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Title: The Starch Content of Roots and the Osmotic Concentration of Expressed Xylem Sap as Predictors of Douglas-Fir Seedling Quality

Abstract approved: ______________________________

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The goal of this study was to evaluate the ability of two seedling quality evaluation methods to predict the field survival of Douglas-fir (*Pseudotsuga menziesii*) seedlings. The starch reserves in seedlings have been suggested as a possible predictor of seedling quality. Starch reserves have been shown to decrease during cold storage, but there has been no concentrated evaluation of their relationship to seedling quality and field survival. This study has investigated the correlation between starch reserves in Douglas-fir seedling roots (and needles) and subsequent field survival. It has also evaluated the ability of measurements of the osmotic concentration of expressed xylem sap to detect seedling damage and predict field survival.

Results show that neither the starch content of roots nor the osmotic concentration of xylem sap is a reliable predictor of Douglas-fir seedling quality. The study also suggested that the starch content of Douglas-fir needles has no significant relationship to seedling quality.

Starch content of roots increased considerably throughout the winter in seedlings growing in nursery beds. Seedlings lifted and cold stored showed large reductions in root starch reserves due to respiration, but did not necessarily exhibit reduced survival potential in the field. Even some seedlings with very low root starch reserves were able to survive on the field site. Any relationship between starch reserves and overall seedling quality is weak at best.

The osmotic concentration of xylem sap also failed to exhibit a significant correlation with field survival. The test was unable to reliably detect seedling damage, especially damage resulting from cold storage or root desiccation. The study demonstrated that severe freezing damage often results in significantly elevated solute concentrations in expressed xylem sap, apparently due to leakage of cell solutes through ruptured cell membranes. However, osmotic concentration of xylem sap below the level associated with severe freezing damage does not necessarily indicate that the seedlings are healthy. The method may be a quick and easy way to detect seedlings damaged by freezing.

The "standard" root growth potential and stress (OSU vigor) tests are still the most reliable techniques to estimate Douglas-fir seedling quality. The best single predictor of field survival in this study was the mean total length of new roots after one month, which accounted for 51 percent of the variability in field survival.

THE STARCH CONTENT OF ROOTS AND THE OSMOTIC CONCENTRATION OF EXPRESSED XYLEM SAP AS PREDICTORS OF DOUGLAS-FIR SEEDLING QUALITY

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TABLE OF CONTENTS

INTRODUCTION 1

LITERATURE REVIEW 7

GENERAL EXPERIMENTAL METHODS AND STANDARD QUALITY
ASSESSMENT TESTS 22

Seedling Source and Lifting 22

Quality-Reducing Treatments 24
Freezing Treatments 25
Root Desiccation 25
Root Submersion 26
Cold Storage 26

Quality Assessment Tests 26
Growth Chamber Performance 27
OSU Vigor Test 27
Root Growth Potential 28
Field Performance 28

Statistical Analysis 29

Results and Discussion 30

STARCH ANALYSIS 44

Materials and Methods 45

Results and Discussion 47

OSMOTIC CONCENTRATION OF EXPRESSED XYLEM SAP 53

Materials and Methods 53

Results and Discussion 54

DISCUSSION AND SUMMARY 60

BIBLIOGRAPHY 64

APPENDICES 78

A. Starch Analysis of Conifer Tissues 78
B. Purification of Amyloglucosidase 84
C. Purification of a-Amylase 89
D. Amyloglucosidase Assay 94
E. a-Amylase Assay 97
F. Preparation of DEAE-Cellulose 101
G. Preparation of Dialysis Tubing 104

LIST OF FIGURES

Figure | Page

1. Flow Chart of Seedling Processing Procedures ... 23
2. Starch Reserve Loss in Seedling Roots During Cold Storage ... 49
3. Root Starch Reserves Increase Throughout Winter in Douglas-Fir Seedlings in the Nursery Bed..... 51
4. Root Starch Content and Field Survival 52
5. Elevated Xylem Sap Osmolarity As Associated With Poor Field Survival ... 59

LIST OF TABLES

Table | Page

1. Experimental Results From the Field Site 31
2. Experimental Results From the Growth Chamber ... 34
3. Root Growth Potential Results 38
4. OSU Vigor Test Results 42
5. Starch Concentration in Seedling Tissues 48
6. Osmotic Concentration of Expressed Xylem Sap ... 55

THE STARCH CONTENT OF ROOTS AND THE OSMOTIC CONCENTRATION OF EXPRESSED XYLEM SAP AS PREDICTORS OF DOUGLAS-FIR SEEDLING QUALITY

INTRODUCTION

Reforestation of harvested land is an essential component of the forest products industry in the Pacific Northwest. As additional acreage is set aside for preservation as wilderness, recreation areas, and protection of endangered species and sensitive sites, it becomes increasingly important to reestablish productive stands on commercial forest sites.

There are numerous factors that affect the success of reforestation efforts, among them are site preparation, planting practices, protection from animal damage, and control of competing vegetation. One especially important factor is the quality of seedlings used in the reforestation effort. If seedlings used for reforestation have been mistreated or damaged prior to planting, the regeneration effort will likely fail, regardless of investments made in other aspects of the reforestation process. The failure of a single plantation can cost thousands of dollars for purchase of additional seedlings, planting costs, vegetation management, and delayed revenues.

Knowledge of the physiological processes of coniferous seedlings has advanced tremendously during the past few decades. This has enabled nurseries to design culturing practices that ensure the production of vigorous, healthy seedlings for outplanting on reforestation sites. Consequently, most coniferous seedlings produced today are of high quality and are readily established on the outplanting site.

Unfortunately, uncontrollable conditions continue to injure and degrade seedlings. Frost early in the season can damage seedlings that have not hardened sufficiently to withstand freezing temperatures. Similarly, failure of cold storage facilities could subject seedlings to excessively low or high temperatures, which adversely affect seedling quality.

Human error can also result in degradation of seedling quality. For example, parking a truck in the sun when hauling bareroot seedlings can result in boxes of seedlings being exposed to excessively high temperatures. Irreversible tissue damage can also result when bareroot seedlings are accidentally left exposed and their roots dry out.

In some instances, seedling damage can be easily detected by visual inspection. Roots torn during the lifting process can be easily identified, and affected seedlings can be culled by nursery personnel. Seedlings whose stems have been girdled by insects or pathogens can also be easily identified and discarded. However, in many cases it is not possible to detect seedling damage through visual inspection. Damage from freezing or root desiccation often produces no visible symptoms until months after the seedlings have been outplanted.

A reliable, rapid method of evaluating seedling quality would greatly assist foresters and landowners in ensuring that only vigorous, healthy seedlings are planted. If low quality seedlings are planted and the plantation fails, initial expenditures will be for naught, and the reforestation process will have to begin again.

A simple, rapid test that could reliably estimate the physiological status of coniferous planting stock would

enable reforestation personnel to match seedlings' field performance potential with their various planting sites. It could also assist nursery managers with the refinement of cultural practices to ensure the production of the highest quality seedlings possible.

Numerous attempts have been made to develop tests to evaluate seedling quality. Initially, the efforts centered on the morphological characteristics of seedlings, generally based on the assumption that a bigger seedling was a better seedling. After studying the performance of morphological grades of southern pines on plantations throughout the south, Wakely (1948) realized that seedling morphological characteristics were often poorly correlated with field performance, and he introduced the concept of physiological grades for seedlings. Since that time numerous attempts have been made to develop tests that identify the physiological status of coniferous seedlings.

One of the earliest methods developed to evaluate seedling physiological status, and probably the most widely used procedure, is the root growth capacity or root growth potential (RGP) technique, first reported by Stone in 1955. This technique evaluates the ability of a seedling to generate new roots when planted in an optimum environment. Numerous studies have shown that the ability of a seedling to grow new roots when planted in an environment favorable to root growth is a good general indicator of the seedling's physiological status, but it often correlates poorly with field performance (Binder et al. 1988, Landis and Skakel 1988, Ritchie and Tanaka 1990).

Another approach to characterizing the overall physiological status of seedlings was developed at Oregon State University (OSU). The OSU vigor test characterizes seedling

quality on the basis of bud burst and survival of potted seedlings placed in a growth-stimulating environment (Herman and Lavender 1979, McCreary and Duryea 1985). This method also measures the ability of seedlings to survive the stresses associated with operational planting, by exposing roots to desiccating conditions before potting and placing in growth-stimulating conditions.

Other seedling quality evaluation procedures have been designed to measure a specific physiological attribute, rather than identify a seedling's overall physiological status. Examples include plant water potential (Joly 1985), frost hardiness (Timmis 1976), dormancy status (Ritchie 1984), and nutrient content of foliage (Landis 1985).

Still other methods have concentrated on measuring the concentration of important biochemical compounds in seedling tissues. Considerable research has been performed on the relationship between seedling quality and carbohydrate reserves (Marshall 1985). Plant growth regulators (PGR's) have also been investigated as possible indicators of seedling quality (Zaerr 1985), but the lack of understanding of the role of PGR's in seedling physiology has hampered investigations of the relationship between PGR levels and seedling quality.

Despite the extensive research efforts to develop methods of assessing seedling quality, there is still no simple technique to reliably predict the survival of outplanted seedlings. The currently available procedures all have shortcomings. Some require expensive, specialized equipment and highly trained technicians to perform the testing. Others take too long to yield reliable assessments to be useful as routine evaluations of seedling quality. Many fail to consistently correlate well with field performance.

An ideal test of seedling quality would have the following characteristics (Zaerr 1985):

- Yield results rapidly.
- Be simple to perform and understand.
- Be cheap and readily accessible to all potential users.
- Reliably assess seedling quality every time.
- Test seedlings nondestructively so that test subjects could be outplanted.
- Quantitatively assess seedling quality, permitting probability values to be assigned to results.
- Be fully diagnostic and able to identify any seedling damage.

It is unlikely that any single procedure could meet all of these criteria. The goal of the research presented in this thesis was to evaluate the usefulness of two seedling quality tests in predicting survival of outplanted Douglas-fir (Pseudotsuga menziesii) seedlings. Specifically, the research objectives were these:

(1) To compare the starch content of roots and/or needles at the time of planting with RGP and OSU vigor test for their ability to assess coastal Douglas-fir seedling quality, as indicated by subsequent survival in the field.

(2) To compare the osmotic concentration of xylem sap at the time of planting with RGP and OSU vigor test for their ability to assess coastal Douglas-fir seedling quality, as indicated by subsequent survival in the field.

This introduction is followed by a literature review on methods of evaluating seedling quality, with special emphasis

on prior studies of starch content and osmotic concentration of xylem sap as they relate to seedling quality.

The third chapter describes the treatments used to create a range of quality in the seedlings and the quality assessment methods used for comparison (RGP and OSU vigor test). The results from these tests are correlated with seedling survival on the outplanting site.

The fourth chapter briefly presents the materials and methods used to determine the starch content of seedling tissue samples. (Starch analysis methods are described in detail in the appendices.) Results from the starch analyses are presented in detail, including correlations with field survival.

The fifth chapter describes the procedures used to measure the osmotic concentration of xylem sap and presents the results of these analyses. It concludes with a discussion of the ability of this test to assess seedling quality and presents correlations with field survival.

The results of all of the research and analyses are summarized in the final chapter. An evaluation of the ability of starch concentration and osmotic concentration of xylem sap to assess seedling quality is presented.

LITERATURE REVIEW

Reforestation personnel have recognized for many years that some seedling lots exhibit higher growth and survival rates when outplanted than other lots of seedlings. This variability in seedling performance has often been attributed to the condition, or quality, of the seedlings at the time of planting. However, there has been considerable debate over the characteristics that a "high quality" seedling should have.

Since the purpose of planting stock is to become successfully established and grow rapidly in a forest plantation, then seedling quality must be defined in terms of a seedling's survival and growth potential on its designated planting site (Ritchie 1984).

Initial attempts to characterize seedling quality were generally based on seedling size, assuming that a bigger seedling is a better seedling. Occasionally, other factors, such as root form, root-shoot ratio, appearance of winter buds, and presence of secondary needles, were also considered (McCreary 1986). Early studies on "seedling quality" reinforced the belief that bigger seedlings exhibit better field performance.

In an early experiment, Paton (1929) investigated the relationship between seedling size and subsequent growth and survival in the field. After studying seedlings from five coniferous species, he concluded that the smallest seedlings in nursery seed beds were small because of an inherent lack of vigor, and that small trees are weaklings and less desirable than larger ones.

Several subsequent studies provided additional evidence to support the assumption that bigger seedlings are better

seedlings, but results were often inconsistent. Chapman (1948) compared field survival and height growth of various sizes, or grades, of 1-0 shortleaf pine (Pinus echinata) and found close correlation between stem caliper and field performance. However, Chapman (1948) also reported that the shortest seedlings had the greatest survival and height growth in some plots. Pomeroy et al. (1949) reported that although larger grades of jack pine (Pinus banksiana) seedlings exhibited better survival and early height growth than smaller seedlings, there was no difference in size after 13 years.

Results such as these lead Wakely to question the validity of using morphological characteristics as a basis for grading forest-tree seedlings. After investigating the performance of southern pine plantations established in the 1920's, 30's, and 40's, Wakely (1949) concluded that morphological grades were not consistently dependable guides to seedling quality. He found that smaller trees sometimes outperformed larger trees, and that seedlings of the same morphological grade, but raised in different nurseries, showed markedly different survival rates when planted on the same site. Based on these observations, Wakely suggested that "a seedling's ability to resist excessive water loss, to take in water, and to extend its root system promptly, might depend far less on its size and form than on its internal chemical or physiological condition--that is, on its physiological grade."

Following up on his initial observations, Wakely (1954) conducted a 2-year study of seedling morphological grades and "showed conclusively that the physiological qualities of seedlings can overbalance the effects of their morphological grades on survival and growth." However, Wakely did not imply that morphology has no influence on subsequent field performance. This is certainly not the case, as many

studies have shown that initial seedling size can greatly affect field survival and growth (Thompson 1985). Wakely, however, was one of the first to recognize that internal physiological characteristics of seedlings can be more important to field performance than seedling morphology.

A study in the Lake States demonstrated that when 2-0 red pine (Pinus resinosa) seedlings were graded into small, medium, and large classes on the basis of stem caliper, the large stock had much better survival than smaller classes after 10 years (Stoeckeler and Limstrom 1950). Based on superior performance of larger seedlings of ponderosa (Pinus ponderosa) and jeffrey pine (Pinus jeffreyi), Fowells (1953) concluded that, in the absence of a better grading system, discarding smaller trees will result in better field survival and increased plantation growth. Although Zaerr and Lavender (1976) reported better survival of Douglas-fir seedlings weighing more than 4 grams than for seedlings weighing less than 4 grams, they also found that the heaviest classes of seedlings exhibited lower survival rates than medium-sized seedlings.

Initial attempts to develop a method of measuring the physiological status of seedlings concentrated on a seedling's ability to generate new roots. Stone (1955) studied the production of new roots on seedlings from five coniferous species and found that although the seedlings all exhibited similar morphological characteristics, production of new roots differed dramatically. Indeed, he found that nearly all seedlings that failed to generate new roots during their initial 60 days in pots died during a subsequent 120-day growth period.

Subsequently, Stone and co-workers at the University of California at Berkely conducted numerous experiments to further characterize the relationship between a seedling's

ability to generate new roots and its outplanting performance. They concluded that the root regenerating potential (RRP) or the root growth potential (RGP) of a seedling is critical to its success in the field (Stone and Schubert 1959a, Stone and Schubert 1959b, Stone et al. 1961, Stone and Benseler 1962). They defined both RRP and RGP as the ability of a seedling to initiate and/or elongate roots when placed in an environment favorable to root growth. RGP was characterized numerically as the number of new roots produced by a seedling during one month in a greenhouse where soil was maintained at 20C (Stone et al. 1963).

Additional studies also identified a number of factors that influenced a seedling's ability to generate new roots. RGP was shown to vary with date of lifting, and a definite periodicity in RGP was identified for several coniferous species (Stone and Schubert 1959c, Stone and Schubert 1959e, Stone et al. 1962, Stone et al. 1963, Krugman et al. 1965, Stone and Jenkinson 1970, Burr et al. 1989). Additional factors found to affect RGP include the length of cold storage (Stone and Schubert 1959d, Stone and Jenkinson 1971, Omi et al. 1991), the nursery where seedlings were produced (Stone et al. 1963), fumigation of nursery seed beds (Krugman et al. 1965), temperature (Krugman and Stone 1966, Binder et al. 1990), and soil moisture (Stone and Jenkinson 1970).

Many other researchers have investigated RGP and its relationship to environmental conditions, physiological properties, and nursery cultural practices during the last two decades. The relationship between carbohydrate reserves and RGP were examined by van den Driessche (1978) and Ritchie (1982), but neither could identify a significant correlation. Zaerr (1967) found that there was little correlation between RGP and auxin concentration in shoots. Day and MacGillivray (1975) confirmed that low soil moisture

content had a detrimental effect on the RGP of white spruce (Picea glauca) seedlings. Repeated nursery root wrenching was shown to increase RGP for Caribbean pine (Pinus caribaea) (Bacon and Bachelard 1978) and Monterey pine (Pinus radiata) (Rook 1969). Nambiar et al. (1979) demonstrated that low soil temperature adversely affects the initiation and elongation of new Monterey pine roots.

There has also been considerable effort to demonstrate a positive correlation between RGP and field survival. Stone and colleagues found that seedlings with high RGP exhibited the best survival when outplanted (Stone 1955, Stone and Schubert 1959a, Stone and Schubert 1959d, Stone et al. 1961). Similar results have been reported by other researchers (Jenkinson and Nelson 1978, Jenkinson 1980, Sutton 1980, Jenkinson and Nelson 1985, Feret and Kreh 1985, McCreary and Duryea 1987), but the relationship between RGP and field survival rates is not always clear (Dunsworth 1986). Ritchie and Dunlap (1980), in a review of the literature, stated that "while it has been difficult to establish a clear cause-effect relationship between RGP and seedling survival after planting, a compelling body of evidence indicates that the two are often very closely correlated."

