Cold Hardiness in Genetically 'Improved' and
Wild Stand Coastal Douglas-fir

by

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ABSTRACT

Breeding for increased growth in coastal Douglas-fir (*Pseudotsuga menziesii* var. *menziesii* (Mirb. Franco)) may affect the level of cold hardiness of seedlings used for reforestation. Cold hardiness was measured in seedlings grown from top-cross, first generation seed orchard, and unimproved wild stand seed throughout one growing season by visual assessment of artificial freeze tests. Some significant differences in freezing damage between genetically improved and wild stand trees were found in spring, fall, and mid-winter. Mitotic index of terminal buds of lateral branches was investigated as an indicator of dormancy, and a negative correlation between mitotic index and cold hardiness was found. During the spring period, there was a significant difference in mitotic index found between the genetic groups. Using the rate of tracheid formation as an indicator of secondary growth, no significant correlations were made between secondary growth and cold hardiness. There were also no significant differences in rate of tracheid formation between the genetic groups. Date of bud burst and rates of shoot extension were related to levels of cold hardiness in the three groups of seedlings. Trees that burst bud earlier completed shoot extension earlier in the season and entered dormancy significantly earlier in the fall. Top-cross trees appeared to extend their growing season later into the fall, thereby gaining a height advantage over wild stand seedlings. These top-cross families do not appear to have an increased late fall frost damage risk, and may have a reduced risk of critical spring frost damage due to delayed growth initiation.

A second study examined the effect of five nutrient treatments varying N:P ratio on cold tolerance of fast and slow growing Douglas-fir families. Trees receiving more N and P grew significantly taller and trees receiving higher levels of N had significantly higher mitotic indices in late fall. Trees with a high N:P ratio were significantly more hardy at all times of the year. Thus, a significant positive correlation existed between hardiness and mitotic index. Increased N and P nutrition may have prolonged the growing season, but did not decrease hardiness.

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CHAPTER 1
INTRODUCTION

Cold hardiness is one of the physiological attributes which determines the environment in which a plant can survive (Sakai and Weiser 1973). Plants that can undergo physiological changes and adapt to freezing temperatures can survive climates which freezing sensitive species find intolerable, and conifers are among the most cold tolerant plants (Sakai and Weiser 1973). Plant cold acclimation is triggered by decreasing photoperiod and decreasing temperatures (Weiser 1970). Hardiness is achieved by alterations in plant membranes, lipids, proteins, and enzyme conformation to prevent injury during exposure to sub-zero temperatures (Sakai and Larcher 1987).

With reforestation comes the demand for seedlings which are not only fast growing, but also are well suited for the local climate in which they are planted. The principal objective of most tree improvement programs is to increase growth rates and increase or maintain wood quality without negatively impacting other physiological properties. Maintenance of tree health includes ensuring that yearly growth and dormancy periodicity traits, such as timing of bud phenology and cold acclimation, are synchronized with regional climate (PNWTIRC 1995). The mid-winter cold hardiness generally observed in Douglas-fir (*Pseudotsuga menziesii* (Mirb. Franco)) is sufficient protection under prevalent winter conditions, however, spring and fall cold hardiness is of great concern in most areas of the Pacific Northwest as these are periods when dormancy is absent and probabilities of damaging freezing events are highest (Timmis et al. 1994). Subsequently, a more thorough understanding of the relationship between levels of cold hardiness and the phenology of the coastal Douglas-fir used in reforestation is required.

With the desire to accelerate growth of trees through artificial means, we may disrupt phenologies well suited to native environmental conditions if genetic correlations exist between growth and adaptive traits. This study examines two aspects of growth enhancement; 1) genetically improved stock selected for higher growth rates, and 2) fertilized stock and their effect on seasonal cold tolerance. A further understanding of the relationships between seasonal cycles of growth and cold hardiness is the final goal.

CHAPTER 2

LITERATURE REVIEW

2.1 Cold hardiness

2.1.1 Freezing damage

2.1.1.1 Membrane damage

Freezing injury in cells is related to irreversible alterations in the membrane structure created by a number of low temperature related events. When the temperature drops below zero, plant cells and the solutions within them remain unfrozen, the solution is 'supercooled'. Without supercooling, intracellular freezing would result. Freezing of this nature is almost always fatal, due to the mechanical destruction of biomembranes resulting from growing ice crystals. Eventually ice will form in the intercellular spaces (extracellular freezing) at a temperature that depends on the plant solution freezing point and the presence of nucleating agents. This creates an osmotic gradient which causes liquid water to be removed from the cell and coalesce with the growing ice crystals outside of the cell (Sakai and Larcher 1987).

As ice forms in the extracellular matrix, the solute concentration within the cell increases and consequently the osmotic potential drops. Extracellular freezing will continue until the osmotic potential is at equilibrium across the membrane and water is no longer drawn from the cell. As substantial amounts of cellular water are removed, the cell becomes increasingly dehydrated (Sakai and Larcher 1987).

The freezing of aqueous solutions of electrolytes may allow large electrical potentials to arise so that a charge separation occurs across the ice interface with potentials of sufficient magnitude to result in lysis of the plasma membrane; the Workman-Reynolds effect (Steponkus et al. 1985). The greatest danger to the cell membrane, however, comes with fluctuations in plant tissue temperature. As dehydration occurs and the cell volume is reduced, the tension in the plasma membrane is reduced to zero and deletion of membrane material occurs via endocytotic vesiculation (Dowgert et. al. 1987). Endocytotic vesiculation itself does not cause injury; however, sufficiently large area reductions of the plasma membrane are irreversible in short periods of time. Should temperatures rise, thawing of the extracellular ice results and the cells are susceptible to lysing during subsequent osmotic expansion that occurs with the resorption of extracellular water. This is referred to as expansion-induced lysis, and is the predominant form of injury to non-acclimated cells (Steponkus 1984).

Cell survival is not only a function of the integrity, but also of the fluidity of the membrane. Membrane fluidity is directly related to temperature. A reduction in temperature initiates a physical change of state in the hydrophobic matrix of the membrane and produces semicrystalline to crystalline lamellae which increase the susceptibility of the membrane to stress (Alberdi and Corcuera1991). Freezing injury is accompanied by the degradation of phophatidyl choline to phoshphatidic acid in the cortex of poplar twigs (Yoshida and Sakai 1974). When the proportion of phosphatidyl choline decreases, there is an increased tendency for a lamellar to hexagonal phase transition (Sakai and Larcher 1987). This leads to a reduction in fluidity and a reduction in the ability of the plasma membrane to carry out normal functions leading to cell death (Sakai and Larcher 1987).

2.1.1.2 Protein damage

Freezing injury is also related to irreversible alterations in protein conformation (Yoshida 1984a). The dehydration stress and mechanical stress of slow extracellular freezing followed by a slow thawing may result in the denaturation of membrane proteins and changes in lipid protein interaction (Sakai and Larcher 1987). This causes the inactivation of K+ and sugar pumps and general breakdown of the membrane system leading to cell death. Many proteins not rendered inactive by denaturation may still change configurationally possibly exposing free sulfhydryl groups (Guy 1990). Freezing dehydration created from the removal of free water in the cell to extracellular ice concentrates the protoplasm. This concentration increases the probability of protein contacting protein. If such contact occurs, aggregation due to intermolecular sulfide bonding is possible due to prior denaturation (Huner et. al. 1982).

2.1.2 Cold acclimation

2.1.2.1 Hardening

To survive in sub-zero environments plants can either avoid freezing or tolerate freezing. Strategies for freezing avoidance include supercooling, modification of intracellular solute concentrations, tolerating tissue desiccation and extratissue freezing. Supercooling provides protection against brief frosts below the freezing point of tissues. In acclimatized tissue, deep supercooling can occur to about -39 °C and can continue for

extended periods (George and Burke 1977). In tissues such as xylem ray parenchyma, there is limited potential for extracellular freezing and supercooling is the only alternative (Sakai and Larcher 1987). Changes in solute concentration in the intracellular solution will allow for greater tolerance of freezing temperatures. The conversion of starch to soluble sugars will decrease the freezing point of the intracellular water (Sakai 1966). Tissue desiccation resulting from extracellular ice formation is a necessary stress to reduce the chance of intracellular freezing (Sakai and Larcher 1987). Extraorgan and extratissue freezing occurs when water is translocated from supercooled organs to ice nucleation centers, and occurs in vegetative buds of some conifers (Sakai 1982).

Strategies for freezing tolerance include dormancy and frost hardiness. Dormancy is the state in which growth does not occur even if the plant is exposed to growth promoting conditions. Some have defined dormancy to be the interval between budset (bud scales present) and bud flushing (Lavender and Hermann 1970), however growth activity occurs within the bud at this time and mitotic activity of the shoot apex is considerable during early leaf primordia initiation (Owens and Molder 1973). Frost hardiness in woody plants is linked to dormancy and occurs in two stages. The first stage occurs at the onset of dormancy as photoperiod decreases, temperatures decrease to 0 °C and most growth processes for the current growing season are completed. Initial hardening is dependent on growth cessation, with growth cessation being identified by an absence of meristematic activity at the shoot tip. Increased meristematic activity or growth rate and frost hardiness have been inversely correlated (Colombo et al. 1989). Shortening photoperiods control the first stage of the hardening process by regulating the rate of hardening (Greer et al. 1989). The second stage is facilitated by temperatures

below zero and a third stage, proposed by some, is induced by temperatures between -30 and -50 °C (Weiser 1970). In addition to the dependence of hardiness on low temperature (van den Driessche 1969) it has also been suggested that sufficient light to allow a certain amount of photosynthesis is required (van den Driessche 1970).

It is important to note that the various plant organs may be induced to harden by different environmental stimuli. For example, while hardening of needles and whole seedlings is increased by a short day treatment, roots of *Picea mariana* hardened only in response to lowering of temperature (Bigras and D'Aoust 1991) This initial independence and eventual reinforcement of the hardening factors (endogenous, short-day, chilling, and freezing) must provide valuable adaptive flexibility to the plant, particularly in harsher environments.

2.1.2.2 Membrane acclimation

Membranes are the primary site of cold injury of plants, therefore their role in acclimation is crucial to the survival of the cell in freezing conditions (Steponkus 1984). Cold acclimated cells reduce the amount of water in the cell to avoid intracellular freezing. During the initial phase of cold acclimation in *Cornus stolonifera* trees, permeability of the plasma membrane increases significantly in response to increasing concentrations of ABA (McKenzie et. al. 1974). This is supported by the significantly increased activity of ATPase from plasma membranes of needles from *Pinus sylvestris*. A high potential for ATPase activity indicates a high uptake of ions lost from increased permeability of the plasma membrane during cold acclimation (Hellergren et. al, 1983).

The degree of fluidity in membranes is believed to be a major factor in determining cold tolerance of plants and the deciding element appears to be the level of desaturation of membrane fatty acids (Williams et. al 1988). The incorporation of unsaturated fatty acids into membrane lipids is thought to be induced by sub-zero temperatures (Senser and Beck 1982b). The preferential accumulation of the unsaturated linoleic acid in all lipids seems to be a generalized mechanism of frost resistance in hardy plants (Latsaque et. al. 1992). In *Picea abies* the ratio of unsaturated to saturated fatty acids increased due to a preferential incorporation of linoleic acid (18:3) and octadecatetraonic acid (18:4) (Senser 1982). This increase in linoleic acid was also found in citrus trees with more than a hundred percent increase of linoleic acid in phospholipids (Norby and Yelenosky 1982).

The specific phospholipids produced during cold acclimation in *Picea abies* are phosphatidyl choline and phosphatidyl ethanolamine (Senser and Beck 1982a). Because phophatidyl choline forms less packed membranes than do other forms of phospholipids, it can significantly increase membrane fluidity. Glycerolipids increased unsaturation by incorporating 18:3 fatty acids in *Pinus strobus* chloroplasts (DeYoe and Brown 1979). In addition to linoleic acid, linolenic acid has been found to increase in cold hardened plants (Yoshida and Sakai 1974). These acids are accumulated because their double bonds create folding carbon chains, which result in the loss of the symmetrical dispersion of the membrane particles, and subsequently increase fluidity (Senser and Beck 1982a). When the phospholipids are not packed as tightly, a drop in temperature will not decrease the fluidity of the membrane, preventing significant loss of membrane function. This fluidity also increases the stability of the plasma membrane to applied electrical fields created by

increased concentrations of ions resulting from the freezing of extracellular water (Steponkus et. al. 1985).

Not only are there qualitative changes in membrane lipids, but quantitative changes occur as well. The maximum levels of phospholipids and galactolipids during the year occurred in the hardening period of *Picea abies* (Senser 1982). Active synthesis of lipids takes place in the cortex of *Populus* twigs during hardening, as has been demonstrated by the incorporation of labeled glycerol and acetate (Levitt 1980). This augmentation of membrane lipids, particularly phospholipids, is thought to be a short day length dependent step (Senser and Beck 1982b). There is a need for a supplementation of phospholipid reserves to replace those degraded during freezing conditions (Yoshida and Sakai 1974). In addition to phospholipids, increases of the glycerolipid component of membranes were also noticed with the increase in freezing tolerance of *Pinus sylvestris* (DeYoe and Brown 1979). The increase in lipid content may be a result of the membrane augmentation of cell organelles at the beginning of the frost hardening process, and the increased number of chloroplasts in frost hardened needle cells (Senser and Beck 1982a). The significant increase of the lipid content increases the lipid to protein ratio, giving the membrane a higher elasticity that enables the cell to tolerate the rapid water influx during thawing (Senser and Beck 1982a). The process of phospholipid supplementation during acclimation periods is also supported by characteristics of dehardening. The decrease of hardiness in the spring is accompanied by large decreases in phospholipids indicating the presence of major membrane associated change from the cold acclimated state (Kedrowski 1980). The increased phospholipid level of acclimated cells may also serve as a reservoir for the repair of the plasma membrane (Yoshida and Sakai 1973).

When large increases of cell volume occur during the rehydration period of the freeze thaw cycle, the ability to increase the surface area of the plasma membrane is crucial for cell survival. Tolerable surface area increment (TSAI) for protoplasts from non-acclimated leaves was lower and strongly dependent on the rate of expansion, while the TSAI from acclimated leaves was significantly greater and independent of the expansion rate (Dowgert and Steponkus 1983). The TSAI level for fifty percent survival of acclimated protoplasts was twice that of non- acclimated cells (Dowgert and Steponkus 1984). The increased tolerance of acclimated plants to osmotic contraction/expansion is due to both an increased expansion potential, and a difference in the behavior of the plasma membrane during contraction. In acclimated cells, osmotic contraction coincides with the formation of numerous polyp-like protuberances that are observed on the outer surface of the plasma membrane (Gordon-Kamm and Steponkus 1982). These protuberances are bounded by and contiguous with the plasma membrane and have a high lipid content (Gordon-Kamm and Steponkus 1982). The exocytotic extrusions are readily reversible which may account for the low incidence of expansion induced lysis observed in acclimated cells (Dowgert and Steponkus 1984). Lethal tensions arise in non-acclimated cells before plasma membrane area has increased by a large fraction; however, the tension of acclimated cells does not rise until isotonic volume is reached (Dowgert et. al. 1987). Non- acclimated cells produce endocytotic vesicles which contain deleted plasma membrane material from osmotic contraction, but the material is not readily available for reincorporation into the plasma membrane as sudden expansion may require (Dowgert and Steponkus 1984).

2.1.2.3 Protein acclimation

Protein metabolism plays a major role in the development of freezing tolerance. An increase in soluble protein is found during the later stages of cold acclimation and this change closely follows frost hardiness (Pomeroy et. al. 1970, Chen and Li 1980). An increased capacity for protein synthesis may arise from an increase in soluble RNA found with cold acclimation (Sarhan and Daoust 1975). An increase in both rRNA and mRNA was found during the hardening period, with new polypeptides arising from the new mRNA (Johnson-Flannegan and Singh 1987). The increase of RNA in *Buxus microphylla* is closely paralleled by an increase in soluble and membrane bound proteins (Gusta and Weiser 1972).

The accumulation of soluble protein accompanies the change in membrane water permeability during acclimation (Kacperska-Palacz et al. 1977). This change in soluble protein concentration could reflect changes in protein synthesis and degradation, or release of proteins from membranes and other bound forms (Brown 1978). In some cases, hardening has been successful even if conditions are present to prevent an increase of soluble proteins, therefore suggesting a possible membrane protein role in cold acclimation (Sakai and Larcher 1987). The role of proteins in frost hardiness is further supported by experiments that inhibit cold acclimation with cycloheximide treatments (Gilmour et al. 1988).

Qualitative protein changes also accompany hardening in woody plants (Craker et al. 1969). Because of the variation in proteins found in different species it is doubtful that

cold acclimation polypeptides are highly conserved in plants (Gilmour et al. 1988). A significant portion of the proteins found in acclimated *Robinia psuedoacacia* were glycoproteins (Brown and Bixby 1975). Glycoproteins are known to have a high water binding capacity, which could play a role in intracellular resistance to ice formation, as an increase in water binding proteins would decrease free cellular water. In total, hardy *Robinia psuedoacacia* seedlings had seventeen proteins different from non hardy seedlings (Bixby and Brown 1975). In fall the appearance of novel proteins in *Pinus lambertiana* and *Pinus monticola* were significantly correlated with frost hardiness in the two species (Ekramoddoullah et al. 1994). Furthermore, a protein absent in late summer accumulated in *Pseudotsuga menziesii* var. *glauca* tissues during the fall. It is thought that this protein may serve as a storage reserve during overwintering (Roberts et al. 1991).

The composition of amino acids in proteins produced during acclimation show a higher degree of hydrophily making them capable of binding and retaining vital water with enough energy to avoid the extreme dehydration and denaturation that occurs during extracellular ice formation (Rochat and Therrien 1975). The increase in relative content of hydrophilic amino acids, such as arginine and glycine, and the decrease of hydrophobic amino acids, such as leucine and phenylalanine, also decreases the possibility of protein aggregation (Shomer-Ilan and Weisel 1975). This high content of hydrophilic amino acids appears to account for the extreme solubility of the proteins in cold acclimated plants (Volger and Heber 1975). Hardy plants possess proteins which remain in their native state at low temperatures and resist sulfhydryl - disulfide interchange, thus decreasing the possibility of inactivation due to intermolecular disulfide bond formation (Huner et. al. 1982).

The presence of new enzymes, increased enzyme activity, and conformational changes of enzymes can be observed in plants undergoing cold acclimation. The increased levels of unsaturated fatty acids at low temperatures may be caused by increased, or new enzyme production at low temperature (Williams et. al. 1988). For example, ribulose-l,5-bisphosphate carboxylase undergoes a conformational change during acclimation which results in a decreased exposure of free sulfhydryl groups even though the total number of free sulfhydryl molecules remains constant (Huner et. al. 1982). Glutathione which protects membranes against peroxidation by free radicals, exists in three forms: a reduced form, an oxidized form, and a mixed disulfide. The concentration of the reduced form significantly increases in *Cornus stolonifera* and *Picea abies* during cold acclimation (Guy and Carter 1982). In addition, the proportion of the two forms of invertase is different in acclimated *Triticum aestivum*, suggesting that one form of invertase replaces the other during cold hardening (Roberts 1979) New isozymic variants for ATPases, esterases, acid phosphatases, leucine aminopeptidases, peroxidases, and some dehydrogenases have also been found in acclimated plants (Guy 1990).

2.1.3 Deacclimation

While cold hardiness is initiated by the decreasing day lengths and temperatures that occur in late fall, de-acclimation in the spring is a result of increasing temperatures, with increasing photoperiod playing a lesser role (Greer and Stanley 1985). Temperature controls the loss of frost hardiness by regulating the rate of dehardening (Greer and Stanley 1985). Rates of dehardening increase considerably with increasing temperatures

indicating the non-linearity of the dehardening with respect to temperature (Repo and Pelkonen 1986). Dehardening may be temporarily delayed by short photoperiods, but otherwise daylength is thought to have little significant quantitative effect. In contrast, some have proposed that photoperiod can play a role beyond that of delaying dehardening, and increasing day length may increase the rate of dehardening (Hawkins and McDonald 1992).

At constant temperatures, the rate of dehardening is initially fixed but declines as the minimum summer frost hardiness is reached. Under variable temperature conditions, mean temperature is better correlated with rate of dehardening than minimum temperature (van den Driessche 1969); however, a low night temperature reduced the dehardening response to higher day temperatures (Eagles and Williams 1992). The effect of chilling temperatures is to widen the temperature range at which bud burst will occur and the greatest increases in flushing occur with increasing temperatures at the low end of the flushing temperature range (Campbell and Sugano 1975).

The effects of warm temperatures on deacclimation have also been found to be dependent on the stage of development in the spring. Dehardening may be a partially reversible process, with the response depending on the degree of hardiness or stage of development (Repo 1991). Increased deacclimation with plant development during the quiescent phase before bud burst results in the loss of the ability of plants to reharden, suggesting a shift in growth and developmental processes and an irreversible change. In other words the further dehardening advances, the less reacclimation is possible (Kobayashi et al. 1983). Bud burst is the critical point for rehardening capability (Fuchigami et al. 1982).

