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PHYSIOLOGICAL SIGNIFICANCE OF ETHYLENE IN NEEDLE ABSCISSION OF ROOT-DETACHED BALSAM FIR (*ABIES BALSAMEA* (L.) MILL.)

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RESUMÉ

L'abscission prématurée des aiguilles constitue un problème majeur pour l'industrie de l'arbre de Noël dans les provinces Atlantiques du Canada. Les objectifs de cette étude étaient donc de déterminer: 1) l'effet de l'éthylène endogène et exogène sur l'abscission des aiguilles, 2) l'effet de l'utilisation d'inhibiteurs de l'activité de l'éthylène sur l'abscission des aiguilles, 3) l'activité des cellulases associées à l'éthylène et l'abscission des aiguilles, 4) l'interaction entre l'éthylène et la variabilité génotypique, et 5) l'effet de l'éthylène sous des conditions de perte d'eau réduite. Nos résultats ont démontré que l'éthylène est impliqué comme signal d'abscission pour les aiguilles du sapin baumier. Il a été constaté que l'évolution de l'éthylène endogène a augmenté à des taux variant de 10,5 à 20,0 $\mu L \cdot g^{-1} \cdot h^{-1}$ suivie immédiatement de l'abscission complète des aiguilles. Selon la concentration utilisée, l'apport exogène d'éthylène a réduit la durée de rétention des aiguilles de 30 à 70%. En absence d'éthylène exogène, l'inhibition de l'éthylène avec AVG ou 1-MCP a retardé respectivement l'abscission des aiguilles de 113 et 73%. D'autre part, l'éthylène a stimulé l'activité des cellulases. Les branches ne démontrant aucun signe d'abscission ont eu une activité cellulase de 0,4 unité alors que les branches induites par l'éthylène exogène et endogène ont eu une activité respective de 4,6 et 3,1 unités. Les génotypes ayant une faible durée de rétention de leurs aiguilles ont libéré 50% plus d'éthylène et ont réagi à l'éthylène exogène plus tôt que les génotypes possédant une durée de rétention élevée de leurs aiguilles. Enfin, le contrôle de la perte d'eau a significativement retardé l'abscission induite par l'éthylène. Ainsi, l'exposition des branches à 90% d'humidité ou à une température de 5°C a diminué la perte d'eau, l'évolution de l'éthylène et l'abscission des aiguilles. Le stockage des branches à une humidité de 90% a été particulièrement efficace augmentant de plus de 3 fois la durée de rétention des aiguilles. Il est ainsi suggéré que le sapin baumier réagisse à de faibles stress hydriques en libérant de l'éthylène. Une exposition de courte durée à l'éthylène est toutefois bénéfique alors qu'une exposition continuelle stimule les enzymes hydrolytiques qui culminent en l'abscission des aiguilles.

ABSTRACT

Post-harvest needle abscission is presenting a major challenge for the Christmas tree industry. Several objectives were tested to determine, 1) the effect of endogenous and exogenous ethylene on needle abscission, 2) the effect of inhibiting ethylene activity, 3) enzyme activity associated with ethylene, 4) ethylene interaction with genotypic variability, and 5) effect of ethylene when water loss is minimized. Ethylene was strongly implicated as the signal for abscission in balsam fir needles. It was found that endogenous ethylene evolution increased to rates between 10.5 and 20.0 $\mu L \cdot g^{-1} \cdot h^{-1}$ immediately preceding complete abscission. Exogenous ethylene reduced NRD by 30 to 70%, dependent on concentration. Inhibition of ethylene action with AVG or 1-MCP delayed abscission by 113% and 73%, respectively, in the absence of exogenous ethylene. Ethylene stimulated cellulase activity. Control branches showing no signs of abscission were found to have 0.4 units of cellulase activity. However, branches experiencing exogenous and endogenous ethylene-induced activity were found to have 4.6 and 3.1 units of cellulase activity, respectively. Low NRD genotypes released 50% more ethylene and responded to exogenous ethylene earlier than high NRD genotypes. Finally, control of water loss significantly delayed ethylene-induced abscission as storage at 90% humidity or 5 °C significantly decreased water loss, ethylene evolution, and, abscission. Storage of branches at 90% humidity was particularly effective, as there was more than a 3-fold increase in NRD. It is proposed that balsam fir responds to small changes in water status by releasing ethylene. Short term exposure to ethylene is actually beneficial, but continuous exposure stimulates hydrolytic enzymes which culminate in abscission.

FOREWORD

A number of the experimental chapters contained in this thesis have been published or accepted to peer-reviewed journals or international conferences. The following describes each work previously published, where they appear in this thesis, and any relevant amendments or commentary. In each case, the primary author (PhD candidate) was responsible for designing and conducting experiments, statistical analysis, and manuscript preparation. Secondary authors (PhD committee) were involved in experimental design, manuscript review, and encourage synthesis of ideas.

(1) MacDonald and Lada RR. 2008. Cold acclimation can benefit only the clones with poor needle retention duration (NRD) in balsam fir. HortScience. 43: 1273 (abstract)

- Chapter 8.2. This was originally presented as cold acclimation effect on balsam fir genotypes, but in the thesis it has been discussed more accurately as a harvest date effect. The needle retention durations reported allowed for classification of balsam fir genotypes.

(2) MacDonald MT, Lada RR, Martynenko AI, Dorais M, Pepin S, Desjardins Y. 2009. Ethylene modulates needle abscission in root-detached balsam fir. HortScience. 44: 1142 (abstract).

- Chapter 5.2. There were no changes from its original format.

