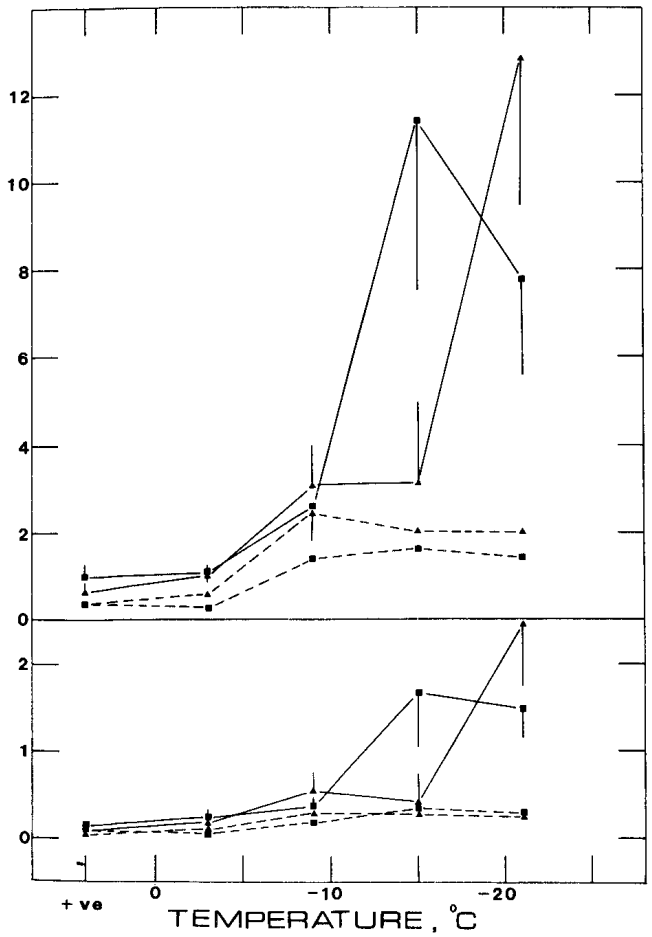

Figure 8.10—*Mean ± SE ethanol and acetaldehyde production in Douglas-fir seedlings after one (▲—▲) and two (■—■) exposures to the various temperatures. Mean (no SE because of small n) ethanol and acetaldehyde production (recovery) three (▲—▲) and five (■—■) days after exposure to the initial temperatures. Modified from Hawkins and DeYoe (1990).*

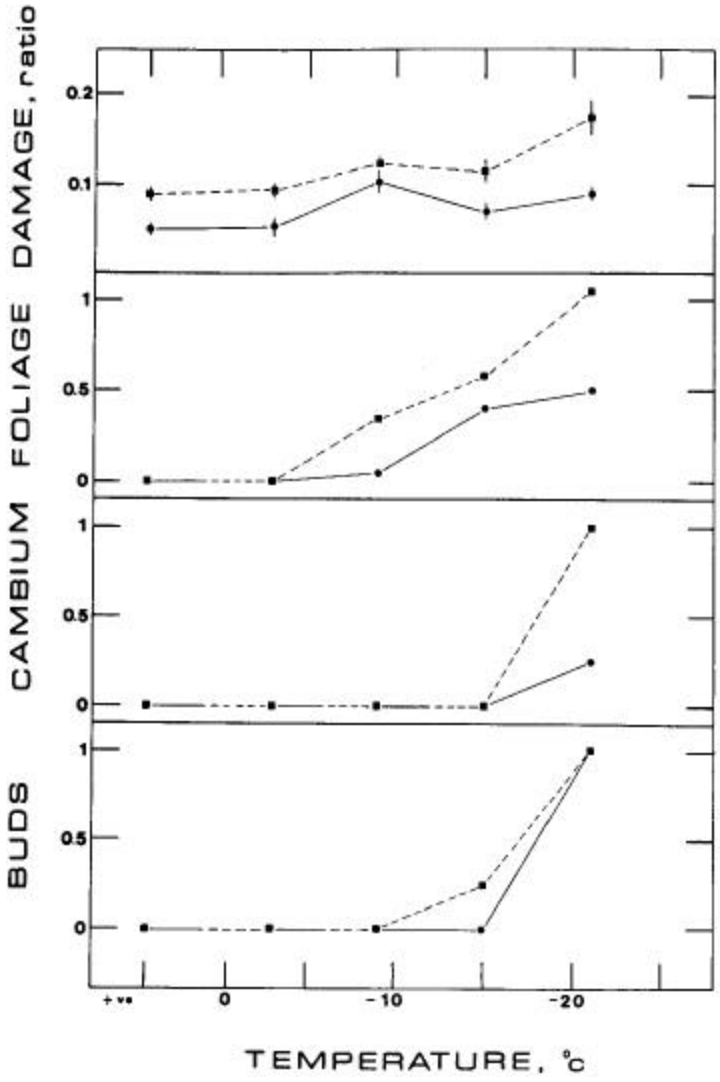

Figure 8.11—Mean ± *SE conductivity damage ratio and mean proportion of foliage, cambia and bud damage in Douglas-fir seedlings after one* **(●—●)** *and two* ***(■—■)*** *exposures to the various temperatures. Modified from Hawkins and DeYoe (1990).*

stress events between the nursery and the planting site once species' baseline values are established.

8.3.2.3 SIVE as a "stress" test

Douglas-fir seedlings were brought from the nursery to the laboratory, placed in the freezer, exposed to five temperatures, and subsamples at each temperature assessed for SIVE, EC, and visual damage after freezing. The remaining stock from each temperature exposure was placed under ambient climatic conditions, the temperature range being o to 9°C. Seventy-two hours after the initial temperature exposure, recovery gas production was determined. Two days later, seedlings from each test temperature were assessed for further recovery. The next day, the remaining seedlings from each temperature were placed in the freezer and re-exposed to the same temperatures as on day zero. Seedlings were reassessed after the second freezing and root production was also determined.

Ethanol and acetaldehyde production increased signify-cantly (Hawkins, unpublished results) between -15 and - 21°C after the first freeze (Figure 8.10). Recovery at all temperatures was greatest after five days (Figure 8.10). Recovery ethanol production was three to four times greater in stock exposed to temperatures of -9°C or less, indicating that while recovery occurs, it is not to pre-stressed levels. The similar level of recovery for seedlings exposed to the three lowest temperatures probably indicates that none of the temperatures constituted a lethal stress even though damage had been done.

Table 8.1 –*Mean number of roots (± SE of mean) produced after one week in a misting (aeroponic) tank under continuous, full spectrum light (= 200 µ mols ? m^{-2} ? s^{-1}) at 23°C for 1+0 Douglas-fir seedlings after two exposures to the temperatures noted.*

Temperature °C	Roots #	SE
+ve	13.3	1.2
-3	11.7	2.4
-9	7.0	2.0
-15	8.0	2.5
-21		0

There was a significant increase in ethanol and acetaldehyde production between -9 and -15°C after the second freeze (Figure 8.10). A similar result was also observed for the other damage assessments (Hawkins, unpublished results) except on the cambium (Figure 8.11).

A large decrease in ethanol production with decreased temperature (as seen between -15 and -21°C) indicates significant lethal damage to the seedling. Foliage, buds, and cambium were completely damaged and conductivity ratio had its greatest value at -21°C. Root growth indicated the same (Table 8.1). No root growth was observed in seedlings exposed twice to -21°C while good root growth was observed at all other temperatures.

While preliminary, the assessment results are encouraging. It appears that once correlations are established with standard tests and the species baseline is increased, a stress test with predictive capabilities can be successfully developed using the SIVE technique and ethanol production as its backbone and other tests, such as F_{VAR} and EC, as adjuncts.

8.3.3 Comparison of the assessment methodologies

While untested operationally, SIVE has shown strong correlations to other established stock quality tests (Hawkins and DeYoe 1990, Hawkins unpublished results). The major advantage of SIVE over the tests with which it was compared is the speed with which results are obtained. Another potential advantage is that it can detect minor physical-mechanical stress events prior to symptoms appearing. Combined, the speed and sensitivity of the SIVE technique make it an excellent candidate for a remedial cultural program in the nursery. SIVE would also be a good screening assessment for seedlings during the storage transportation phase.

To date, the majority of SIVE analyses have been done destructively and this limits the utility of the test. However, this was for technical convenience rather than necessity. In the future in our laboratory, there will be a gradual move from destructive to non-destructive sampling. The present gas chromatograph (GC) program limits the number of samples which can be done in a day (four per hour), thereby increasing sample costs. Alternative column types and GC programs, and detection systems, are being investigated to overcome this problem. Once done, the major hurdle to SiVE testing will be in the capital cost of a GC. This may, in the short-term, restrict the SIVE test to fee for service, seedling quality assessment laboratories.