Dehardening is a much faster process than hardening (Aronsson 1975). The relatively short period required means that field survival is potentially very sensitive to the occurrence of dehardening conditions (Gay and Eagles 1991). However, mid-winter solar warming to temperatures above the freezing point is unlikely to result in dehardening and subsequent freezing injury, because warming is infrequent, of short duration, and does not always raise needle temperature above the freezing point (Strimbeck et al. 1993). Again, individual organs respond to different environmental cues. The warming of soil advanced the date of growth initiation and warming of the air increased the rate of bud development once initiated (Sorenson and Campbell 1978).

Two theories exist for a molecular basis of deacclimation. The first possibility is that it is essentially the reverse of acclimation. Dehardening may be due to the cessation of cold induced gene transcription and synthesis of cold induced polypeptides. This is supported by studies showing disappearance of cold-induced gene products during deacclimation (Martin et al. 1978). The second theory is that deacclimation is an active process requiring the expression of specific genes to reverse some of the biochemical and physiological consequences of acclimation. No direct evidence for this theory has been gathered to this point (Howarth and Ougham 1993).

2.1.4 Variation in hardiness, phenology, and breeding effects

2.1.4.1 Natural variation in hardiness

Significant variation exists in natural populations of many conifer species, including *Pseudotsuga menziesii* var. *menziesii* (Menzies and Holden 1981, Skroppa 1991, Aitken

et al. 1996, Aitken and Adams 1996). Variation in hardening requirements is dependent on the origin of the plant, thus injuries may occur when trees are planted on sites with a more severe climate than that of their seed source. A more southern population may begin autumn cold acclimation later in the season than a northern population due to the photoperiod and temperature requirements for acclimation. For example *Picea sitchensis* from Alaskan provenances acclimates in September, in response to shortening daylengths alone, whereas Oregon provenances do not harden until November after repeated frosts (Cannell and Sheppard 1982). In Scandinavia, *Picea abies* populations moved to the north can never reach the same degree of hardiness as the local populations (Dormling 1982).

In terms of dehardening, the quantitative effect of treatments of photoperiod, chilling, and flushing temperature on the rate of bud development are complex and differ among provenances, giving rise to many interactions. General responses are similar for all provenances, however increases in the rate of dehardening associated with photoperiod appear to depend more strongly on provenance, while interaction with flushing temperatures is still important (Campbell and Sugano 1975). Several weeks before bud burst, shoots of *Picea sitchensis* dehardened in response to warm temperatures, with southerly provenances doing so before northerly ones. All provenances burst bud at the same time and were equally frost susceptible at this time (Cannell and Sheppard 1982).

2.1.4.2 Cold hardiness and tree breeding

Breeding specifically for cold hardiness is possible. Within provenances, individual seedlings exhibit a wide range of frost tolerance. This significant variation allows for genetic screening for frost tolerance (Menzies and Holden 1981). Some studies indicate polygenic inheritance of frost hardiness, and large within population variation offers good opportunities for hardiness breeding (Norell et al. 1986). The main traits which can be used to select for frost hardy genotypes are the rate of autumn hardening, the time of pre-bud burst dehardening, and the time of bud-burst (Cannell and Sheppard 1982). It has been noted that in *Pseudotsuga menziesii* var. *menziesii*, although significant family variation in fall cold hardiness is found, individual heritability is relatively low (Aitken et al. 1996). In contrast, individual heritabilities for spring cold hardiness are quite high (Aitken and Adams 1997).

Cold hardiness is usually a trait secondary to growth rate in selection programs. In the selection of fast growing trees, the level of cold hardiness and other phenological traits may be compromised. Faster growing trees selected for breeding programs must attain a size advantage through one, or a combination of : 1) initiation of growth earlier in spring; 2) prolonging the growing season into the autumn; or 3) growing at a faster rate during the growing season (Aitken and Adams 1995a). It appears that the differences in total growth between trees are caused mostly by variation in the length of the growth period, while rate of growth is nearly constant (Dormling 1982). However, lengthening the growing season increases the potential for frost damage (Aitken and Adams 1995a).

A key element to any successful breeding program is the maintenance of tree health. This includes ensuring synchronization of cold acclimation, deacclimation, and growth phenology with the local climate (Sakai and Larcher 1987). In *Pseudotsuga*

menziesii var. *menziesii*, bud burst is under moderate to strong genetic control indicating that this trait could be readily altered in breeding programs. Timing of both bud burst and bud set were correlated with growth in seedlings, and selection for greater growth is expected to accelerate bud burst and delay bud set (Adams and Li 1992). The practical implications of shifting cambial phenology in breeding programs are probably minimal in terms of frost damage as projected changes are of the magnitude of a few days. Weak genetic correlations have been found to exist between date of bud burst and dates of diameter growth initiation and cessation (Li and Adams 1994).

The effects of selection for growth rate on annual developmental cycle traits were investigated in coastal and Cascade mountain populations of *Pseudotsuga menziesii* var. *menziesii* and *glauca* (Aitken and Adams 1995a). In this study family, selection for increased height growth was expected to increase the likelihood of second flushing. In the coastal group, selection for increased height delayed bud flush and shoot dehardening. In the Cascade population, increased growth was correlated with earlier bud flush and lower shoot cold hardiness in the spring. Aside from a second flush, selection for height growth is thought to have little effect on fall cold injury (Aitken and Adams 1995a). This balance between selection for high growth potential in mild environments and selection for cold hardiness in severe environments occurs naturally as populations adapt to their setting. Resistance to damage by cold temperature is an indication of the general fitness of a family and better growth may be a consequence of resistance to extreme cold (Rehfeldt 1982b). Even if hardiness is not strongly related to field performance (yield) it may be used in early testing so that adaptation to unfavorable freezing events is maintained or improved (Sulzer et al. 1993).

In recent years yet another factor has been included in the final determination of seedling hardiness; the conditions of reproduction. Controlled cross seedlings produced at lower latitudes than their place of origin were less hardy than natural stand seedlings originating from the same latitude as their parents (Skroppa et al. 1994). Seedlings from crosses in the north are generally more frost hardy than their full sibs from the south, and this is most likely related to differences in photoperiod between seed orchards. The effect of the environment may be specific to female flowering (Johnsen et al. 1996). It is suggested that altered performance of progenies could be due to an activation of regulatory mechanisms affecting the expression of genes controlling adaptive traits.

2.1.5 Effect of nutrition on cold hardiness

Examining the body of research concerning nutrition and cold hardiness, contradictions are found for every study. One fact remains constant, however, a nutritionally balanced plant can tolerate greater levels of stress than a plant with a nutrient imbalance or deficiency (Klein et al. 1989). There is also evidence that hardiness is influenced by the duration and timing of the fertilizer application period. Significant differences in cold tolerance associated with time of nitrogen (N) application were detected in autumn and winter, but not in spring. *Picea rubens* seedlings receiving N in mid or late summer were as hardy or hardier than seedlings fertilized in early summer, regardless of the concentration of fertilizer (DeHayes et al. 1989). Supporting this, *Pseudotsuga menziesii* var. *menziesii* seedlings that received the bulk of fertilizer early in the season were found to be the least frost hardy. In contrast, seedlings that received a

constant level of fertilization throughout season were most hardy, while seedlings that received the bulk of fertilizer later in the season were of intermediate hardiness (Hawkins et al. 1995).

N fertilization impacts growth patterns most significantly and subsequently has been intensively studied in relation to cold hardiness. N has been found to advance bud break in the first summer after planting, but otherwise, to have no negative effects on the frost hardiness of several conifers (Benzian et al. 1974). This was supported by a study of frost tolerance of *Juniperus chinesis* shoots and roots which were unaffected by N application, although spring growth of shoots was greater (Bigras and Rioux 1989). In the fall, plants with high levels of N may take longer to become dormant. Fertilization can extend the growth period into autumn, thus affecting the time of bud set and decreasing frost hardiness so that seedlings are damaged by early frosts (Christersson 1977). However, in an example with *Larix leptolepis*, hardiness increased when fertilization was continued until October. Shoot growth cessation was delayed by prolonged fertilization, but this did not result in lower hardiness. It was suggested that only if temperature was higher than normal during acclimation would cessation of growth be too late for optimal hardiness to develop (Hansen 1992). It has also been proposed that fertilization may affect hardiness only when plants are grown with nutrients at levels which do not stimulate the highest growth rates (Pellett and Carter 1981)

When seedlings are cold hardy, N application often has little effect or may slightly increase hardiness, therefore timing of fertilization is critical (van den Driessche 1991). Applications of N applied to plants late enough in the season to increase foliar nutrient concentrations without further increasing growth decreased fall frost damage in *Picea*

sitchensis and *Tsuga heterophylla* (Benzian 1966). On the contrary, hardy *Pinus sylvestris* seedlings with the highest nitrogen content, showed a decrease in frost hardiness of 3 °C (Hellergren 1981). An increase in N fertilization may create an increase in cellular injury, mainly in the plasmalemma and in the tonoplast.(Soikkeli and Karenlampi 1984). It is thought that a high N content can influence hardiness through an increase in cell size and a higher degree of vacuolization within the cell. Increased vacuolization would cause a rise in intracellular ice crystal formation at lower temperatures (Levitt 1980). Furthermore, the level of freezing may also play a role in determining the level of damage. In *Pinus sylvestris*, greater hardiness occurred in plants with greater N supply in tests with temperatures creating maximum damage. At less damaging temperatures there was a tendency for lower hardiness with increasing nitrogen concentrations (Aronsson 1980) giving support for an interaction between temperature and N supply.

It is unlikely that phosphorus (P) has much effect on winter cold hardiness (van den Driessche 1991). It has been found that neither spring frost tolerance nor spring growth is affected by P in *Juniperus chinesis* (Bigras and Rioux 1989). Furthermore, there was no evidence of a N x P interaction in *Picea rubens* (DeHayes et al. 1989). However, some have found a high P content to increase seedling damage from frost. This effect was attributed to an extended growth period compared to seedlings with low P supply (Malcolm and Freezaillah 1975).

Since frost hardiness has been correlated with cell membrane permeability to water, different potassium (K) treatments may result in differing water permeability and subsequent variation in intracellular freezing. Support for this theory has come from observations of drastically reduced fall frost damage in *Picea sitchensis* and *Tsuga*

heterophylla fertilized with K (Benzian 1966). However, different K contents in the shoots of unhardened and 3 week hardened seedlings of *Pinus sylvestris* were found to have no effect on frost hardiness development (Christersson 1973). In addition Bigras and Rioux (1989) observed that neither spring frost tolerance nor spring growth is affected by K in *Juniperus chinesis*. K concentrations are important in drought resistance, and water stress often occurs with low temperatures, subsequently the effects of K on cold acclimation may be indirect (Aronsson 1980).

2.2 Mitotic index

The mitotic index describes the percentage of cells in division at the time of fixation. Meristematic activity or growth rate and frost hardiness have been negatively correlated (Colombo et al. 1989). The growth cycle of the vegetative apex can be subdivided into five stages: 1) dormancy 2) early bud-scale initiation 3) late bud scale initiation and apical enlargement 4)early rapid leaf initiation and 5) late slow leaf initiation (Owens and Molder 1973) followed by dormancy. In all stages but dormancy, there is some degree of mitotic activity and plant tissue is susceptible to freezing damage. There is very little to no mitosis occurring during dormancy. During active phases, the peripheral zone is mitotically more active than the apical zone, but the apical zone is not quiescent. During the bud scale initiation phase, growth begins and cell divisions first appear in the peripheral zone, then in the rib meristem, then finally in the apical zone. Mitotic index continues to increase throughout the apical elongation phase of bud -scale initiation and begins to decrease with the transition to leaf initiation. Entering fall and late leaf initiation,

mitotic frequency drops off to near zero. In the dormant stage, the dormant bud with bud scales enclosing the stem tip has all the leaf primordia for the following season. Changes in mitotic frequency in the apical zones follow changes in the growth and development of the respective zones during the annual cycle (Owens and Molder 1973). Initial hardening is dependent on growth cessation, or lack of meristematic activity at the shoot tip, and does not require low temperatures to proceed (Colombo et al. 1989).

Growth and related mitotic activity ceases earliest in plants with low nitrogen supply. It was found that fertilization at planting increased mitotic index, but fertilized seedlings entered dormancy at the same time as unfertilized seedlings (Carlson et al. 1980).

2.3 Techniques for assessing cold hardiness

The most integrated form of testing involves whole-plant freezing. This subjects complete plants, with roots insulated, to sub-zero test temperatures. Plants are then placed in normal growing conditions until damage is manifested, usually with results appearing in 7-14 days (Burr et al. 1990). To make determination of cold hardiness much less cumbersome, artificial freezing of detached twigs mirrors the relative amounts of damage expected for intact plants subjected to natural freezing (Rehfeldt 1986). Independent of the method chosen to test for hardiness, it is important to assess the cold hardiness of tissue types separately as differential hardening will occur (Sakai and Weiser 1973).

A common method of freeze testing tissues is the electrolyte leakage test. Tissue cuttings are subjected to freezing temperatures in treatment tubes. After thawing, water is added to each tube, shaken, and the conductivity of the solution is then measured. The tube and contents are then placed in a boiling water bath to induce maximum levels of membrane damage and the conductivity is remeasured. An index of injury is used to convert the percentage of electrolytes released in unfrozen controls (0%) compared to frozen samples as a percentage of boiled tissue (100%) (Burr et al. 1990)

The visual assessment technique is perhaps the simplest of the analytical methods. Its simplicity and brevity allows many more samples to be analyzed in a given amount of time than does the electrolyte leakage test. Plant cuttings are frozen to test temperatures and then are placed in humid conditions at room temperature and discoloration is observed. Foliar damage usually is evident within 3 days following freeze testing. With cambial browning, symptoms develop within 7-10 days. Since hardiness develops from top to bottom of the stem in many conifers, description of cambial damage based on portion of stem affected is important. Damage to the cambium on the terminal leader may not result in seedling mortality, however, damage at ground line, common in early autumn frosts, is lethal (Keates 1990). A significant correlation between the electrolyte leakage technique and the visual assessment technique has been found (Shortt et al. 1996).

Differential thermal analysis (DTA) involves the measurement of exothermic (heat releasing) events involved when water freezes. This technique has advantages when monitoring a freeze thaw cycle. Excised tissue is placed in a cylinder of aluminum foil connected to a differential thermocouple. A similar thermocouple and aluminum without any tissue is used as a reference. The thermocouples are individually placed in plastic

tubes and inserted into an aluminum block to provide uniform cooling. A high temperature exotherm (HTE) will occur with the freezing of extracellular water and a low temperature exotherm (LTE) occurs when freezing of supercooled intracellular water occurs (Burr et al. 1990). An alternative to the DTA is the electrical impedance method. By sending an electronic signal through the plant the ionic permeability of frost injured cell membranes can be measured by a decrease in electrical impedance of stem tissue (Keates 1990).

Several tests monitor the photosynthetic apparatus as an indicator of plant health and freezing damage. Chlorophyll fluorescence parameters have shown a significant linear relationship to needle freezing damage and seedling survival (Fisker et al. 1995). One advantage of chlorophyll fluorescence is the capability of detecting non-visible damage to seedlings. Decreases in chlorophyll fluorescence in needles indicates that photoinhibition occurs during prolonged frost hardening (Strand and Oquist 1988). Infrared gas analysis can be used to measure rates of photosynthesis and compare post freezing photosynthesis with rates prior to freeze testing. This method has shown to be most effective when seedlings are less hardy and is less useful as seedlings become acclimated (Keates 1990). Enzyme inactivation measured by the tetrazolium chloride test monitors the reduction in enzyme activity due to freezing damage. Again, the dehydrogenase activity measured is greatest when tissues are least hardy. This test may be most useful in root tissue (Keates 1990).

Screening for spring frost hardiness can be effectively accomplished by freeze-testing a single tissue type, or by assessing date of bud burst (Aitken and Adams 1997). The plant's phenology can be an accurate guide for determining its relative spring hardiness. Fall hardiness screening is considerably more complex, as bud and shoot

phenology are poor predictors of fall cold hardiness, and genetic correlations among tissue types are only moderate (Aitken and Adams 1995b). Frost hardiness can also be estimated by testing the root growth capacity which has shown a clear positive relationship with hardiness (Simpson 1990).

CHAPTER 3

HARDINESS STUDY ON REALIZED GAIN TREES

3.1 Introduction

Genetic selection and orchard seed production result in large amounts of genetically 'improved' seed for reforestation. In British Columbia, genetic testing of coastal Douglas-fir (*Pseudotsuga menziesii* var. *menziesii* Mirb. Franco) since the mid seventies has led to the identification and selection of clones which produce progeny exhibiting superior volume growth (Heaman and Woods 1989). The Douglas-fir trees sampled in this study were planted in 1993 for trials designed to estimate realized gain from the British Columbia Ministry of Forests breeding programs. The primary goal of this program was to develop generalized predictions of unit-area volume gains for a range of genetic levels estimated from individual tree progeny tests (Woods1992).

Early spring cold hardiness is of great concern in most areas of the Pacific Northwest as this is the period when dormancy is broken and tree growth resumes. It has been observed that this is the time of year when trees are most damaged by low temperatures (Jack Woods, B.C. Min. of Forests, pers. comm.). This study was initiated to analyze the hardiness of Douglas-fir stock from the Ministry of Forests breeding program and to ensure that the 'improved', faster growing trees did not have an increased susceptibility to freezing during the most critical periods of the year. The objectives of this study were as follows:

- To compare cold hardiness in Douglas-fir trees produced from wild stand seed with those resulting from breeding programs.

- To investigate relationships between mitotic index in lateral buds and cold hardiness in wild stand and improved Douglas-fir.
- To study date of bud burst and patterns of shoot extension in relation to cold hardiness in wild stand and improved Douglas-fir
- To analyze the progression of secondary growth in relation to cold hardiness in wild stand and improved Douglas-fir.

3.2 Material and Methods

SEEDLINGS

The Douglas-fir seedlings investigated were of three genetic levels which originated from the following sources: 1) wild stand seed collected from natural stands, 2) first generation seed orchard seed, and 3) top cross seed. Parent trees with phenotypic superiority were selected from wild stands and included in a first generation seed orchard and breeding population. Collection locations were distributed throughout the Maritime seed zone south of 51 °N. Random mating of these trees produced first generation seed orchard seed. These orchards currently account for most of the seed used to produce reforestation stock in the Maritime seed zone. Seedlings from controlled crosses in the first generation seed orchard were grown in progeny tests. The top cross trees are the offspring of parents which produced progeny with the best growth based on 12-year volume data from existing progeny tests. Those top parents were then crossed again and top cross seed is the result. Top cross seed will have growth potential approximately equal to a second generation seed orchard. The breeding values based on stem volume of

the wild stand families were 0, while the seed orchard families were estimated at 2. The top cross families had breeding values ranging from 17 to 19 (J. Woods pers. comm.).

Seedling stock was grown in styroblock 615 containers, sown with one seed per cavity at the B.C. Ministry of Forests Cowichan Lake Research Station in 1992. Each family within orchard or wild stand seedlot was grown in a separate styroblock. Block organization in the nursery was random. Stock was overwintered in the greenhouse and lifted in mid-February 1993. Seedlings were mattock or shovel planted, depending upon site conditions, between mid-February and mid-March 1993. Dead and weak trees were replaced with extra stock which was transplanted from nursery beds. In this cold hardiness study five families within each genetic level were sampled from the realized gain trials.

SITES

Two test sites of the many sites planted with realized gain trials were monitored for this study. Locations were chosen to represent the breadth of the geographic distribution of Douglas-fir within the seed zone. One site, Holt Creek, is on southern Vancouver Island, (48°45'N, 123°50'W, elevation 120m) near Lake Cowichan. The site has a north east aspect with moderate slope. The other site is close to the Chehalis River (49°22'N, 121°58'W, elevation 390m) near Mission. This site has a west south west aspect and is also moderately sloped. Vegetation was controlled through manual cutting to ensure trees remain free to grow through to rotation age. Natural regeneration was also removed

Two blocks on each site were sampled for this study. Blocks were arranged in a split plot design. Blocks consist of three, 100-tree plots (10 by 10 trees). Each of the three main plots contain seedlings from either the wild stand, seed orchard, or top-cross seedlots. The number of family subplots per genetic level varied from thirteen for top-cross, eight for wild stand, to seven for seed orchard. The allocation of genetic levels to plots within blocks, and of seedlings from each family was random. Five families per seedlot were selected randomly for inclusion in the cold hardiness study. Spacing of the trees is 3 m by 3 m, with a variation of up to 1 m to allow for optimum planting location.

METHODS

Frost Hardiness

COLLECTION OF MATERIAL

Secondary lateral shoot tips from the previous year's whorl were clipped from each study tree and placed in individual plastic bags with their identity label. All samples were stored in a refrigerator and underwent the freezing protocol within 24 hours of collection. Study trees were marked with flagging tape and metal identification tags on the first sampling. The same trees were sampled on all successive dates.