(3) MacDonald MT, Lada RR, Dorais M, Pepin S, Desjardins Y, Martynenko AI. 2010. Ethylene exposure duration affects postharvest needle abscission in balsam fir (Abies balsamea L.). HortScience. In Press.

- Chapter 5.2. In addition to regression analysis, data in manuscript was presented in a table

(4) MacDonald MT, Lada RR, Martynenko AI, Dorais M, Pepin S, Desjardins Y. 2010. Ethylene triggers abscission in root-detached balsam fir. Trees. 24: 879-886.

- Chapters 5.3, 5.4, and all of Chapter 6. These experiments were combined in a single manuscript to discuss the role ethylene has in needle abscission.

(4) MacDonald MT, Lada RR, Martynenko AI, Dorais M, Pepin S, Desjardins Y. 2010. Is there a relationship between ethylene evolution, ethylene sensitivity, and needle abscission in root-detached balsam fir? 28th International Horticultural Congress, Aug. 22-27.

- Chapters 8.3, 8.4. Discussion was expanded from original presentation.

(5) MacDonald MT, Lada RR, Dorais M, Pepin S, Desjardins Y, Martynenko AI. 2010. Is there a relationship between ethylene evolution, ethylene sensitivity, and needle abscission in root-detached balsam fir? Acta Hort. In Press.

- Chapters 8.3, 8.4. Material presented at the 28th International Horticultural Congress was invited to be submitted as a manuscript.

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What nobler employment, or more valuable to the state than that of the man who instructs the rising generation
- Marcus Tullius Cicero (106 BC – 43 BC)

It is the supreme art of the teacher to awaken joy in creative expression and knowledge
- Albert Einstein (1879 – 1955)

People can come up with statistics to prove anything. 14% of all people know that
- Homer Simpson (1989 – present)

This work is dedicated to my loving wife, Lori Anne

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ABA	abscisic acid	EIC	ethylene incubation chamber
ACC	aminocyclopropane-1-carboxylic acid	IAA	indole-3-acetic acid
AdoMet	adenosyl methionine	iPA	isopentenyladenosine
Ambiol	2-methyl-4-[dimethylaminomethyl]-5-hydroxybenzimidazole	iPMP	isopentenyladenosine-5-monophosphate
AMP	adenosine monophosphate	JA	jasmonic acid
ANOVA	analysis of variance	LOX	lipoxygenase
AOC	allene oxide cyclase	MCP	1-methylcyclopropene
AOS	allene oxide synthase	MII	membrane injury index
AVG	aminoethoxyvinylglycine	mRNA	messenger ribonucleic acid
AWU	average water use	MTA	methylthioadenosine
cDNA	complimentary DNA	NADPH	nicotinamide adenine dinucleotide phosphate
CMC	carboxymethylcellulose	NRD	needle retention duration
DACP	diazocyclopentadiene	PBS	phosphate buffer solution
DMAPP	dimethylallyl-pyrophosphate	PG	polygalacturonase
DNA	deoxyribonucleic acid	ROS	reactive oxygen species
DPE	day of peak ethylene	TRIS	tris(hydroxymethyl)aminomethane
EC_k	electrical conductivity (final)	tRNA	transfer ribonucleic acid
EC_o	electrical conductivity (initial)	XPP	xylem pressure potential

1.0 INTRODUCTION

Christmas trees and greenery are an important specialty horticulture industry. Originally, most trees were harvested from natural forest stands and then transported to urban areas for sale. Though commercialization began in 1851, over 90% of all trees were harvested from natural stands until the late 1940s (Albers and Davis 1997). It was after World War II that the market began to shift, trees were grown in plantations, and cosmetic improvements such as shearing were implemented in response to consumer demand (Davis 1996). Today, more than 95% of all Christmas trees come from plantations. The most common tree species sold are balsam fir (*Abies balsamea*, L.), Douglas fir (*Pseudotsuga menziesii*, Mirb.), white spruce (*Picea glauca*, Schwer.) and eastern red cedar (*Juniperus virginiana*, L.). It is estimated that 3-6 million trees are produced annually in Canada (Statistics Canada 2008), 28-30 million trees in United States (NCTA 2008), and 50-60 million in Europe (Davis 1996; Frampton and McKinley 1999). Overall, the worldwide market value has been estimated at $1.85 billion with a contribution of $50 to $100 million from Canada (Albers and Davis 1997; Statistics Canada 2008).

The balsam fir is typically found in eastern and central Canada (Fig. 1) and is the principal Christmas tree species grown in Atlantic Canada, preferred by consumers for its unique fragrance and needle characteristics. In Nova Scotia alone there is approximately 10,000 ha grown, with about 1.5 – 2.0 million trees harvested annually valued at approximately $30 million. In all of Atlantic Canada, over 3 million trees are harvested, which is worth $72 million annually (CTCNS 2008). Currently, the majority of Christmas trees are exported to the United States, though there is also some market in Puerto Rico, Brazil, Panama, Venezuela, Mexico, and Japan.

Figure 1: Map of Canada and United States. Natural occurring balsam fir can be found in the green shaded region, largely in eastern and central Canada (adapted from Little (1999))

Despite the fact that the Christmas tree industry is a billion dollar industry worldwide, and a multimillion dollar industry locally, needle abscission is presenting a major challenge for producers. The increasing demand for these trees in foreign markets combined with the desire for trees by United States Thanksgiving as a family holiday tree means more trees will be harvested at an earlier date, possibly resulting in poor needle retention characteristics in those trees (Mitcham-Butler et al. 1988). In addition, the reduction in needle retention of Christmas trees have been attributed to warm fall temperatures during harvest in mid to late October (Chastagner and Riley 2003). While needle losses occur during harvesting, handling, transportation, and at display stands, extensive needle loss after consumer purchase has become a matter of great concern for the industry. The industry suffers huge economic losses due to the reduced marketability of the trees caused by poor needle retention characteristics. Competition from artificial trees adds to the real-tree marketing complexity, as consumers have developed zero tolerance for needle abscission.