Regardless, SIVE is a test with great potential. If coupled to other tests, the SIVE technique could be developed during the next decade to become an important member of

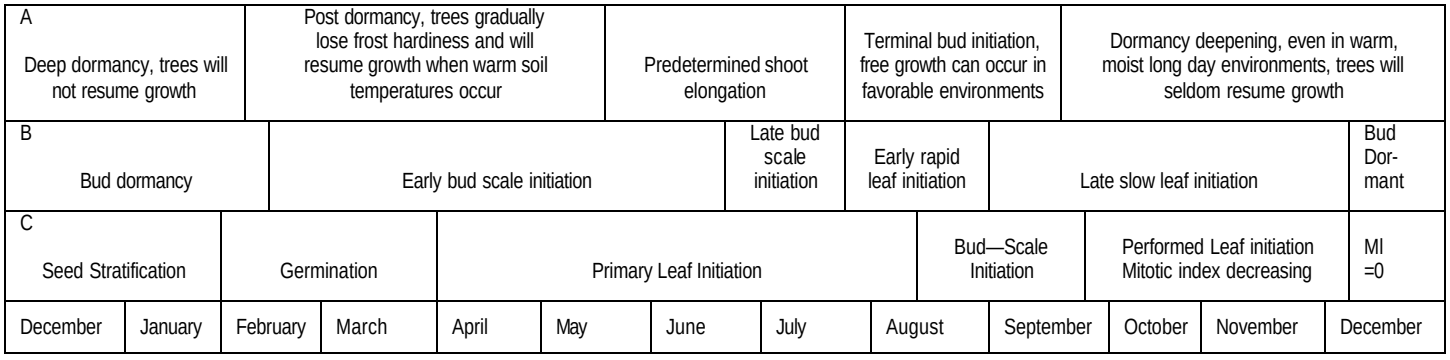

Figure 8.12—*A general comparison of annual physiological events (A), growth patterns of young and mature trees (B), and events of growth and development in first year seedlings (C). Also shown in (C) is the mitotic index state in the terminal bud. Modified from Carlson et al. (1980) and Fielder & Owens (1989).*

the battery of predictive tests which will ensure seedling quality and plantation success.

8.4 Mitotic Index (MI)

8.4.1 Historical theory

There has been considerable literature devoted to both the definition of dormancy and its developmental stages (see Carlson et al. 1980 for review). In conifers, dormancy is generally defined as any case in which elongation does not take place in a tissue predisposed to do so (Doorenbos 1953, Cleary et al. 1978). Owens and Molder (1973), in describing the annual growth cycle of mature Douglas-fir buds, defined dormancy as the absence of cell divisions in the apex. The work of Owens (1968) and Owens and Molder (1973) shows that the mitotic frequency (percentage of dividing cells in ten percent of apical volume of five median sections) clearly decreases with the onset of dormancy and becomes approximately zero (no cell divisions). This corresponds to the period of deep dormancy (Lavender and Cleary 1974) and lasts several months (Figure 8.12) and appears to be closely correlated with seedling resistance to stress (Lavender 1985).

Because the methodology of Owens (1968) for determining mitotic frequency was lengthy and fairly complex, Carlson et al. (1980) developed a bud squash method which is a comparatively rapid assessment of bud nuclear activity. In this method, buds are squashed on a microscope slide, stained, and the number of cells in division expressed as a percentage of all cells counted. This has been coined the "bud squash" method (Table 8.2) and has been used extensively since its development.

Table 8.2—*The bud squash method for determining mitotic activity in conifer seedlings. After Car/son et al. (1980).*

Step	Description of Step
A	Remove buds from seedlings, remove bud scales.
B	Fix buds in McClintoks [Conc. acetic acid: 100% ETOH; 1:3 v/v] for a minimum of 6h.
C	Hydrolyse buds in Warmk's solution [95% ETOH: Conc. HCL; 1:1 v/v] for 10-25 min[1].
D	Place buds into Carnoy's [100% ETOH: Conc. acetic acid: Chloroform; 6:1:3 v/v/v] solution for 5-20 min[2].
E	Stain buds with orcein or acetocarmin[3] 10-20 min. Halfway through the staining place a cover slip over the bud and press firmly down to flatten the bud to a near single layer on the slide. Heat the slide over an open flame but not to boiling.
F	Count the number of cells using a microscope with a 12.5X ocular equipped with a counting grid and a 40X objective lens[4].

1 To dissolve material between cell walls.

2 To counteract softening.

3 Preparation of 0.5% acetocarmin solution. 550mg in 45% acetic acid. Heat to boiling point, remove from heat, add dye, stir, and cool. After solution has cooled, filter through Whatman #1 or similar type filter paper.

4 To determine the onset of bud dormancy precisely the most mitotically active area of the squash should be counted.

8.4.2 Applications of mitotic index

Using the bud squash technique, Carlson et al. (1980) viewed a steady decrease in meristematic cell activity of coastal Douglas-fir during the fall. Cell divisions became essentially zero by December 15. Binder (1983) observed that for coastal Douglas-fir, cell divisions or MI became zero on about December 15 while in interior seedlots, activity ceased one month earlier, even though the seedlots were grown under similar conditions at the same location (Figure 8.13). Activity rapidly increased about the middle of February in greenhouse container stock and in early March for bareroot stock (Figure 8.13). Fielder and Owens (1989) in a detailed developmental study using sectioned embryonic shoots of coastal and interior Douglas-fir (Figure 8.14), confirmed the findings of Binder (1983). Carlson (1985) found that MI on loblolly pine ranged from 17 percent in midsummer to 3 percent in midwinter. Comparative studies of three, open-pollinated, loblolly pine families showed significant differences in MI at several points between late September and early March. These results are discussed in terms of loblolly pine bud dormancy (Carlson 1985). An MI study comparing western hemlock seedlings lifted and placed in cold storage in mid-November against seedlings that remained in the greenhouse was conducted by O'Reilly and Owens (1989). They observed that MI was zero by December 23 in the former, while MI of the latter went to zero on January 13 and for less than one month.

O'Reilly and Owens (1987) did an extensive study on morphology, including MI, on seven provenances of lodgepole pine from 50 to 60° N latitude and planted at one interior location near Prince George, British Columbia. (53° 46' N latitude.). Their data indicate that terminal apices of six of seven provenances were active mitotically by the end of March and began to decline in

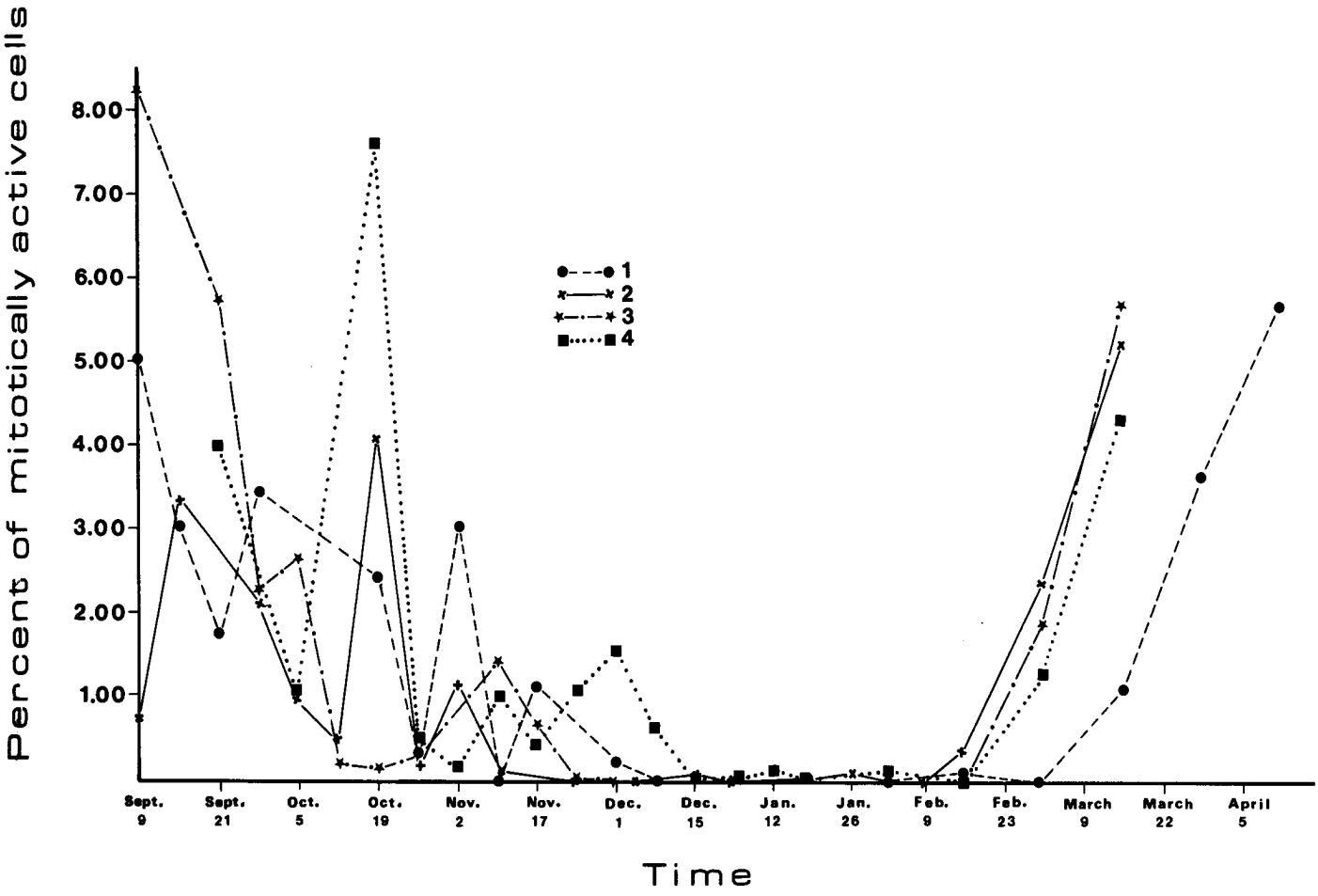

Figure 8.13—*Mitotic activity of two coastal (1 bareroot, 4 = container) and two interior (2 = drybelt and 3 = wetbelt) Douglas-fir seedlots from September through April. All seedlings were held either in the greenhouse or the field under ambient conditions during the test period.*