FREEZING PROTOCOL

The freezing protocol followed Aitken and Adams (1995b). Shoot tips were trimmed to 5 cm and were placed on damp cheesecloth with buds placed nearest the edge. The cheesecloth was then folded over the cuttings and packaged in flattened aluminum foil with open ends to encourage ice nucleation. The packets were hung from a rack in a

Forma Scientific (Marietta, Ohio, USA) Biofreezer with air space around all packets, and held for a minimum of five hours at -2 °C. Packets containing control shoots were removed at this time. The remaining packets were then cooled at 5°C/hour to each of three test temperatures. Samples were maintained at each test temperature for an hour and then packets were removed to a refrigerator. Three temperatures were selected to create a range of damage for needle tissue, and hopefully a range for bud and cambium tissue as well. A test freezing run two weeks prior to sampling determined the temperature range. Temperature control programs were manipulated using a Caltech Scientific (Richmond, B.C.) model 8000-controller. After freezing, the packets were placed in the refrigerator overnight and the cheesecloth and samples were removed from the foil the following day. The samples, still in folded cheesecloth, were put into plastic bags and left at room temperature for one week before scoring damage. Table 1 describes the sampling design for cold hardiness assessment.

VISUAL SCORING

The visual scoring method followed Aitken and Adams (1995b). The needles were scored to the nearest 10% damage. Olive green, brown, and gray discolored needles were considered to be damaged. The stem was sliced in half lengthwise and the area of yellow-brown discolored cambium was distinguished from its healthy green counterpart and assessed to the nearest 10%. The terminal buds were also sliced in half and the needle primordia were scored as either undamaged (green), moderately damaged (green and brown mixed), or completely damaged (brown or black).

Mitotic Index

FIXATION AND STAINING

The terminal vegetative buds from secondary lateral branches were removed from half of the cuttings before freezing. Table 1 describes the sampling design for mitotic index assessment. Bud scales were removed using a razor blade. Shoot apices and roughly 5 mm of the subtending shoot was dissected and if subtending leaf primordia covered the apex, all but one ring of primordia were removed. This was done using a triple O insect pin mounted on a needle probe. Grob and Owens (1993) suggest leaving the last few bud scales to protect the apex from the mechanical damage of processing, however, this was found to prevent adequate staining more often than it conferred a benefit in protection, therefore all bud scales were removed.

The fixation and staining protocol followed Grob and Owens (1993). Apices and the subtending shoot were fixed in 10% neutral formalin (adjusted for pH 7.0 neutrality with 1N NaOH) for a minimum of 2 hours in a refrigerator (0-4 °C). Samples were kept in vials throughout the fixation and staining procedure. Fixation longer than 2 hours was not detrimental if samples were kept at this low temperature. Samples were then washed 3 times in refrigerated distilled water over a 24 hour period to remove all fixative from the bud tissue. Prior to hydrolysis, samples were washed once in room temperature (≈20 °C) distilled water to raise the tissue temperature; 20 °C is the standard temperature for the hydrolysis step of the procedure. As lab temperature seldom deviated more than a few degrees from this temperature, a water bath was not required to maintain this temperature. Apices were then hydrolyzed with 5N HCl at room temperature for 50 minutes to produce

an adequate balance between hydrolysis and stain receptivity. After hydrolysis, apices were washed once in distilled water. They were then stained with Schiff's reagent and kept for a minimum of two hours in the dark at room temperature. Schiff's reagent stains DNA a deep reddish brown. Staining for periods exceeding two hours did not seem to affect the intensity of the staining. Specimens were then rinsed three times at 10 minute intervals in sulphur dioxide (SO_2) water. This procedure lightened the stain in the cell cytoplasm subsequently increasing the contrast between the nuclear material and the cytoplasm. Apices were then washed in refrigerated distilled water and stored in distilled water in the refrigerator for up to 4 days before squash preparation.

SQUASH PREPARATION

Shoot apices stained darker than the surrounding leaf primordia tissue and were easily located under 16X magnification of a Wild (Heerbrug, Switzerland) M3C dissecting microscope. Any primordia blocking the apex were removed, followed by exterior tissue immediately subtending the apical dome. The apex was then dissected with the tips of two insect pins and placed in a small drop of 45% acetic acid on a microscope slide. If excess acetic acid was used, shearing of the squash with the coverslip was likely. A coverslip was placed on the apex, with one edge of the coverslip lowered first to create an initial squash. The eraser end of a wood pencil was used to apply moderate vertical pressure to completely squash the apical tissue into a monolayer.

SAMPLING OF SQUASHES

The sampling of squashes followed Grob and Owens (1993). Observations of the squashes were done using a Leitz (Wetzlar, Germany) Laborlux S light microscope with 10x ocular, 20x and 40x objective lenses. The squashed area apex was located with the 20x objective and scanning was done with the 40x objective. Horizontal scanning was guided with a Leitz 10 x 10 mm, square grid ocular micrometer with divisions at 1 mm intervals. The intersection of the center vertical and horizontal lines creates the center point of the grid. Scanning began by aligning the top horizontal line on the top border of the squashed cell conglomeration. The center point was then moved horizontally to the left until it was aligned with the left border of the squash. Moving from left to right, any nuclei or chromosomes that made contact with the center point of the grid intersections were recorded as dividing or non-dividing. Cells that contained clearly defined visible condensed chromosomes (clearly prophase- telophase) were counted as dividing. Only one nucleus of an anaphase or telophase combination was counted if either one or both came into contact with the scanning line. Tannin filled pith cells and oblong procambial cells were easily differentiated and not included in the count. Squashes significantly polluted with these cell types were not scanned. Following a complete horizontal scan, the center horizontal line was moved down to the position where the bottom horizontal line had previously been aligned (200 µm distance Grob and Owens 1993) . The horizontal scanning procedure was then repeated with the center point again being aligned to the left side of the squash. Scans were made until readjustment of the center horizontal line was beyond the bottom of the squash. Mitotic index was calculated as follows:

Mitotic index = (#dividing cells/ total counted cells (dividing and non-dividing) x 100

A minimum of 2 mitotic indices per tree per sampling date were calculated depending on the success of the staining and squashing.

Bud Burst

A visual assessment of stage of shoot development was used to score degree of bud burst on two spring dates at the Holt site and on one date for the Chehalis site. The average stage of development on each tree was assessed and one bud burst score was given for each of the 60 trees surveyed at each site and sampling date. The criteria for the ranking of the buds on a scale developed for this project is described in Table 2.

Shoot Elongation

The length of the leader and an identified lateral from the first whorl (current year) was monitored on each sample tree in the field sites. At each post bud burst sampling, the length of the new shoots was measured with calipers. Shoot length was measured from bud scale scar to terminal bud tip. Using the final shoot length, percent growth complete on each sampling date, and also during a single period, was calculated for all 60 trees on each site. In early fall, lammas growth was recorded as either present (1) or absent (0) on lateral branches.

Secondary Growth (tracheid observation)

Secondary growth in branches of Douglas-fir seedlings has been separated into six stages: 1) earlywood formation, 2) latewood formation, 3) cessation of cambial cell division, 4) dormancy, 5) cambial cell expansion, and 6) resumption of cell division (Rensing and Owens 1994). Earlywood formation occurs until July in Victoria. Differences in wall thickness and cell shape distinguish cambial cells from tracheids in early differentiation. In late July, latewood formation is dominant. Cambial cell division ceases in early September, with tracheid differentiation continuing until the end of October (Rensing and Owens 1994).

The degree of tracheid formation in the laterals from the first whorl was monitored as an indicator of secondary growth. At each post bud burst sampling, thin cross sections of the new shoot from secondary lateral branches were taken. The cross sections were taken with a razor blade 5 cm back from the bud of the shoots used for freeze testing. Samples were placed in a drop of toluidine blue on a microscope slide and topped with a slide cover. Toluidine blue to stain cellulose was used to enhance cellular identity. Cross sections were thin enough to easily observe cellular identity under the light microscope. At each sampling date, the number of tracheids formed across a radius was counted. Only clearly defined tracheids were counted to avoid confusion with the cambial zone. Using the final tracheid number as a total for the year's development, percent secondary development on each sampling date could be calculated. For each sampling, two tracheid counts were made for each of the 60 trees at each site.

FIELD SITE SAMPLING

The field site sampling and assessment schedule is summarized in Table 3.

STATISTICAL ANALYSIS

An arcsin-square root transformation was done for all percentage data. Transformed data were analyzed by ANOVA (PROC ANOVA (SAS 1988)). Hardiness, mitotic index, primary, and secondary development was compared among genetic levels using the following model:

$$Y_{ijklm} = \mu + B_i + b_{(i)} + G_j + BxG_{ij} + F|G_{(j)k} + BxF|G_{i(j)k} + e_{(ijk)l} + s_{(ijkl)m}$$

where

μ is the overall mean

B_i is the effect of blocking (i = 1, 2)

$b_{(i)}$ is the blocking restriction error (i = 1, 2)

G_j is the effect of the genetic level (j = 1, 2, 3)

BxG_{ij} is the effect of the interaction between block and genetic level

$F|G_{(j)k}$ is the effect of the family nested within genetic level (k = 1, 2 , 3, 4, 5)

$BxF|G_{i(j)k}$ is the effect of the interaction between block and family nested within genetic level

$e_{(ijk)l}$ is the sampling error among trees within family plots

$s_{(ijkl)m}$ is the sub sample error among samples from individual trees

All factors were fixed except for error terms. Hardiness between sites or dates was not compared statistically as damage was considered to be dependent on the date of sampling, and test temperatures differed among dates. Temperature was not included in

the model as some test temperatures did not create a range of damage in the samples. Thus data from the temperature resulting in the greatest spread of damage was used in ANOVA calculations.

Correlations between cold hardiness, mitotic index, and degree of primary and secondary growth were calculated with PROC CORR (SAS 1988). For correlations, all samples were pooled from the three genetic levels.

A linear regression of transformed needle damage at each test temperature was used to calculate the temperature resulting in 50% damage (LT50) for each genetic level on each sampling date.

3.3 Results

The yearly patterns of cold hardiness, mitotic index, primary shoot extension, and secondary growth give a clear picture of the transition from dormancy to high growth activity and back to dormancy (Figure 1). The relationships of these properties are clearly illustrated as the trees follow their annual cycle. In winter, trees are at very hardy, while mitotic activity is very low. Entering the growing season, hardiness decreases, and mitotic activity increases. During the growing period, shoots develop and hardiness is at the annual low. Into the autumn, growth is complete and mitotic index decreases, while hardiness increases.

Figure 2 describes changes in needle hardiness in the top-cross, seed orchard, and wild-stand performance groups individually throughout the year at the Holt and Chehalis

sites. By following the hardiness levels of shoot tissues throughout the year, it was possible to determine if a particular genetic group is more susceptible to freezing damage in a particular period.

TISSUE FREEZING

The first sampling dates in early spring, 1996 produced intermediate needle freezing damage at -32 °C for trees from the Holt site (Table 4) and at -28 °C for trees from the Chehalis site (Table 5), thus needle tissue from the Holt site appeared hardier. Needle freezing damage was significantly different among genetic levels at both sites. At Holt, hardiness of wild stand trees was not significantly different from top-cross trees, but both groups were less hardy than seed orchard trees. At Chehalis, hardiness of seed orchard trees was not significantly different from that of wild stand or top cross trees, but wild stand trees were less hardy than the top-cross trees. At this sampling, the test temperatures resulted in 100% damage for bud tissue (Tables 6, 7), but no damage for cambium tissue (Tables 8, 9) at both sites.

The second sampling dates (late March, early April 1996) produced intermediate needle freezing damage at -18°C for trees from the Holt site (Table 4) and at -16 °C for trees from the Chehalis site (Table 5). Needle damage was not significantly different among the genetic levels at Holt, but was significantly different among the genetic levels at Chehalis. At Chehalis the hardiness of seed orchard trees was not significantly different from wild stand trees, but was greater than that of the top-cross trees. Wild-stand trees did not display statistically different hardiness from top-cross trees. At this sampling, the

maximum freezing temperature of -22 °C for Holt and -20 °C for Chehalis created no damage in either bud or cambium tissues (Tables 6,7,8, 9).

The third sampling dates (late April, early May) produced intermediate needle freezing damage at -10 °C for trees from the Holt site (Table 4) and at -8 °C for trees from the Chehalis site (Table 5). Needle damage was not significantly different among genetic levels at Holt nor at Chehalis. A range of bud damage was created at the maximum test temperatures of -14 °C for Holt and -12 °C for Chehalis (Tables 6, 7). Bud freezing damage was significantly different among genetic levels at Holt, but not at Chehalis. At Holt the ranking of bud damage from most damage to least was wild stand trees, seed orchard trees, then top-cross trees. A small range of cambium damage was created by the maximum test temperature of -14 °C for Holt and -12 °C for Chehalis site trees. Cambium freezing damage was not significantly different among genetic levels for Holt or Chehalis (Tables 8, 9).

The fourth sampling dates (late May, early June) produced intermediate tissue freezing damage at -6 °C for trees at both sites (Tables 4, 5). Needle damage was not significantly different among genetic levels in Holt, or Chehalis site trees, and neither was cambium freezing damage (Tables 8, 9). Since the terminal buds had burst and new terminal buds had not yet formed, there was no bud damage scoring.

The fifth and sixth sampling dates in summer produced intermediate needle freezing damage at -6 °C for both sites (Tables 4,5). Needle damage was not significantly different among the genetic levels at Holt, or at Chehalis.

The seventh sampling in October, 1996 produced intermediate needle freezing damage at -10 °C for Holt (Table 4) and at -14 °C for Chehalis (Table 5) trees. Needle

damage among the genetic levels was significantly different at the Holt site, but not at the Chehalis site. At Holt, the top-cross trees and seed orchard trees were of statistically similar needle hardiness, while the wild stand trees were statistically more hardy than the other two genetic groups (Figure 3). A range of bud damage was created at a test temperature of -14 °C for the Holt site and -18 °C for the Chehalis site trees. Bud freezing damage was not significantly different among genetic groups for Holt, or for Chehalis trees (Tables 6, 7). Cambium freezing damage was evident at a test temperature of -14 °C and was not significantly different among genetic levels at the Holt site (Table 8). At the Chehalis site, freezing at test temperatures of -18 °C did not produce any cambium damage (Table 9).

The eighth sampling date (November) produced intermediate needle freezing damage at -20°C for the Holt site (Table 4). Needle damage was not significantly different among genetic groups. At this sampling, the minimum test temperature of -24°C created no damage for bud or cambium tissue (Tables 6, 8). There was no November 1996 sampling at the Chehalis site due to road inaccessibility.

The ninth sampling date, in early spring 1997, produced intermediate needle freezing damage at -20 °C for the Holt site (Table 4). Needle damage was not significantly different among genetic groups. A range of bud damage was created at a test temperature of -20 °C. Bud damage was not significantly different among genetic groups (Table 6). There was no cambium damage at the minimum test temperature of -24 °C. There was no March 1997 sampling planned for the Chehalis site.

The April 1997 sampling produced intermediate needle freezing damage at -14 °C for the Holt site (Table 4). Needle freezing damage was not significantly different among

the genetic groups. A small range of bud freezing damage was created at the -20 °C test temperature (Table 6). There was no cambium damage at the minimum test temperature of -18 (Table 8). The samples collected in April 1997 from the Chehalis site could not be analyzed due to equipment failure.

Although there was often no statistically significant difference in freezing damage among genetic levels, in the early spring (February and March) wild stand trees tended to be least hardy, and top cross and seed orchard trees most hardy. In the early fall (October and November), however, top cross trees were the least hardy and wild stand trees were the most hardy in all but one sampling.

MITOTIC INDEX

A significant difference in mitotic index between performance groups was found on the March 1996 sampling date for the Holt site only (Table 10). On this date at Holt there was no statistical difference in mitotic index of trees from wild stand seed and from seed orchard seed, but the top-cross trees had a significantly lower mitotic index. There were no significant differences in mitotic index for any other samples and no consistent pattern in ranking of the performance levels by mitotic index.

BUD BURST

Bud development did not differ significantly among performance groups on any of the three sampling dates at either site (Table 11). The general trend was for wild stand trees to have the most advanced bud development followed by seed orchard, and top cross trees. There were significant correlations between the progression of bud burst at

samplings 11 and 12 in early and late May 1997 and the needle freezing damage for both March and April 1997 for the Holt site (Table 12). Needle freezing data from April 1996 (sampling 3) was also significantly correlated to bud burst progression during 1997 (Table 13). As there was not a successful freezing sampling at Chehalis in April 1997 no correlation with bud burst in the same year could be made. The needle freezing data from 1996 (sampling 3) was not significantly correlated with bud burst progression at the Chehalis site (Table 13).

LEADER GROWTH

There were no significant differences among genetic levels in the length of new shoots on any sampling date or site. In five of six samples, however, top cross trees had longer shoots on average. Wild stand trees appeared to have the fastest rate of growth early in the year as the percentage of growth completed was greatest in that group in three out of four early sampling dates.

At the end of May 1996 (sampling 4) at the Holt site there were no significant differences among the genetic groups in leader height ($p=0.1092$)(Figure 4a), or percentage of total growth complete to that date ($p=0.0662$)(Figure 5a). At the Chehalis site, there was also no significant difference among genetic groups in leader height ($p=0.2928$)(Figure 4b), or percentage of total growth in early June, 1996 ($p=0.5999$)(Figure 5b). At the Holt site in May, ranking of genetic groups by new leader growth from most to the least was wild-stand, seed orchard, and top-cross (Figure 6). Although differences among genetic groups were very small, leader height and percentage

growth were generally greatest in wild stand trees followed by seed orchard and top cross trees.

The fifth sampling at the Holt site in July 1996 found no significant difference among genetic groups in leader height (p=0.8654)(Figure 4a), percentage of growth to that date (p=0.9070)(Figure 5a), or new growth during that period (p=0.5106)(Figure 6a). At the Chehalis site, there was also no significant difference among genetic groups in leader height (p=0.1201)(Figure 4b), percentage of growth to that date (p=0.7780)(Figure 5b), or new growth during that period (p=0.1324)(Figure 6b). On average, top cross trees showed slightly greater leader growth in the June to July 1996 period on both sites.

The sixth sampling at the Holt site in late August 1996 found no significant difference among genetic groups in leader height (p=0.7357); however, the ranking of leader height from tallest to shortest among genetic groups was top-cross, seed orchard, and wild-stand (Figure 4a). At the Chehalis site in early September 1996, there were also no significant differences in leader height (p=0.1058), but the ranking of leader height from tallest to shortest among genetic groups was top-cross, wild-stand, and seed-orchard (Figure 4b). There was little spread between genetic levels in the percentage of total leader growth complete by that date at both sites (Figure 5). Final leader length was significantly correlated to final lateral length at both Holt (r=0.6058, p=0.0044) and Chehalis (r=0.6334, p=0.0012).

At the October 1996 sampling dates lammas growth was measured as either present or absent. At the Holt site lammas growth was noted in both top-cross (0.10) and seed orchard (0.10) groups, but was absent in the wild stand group. At Chehalis, lammas

growth was present in top-cross (0.10), but was absent in seed orchard and wild stand groups.

SECONDARY DEVELOPMENT

There were no significant differences in the degree of secondary development among genetic groups at sampling date 5 in July 1996 for the Holt or Chehalis sites (Table 14). The degree of secondary development in July was not correlated with freezing damage at samplings 5, 6, or 7 (July to October) for the Holt, or the Chehalis site (Table 15). The number of tracheids formed by the end of the summer at sampling 6 was used as the final tracheid count (100%).

CORRELATION OF MITOTIC INDEX, TISSUE DAMAGES, GROWTH, AND DEVELOPMENT

In late March and early April 1996 (sampling 2), there were significant correlations of needle freezing damage with mitotic index for all families pooled from the three genetic levels for both Holt and Chehalis trees (Figure 7a,b). Bud and cambium damage were not successfully measured at this date and a relationship to mitotic index could not be determined.

One month later (late April, early May), there were significant correlations of needle freezing damage with mitotic index for Holt trees ($r=0.6440$, $p=0.0053$), but not for Chehalis trees ($r=0.4167$, $p=0.0578$). At this time, mitotic index of terminal buds on lateral shoots prior to bud flush was not significantly correlated with either bud, or cambium freezing damage at Holt or Chehalis sites (Table 16). Needle damage at

sampling 3 was not significantly correlated with either bud, or cambium tissue damage at Holt, but it was significantly correlated with cambium damage at Chehalis.

At sampling 4, in late May and early June 1996, there was a significant correlation between needle damage and cambium tissue damage due to freezing at both Holt and Chehalis sites (Table 17). There was no significant correlation between new leader growth and needle damage at Holt, however there was a significant correlation at Chehalis. Shoot growth was not correlated with cambium damage for Holt, but it was for Chehalis trees (Table 17).

In October of 1996 (sampling 7) there were no significant correlations between needle freezing damage and mitotic index of the newly formed terminal buds on lateral shoots for Holt ($r=0.3910$, $p=0.1499$) or Chehalis sites ($r=0.3564$, $p=0.0974$). At Holt, mitotic index was not significantly correlated with bud or cambium freezing damage, or final leader length (Table 18). At Chehalis, mitotic index was not significantly correlated with bud freezing damage, or final leader growth (Table 18). At Holt, needle damage was significantly correlated with bud, and cambium freezing damage, but not with final shoot growth. There was also no correlation between bud or cambium damage and final leader growth (Table 18). At Chehalis there was a significant correlation of bud damage with final leader growth. At Chehalis there was no cambium damage.