Leaf or needle abscission can be influenced by several factors, such as climate, excessive nutrition, air pollution, genetic variations, cold acclimation, or dehydration. Due to the

fact that Christmas trees exist as a root detached system, dehydration or cavitation of xylem was hypothesized to be the most important factor in accelerating abscission. It is well documented that drought conditions can cause premature senescence and abscission in various Christmas tree species (Chastagner 1986). In poplar trees there was a linear relationship between soil water potential and leaf abscission, where abscission increased as soil water potential decreased (Pallardy and Rhoads 1997). In conifers, it was shown that when trees experience xylem pressure potentials (XPP) above their threshold damage levels (usually in the range of -3 MPa to -4 MPa), it can accelerate needle drop (Hinesley and Snelling 1991). However, Rajasekaran et al. (2005a) have found that xylem pressure potential and branch water content remain relatively consistent in balsam fir in a natural system as compared to a root detached system. It was suggested that while water flux is important to prevent dehydration induced senescence and needle drop, hydration alone cannot prevent needle drop implying that xylem pressure potential may not be the biophysical trigger for needle drop.

Rajasekaran et al. (2005a) presented the hypothesis that dehydration beyond a certain threshold would have stimulated synthesis of certain signals such as ethylene. Ethylene production also increases as a response to several abiotic stresses, such as limited light or mechanical stress, which may occur during handling and storage of trees. It has been established in several species that ethylene disrupts the basipetal transport of auxin, which can modify ethylene sensitivity of a plant (Beyer and Morgan 1971). Ethylene then stimulates the synthesis of several cell wall degrading enzymes, which are speculated to facilitate abscission (Sexton et al. 1985). Though ethylene has been a well studied topic, there have been no studies to date to investigate ethylene's potential role in balsam fir needle abscission.

Ultimately, little is understood regarding needle abscission in balsam fir. It is widely known that after harvest, needle abscission will occur that would not have occurred if a tree was left untouched. The actual signal and mechanism of abscission in balsam fir is unknown. The purpose of this research is to determine the role ethylene may have in the physiological changes from root detachment (harvest) to abscission.

2.0 LITERATURE REVIEW

2.1 Senescence and abscission in plants

2.1.1 Progression of senescence

Senescence is a complex, highly regulated, developmental phase in the life of a leaf that results in the coordinated degradation of macromolecules and the subsequent mobilization of components to other parts of the plant (Buchanan-Wollaston 1997). As such, senescence can be considered as the progressive death of cells of an organ or organism. Relating to leaves or needles, senescence is a process that must occur in normal development and usually involves cessation of photosynthesis, disintegration of chloroplasts, break down of leaf proteins, loss of chlorophyll, and removal of amino acids (Smart 1994).

There are three phases that occur during the lifespan of a leaf. At first, there is rapid expansion, incorporation of nitrogen and carbon, and protein synthesis to allow a leaf to reach maximum photosynthetic potential. The second stage occurs once the leaf has reach maximum photosynthetic potential, during which time the leaf is a benefit for the plant. Carbon accumulation is high and protein turnover is consistently low. Eventually, internal or external conditions initiate leaf senescence, where there is a massive relocation of carbon, nitrogen, and minerals to other parts of the plant (Buchanan-Wollaston 1997).

Physical indications of senescence occur much later than the actual onset, but are usually characterized when the mesophyll tissue begins to lose its greenness and turn to yellow or red. The color change is due to both preferential degradation of chlorophylls compared to carotenoids and synthesis of new compounds, such as anthocyanins and phenolics (Matile 1992). The ultimate consequence of leaf senescence is often abscission (Smart 1994).

In addition to the programmed type of leaf senescence, which takes place during plant development, the degradation of macromolecules and mobilization of cellular component

from leaves can also occur in response to external environmental stresses. Plants have to respond rapidly to deteriorating environmental conditions since, unlike animals, they cannot move in order to find a more favorable situation. One response that plants can make is to remove those parts of the plant that are not essential. For example, a diseased leaf will senesce, die and drop off the plant, thus helping to prevent spread of disease and allowing the rest of the plant to continue in its development (Buchanan-Wollaston 1997). Similarly, nitrogen deficiency, light limitation, and drought stress will initiate the onset of senescence which may result in early seed development or reduce photosynthetic requirements, potentially allowing a plant to survive throughout the stressful period (Pallardy and Rhoads 1997). However, a difference from the natural and programmed senescence is that, in these cases, the processes may be reversible if the stress conditions are relieved before senescence has progressed beyond a certain point (Stoddart and Thomas 1982).

2.1.2 Abscission

Abscission is the term used to describe the process of natural separation of organs (i.e. leaves, flowers, fruit) from the parent plant, typically occurring after senescence (Taylor and Whitelaw 2001). Though abscission is often linked to senescence, it is possible for abscission to occur independently from senescence (Abeles et al. 1992; Morgan and Drew 1997).