The research on the correlation between RGP and field survival has also lead to the development of the concept of "lifting windows" for coniferous seedlings. By studying the effects of various lifting dates and cold storage on RGP and subsequent survival, researchers have identified periods of time during the year (lifting windows) when seedlings can be lifted, stored, and outplanted with little degradation in quality. Extensive research of this phenomenon has shown that lifting windows vary not only for different species, but also by seed source (Jenkinson and Nelson 1978, Jenkinson 1980) and location of nursery (Hermann et al. 1972, Jenkinson 1984).

Although there has been extensive research on the relationship between RGP and field survival, there is little data that clearly demonstrate that poor survival is closely correlated with low RGP, except for poor survival due to untimely lifting and cold storage. Feret et al. (1985) demonstrated, however, that both RGP and field survival of loblolly pine (Pinus taeda) seedlings are affected by varying the temperature and duration of cold storage. McCreary and Duryea (1987) reported that field survival of seedlings subjected to damage from freezing, hot storage, root desiccation, and root submersion in hot water was closely correlated with RGP.

Little research has been done on the relationship between RGP and seedling growth in the field. Significant correlations between height growth and RGP have been reported for both jack pine and white spruce, but the tremendous variability in RGP measurements tended to obscure the relationship (Sutton 1980). The relationship between RGP and first-year height growth has been reported to be curvilinear for both loblolly pine (Feret et al. 1985) and lodgepole pine (Pinus contorta) (Burdett et al. 1983). Significant correlations between RGP and both first- and second-year height growth have been reported for loblolly pine (Feret and Kreh 1985) and Douglas-fir (McCreary and Duryea 1987).

Although RGP is clearly a good indicator of seedling quality, it is only a fair predictor of outplanting survival (Binder et al. 1988, Landis and Skakel 1988, Ritchie and Tanaka 1990). RGP is a point-in-time assessment of seedling quality, providing an indication of high stress resistance or seedling damage. RGP is hypothesized to reflect overall seedling quality due to its relationship with cold hardiness and bud dormancy (Ritchie 1985, Tinus et al. 1986, Burdett 1987, Burr et al. 1989, Ritchie and Tanaka 1990). However,

like other quality assessment tests, RGP does not factor in planting or site quality, which significantly impact outplanting success.

The use of RGP as a predictor of seedling quality is widespread, but there are many problems with this method. The technique lacks accuracy, precision, and repeatability (Binder et al. 1988, Ritchie and Tanaka 1990). RGP test conditions have a tremendous impact on the production of new roots, and optimum conditions vary with the species tested (Binder et al. 1990). Within-test root production is highly variable, and mean RGP values are often poorly correlated with outplanting survival and growth.

Another drawback of RGP is the length of time required to complete the test. Most studies of RGP have used evaluation intervals of 21 to 30 days--an unacceptable delay for most operational decisions regarding whether or not to discard a batch of possibly damaged seedlings. Hydroponic and aeroponic methods of estimating RGP have been developed that yield results in as little as 7 days for some species (Burdett 1979, Rietveld and Tinus 1990).

Even if useful RGP assessments can be made after a reasonably short time period, the technique is tedious and time consuming. The best predictor of field performance has been shown to be the average number of new roots per seedling (McCreary and Duryea 1987), a measurement that can take over an hour for vigorous, healthy seedlings. Recent research has concentrated on less time-consuming methods of estimating RGP, such as semiquantitatively scaling seedlings on the approximate number of new roots (Burdett 1979, Dolata 1986), measuring root volume (Burdett 1979), and measuring root area index (Rietveld 1986, Rietveld and Tinus 1990). Although some of these alternative methods appear promising,

the most rapid RGP assessment techniques require expensive, specialized equipment.

Another method to evaluate seedling quality is the OSU vigor test, developed at Oregon State University over the last 25 years. This procedure monitors bud burst and survival of seedlings potted and maintained in a growth-stimulating environment. Half of the test seedlings are first placed in a "hot, dry" room [32C., 30 percent relative humidity (R.H.)], for 15 minutes before potting. The method was designed to simulate seedling stress associated with lifting, planting, and initial establishment in the field. It is theorized that drying seedling roots prior to potting causes weak, low-quality seedlings (which would likely die if outplanted) to die or exhibit delayed bud burst in the growth chamber. A rating system, based on mortality and time of bud burst, has been developed to predict field performance (McCreary 1986).

The method was first described by Hermann and Lavender (1979). It has since been evaluated by several researchers, primarily at OSU, with mixed results. A positive correlation between growth room survival of stressed seedlings and field survival was reported by Lavender et al. (1980), but the correlation was weak. McCreary and Duryea (1985) reported positive correlations between field survival and survival of both stressed and unstressed seedlings in the growth room. The method was also used operationally for several years and exhibited significant correlations between field survival and growth room survival of both stressed and control seedlings of several species (McCreary 1986). In contrast, Omi et al. (1986) reported poor relationships between field survival and OSU vigor test ratings for Douglas-fir. McCreary and Duryea (1987) reported that growth room survival of both stressed and unstressed seedlings was highly correlated with field performance.

In an experiment comparing the predictive ability of three different quality evaluation techniques, McCreary and Duryea (1987) found that the OSU vigor test had the highest correlation with both first- and second-year field survival. Surprisingly, the study also indicated that survival of both stressed and unstressed seedlings in the growth chamber predicted field performance equally well. The authors consequently concluded that growth room survival of either stressed or unstressed seedlings after 6 weeks could be used to predict field performance, and simply potting seedlings and monitoring their survival and bud burst in a growth room would suffice for the vigor evaluation.

The results from McCreary and Duryea (1987) indicate that field performance can be projected from seedling survival in a growth-stimulating environment. These results have been contradicted by other researchers (Lavender et al. 1980, Omi et al. 1986), suggesting that the technique cannot be relied on to evaluate seedling quality. In addition, the technique requires the use of expensive growth chambers, and results are unavailable for 6 to 8 weeks.

Numerous alternative methods for assessing seedling quality have been investigated during the last two decades. Most rely on measuring a specific physiological attribute, rather than characterizing the overall physiological quality of seedlings. Although these "material attributes" are generally more easily measured, the results frequently have rather low predictive value, unless the measurements are outside of the normal range (Ritchie 1984).

Plant water potential, or plant moisture stress (PMS), is one material attribute that is routinely measured in seedling production facilities to assess seedling physiological status. Most commonly, measurements of PMS are made with a pressure chamber, or "pressure bomb" (Ritchie and

Hinckley 1975, Cleary and Zaerr 1980). PMS measurements are used by nursery personnel to properly schedule irrigation (Zaerr et al. 1981, Cleary et al. 1986) and other culturing practices, including root wrenching and lifting (Burdett and Simpson 1984, Edgren 1984). Measurements of PMS are also used to determine if cold-stored seedlings have adequate moisture content (Cleary and Zaerr 1980). These uses of PMS measurements simply help to ensure that seedling quality is maintained in the nursery and until seedlings are out-planted, but do not actually assess seedling quality.

PMS measurements have also been proposed as a method of assessing seedling frost hardiness and general seedling quality. Bixby and Brown (1974) found an initial decrease in PMS following the freezing of black locust (Robinia pseudoacacia). Similar results were also reported for other species by Timmis (1976). Day and MacGillivray (1975) found that increased PMS readings were exhibited by white spruce seedlings with low RGP measurements. Similarly, significant correlations between RGP and PMS readings have been reported for several hardwood species following cold storage (Webb and von Althen 1980).

In a study of the use of PMS to evaluate seedling quality, McCreary and Duryea (1987) found a significant correlation between changes in PMS of potted seedlings over an 8-day period and field performance. The authors found that the higher the percentage of seedlings whose PMS values were greater than 0.5 megapascals (MPA) or less than 3.0 MPA on the eighth day after potting, the greater the field survival and growth. Interestingly, this research identified a stronger correlation between the PMS evaluation and both first- and second-year field survival, than between RGP and field survival. The strongest correlation with field survival was exhibited by the OSU vigor test. Of the three techniques evaluated, the PMS evaluation had the weakest

correlation with height growth of outplanted seedlings. Clearly, PMS measurements provide a useful assessment of seedling quality, but additional research is required to clarify the relationship between PMS and field performance.

Other methods of evaluating seedling quality have been developed and tested, some have even been used operationally. Examples include assessment of frost hardiness (Timmis 1976, Wallner et al. 1982, Glerum 1985, Burr et al. 1986, Burr et al. 1987, Laacke et al. 1987, Burr et al. 1989), bud dormancy (Lavender 1985, Tinus et al. 1987), root respiration (McCreary and Zaerr 1987), the oscilloscope technique (Askren and Hermann 1979, Holbo et al. 1981), infrared thermography (Weatherspoon and Laacke 1985, Laacke et al. 1987, Orlander et al. 1989), electrical resistance or Shigometer test (McCollough and Wagner 1987), stress-induced volatile emissions (Hawkins and Binder 1990), and chlorophyll fluorescence (Hawkins and Lister 1985, Vidaver and Binder 1987, Vidaver et al. 1988, Hawkins and Binder 1990). Although some of these tests show potential, none has been found to be completely satisfactory at this time.

Two techniques for assessing seedling quality that have shown promise in previous experiments and yield results in a short time are the starch content of seedlings and the osmotic concentration of xylem sap. Extensive investigations of carbohydrate status and its relationship to various physiological attributes have been performed, but little research has investigated the correlation between starch content and seedling quality. In addition, interpretation of the literature is hampered by the diversity of carbohydrate extraction and measurement techniques utilized, which determines the extent of carbohydrate extraction and the actual compounds measured.

For many years researchers have thought that high levels of reserve carbohydrates were necessary for initiation of new root growth (Wakely 1948, Ritchie 1982). However, it is now apparent that factors other than carbohydrate reserves affect RGP.

Van den Driesschhe (1978) found that RGP in red pine increased throughout fall to a peak in midwinter, before decreasing in early spring. In contrast, starch content of red pine stems and roots was relatively unchanged throughout this time period. Starch content of needles did increase throughout fall, but declined to a minimum during midwinter (when RGP was highest), before increasing again in early spring. Starch reserves in white spruce exhibited a similar pattern of change as that found in red pine, but RGP patterns were quite different. White spruce RGP was very high in late summer before dropping to a low level, followed by a gradual increase through the winter months. Van den Driessche concluded that there was little relationship between starch reserves and RGP.

In a study of cold-stored Douglas-fir seedlings, Ritchie (1982) found that total nonstructural carbohydrates declined gradually throughout the storage period, while RGP peaked after 6 months of storage before declining with extended storage. These results do not support a direct relationship between carbohydrate reserves and RGP.

In the same study, Ritchie (1982) measured the total nonstructural carbohydrate reserves of Douglas-fir seedlings lifted at various times. He found that roots and stems showed a gradual increase of carbohydrate reserves throughout winter, reaching the highest levels in early spring. In contrast, total nonstructural carbohydrates in foliage peaked in late January before declining in February, then increased again in March. RGP of these seedlings was low in

early winter before peaking in January, followed by a gradual decline during late winter and early spring.

Similarly, Rose and Whiles (1985) found no relationship between the initial starch content of lateral roots and RGP of loblolly pine seedlings. In this study, nondestructive sampling techniques were used to gather root samples for starch analysis, and the same seedlings were used to determine RGP and then measure starch content again after the RGP test. Although the authors did find a correlation between RGP and root starch content after the RGP test, the coefficient of determination was very low (R^2=0.32).

Witherspoon and Lumis (1986) found that little-leaf linden (Tilia cordata) lifted and planted in the fall exhibited significantly higher RGP than seedlings either lifted in fall and planted in spring or lifted and planted in spring. However, the difference in root starch content of the seedlings was not sufficient to account for the large difference in RGP. Their research did confirm a loss in root starch content during cold storage of the fall-lifted seedlings that were subsequently planted in spring.

Rose (1992) found no relationship between root starch content of loblolly pine seedlings and RGP. Measurements taken over a 30-day period showed that RGP was not related to the percentage of starch initially in the roots, nor was it related to the starch content of new white lateral roots. The study did find that seedlings that produced new roots generally had more starch in all plant components than seedlings without new roots.

These studies clearly show that there is little or no relationship between RGP and carbohydrate reserves of seedlings. Experiments with shading, girdling, and defoliation suggest that root growth is dependent not on reserve

carbohydrates, but on current photosynthates (Richardson 1958, Zaerr et al. 1973, van den Driessche 1978, Marshall 1984). These results do not, however, rule out the possibility that starch reserves could be an indicator of general seedling quality, since only two of the studies evaluated field survival, and in both studies almost all of the seedlings survived (Ritchie 1982, Witherspoon and Lumis 1986).

It is commonly thought that RGP is related to bud dormancy and is primarily an indication of a seedling's readiness to grow (Ritchie and Dunlap 1980, Burr et al. 1989, Ritchie and Tanaka 1990). Carbohydrate status probably is more a reflection of a seedling's ability to withstand stress, such as cold storage, when respiratory losses are not replenished by photosynthesis (Marshall 1985). If this is the case, then carbohydrate reserves may only be important for seedling survival when they are forced to draw heavily on their reserves (Little 1970).

Carbohydrate reserves have been shown to decline during cold storage of many species, including jeffrey pine and ponderosa pine (Hellmers 1962, Omi 1990, Omi and Rose 1990); loblolly pine (Gilmore 1961); mugo pine (Pinus mugo) and Monterey pine (McCracken 1979); black walnut (Juglans nigra), northern red oak (Quercus rubra), white ash (Fraxinus americana), and yellow-poplar (Liriodendron tulipifera) (Rietveld et al. 1982); Scots pine (Pinus sylvestris) (Puttonen 1986); little-leaf linden (Witherspoon and Loomis 1986); and Douglas-fir (Ritchie 1982). Several of these studies demonstrated a correlation between carbohydrate reserves and survival (Gilmore 1961, Hellmers 1962, Rietveld et al. 1982, Puttonen 1986). Puttonen (1986) showed that Scots pine seedlings exhibited significant mortality if total carbohydrate reserves dropped below 2 percent of dry matter during storage, and suggested that this may be a threshold level for seedling survival.

These studies clearly demonstrate that starch levels decline during cold storage due to respiratory loss, and suggest a relationship between starch, or at least total carbohydrate reserves, and seedling survival. Since field survival is the ultimate test of seedling quality, this suggests that starch levels may be a reliable indicator of seedling quality. However, the lack of a relationship between RGP and carbohydrate reserves contradicts a clear linear relationship and indicates that if starch reserves impact on seedling quality it may be in the form of a threshold level, as suggested by Puttonen (1986).

There has been very little research performed on the relationship between the osmotic concentration of xylem sap and seedling quality. A preliminary study by Joly (1985) showed that lethal temperatures, both high and low, resulted in an increased osmotic concentration of expressed xylem sap. Joly concluded that the elevated osmotic concentration of expressed xylem sap in seedlings killed by lethal temperatures is due to disruption of the cell membranes, permitting solute leakage from the cell contents. Undamaged seedlings showed very low osmotic concentrations, suggesting that high osmotic concentrations of expressed xylem sap may be a reliable indicator of seedling damage and an estimator of seedling quality. Obviously, much more research is needed to clarify the relationship between the osmotic concentration of xylem sap and seedling quality.

GENERAL EXPERIMENTAL METHODS AND STANDARD QUALITY ASSESSMENT TESTS

The preceeding literature review describes many of the previously investigated tests for evaluating seedling quality and includes an indepth discussion of the most common operationally used tests currently available. To enhance the investigation of the ability of starch content and osmotic concentration of xylem sap to estimate seedling quality, several of the "standard" quality assessment tests were run concurrently. This permitted comparison of results from the new methods with the predictive ability of "standard" tests, in addition to analyzing the new methods' ability to estimate seedling field survival. The "standard" quality assessment tests chosen for comparison include root growth potential (RGP), OSU vigor test, and growth room survival.

This chapter provides a description of the experimental design, seedling stock, and quality-reducing treatments used in this investigation. An overview of the seedling processing procedures utilized in this study is illustrated in Figure 1. The chapter also presents results from the "standard" quality assessment tests and the outplanting site, including a statistical analysis of the predictive ability of the "standard" tests.

Seedling Source and Lifting

The study was performed over two planting seasons: February to June 1985 and October 1985 to March 1986. Seedling stock utilized was 2-0 bareroot Douglas-fir seedlings grown at the D. L. Phipps State Forest Nursery near Elkton, Oregon. The first portion of the project consisted of 2,200 seedlings lifted on February 6, 1985, grown from seed originating in Oregon seed zone number 252.