mid-August. They were bud dormant by mid- to late-September. Differences among provenances in this regard were significant.

Macey (1982) reported that MI correlated well with frost hardiness in white spruce and that the technique may be used to predict the ability of seedlings to withstand cold storage. Because seedlings exposed to short days and warm temperatures into early winter formed mitotically inactive buds but flushed when exposed to favorable conditions, this worker suggests that MI does not reflect bud dormancy status. Therefore, MI cannot be used to predict lifting date. While this would appear to compromise the test's predictive capability, this is not so from an operational point of view. Operationally grown stock would never be exposed to such conditions. Therefore, the situation should never arise. Also, frost hardiness of black spruce seedlings exposed to different environments has been correlated with MI of the embryonic shoot (Colombo et al. 1989).

Dunsworth and Hartt (1987) found MI sensitive enough to discriminate among a variety of Douglas-fir seedlots. They indicate further study is required to correlate mitotic indexing with other physiological parameters in order to determine optimal lifting and storage times with respect to bud dormancy.

Dunsworth and Kumi (1982) applied the concept of MI to study root activity. They found the technique was sensitive enough to detect seasonal variability in root activity of both Douglas-fir and amabilis fir. Their data indicate that high elevation natural amabilis fir was considerably more active and reached highest activity two weeks before natural Douglas-fir from low elevation. The technique may be useful to discriminate among stocktypes but this requires further study. Using mitotic indexing, Dunsworth (1989) also demonstrated that peak activity in both natural Douglas-fir and western hemlock for both spring and fall could be bracketed by soil climate conditions above -1 bar soil tension and 4°C. Based on mitotic

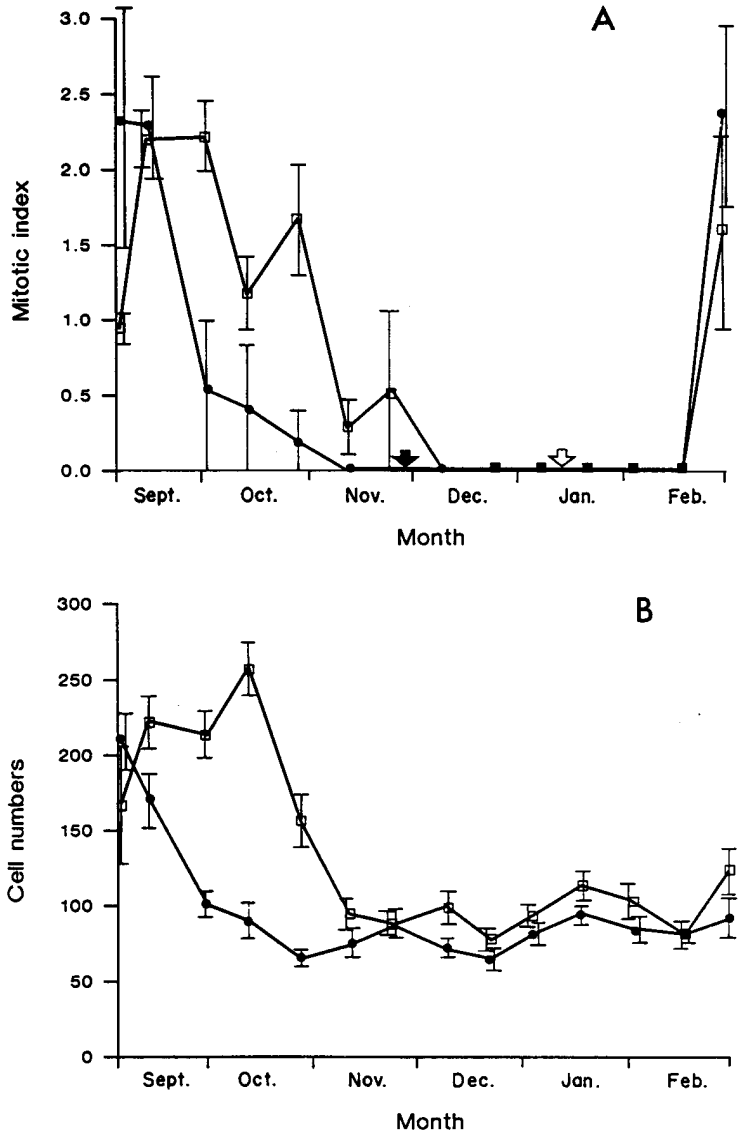

Figure 8.14—*Average ± 1 SE mitotic index (A) and average number of cells (B) per median section, based on four to nine apices per collection of coastal (■) and interior (●) Douglas-fir. End of cell division in subtending leaf primordia indicated by an open and a closed arrow for coastal and interior varieties, respectively. From Fielder and Owens (1989); with permission.*

activity of roots, a survival and growth advantage of 10 percent to 15 percent can be gained by planting within this hypothetical window.

8.4.3 Summary of MI application

After the initial purchase of the microscope, MI becomes a relatively inexpensive test to be done by a trained individual. The lack of wide operational use is probably due to its apparent complexity and lack of applied operational publications (method outlined in Table 8.2). However, this should not detract from the test. There are sufficient data to suggest that MI could play an important role in the optimization of stock quality during the bridging phase (lifting to planting hole), in conjunction with testing of seedling stress resistance. For example in Douglas-fir, MI should remain at or near 0 for 7 consecutive days prior to lifting and storage and the seedlot at -18°C should have less than 25 percent foliage damage (i.e., LT_{25}).

8.5 Electrolyte Conductivity (EC)

8.5.1 Historical theory

The measurement of electrolyte conductivity (leakage) from stressed plant tissue to assess viability was developed by Dexter et al. (1930, 1932). The technique is based on the assumption that whatever the cause of injury to the plant, the result is always a loss of semipermeability of the protoplasmic membrane (Wilner 1960). This results in ion (electrolytes) flow out of the cells and it can be measured using a good quality conductivity meter. The amount of electrolytes which diffuse is assumed to be proportional to the injury. It should be noted here, in caution,

that cell rupture and loss of semipermeable characteristics are generally inferred to be synonymous (see Palta and Li 1978). This, however, is not the case. The loss of plasma membrane semipermeability, as opposed to mechanical rupture, can be affected by other means (Steponkus 1984).

8.5.2 Electrolyte conductivity and cold—hardiness

Most studies that use electrolyte leakage as an indicator of stress damage have used it to measure, for example, relative ratings of cold hardiness of both shoots and roots in several woody species (Wilner 1955, 1959, Wilner and Vaartaja 1958). Flint et al. (1967) improved the technique and developed the equation now known as the Index of Injury(I_t). See also Colombo and Glerum (1984), Colombo et al. (1984), and Glerum (1985).

$$I_t = 100[(R_t-R_0)/(1-R_0)]$$

where: $R_t = L_t/L_{k2}$ and $R_0 = L_0/L_{k1}$, and

I_t = Index of injury resulting from exposure to freezing temperatures.
R_t = Fractional release of total electrolytes from sample exposed to freezing temperature (t0C).
R_0 = Fractional release of electrolytes from unfrozen sample.
L_t = Specific conductivity of leachate from sample frozen to temperature (t0C).
L_{k2} = Specific conductivity of leachate from sample frozen to temperature (t0C) and then heat killed.
L_0 = Specific conductivity of leachate from unfrozen sample.
L_{k1} = Specific conductivity of leachate from unfrozen sample after heat killing.