In many cases, abscission occurs naturally and is beneficial to a plant. As a plant grows, lower leaves may eventually be shaded by higher canopy. As such, it is beneficial for a plant to shed those leaves before their metabolic cost exceed the amount of carbon that can be fixed (Brown 1997). Leave are often shed in periods of water deficit for similar reasons (Taylor and Whitelaw 2001). A final example might be found in the infection of a plant leaf, whereby abscission would be useful in preventing spread of infection to the rest of the plant (Sexton and Roberts 1982).

Abscission is a highly regulated process involving multiple changes in cell structure, metabolism, and gene expression. Early research revealed that abscission normally occurs

at highly predictable positions where cells are morphologically distinct from the rest of an organ (Brown and Addicott 1950). This area is commonly referred to as the abscission zone and is a fascinating area of study due to physical, physiological, and biochemical changes that take place during abscission (Harris et al. 1997).

2.1.3 Histology of the abscission zone

The cells that comprise the abscission zone are often morphologically distinguishable before abscission, typically identified as small, square-shaped cells containing a dense cytoplasm (Sexton and Roberts 1982). Cells in the abscission zone appear to have lost the ability to enlarge and become vacuolated as part of normal growth and development. Rather, those cells retain the ability to enlarge only during the abscission process. The actual number of cells involved in forming the abscission zone differs between species. For example, abscission in tomato flowers takes place within two discrete cell layers while abscission occurs in over 50 cell layers in a species of elderberry, *Sambucus nigra* (Tabuchi and Arai 2000; Taylor and Whitelaw 2001).

The separation of plant organs from the parent plant is often generated by mechanical forces, which, in combination with a weakened cell wall, allow for efficient shedding of an organ (Sexton et al. 1985). While in some instances mechanical forces can be caused by wind or abrasion, it is also possible for cells in the abscission zone to generate their own mechanical forces. Rounded cells are often observed on a micrograph of abscised cells, which presumably result from the release of turgor pressure from neighboring cells (Brown 1997). Even before abscission, it is possible that there may be cell expansion, as the ability of the cell wall to contain turgor force is compromised by the action of cell wall hydrolases. The subsequent rounding of the cells generates force perpendicular to the abscission zone, causing enough force to separate tissues, rupture the xylem, and cause abscission (Fig. 2) (Sexton et al. 1985). Wright and Osborne (1974) postulated that the hydrolytic enzymes might also induce cell expansion, as it was found the enzyme activity is increased immediately before cell expansion.

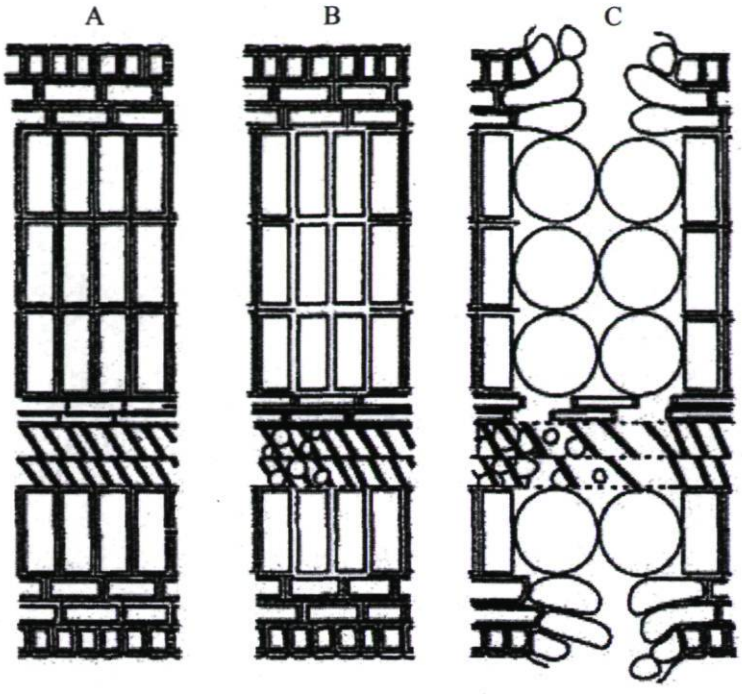


Figure 2: Diagrams of longitudinal sections showing leaf abscission. A) Separation zone before abscission begins; B) Early stage of abscission showing the weakening of middle lamella after increases in hydrolytic enzymes; C) Final stage of abscission characterized by complete breakdown of lamella, cell expansion, and rupture of any remaining connective tissues. Modified with permission from Sexton et al. (1985)

2.1.4 Enzymes linked to abscission

Anatomical studies of abscission have identified almost complete disappearance of the middle lamella and extensive swelling and disorganization of the microfibrils in the primary wall of abscission zone cells. These changes result from the activity of several hydrolytic enzymes, such as pectinase or cellulase, which hydrolyze several cell wall constituents to weaken the cell wall (Sexton et al. 1985). Although much research has been done on enzymes related to abscission, the exact role of the various enzymes has been difficult to establish for various reasons, such as the limited thickness of the abscission zone, the fact that only certain members of enzyme families are involved, dependence on ions, pH, and co-factors, and potential interactions with other proteins (Brown 1997).

Cellulase has been of particular interest since it has consistently been correlated with abscission (Abeles et al. 1992). Several theories have been presented to account for the correlation between cellulase and abscission. Sexton and Redshaw (1981) proposed that

cellulase is involved in the turnover of hemicelluloses, which would cause cell expansion observed in abscission zones. Brummell et al. (1994) proposed that cellulase demonstrates activity against xyloglucans, which would help in cell separation. Finally, Addicott (1982) proposed that cellulase may not induce abscission, but instead is necessary for wound healing responses necessary to prevent infection after abscission.