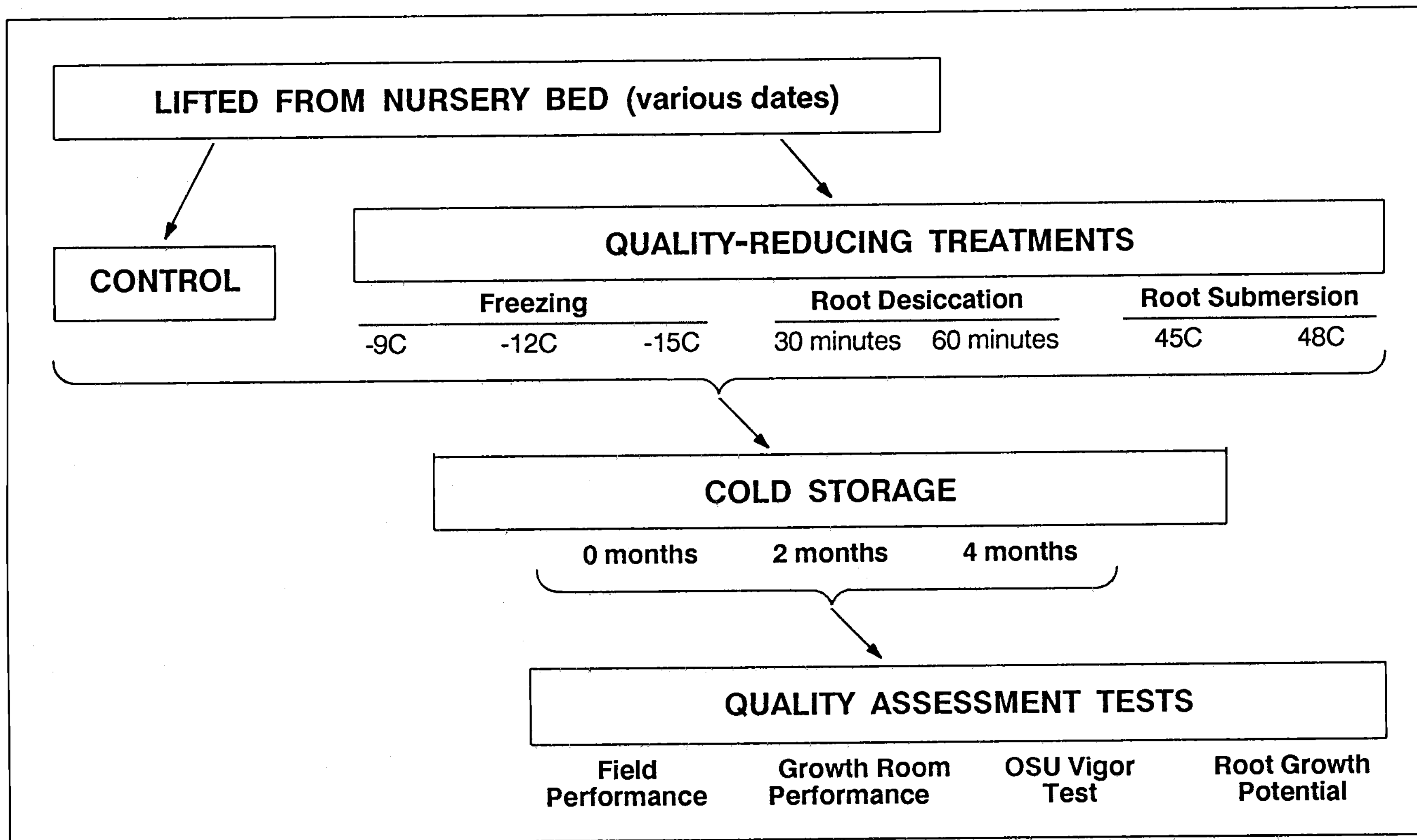

Figure 1. Flow Chart of Seedling Processing Procedures. After lifting, seedlings were split into groups. Each group received one quality-reducing treatment. They were then divided into three subgroups for the storage component (not all lifts were subjected to storage). After storage, the subgroups were further divided for quality assessment.

The second part consisted of seedlings from four lifting dates: 1,100 seedlings on October 22, 1985; 200 seedlings on December 5, 1985; 1,500 seedlings on January 17, 1986; and 900 seedlings on March 10, 1986. All of these latter seedlings were grown from seed collected in Oregon seed zone number 491.

Seedlings were inspected after lifting and graded to ensure relative uniformity. All damaged seedlings (other than minor root damage) and seedlings with multiple tops were discarded. Seedlings from the first season were generally smaller than those from the second season. Consequently, the minimum seedling stem caliper retained was 3 mm for the first season and 4 mm for the second season.

The seedlings were then randomly divided into groups for application of quality-reducing treatments. Roots of the seedlings were washed to remove any clinging soil, then pruned to 25 cm with a paper cutter. Excess water was shaken off the roots before placing the seedlings into double plastic bags and cold storage until treatment and planting.

Quality-Reducing Treatments

Seedlings were subjected to one of several quality-reducing treatments to create a range of quality in the study population. Treatments chosen included the following:

- Freezing to -9C, -12C, or -15C.
- Root desiccation for 30 minutes or 60 minutes.
- Root submersion in 45C or 48C water.
- Cold storage for 60 days or 120 days.
- Control (no quality-reducing treatment).

Only some of the treatments were applied to any single lift of seedlings, and no group of seedlings was subjected

to more than two of the quality-reducing treatments (one of which was always cold storage). A brief description of each treatment follows.

Freezing Treatments

Freezing treatments were performed in a Kalt freezing chamber that was programmed to equilibrate at 4C for 1 hour, then decrease by 2C per hour to a final temperature of -9C, -12C, or -15C, where it remained for 1 hour. Seedlings subjected to freezing treatments were enclosed in plastic bags, 30 to 40 seedlings per bag to ensure even freezing, and placed in a single layer in the freezing chamber. Upon reaching the target temperature, individual bags of seedlings were carefully removed and placed in a cold room (4C) to thaw slowly. Freezing treatments simulate the damage resulting from frost in the field or the failure of a cold storage facility.

Root Desiccation

Root desiccation was simulated in a controlled atmosphere room maintained at 35C, with 35 percent relative humidity (R.H.). Seedlings to be treated were subdivided into groups of 30 for easier handling, then their roots were gently patted dry with absorbent cloths. Root desiccation was performed by hanging seedlings singly on a rope stretched across the hot, dry room, and held upright by clothespins. After the designated drying time, seedlings were sequentially removed from the rope and placed in a bucket of water for 5 minutes to rehydrate the roots (Hermann 1967, McCreary and Duryea 1985). Excess water was shaken off the roots and the seedlings were placed back into the cold room until planted. These treatments simulate seedling damage that may result from root exposure during lifting and planting operations.

Root Submersion

These treatments were performed in a large sink filled with hot water, the temperature of which was carefully monitored and maintained within 1 degree of the desired temperature. Seedlings subjected to root submersion were initially separated into groups of 40 seedlings, and a twist-tie was placed around each group. Roots of the seedlings were placed into the hot water (45C or 48C) for exactly 15 minutes. After removal from the hot water, excess water was shaken off the roots, and the treated seedlings were returned to the cold room until planted. These treatments were designed to simulate the rapid heating that can occur when seedlings are improperly stored or left exposed to direct sunlight.

Cold Storage

Cold storage was performed in a cold room maintained at 4C, plus or minus 2C. Seedlings were placed in double plastic bags, and the bags stored on wooden racks to maintain uniform cooling. Stored seedlings were inspected monthly, and their roots were moistened, if necessary. Mold growth was not a problem on seedlings stored for 60 days, but was apparent on some of the previously treated seedlings that were subsequently stored for 120 days. However, mold growth was generally minimal, and no chemicals were used to inhibit molding.

Quality Assessment Tests

At the time of planting, each group of treated seedlings was randomly subdivided into groups of 20 seedlings for quality assessment. In addition to field performance, three "standard" tests were used in this study.

They were growth chamber performance, root growth potential, and the OSU vigor test. Results from each test were used to formulate regression equations for predicting field survival and to provide a basis for comparison with the two quality assessment tests being evaluated.

Growth Chamber Performance

Seedlings utilized for growth chamber performance were planted five per pot in forest soil and placed in the growth chamber. A single growth chamber was used throughout this study. Pots were watered every other day to maintain the soil near field capacity. The growth chamber was maintained at 20C with a 16-hour photoperiod.

Seedling buds were monitored biweekly throughout the growth period and rated on the following scale of 0 to 5:

Rating	Bud Condition
0	dormant--dark brown
1	scales beginning to lighten
2	scales light brown, tip of bud yellow
3	bud burst--needles just visible
4	needles exposed and expanding
5	flush--needles expanded and stem visible

Data were collected on the terminal and most advanced lateral buds on each seedling. At the end of the growth period (approximately 60 days), the final condition of each seedling was recorded. A seedling was classified as dead if the cambium at the root collar was brown and desiccated.

OSU Vigor Test

This test is based on the comparison of bud burst and survival of stressed and unstressed seedlings placed in a

growth-stimulating environment. The handling of unstressed seedlings is described above. Stressed seedlings were subjected to root desiccation for 15 minutes in a hot, dry room (35C, 35 percent R.H.) (Hermann and Lavender 1979, McCreary and Duryea 1985). After rehydration of their roots, seedlings were planted five per pot and placed in the growth chamber. Bud burst and survival were monitored as described above.

Root Growth Potential

Before planting seedlings for determination of RGP, they were inspected for new root growth, and new active root tips were removed, if present. Seedlings were then potted five per pot and placed in the growth chamber. After 1 month of growth (28 days the first season, 30 days the second season), seedlings were removed from the pots, their roots washed, and all new white root tips greater than 5 mm in length were measured and recorded (Ritchie 1985).

Field Performance

The outplanting site was located in the OSU Forest Genetics Nursery, 11 km north of Corvallis, Oregon. The field plot was laid out in blocks of four rows, 0.3 m apart. Five seedlings were planted per row, at 0.3 m spacing. Treatments were randomly assigned to individual rows to negate any variation in microsite conditions within the field plot. On each planting date, all seedlings were planted with a shovel in a single day.

Vegetation on the field site was controlled manually. Seedlings were protected from deer damage through the application of Big Game Repellant (Powder-BGR-P, Deer-Away, Minneapolis, Minnesota) at the time of bud burst. This precaution was insufficient to prevent all damage, however, and

several seedlings received significant browsing. Pocket gophers on the site were controlled through poison baiting (Gopher Bait, ORCO, Eugene, Oregon), and damage from these animals was limited to one seedling.

Buds of the outplanted seedlings were monitored regularly throughout spring and early summer, the period of active shoot elongation. Survival was determined on the following November 1st, after the commencement of the fall rains.

Statistical Analysis

Survival data collected on the seedlings were converted to an average percent survival for each treatment and quality assessment test. These data were then normalized through an arcsine square root transformation for use in the formation of predictive regression equations.

Data collected on date of bud burst and bud flush were evaluated as the number of days from planting until burst and flush for the terminal and most advanced lateral buds. The mean number of days until bud burst and bud flush was calculated for each treatment and quality assessment test, then transformed by taking the square root. For evaluation with the OSU vigor test, the percentage of stressed and unstressed seedlings that had experienced bud burst after 8 weeks was also calculated.

RGP data were evaluated as the total number of new roots greater than 5 mm in length, as well as the total combined length of all new roots. Means were calculated for each treatment, and the total number of new roots was transformed by taking the square root. These results were used in the formation of regression equations.

Through stepwise linear regression of field survival on the data from each quality assessment test, the "best" predictive equation was formulated for each method. Predictive variables in each equation were selected by comparison of F-values; those variables with the highest F-values (and, consequently, the highest correlation with field survival) were included in the equation if the correlation was significant ($P \leq 0.10$). The "best" equation was chosen on the basis of the highest R^2 value. Residual plots were formed for each of the "best" regression equations to investigate whether the equation adequately fit the data.

Results and Discussion

Table 1 presents the complete results from this study on the field site, while Table 2 presents the same data from the growth chamber. The use of quality-reducing treatments was very successful in creating a wide range of quality within the study population, both in the field and the controlled environment chamber.

In general, seedling survival on the outplanting site was considerably less than in the growth chamber. Although this is rather common, due to the less than optimum conditions in the natural environment, field survival in this study was adversely impacted by several factors.

In the first season of this study, the cold storage of the seedlings delayed planting until mid-April and mid-June, well past the optimum time for planting. To make things even worse, the weather during the summer of 1985 was very hot and dry. Consequently, only 3 of the 160 seedlings planted in June 1985, after 4 months of cold storage, survived until November 1985.

Adverse weather conditions also contributed to reduced field survival during the second season of the study.

Table 1. Experimental Results From the Field Site.

LIFT DATE	TREATMENT APPLIED[1]	COLD STORAGE (Months)	PERCENT SURVIVAL	MEAN DAYS TO BUD BURST		MEAN DAYS TO BUD FLUSH	
				Lateral	Terminal	Lateral	Terminal
1-85	None	0	60	16.8	31.9	26.7	38.9
1-85	30 mins. RD	0	30	28.9	41.1	37.1	48.5
1-85	60 mins. RD	0	35	27.7	41.0	36.6	47.4
1-85	-9C FR	0	40	14.1	27.8	22.9	35.7
1-85	-12C FR	0	15	17.1	37.8	25.3	41.9
1-85	-15C FR	0	0	12.3	28.7	20.3	29.0
1-85	45C RS	0	45	17.1	27.9	24.8	34.0
1-85	48C RS	0	60	17.2	38.1	25.3	43.9
1-85	None	2	0	12.8	19.2	18.6	25.6
1-85	30 mins. RD	2	20	16.7	19.7	21.5	24.0
1-85	60 mins. RD	2	15	20.9	28.1	26.4	34.1
1-85	-9C FR	2	45	18.4	17.2	23.0	21.3
1-85	-12C FR	2	0	26.7	31.0	33.5	34.0
1-85	-15C FR	2	0	11.7	13.0	19.0	21.0
1-85	45C RS	2	40	17.3	24.4	24.7	30.2
1-85	48C RS	2	50	13.8	22.2	20.1	27.5
1-85	None	4	5	8.0	9.8	12.9	13.9
1-85	30 mins. RD	4	0	10.8	14.4	16.4	19.4
1-85	60 mins. RD	4	0	25.4	22.5	27.8	28.9

- - Continued. See footnote at end of table. - -

Table 1. Experimental Results From the Field Site (Continued).

LIFT DATE	TREATMENT APPLIED[1]	COLD STORAGE (Months)	PERCENT SURVIVAL	MEAN DAYS TO BUD BURST		MEAN DAYS TO BUD FLUSH	
				Lateral	Terminal	Lateral	Terminal
1-85	-9C FR	4	0	9.6	11.3	14.1	16.0
1-85	-12C FR	4	0	17.5	13.0	24.0	16.0
1-85	-15C FR	4	0	10.0	N/A	N/A	N/A
1-85	45C RS	4	10	7.9	9.4	12.7	13.5
1-85	48C RS	4	0	11.6	13.2	15.7	17.1
10-85	None	0	15	67.3	101.0	84.2	104.0
10-85	-9C FR	0	0	100.0	N/A	110.0	N/A
10-85	-12C FR	0	0	N/A	N/A	N/A	N/A
10-85	-15C FR	0	0	50.0	N/A	54.0	N/A
10-85	15 mins. RD	0	10	71.8	N/A	80.7	N/A
10-85	30 mins. RD	0	15	71.7	N/A	86.0	N/A
10-85	60 mins. RD	0	0	N/A	N/A	N/A	N/A
10-85	None	2	55	42.7	72.9	51.0	77.9
10-85	None	4	20	28.8	33.2	34.4	37.8
12-85	None	0	75	32.7	90.8	46.7	97.3
1-86	None	0	90	19.0	38.3	27.0	44.0
1-86	-9C FR	0	60	28.1	40.7	38.0	46.3
1-86	-12C FR	0	90	24.4	35.7	31.5	42.4
1-86	-15C FR	0	35	24.6	39.1	34.7	44.8

- - Continued. See footnote at end of table. - -

Table 1. Experimental Results From the Field Site (Continued).

LIFT DATE	TREATMENT APPLIED[1]	COLD STORAGE (Months)	PERCENT SURVIVAL	MEAN DAYS TO BUD BURST		MEAN DAYS TO BUD FLUSH	
				Lateral	Terminal	Lateral	Terminal
1-86	15 mins. RD	0	70	22.3	38.3	29.7	44.6
1-86	30 mins. RD	0	60	30.5	43.1	37.9	49.1
1-86	60 mins. RD	0	35	39.9	46.7	49.8	52.9
1-86	None	2	95	14.3	20.3	20.7	25.2
1-86	-9C FR	2	15	18.7	22.1	26.1	27.0
1-86	-15C FR	2	0	37.7	51.5	46.8	55.5
1-86	15 mins. RD	2	65	19.2	23.8	25.7	28.0
1-86	30 mins. RD	2	30	25.4	27.9	31.3	32.4
1-86	60 mins. RD	2	0	38.6	26.3	47.6	30.0
3-86	None	0	100	5.0	12.9	14.0	17.7
3-86	-9C FR	0	20	4.6	12.4	18.6	19.5
3-86	-12C FR	0	15	5.1	13.3	15.6	21.0
3-86	-15C FR	0	0	7.1	13.2	12.4	13.5
3-86	15 mins. RD	0	85	5.2	15.3	14.9	20.5
3-86	30 mins. RD	0	45	6.1	28.4	37.1	32.8
3-86	60 mins. RD	0	0	18.6	15.0	51.3	21.0

[1] Abbreviations used: RD = Root Desiccation; FR = Freezing; RS = Root Submersion; C = Degrees Centigrade; N/A = Not Applicable.

Table 2. Experimental Results From the Growth Chamber.