Early estimates of cold-hardiness using leakage were done in Douglas-fir (van den Driessche 1969, 1976), Scots pine (Aronsson and Eliasson 1970), Monterey pine (Green and Warrington 1978), and black and white spruce (Colombo et al. 1981, 1989). Burr et al. (1986) found freeze-induced electrolyte leakage of needle tissue to be a better predictor of cold hardiness than differential thermal analysis and even the whole plant test (visual damage) in Douglas-fir, ponderosa pine, and Engelmann spruce. According to Burr et al. (1986) the electrolyte leakage test, with the exception of the last week of deacclimation, tends to be somewhat more conservative than the whole plant freeze test. The LT_{50} (temperature at which 50 percent of the samples are killed) occurs at a higher temperature. Nevertheless, they (Burr et al. 1986) state that the EC test is the most precise of the three and detects slight changes in tissue cold-hardiness. Berrang and Steiner (1986) found they could detect seasonal differences in cold tolerance of needles, stems, and male and female strobili in pitch pine using this technique. However, van den Driessche (1976) found that hardiness level prediction of mean conductivity percent did not fully agree with controlled-environment survival results obtained from whole Douglas-fir seedlings after freezing tests.

Freeze-induced electrolyte leakage of shoot tips is used operationally for monitoring frost-hardiness of stock in extended greenhouse culture in Ontario (Colombo et al. 1984, Colombo and Cameron 1986).

In general, the electrolyte leakage method works well to detect tissue cold hardiness either as a direct test of cold hardiness or as a reaction to cold stress (Flint et al. 1967, Colombo et al. 1984). Two important advantages of the technique are: it is useful for measurement of all conifers, and a great many samples can be measured concurrently with no increase in equipment (Burr et al. 1986).

In this regard, the results of Zhang and Willison (1987) are very interesting. Using cultures of brome grass to measure cold-hardiness they found that electrolyte leakage always underestimated the frost hardiness by comparison to fluorescein diacetate (FD) vital staining. Fluorescein diacetate tests for metabolic activity, that is, the capacity of cells to display esterase activity. They found that there was a difference in ions leaked after 18 hours in deionized water and leakage after 1 hour in deionized water. They termed this differential percent leakage (DPL). They found that one-half the maximum DPL (DPL_{max}) was very similar to LT_{50} estimates of frost damage using the FD method. The value of the correlation between LT_{50} by DPL_{max} and LT_{50} by FD is just over 0.97. The physiological basis of the DPL_{max} effect apparently is that frost-killed cells, on thawing, leak electrolytes rapidly while living cells with intact plasma membranes leak ions slowly. The rationale is that as more cells are damaged, the difference in leakage in relation to deionized water immersion time will decrease. Thus, if the maximum difference (DPL_{max}) corresponds to 100 percent living cells, then one-half of this difference corresponds to 50 percent living cells. To our knowledge, this version of electrolyte leakage has not yet been applied to conifers and if used, may yet further improve the sensitivity of the test.

8.5.3 Electrolyte conductivity and other assessments

Electrolyte leakage has been used to assess genetic variations in cold tolerance (Kolb et al. 1985, Raymond et al. 1986) of tree species. It has also been used to assess damage to trees from air pollutants (Keller 1986, Leith et al. 1989) and other stresses such as leaf desiccation (Leopold et al. 1981). The technique has worked fairly well in such studies. Keller (1986), for example, reports that sulphur dioxide fumigations increased measurable leachate conductivity even at concentrations causing no visible symptoms of injury. Kolb et al. (1985) also commented that the technique may have practical value for tree improvement programs if differences remain reasonably consistent between years, as their results indicate.

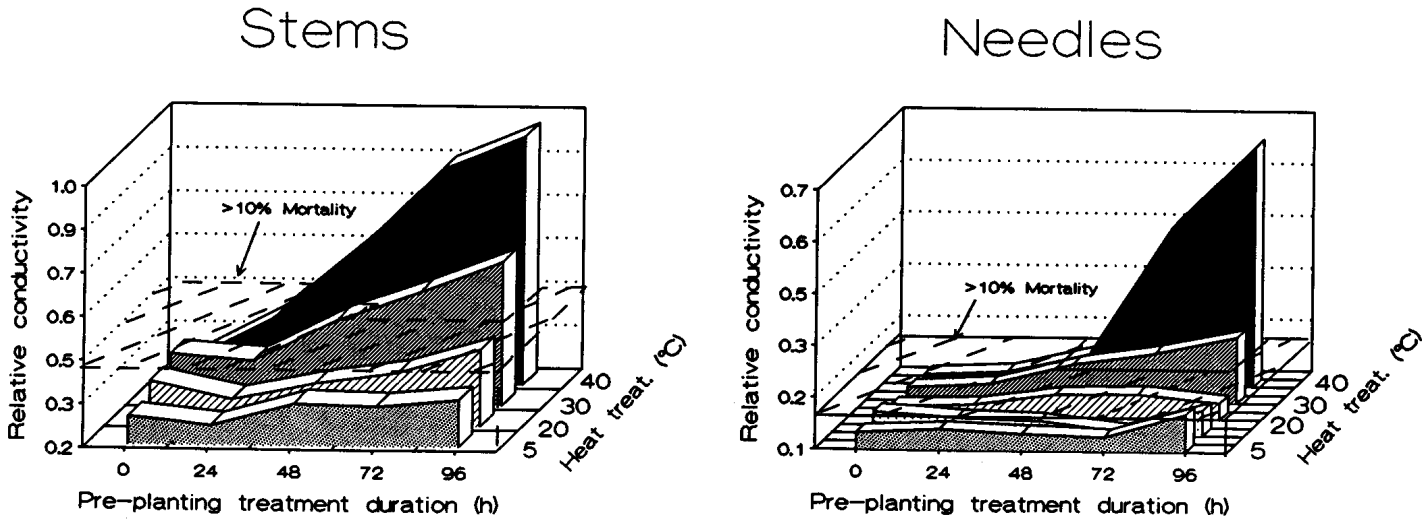

Figure *8.15*—*Relative conductivity of white spruce stems and needles after 5, 20, 30 and 40°C heat treatments for 0, 24, 48, 72 and 96h. The 10 percent mortality rate from outplanted test plots is indicated. Modified from Binder and Fielder (1990).***

8.5.4 Electrolyte conductivity for stress evaluation in conifers

Electrical conductivity has been recently used to evaluate heat stress resistance in white spruce seedlings prior to planting (Binder and Fielder 1990).

8.5.4.1 Heat-treated stem and needle segments

After removal from cold storage, boxed seedlings were heated to 5, 10, 20, 30 and 40°C for 0, 24, 48, 72 and 96 hours. The conductivity of leachates were determined for untreated and killed controls, and frozen and frozen/killed treatments after incubation for 24 hours at 25°C. The I_t was calculated after Flint et al. (1967).

Temperature treatment and duration affected the fractional release of electrolytes from stem and needle segments (Figure 8.15). The rate of increase of mortality over time was dependent upon the treatment temperature. The results for needle and stem segments were similar except the response was greater and conformed closer to the expression of mortality at lifting time in the case of stem segments (Figure 8.15). Electrolyte leakage from needle segments was strongly correlated with field needle damage after 14 days (Figure 8.15). Fractional release of electrolytes above 0.5 for stem segments indicated a potential for mortality >10 percent for all temperature treatments. There appears to be close agreement between electrolyte leakage from stem and needle segments to high post-cold storage temperatures, and field results. This is opposed to the findings of van den Driessche's (1976) growth chamber survival assessment where changes in EC did not fully reflect field survival.

The development of damage over 48 hours at 40°C (Figure 8.1 5) indicates this was not due to direct primary heat injury (i.e., heatshock). It would suggest temperature and time of exposure were interacting to intensify indirect heat injury. Direct heat injury is manifested in the order of minutes or hours following the exposure (Levitt 1980). Field results show that needle damage was evident after 72 hours at 40°C, but not after 48 hours. Needle damage after the 48 hours at 40°C treatment increased over the season, this being consistent with the long-term effects of indirect heat injury.

The difference in response of stem and needle segments to post-cold storage heating also indicates the most probable chief cause of mortality. Electrolyte leakage from needle segments corresponded most closely to percent field needle damage after 14 days. On the other hand, electrolyte leakage from stem segments corresponded most closely with mortality at lifting time. These results suggest that membrane damage to stem tissues (i.e., cambium and conducting tissues) may be more important to eventual survival than damage to needle cells. Survival is dependent upon healthy conducting tissues.