It has been difficult to identify the precise role of cellulase in abscission because there are so many different forms, but not all forms may be involved with abscission. A study on bean leaf abscission found the presence of several different cellulase isozymes, but only the isozyme with an isoelectric point of 9.5 increased during abscission (Durbin et al. 1981). Antibodies specific to the 9.5 cellulase demonstrated that cellulase was not present before abscission, increased in relation to a decrease in leaf attachment break strength, and was localized in the abscission zone (De Campillo et al. 1996; Durbin et al. 1981; Sexton et al. 1980). Further complications arose from recent work that identified that cellulases are a large and diverse gene family. For example, cellulase in tomatoes is controlled by at least 6 different genes (Lashbrook et al. 1994).

Pectinases have been much more difficult to identify, but are expected to be involved due to anatomical studies showing the dissolution of the middle lamella during abscission (Addicott 1982). The major pectinase studied, with respect to abscission, is polygalacturonase. In several studies, an increase in polygalacturonase coincided with ethylene-induced abscission (Greenberg et al. 1975; Taylor et al. 1990), though polygalacturonase only increased after abscission in tomatoes (Tucker et al. 1984) and not at all in rape pod (Meakin and Roberts 1990). More sensitive tests eventually did reveal a correlation between polygalacturonase and abscission in rape pod (Petersen et al. 1996).

Besides cellulases and pectinases, several other enzymes have been associated with abscission. These include peroxidases, oxidases, chitinases, and β-1,3-glucanase (Sexton et al. 1985). The exact role of these enzymes has yet to be determined.

2.2 Abiotic factors causing senescence and abscission

2.2.1 Genotypic and phenotypic variation influences on senescence and abscission

There are distinct differences in needle abscission characteristics among conifer species. When different species of potential Christmas trees, such as, Atlantic white cedar (*Chamaecyparis thyoides* L.), Arizona cypress (*Cupressus arizonica* var, glabra), eastern white pine (*Pinus strobus* L), Leyland cypress (*Cupressocyparis leylandii* A. B. Jacks & Dallim), and Virginia pine (*Pinus virginiana* L) were analyzed for their post harvest qualities, it was found that the hydration, rehydration, and change in mass characteristics differed considerably among species (Hinesley and Snelling 1997). Similarly, Chastagner and Riley (2003) reported that Nordmann fir (*Abies nordamnniana*) trees had high quality foliage and better needle retention characteristics than Noble fir (*Abies procera*) trees while Bates et al. (2004) found Fraser firs (*Abies fraseri*) to have superior post harvest qualities such as longer drying time, longer needle retention, and lasting color when compared to Canaan firs (*Abies intermedii*). A list of superior qualities and the corresponding ratings for selected species can be seen in Table 1.

Table 1: Selected characteristic ratings of species commonly used as Christmas trees (Johnson 1996). Ratings range from A (representing an excellent score) to F (representing a very poor score)

Species	Fragrance	Color	Twig stiffness	Shipping qualities	Pest resistance	Needle retention
White pine	B	B	C	A	D	A
Scotch pine	C	A – F	A	C	F	A
Virginia pine	C	B – F	A	C	E	D – F
Austrian pine	C	B	A	F	D	A
Fraser fir	A	A	D	A	B	B
Balsam fir	A	A	D	A	B	B
White fir	B	B	C	A	B	B
Douglas fir	B	A	D	A	B	B
Norway spruce	C	C	C	B	D	E
Blue spruce	C	A	A	D	D	C
White spruce	E	B	B	B	C	D

Kubiske et al. (1990) studied the intraspecific influence of Douglas fir (*Pseudotsuga menziesii*) trees grown at Pennsylvania and west coast regions and concluded that coastal trees had poor post-harvest qualities and withstanding abilities to sub-freezing temperatures than the Pennsylvania grown trees. Similar intraspecific variations in the

needle longevity of several evergreen species were observed by Nebel and Matile (1992) and Jalkanen et al., (1995). Chastagner and Riley (2003), from their experiments with Canaan fir, found that needle retention is a highly hereditary characteristic and suggested that breeding strategies could help in identifying genotypes with superior needle retention traits.

As senescence is a key degenerative process that controls the post harvest quality of all plant systems, Arabidopsis has been established as a model plant system to elucidate the underlying molecular mechanisms and physiological processes of senescence. Lim and Nam (2005) studied the molecular aspects of senescence in Arabidopsis and revealed that senescence is a genetically controlled degenerative process. They have highlighted the potential for screening genotypes to ensure better post harvest quality in plant systems. Woo et al., (2004) found that mutants of Arabidopsis, such as ore1, ore2 and ore3 were tolerant to oxidative stress instigated by senescence processes. These findings indicate a potential for existence of genotypes with superior tolerance to senescence reactions and consequentially longer needle retention capabilities. However, the knowledge on genetic make up of ecotypes in governing the senescence processes in Christmas trees are very limited. Studies investigating genotypic influences on the senescence processes would provide opportunity to identify superior genotypes with better post harvest qualities.

2.2.2 Post-harvest storage conditions influence on needle abscission

Environmental conditions before and after harvest may have a significant bearing on the post harvest quality of Christmas trees. Vapor pressure deficit, temperature, and light intensity are critical factors that could radically affect the post harvest qualities of Christmas tree. Jalkanen et al. (1995) found that needle longevity in *Pinus sylvestris* was negatively correlated with summer temperatures. It was concluded that high temperatures increased vapor pressure deficit in atmospheric air, escalated transpiration losses, and subsequently increased the xylem pressure potential in the trees. Other contributing environmental factors include wind and snow cover, where it has been shown that conifer plants covered with snow had less leaf mortality than those exposed to abrasive wind forces during winter periods (Hadley and Smith 1983).