LIFT DATE	TREATMENT APPLIED[1]	COLD STORAGE (Months)	PERCENT SURVIVAL	MEAN DAYS TO BUD BURST		MEAN DAYS TO BUD FLUSH	
				Lateral	Terminal	Lateral	Terminal
1-85	None	0	80	16.8	31.9	26.7	38.9
1-85	30 mins. RD	0	75	28.9	41.1	37.1	48.5
1-85	60 mins. RD	0	65	27.7	41.0	36.6	47.4
1-85	-9C FR	0	45	14.1	27.8	22.9	35.7
1-85	-12C FR	0	35	17.1	37.8	25.3	41.9
1-85	-15C FR	0	0	12.3	28.7	20.3	29.0
1-85	45C RS	0	90	17.1	27.9	24.8	34.0
1-85	48C RS	0	80	17.2	38.1	25.3	43.9
1-85	None	2	100	12.8	19.2	18.6	25.6
1-85	30 mins. RD	2	100	16.7	19.7	21.5	24.0
1-85	60 mins. RD	2	80	20.9	28.2	26.4	34.1
1-85	-9C FR	2	15	18.4	17.3	23.0	21.3
1-85	-12C FR	2	10	26.7	31.0	33.5	34.0
1-85	-15C FR	2	10	11.7	13.0	19.0	21.0
1-85	45C RS	2	90	17.3	24.4	24.7	30.2
1-85	48C RS	2	100	13.8	22.2	20.1	27.5
1-85	None	4	80	8.0	9.8	13.0	13.9
1-85	30 mins. RD	4	80	10.8	14.4	16.4	19.4
1-85	60 mins. RD	4	60	25.4	22.5	27.8	28.9

- - Continued. See footnote at end of table. - -

Table 2. Experimental Results From the Growth Chamber (Continued).

LIFT DATE	TREATMENT APPLIED[1]	COLD STORAGE (Months)	PERCENT SURVIVAL	MEAN DAYS TO BUD BURST		MEAN DAYS TO BUD FLUSH	
				Lateral	Terminal	Lateral	Terminal
1-85	-9C FR	4	40	9.6	11.3	14.1	16.0
1-85	-12C FR	4	10	17.5	13.0	24.0	16.0
1-85	-15C FR	4	0	10.0	N/A	N/A	N/A
1-85	45C RS	4	85	7.9	9.4	12.7	13.5
1-85	48C RS	4	65	11.6	13.2	15.7	17.1
10-85	None	0	100	67.3	101.0	84.2	104.0
10-85	-9C FR	0	5	100.0	N/A	110.0	N/A
10-85	-12C FR	0	0	N/A	N/A	N/A	N/A
10-85	-15C FR	0	0	50.0	N/A	54.0	N/A
10-85	15 mins. RD	0	70	71.8	N/A	80.7	N/A
10-85	30 mins. RD	0	15	71.7	N/A	86.0	N/A
10-85	60 mins. RD	0	10	N/A	N/A	N/A	N/A
10-85	None	2	45	42.8	72.9	51.0	77.9
10-85	None	4	35	23.8	33.2	34.4	37.8
12-85	None	0	100	32.7	90.8	46.7	97.3
1-86	None	0	100	19.0	38.4	27.0	44.0
1-86	-9C FR	0	75	28.1	40.7	38.0	46.3
1-86	-12C FR	0	100	24.4	35.7	31.5	42.4
1-86	-15C FR	0	80	24.6	39.1	34.7	44.9

- - Continued. See footnote at end of table. - -

Table 2. Experimental Results From the Growth Chamber (Continued).

LIFT DATE	TREATMENT APPLIED[1]	COLD STORAGE (Months)	PERCENT SURVIVAL	MEAN DAYS TO BUD BURST		MEAN DAYS TO BUD FLUSH	
				Lateral	Terminal	Lateral	Terminal
1-86	15 mins. RD	0	100	22.3	38.3	29.7	44.6
1-86	30 mins. RD	0	95	30.5	43.1	37.9	49.1
1-86	60 mins. RD	0	70	39.9	46.7	49.8	52.9
1-86	None	2	100	14.4	20.3	20.7	25.3
1-86	-9C FR	2	70	18.7	22.1	26.1	27.0
1-86	-15C FR	2	20	37.7	51.5	46.8	55.5
1-86	15 mins. RD	2	100	19.2	23.8	25.7	28.1
1-86	30 mins. RD	2	90	25.4	27.9	31.3	32.4
1-86	60 mins. RD	2	50	38.6	26.3	47.6	30.0
3-86	None	0	100	5.0	12.9	14.0	17.7
3-86	-9C FR	0	20	4.7	12.4	18.6	19.5
3-86	-12C FR	0	20	5.1	13.3	15.6	21.0
3-86	-15C FR	0	0	7.1	13.2	12.4	13.5
3-86	15 mins. RD	0	100	5.2	15.3	15.0	20.5
3-86	30 mins. RD	0	90	6.1	28.4	37.1	32.8
3-86	60 mins. RD	0	65	18.6	15.0	51.3	21.0

[1]Abbreviations used: RD = Root Desiccation; FR = Freezing; RS = Root Submersion; C = Degrees Centigrade; N/A = Not Applicable.

Shortly after planting the seedlings lifted in October 1985, the weather turned unseasonably cold and dry. Many of the seedlings had not sufficiently hardened to withstand the below freezing temperatures, and only 8 of the 140 seedlings planted at that time survived until November 1986. In contrast, 55 percent of the October-lifted seedlings planted in the field after 2 months cold storage survived until November 1986.

Previous investigators have demonstrated a very high correlation between growth chamber and field performance (McCreary and Duryea 1985). A highly significant relationship was also exhibited in this study, but the correlation between field survival and growth chamber survival was considerably less than in previous studies (r=0.69). Indeed, the residual plot from regression of growth room survival on field survival (both normalized by an arcsine squareroot transformation) showed heteroscedasticity, which was not eliminated through weighted least squares regression. The severe conditions on the field site, which resulted in reduced survival, certainly contributed to this rather weak correlation. The lack of a strong relationship between growth chamber and field survival simply demonstrates, once again, that many factors besides seedling quality affect growth and survival in the natural environment (McCreary 1986).

Results from the RGP evaluation are shown in Table 3. As found in previous investigations, there was a highly significant relationship between RGP and seedling survival, both in the growth chamber and on the field site. The RGP measurement that predicted growth chamber survival best was the mean number of new roots greater than 5 mm in length (normalized by taking squareroot), which accounted for 73 percent of the variability in growth chamber survival (R^2= 0.73). In contrast, the mean total length of new roots had the greatest correlation with field survival (R^2=0.51), but accounted for only a little over half of the variability.

Table 3. Root Growth Potential Results.

LIFT DATE	TREATMENT APPLIED[1]	COLD STORAGE (Months)	PERCENT SURVIVAL	MEAN NUMBER OF NEW ROOTS	MEAN TOTAL LENGTH (cm) OF NEW ROOTS
1-85	None	0	100	96.8	1,033
1-85	30 mins. RD	0	100	60.9	799
1-85	60 mins. RD	0	95	22.3	269
1-85	-9C FR	0	100	104.6	1,016
1-85	-12C FR	0	95	62.3	655
1-85	-15C FR	0	40	0.0	0
1-85	45C RS	0	100	27.6	396
1-85	48C RS	0	100	44.1	519
1-85	None	2	100	110.4	1,512
1-85	30 mins. RD	2	100	156.5	1,735
1-85	60 mins. RD	2	90	47.5	567
1-85	-9C FR	2	100	58.0	598
1-85	-12C FR	2	50	27.0	275
1-85	-15C FR	2	0	0.0	0
1-85	45C RS	2	85	108.2	1,505
1-85	48C RS	2	100	154.6	1,965
1-85	None	4	100	94.9	1,117
1-85	30 mins. RD	4	100	109.5	1,218
1-85	60 mins. RD	4	85	43.2	445

- - Continued. See footnote at end of table. - -

Table 3. Root Growth Potential Results (Continued).

LIFT DATE	TREATMENT APPLIED[1]	COLD STORAGE (Months)	PERCENT SURVIVAL	MEAN NUMBER OF NEW ROOTS	MEAN TOTAL LENGTH (cm) OF NEW ROOTS
1-85	-9C FR	4	60	47.0	460
1-85	-12C FR	4	35	22.0	226
1-85	-15C FR	4	5	0.0	0
1-85	45C RS	4	100	98.5	1,172
1-85	48C RS	4	85	105.3	1,323
10-85	None	0	95	66.2	906
10-85	-9C FR	0	10	0.0	0
10-85	-12C FR	0	45	0.0	0
10-85	-15C FR	0	35	0.0	0
10-85	15 mins. RD	0	85	45.2	647
10-85	30 mins. RD	0	75	0.0	0
10-85	60 mins. RD	0	80	0.0	0
10-85	None	2	55	24.7	428
10-85	None	4	45	20.9	330
12-85	None	0	100	185.8	2,731
1-86	None	0	100	141.0	2,042
1-86	-9C FR	0	70	74.5	902
1-86	-12C FR	0	100	109.5	1,565
1-86	-15C FR	0	95	74.5	948

- - Continued. See footnote at end of table. - -

Table 3. Root Growth Potential Results (Continued).

LIFT DATE	TREATMENT APPLIED[1]	COLD STORAGE (Months)	PERCENT SURVIVAL	MEAN NUMBER OF NEW ROOTS	MEAN TOTAL LENGTH (cm) OF NEW ROOTS
1-86	15 mins. RD	0	100	154.0	2,343
1-86	30 mins. RD	0	100	119.3	1,857
1-86	60 mins. RD	0	100	23.4	293
1-86	None	2	100	182.7	2,492
1-86	-9C FR	2	95	59.8	785
1-86	-15C FR	2	60	0.4	3
1-86	15 mins. RD	2	100	160.2	2,285
1-86	30 mins. RD	2	100	134.3	1,803
1-86	60 mins. RD	2	80	14.1	162
3-86	None	0	100	153.0	1,978
3-86	-9C FR	0	45	17.3	221
3-86	-12C FR	0	15	3.9	35
3-86	-15C FR	0	5	0.0	0
3-86	15 mins. RD	0	100	205.2	2,408
3-86	30 mins. RD	0	100	42.4	376
3-86	60 mins. RD	0	60	1.4	12

[1]Abbreviations used: RD = Root Desiccation; FR = Freezing; RS = Root Submersion; C = Degrees Centigrade; N/A = Not Applicable.

The strong relationship between RGP and seedling survival was clearly demonstrated in this investigation, but it is also clear that more than the ability to grow new roots is essential for seedling survival, especially in the natural environment.

Table 4 shows the results from the OSU vigor test. In many cases the test successfully identified lots of seedlings with poor seedling quality that subsequently exhibited low survival rates in the field, confirming the value of this quality assessment test. On the other hand, the test identified several seedling lots as good to excellent in quality, but field survival was very poor. The severe conditions on the field site certainly contributed to this lack of correlation.

The mixed results from these "standard" quality assessment tests are not surprising and clearly demonstrates the difficulty in predicting field performance of Douglas-fir seedlings. The strong correlation between the test results and actual field survival indicates that the quality attributes assessed by these tests (i.e., root growth, stress resistance), are important components of seedling quality. However, the results also clearly indicate that many other factors, in addition to seedling quality, affect a seedling's field performance.

Table 4. OSU Vigor Test Results.

LIFT DATE	TREATMENT APPLIED[1]	COLD STORAGE (Months)	GROWTH ROOM PERCENT SURVIVAL		DIFFERENCE IN PERCENT BUD BURST	PREDICTED QUALITY	FIELD PERCENT SURVIVAL
			Unstres'd	Stressed			
1-85	None	0	80	75	5	Fair	60
1-85	30 mins. RD	0	75	80	15	Fair	30
1-85	60 mins. RD	0	65	45	25	Poor	35
1-85	-9C FR	0	45	85	-5	Poor	40
1-85	-12C FR	0	35	20	30	Poor	15
1-85	-15C FR	0	0	0	40	Poor	0
1-85	45C RS	0	90	70	10	Fair	45
1-85	48C RS	0	80	70	15	Fair	60
1-85	None	2	100	100	5	Excellent	0
1-85	30 mins. RD	2	100	90	10	Good	20
1-85	60 mins. RD	2	80	10	45	Poor	15
1-85	-9C FR	2	15	30	-30	Poor	45
1-85	-12C FR	2	10	35	-30	Poor	0
1-85	-15C FR	2	10	0	15	Poor	0
1-85	45C RS	2	90	100	-5	Good	40
1-85	48C RS	2	100	100	-10	Excellent	50
1-85	None	4	80	80	0	Fair	5
1-85	30 mins. RD	4	80	55	15	Poor	0
1-85	60 mins. RD	4	60	35	35	Poor	0

- - Continued. See footnote at end of table. - -

Table 4. OSU Vigor Test Results (Continued).

LIFT DATE	TREATMENT APPLIED[1]	COLD STORAGE (Months)	GROWTH ROOM PERCENT SURVIVAL		DIFFERENCE IN PERCENT BUD BURST	PREDICTED QUALITY	FIELD PERCENT SURVIVAL
			Unstres'd	Stressed			
1-85	-9C FR	4	40	60	15	Poor	0
1-85	-12C FR	4	10	15	-5	Poor	0
1-85	-15C FR	4	0	0	5	Poor	0
1-85	45C RS	4	85	85	10	Good	10
1-85	48C RS	4	65	75	20	Fair-Poor	0
10-85	None	0	100	70	15	Fair-Poor	15
1-86	None	0	100	100	0	Excellent	90
3-86	None	0	100	100	0	Excellent	100

[1]Abbreviations used: RD = Root Desiccation; FR = Freezing; RS = Root Submersion; C = Degrees Centigrade; N/A = Not Applicable.

STARCH ANALYSIS

Previous research has identified several compounds, including starch, sugars, hemicelluloses, fats, and fatty acids, that act as energy reserves in seedlings (Kreuger and Trappe 1967, Glerum 1980, Distelbarth et. al. 1984, Loescher et al. 1990). The contribution of hemicelluloses to energy reserves is still unclear, and the difficulty in characterizing and measuring these compounds eliminated them from consideration as an indicator of seedling quality. Fats and fatty acids are known to be important energy reserves in coniferous species, and in Douglas-fir specifically (Krueger and Trappe 1967, Glerum 1980), but the level of these reserves does not change dramatically through the year. It seems unlikely that these compounds would provide an estimation of seedling quality.

On the other hand, starch and sugar reserves have been shown to fluctuate considerably from season to season and have been suggested as a reliable indicator of seedling quality (Krueger and Trappe 1967, Marshall 1985, Puttonen 1986). Previous studies with Douglas-fir have shown that total nonstructural carbohydrate reserves increase throughout winter before decreasing with the onset of spring growth (Ritchie 1982). Krueger and Trappe (1967) demonstrated that sugar concentrations peak during mid-winter, while starch content peaks several months later.

In this study, we chose to concentrate solely on starch reserves as a predictor of seedling quality; therefore, it was essential that the methods utilized were specific for starch. Many methods of measuring carbohydrate reserves have been developed over the years (Heinze and Murneek 1940, Krueger and Trappe 1967, Ebell 1969, Beutler 1984, Rose et al. 1991), but only enzyme analyses are specific for starch.

The enzymic hydrolysis method described by Haissig and Dixon (1979) was chosen as the safest, easiest, and most reliable starch measurement technique and was utilized in this study. Minor modifications were made to streamline the method without sacrificing reliability and accuracy (Beutler 1984, Pazur 1985, Rose et al. 1991).

This chapter briefly describes the methods used to measure starch. A full description of the enzyme purification and starch measurement techniques is included in the appendices. Results from the starch analyses are also presented in this chapter, and the relationship of starch to seedling quality is evaluated.

Materials and Methods

Tissue samples for starch analysis were collected from the seedlings immediately before planting (1 day before planting on the field site). Each sample consisted of several roots or needles removed from each seedling and pooled over a group of five seedlings (each pot in the growth chamber or row in the field consisted of five seedlings). Subsequently, tissue samples for many of the treatments were pooled over a group of 20 seedlings prior to analysis.

Tissue samples were placed in plastic bags and immediately frozen to -20C. Tissue samples were transferred to a -80C freezer for long-term storage within 8 hours of collection.

In preparation for starch measurement, tissue samples were transferred to glass vials and steamed for 5 to 10 minutes to denature the native enzymes (Loomis 1985). The tissue samples were then dried by lyophilization and ground to pass through a 40-mesh screen.

Soluble sugars, pigments, and other interfering compounds were subsequently extracted with a methanol:chloroform: water solution at least three times to ensure complete extraction. Extracted tissue samples were dried overnight at 50C to evaporate any residual solvent.

Starch was solubilized by mixing the extracted tissue pellet with 0.1 N sodium hydroxide solution and incubating at 50C for 30 minutes. This solution was neutralized with 0.1 N acetic acid in preparation for starch digestion.

Starch was digested by incubating the solubilized tissue solution with purified a-amylase and amyloglucosidase for 24 hours at 50C to 55C. This procedure converts the starch from a polymer to free glucose molecules.

After conversion of the starch to glucose, an aliquot of the solution was transferred to a test tube and diluted to fall within the range of the glucose standard curve. Glucose concentration was measured through a coupled reaction involving glucose oxidase and peroxidase, which results in the oxidation of o-dianisidine, producing a reddish color upon the addition of concentrated sulfuric acid. One molecule of o-dianisidine is oxidized for each molecule of glucose in the sample solution. Quantification of the color intensity with a spectrophotometer and comparison with the concurrently run glucose "standard curve" permits accurate calibration of the glucose measurements.

Glucose values were converted to starch equivalents and percent dry weight for statistical analysis. Means were calculated for each sample (samples were analyzed for starch content at least three separate times) and for each treatment. The data were normalized through an arcsine squareroot transformation for use in predictive regression equations.

Results and Discussion

The results from the starch measurements are shown in Table 5. Treatments for analysis of starch content were carefully chosen to evaluate the effects of various seedling stress factors on starch content, as well as the relationship of starch reserves and seedling quality. Due to the lack of a significant correlation between starch content and seedling quality, starch measurements were not performed on all treatments.