8.5.4.2 Frost—hardiness testing by index of injury method

The hardiness of stems and needles can be determined by electrolyte conductivity. Binder and Fielder (1990) determined the temperature increase (i.e., to less negative value) to I_{50} (50 percent index of injury) for stem and needle segments of boxed, post-cold stored white spruce exposed to various heat treatments for up to 96 hours (Table 8.3). The extrapolated I_{50} for control stems and needles was -100°C and -79°C, respectively. Results sug-

Table *8.3*—*Average percent temperature increase (i.e., less negative) in the index of injury of 50% (I_{50}) to stem and needle segments as a result of exposure of boxed white spruce seedlings to 5, 10, 20, 30 and 40°C temperature for up to 96 h*. Freezing temperatures were determined from interpolation and extrapolation from regressions of index of injury with freezing treatments to -12, -20, -28 and -36°C. Regressions were fitted through 4 points with 3 measurements at each point (modified from Binder and Fielder 1990).*

Temperature Treatment °C	% of Control in I_{50} Stems	over 96 h Needles
5	100	100
10	59	58
20	36	44
30	26	32
40	35 (to 48 h)**	58 (to 48 h)

* Seedlings received 8 days of thawing at 5 ℃ before being heat treated

**There were no live seedlings after 48h at 40℃.

gest that for both stems and needles, temperatures in excess of +5°C applied to stock thawed from the -2°C of storage reduced frost-hardiness. The magnitude of the reduction depends on the amount of heat and its duration. The reduction in I_{50} at 40°C after 48 hours was similar to 20°C and 10°C for 96 hours for stems and needles, respectively. Differences in frost hardiness between stems and needles after 48 hours at 40°C may point to a greater thermotolerance of needles (needles are subjected to more direct thermal heating than stems), at least initially. Evidence suggests (Levitt 1980) that thermotolerance and winter freezing tolerance are related since high reduction capacity of the membrane is important to both.

8.5.5 Assessment of electrolyte conductivity

Electrolyte leakage has a wide range of potential applications in stock quality testing. Some of its advantages are that large numbers of samples can be done in a short period of time, it is statistically valid, a small tissue sample is required, and it is highly sensitive. However, disadvantages are that it is destructive and seasonal baseline trends have not been described for nursery monitoring programs.

Because of its mechanical simplicity, statistical rigor, and low initial cost of equipment, EC is a test that could be used increasingly in nursery and field diagnostic situations. EC provides good support data to almost all physiological tests. This test should be a part of any integrated assessment program.

8.6 Other Tests

8.6.1 Triphenyl tetrazolium chloride (TTC)

The earliest reports of triphenyl tetrazolium chloride (TTC) as a test of tissue viability were by Roberts (1951, 1957), Parker (1951, 1953), Larcher and Eggarter (1960) and Purcell and Young (1963). These tests were generally of a qualitative nature. If color was observed, the tissue was considered to be viable. Steponkus and Lanphear (1967) refined the technique so that it was quantitative and could be analyzed statistically. This allowed testing of small

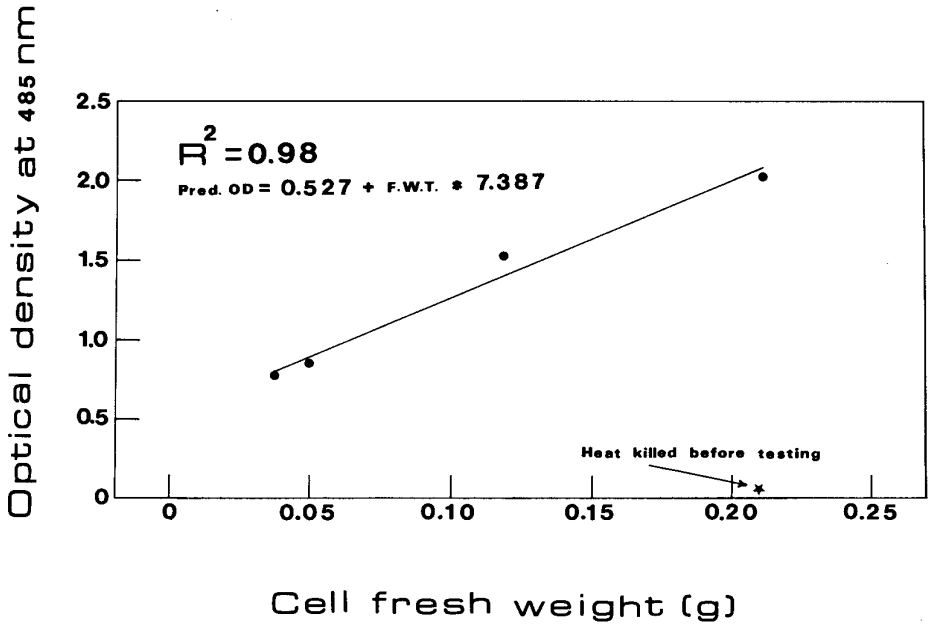

Figure 8.16—*The regression correlation (r^2 = 0.97) between TTC reduction and Douglas-fir callus tissue fresh weight.*

pieces of tissue, the results of which were used to predict the future viability of the whole plant.

The technique estimates the activity of live tissue which has the capacity to display dehydrogenase activity. The test is, therefore, one of metabolic viability and is similar to the fluorescein diacetate method (Section 8.5.2). Dehydrogenase class enzymes are able to alter TTC, which is colorless and soluble in water, to its derivative, formesan, which is red and soluble in alcohol and can be boiled out of the tissue and measured optically in a spectrophotometer (Steponkus and Lanphear 1967). The procedure is outlined in Table 8.4.

There is a good correlation (r^2) between the amount of TTC reduced and tissue fresh weight using this method (Figure 8.16). Using this method, Sugawara and Sakai (1978) measured cold acclimation of callus cultures of Jerusalem artichoke. When calluses were hardened at 0°C for 18 days and then frozen to temperatures ranging from -3 to -20°C, they found a very good reciprocal correlation between TTC reduction rate and amino acid releases, and a parallel correlation between TTC reduction rate and regrowth after freezing. Chen and Gusta (1983) found good agreement between the TTC test, the fluorescein diacetate test (enzymatic and membrane permeability) and a regrowth test after freezing of cell suspension cultures of winter wheat and a winter rye. Zhang and Willison (1986) used the fluorescein diacetate method and adjusted electrolyte leakage method (see Section 8.5.2) to assess freeze damage and found close agreement between the two tests. Altmann (1969) used a variation of the TTC test to study freeze stress. The substrate is nitroblue tetrazolium and the product of the dehydrogenase activity is the green dimethyl-formamide molecule (c.f. Timmis 1976).

Table 8.4—*The TTC procedure as modified from the findings of Steponkus and Lanphear (1967), Withers (1978) and Binder (1981).*

Step	Description
A	X% Buffered TTC[1] in 78% $NaHPO_4$+ 22%KH_2PO_4 + Wetting agent.
B	Add above solution to small pieces of tissue (mg).
C	Vacuum infiltrate.
D	Incubate at 30°C for 15h.
E	Drain off TTC.
F	Wash with distilled water.
G	Extract with hot ethanol (95%) for 30 min. in water bath.
H	Bring to volume with 95% ethanol[2].
I	Read in spectrophotometer[3].

1 Use between 0.1 and 0.7% TTC depending on the amount of tissue.
2 Amount of tissue should be enough to produce optical density readings below 2 absorbance units at 485 nm.
3 Use 430 nm (Steponkus and Lanphear 1967) or 485 nm (Withers 1978; Binder 1981)

There are few reports in the literature which directly use TTC as a stress test in conifers. Timmis (1976) compared leaf segment flotation, seedling water stress, photosynthesis, impedance ratio, and needle dehydrogenase activity (TTC) to detect live and dead Douglas-fir seedlings after freezing stress during five different frost hardening stages. He found that TTC was the second best method of detecting live and dead seedlings (76 percent on average; the impedance test was first at 87 percent on average). However, the TTC test could not distinguish between dead and live groups when these seedlings were exposed to night frosts (i.e., quiescence after rest) when frost hardiness was maximum. The TTC test was not predictive of frost damage at any stage of frost hardiness if stem segments were used. The method has been used to estimate cell viability of Douglas-fir suspension cultures after freezing to -196°C (Binder 1981). The method has also been used to estimate the seasonal variation in root hardiness of container-grown Norway spruce, Scots pine, and lodgepole pine (Lindström and Nyström 1987). The test was able to distinguish between fall hardening and spring dehardening of roots, as well as distinguishing species differences in this regard. The test can also detect differences between cold hardiness of mature and young roots of the same species (Lindström and Mattsson 1989).

Binder and Fielder (1990) used TTC as an indicator of sensitivity to heat stress in white spruce buds after cold storage (Figure 8.1 7). Enzyme activity was significantly reduced after 12 hours at 40 and 60°C but not at 20 and 30°C. Activity was reduced after 24 and 48 hours at 20 and 30°C but this was not significant compared to 1 2 hours. Respiration measurements on these buds were erratic. Only buds treated at 60°C showed no respiration after 12 hours. Respiration was also significantly depressed after a 40°C treatment for 48 hours. Apparently the HG test is a better diagnostic measure of heat stress in buds than is respiration. However, what possible correlation there is between the buds increasing lack of ability to reduce HG with temperature and time and ability to flush, extend needles or elongate is not known at this time.