Abundant evidences exist for longer needle longevity in spruce, pine and fir grown under higher elevations and higher latitudes (Reich et al. 1996; Schoettle and Fahey 1994). For instance, the needle longevity of *Pinus contorta* increased by 3.5 years for an elevation difference of 400m under natural conditions (Schoettle 1990). Reich et al. (1996) suggested that the enhanced needle longevity is a consequence of decreasing growth rates and photosynthetic rate of evergreen species under resource limited or stress imposed conditions. Conservation of nutrients such as, sugars, and starches are also suggested to contribute to enhanced needle longevity (Beck et al. 2004). However, needle longevity decreases by approximately 27-30% when trees are supplied excess N and K (Balster and Marshall 2000; Amponsah et al. 2005).

Dehydration triggers senescence and it may affect post harvest needle retention unless transpiration is controlled. Hinseley and Snelling (1991) studied several species for their post-harvest qualities and they found that Atlantic white cedar dried rapidly after harvesting but failed to rehydrate completely on stands, resulting in rapid loss of needles mass. Weather conditions, such as temperature, wind, or humidity, during and immediately after harvesting of trees can also deteriorate the post harvest quality significantly. Loss of moisture from trees harvested during warm temperature hours can be rapid which could lead to heavy needle losses (Hinesley and Chastagner 2004). It was suggested that baling of trees with moisture proof covers during storage and transportation would decrease the moisture loss and hence indirectly improve the needle retention qualities, though increases in temperature could prevent this from being a viable option. Transportation inside refrigerated containers has been found to decrease moisture loss due to decreased vapor pressure deficit. Controlled atmospheric storage is another alternative, usually involving storage at cool temperatures (approximately 5°C) with modified O_2/CO_2 ratios, that has been known to significantly reduces senescence in fruits, vegetables, and flowers (Gorny et al. 2002). While detailed information on the post harvest storage and transport conditions is not available for balsam fir, controlled atmospheric storage has been shown to effectively reduce the rates of oxidation,

transpiration, respiration, and ethylene production in order to delay senescence (Gorny 1997).

2.2.3 Cold acclimation influence on needle abscission

Cold hardening or frost hardiness is environmentally induced with photoperiod controlling the first phase of hardening and low temperature controlling the second, dormant phase of hardening (Greer et al. 2000). Recently, freeze hardening of plants during late fall and early winter was found to predispose plants towards better needle retention, though it was noticed that the effect was greatly diminished in genotypes with high initial needle retention (MacDonald and Lada 2008). Several biochemical and physiological changes were found to occur prior to, and after cold hardening. These processes include changes in membrane composition and fluidity (Quinn 1985), accumulation of sugars, starches (Beck et al. 2004), cold-induced proteins (Close 1997) and boosting of free-radical scavenging potentials of cell (Tao et al. 1988). Accumulation of sucrose and glucose during autumn months was found to be a requirement for frost hardiness during winter. Physiologically, drought tolerance and cold hardiness were found to co-occur in Norway spruce (Blodner et al. 2005). This phenomenon indicates that cold hardiness may potentially render drought tolerance and consequently influence the needle retention characteristics.

Although the exact mechanism for the co-existence of tolerance to cold temperatures and drought is unknown, evidence exists that cold hardening enhances needle retention qualities of many conifers. In fact, delay in the onset of cold temperatures can result in ineffective cold hardening and subsequently result in poor needle retention (Chastagner and Riley 2003). Although research findings exist on several physiological mechanisms of cold hardening on carbohydrate metabolism, there is limited information available on the direct influence of cold hardening on needle retention. Rajasekaran and Thiagarajan (2006) conducted an experiment comparing the effects of artificial cold hardening and natural cold hardening on needle retention in balsam fir. It was found that artificial cold hardening at 0°C and 5°C in a one year old, root-intact balsam fir seedling facilitated needle retention better than the root-detached seedling suggesting that an yet unknown

factor could facilitate cold acclimation leading to enhanced needle retention. Seedlings that were exposed to temperatures below 0°C, despite gradual acclimation, resulted in needle loss. However, balsam fir that were exposed to natural cold acclimation, that is, grown in an orchard over the autumn months, received benefits to needle retention even in subzero temperatures (MacDonald and Lada 2008).

2.2.4 Dehydration influence on needle abscission

Water deficit conditions and other stresses that cause a deficiency in water, including salt and cold temperatures, can also promote abscission as a result of a decline in the growth and vigour of the plant. Abscission due to drought stress is a common occurrence in deciduous species grown in tropical and subtropical climates, and is necessary to reduce the rate of transpiration. As water deficit develops, there is a rapid decline in leaf expansion concomitant with a reduction in the rate of photosynthesis (Jones et al. 1980; McCree 1986) mainly due to stomatal closure (Chaves 1991). Stomata regulate gas exchange rate in leaves and thereby transpiration and photosynthesis and as a consequence may also promote leaf senescence and abscission (Thimann et al. 1982).

Post harvest water relations have been studied extensively in many root-detached conifers (Bates et al. 2004). Water relations are traditionally monitored either by the percent moisture content or xylem pressure potential of the branches. The XPP is an indicator of tension at which water is held in the xylem conduits and values above certain threshold, depending on the species, can induce water stress, which may perhaps accelerate needle loss and lead to discoloration, with a tendency to defoliate under rehydration (Hinesley and Snelling 1991). The damage threshold moisture potentials in relation to needle loss have been well documented in several pine and spruce species, which generally range from -3 MPa to -4 MPa, and the threshold levels may vary between species (Heiligmann and Brown 2005; Hinesley and Snelling 1997; Chastagner and Riley 2003).