At both Holt and Chehalis sites, there was a significant negative correlation between needle freezing damage in October at sampling 7 and the percentage of total growth that had occurred to samplings 4 and 5 (June and July) (Table 19). At sampling 8 in November 1996, there was a significant correlation between needle freezing damage and mitotic index for Holt ($r=0.6330$, $p=0.0012$).

In March 1997 at sampling 9, there was no significant correlation between needle freezing damage and bud damage for Holt ($r=0.3538$, $p=0.3407$). In April 1997, at sampling 10, needle freezing damage was significantly correlated to the sampling 9 needle damage ($r=0.5304$, $p=0.0420$) for the Holt site. There was no significant correlation between needle freezing damage and bud damage ($r=0.2179$, $p=0.7186$) at that time.

3.4 Discussion

In winter the freezing tolerance of the trees in the study was high and subsequently the temperatures causing 50% damage (LT50) were relatively low. Trees were dormant and growth processes were at a minimum. This is reflected in the mitotic index which was low or zero. Entering spring, the LT50's increased as the trees deacclimated and dormancy was broken in response to the increasing daylengths, and to a lesser degree, increasing temperatures (Greer and Stanley 1985). Mitotic indices rose rapidly as apical cellular division was initiating shoot development. During late spring and summer LT50's were at the yearly low with deacclimation fully complete and dormancy completely broken. Bud burst had occurred, and primary and early secondary growth were underway. Mitotic index would be at a maximum during this period. Entering the fall, acclimation began and LT50's slowly declined. Primary growth and the bulk of secondary growth was complete. Trees shift from the growth phase into dormancy using decreasing day lengths and temperatures as environmental cues (Weiser 1970). Mitotic index declined as shoot apices began to decrease activity and enter the rest phase for safe overwintering. The specific qualities are detailed at regular intervals throughout the year below.

WINTER

In late winter (sampling 1, February), trees were still very cold hardy. Although there were differences in hardiness among the genetic groups, the artificial LT50's are so low for all groups that freezing damage in the field during this period is unlikely. The

climate of coastal B.C. is mild and such severe conditions are improbable (Aitken and Adams 1996). To determine actual cold hardiness tissue hardiness would have to be calibrated with whole plant freeze testing. Separate tissue testing has predicted whole plant LT0 to LT10 values and subsequently the needle hardiness tests may show less hardiness than is actually present (Burr et al. 1990). At mid-winter, bud hardiness was less than that of needle tissue, however cambium tissue was undamaged even at the lowest test temperatures of -32°C. It has been suggested that in lab experiments, bud tissue is damaged at warmer temperature than needle or stem tissue because of the rapid rate of cooling in the artificial freeze tests (Aitken and Adams 1996). It is thought that bud tissue may avoid freezing damage by supercooling rather than by the extracellular freezing that protects stem and needle tissue (Sakai 1982). Subsequently, a more gradual freezing process may elicit more accurate results.

SPRING

In early spring (sampling 2, late March), tissues had started to deharden in response to environmental cues as indicated by the LT50s averaging approximately 12°C warmer than for the previous sampling. Wild-stand trees either showed the highest or intermediate levels of damage, while top-cross trees displayed either intermediate or the least amount of damage. Wild stand trees always showed more visible damage than the top-cross group suggesting that the wild stand group had undergone more deacclimation by this date. Test temperatures created no damage in bud and cambium tissues. When compared to the previous sampling, this may indicate that bud tissues deharden at different rates than the needle tissues, while cambium tissue is still very hardy. With the transition

from dormancy to growth in its initial stage, wild stand trees showed an increased activity in the shoot apex. Top-cross shoot buds were delayed in their spring development and less mitotically active than the wild stand buds. This pattern was seen at both sites. Significant correlations were found between spring mitotic index and freezing damage. This supports the idea that the trees which lost dormancy earliest and initiated growth earliest as indicated by increased apical activity were the most susceptible to freezing damage.

By mid-spring (sampling 3, early May), deacclimation was almost complete in needle tissues with LT50's rising another 10°C. There was very little variation in needle damage among the genetic groups and all groups were roughly of the same hardiness level. The exception may be the seed orchard group at Holt which manifested less damage than the other groups. Wild-stand trees either showed the most or intermediate levels of damage in needle tissue, while top-cross trees displayed either intermediate or the least amount of damage. Bud deacclimation was well underway at this point. As bud burst approached, the transition from hardiness to the freezing susceptible growth phase was nearly complete. Wild stand trees always showed more visible damage to buds than the top-cross group suggesting that the wild stand group had undergone more bud deacclimation to this date. This follows the pattern of deacclimation in needle tissue, but with a delayed initiation. Cambium tissue was still much hardier than the other tissue types. As the generative tissue for the stem, it is proposed that this may be a mechanism to ensure its survival above all.

In mid-spring (early May) all performance groups were very similar in their mitotic activity signifying that all trees were at or nearing their complete release from dormancy.

The mitotic index was up to twice that of a month prior. For Chehalis, the seed orchard trees may have been lagging in their transition to growth (similar to top-cross at the Holt site), although there was still a 25% increase in mitotic index. Again, positive correlations were found between mitotic index and needle damage. Mitotic index was not correlated with either bud or cambium damage at the Holt or Chehalis sites.

Needle damage was not correlated with either bud or cambium tissue damage at the Holt site in mid spring. At Chehalis there was also no relationship between needle and bud damage, however, needle and cambium damage were correlated. At Holt, bud and cambium damage were unrelated, however a relationship was found at Chehalis. Previously, this disparity in hardiness among tissues has been seen in fall hardiness (Aitken et al. 1996), but spring hardiness has been shown to be highly correlated among tissues (Aitken and Adams 1997).

In late spring (sampling 4, early June) there was very little variation in needle damage as deacclimation was complete with LT50's at the -5°C plateau for Chehalis trees, while trees for the Holt site were only 1.5°C more cold tolerant.. Hardiness was at its low for the year and needle tissue was highly susceptible to freezing damage. There was no bud tissue as the buds had burst to form the year's new growth. Cambium freezing damage of the new shoots was high and did not differ among the genetic levels at the Holt or Chehalis sites, thus the new shoots had little cold tolerance. A significant relationship was found between needle and cambium damage for trees at both Holt and Chehalis sites. On this date the new shoots were extremely green and tender, thus, there was little difference between the needle and stem tissue in lignification, and the tissues had similar levels of damage. At Holt, needle and cambium damage were found to be unrelated to the

new shoot length. This was not the case at Chehalis where both needle and cambium damage had a negative relationship with the shoot growth, thus as the shoots lengthened, their hardiness increased. This is consistent with previous research which found during bud burst the newly emerging shoots were hardy to only -3 to -5°C until they were about 3.5cm when they became hardy from -5 to -10°C (Cannell and Sheppard 1982).

By monitoring the leader growth throughout the year it was possible to determine the periods when growth occurred and if there were differences among the genetic levels. A reliable measure of early summer phenology is the shoot elongation rhythm itself (Nilsson and Walfredsson 1994). At both Holt and Chehalis, wild stand trees appeared to begin shoot growth slightly earlier than the top-cross trees, but the top-cross trees had the longest shoots by the end of the growing season.

SUMMER

During the summer months (samplings 5 and 6), the trees were in the midst of the growing season; dormancy was non-existent, and with it almost all cold hardiness. In July, the Holt trees reached the summer low in cold tolerance; however the Chehalis trees displayed a similar, but slightly greater hardiness than the month before. During August, trees on both sites appeared to have initiated the earliest stage of acclimation as LT50's fell 1.5°C from the summer high. In terms of summer hardiness, it was expected that developing shoots would have little freezing resistance during the period between bud burst and the end of shoot elongation (Dormling 1982). In the absence of any substantial hardiness, little variation in needle cold tolerance was observed. This was similar to a previous study *Pinus sylvestris* which indicated that clonal variation in needle cold

tolerance was significant throughout the year except during the summer (Nilsson and Walfredsson 1994).

At Holt during June, similar shoot extension properties were found among the genetic groups. Length of new growth during this period was greatest in the top-cross group which also had the greatest percentage growth during this interval. At Chehalis, top cross trees had the longest new shoots, with seed orchard trees just ahead in their percentage of total growth complete. Between late May/ early June and mid-July was the period of greatest growth for trees on both sites.

Since the primary growth phenology was linked to spring cold hardiness, it might be expected that extended secondary growth would be negatively correlated with fall freezing hardiness. However, there were no significant correlations between the degree of secondary development and any freezing tests in the summer or later into the fall for either site. There are three possibilities that result from this analysis of secondary development: 1) that it is not a reliable indicator of secondary growth phenology, 2) secondary growth phenology has no correlations to cold acclimation, and 3) the sample size and design were inadequate to detect a significant correlation. It seems unlikely that any growth phenology would be unrelated to cold hardiness, and subsequently the method of measurement and the scale of the study may be at fault.

In late August, shoot extension was complete for both sites with the top cross trees having produced the most growth at both Holt and Chehalis sites. Top-cross trees also experienced the most absolute growth between July and August, but as a percentage of total annual growth, the seed orchard and wild-stand groups showed the highest rates of shoot extension during this period for Holt and Chehalis sites, respectively. The main

result from the primary growth analysis is that the wild-stand group grew at a faster rate early in the season. During the early growth periods, the wild stand trees completed a slightly greater proportion of their total growth and had longer shoots at Holt. The top-cross genetic group had greater absolute growth later in the year, but it did not have proportionally higher growth than the other groups. From this result, it is questionable that growth rates are similar in all groups and that differences in the growth period produced the differences in overall performance as has been previously stated (PNWTIRC 1995). Though none of these results were statistically significant, the trends were consistent, but not without exception.

FALL

Johnsen and Ostreng (1994) found that seed orchard progenies were taller, formed terminal buds later in the season, and subsequently were more damaged by fall frost than wild stand trees. Some have disagreed with this (Nilsson and Walfredsson 1994). A prior study in western Washington found there was no relationship between tree height and cold injury in the coastal zone, while in the Cascade zone, taller trees did appear to be more susceptible, but the association was weak (Aitken et al. 1996). In this study there was little support for an association between tissue hardiness and overall shoot growth, as the only correlation occurred in Chehalis trees in October 1996 between bud hardiness and shoot length.

During early October (sampling 7), the Holt trees were well into fall hardening, but were not as hardy as the Chehalis group based upon LT50 comparisons. In this study, the taller top cross genetic group had the most needle damage in samples from both sites, with

the wild stand group the least damaged. This would suggest that the top cross group was slower to become dormant when compared to the seed orchard or wild-stand groups. This is further supported by the fact that the top-cross group displayed either the most, or intermediate bud damage when compared to the other groups. Damage of buds at this sampling was much less than that of needles, which would indicate that the buds were hardening on a different schedule than the needle tissue. October cambium damage was minimal at both sites and this tissue was the hardiest of those studied, as it was during the earliest months of the year. For both sites, needle damage was linked to bud damage and it was also correlated with cambium damage at the Holt site. This was not the case in the spring and perhaps supports a separate process theory for the acclimation and deacclimation processes (Howarth and Ougham 1993).

At Holt, the mitotic activity was still relatively high in October, at similar levels to the late spring sampling. This indicated that the trees were not fully dormant. At Chehalis it was similar, with relatively high apical activity considering the tree's higher levels of cold tolerance. There was no strong relationship between mitotic index and needle, bud or cambium damage, or shoot growth for either Holt or Chehalis in early fall. In this study there was a correlation among tissue damages during this period. Previously, genetic correlations among needle, stem and bud tissue for fall cold damage have been found to be weak (r=0.16-0.58), and it has been suggested that the genes that control hardening could be different for different tissues (Aitken et al. 1996). If this were true, evaluation of a single tissue is probably not adequate for assessing overall cold hardiness of genotypes.

Needle freezing damage scored in October 1996 was negatively correlated with the percentage of total growth that had occurred in late spring and early summer (sampling 4

and 5) for both Holt and Chehalis trees. Therefore, the trees that had completed more of their growth earlier in the season were more hardy during early fall. Although the differences in hardiness between the genetic groups may have been minimal, for all the study trees as a whole this relationship is strong. This begs the question: why would faster growing trees continue to grow into the fall versus initiating growth earlier in the spring? It has been shown that damaging spring frosts are two to three times more frequent than fall frosts (Timmis et al. 1994). If this is the case, then selection for trees growing later into the season, rather than earlier, would be strong. It would be beneficial to extend the growing season into the least 'dangerous' season of the year in areas without pronounced drought.

Nilsson and Walfredsson (1994) stated that negative correlations between height growth and needle cold tolerance in autumn indicate that early cold acclimation and increased survival are not easily combined with maximum growth. This statement may be modified to include only late season growth based on the results of this study. Genetic correlation for growth potential and bud set suggest that a network of intercorrelated traits exists within and among populations (Rehfeldt 1982a). This network appears keyed to adaptation to cold; high growth potential is related to delayed bud set, but late bud set correlates with high damage from early fall frosts. It appears that trees which have completed more of their growth earlier in the year would have an earlier bud set, and subsequently would be less susceptible to fall freezing damage.

For Holt in late fall, (sampling 8, November) the top-cross performance group again displayed the most damage adding support to the suggestion that it began hardening at a later date than the other groups. Needle tissue hardiness was near the highest level

measured, while bud tissue hardiness was below the needle damage temperature range. The cambium maintained its greater level of cold tolerance. Winter cold hardiness has not been found to be correlated with autumn cold hardiness (Nilsson and Walfredsson 1994) which may explain a difference in ranking when compared to the February sampling. Holt trees showed a decline in mitotic activity as trees were in transition to dormancy. Top cross trees appeared to delay the transition from growth to dormancy in comparison with those in the wild stand group. Mitotic index and needle damage were again correlated during this period. This supports the proposition that the top-cross trees extended their growing season later into the fall period.

SPRING 2

In the second year of the study, mid spring (sampling 9, March 1997) needle damage at Holt followed a similar early-spring pattern when compared to the previous year. Wild-stand trees displayed the highest levels of needle and bud damage, while top-cross trees were the most hardy. This is consistent with previous findings which suggest family rankings for fall cold hardiness are expected to be relatively consistent over sites and years (Aitken and Adams 1996). Cambium tissue was still hardiest illustrating the consistency of tissue hardiness in rankings from year to year. The second year samplings (9 and 10) found the extent of needle freezing damage to be unrelated to bud freezing damage. This was also similar to the results found in the previous year of the study.

By examining the progression of bud burst it was possible to gauge the phenological stage of the shoot in terms of its growth initiation. Previously, timing of bud burst and bud set have been shown to be only weakly correlated with fall cold injury in

saplings (Aitken et al. 1996). However, bud burst and spring cold hardiness are strongly correlated in seedlings (Greg O'Neill Ph.D. in prep. OSU). The late spring survey (sampling 11, early May) at Holt showed slightly advanced development in bud burst in the wild stand group when compared to the top-cross group. The difference was not large, but was consistent with the trend of top-cross trees being less phenologically advanced at this stage of the year. The significant correlations between the progression of bud burst on this date and the needle freezing damages at both spring samplings illustrate the link between an advanced growth phenology and the dehardening process. Similar patterns can be expected from year to year and this is supported by the significant correlation of spring needle freezing data from 1996 with bud burst progression in 1997. The second bud burst sampling (sampling 12, late May) for Holt mirrored the survey two weeks prior. Significant correlations between the progression of bud burst and the needle freezing damage at both spring samplings show a direct relationship between the loss of hardiness and the initiation of growth processes. Needle freezing data from 1996 also correlates to needle freezing damage of the same trees in 1997 supporting the constancy of relative phenologies from year to year. Chehalis trees were similar to Holt trees with respect to the slower development of the top-cross trees, however, the seed orchard group had the most advanced bud burst at Chehalis whereas the wild stand trees were most advanced at Holt. For Chehalis, spring needle freezing data from 1996 did not show a clear relationship with the bud burst patterns of 1997.

Bud burst was delayed in the top-cross seedlings. The late bud flushing might allow for the avoidance of spring frost damage for this group (Dormling 1982). It is possible that this is a result of the extended growth into the fall leaving insufficient time

for bud maturation before entering dormancy. The consequence of this would be a delay in the spring flush as buds complete maturation.

Table 1. Sampling design of realized gain hardiness study

Cold hardiness	
5 families /genetic level (2 trees/ family) 3 genetic levels /block 2 blocks /site	TOTAL = 60 trees /site
60 trees x 8 cut samples (2 per test temperature and control)	= 480 cuttings/site /date
Mitotic Index	
5 families /genetic level (2 trees/ family) 3 genetic levels/ block 2 blocks /site	TOTAL = 60 trees /site /date
60 trees x 4 terminal lateral buds /tree	= 240 squashes /site /date

Table 2. Scheme used to estimate the stage of terminal bud flushing (bursting)

Score	Description
1	A closed, very compact bud (dormant)
2	A moderately swollen, but still entirely enclosed and relatively compact bud
3	A swollen bud that had undergone significant extension, but still had completely intact bud scales
4	A swollen bud, elongated, with only partially (less than 1/3) disturbed bud scales and needles visible
5	An elongated bud, with most of the bud scales disturbed
6	An elongating shoot completely free of bud scale enclosure
7	A shoot that was completely free of bud scales and significantly elongated

Table 3. Sampling schedule for the Holt Creek and Chehalis River sites.

Site	Year	Sampling Date	Test Temp (°C)	Assessment
HOLT CREEK				
	1996			
		1 16-Feb	-28,-32,-36	Cold tolerance
		2 20-Mar	-14,-18,-22	Cold tolerance and mitotic index
		3 25-Apr	-6,-10,-14	Cold tolerance and mitotic index
		4 30-May	-6,-10	Cold tolerance and 1° development
		5 11-Jul	-6	Cold tolerance, 1° and 2° development
		6 28-Aug	-6	Cold tolerance, 1° and 2° development
		7 10-Oct	-6,-10,-14	Cold tolerance and mitotic index
		8 14-Nov	-16,-20,-24	Cold tolerance and mitotic index
	1997			
		9 21-Mar	-16,-20,-24	Cold tolerance
		10 10-Apr	-10,-14,-18	Cold tolerance
		11 8-May		Bud burst
		12 20-May		Bud burst
CHEHALIS RIVER				
	1996			
		1 25-Feb	-28,-32,-36	Cold tolerance
		2 3-Apr	-12,-16,-20	Cold tolerance and mitotic index
		3 8-May	-8,-12,-16	Cold tolerance and mitotic index
		4 8-Jun	-6,-10	Cold tolerance and 1° development
		5 17-Jul	-6	Cold tolerance, 1° and 2° development
		6 8-Sep	-6	Cold tolerance, 1° and 2° development
		7 18-Oct	-10,-14,-18	Cold tolerance and mitotic index
	1997			
		12 27-May		Bud burst

Table 4. Ranking of genetic group mean needle cold hardiness throughout 1996-97 for Holt Creek. Values are damage means of all selected trees within genetic groups with standard error of means in brackets (WS= wild stand, SO=seed orchard, TC=top cross)

Sampling	Date	Temp (°C)	Group	Damage (%)	LT50 (-°C)	Pr>F[2]
1	16-Feb '96	-32	WS	60.8 (4.2)a[1]	32.7	**p=0.0057**
			TC	60.7 (3.2)a	33	
			SO	39.8 (3.5)b	34.8	
2	27-Mar '96	-18	WS	67.6 (5.8)a	19.1	p=0.1777
			TC	61.1 (5.4)a	19.8	
			SO	55.2 (5.6)a	20.3	
3	25-Apr '96	-10	TC	63.0 (4.2)a	9.6	p=0.1925
			WS	60.5 (4.0)a	9.7	
			SO	50.0 (5.6)a	10	
4	30-May '96	-6	SO	34.0 (5.2)a	6.6	p=0.4802
			WS	29.1 (4.2)a	6.7	
			TC	23.8 (4.5)a	6.8	
5	11-Jul '96	-6	WS	87.5 (3.8)a	5.1	p=0.6951
			SO	85.7 (3.8)a	5.1	
			TC	83.9 (3.2)a	5.2	
6	28-Aug '96	-6	TC	42.7 (3.9)a	6.3	p=0.7694
			SO	38.2 (5.0)a	6.6	
			WS	36.5 (3.6)a	6.7	
7	10-Oct '96	-10	TC	62.0 (4.0)a	8.7	**p=0.0092**
			SO	50.2 (5.4)a	8.8	
			WS	30.3 (4.5)b	12.1	
8	14-Nov '96	-20	TC	24.2 (2.8)a	24.2	p=0.2762
			SO	19.7 (2.5)a	26.4	
			WS	17.8 (2.7)a	27.3	
9	21-Mar '97	-20	WS	51.3 (4.9)a	20.8	p=0.2030
			SO	48.0 (4.3)a	21.2	
			TC	36.3 (3.7)a	21.5	
10	10-Apr '97	-14	WS	56.5 (4.9)a	14.1	p=0.6334
			TC	53.3 (3.8)a	14.1	
			SO	50.5 (5.2)a	14.3	

[1]means followed by the same letter are not significantly different (p=0.05) within sampling date
[2]probability of mean transformed % freezing damage differing significantly among genetic levels

Table 5.Ranking of genetic group mean needle cold hardiness throughout 1996-97 for Chehalis. Values are damage means of all selected trees within genetic groups with standard error of means in brackets (WS= wild stand, SO=seed orchard, TC=top cross).