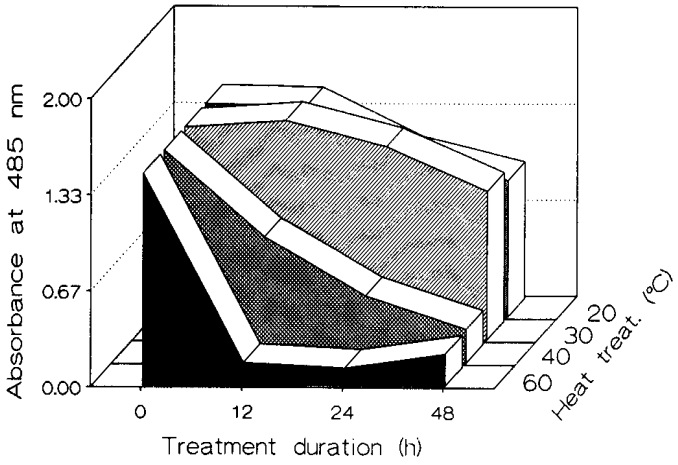

Figure 8.17—*TTC reduction in white spruce after 20, 30, 40 and 60°C heat treatments for 12, 24 and 48h. Results based on three replicates, each of six buds. At 20 and 30°C, the results are not significantly different. Results at 40 and 60°C after 12 and 24h were significantly different from each other and 20 and 30°C. After 48h, there was no difference between 40 and 60°C.*

Even though laboratory-based, TTC could be incorporated into a stress test to assess for functional levels of enzyme activity. Historically, this test has not been used to its fullest extent. During the planting of stock from storage, it could be an important aid in discriminating between viable, moribund, and dead stock. Clearly, there is a need to define the range and conditions under which this test can be used and which tests are best used in conjunction with it.

8.6.2 Days to bud break (DBB)

Days to bud break can be used to assess the level of dormancy in conifer seedlings (Ritchie 1984a, Ritchie et al. 1985). In the simplest terms, the longer it takes for buds to break, the greater the level of dormancy (Campbell 1978, Ritchie 1984a, Lavender 1985

Table *8*.5—*Days to budbreak at lift, during storage and at planting in a white spruce seedlot exposed to four different photoperiod durations to control height growth during the nursery cultural phase. Modified from Hawkins and Draper (1990).*

Photoperiod h	November 88 lift	DBB January 89 Store	May 89 plant
13	15.1	10.8	7.9
15	20.2	11.7	9.5
17	20.0	12.6	9.7
19$	24.1	13.7	9.7

$ Ambient photoperiod at Red Rock Research Station, near Prince George, B.C., = 54°N. latitude

To assess DBB, stock is placed in an optimizing environment, similar to that described for RGC (c.f. Binder et al. 1990) and the number of days required for the terminal bud scales to part and expose new, green needles is recorded. Ritchie (1986) used the days to bud break to develop a linearized "dormancy release index" (DRI). This index is calculated as the ratio of the number of days required to force bud burst in a fully chilled seedling over that in the seedling of interest. According to Hermann (1967) and Ritchie (1984a), a fully chilled coastal Douglas-fir seedling can be force-flushed in a minimum of 10 days. Therefore in Washington state, the Douglas-fir DRI is written as: DRI = 10/DBB.

However, for other geographic locales, the DRI numerator must be redefined. The DBB assessment, though time-consuming, can provide valuable information about the effect of nursery cultural treatments on seedling dormancy intensity. Days to bud break has been used to define the relationship between bud dormancy, cold hardiness and stress resistance (Ritchie 1986) as well as root growth potential (Burr et al. 1986, 1989) in some western conifers. For example, according to Ritchie (1986), in Douglas-fir, maximum stress resistance and hence survival potential falls somewhat beyond the peak of dormancy after several hundred hours of chilling exposure.

When photoperiod is used to control height growth (photoperiod is often viewed as a "non-stress" method of achieving seedling height control) in white spruce, it can have a significant impact on DBB between nursery treatments (Table 8.5). This phenomenon has been reported for other spruce species and the result can be a significant perturbation on bud phenology and diminished field performance (Hawkins and Hooge 1988, Odlum and

Colombo 1988). The range of DBB at planting presented (Table 8.5) is much smaller than previously described (Hawkins and Hooge 1988). Whether the smaller range in DBB is of significance, under field conditions, has yet to be determined.

As dormancy intensity plays a crucial role in seedling establishment (Ritchie 1984b), nursery cultural modifications should be assessed for their impact on DBB, especially considering that cultural modifications, such as blackout, can elicit such marked performance responses. Douglas-fir seedlings in mid-dormancy release (i.e., that region between maximum dormancy [buds do not flush] and quiescence [buds flush] when placed in a growth permissive environment), apparently, are most resistant to stress (Ritchie 1986).

While DBB is inexpensive, simple and straightforward to conduct and assess, it seldom is done on an operational basis—presumedly because of the time involved (a minimum of 30 to 80 days depending on the species and the requirement to assess bud development at regular intervals). Perhaps, in the future, the time factor may be overcome by the establishment of correlations between DBB and some of the more rapid tests. Ritchie (1989) has shown that accumulation of about 1,400 natural chilling hours (air temperature below 6°C) equates with both DBB and DRI. Regardless of the time aspect, DBB is a test which should be encouraged operationally because of the valuable information it yields. Recently, Ritchie (1989) has outlined a strategy in which freeze or cold storage of conifer stock can be used to manipulate the release of dormancy and hence maximize seedling physiological quality at the time of planting. As a target in Douglas-fir, the DRI value should be between 0.25 and 0.40 (Ritchie 1989).

8.6.3 Phytogram

A protocol which may prove to be of immediate utility to bare root nurserymen and field foresters is the phytogram response. A noble metal (palladium) electrode is placed in the stem or lateral branch of the tree and a reference electrode is placed in the soil (Gensler 1980, 1986, 1988, 1990). The two electrodes yield a dynamic extracellular electropotential for the tree. A phytogram is a plot of the continuous measure of the extracellular electropotentials obtained from the tree.

Three zones of electropotential are found in plants (Gensler 1989ab). The normal range is from 300 to 700 mV. In this range a diurnal pattern is exhibited, rising in the morning to an afternoon plateau and then declining until the morning rise. The second zone is from 0 to 300 mV and is termed the hypo-potential range (Gensler 1989a). This range is characteristic of wet soil conditions. Time spent in this range is usually short but under prolonged saturated conditions, the potential will remain in this range. Seven hundred mV up to 1,400 mV is the hyper-potential range, and movement into and out of this range is very rapid and is termed "spiking" (Gensler 1990). This range is entered only under relatively specific combinations of temperature and light (high stress). Not only are the dynamic potentials valuable, but daily, seasonal, and annual activity or vigor indices can be constructed (Gensler 1990). This provides the user with a number that can be used to compare two treatments, sites, or species separated in space and time.

Hypotheses have been put forward and related to empirical results for the three ranges of electropotential discussed above. In the hypo-potential range, the hypothetical causative reactant is the ethanol/acetaldehyde couple (Gensler 1989a). This assumes anaerobic root zone conditions. The oxygen hypothesis has been developed to deal with potentials in the normal range (Gensler 1986, 1988). The electropotential is a measure of the extracellular electrolyte concentration in this range. Oxygen diffuses to the extracellular spaces during active photosynthesis and away from the spaces during active respiration, thus accounting for the diurnal pattern. Hydrogen peroxide is the hypothetical causative reactant for the hyper-potential range (Gensler 1989a). Due to excess energy, a superoxide radical is formed and is then converted to hydrogen peroxide, preventing physical damage to the plant.

Regardless of what causes the potential to be generated, this technique has been successfully used as a water management tool for cotton crops in Arizona (Gensler 1983, 1988, 1989b). This indicates the technique has the ability to be used as an aid in monitoring the "physiological" impact and time line of droughting in bare root seedlings. The phytogram approach has also been used to distinguish between field site types and levels of tree vigor in established Douglas-fir plantations (Gensler 1990). This suggests the technique may also have a place in assisting the field forester with his silvicultural decision-making. However, these areas require an expanded understanding and application of the phytogram.

Because of its inherent diurnal periodicity, the phytogram technique could play a vital role is in container nursery crop management. This area is a must for research because it offers the opportunity of assigning phytogram (physiological) indices to nursery crops. Nursery phytogram indices in conjunction with other physiological assessments would allow stock to be better matched to its planting site, resulting in enhanced performance and reinforcing the target seedling concept.