It is important to note that although hydration can be a factor in accelerated needle loss, hydration alone cannot retain needles. Bates et al. (2004) studied the effect of hydration in Canaan and Fraser fir and concluded that XPP values did not relate with needle fall

characteristics. Instead, it was proposed that a multitude of factors govern needle retention and color characteristics. Similar results have been found by Rajasekaran et al. (2005a), where XPP values were found to vary significantly between and within *Abies balsamea* populations. However, there was no relationship between needle fall and XPP. Also, Rajasekaran and Thiagarajan (2006) found that while XPP is fairly consistent among the treatments that retained or lost needles, water consumption has been high with the ones that lost large amounts of needles indicating that the needle loss is triggered by factors other than just the biophysical factor such as XPP.

Water use by a plant also indicates the health status of the tree. Proper hydration ensures adequate supply of moisture to meet the demand of transpiration and basic physiological functions. In general, a tree weighing around 5 kg can consume approximately 1L of water per day (Rajasekaran et al. 2005a). However, despite a large initial uptake, constant consumption of water indicates normal water status maintenance activity. Abnormal water status can be observed in those trees with very low water consumption, which indicates embolism that may lead to irreversible drying. In addition, excessive water consumption is also indicative of enhanced transpiration or poor stomatal control, which may perhaps be due to low synthesis, supply, or release of ABA (Rajasekaran et al. 2005b).

While water flux is important to prevent dehydration induced senescence and needle drop, hydration alone cannot prevent needle drop suggesting that a lack of some root derived factor that prevents or delays senescence. For example, cytokinins are believed to be synthesized in meristematic tissues such as root apices then transported to needles via the xylem (Srivastava 2002). A decline in cytokinin supply can increase ethylene sensitivity, which may initiate the senescence and continued increase in ethylene may trigger needle abscission (Meir et al. 2007; 2010) . However, there is no information on the ethylene dynamics in root detached balsam fir. It is not known whether ethylene is the triggering factor in needle drop.

2.3 Role of plant growth regulators in senescence and abscission

2.3.1 Abscisic acid

Abscisic acid (ABA) was first discovered and isolated by Okhuma et al. (1963). Since ABA was thought to play a role in abscission, the isolated compound was called abscisin II. Simultaneously, Cornforth et al. (1965) isolated a dormancy hormone in angiosperms. Comparison of the infrared spectrum revealed that abscisin II and the dormancy hormone were identical, and the compound was called ABA.

At the cellular level, there is evidence that biosynthesis of ABA occurs in the chloroplast and in roots (Milborrow and Lee 1998). Both types of root tissue, stele and cortex, possess an equal capacity to synthesize ABA even at water losses of 50% or more. The largest accumulation of ABA is in the root tips, likely due to low vacuolization of root tip cells with a high percentage of cytosol, the compartment where ABA is formed (Hartung et al. 2002). Chloroplast synthesized ABA can be loaded to the phloem and transported to the roots, where it may be stored or circulated through the xylem vessels (Jeschke et al. 1997).

The first step in ABA synthesis is the combination and rearrangement of pyruvate and glyceraldehydes phosphate to give isopentenyl diphosphate, which can be elaborated into carotenoids (Milborrow 2001). Through oxidative cleavage the 40-carbon carotenoids can be broken down into a 15-carbon xanthoxin molecule. Xanthoxin is oxidized and desaturated at the 2' and 3' bond to form abscisic aldehyde, which is then oxidized to ABA (Fig. 3) (Schwartz et al. 2003). Catabolism of ABA occurs through the hydroxylation of the 8' position by ABA 8' hydrolase to yield an unstable intermediate, quickly rearranging to phaseic acid (Milborrow 2001). Regulation of ABA formation under stress is dependent on the type of plant tissue. There is a rapid and sudden increase (approximately 40 fold) in leaf tissue ABA (Milborrow 2001), a slow and progressive increase in root tissue ABA (Cornish and Zeevart 1985), and no increase in fruit tissue ABA under drought stress (Milborrow 2001).