The results are very interesting, but generally not surprising. The quality-reducing treatments (other than cold storage) did not significantly alter the starch content of seedling tissues, but did greatly reduce both growth chamber and field survival. This clearly shows that starch content alone is not a reliable predictor of seedling quality. Injuries that greatly reduce seedling quality cannot be identified by measuring starch reserves. The correlation between starch content (arcsine squareroot of mean percent dry weight) and field survival (arcsine squareroot percent survival) was insignificant (R^2=0.07, P=0.31), as was the correlation between starch content and growth room survival (R^2=0.09, P=0.25).

Cold storage resulted in a significant decrease in the starch content of seedling roots, but did not show a strong relationship between starch content and seedling quality. Figure 2 illustrates the decrease in starch content during cold storage for three lifting dates. Seedlings from the 1985-86 season utilized their root starch reserves at a faster rate than those from the previous season. Seedlings lifted in January 1985 utilized their root starch reserves at a relatively constant rate of approximately 0.5 percent of dry weight per month of cold storage, while those lifted in January 1986 utilized their root starch reserves about 3.5 times faster during 2 months of cold storage.

Table 5. Starch Concentration in Seedling Tissues.

LIFT DATE	TREATMENT APPLIED[1]	COLD STORAGE (Months)	MEAN PERCENT STARCH		PERCENT SURVIVAL	
			Roots	Needles	Field	Growth Chamber
1-85	None	0	5.41	0.11	60	80
1-85	60 mins. RD	0	5.40	0.11	35	65
1-85	-15C FR	0	5.20	N/A	0	0
1-85	45C RS	0	5.36	0.12	45	90
1-85	None	2	4.37	0.12	0	100
1-85	None	4	3.30	0.11	5	80
10-85	None	0	2.27	N/A	15	100
10-85	-15C FR	0	2.79	N/A	0	0
10-85	60 mins. RD	0	2.53	N/A	0	10
10-85	None	2	0.11	N/A	55	45
10-85	None	4	0.08	N/A	20	35
12-85	None	0	2.53	N/A	75	100
1-86	None	0	6.19	N/A	90	100
1-86	-15C FR	0	6.28	N/A	35	80
1-86	None	2	2.71	N/A	95	100
3-86	None	0	10.78	N/A	100	100

[1]Abbreviations used: RD = Root Desiccation; FR = Freezing; RS = Root Submersion; C = Degrees Centigrade; N/A = Not Applicable.

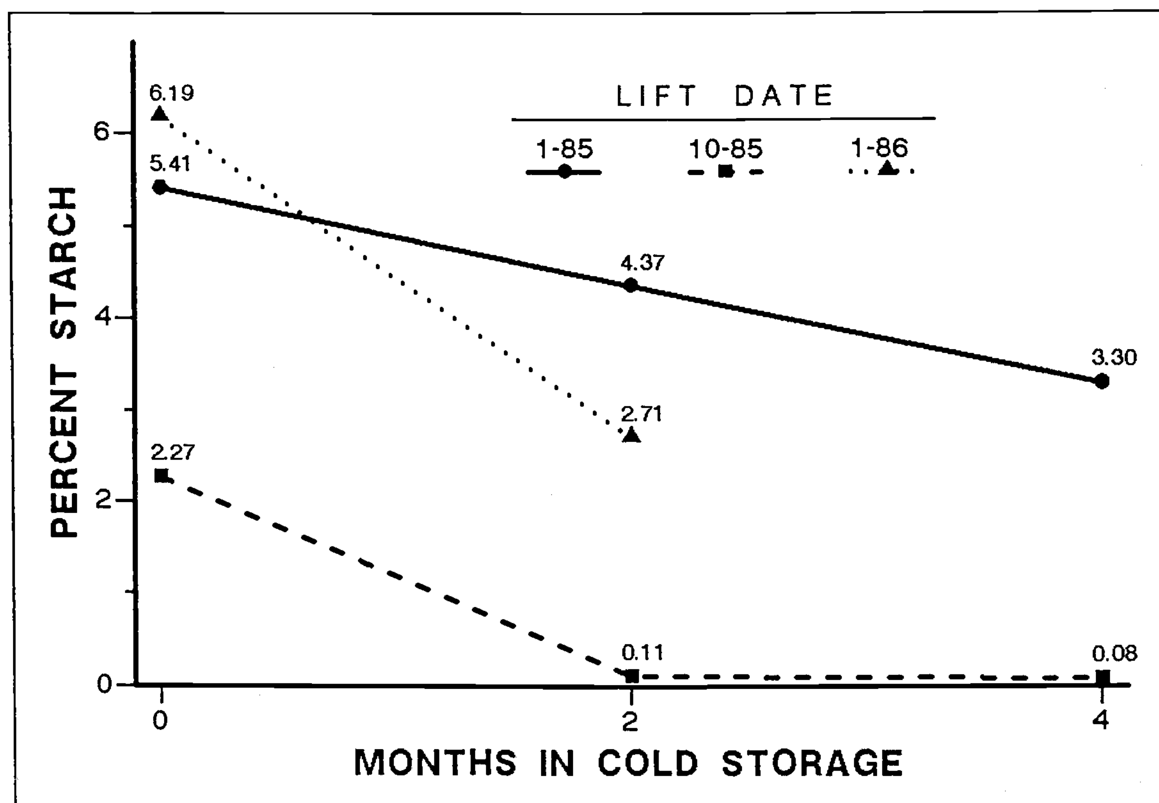

Figure 2. Starch Reserve Loss in Seedling Roots During Cold Storage. Data shown are for control seedlings and those subjected only to cold storage. Loss of starch reserves during cold storage due to maintenance respiration can be substantial.

Seedlings lifted in October 1985 utilized their root starch reserves at the rate of approximately 1 percent of dry weight per month during the first 2 months of cold storage, at which time they reached such a low level that, apparently, remaining starch could not be utilized for maintenance respiration, and an alternative energy reserve was drawn upon during subsequent storage. After 4 months of cold storage, the seedlings had less than 0.1 percent starch in their roots, and yet 20 percent of these seedlings survived in the field (35 percent in the growth chamber). This appears to contradict the idea that a threshold level of starch reserves is needed for seedlings to survive (Marshall 1985, Puttonen 1986), unless that threshold is very low.

The relationship between the loss of root starch reserves during cold storage and subsequent survival was evaluated by regressing mean percent starch (arcsine square-root normalization) on seedling survival (arcsine squareroot normalization) for the controls and cold storage treatments only. There was no significant relationship between starch content of cold-stored seedlings and field survival (R^2= 0.02, P=0.76). However, there was a significant relationship between the starch content of cold-stored seedlings and growth chamber survival (R^2=0.56, P=0.03), suggesting that starch reserves may indeed be an indicator of seedling quality following cold storage.

Omi (1990) found that the starch reserves of ponderosa pine seedlings lifted in the fall and stored below freezing until planting in spring were poorly related to root initiation and first-year field growth. However, starch reserves at the time of planting were significantly related to first-year field survival.

While these results suggest that starch reserves of cold-stored seedlings are an important component of seedling qaulity, they also show that starch content alone does not provide an adequate assessment of seedling quality. The relationship between starch reserves and field survival is weak, at best, and additional information (such as RGP) is required to adequately assess seedling quality.

This study has confirmed that starch reserves increase during the months preceding spring growth, as previously reported (Krueger and Trappe 1967, Ritchie 1982). Figure 3 illustrates the increase of root starch reserves in seedlings in the nursery bed from October 1985 to March 1986. In contrast to Krueger and Trappe (1967), root starch reserves began to increase during mid-winter and continued to increase until at least early spring. Since total

carbohydrate reserves were not measured during this study, it was not possible to determine whether the increase in starch reserves was due to winter photosynthesis or the conversion of free sugars to starch.

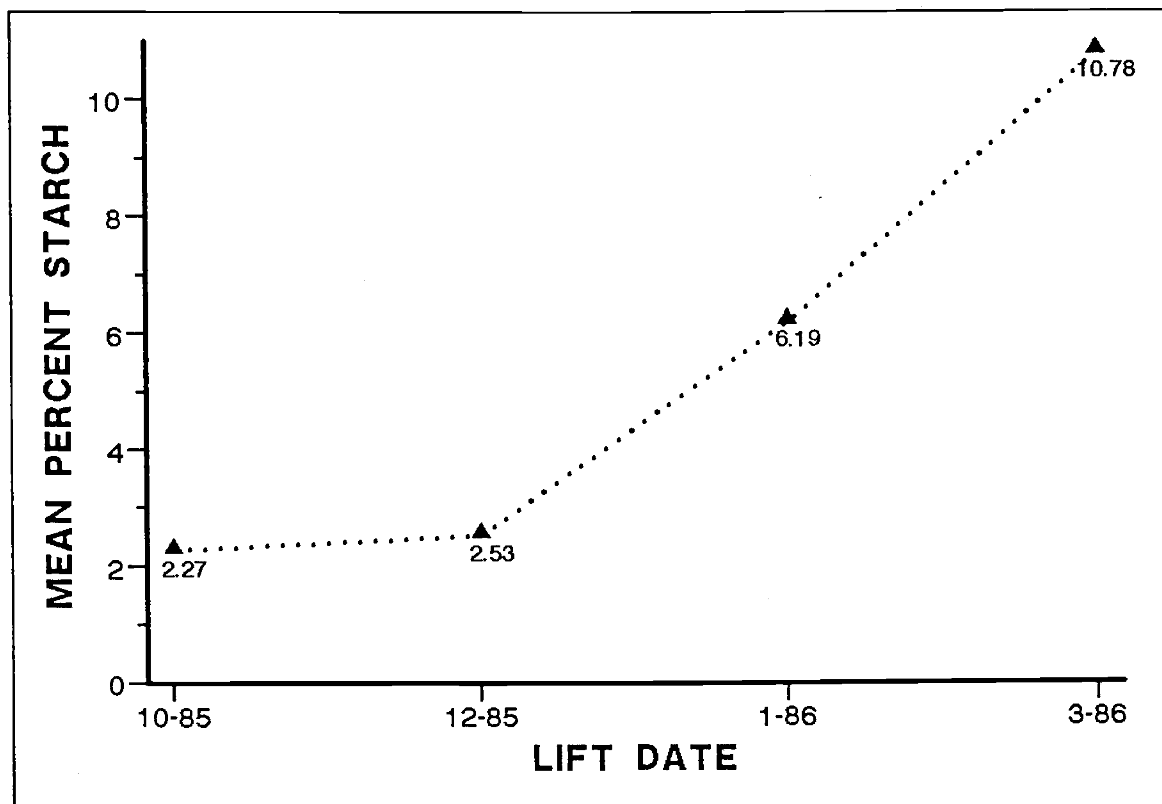

Figure 3. Root Starch Reserves Increase Throughout Winter in Douglas-Fir Seedlings in the Nursery Bed.

The analysis of the starch content of needles from Douglas-fir seedlings indicated that starch is not a significant energy reserve in this tissue, at least not during winter. Measurements of the starch content of needles from seedlings lifted in January 1985 showed that starch reserves averaged approximately 0.1 percent of dry weight and did not decrease during cold storage. These results contradict the findings of other researchers and suggest that previous investigations have been measuring compounds other than starch. It is of course possible that the method utilized

in this study failed to access the starch reserves present in needles, but this seems unlikely.

Figure 4 clearly illustrates the lack of a relationship between starch reserves and field survival. For example, while root starch content did not increase significantly from October to December 1985, the survival of outplanted seedlings increased from 15 percent in October to 75 percent in December. Obviously, starch reserves alone cannot be relied upon to evaluate the quality of Douglas-fir seedlings.

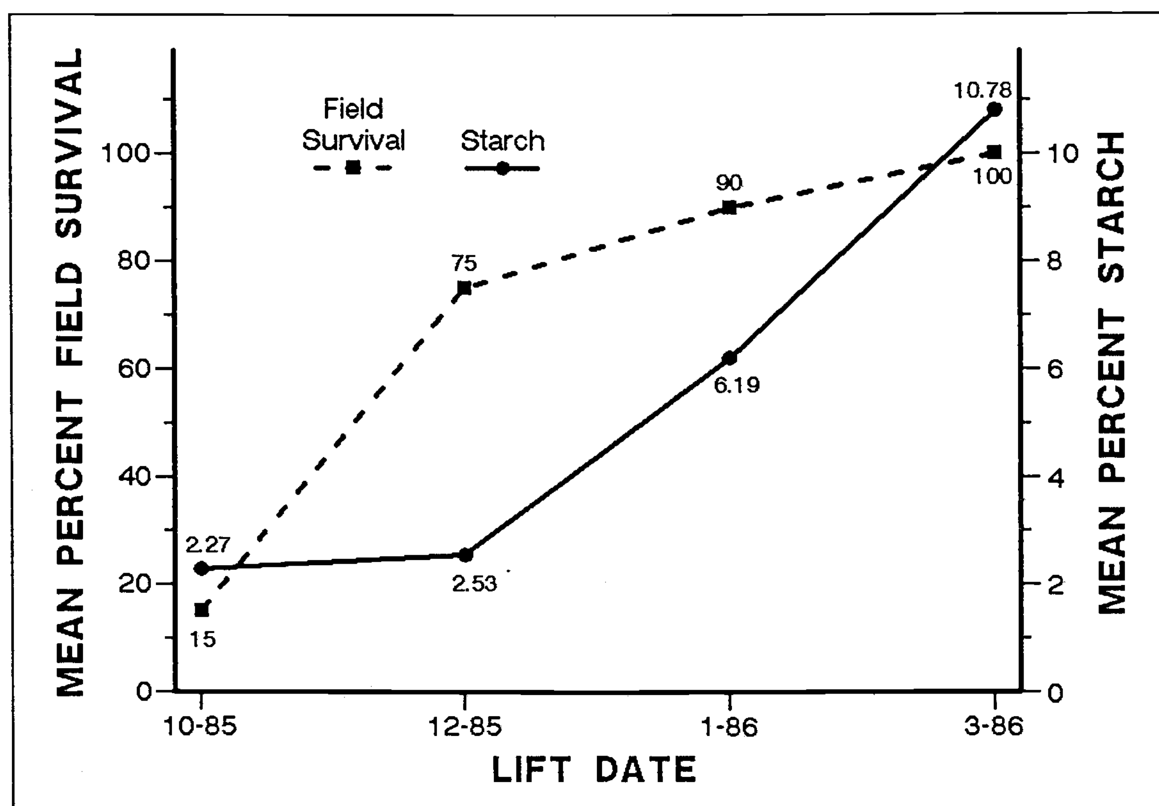

Figure 4. Root Starch Content and Field Survival. Data shown are for control seedlings only (no quality-reducing treatments). Root starch reserves increase throughout winter in seedlings in the nursery bed, but are poorly correlated with field survival. Starch content alone does not adequately characterize seedling quality.

OSMOTIC CONCENTRATION OF EXPRESSED XYLEM SAP

Little is known about the relationship between seedling quality and the concentration of solutes in xylem sap. It is known that the xylem functions primarily as a conduit for water from the roots to needles. Since water is the primary commodity transported by xylem tissue, the concentration of solutes in xylem sap is very low in healthy plants, and mineral salts make up the bulk of the dissolved solutes.

When a cell membrane is damaged, such as can be caused by freezing or excessive heat, the integrity of the membrane is frequently compromised, and the contents of the cell can leak out. Previous research by Joly (1985) indicated that leakage of cell solutes from damaged cells considerably increased the osmotic concentration of xylem sap. This relationship, if shown to be consistent, could be a useful indicator of seedling damage.

The tremendous benefit of this technique is the short period of time required to complete the test--less than 5 minutes. The equipment required is relatively inexpensive and easy to use. Interpretation of the results could be as simple as comparing the readout on the osmometer with a chart correlating osmotic concentration and predicted seedling quality. In fact, if it could be shown that the osmotic concentration of xylem sap accurately and realiably predicts seedling quality and is able to identify all types of seedling damage, it would meet the criteria of an ideal test of seedling quality (Zaerr 1985).

Materials and Methods

Seedlings were tested for the osmotic concentration of their xylem sap 1 day before being planted. To complete the measurement, a small undamaged branchlet was removed from

the seedling, and approximately 1/2 inch of the bark and phloem tissue was removed from the cut end. This ensured that the sample would not be contaminated by phloem exudate.

The branchlet was then inserted through a one-hole stopper and installed into a pressure chamber (PMS Instruments Co., Corvallis, Oregon). Chamber pressure was then increased, forcing xylem sap from the tissue and onto a dry filter paper disk placed on the end of the stem. When the disk became saturated with expressed xylem sap, it was immediately transferred to the sample chamber of a vapor pressure osmometer (Wescor Inc., Logan, Utah) for determination of osmotic concentration (total concentration of solute in the sap). Measurement of osmolarity proceeds automatically until the osmotic concentration (in millimoles of solute per kilogram of solvent) appears on the display (approximately 90 seconds).

Results and Discussion

Results (Table 6) from the investigation of the relationship between seedling quality and the osmotic concentration of xylem sap were rather disappointing. While it appears that membrane damage severe enough to greatly reduce seedling quality may be accompanied by a significant increase in xylem sap osmolarity, it is also apparent that lethal damage is not always associated with increased osmotic concentration of the xylem sap.

While the relationship between seedling survival in the growth chamber and the osmotic concentration of expressed xylem sap was highly significant ($P=0.0008$), the correlation was quite weak ($R^2=0.18$). Squaring the mean osmotic concentration improved the correlation a little, but not substantially ($R^2=0.20$). The relationship between field survival and the osmotic concentration of xylem sap was insignificant.

Table 6. Osmotic Concentration of Expressed Xylem Sap and Its Relationship to Seedling Survival.