Sampling	Date	Temp (°C)	Group	Damage (%)	LT50 (-°C)	Pr>F[2]
1	23-Feb '96	-28	WS	64.8 (3.9)a[1]	27.8	**p=0.0277**
			SO	52.3 (4.8)ab	28.2	
			TC	37.2 (4.8)b	29.9	
2	3-Apr '96	-16	SO	47.5 (4.6)a	15.4	**p=0.0306**
			WS	38.0 (4.7)ab	16.7	
			TC	25.0 (2.7)b	17.8	
3	3-May '96	-8	TC	56.8 (4.3)a	5.4	p=0.7139
			SO	53.5 (4.6)a	6.1	
			WS	50.8 (5.0)a	6.6	
4	8-Jun '96	-6	TC	63.8 (5.6)a	4.9	p=0.9980
			SO	63.2 (6.1)a	5.0	
			WS	63.0 (6.3)a	5.0	
5	17-Jul '96	-6	TC	57.5 (5.4)a	5.7	p=0.7985
			WS	55.5 (5.1)a	5.7	
			SO	51.8 (5.7)a	5.9	
6	6-Sep '96	-6	WS	44.8 (5.5)a	6.2	p=0.7459
			TC	40.0 (4.5)a	6.5	
			SO	37.2 (5.4)a	6.7	
7	18-Oct '96	-14	TC	53.8 (3.7)a	13.5	p=0.1144
			SO	43.2 (4.0)a	14.8	
			WS	36.2 (4.3)a	15.4	

[1]means followed by the same letter are not significantly different (p=0.05) within each sampling date

[2]probability of mean transformed % freezing damage differing significantly among genetic levels

Table 6.Ranking of genetic group bud cold hardiness throughout 1996-97 for Holt Creek. Values are damage means of all selected trees within genetic groups with standard error of means in brackets (WS= wild stand, SO=seed orchard, TC=top cross)

Sampling	Date	Temp (°C)	Group	Damage (%)	Pr>F[2]
1	16-Feb '96	-28	WS	100a[1]	p=0.9999
			SO	100a	
			TC	100a	
2	27-Mar'96	-22	WS	0a	p=0.9999
			SO	0a	
			TC	0a	
3	25-Apr '96	-14	WS	94.8 (3.4)a	**p=0.0195**
			SO	80.2 (6.4)b	
			TC	65.1 (7.6)c	
7	10-Oct '96	-14	SO	13.9 (7.5)a	p=0.1583
			TC	12.5 (6.1)a	
			WS	0.0 (0)a	
8	14-Nov'96	-24	WS	0a	p=0.9999
			SO	0a	
			TC	0a	
9	21-Mar'97	-24	SO	45.1 (6.8)a	p=0.1945
			WS	27.5 (6.5)a	
			TC	25.2 (7.2)a	
10	10-Apr '97	-14	SO	12.6 (4.9)a	p=0.2020
			WS	12.5 (3.8)a	
			TC	4.3 (5.2)a	

[1]means followed by the same letter are not significantly different (p=0.05) within each sampling date

[2]probability of mean transformed % freezing damage differing significantly among genetic levels

Table 7.Ranking of genetic group mean bud cold hardiness throughout 1996-97 for Chehalis. Values are damage means of all selected trees within genetic groups with standard error of means in brackets (WS= wild stand, SO=seed orchard, TC=top cross)

Sampling	Date	Temp (°C)	Group	Damage (%)	Pr>F[2]
1	23-Feb '96	-32	WS	100a[1]	p=0.9999
			SO	100a	
			TC	100a	
2	3-Apr '96	-20	WS	0a	p=0.9999
			SO	0a	
			TC	0a	
3	8-May '96	-12	WS	35.7 (7.6)a	p=0.9395
			TC	35.7 (5.7)a	
			SO	30.1 (7.3)a	
7	18-Oct '96	-14	TC	20.2 (9.2)a	p=0.2213
			WS	14.8 (8.2)a	
			SO	5.5 (5.1)a	

[1]means followed by the same letter are not significantly different (p=0.05) within each sampling date

[2]probability of mean transformed % freezing damage differing significantly among genetic levels

Table 8.Ranking of genetic group cambium cold hardiness throughout 1996-97 for Holt Creek. Values are damage means of all selected trees within genetic groups with standard error of means in brackets (WS= wild stand, SO=seed orchard, TC=top cross)

Sampling	Date	Temp (°C)	Group	Damage (%)	Pr>F[2]
1	16-Feb '96	-36	WS	0a[1]	p=0.9999
			SO	0a	
			TC	0a	
2	27-Mar'96	-22	WS	0a	p=0.9999
			SO	0a	
			TC	0a	
3	25-Apr '96	-14	TC	24.9 (4.0)a	p=0.2622
			SO	20.1 (4.7)a	
			WS	10.3 (3.2)a	
7	10-Oct '96	-14	SO	5.6 (5.3)a	p=0.5133
			TC	5.3 (3.4)a	
			WS	0.0 (0)a	
8	14-Nov'96	-24	WS	0a	p=0.9999
			SO	0a	
			TC	0a	
9	21-Mar'97	-20	WS	0a	p=0.9999
			SO	0a	
			TC	0a	
10	10-Apr '97	-18	WS	0a	p=0.9999
			SO	0a	
			TC	0a	

[1]means followed by the same letter are not significantly different (p=0.05) within each sampling date

[2]probability of mean transformed % freezing damage differing significantly among genetic levels

Table 9.Ranking of genetic group mean cambium cold hardiness throughout 1996-97 for Chehalis. Values are damage means of all selected trees within genetic groups with standard error of means in brackets (WS= wild stand, SO=seed orchard, TC=top cross)

Sampling	Date	Temp (°C)	Group	Damage (%)	Pr>F[2]
1	23-Feb '96	-34	WS	0a[1]	p=0.9999
			SO	0a	
			TC	0a	
2	3-Apr '96	-20	WS	0a	p=0.9999
			SO	0a	
			TC	0a	
3	3-May '96	-12	WS	22.2 (5.9)a	p=0.4802
			TC	7.5 (3.8)a	
			SO	2.7 (1.7)a	
7	18-Oct '96	-18	TC	0a	p=0.9999
			WS	0a	
			SO	0a	

[1]means followed by the same letter are not significantly different (p=0.05) within each sampling date

[2]probability of mean transformed % freezing damage differing significantly among genetic levels

Table 10. Mitotic indices of terminal buds on lateral shoots for two sites throughout 1996. Values are means of all trees within each genetic group and standard errors of the mean are bracketed (WS-wild stand, SO-seed orchard, TC-top-cross)

Sampling	Date	Site	Group	Mitotic Index (%)	Pr>F[2]
2	27-Mar	Holt	WS	1.4 (0.16)a[1]	**p=0.0220**
			SO	1.4 (0.17)a	
			TC	0.9 (0.18)b	
	3-Apr	Chehalis	WS	1.9 (0.11)a	p=0.1670
			SO	1.6 (0.16)a	
			TC	1.6 (0.13)a	
3	25-Apr	Holt	TC	2.8 (0.13)a	p=0.3963
			SO	2.7 (0.15)a	
			WS	2.6 (0.13)a	
	8-May	Chehalis	WS	2.4 (0.12)a	p=0.1735
			TC	2.3 (0.17)a	
			SO	2.1 (0.15)a	
7	10-Oct	Holt	SO	2.4 (0.16)a	p=0.3907
			TC	2.2 (0.17)a	
			WS	2.1 (0.18)a	
	18-Oct	Chehalis	SO	2.5 (0.21)a	p=0.6764
			WS	2.3 (0.23)a	
			TC	2.1 (0.22)a	
8	14-Nov	Holt	TC	1.3 (0.14)a	p=0.1766
			SO	1.1 (0.10)a	
			WS	1.0 (0.13)a	

[1]means followed by the same letter are not significantly different (p=0.05) within each sampling date

[2]probability of mean transformed mitotic index differing significantly among genetic levels

Table 11. Scoring of bud development during spring 1997. Values are means of all trees within each genetic group with standard error of the mean in brackets (WS-wild stand, SO-seed orchard, TC-top cross).

Sampling	Date	Site	Group	Score	Pr>F[2]
11	8-May	Holt	TC	2.31 (.14)a[1]	p=0.3803
			SO	2.35 (.16)a	
			WS	2.56 (.14)a	
12	20-May	Holt	TC	5.75 (.21)a	p=0.1349
			SO	5.83 (.31)a	
			WS	6.38 (.22)a	
	27-May	Chehalis	TC	5.88 (.27)a	p=0.4484
			WS	6.08 (.17)a	
			SO	6.15 (.22)a	

[1]means followed by the same letter are not significantly different (p=0.05) within each sampling date
[2]probability of bud development score differing significantly among genetic levels

Table 12. Correlations (r) of bud burst and freezing damage for grouping of all samples from the three genetic levels at Holt during spring 1997 (n=60)

	Freezing 9 (21-Mar)	Freezing 10 (10-Apr)	Budburst 11 (8-May)	Budburst 12 (20-May)
Freezing 9	XXXX	0.5304	0.5892	0.5256
	XXXX	**p=0.0420**	**p=0.0208**	**p=0.0442**
Freezing 10		XXXX	0.6728	0.7552
		XXXX	**p=0.0060**	**p=0.0011**
Budburst 11			XXXX	0.8622
			XXXX	**p=0.0001**

Table 13. Correlation (r) of May 1997 bud burst and April 1996 freezing damage for all samples from the three genetic levels for the Holt and Chehalis sites (n=60).

Site	Date	Correlation	Pr>F
Holt	8-May	0.6613	**p=0.0072**
	20-May	0.6408	**p=0.0101**
Chehalis	27-May	0.3505	p=0.2003

Table 14. Secondary growth as estimated by tracheid development for the three genetic groups with standard error of the means in brackets (WS-wild stand, SO-seed orchard, TC-top cross)

Sampling	Date	Site	Group	% Dev	Pr>F[2]
5	11-Jul	Holt	SO	80.0 (2.3)a[1]	p=0.1081
			TC	76.0 (1.5)a	
			WS	72.4 (1.9)a	
	17-Jul	Chehalis	WS	73.6 (1.3)a	p=0.8674
			SO	73.0 (1.6)a	
			TC	72.5 (1.2)a	
6	28-Aug	Holt	WS	100	XXX
			SO	100	
			TC	100	
	8-Sep	Chehalis	WS	100	XXX
			SO	100	
			TC	100	

[1]means followed by the same letter are not significantly different (p=0.05) within each sampling date

[2]probability of mean % tracheid development differing significantly among genetic levels

Table 15. Correlations (r) between secondary development (% dev) on the fifth sampling and freezing damage for Holt and Chehalis sites in 1996 (Holt-normal, Chehalis-italicized). Data were pooled to include all three genetic levels (n=60).

	% Dev (5)	Freezing 5	Freezing 6	Freezing 7
% Dev (5)	XXXX XXXX	0.2078 p=0.6610	-0.1334 p=0.8572	0.2007 p=0.6872
Freezing 5	*-0.1389* *p=0.8520*	XXXX XXXX	0.2738 p=0.4260	0.4069 p=0.0824
Freezing 6	*0.1503* *p=0.8080*	*0.2886* *p=0.3654*	XXXX XXXX	0.2425 p=0.5322
Freezing 7	*0.0964* *p=0.9198*	*0.6183* ***p=0.0023***	*0.5957* ***p=0.0079***	XXXX XXXX

Table 16. Correlations (r) between tissue damages and mitotic index for Holt and Chehalis sites at sampling 3 in late April and early March (Holt-normal Chehalis-italicized). Data were pooled to include all three genetic levels (n=60)

	Needle	Bud	Cambium	Mitotic Index
Needle	XXXX XXXX	0.3713 p=0.1331	-0.1136 p=0.8887	0.6440 **p=0.0053**
Bud	*0.3295* *p=0.2376*	XXXX XXXX	0.1797 p=0.7258	-0.1377 p=0.8370
Cambium	*0.4731* ***p=0.0139***	*0.5474* ***p=0.0009***	XXXX XXXX	0.0969 p=0.9189
Mitotic Index	*0.4168* *p=0.0578*	*0.2486* *p=0.5025*	*0.4331* *p=0.0604*	XXXX XXXX

Table 17. Correlations (r) of tissue damages and new shoot length for Holt and Chehalis sites at sampling 4 in late May and early June (Holt-normal, Chehalis-italicized). All data were pooled for the three genetic levels (n=60).

	Needle	Cambium	Shoot Growth
Needle	XXXX XXXX	0.8845 **p=0.0001**	-0.3777 p=0.2852
Cambium	*0.8711* ***p=0.0001***	XXXX XXXX	-0.1957 p=0.7749
Shoot Growth	*-0.5736* ***p=0.0102***	*-0.5233* ***p=0.0342***	XXXX XXXX

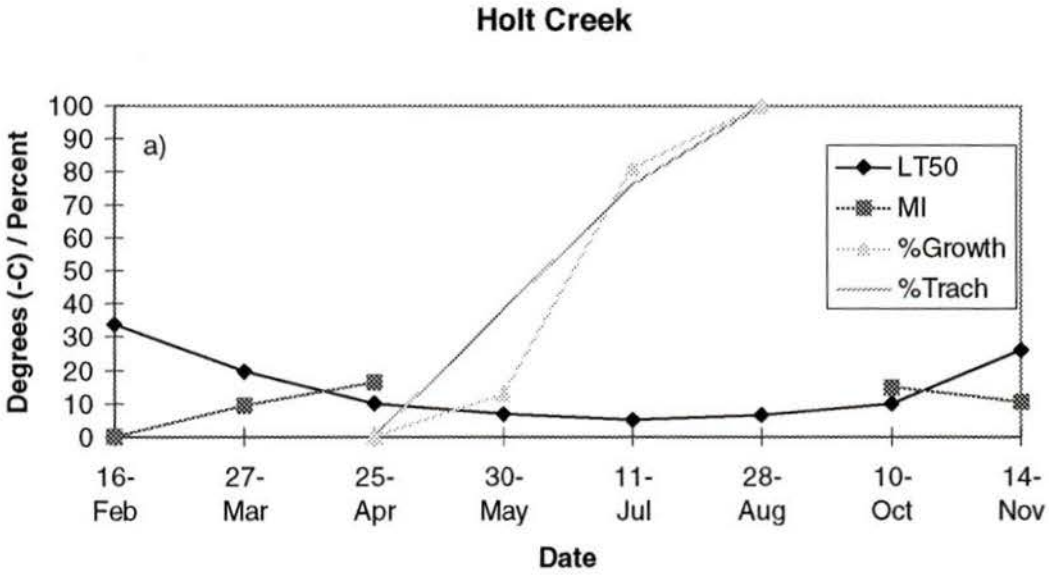
Table 18. Correlations (r) of tissue damages, mitotic index, and final shoot length for Holt and Chehalis at sampling 7 in mid-October 1996 (Holt-normal, Chehalis-italicized). All data were pooled for the three genetic levels (n=60)

	Needle	Bud	Cambium	Mitotic Index	Shoot Growth
Needle	XXXX XXXX	0.6328 **p=0.0018**	0.5211 **p=0.0392**	0.3569 p=0.4098	-0.3968 p=0.2599
Bud	*0.5685* ***p=0.0125***	XXXX XXXX	0.7664 **p=0.0001**	0.2625 p=0.6568	0.4102 p=0.2411
Cambium	*N/A*	*N/A*	XXXX XXXX	0.2645 p=0.6518	0.3935 p=0.2681
Mitotic Index	*0.5182* *p=0.0674*	*0.1709* *p=0.8784*	*N/A*	XXXX XXXX	0.2507 p=0.6999
Shoot Growth	*0.3059* *p=0.4924*	*0.5500* ***p=0.0234***	*N/A*	*-0.3689* *p=0.4813*	XXXX XXXX

Table 19. Correlation (r) between percent growth complete in June (4) and July (5) and freezing damage in mid-October (7) for the Holt and Chehalis sites in 1996. Data were pooled for all three genetic groups (n=60).

Site	Factor	%Growth4	%Growth5
Holt	Damage 7	-0.5598 **p=0.0187**	-0.5283 **p=0.0355**
Chehalis	Damage 7	-0.6573 **p=0.0007**	-0.5543 **p=0.0189**

Figure 1.Average needle cold hardiness (LT50), transformed mitotic index (MI%), percentage of leader growth complete (%Growth) and percent tracheids formed (%Trach) for all genetic groups for the a) Holt Creek and b) Chehalis River sites (1996).



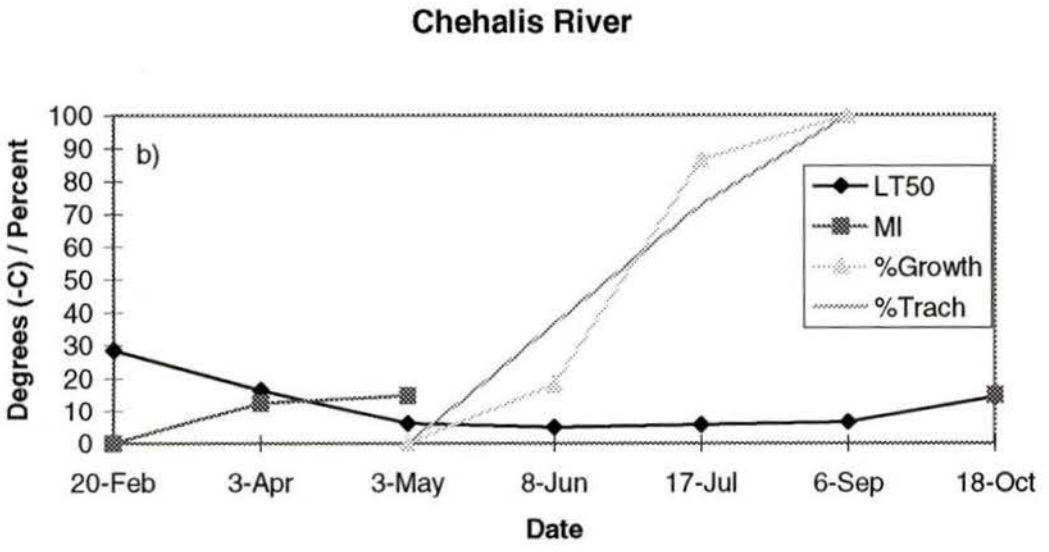
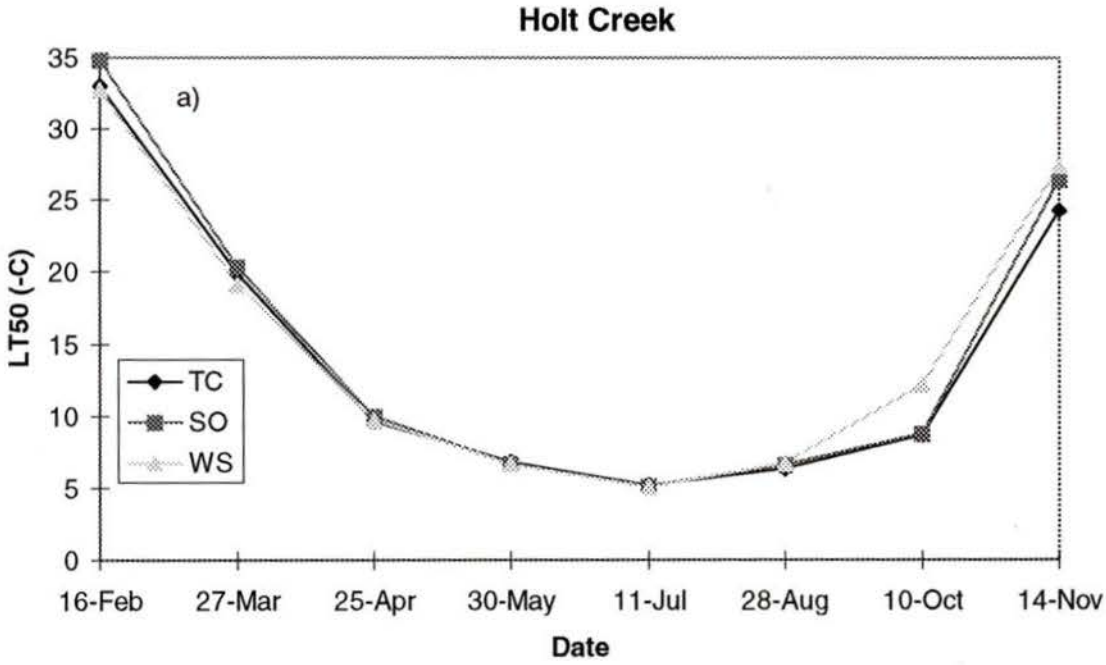


Figure 2. Cold hardiness (LT50) of needle tissue for the wild stand (WS), seed orchard (SO), and top-cross (TC) genetic groups for the a) Holt Creek and b) Chehalis River sites throughout 1996.