8.7 Toward 2000

The importance of the physiological state of a seedling as a component of "quality" is accepted today without ques-

Table 8.6--*Description and rating scale of the nine criteria used for evaluating seedling stock quality tests. This is based on the conceptual framework proposed by Zaerr (1985). Two criteria added to Zaerr's list are basis of the test, that is what is it measuring, and predictiveness of the test, because of its relationship to plantation success.*

Criterion	Scale	Description
BASIS:	What is the test based on?	
	0	Non-physiological
	1	Physiology is inferred
	2	Physiology is directly measured
RAPID:.	Time with which results are available	
	1	>1 week
	2	1-7days
	3	2-24h
	4	<2h
SIMP:	Simple, Ease of understanding/use, all levels of operation.	
	1	Requires a researcher
	2	Requires a forestry/nursery professional
	3	Requires a technician
	4	No formal training required
CHEAP:	Cheap and accessible to all potential users.	
	1	Available only in a research laboratory
	2	> $1,000 and available in the marketplace
	3	$100 -$1,000 and available in the marketplace
	4	<$100 and available in the marketplace
RELI:	Reliable, the test works every time.	
	1	It works every time but is seasonally limited
	2	It works every time
NON-D:	The test is non-destructive.	
	1	A non-tested sample is outplanted (destructive test)
	2	A portion of the plant is removed for testing and the plant outplanted
	3	Same plant tested and outplanted
QUANT:	The test is quantitative not qualitative.	
	1	The test is neither precise or repeatable
	2	Precise or repeatable
	3	Precise and repeatable
DIAG:	Diagnostic, cause of any past seedling damage is indicated.	
	1	No specific diagnostic ability
	2	Physiological system specific diagnostic ability
	3	Multisystem diagnostic ability
	4	Cause of any seedling damage is indicated
PRED:	Predictive, future performance of the seedling indicated.	
	1	No indication of performance potential
	2	Potential indicator of performance
	3	Predictor of performance

tion (Ritchie 1984b, Duryea 1985a, Puttonen 1989). However, as recently as 1989, Ritchie (1989) pointed out there still is no consensus among workers as to what "physiological quality" means—even though the major concepts and principles of physiological quality are well documented (Duryea 1985a, Puttonen 1989). Therefore, one is forced to conclude that cohesive guidelines for the application of these concepts and principles to forest regeneration management are still required. We must define what management value is in the test results. In practical terms, why do we want to do the test and what do we expect to gain from it?

Zaerr's (1985) astute characterization that an ideal vigor test must be rapid, simple, accessible, reliable, non-destructive, quantitative, and diagnostic is even more crucial today. He (Zaerr 1985) aptly stated ". . . these characteristics can serve as goals for developing new methods and as benchmarks for judging existing techniques."

Several of the tests described in previous sections, as well as others (see Puttonen [19891 for a more detailed list), are evaluated using a modification of Zaerr's (1985) criteria (Table 8.6). Whether all nine criteria and the rating scales used are valid is open to debate. The criteria guidelines suggested here are meant only as a starting point to evolve from, not an end point. For instance, compared to a $25,000 regeneration program, "simple" and "cheap" (Table 8.6) become less important when a $1,000,000 program is at issue.

Clearly, based on our modified criteria, none of the tests, alone, go very far toward achieving high scores or maximum sum/products of 29/27648 (Table 8.7). There are three major surprises in Table 8.7: how poorly standard tests such as RGC rate, how well the EC technique rates compared to those in widespread operational use, and how the "up and coming" tests still have a distance to go to reach the level of the "ideal" single test. For simplicity, none of the tests were evaluated as a battery. Seedling stock quality tests must be evaluated, both alone and in conjunction with other tests that are frequently used with it. All seedling stock quality assessments must be evaluated using the same criteria, not different criteria for different tests.

Since seedling quality must potentially be capable of being evaluated at any stage from nursery tenure through planting, the utility and applicability of specific tests must be rigorously defined. The development and expression of root growth potential by Ritchie and Dunlap (1980) is an excellent example. A compiled list of specific physiological tests in relation to diagnostic utility at various cultural or lifting phases would be useful to forest nursery personnel, field foresters, and academics alike. This process has, in part, been initiated with *Evaluating Seedling Quality*

Table 8.7—*Sum and product rankings (maximum sum and product are 29 and 27648) of various stock quality tests using the nine criteria and their rating scales outlined in Table 8.6. The greater the sum and the product the greater the utility of the individual test. Evaluations are based on technology at the time of writing, not where it looks to be headed. RGC and morphology were the benchmarks for this work.*

TEST	BASIS	RAPID	SIMP	CHEAP	RELI	NON-D	QUANT	DIAG	PRED	SUM	PRODUCT
Morph[a]	0	4	4	4	2	3^{σ}	3	1	1	22	0
RGC	1	$1^{ß}$	4	3	1	1^{Σ}	1	1	2	15	24
MI	1	3	3	3	2	2	2	1	1	18	216
F_{VAR}	2	4	2	1^{π}	2	3	2^{τ}	2	2	20	768
EC	1	2	3	3	2	2	3	2	2	20	864
DBB	1	1	4	3	1	1^{Σ}	2	1	2	16	48
SIVE	2	4	2	1	2	1^{μ}	3	2	2	18	384
Phytogram	1	2^{Γ}	2	2	2	3	2	2	2	18	384
TTC	2	2	3	2	2	2	3	2	1	19	576

a, Abbreviations: Morph, morphology; RGC, root growth capacity; MI, mitotic index; F_{VAR}, variable chlorophyll fluorescence; EC, electrical conductivity; DBB, days to bud break; SIVE, stress-induced volatile emissions; and TTC, triphenyl tetrazolium chloride.

ß, Test can be done in seven days but generally is longer.

Γ, About 3-7 days for wound to heal around electrode and for the signal to stabilize, then it is instantaneous, a 4; sum/product would then be 20/768.

π, There are a limited supply of instruments in operational use, could be viewed as a 2, resulting in a sum/product of 22/2304.

σ, If masses are done, this value becomes a 1, sum/product of 20/0.

Σ, After the assessment is done the stock can be outplanted but this is not done operationally.

μ, This can be done non-destructively too, resulting in a sum/product of 19/576.

τ, F_{VAR} transient quantification under analytical review.

(Duryea 1985a) and Purtonen's (1989) review. We propose, over the next ten years, that a handbook of rigorously defined tests displaying their utility and applicability be compiled. A periodic update of this handbook would serve two important functions. First, it would provide a useful dictionary of current tests; second, it would suggest areas, either with a test, its intent, or a particular species, in which gaps exist and more information is required.

In the preceding sections, we have generally viewed the physiology-based tests on an individual basis. This is, however, a misrepresentation of how they should be used. Tests looking at different seedling systems must be incorporated into a test battery so as to allow overall seedling health and vigor to be established. Because of the application of individual tests, to date, we have no stock quality test(s) which can predict actual field performance (c.f. Lavender 1989). In Proser's (1958) terminology, acclimation is plant adaptation to a single factor while acclimatization is adaptation to a complex of environmental factors. To date, what we do have is a series of tests which indicate seedling potential of performance or