LIFT DATE	TREATMENT APPLIED[1]	COLD STORAGE (Months)	MEAN OSMOTIC CONCENTRATION (mmol/kg)	PERCENT SURVIVAL	
				Field	Growth Chamber
1-85	None	0	31.6	60	80
1-85	30 mins. RD	0	33.8	30	75
1-85	60 mins. RD	0	35.3	35	65
1-85	-9C FR	0	32.5	40	45
1-85	-12C FR	0	44.0	15	35
1-85	-15C FR	0	71.5	0	0
1-85	45C RS	0	34.8	45	90
1-85	48C RS	0	32.3	60	80
1-85	None	2	30.9	0	100
1-85	30 mins. RD	2	37.4	20	100
1-85	60 mins. RD	2	37.0	15	80
1-85	-9C FR	2	35.4	45	15
1-85	-12C FR	2	39.4	0	10
1-85	-15C FR	2	45.3	0	10
1-85	45C RS	2	32.0	40	90
1-85	48C RS	2	42.5	50	100
1-85	None	4	26.6	5	80
1-85	30 mins. RD	4	26.6	0	80
1-85	60 mins. RD	4	30.0	0	60

- - Continued. See footnote at end of table. - -

Table 6. Osmotic Concentration of Expressed Xylem Sap and Its Relationship to Seedling Survival (Continued).

LIFT DATE	TREATMENT APPLIED[1]	COLD STORAGE (Months)	MEAN OSMOTIC CONCENTRATION (mmol/kg)	PERCENT SURVIVAL	
				Field	Growth Chamber
1-85	-9C FR	4	31.8	0	40
1-85	-12C FR	4	28.4	0	10
1-85	-15C FR	4	34.4	0	0
1-85	45C RS	4	32.4	10	85
1-85	48C RS	4	35.4	0	65
10-85	None	0	40.5	15	100
10-85	-9C FR	0	88.4	0	5
10-85	-12C FR	0	74.2	0	0
10-85	-15C FR	0	135.4	0	0
10-85	15 mins. RD	0	54.4	10	70
10-85	30 mins. RD	0	57.1	15	15
10-85	60 mins. RD	0	56.9	0	10
10-85	None	2	40.8	55	45
10-85	None	4	49.6	20	35
12-85	None	0	47.9	75	100
1-86	None	0	43.6	90	100
1-86	-9C FR	0	42.3	60	75
1-86	-12C FR	0	42.9	90	100
1-86	-15C FR	0	42.2	35	80

- - Continued. See footnote at end of table. - -

Table 6. Osmotic Concentration of Expressed Xylem Sap and Its Relationship to Seedling Survival (Continued).

LIFT DATE	TREATMENT APPLIED[1]	COLD STORAGE (Months)	MEAN OSMOTIC CONCENTRATION (mmol/kg)	PERCENT SURVIVAL	
				Field	Growth Chamber
1-86	15 mins. RD	0	43.7	70	100
1-86	30 mins. RD	0	46.5	60	95
1-86	60 mins. RD	0	47.0	35	70
1-86	None	2	52.0	95	100
1-86	-9C FR	2	54.3	15	70
1-86	-15C FR	2	49.3	0	20
1-86	15 mins. RD	2	53.1	65	100
1-86	30 mins. RD	2	60.3	30	90
1-86	60 mins. RD	2	65.8	0	50
3-86	None	0	48.0	100	100
3-86	-9C FR	0	62.6	20	20
3-86	-12C FR	0	70.4	15	20
3-86	-15C FR	0	88.4	0	0
3-86	15 mins. RD	0	52.8	85	100
3-86	30 mins. RD	0	61.5	45	90
3-86	60 mins. RD	0	70.2	0	65

[1]Abbreviations used: RD = Root Desiccation; FR = Freezing; RS = Root Submersion; C = Degrees Centigrade; N/A = Not Applicable.

Obviously, many types of seedling damage that significantly reduce quality have little negative impact upon the integrity of cell membranes (e.g., root desiccation). To complicate matters further, the data suggest that seedlings that have suffered membrane damage resulting in elevated xylem sap osmolarity (i.e., freezing) are able to recover leaked solutes during prolonged cold storage. It is possible that seedlings are repairing damaged cell membranes during cold storage, but it seems more likely that undamaged cells are responsible for the reabsorption of leaked solutes. If damaged membranes were being repaired, this should be reflected as improved survival following cold storage, but this was not true in most cases.

The osmotic concentration of xylem sap is clearly not a reliable predictor of seedling quality, but it may be useful in identifying seedlings that were damaged by freezing temperatures if measurement of xylem sap osmolarity is performed soon after the damage was sustained. In this investigation, seedlings that had xylem sap osmolarity exceeding 60 mmol/kg exhibited very poor survival in the field (Figure 5) and generally in the growth chamber as well. It appears safe to say that seedling lots that exhibit mean osmotic concentration of their xylem sap that exceeds 60 mmol/kg are generally of poor quality and should probably be discarded.

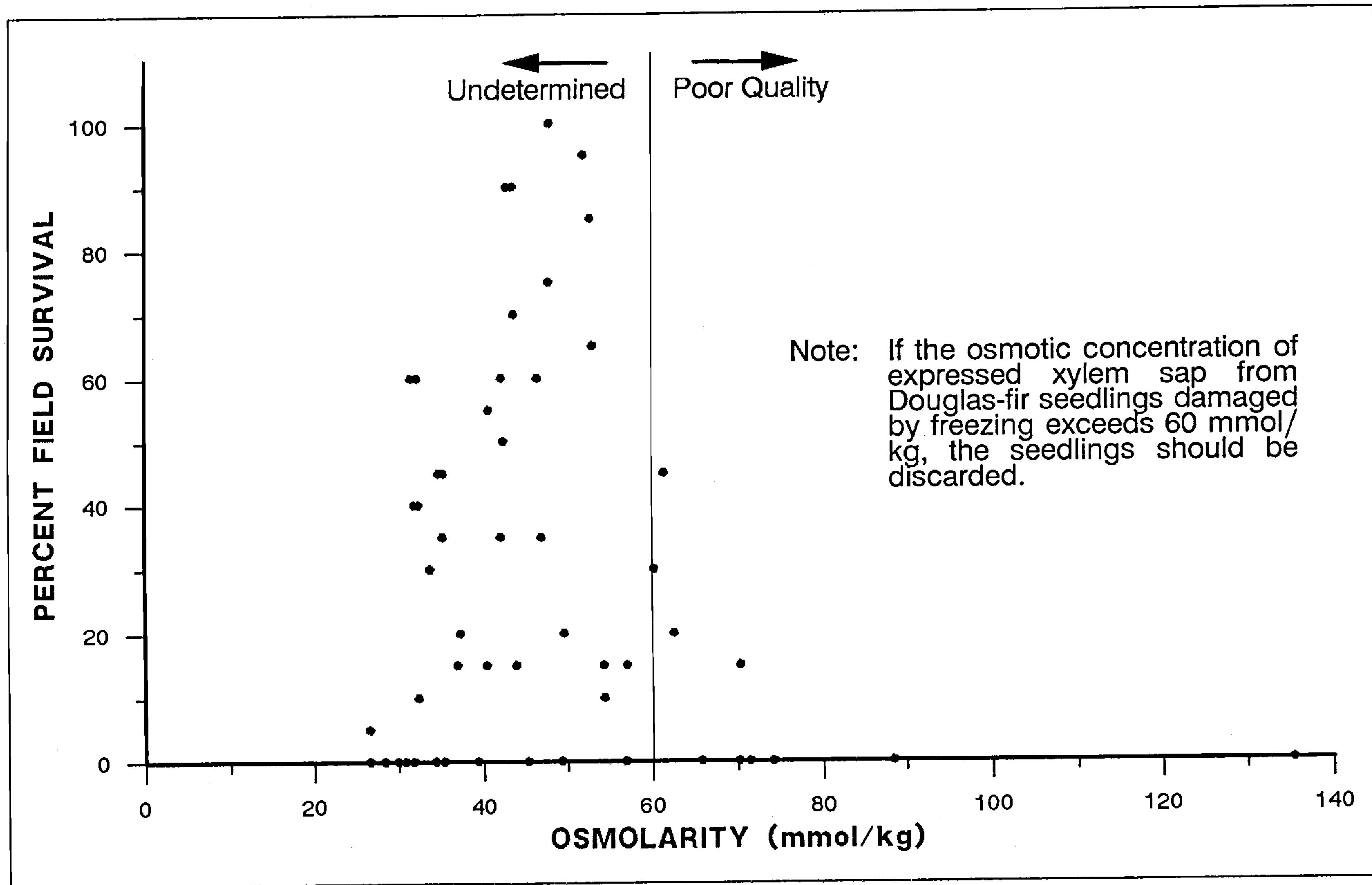

Figure 5. Elevated Xylem Sap Osmolarity Is Associated With Poor Field Survival.

DISCUSSION AND SUMMARY

The project described in this thesis was designed to assess the ability of the osmotic concentration of xylem sap and the starch content of roots and/or needles to estimate Douglas-fir seedling quality. As a benchmark of the ability of these new methods to predict field survival, two "standard" quality assessment tests were run simultaneously. The predictive ability of the RGP and OSU vigor test methods has previously been demonstrated (McCreary 1986).

Results indicate that neither the starch content of roots nor the osmotic concentration of expressed xylem sap is a reliable predictor of Douglas-fir seedling quality. The starch content of Douglas-fir needles apparently has no relationship to seedling survival; needle starch levels were very low in mid-winter, with no change during cold storage.

The starch content of Douglas-fir roots proved to have no significant relationship with seedling survival in either the field or controlled environment chamber. This result was not surprising, as previous research had indicated that if starch reserves have an impact on seedling quality, it is due to a threshold level below which seedlings would die. This study provided data that suggest that if a threshold level of starch reserves is indeed required for seedling survival, it must be extremely low. Several cold-stored seedlings with starch reserves averaging less than 0.1 percent dry weight were able to survive on the field site.

The surprising result with respect to the starch content of roots was the total lack of a relationship (R^2=0.02) between starch reserves following cold storage and field survival. The fact that root starch reserves after cold storage were significantly correlated with growth chamber survival (R^2=0.56) makes this even more puzzling. It is likely that

the harsh conditions on the field site during both seasons of this study caused the death of many seedlings that would have survived "normal" conditions and, consequently, overshadowed a possible relationship between root starch reserves of cold-stored seedlings and field survival. Even so, root starch reserves do not appear to be a useful predictor of general seedling quality, and it is probably not worth the expense to set up a lab to measure seedling starch reserves for the purpose of estimating seedling quality.

The osmotic concentration of expressed xylem sap also failed to reliably predict field survival of Douglas-fir seedlings. It did exhibit a weak correlation with seedling survival in the growth chamber, and the method was capable of identifying seedlings with severe damage from freezing.

When a cell is frozen, ice crystals frequently damage the cell membrane, permitting the leakage of solutes from inside the cell into extracellular fluids. When many cells are damaged through severe freezing, solute leakage is sufficient to elevate the osmotic concentration of xylem sap. This can be easily measured with a vapor pressure osmometer, and this study showed that seedling lots with average xylem sap osmolarity exceeding 60 mmol/kg have very poor survival potential and should probably be discarded. Only seedlings exposed to lethal freezing temperatures exhibited osmotic concentrations of xylem sap that exceeded 60 mmol/kg.

The benefit of the xylem sap test is the rapid availability of the results. With this method a nursery manager could test 20 to 30 seedlings within an hour and know immediately whether the previous night's severe frost significantly damaged the seedlings. The only problem with the scenario is that osmolarity readings for xylem sap below 60 mmol/kg do not guarantee that the seedlings are healthy. Consequently, it seems that this method of estimating

seedling quality will be of limited use to the reforestation industry.

It appears that the RGP and OSU vigor test methods are still the most reliable techniques to estimate seedling quality. In this study, the overall "best" regression equation to predict Douglas-fir seedling survival combined measurements from both of these tests as follows:

NORMSURV = -0.1567 + 0.0003034 (MEANLNGTH) - 0.1634 (SQTLATBRST) + 0.1919 (SQTTERFLSH)

where: NORMSURV = arcsine squareroot of mean percent survival
MEANLNGTH = mean total length of new roots (in mm)
SQTLATBRST = squareroot mean days to lateral burst
SQTTERFLSH = squareroot mean days to terminal flush

Variable	Partial R^2	Model R^2	$F_{(3,43)}$	P
MEANLNGTH	0.427	0.427	28.19	0.0001
SQTLATBRST	0.062	0.489	7.32	0.0097
SQTTERFLSH	0.074	0.564	13.17	0.0008

This equation accounted for 56 percent of the variability in field survival (R^2=0.56, $F_{(3,43)}$=18.50, P=0.0001), but it probably isn't worth the effort needed to complete both RGP and OSU vigor test assessments.

The best single predictor of field survival was the mean total length of new roots (in mm), which accounted for 51 percent of the variability in field survival. If a reliable assessment of seedling quality is needed, it appears that RGP is still the best method available.

One problem with all of the currently available methods is the tremendous variability in seedling response. Although the mean total number of new roots was significantly correlated with field survival, there was tremendous variability in the total length of new roots produced by seedlings that were treated identically. Since it is impossible to eliminate the variability in seedling response, it is necessary to sample quite large numbers of seedlings to obtain an accurate assessment of the overall quality of the seedlings. This is quite costly and very tedious.

As has been shown in previous investigations, there is no quality assessment test that can precisely predict seedling survival in the field. The excellent correlations with growth chamber survival demonstrated in this and other studies indicate that many of the available methods can provide a reliable estimation of seedling quality. The problem is that seedling quality is only one of many factors that affect seedling survival in the natural environment. It is known that weather, competition from other vegetation, animal browsing, and other factors also significantly impact upon field survival. Until the influence of these environmental factors on seedling survival can be measured and characterized, plantation success or failure cannot be accurately predicted.

The RGP and OSU vigor test provide adequate estimations of general seedling quality and are the best quality assessment tests available at this time. Future research should concentrate on developing a "battery" of simple tests, such as the osmotic concentration of xylem sap, which can be performed quickly and, when used concurrently, yield a reliable assessment of seedling quality. The goal is to ensure that only vigorous, healthy seedlings are planted, increasing the probability of successful plantation establishment.

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APPENDICES

Appendix A

STARCH ANALYSIS FOR CONIFER TISSUES

Adapted from Haissig and Dickson 1982, 1979; Ebell 1969.

Solutions:

1) methanol:chloroform:water, 12:5:3 v/v/v (MCW solution)--mix 1200 ml methanol, 500 ml chloroform, and 300 ml deionized distilled water (ddH_2O). Use reagent grade solvents, or better.

2) 0.1 N sodium hydroxide--dissolve 4.00 g sodium hydroxide (NaOH) in 1000 ml ddH_2O.

3) 0.1 N acetic acid--add 5.75 ml glacial acetic acid (HAc) to 950 ml ddH_2O. Mix well and bring to a total volume of 1000 ml with additional ddH_2O.

4) 30% sodium hydroxide--dissolve 30.0 g NaOH in 100 ml ddH_2O.

5) 0.05 M sodium acetate buffer, pH 5.1--add 2.84 ml glacial acetic acid (HAc) to about 900 ml ddH_2O. Adjust to pH 5.1 with 30% sodium hydroxide (solution #4). Bring to total volume of 1000 ml with additional ddH_2O.

6) 0.1 M sodium phosphate buffer, pH 7.0--dissolve 8.7 g dibasic sodium phosphate (Na_2HPO_4) and 5.3 g monobasic sodium phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$) in 1000 ml ddH_2O.

7) a-amylase/amyloglucosidase digestion solution--this solution should contain 2 uM units/ml of amyloglucosidase (from Aspergillus niger) and 400 uM units/ml of a-amylase (from A. oryzae) in 0.05 M NaOAc buffer, pH 5.1 (solution #5). Use purified, assayed enzymes to prepare this solution (see the appropriate purification and assay procedures in Appendices B-E).

8) glucose oxidase/peroxidase/o-dianisidine solution--dissolve 100.0 mg o-dianisidine dihydrochloride in 10.0 ml ddH_2O. (This step is needed, as the dye does not dissolve well in the final buffer.) Mix the 10.0 ml o-dianisidine solution with 990 ml 0.1 M sodium phosphate buffer, pH 7.0 (solution #6). Add the appropriate amount of glucose oxidase (GOD) and peroxidase (POD), to yield a final concentration of 5 units GOD/ml and 1 unit POD/ml, and mix well. [See the bottle labels for the enzyme assays. The approximate amounts of enzymes to add are 44-45 mg glucose oxidase (from A. niger, type X, Sigma no. G-8135 or G-7141) and about 5 mg peroxidase (from horseradish, type II, Sigma no. P-8250).] The final solution is 0.16 mM o-dianisidine in 0.1 M sodium phosphate buffer, pH 7.0, containing about 5 units GOD/ml and 1 unit POD/ml. This solution is stable for up to 1 month if stored in the refrigerator in a brown bottle.

9) 75% sulfuric acid--place 250 ml ddH_2O in a heavy glass acid stock bottle and very slowly (over an hour or two) add 750 ml sulfuric acid (H_2SO_4) while stirring slowly with a magnetic stir bar. The dilution of acid generates considerable heat and must be done slowly. Wear rubber gloves, safety glasses, and a lab coat. Be careful; too much heat can cause the stock bottle to break, but <u>do not</u> try to cool it with water.

Procedures:

Immediately after collection, the fresh tissue for starch analysis should be cooled to -20C (or -80C if available). Fresh tissue can be stored for months if maintained at -80C. The tissue is then steamed (at ambient atmospheric pressure) for 5-10 minutes to denature the native enzymes (Loomis 1985). After steaming the tissue is dried by lyophilization (approximately 3 days). The tissue is then ground with a Wiley mill to pass through a 40-mesh screen and stored at -20C (-80C is preferable) until analyzed.