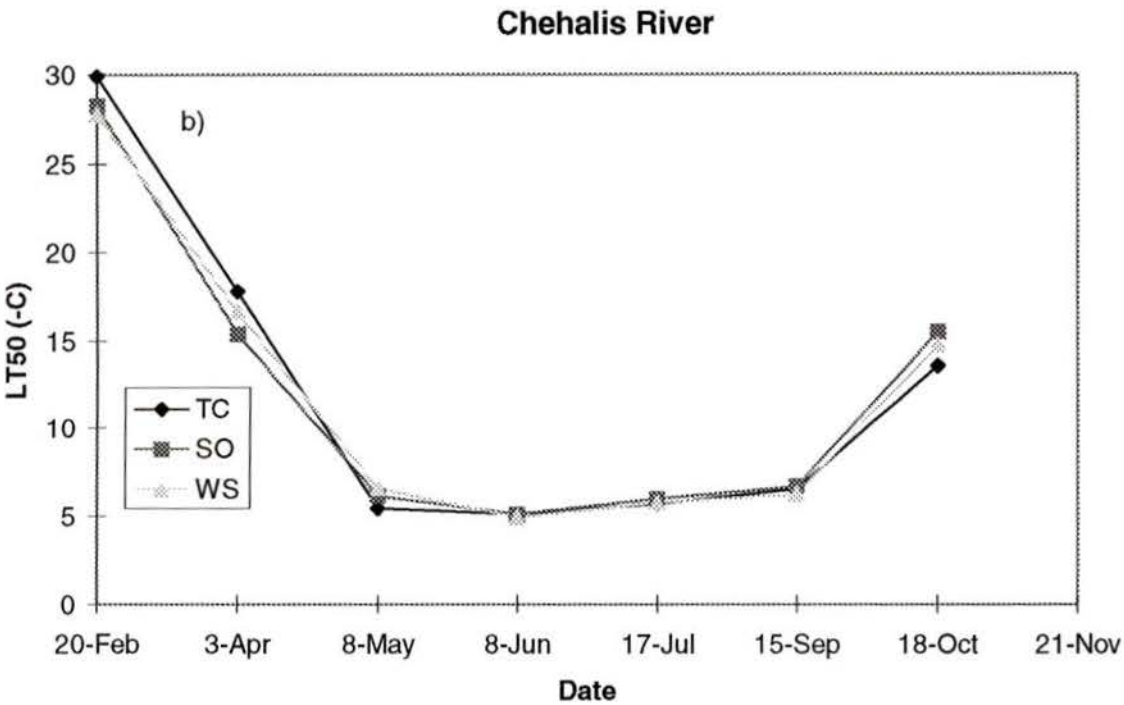


Figure 3.Mean needle freezing damage in Aug./Sept., Oct., and Nov. for trees of top cross, seed orchard, and wild stand genetic groups from both Holt and Chehalis sites. Error bars represent standard error of means. Means described with the same letter are not significantly different (p=0.05).

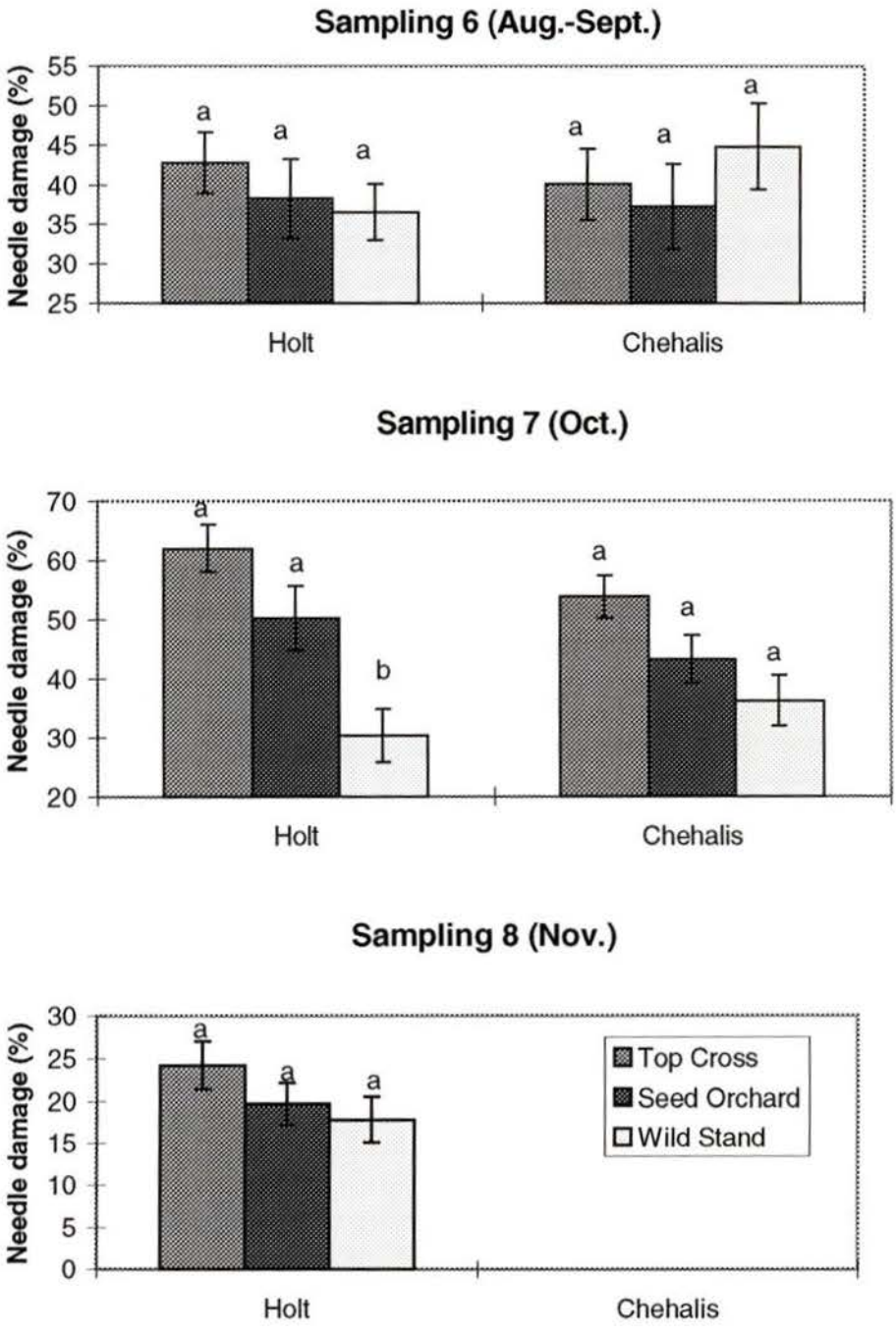


Figure 4. Leader growth of the genetic groups at the a)Holt and b)Chehalis sites for 1996 (WS-wild stand, SO-seed orchard, TC-top cross)

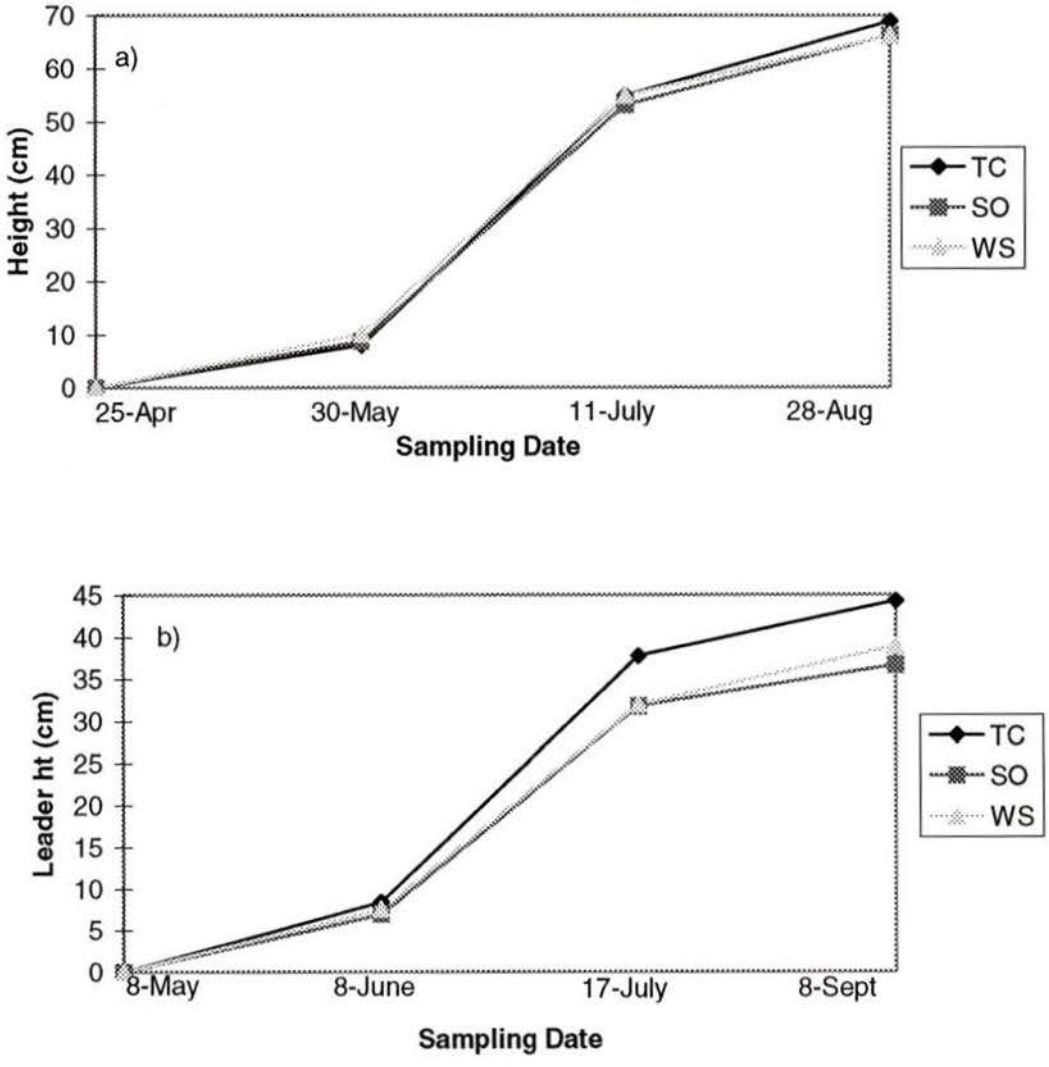
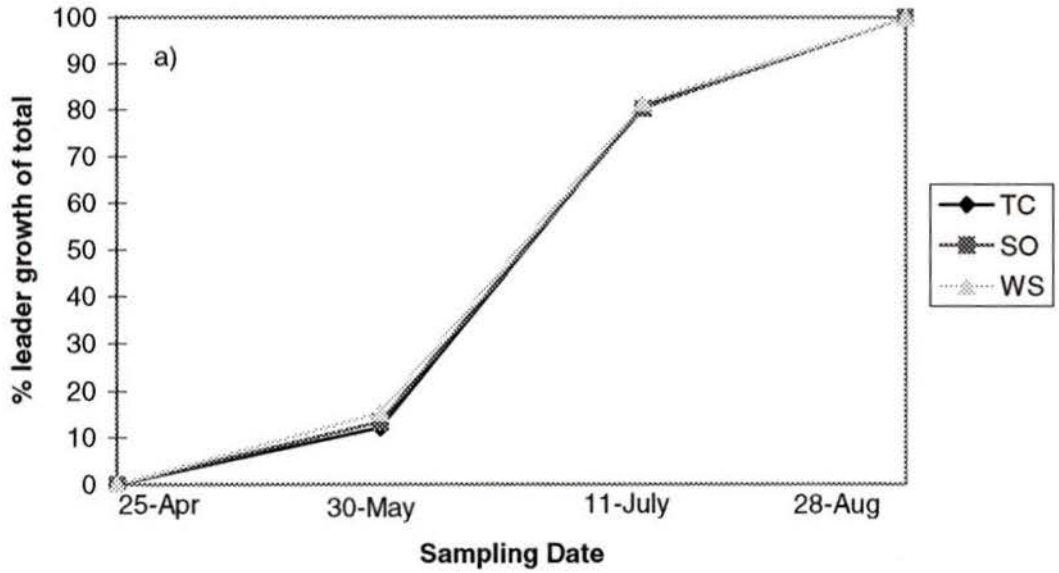


Figure 5. Percentage of total growth of leader height of in the genetic groups at the a) Holt and b) Chehalis sites for 1996 (WS-wild stand, SO-seed orchard, TC-top cross)



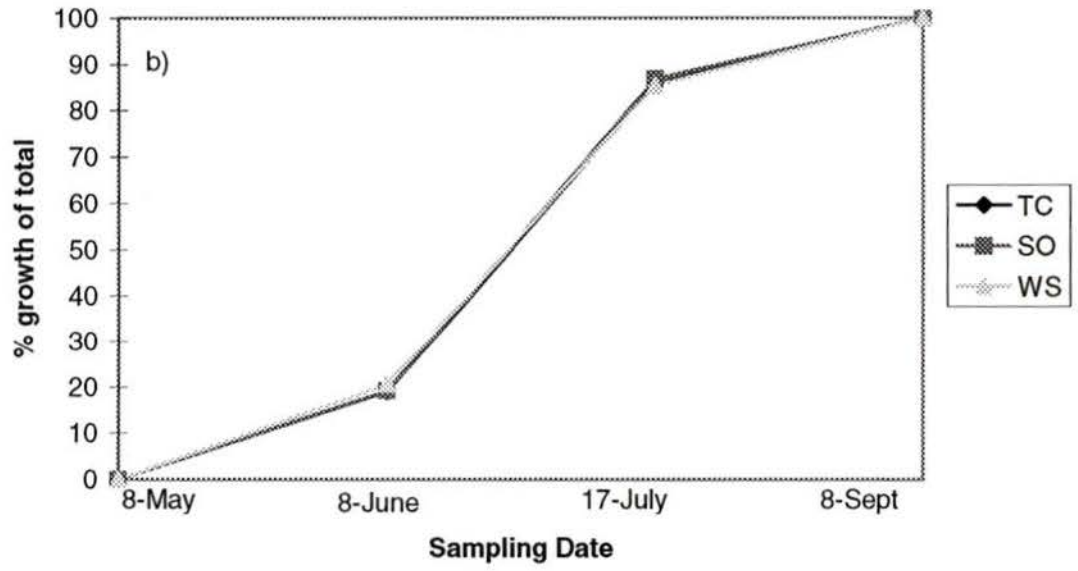
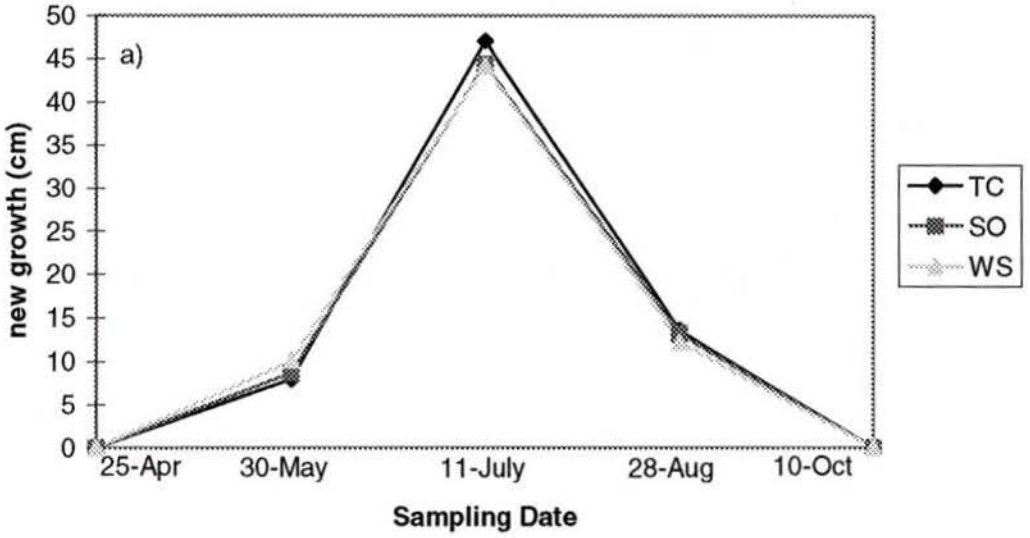


Figure 6. New leader growth during each sampling period at the a) Holt and b) Chehalis sites for 1996 (WS-wild stand, SO-seed orchard, TC-top cross)



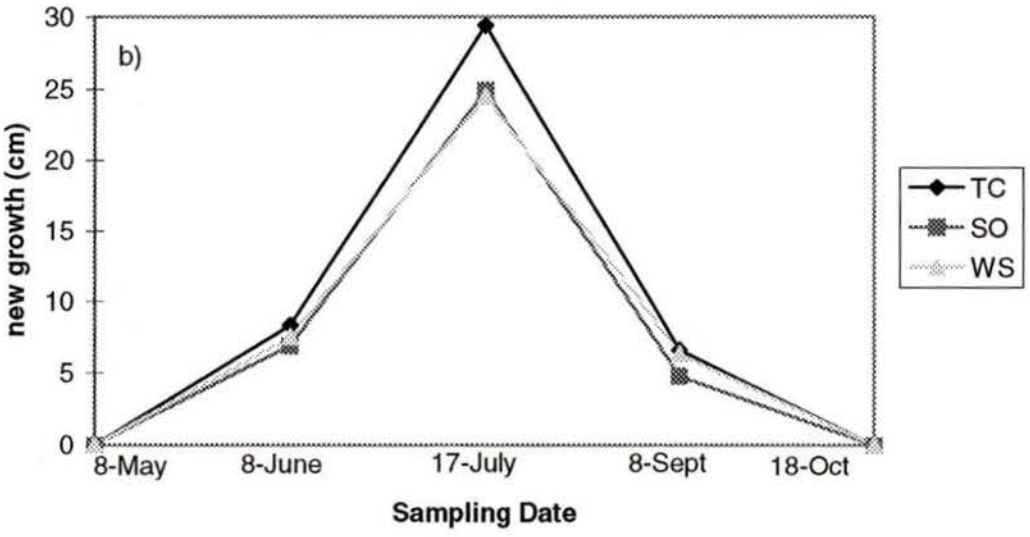
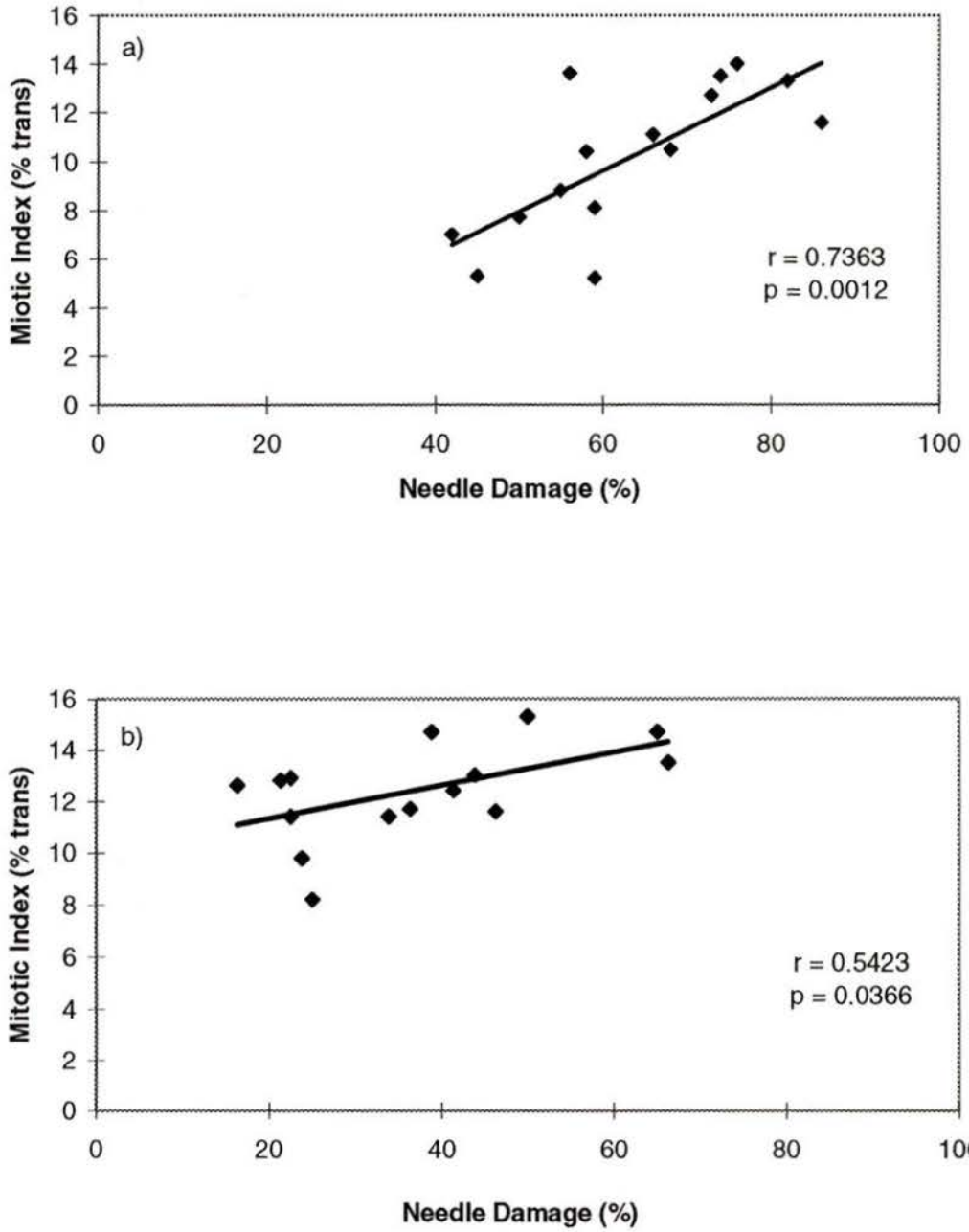


Figure 7. Mitotic index plotted against needle freezing damage for both a)Holt and b) Chehalis sites at sampling 2 in late March and early April 1996



CHAPTER 4

THE INFLUENCE OF N AND P SUPPLY ON FROST HARDINESS

4.1 Introduction

There is conflicting evidence concerning the effects of nutrients on frost hardiness. Complications in interpretation arise from variation in the duration and timing of the fertilizer application period. Significant differences in cold tolerance associated with time of N application in *Picea rubens* have been found in autumn and winter, but not in spring. N applications in mid or late summer resulted in seedlings as hardy or hardier than those fertilized in early summer (DeHayes et al. 1989). With *Pseudotsuga menziesii* var. *menziesii*, seedlings that received the bulk of fertilizer early in the season were found to be least frost hardy, seedlings that received a constant level of fertilization throughout season were most hardy, while seedlings that received the bulk of fertilizer later in the season were of intermediate hardiness (Hawkins et al. 1995). When seedlings are cold hardy, N application often has little effect or may slightly increase hardiness, therefore timing of fertilization is critical (van den Driessche 1991).