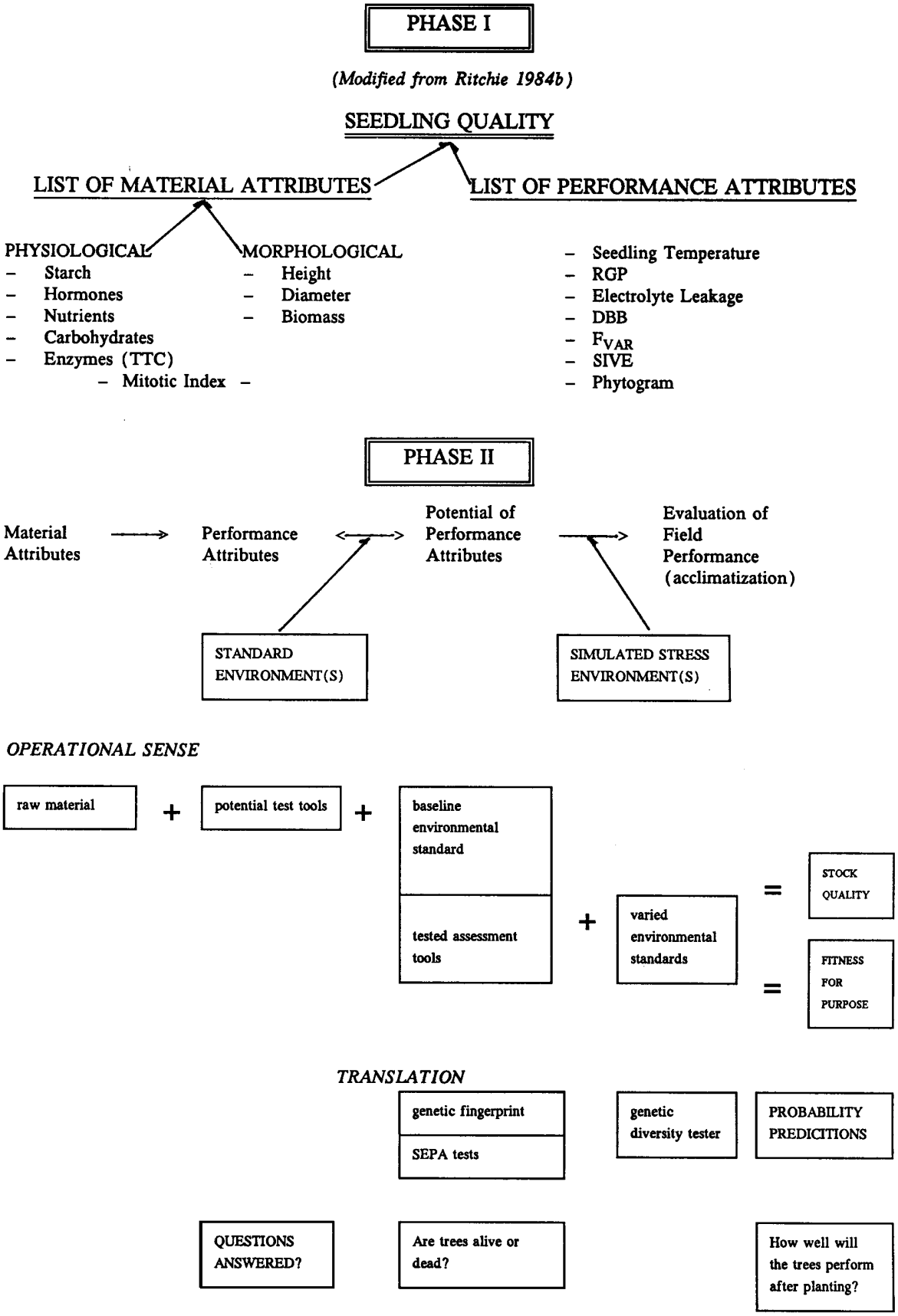

Figure 8.18—*A model for testing or determining seedling quality starting with a static phase I based on Ritchie's (1984b) conceptual framework for evolving to phase Iiwhich is predictive or dynamic in nature.*

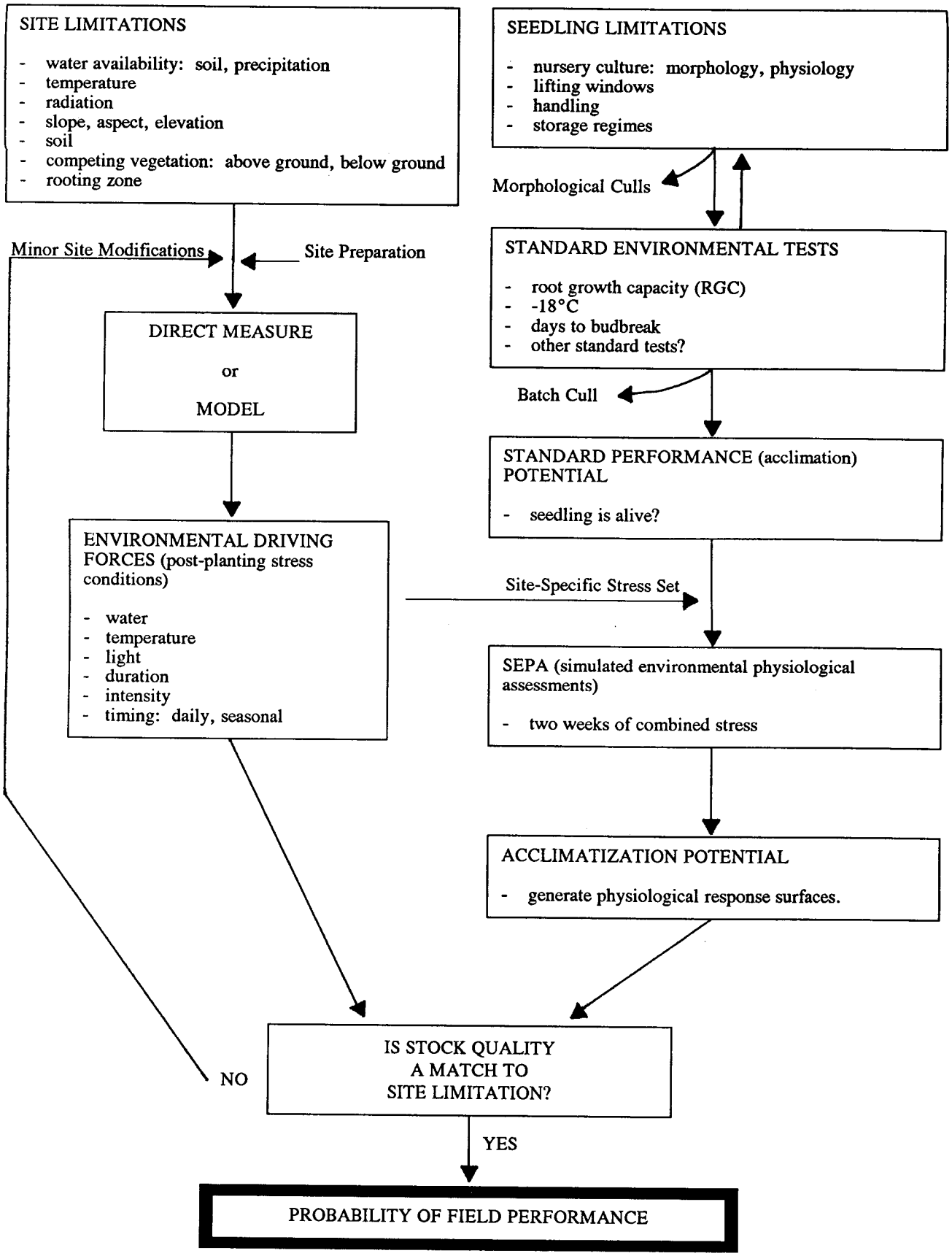

Figure 8.19—A generalized operational model of the Phase II concept (presented in Figure 8.18). Specific SEPA (simulated environmental physiological assessments) tests and environmental parameters depend on management objectives. Glen Dunsworth is thanked for his contributions to this figure.

acclimation (c.f. Sutton 1979, Ritchie 1984b, Puttonen 1989). However, as Puttonen (1989) indicated, prediction of seedling performance in the field (acclimatization) is the ultimate objective of seedling testing. The goal in the coming decade is to extend the potential of performance indicator assessments to prediction of acclimatization.

Based on Ritchie's (1984b) conceptual development of seedling material and performance attributes (Figure 8.18, Phase I), we propose to extend the use of this model from Phase I, a potential performance indicator (i.e., acclimation), to Phase II, a performance predictor (i.e., acclimatization). The largest difference between Phase I and Phase II is that Phase II relies on a sound basic, theoretical understanding of what is being measured. By definition, Phase I assessments must be carried out in strictly defined standardized environments and are unbiased. Phase II measurements will be conducted in a variety of environments and the specific physiological assessments will be based on management objectives, e.g., stock allocation and reallocation. In the Phase II context, Ritchie's (1984b) performance attributes are viewed as a potential of perforance, under standard defined conditions. While responses derived under standard conditions allow system performance function to be ascertained (put simply, the seedling is alive and performs under standard conditions, or it is dead), the ability to predict actual field performance of the tested stock is marginal at best. Witness the RGC controversy. Despite the vast sums riding on RGC test results, there still is uncertainty as to what is being measured and how RGC results relate to field performance (Burden 1987, Binder et al. 1988, Landis and Skakel 1988). We propose that a series of simulated, environmental-physiological assessments (SEPA) tests be conducted under varied environmental, perhaps stressful, conditions, so that seedling response surfaces can be generated. The response surfaces can then be used in probability based projections to answer that very important question: *How will the trees perform after planting?* This amounts to developing genetic-environment interaction performance ratings for a range of species. Clearly, realization of the completion of Phase II will require a comprehensive, well planned, multidisciplinary team approach.

In Figure 8.18 (Phase II), we present a hypothetical scheme using the SEPA approach to give an indication of how battery assessments could be done. The choice of assessments and environmental conditions used will depend on the management objectives for that stock (Figure 8.19). For example, Grossnickle et al. (1988) used a battery approach when looking at material and performance attributes—specifically, drought avoidance, drought tolerance, and cold tolerance. Environmental conditions could simulate specific low, moderate, or high stress sites. A high stress site would be a steep southerly aspect and the environmental variables could be high temperature, high insolation, and low soil water potential (due to drought or low soil temperatures). Stock would undergo a two week acclimatization under these conditions with SIVE/F_{VAR} monitoring throughout, followed by evaluation. Post environmental stress evaluations could include RGC, F_{VAR}, EC, TTC, etc. In the short term, this would provide a response surface on which to base reforestation establishment decisions. In the long term, such tests performed in conjunction with field growth measurements will provide base values that ensure field performance of specific stocktypes. It must be demonstrated (cost effectiveness) to both producers and consumers of seedlings that the cost of ensuring seedling health is minor compared to the cost of plantation failure.

The mandated mission of stock quality physiologists for the next decade should be to move stock quality tests toward a greater score on all nine points (Tables 8.6 and 8.7) and to redefine rating criteria. This will be accomplished by test refinement, by developing and establishing new tests which meet the specific rating criteria, but above all, by integrating tests together so that seedling fitness (acclimatization) rather than plant system health (acclimation) is evaluated. When predictive test batteries with their corresponding goals and implications are achieved, the target seedling will no longer be a management concept but a physiological fact.

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