Place chromic acid-washed 15 ml centrifuge tubes in oven overnight to ensure that they are completely dry. Cool to room temperature in a desiccating chamber, then weigh to the nearest 0.1 mg. Place tissue samples (50-100 mg, preferably use 100 mg) into the dried, weighed centrifuge tubes. Place the tubes and samples into the 50C oven overnight to remove any remaining moisture in the sample. Cool the tubes and samples to room temperature in the desiccating chamber, then weigh to the nearest 0.1 mg to obtain the tissue dry weight.

Add 5.0 ml MCW solution (solution #1) to each sample to suspend the tissue (use repipeter if available). Sonicate for 10 seconds (or use Vortex mixer on lowest setting). [These steps should be done in a hood; wear rubber gloves, safety glasses, and a lab coat. Chloroform is reported to be carcinogenic.]

After 10 minutes at room temperature (20 minutes if vortexed), the tubes are centrifuged at 1100g for 10 minutes. Remove the supernatant by aspiration or by hand using a Pasteur pipet. Work in the hood and be careful not to remove any of the tissue sample while aspirating the MCW solution. Save this solution for analysis of soluble sugars, if desired. Repeat the extraction with MCW solution

and centrifugation 2 to 3 more times until the MCW solution is clear. (Root and stem tissue should be extracted at least 3 times, needles at least 4 times.) Combine all of the MCW extracts for the determination of soluble sugars, if desired. Place the extracted tissue samples in the 50C oven overnight to evaporate residual MCW.

Remove the tubes from the oven and add 4.0 ml of 0.1 N NaOH (solution #2). Stopper and mix on the Vortex mixer until the pellet is broken up and suspended within the solution. Incubate in the 50C oven for 30 minutes with occasional swirling. The starch should be solubilized during this procedure. After 30 minutes the sample solution is neutralized and adjusted to pH 5.1 by addition of 5.0 ml of 0.1 N acetic acid (solution #3). The starch is now dissolved in a 0.05 M sodium acetate (NaOAc) buffer, pH 5.1, and ready for enzyme digestion.

Add 1.0 ml of the a-amylase/amyloglucosidase digestion solution (solution #7) to each of the tubes. Stopper the tubes, mix the tissue/enzyme solution well (Vortex mixer), and incubate for 24 hours at 50C to 55C. The tubes should be checked after reaching the incubation temperature to insure that no stoppers have been pushed out via thermal expansion. Occasional mixing during the starch digestion is not necessary, but can be done if desired.

After digestion, mix the sample solutions well (Vortex mixer), and centrifuge at 1100g for 10 minutes. Transfer two 0.5 ml aliquots (to replicate the glucose determinations and check your precision) of the diluted sample solution to small test tubes (approximately 10 ml); use 0.05 M NaOAC buffer, pH 5.1 (solution #5) to prepare the dilutions. Dilution is necessary to get the sample solutions into the range of the glucose standard curve. The appropriate dilution depends on the anticipated amount of starch in the

sample: a 1:2 dilution is good for low starch tissue (up to 1.5% starch, i.e. needles), a 1:5 dilution is suitable for tissue with 1-4.5% starch, a 1:10 dilution is appropriate for tissues with 4-9% starch, and a 1:20 dilution is suitable for high starch tissues (9-18% starch). (Some tissues with even higher starch content, will require more than a 1:20 dilution. If the sample size is smaller than 100 mg, a smaller dilution will be needed.) Add 5.0 ml of the glucose oxidase/peroxidase/o-dianisidine solution (solution #8) to each 0.5 ml sample aliquot. Stopper, mix well, and incubate at 37C for 30 minutes. [The temperature must be controlled carefully, as GOD is inactivated at 39C (Bentley 1955). An alternative procedure is incubation at room temperature for 45 minutes.] The glucose standards should be treated in the same manner (two 0.5 ml aliquots of each concentration plus 5.0 ml solution #8).

Transfer the tubes to a cold water bath and rapidly add 1.0 ml 75% sulfuric acid (solution #9) to each tube to stabilize the color formed (cut off about 1 mm of the pipet tip to ease the pipetting of the viscous acid). After the tubes have cooled, stopper, mix well with the Vortex mixer, and read the absorbance at 525 nm versus a buffer-reagent blank. Determine the glucose concentration by comparison with the glucose standard curve run simultaneously (run 10, 20, 40, 60, 80, and 100 ug/ml glucose standards).

The glucose values are converted to starch equivalents by multiplying the glucose concentration by 0.9 (Pazur 1987). The starch equivalents are converted to percent dry weight by dividing by the dry weight of the corresponding tissue sample.

<u>Glucose Standard Curve Solutions:</u>

1) 0.05 M sodium acetate buffer, pH 5.1, with 0.1% benzoic acid--add 2.84 ml glacial acetic acid to about 900 ml

ddH_2O. Adjust to pH 5.0 with 30% NaOH. Add 1.000 g benzoic acid and mix until completely dissolved. Then add a few more drops of 30% NaOH to achieve a final pH of 5.1. Adjust to 1000 ml with additional ddH_2O.

2) 1% glucose solution--dissolve 0.100 g anhydrous glucose in 10.0 ml 0.05 M NaOAc buffer, pH 5.1, with 0.1% benzoic acid (solution #1).

3) 0.1% glucose solution--mix 5.0 ml 1% glucose solution (solution #2) with 45.0 ml solution #1.

4) 100 ug/ml glucose standard--mix 10.0 ml 0.1% glucose (solution #3) with 90.0 ml solution #1.

5) 80 ug/ml glucose standard--mix 8.0 ml 0.1% glucose with 92.0 ml solution #1.

6) 60 ug/ml glucose standard--mix 6.0 ml 0.1% glucose with 94.0 ml solution #1.

7) 40 ug/ml glucose standard--mix 4.0 ml 0.1% glucose with 96.0 ml solution #1.

8) 20 ug/ml glucose standard--mix 2.0 ml 0.1% glucose with 98.0 ml solution #1.

9) 10 ug/ml glucose standard--mix 1.0 ml 0.1% glucose with 99.0 ml solution #1.

10) Blank--use 0.5 ml aliquots of solution #1.

The glucose solutions should be stored in the refrigerator and are stable for months (when prepared with benzoic acid).

Appendix B

PURIFICATION OF AMYLOGLUCOSIDASE

Adapted from Pazur et al. 1984, Pazur and Ando 1959.

Solutions:

1) 0.04 M calcium acetate--dissolve 0.352 g calcium acetate monohydrate ($Ca(OAc)_2 \cdot H_2O$) in 50.0 ml deionized distilled water (ddH_2O).

2) 0.1 M citric acid--dissolve 19.21 g anhydrous citric acid (or 21.014 g monohydrate) in 1000 ml ddH_2O.

3) 0.2 M dibasic sodium phosphate--dissolve 28.392 g dibasic sodium phosphate (Na_2HPO_4) in 1000 ml ddH_2O. (It's best to add the phosphate to the water, or large, hard-to-dissolve clumps will form).

4) 0.1 M citrate/phosphate buffer, pH 8.0--mix 4.7 ml 0.1 M citric acid (solution #2) with 120 ml 0.2 M Na_2HPO_4 (solution #3). This is only an approximation and may need adjustment.

5) 0.05 M sodium acetate (NaOAc) buffer, pH 5.1--add 2.84 ml glacial acetic acid to approximately 900 ml ddH_2O. Adjust the pH to 5.1 with 30% sodium hydroxide (NaOH) and bring to a final volume of 1000 ml with additional ddH_2O.

0.05 M Citrate/Phosphate Buffers:

6) pH 8.0--mix 19.0 ml 0.1 M citric acid (solution #2), 481 ml 0.2 M Na_2HPO_4 (solution #3), and 500 ml ddH_2O. (This yields pH 7.98 at room temperature).

7) pH 6.0--mix 48.0 ml 0.1 M citric acid (solution #2), 77.0 ml 0.2 M Na_2HPO_4 (solution #3), and 125 ml ddH_2O. (These volumes are only approximate and may need further refinement. Adjust the pH with phosphoric acid if necessary).

8) pH 4.0--mix 79.0 ml 0.1 M citric acid (solution #2), 46.0 ml 0.2 M Na_2HPO_4 (solution #3), and 125 ml ddH_2O. (This should yield pH 4.0 at room temperature, but the volumes may need minor adjustments.)

Column Preparation:

Pour a 50 cm^3 column of prepared DEAE-cellulose (Whatman DE-32) using vacuum to obtain good packing. [The DEAE-cellulose should first be prepared using the appropriate buffer (0.05 M citrate/phosphate, pH 8.0) following the procedures detailed in Appendix F. An alternative is the use of pre-prepared DEAE-cellulose (Whatman DE-52), which has been equilibrated with the desired buffer before use.] The column is poured in a 60 cm^3 disposable syringe barrel. Use two pieces of Whatman #4 filter paper at the bottom of the syringe (one small paper-punch-size piece to cover the central opening, another piece the size of the syringe barrel on top of the first), and one piece on top of the packed column (the size of the syringe barrel).

Wash the column thoroughly with about 500 ml of 0.05 M citrate/phosphate buffer, pH 8.0 (solution #6), using a peristaltic pump. Begin washing at a 0.75-1.0 ml/minute flow rate to ensure good packing of the column. The flow rate can be increased to about 2 ml/minute after an hour of washing. The column can be stored overnight at room temperature if the system is tightly closed to prevent column desiccation.

Enzyme Preparation:

Dissolve 5.0 g of the crude amyloglucosidase (from *Aspergillus niger*, lyophilized powder, Sigma no. A-3423 or equivalent) in 50 ml ddH_2O. Filter through Whatman #4 filter paper. Rinse beaker with 2-5 ml ddH_2O, and pour through the funnel to rinse it.

Add 10.0 ml of 0.04 M calcium acetate (solution #1) to the filtrate and mix well. [The Ca^{+2} ion stabilizes the amyloglucosidase and causes the precipitation of some protein impurities (Pazur 1987).] Centrifuge at 1100g for 10 minutes. Decant the supernatant into prepared dialysis tubing and dialyze versus ddH_2O for 24 hours or overnight in the refrigerator (see Appendix G for preparation of dialysis tubing). Discard filter paper and precipitate.

Enzyme Separation:

Wash the column with an additional 100 ml of 0.05 M citrate/phosphate buffer, pH 8.0 (solution #6), immediately prior to enzyme application. Allow the buffer to run down to the top of the column before applying the enzyme solution, but do not allow the column to run dry (or it may need to be discarded).

Mix the dialyzed amyloglucosidase solution with an equal volume of 0.1 M citrate/phosphate buffer, pH 8.0 (solution #4). Carefully layer the amyloglucosidase solution onto the top of the column with a Pasteur pipet to establish a small reservoir of solution to buffer the drops. Apply the remaining enzyme solution to the column using the peristaltic pump and a 0.75 ml/minute flow rate. A slow flow rate during application is essential for proper bonding of the enzyme to the column matrix (Cooper 1977).

After all of the amyloglucosidase solution has been applied to the column, run it down to the top of the column. Layer 0.05 M citrate/phosphate buffer, pH 8.0 (solution #6), onto the top of the column using a Pasteur pipet. Then wash the column with 250 ml of this solution using a 2 ml/minute flow rate (all further elution of the column will be done using a 2 ml/minute flow rate). This will elute the glucotransferase and an a-amylase that are present in the enzyme mixture.

After washing the column with solution #6, let the solution run to the top of the column as before. Using a Pasteur pipet, layer 0.05 M citrate/phosphate buffer, pH 6.0 (solution #7), onto the column. Wash the column with 250 ml of this buffer. The first 140 ml of eluate after switching to pH 6.0 may be discarded. At that point (about 70 minutes after application of the pH 6.0 buffer), collect the next 80 ml of eluate (approximately 40 minutes of running); this is the amyloglucosidase II fraction.

After elution with solution #7 is complete, run the solution to the top of the column, and switch to 0.05 M citrate/phosphate buffer, pH 4.0 (solution #8), as before. The first 100-110 ml (about 50-55 minutes of running) of eluate after switching to pH 4.0 may be discarded. At that point collect the next 100 ml of eluate (approximately 50 minutes of running); this is the amyloglucosidase I fraction and the bulk of the crude enzyme preparation. After elution of this fraction the separation is complete and the column may be discarded.

Final Amyloglucosidase Purification:

The amyloglucosidase is further purified by acetone precipitation. Cool the amyloglucosidase II fraction to near freezing, then adjust to pH 4.0 with phosphoric acid; this

is the isoelectric point of amyloglucosidase II. Mix with an equal volume (80 ml) of ice cold (-20C) acetone, and readjust to pH 4.0 (acetone affects the pH). Cover the solution and place in -20C freezer for a few hours or overnight to aid precipitation.

Recover the amyloglucosidase II by centrifuging at 1100 g for 10 minutes; discard the supernatant. Suspend the precipitate in a small amount of 0.05 M NaOAc buffer, pH 5.1 (solution #5), and transfer to a plastic test tube. Assay the solution for activity (see procedure in Appendix D), then place in -20C freezer for storage.

Cool the amyloglucosidase I fraction to near freezing and adjust to pH 3.5, the isoelectric point of amyloglucosidase I, with phosphoric acid. Mix with an equal volume (100 ml) of ice cold acetone, and readjust to pH 3.5. Cover and place the solution in -20C freezer for a few hours or overnight, as above. Recover the amyloglucosidase I by centrifugation as above, and suspend in 0.05 M NaOAc, pH 5.1 (solution #5). After assaying for activity (Appendix D), store the solution in a plastic tube in -20C freezer until needed.

Appendix C

PURIFICATION OF a-AMYLASE

Adapted from Takagi et al. 1971, Toda and Akabori 1963, Tsugita et al. 1959.

Solutions:

1) 0.1 M sodium acetate buffer, pH 7.5--dissolve 4.0 g sodium hydroxide (NaOH) in 950 ml deionized distilled water (ddH_2O). Adjust to pH 7.5 with glacial acetic acid (HAc). [The pH will decrease very slowly during the addition of the first 5 ml, but will then change rapidly. Be careful not to overshoot the desired pH.] Bring to 1000 ml total volume with additional ddH_2O.

2) 0.2 M sodium acetate buffer, pH 7.5--dissolve 4.101 g anhydrous sodium acetate (NaOAc) in 250 ml ddH_2O. Adjust to pH 7.5 with 10% glacial acetic acid; approximately 2-3 drops will be needed.

3) 0.4 M sodium acetate buffer, pH 7.5--dissolve 8.203 g anhydrous NaOAc in 250 ml ddH_2O. Adjust to pH 7.5 with 10% glacial acetic acid; approximately 5-6 drops will be required.

4) 0.25 M calcium acetate--dissolve 2.20 g calcium acetate monohydrate ($Ca(OAc)_2 \cdot H_2O$) in 50.0 ml ddH_2O.

5) 1.0 M sodium hydroxide--dissolve 10.0 g sodium hydroxide (NaOH) in 250 ml ddH_2O.

6) 0.05 M sodium acetate (NaOAc) buffer, pH 5.1--add 2.84 ml glacial acetic acid to approximately 900 ml ddH_2O.

Adjust to pH 5.1 with 30% sodium hydroxide (NaOH) and bring to a final volume of 1000 ml with additional ddH_2O.

Column Preparation:

Pour a 50 cm^3 column of prepared DEAE-cellulose (Whatman DE-32), using vacuum to obtain good packing. [The DEAE-cellulose should first be prepared using the appropriate buffer (0.1 M NaOAc, pH 7.5), following the procedures detailed in Appendix F. An alternative is the use of pre-prepared DEAE-cellulose (Whatman DE-52), which has been equilibrated with the desired buffer before use.] The column is poured in a 60 cm^3 disposable syringe barrel. Use two pieces of Whatman #4 filter paper at the bottom of the syringe (one small paper-punch-size piece to cover the central opening, another piece the size of the syringe barrel on top of the first), and one piece on top of the packed column (the size of the syringe barrel).

Wash the column thoroughly with about 500 ml of 0.1 M sodium acetate buffer, pH 7.5 (solution #1), using a peristaltic pump. Begin washing at a flow rate of 0.75-1.0 ml/minute to ensure good packing of the column. The flow rate can slowly be increased to 2.0 ml/minute after an hour of washing. The column can be stored overnight at room temperature if the system is tightly closed to prevent column desiccation.

Enzyme Preparation:

Dissolve 2.000 g a-amylase powder (from Aspergillus oryzae, crude preparation type X-A, Sigma no. A-0273 or equivalent) in 10.0 ml ddH_2O in a small beaker, using a stainless steel spatula. This takes quite a while and a lot of stirring, but it all will eventually go into solution.

Add 10.0 ml of 0.25 M calcium acetate (solution #4) to the a-amylase solution and mix thoroughly. Filter through Whatman #4 filter paper. Rinse the beaker with a few drops of ddH_2O and pour this through the filter paper to rinse it.

Transfer the filtered enzyme solution to prepared dialysis tubing and dialyze versus ddH_2O overnight in the refrigerator (see preparation procedure in Appendix G). Discard the filter paper and precipitate.

Enzyme Separation:

Wash the column with an additional 100 ml of 0.1 M NaOAc buffer, pH 7.5 (solution #1), at a 2 ml/minute flow rate before applying the enzyme solution. Allow the buffer to run down to the top of the column before applying the enzyme solution, but do not allow the column to run dry (or it may need to be discarded).

Remove the enzyme solution from the dialysis tubing and determine its volume. Add sufficient anhydrous sodium acetate powder to the enzyme solution to yield a final concentration of 0.1 M NaOAc [i.e. for 30 ml of enzyme solution, add 0.246 g anhydrous NaOAc]. The pH of this solution will be about 6.6, and should be adjusted to 7.5 by the addition of 1 M NaOH (solution #5); about 2-3 drops will be required.