N fertilization has the potential to impact growth patterns and thus cold hardiness. In the fall, plants with high levels of N may take longer to become dormant. Subsequently, fertilization can extend the growth period into autumn decreasing frost hardiness (Christersson 1977). However, in an example with *Larix leptolepis*, hardiness increased when fertilization was continued until October. Shoot growth cessation was delayed by prolonged fertilization, but this did not result in a lower hardiness (Hansen 1992).

It is unlikely that phosphorus has much effect on winter cold hardiness (van den Driessche 1991). However, some have found a high P content to increase seedling damage from frost. This effect was attributed to an extended growth period compared to seedlings with low P supply (Malcolm and Freezaillah 1975); a similar effect as attributed to N fertilization.

The primary objective of this study was to observe the influence of nutrient supply on cold hardiness in fast and slow growing families of Douglas-fir. The effects on cold hardiness of nutrients promoting growth was compared with the effect of genetic differences in growth.

4.2 Materials and Methods

SEEDLINGS

The trees used for the nutrition study were surplus from a nutrient retranslocation experiment (Hawkins et al. 1998). Seedlings were produced from ten crosses of Douglas-fir originating from the B.C. Ministry of Forests' Tree Improvement Program. Five crosses were expected to produce fast growing progeny with breeding values between 21 and 40, while the remaining five crosses were expected to produce slow growing progeny with breeding values between -15 and -18 (J. Woods pers. comm.)

All seed was sown in late June 1993 in 415 styroblocks in a 3:2 peat:perlite mix with 3.33 kg dolomite lime and 1.32 kg micromix per m^3 soil incorporated. The styroblocks were placed randomly in a greenhouse and misted frequently for two weeks following germination.

METHODS

Three weeks after germination, nutrient treatments began. Five nutrient treatments varied nitrogen (N): phosphorus (P) ratio in the following concentrations (mgL^{-1}): 20:20, 100:4, 100:20, 100:60, and 250:20. Nitrogen was supplied as NH_4NO_3, phosphorus as $NaH_2PO_4H_20$, and potassium was added to all treatments as K_2SO_4 at 100 mgL^{-1}. One litre of nutrient solution was applied weekly to the 45 seedlings in each of four styroblocks per treatment until the end of September, and seedlings were irrigated as needed.

The plants were maintained in the greenhouse until May 1994 when the seedlings were repotted into 1.1 L Polybags (Menne Nursery Corp., Amherst, N.Y.) in sand mixed with dolomite lime and micromix in the proportions described above. The nutrient X genetic gain groups were arranged randomly on a gravel bed outdoors. Nutrient treatments continued throughout the summer twice weekly, and additional water was supplied as needed. In May 1995, seedlings were repotted in 3 L pots in sand mixed with dolomite lime and micromix as described above. Nutrient treatments continued twice weekly, and additional water was supplied as needed. In May 1996, trees were repotted as before in 10 L pots with the same maintenance schedule.

Frost hardiness assessment was conducted in the autumn of 1995 and the spring and autumn of 1996 (Table 20). The protocol described in Chapter 3 was followed for determining freezing damage, mitotic index, and shoot elongation. Bud burst in the spring of 1996 was scored as either 0 for an intact bud and 1 for a burst bud. Lammas growth in the fall of 1996 was scored as 1 for present and 0 for absent.

EXPERIMENTAL DESIGN

The experimental design is summarized in Table 21. All trees chosen for cold hardiness analysis were marked with tape on the initial sampling. The same trees were sampled on following dates.

STATISTICAL ANALYSIS

An arcsin-square root transformation was done for all comparisons of percentage data. Transformed data was analyzed by ANOVA (PROC ANOVA (SAS 1988)). Hardiness, mitotic index, and primary development was compared among nutrient treatments and performance levels using the following model:

$$Y_{ijk} = u + T_i + P_j + TxP_{ij} + e_{(ijk)l} + s_{(ijkl)m}$$

u is the overall mean

T_i is the effect of the nutrient treatment

P_j is the effect of the performance level (fast or slow growing family)

TxP_{ij} is the effect of the interaction between treatment and performance level

$e_{(ijk)l}$ is the sampling error

$s_{(ijkl)m}$ is the sub sample error

All effects were fixed except for error terms. Temperature was not included in the model as some test temperatures did not create a range of damage in the samples. Thus data from the temperature resulting in the greatest spread of damage was used in ANOVA calculations.

4.3 Results

TISSUE FREEZING

Intermediate needle damage on the first sampling in November 1995 was produced by temperatures of -16°C. Needle damage was significantly different among the nutrient treatments (p=0.0063)(Figure 8), but there was no difference between fast and slow growing families (p=0.1480). Overall fast growing family trees (53.7%(±5.7SEM)) displayed more damage than slow growing trees (36.1%(±5.9)). There was not a significant treatment by performance group interaction (p=0.4599).

Intermediate needle damage on the second sampling in April 1996 was created by a test temperature of -10°C. Needle damage was significantly different among the nutrient treatments (p=0.0001)(Figure 9), but not between fast and slow growing families (p=0.6935). On average, fast growing trees displayed slightly more damage (78.8%(±4.1)) than slow growing trees (72.2%(±3.9)). There was no significant treatment by performance group interaction (p=0.2475).

Bud damage was significantly different among the nutrient treatments in April of 1996 (Table 22), but not among fast and slow growing families (p=0.3271). There was a significant treatment by performance group interaction (p=0.0044). For the 20:20 treatment, fast growing families were less damaged than the slow growing families (Figure 10). The 100:4 and 100:20 treatment fast growing trees were more damaged than slow growing trees. The 100:60 and 250:20 treatments had no freezing damage for either fast or slow growing families.

Cambium damage in April 1996 was also significantly different between nutrient treatments (Table 22), but not between fast and slow growing families (p=0.2428). There

was no treatment by performance group interaction (p=0.2062). Fast growing trees generally displayed less damage (66.8%(±6.1)) than slow growing trees (74.5%(±6.2)).

Sampling 4 (Oct. 30, 1997) produced intermediate needle damage at -12 °C. Needle damage was significantly different among the nutrient treatments (p=0.0001)(Figure 11), but not among fast and slow growing families (p=0.0533). There was no significant treatment by performance group interaction (p=0.0919). In general, fast growing trees displayed more damage (70.4%(±5.0)) than slow growing trees (57.4%(±3.9)).

Bud damage was significantly different among the nutrient treatments (Table 22), but not between fast and slow growing families (p=0.1432). There was a significant treatment by performance group interaction (p=0.0219). For the 20:20 treatment, fast growing families were more damaged than the slow growing families (Figure 12). The 100:4 treatment fast growing trees were least damaged. Fast and slow growing trees in 100:20 and 100:60 nutrient regimes had roughly similar levels of damage. The 250:20 treatment had no freezing damage for either fast or slow growing families.

Cambium damage in October was also significantly different among nutrient treatments (Table 22), but not between fast and slow growing families (p=0.1561). There was no significant treatment by performance group interaction (p=0.2756). Fast growing trees displayed more damage (43.5%(±6.9)) than slow growing families (31.4%(±7.7)) on average.

MITOTIC INDEX

The first sampling in November, 1996, found significant differences in mitotic index between the treatment groups (p=0.0003)(Figure 13), but not between fast and slow growing families (p=.7546). There was no significant treatment by performance group interaction (p=0.1016).

BUD BURST

Bud burst scored at the second sampling in April, 1996, differed among nutrient treatments. Bud burst had occurred in both 100:4 (0.61) and 20:20 (0.20) treatments, but was absent in 100:20 (0), 100:60 (0), and 250:20 (0) treatments. Fast growing trees had marginally more advanced bud burst (0.19) than the slow growing trees (0.13).

LEADER GROWTH

There was a significant difference found on the third sampling in September 1996 in the height of the trees subjected to the different nutrient regimes (p=0.0001)(Figure 14). There was no difference in the height of fast and slow growing families (p=0.1018). There was not a significant treatment by performance group interaction (p=0.7016). Overall fast growing trees (67.9cm(±4.9)) were slightly taller than the slow growing trees (62.5cm(±4.9)).

The nutrient treatments also produced significant differences in the length of the 1996 leader (p=0.0006)(Figure 14), however, there was not a significant difference in new leader growth between fast and slow growing families (p=0.7715). There was no significant interaction between treatment and performance group (p=0.9634). Overall,

fast growing families (9.3cm(±1.1)) displayed similar growth over the 1996 growing season as the slow growing families (10.2cm(±1.3)).

Lammas growth was present in 100:20 (0.37), 100:60 (0.30), and 250:20 (0..22) treatments, but was absent in the 20:20 and 100:4 treatments. There were more trees with a second flush in fast (0.23) than slow (0.13) growing families.

CORRELATION OF TISSUE DAMAGE, MITOTIC INDEX, AND SHOOT EXTENSION

A significant negative correlation between mitotic index and needle damage was found in November, 1995 (Figure 15).

There were no significant correlations found between needle damage and bud damage on the second sampling in April, 1995 (Table 23). However, there was a significant correlation between needle and cambium damage. Bud and cambium damage were not correlated.

At the fourth sampling in October, 1996, there were significant correlations among needle, bud, and cambium damage, but not between needle damage and shoot growth evaluated in September (Table 24). Bud damage was significantly correlated to cambium damage, but not with shoot growth. Cambium damage was not correlated with shoot growth.

4.4 Discussion

FALL 1995

Late fall (sampling 1) found differences in needle hardiness among the nutrient treatments, but not between fast and slow growing families. Only the N20:N20 and the N250:P20 treatments differed significantly, however in general, the treatments with a high N:P ratio were more hardy. The 250:20 treatment maintained mitotic activity later into the fall when compared to the 20:20 or the 100:4 treatments. These results support the idea that an increased level of a balanced nutrient supply delays dormancy in the fall and extends the growing season. It has previously been shown that mitotic activity ceases earliest in plants with low nitrogen supply (Carlson et al. 1980). Trees with high mitotic index due to high N supply did not suffer increased freezing damage, and this contradicts previous work which suggests that well fertilized trees will be the least hardy (Aronsson 1980, Christersson 1977). This is most likely a consequence of the trees being healthier and more vigorous and generally more resistant to stress (Klein et al. 1989).

SPRING & SUMMER 1996

In mid-spring of the following year (sampling 2), there were differences in needle damage among the nutrient treatments. The ranking of needle damage by nutrient treatment was similar to that found in the autumn sampling. Bud and cambium damage was also significantly different among the nutrient treatments. There was no correlation

between bud damage and needle or cambium damage, but there was a correlation between needle and cambium damage.

Bud burst was most advanced in 100:4 trees, followed by 20:20 trees. Bud burst was delayed in treatments 100:20, 100:60, and 250:20. The higher level of nutrition in these groups appears to create this delay. This may be a result of the extended development of buds in the 100:20, 100:60, and 250:20 trees in the fall leaving insufficient time for bud maturation before entering dormancy. The consequence is a delay in the spring as buds complete maturation before bursting (Dormling 1982). This is corroborated by the mitotic index data from the previous fall which showed the groups given higher nutrient levels to have higher mitotic activity. These results differ from a previous study showing that nitrogen advanced bud break during the following year, but had no negative effects on the frost hardiness of several conifers (Benzian et al. 1974). In the 1974 study however, N fertilization was initiated in September and subsequently did not follow the same constant fertilization schedule as implemented in this study.

The ranking of tree height and annual growth were similar to the ranking of hardiness levels. There was a clear separation of tree height between trees in the 20:20 and 100:4 treatments which were much smaller than those in 100:20, 100:60, and 250:20 treatment groups. The moderate to high levels of N in conjunction with moderate to high levels of P produced the tallest trees and the greatest annual growth. While freezing damage was obviously different among nutrient treatments, there was seldom any significant difference between fast and slow growing families. This could be explained by the lack of any observed difference in the primary growth, and perhaps phenological, characteristics between the fast and slow growing trees.

FALL 1996

In the fall of the second year (sampling 4) freezing damage patterns were similar to spring and the previous fall. Needle, bud, and cambium damage differed among the nutrient treatments. These results are somewhat confusing. Why have the trees given more fertilizer remained hardiest well into the fall? There may be an interaction between two factors coming into play: 1) extension of growth activity by fertilization and 2) improved plant health due to fertilization. In the fall, trees fertilized with higher levels of nitrogen may enter dormancy later (Christersson 1977). Extending the growth period will delay bud set and the frost hardiness will be lower so that seedlings may be damaged by lower freezing temperatures. In this study, however, 250:20 trees, had the highest amount of N fertilization, and produced the largest and most vigorously growing trees. The cold hardiness studies without exception showed the 250:20 trees to be the least freezing susceptible. This supports studies which showed that seedlings grown with adequate nutrition were less damaged by freezing than nutrient deficient seedlings (Klein et al. 1989). A nutrient starved tree will grow at a lower rate and may be unable to acclimate due to the expense required for modifying membranes, proteins etc. 'Luxury' uptake of macroelements by plants is generally considered to reduce hardiness, however, trees must reach an optimum N status for hardening (Bigras et al. 1997). It appears that even though the trees with more N and P are growing at higher rates later into the fall, their overall health is so much better that even in a semi-acclimated state, they have more cold tolerance than the less fertilized seedlings. However, the 100:20 and 100:60 treatment

tress were less hardy than the 100:4 treatment. It is possible that the higher N:P ratio and/or the low P (Malcolm and Freezaillah 1975) level may be a factor contributing to the greater hardiness.

The level of freezing may also play a role in determining the level of damage. In *Pinus sylvestris*, greater hardiness occurred in plants with greater nitrogen supply in tests with temperatures creating maximum damage. At less damaging temperatures there was a tendency for lower hardiness with increasing nitrogen concentrations (Aronsson 1980) giving support for an interaction between temperature and nitrogen supply. The test temperatures in this study were selected to created an intermediate level of visual damage which on a physiological level may have been relatively high. Subsequently, the trees with higher fertilization levels might have been favored in these hardiness tests.

Table 20. Sampling schedule for the nutrition and hardiness study.

Year	Sampling	Date	Analysis
1995			
	1	Nov 16	Cold tolerance and mitotic index
1996			
	2	Apr 18	Cold tolerance and bud burst
	3	Sep 25	Primary development
	4	Oct 30	Cold tolerance

Table 21. Experimental design of nutrition and hardiness study

Cold hardiness

10 trees (5 fast growing + 5 slow growing) /nutrient treatment
5 nutrient treatments

TOTAL = 50 trees

50 trees x 8 cut samples (2 per test temperature and control)

= 400 cuttings /date

Mitotic Index

10 trees / nutrient treatment
5 nutrient treatments

TOTAL = 50 trees

50 trees x 4 terminal buds / tree

= 200 squashes /date

Table 22. Cold hardiness of bud, and cambium tissue between five nutrient treatments in 1996 with standard error of mean in brackets

Sampling	Date	Test Temp. (-°C)	Tissue	Treatment (N:P)	Damage %	Pr>F[2]
2	18-Apr	-14	Bud	20:20	40.0 (8.2)a[1]	p=0.0073
				100:4	12.5 (7.1)b	
				100:20	11.2 (6.8)b	
				100:60	0 (0)b	
				250:20	0 (0)b	
		-18	Cambium	100:4	100 (0)a	p=0.0046
				20:20	85.2 (5.3)a	
				100:20	5.1 (8.5)b	
				100:60	0.3 (7.9)b	
				250:20	9.7 (3.4)b	
4	30-Oct	-16	Bud	100:4	69.2 (6.3)a	p=0.0055
				20:20	30.9 (7.1)a	
				100:20	28.3 (6.2)ab	
				100:60	1.1 (1.7)ab	
				250:20	0 (0)b	
		-16	Cambium	100:4	87.5 (4.9)a	p=0.0001
				20:20	77.8 (6.8)a	
				100:20	18.8 (6.4)bc	
				100:60	3.8 (4.2)bc	
				250:20	3.6 (3.1)c	

[1]means followed by the same letter are not significantly different (p=0.05) within each sampling date

[2]probability of mean transformed % freezing damage differing significantly among nutrient treatments

Table 23. Correlations (r) between needle, bud, and cambium damage in trees from five nutrient treatments in April 1996 (n=50)

	Needle	Bud	Cambium
Needle	XXXX XXXX	-0.1819 p=0.7438	0.7100 **p=0.0001**
Bud		XXXX XXXX	0.4050 p=0.1027
Cambium			XXXX XXXX

Table 24. Correlations (r) between needle, bud, and cambium tissue damage between treatment and performance groups for the nutrition study in October 1996 (n=50)

	Needle	Bud	Cambium	Shoot Growth
Needle	XXXX XXXX	0.6276 **p=0.0119**	0.6632 **p=0.0045**	-0.3375 p=0.4899
Bud		XXXX XXXX	0.8097 **p=0.0001**	0.5365 p=0.0756
Cambium			XXXX XXXX	0.3388 p=0.4861
Shoot Growth				XXXX XXXX

Figure 8. Needle freezing damage in November 1995 for the nutrition study. Error bars represent standard error of means. Means described with the same letter are not significantly different (p=0.05)

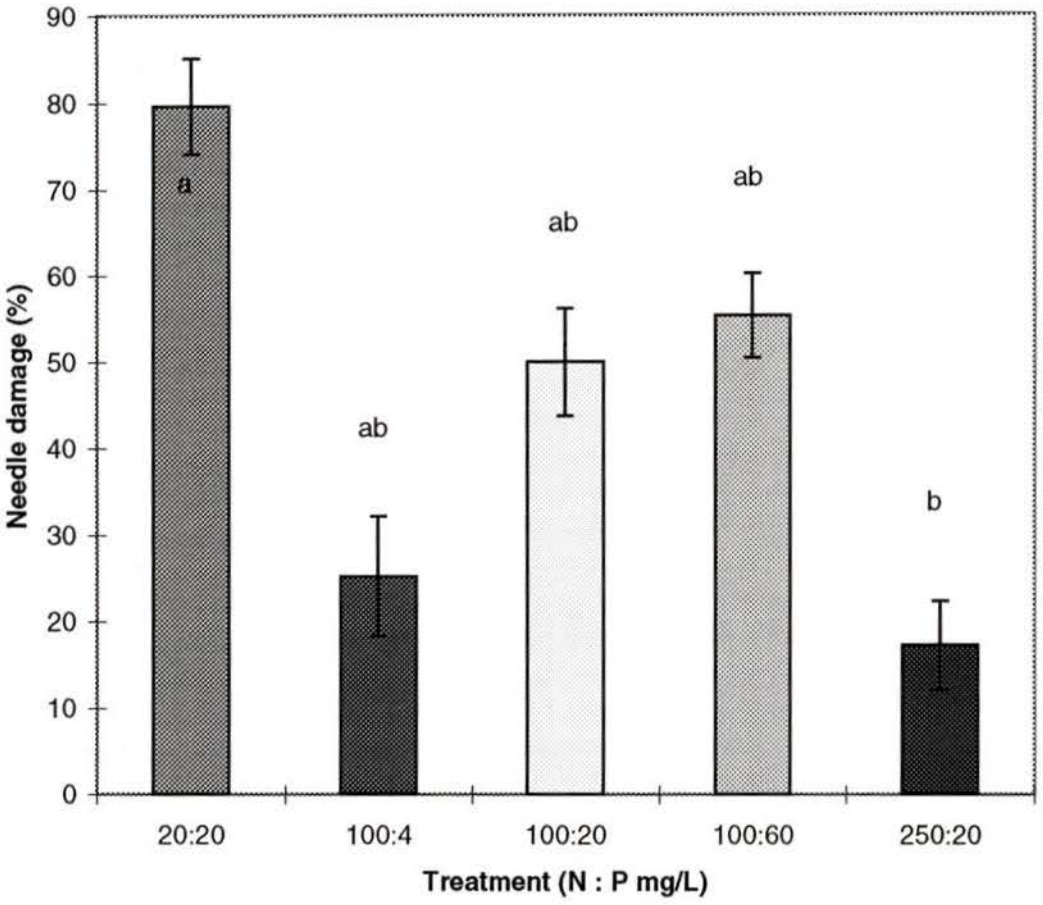


Figure 9. Needle freezing damage at sampling 2 in April 1996 for the nutrition study. Error bars represent standard error of means. Means described with the same letter are not significantly different (p=0.05)

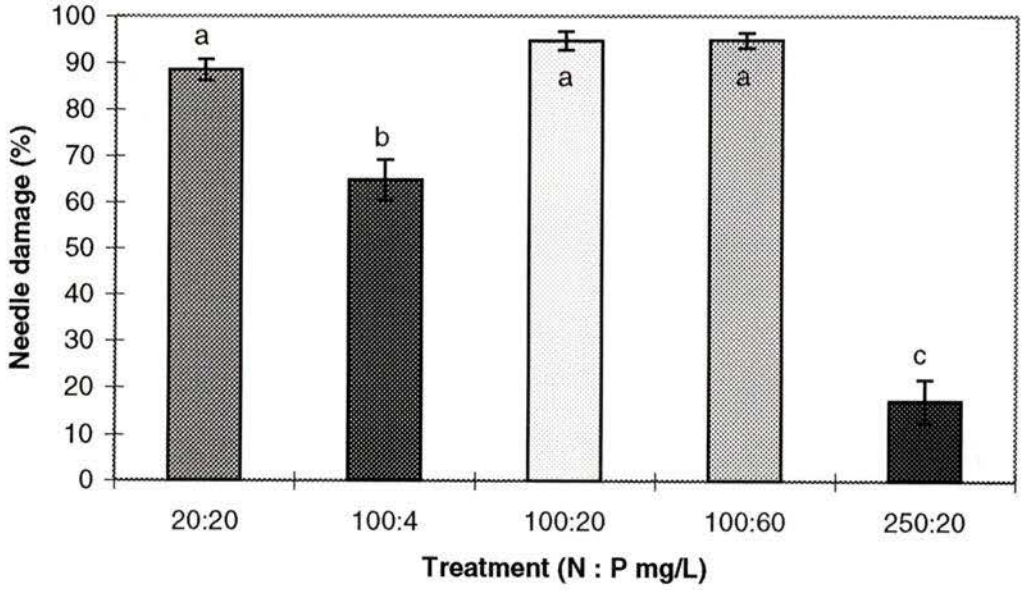


Figure 10. Bud damage among fast and slow growing families describing the significant nutrient treatment x performance group interaction in April 1996. Error bars describe standard error of means.

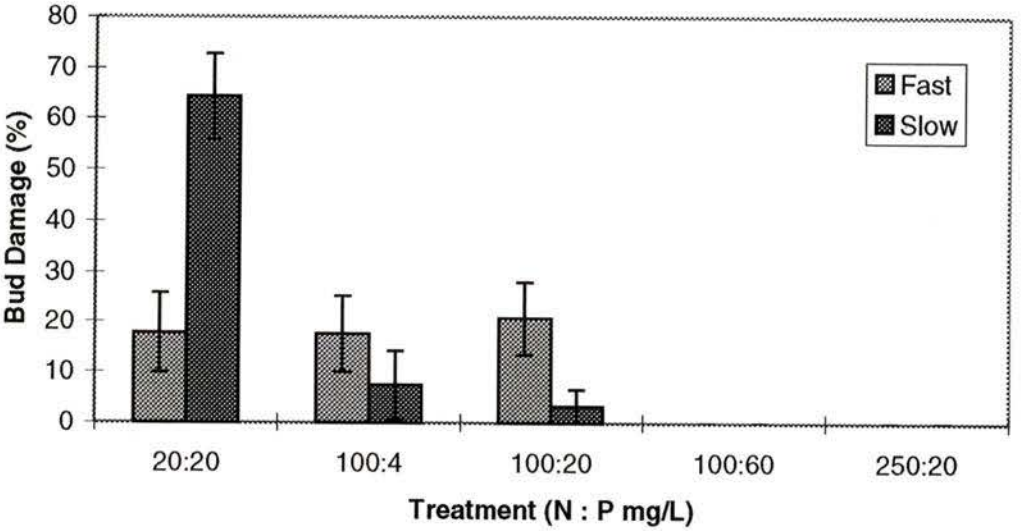


Figure 11. Needle freezing damage at sampling 4 in October 1996 for the nutrition study. Error bars represent standard error of means. Means described with the same letter are not significantly different (p=0.05)

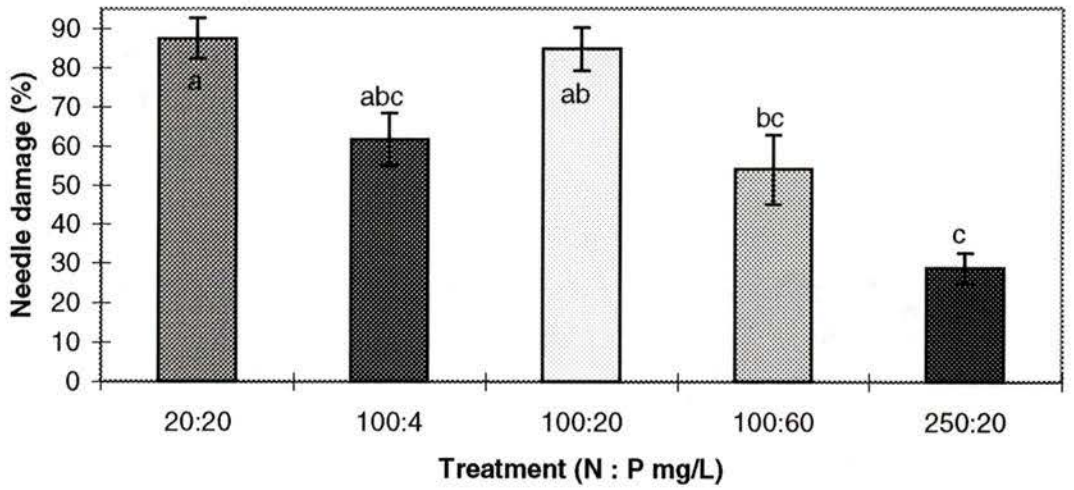


Figure 12.Bud damage among fast and slow growing families describing the significant treatment x performance group interaction in October 1996. Error bars describe standard error of means.

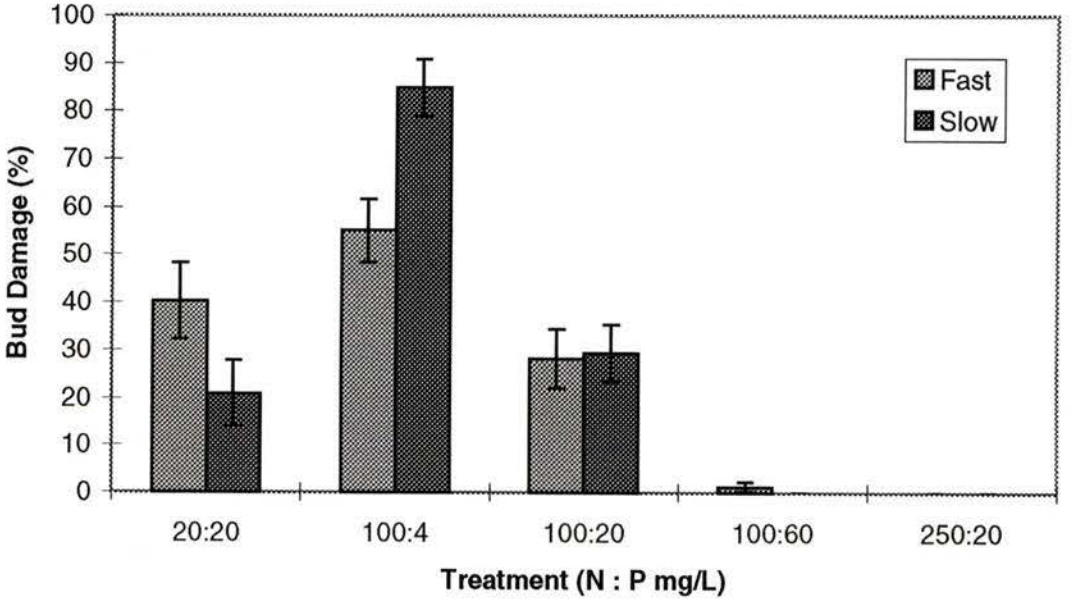


Figure 13. Mitotic index of terminal lateral shoots for the nutrition study in Nov. 1995. Error bars represent standard error of means. Means described with the same letter are not significantly different (p=0.05)

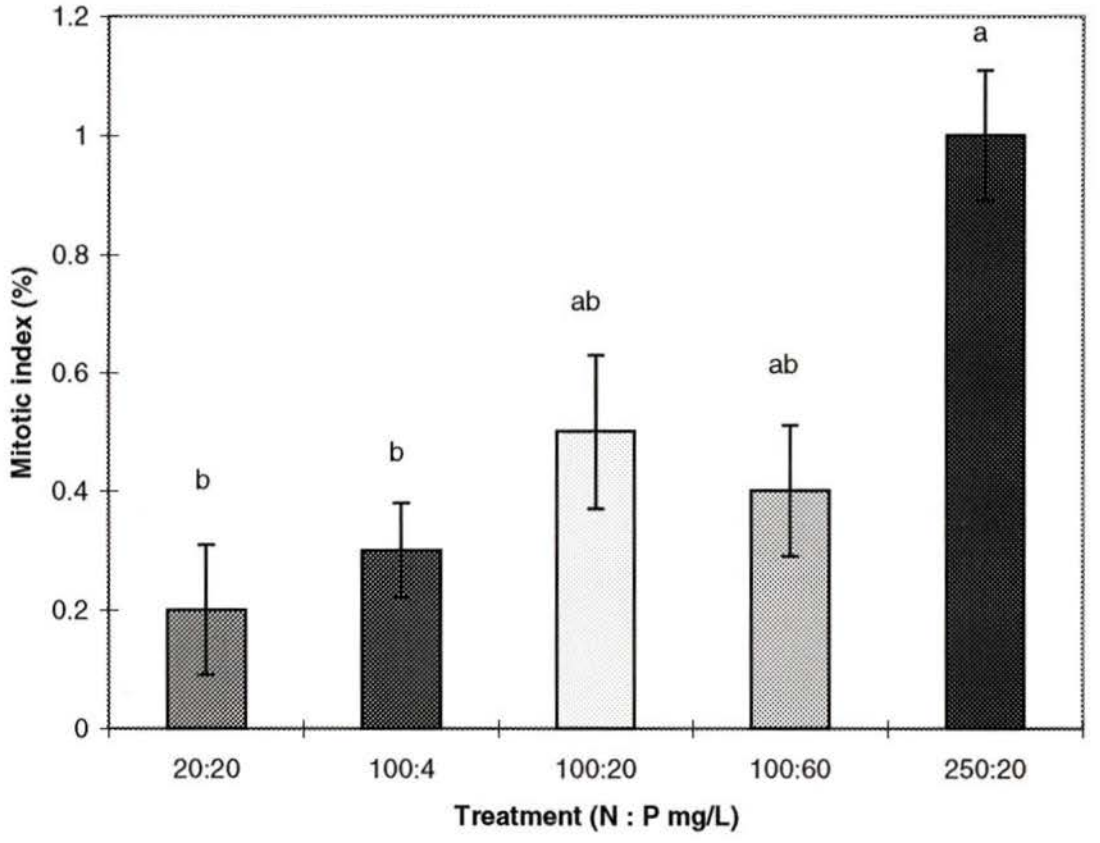


Figure 14. Tree height and new growth during the season for the nutrition study in 1996. Error bars represent standard error of means. Means described with the same letter are not significantly different (p=0.05)

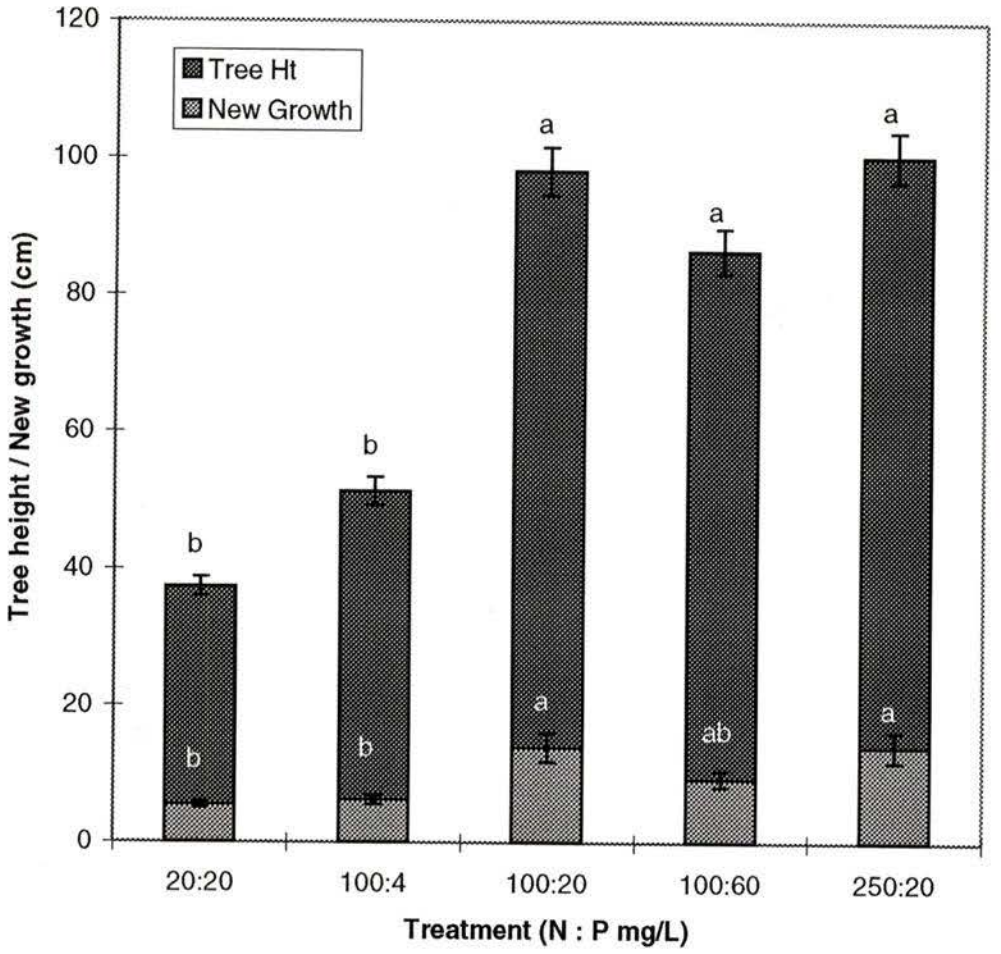
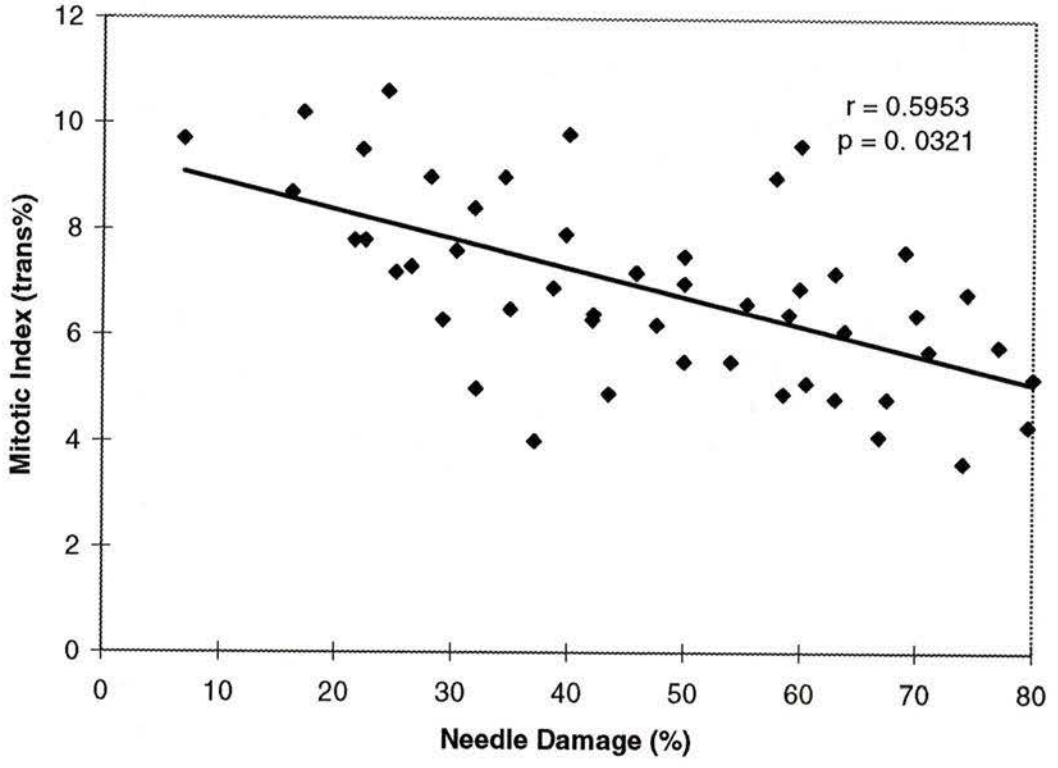


Figure 15. Mitotic index plotted against needle freezing damage for the five nutrient treatments in November 1995. Values are means of all samples within each treatment



CHAPTER 5

CONCLUSIONS

When the hardiness of Douglas-fir trees grown from wild stand seed and those produced from breeding programs were compared, differences were often not significant, however, trends were generally consistent. In spring, wild stand trees lost hardiness earlier, while in the early fall, top-cross trees acclimated later. By late fall, genetic groups had similar hardiness levels. Needle tissue was usually the most susceptible to freezing damage, while cambium tissue was the hardiest without exception.

Mitotic index, an indicator of dormancy, was linked to cold hardiness. A significant, positive correlation was found between mitotic index and freezing damage for all study trees pooled. Wild stand trees appeared to lose dormancy earlier in the spring, while the top cross trees entered dormancy later in the fall. In terms of growth initiation, the trend of wild stand trees bursting bud earliest was consistent when compared to the top cross and seed orchard trees. Top-cross trees, without exception, displayed delayed bud burst, which was consistent with a lower early spring mitotic index and a lower rate of growth in early summer. Date of bud burst did showed significant correlations with spring cold hardiness.

There were no significant differences in growth between wild stand trees and trees from breeding programs in this study. However, across the whole realized gain trial the increased growth displayed by the 'improved' trees is highly significant ($p<.0001$) (Jack Woods pers. comm.). In five of six samplings and in final growth for the year, the top cross trees did display the most growth. Shoot extension phenology during the summer

was associated with fall hardiness for all study trees. Trees that had completed more of their growth earlier in the summer were more hardy in the early fall. Secondary growth phenology as measured in this study showed no correlation to hardiness.

In the nutrition study, trees receiving more N and P grew taller, and trees with a high N:P ratio were more hardy at all times of the year. Trees receiving more N had higher mitotic indices in late fall, but these same trees were more tolerant to freezing temperatures than their less fertilized, less mitotically active counterparts. In this case, nutrition may have prolonged the growth season, but did not decrease hardiness. Amongst the nutrition study trees, family performance groups (fast and slow growing) did not differ significantly in hardiness, mitotic index, or growth.

Genetically faster growing trees from the realized gain study started growth later and grew later. This may result in a decreased risk of spring frost damage, but an increased risk of fall freezing damage. As the chance of damaging spring frosts is two to three times more likely than damaging fall frosts (Timmis et al. 1994) this cannot be considered a setback to tree improvement. Fast and slow growing families in the nutrition study showed no significant difference in growth or frost hardiness, and this may be a consequence of their young age. Faster growth due to improved nutrition resulted in trees growing later into the year, but they were still cold hardy. Thus effects on hardiness produced by faster growth may differ depending on whether the accelerated growth is due to environmental or genetic factors.

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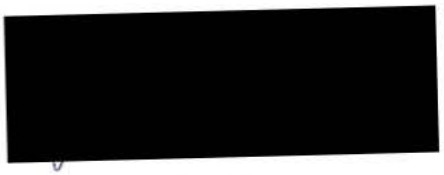
Author 

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April 3, 1998