The a-amylase solution should be carefully layered onto the top of the column with a Pasteur pipet to establish a small reservoir of solution (7-10 ml) to buffer the falling drops. Be careful not to disturb the surface of the column. Apply the remaining enzyme solution via the peristaltic pump, using a 0.75 ml/minute flow rate. A slow flow rate during application is essential for proper bonding of the enzyme to the column matrix (Cooper 1977).

After all of the enzyme solution has been applied to the column, run it down to the top of the column. Carefully layer 0.1 M NaOAc buffer, pH 7.5, onto the column using a Pasteur pipet. Reattach the peristaltic pump and wash the column with 200-250 ml of solution #1 using a 2 ml/minute flow rate. [All further elution of the column will be done at this flow rate.] This will elute some of the undesirable compounds from the column.

After washing the column with solution #1, let the buffer run down to the top of the column as before. Using a Pasteur pipet, layer 0.2 M NaOAc buffer, pH 7.5 (solution #2), onto the top of the column. Wash the column with about 200 ml of this buffer. This buffer will elute an amyloglucosidase from the column (Fleming 1968).

After washing with solution #2, run the solution to the top of the column, and switch to 0.4 M NaOAc buffer, pH 7.5 (solution #3), as before. The first 30 ml of eluate following the switch to solution #3 should be discarded. Collect the next 75 ml of eluate; this contains the a-amylase, and the bulk of the activity of the crude enzyme mixture. Upon elution of this fraction, the separation is complete and the column may be discarded.

Final a-Amylase Purification:

Add the appropriate amount of calcium acetate monohydrate to the a-amylase solution to yield a final concentration of 0.025 M (add 0.331 g Ca $(OAc)_2 \cdot H_2O$ to 75 ml a-amylase solution). [The Ca^{+2} ion is essential for the stability of a-amylase (Allen and Spradlin 1974, Robyt and Whelan 1968).] After solubilization of the $Ca(OAc)_2 \cdot H_2O$, adjust the solution to pH 4.2, the isoelectric point of a-amylase, by addition of glacial acetic acid.

Precipitate the a-amylase by the addition of ice cold acetone to a final concentration of 60% (add 113 ml of acetone to the a-amylase solution). Further addition of glacial acetic acid will be required to readjust the pH to 4.2 after adding the acetone. Cover with parafilm and place the solution in -20C freezer for a few hours to aid precipitation. Recover the a-amylase by centrifugation at 1100 g for 10 minutes. Discard the supernatant.

Suspend the precipitate in 0.05 M sodium acetate buffer, pH 5.1 (solution #6), to which a tiny pinch of sodium chloride (NaCl) has been added. [The chloride ion is essential for maximum a-amylase activity (Robyt and Whelan 1968).] Transfer the a-amylase suspension to plastic tubes. Assay the solution for activity (see procedure in Appendix E), then store in -20C freezer until needed.

Appendix D

AMYLOGLUCOSIDASE ASSAY

Adapted from Sigma Diagnostics 1984, Pazur et al. 1971.

Solutions:

1. 0.05 M sodium acetate (NaOAc) buffer, pH 5.1--mix 2.84 ml glacial acetic acid with approximately 900 ml deionized distilled water (ddH_2O). Adjust to pH 5.1 at room temperature with 30% sodium hydroxide (NaOH) and bring to a final volume of 1000 ml with additional ddH_2O.

2) 0.21 mM o-dianisidine/0.05 M NaOAc buffer, pH 5.1--dissolve 13.2 mg o-dianisidine dihydrochloride in 2.0 ml ddH_2O (this solution is stable for about three months in the refrigerator). Mix 1.0 ml of this solution with 99.0 ml 0.05 M NaOAc, pH 5.1 (solution #1). This final solution is stable for up to one month if stored in a brown bottle in the refrigerator.

3) 1% starch solution--dissolve 0.100 mg soluble potato starch in 10.0 ml 0.05 M NaOAc, pH 5.1 (solution #1). Bring the solution to a gentle boil while continuously stirring with a magnetic stir bar. The final solution should be translucent with a slight whitish tint. Cool to room temperature and return to 10.0 ml total volume, if necessary.

4) Glucose oxidase (GOD)/peroxidase (POD) solution--dissolve the appropriate amount of GOD and POD in 0.05 M NaOAc, pH 5.1 (solution #1). [See the assay on the bottle label. The approximate amounts needed are 1 mg glucose oxidase (from Aspergillus niger, type X, Sigma

no. G-8135 or G-7141) per 2 ml of solution and 1 mg peroxidase (from horseradish, type II, Sigma no. P-8250) per 3 ml of solution.] A final concentration of 50-60 units of each enzyme per ml is desired. Make 2-4 ml of this solution; it is stable for at least 3 months if stored in the refrigerator.

5) Amyloglucosidase solution--prepare a dilution of the amyloglucosidase sample to be assayed using solution #1, containing 0.5-1.0 units/ml. Start with a 1:10 dilution and dilute further as needed.

Assay Procedure:

In a 3 ml optical glass cuvette (Markson #1-G-10 or equivalent), or small test tube if using spectrophotometer with sipper, mix 2.40 ml 0.21 mM o-dianisidine solution (solution #2), 0.50 ml 1% starch solution (solution #3), and 0.10 ml of the glucose oxidase/peroxidase solution (solution #4). This solution will stain the cuvettes, but can be cleaned with methanol, ethanol, or a sodium hydroxide solution.

After mixing thoroughly, equilibrate at 25C (or room temperature) and check the absorbance at 500 nm to ascertain that it is steady, then calibrate the spectrophotometer with the solution.

At time 0, add 0.10 ml of the amyloglucosidase solution (solution #5) to the above mixture. Mix well by vortexing or inversion and place the cuvette into the spectrophotometer. Follow the increase in absorbance at 500 nm over 10-15 minutes, recording the A_{500} at 15-20 second intervals. Dilute the enzyme solution further, if necessary, so that the rate of increase in absorbance is linear (concentrated enzyme solutions exhibit exponential rate increase). Use the

maximum linear rate of absorbance increase to calculate the activity.

There is a 1:1 ratio of o-dianisidine oxidized to D-glucose liberated from starch.

uM units/ml amyloglucosidase solution =

$(A_{500}/\text{minute})(3.1\ \text{ml})(\text{dilution factor})/(7.5)(0.1\ \text{ml})$

Where: 3.1 ml = volume of assay mixture

0.1 ml = volume of amyloglucosidase solution in assay mixture

7.5 = mM extinction coefficient for oxidized o-dianisidine

dilution factor = 200, for 1:200 dilution (and so forth)

IU units amyloglucosidase/ml =

(uM units/ml)(0.18 mg/uM glucose)

Where: molecular weight of glucose = 180.16 g/mole

Appendix E

a-AMYLASE ASSAY

Adapted from the Worthington Manual 1977, Bernfeld 1951.

Solutions:

1) 0.02 M sodium phosphate buffer, pH 6.9, with 0.006 M sodium chloride--dissolve 2.839 g dibasic sodium phosphate (Na_2HPO_4) and 0.351 g sodium chloride (NaCl) in 950 ml deionized distilled water (ddH_2O). Adjust to pH 6.9 with 1 M hydrochloric acid (HCl), and bring to a final volume of 1000 ml with additional ddH_2O. This solution is stable for at least a year if kept refrigerated.

2) 2 N sodium hydroxide--dissolve 8.000 g sodium hydroxide (NaOH) in 100 ml ddH_2O. This solution is stable for years at room temperature.

3) 3,5-dinitrosalicylic acid reagent--dissolve 1.000 g 3,5-dinitrosalicylic acid in 20.0 ml 2 N NaOH (solution #2). Add the dye slowly while stirring vigorously with a magnetic stir bar. Dissolving the dye can be aided by adding up to 50 ml ddH_2O. When the dye is completely solubilized, add 30.0 g potassium sodium tartrate tetrahydrate (Rochelle salt) slowly, while continuously stirring. After complete solubilization, bring the total volume of the solution to 100 ml with additional ddH_2O. Flush the flask with nitrogen gas (N_2) and rapidly cover tightly with parafilm to exclude carbon dioxide (CO_2), which causes rapid decomposition of the dye [this step is unnecessary if the solution will be used immediately and then discarded]. This

solution is stable for up to 2 weeks if kept covered and stored in the refrigerator.

4) 1% starch solution--dissolve 0.500 g soluble starch in 50.0 ml 0.02 M sodium phosphate buffer, pH 6.9, with 0.006 M NaCl (solution #1). Bring to a gentle boil while continuously stirring with a magnetic stir bar. The final solution should be translucent, with a slight whitish tint. Cool to room temperature and return to 50.0 ml total volume with ddH_2O, if necessary.

5) a-amylase solution--a concentration of 100-250 units a-amylase per ml of solution is needed for this assay. At least three concentrations within this range are required for accurate determination of the a-amylase activity, so prepare a range of dilutions (i.e. 1:10, 1:50, 1:100, 1:500, 1:1000) for each enzyme preparation to be assayed; use solution #1 to prepare the dilutions.

Maltose Standard Curve:

100 mM maltose stock solution--dissolve 0.360 g maltose monohydrate in 10.0 ml solution #1. This solution is stable for several weeks if stored covered in the refrigerator.

10 mM maltose standard--mix 1.0 ml 100 mM maltose stock with 9.0 ml solution #1. Prepare two 1.00 ml aliquots for the standard curve.

7.5 mM maltose standard--mix 750 ul 10 mM maltose standard with 250 ul solution #1. Prepare two 1.00 ml aliquots.

5.0 mM maltose standard--mix 500 ul 10 mM maltose standard with 500 ul solution #1. Prepare two 1.00 ml aliquots.

2.5 mM maltose standard--mix 250 ul 10 mM maltose standard with 750 ul solution #1. Prepare two 1.00 ml aliquots.

1.0 mM maltose standard--mix 100 ul 10 mM maltose standard with 900 ul solution #1. Prepare two 1.00 ml aliquots.

0.0 mM maltose standard (blank)--use two 1.00 ml aliquots of solution #1.

<u>Assay Procedure:</u>

Transfer 0.50 ml aliquots of the enzyme dilutions prepared above to large test tubes (>12 ml capacity). It is best to run duplicates of each enzyme dilution. Also run a blank containing 0.5 ml of solution #1. Equilibrate all tubes at 25C (room temperature is okay).

At timed intervals [10 sec is good; 5 sec is too short for pipetting], add 0.50 ml 1% starch solution (solution #4) to each tube containing an enzyme dilution (and the solution #1 blank), and mix well on a Vortex mixer. [Cut about 1 mm of the pipet tip off to increase the bore and allow faster transfer of the solutions.]

Incubate the tubes at 25C (or room temperature) for <u>exactly 3 minutes</u>. Then, at the same timed interval as before, sequentially add 1.00 ml of the 3,5-dinitrosalicylic acid (DNS) reagent to each tube. The DNS reagent should be at the same temperature as the enzyme/starch solutions. Immediately mix the solutions thoroughly on a Vortex mixer; this stops the enzyme reaction.

At this point, add 1.00 ml of the DNS reagent to each of the tubes comprising the maltose standard curve and mix thoroughly. [The maltose standard curve should be run in duplicate--

run 2 tubes of each maltose concentration, each containing 1.00 ml of the appropriate maltose solution.]

Incubate all of the tubes in a boiling water bath for 5 minutes (boil all of the tubes at once, if possible, to ensure the same amount of time in the water bath for all tubes). Cool the tubes to room temperature, and add 10.0 ml of ddH_2O to each tube (use a repipeter, if available). Mix the contents thoroughly using a Vortex mixer and/or tube inversion. Nothing in this assay is extremely harmful, so general safety procedures are adequate.

Determine the absorbance of each tube at 540 nm using the 0.0 mM maltose standard as the blank. It may be necessary to correct for absorption due to the starch solution, by subtracting the absorbance of the buffer/starch blank from the absorbances of the enzyme solutions (not the maltose standard curve). However, the absorbance of the starch solution is usually negligible and generally can be ignored.

The liberation of maltose (in umoles) by the enzyme solutions is determined from the maltose standard curve using linear regression analysis. A maltose standard curve must be processed each time the assay is performed, concurrently with the enzyme preparations.

units/ml a-amylase solution = (umoles maltose liberated)
(dilution factor)/(0.5 ml)(3 minute)

Where: 0.5 ml = volume of enzyme solution used
3 minutes = time allowed for starch digestion
dilution factor = 100, for 1:100 dilution
(and so forth)

Appendix F

PREPARATION OF DEAE-CELLULOSE

Adapted from McDonald 1985, Cooper 1977.

Solutions:

1) 0.5 N sodium hydroxide--dissolve 60.000 g sodium hydroxide (NaOH) in 3000 ml deionized distilled water (ddH_2O).

2) 0.5 N hydrochloric acid--add 64.7 ml 36% (11.6 N) hydrochloric acid (HCl) to 1435 ml ddH_2O.

3) 0.2 M sodium acetate buffer, pH 7.5--dissolve 12.303 g anhydrous sodium acetate (NaOAc) in 750 ml ddH_2O. Adjust to pH 7.5 with 10% glacial acetic acid (HAc); approximately 6 drops will be required.

4) 0.1 M sodium acetate buffer, pH 7.5, with 0.1% sodium azide--dissolve 2.051 g anhydrous NaOAc in 250 ml ddH_2O, and adjust to pH 7.5 with approximately 1 drop of 10% HAc. Add 0.250 g sodium azide (NaN_3) and stir to dissolve.

5) 0.1 M citrate/phosphate buffer, pH 8.0--dissolve 0.548 g anhydrous citric acid and 20.485 g dibasic sodium phosphate (Na_2HPO_4) in 750 ml ddH_2O.

6) 0.05 M citrate/phosphate buffer, pH 8.0 with 0.1% sodium azide--dissolve 0.091 g anhydrous citric acid and 3.414 g Na_2HPO_4 in 250 ml ddH_2O. Add 0.250 g NaN_3 and stir to dissolve.

Procedure:

Suspend 100 g dry DEAE-cellulose powder (Whatman DE-32) in approximately 1500 ml of 95% ethanol in a large beaker. Gently stir the slurry occasionally with a glass stirring rod (DEAE-cellulose fragments easily, so stirring should be kept to a minimum). After 30 minutes filter the slurry through a large Buchner funnel (using Whatman qualitative filter paper #1 or #4) to remove the ethanol.

Suspend the DEAE-cellulose in 1500 ml 0.5 N NaOH and stir occasionally for 1 hour. Filter the slurry as before, and wash extensively in the funnel with tap water.

Suspend the DEAE-cellulose in 1500 ml 0.5 N HCl and stir occasionally for 30 minutes. Filter as before and wash extensively with tap water.

Suspend the DEAE-cellulose in 1500 ml 0.5 N NaOH and stir occasionally for 30 minutes. Filter as before and wash with tap water until the pH is reduced to 8.0.

At this point the DEAE-cellulose is ready to be equilibrated with the initial buffer to be used in the enzyme purification procedure. Suspend half of the material in 750 ml 0.2 M NaOAC buffer, pH 7.5 (solution #3; for the purification of a-amylase), and the other half in 750 ml 0.1 M citrate/phosphate buffer, pH 8.0 (solution #5; for the purification of amyloglucosidase). [A concentrated buffer is used for the equilibration of the DEAE-cellulose.] Stir the suspensions gently for at least 3 hours while monitoring the pH. Maintain the pH at its initial level (7.5 or 8.0) by the addition of the appropriate acid solution (acetic or citric), if necessary.

After the pH has stabilized, quit stirring and allow the DEAE-cellulose to settle. Any fines that have been generated during the procedure will float to the top of the slurry and should be removed at this time by aspiration, or with a Pasteur pipet. Filter the slurries as before, and wash with tap water.

Re-suspend the DEAE-cellulose in approximately 150 ml of the appropriate buffer containing 0.1% NaN_3 (solutions #4 and #6). The slurry should again be allowed to settle and any fines removed as before. For proper column packing, the excess buffer after settling of the ion exchanger should be approximately 20% of the total volume. Add additional buffer if necessary. Store the prepared DEAE-cellulose at 4C until ready to use. Resuspend the DEAE-cellulose before pouring the column.

Appendix G

PREPARATION OF DIALYSIS TUBING

Adapted from McDonald 1985, Cooper 1977.

Solutions:

1) 5mM (ethylenedinitrilo)-tetraacetic acid--dissolve 1.396 g (ethylenedinitrilo)-tetraacetic acid disodium salt (EDTA) in 750 ml deionized distilled water (ddH_2O).

2) 1 M sodium hydroxide--dissolve 10.0 g sodium hydroxide (NaOH) in 250 ml ddH_2O.

Procedure:

Cut the dry dialysis tubing into short lengths appropriate for the amount of solution to be dialyzed. Moisten one end of the tubing and tie it into a double knot. Be gentle or the tubing may be damaged.

In a 1000 ml wide-mouthed Erlenmeyer flask, bring the 5mM EDTA solution (solution #1) to a boil while stirring slowly with a magnetic stir bar. Adjust the solution to pH 8.0 with 1 M NaOH (solution #2); about 6 ml will be needed.

Add the pieces of dialysis tubing to the boiling 5 mM EDTA solution, pH 8.0, and boil for 15 minutes with occasional stirring (use a long glass stirring rod). Be careful not to damage the tubing.

All further handling of the tubing should be done while wearing rubber gloves. This protects the boiled dialysis

tubing from contamination by protease enzymes on your hands. Rinse the tubing thoroughly with tap water. Follow this with a thorough rinsing in ddH_2O.

Store the prepared dialysis tubing in ddH_2O in the refrigerator (4C). If kept covered and well-hydrated, the prepared tubing will remain in good condition for a year or more.