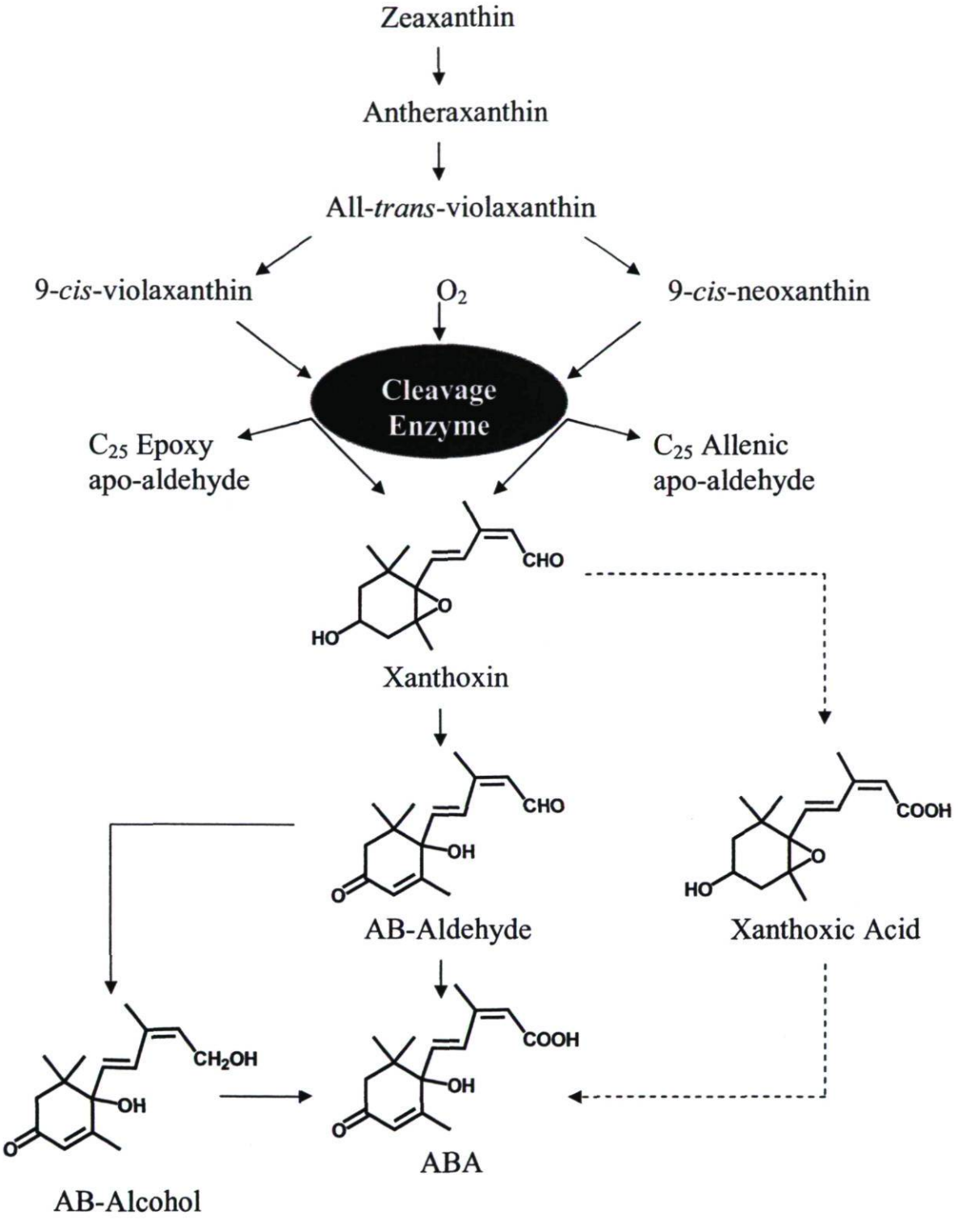


Figure 3: Biosynthesis of abscisic acid from carotenoid precursors in higher plants. Solid lines indicate known pathways or pathways with strong evidence, while broken lines indicate minor or speculative steps. (Cutler and Krochko 1999)

A primary role of ABA is the ability to regulate stomatal aperture, which involves the orchestration of ion channel activities at the plasmalemma and tonoplast (MacRobbie 1995). Closure of stomata is caused by low (10^{-7} M) concentrations of ABA and is initiated within 5 minutes. Upon withdrawal of the hormone supply, reopening also starts within 5 minutes. Gas analysis of leaves treated with ABA allows one to distinguish effects on the stomatal apparatus from inhibition of photosynthesis and to conclude that ABA acts on stomata directly (Cummins et al. 1971). The signal transduction pathway responsible for relaying the ABA signal to its target cation and anion channel (Blatt and Thiel 1993) seems to be preformed and comprised of ABA-receptors, protein kinases, and protein phosphatases (Pei et al. 1997).

With respect to leaf senescence and abscission, ABA is typically considered to accelerate the process. Cracker and Abeles (1969) found that low concentrations ($5x10^{-4}$ to $5x10^{-6}$ M) of ABA accelerated abscission and decreased the amount of mechanical force required to shed leaves of cotton explants. It was concluded that ABA increased the activity of cellulases, thereby promoting weakening of the cell walls in the abscission zone. It was later found that endogenous ABA increases at early stages in senescence, then decreases prior to abscission, which could support the theory that ABA triggers hydrolytic enzyme activity (Even-Chen and Itai 1975). However, in a long term experiment involving the daily spraying of 0.1 mM ABA onto wheat plants for 84 days, it has been noted that ABA does not promote leaf senescence or abscission under these conditions. Instead, ABA was found to actually increase leaf longevity (Hall and McWha 1981).

The bulk of the research suggests that ABA exhibits strong abscission-promoting activity in isolated tissue explants but is erratically active in whole plant assays (Addicott 1983; Flores and Dorffling 1990). A study by Suttle and Abrams (1993) was designed specifically to compare the effect of ABA on cotton explants to intact seedlings. Within 40h, complete abscission occurred in explants treated with ABA, while there was no effect on abscission in intact seedlings after single or daily application of ABA. The physiological bases for the attenuated activity of exogenous ABA in whole plants are

unknown, but have been ascribed to its inherent chemical and metabolic instability, limited translocation, or poor uptake (Addicott 1983).

There is strong evidence that ABA is also linked to ethylene in inducing senescence and abscission. Cracker and Abeles (1969) and Morgan (1984) found that application of exogenous ABA induced ethylene production, which has been linked to abscission. In addition, it was found that when ABA was used in conjunction with the ethylene antagonist silver thiosulfate, all abscission related effects of ABA were completely abolished (Suttle and Abrams 1993).

2.3.2 Cytokinins

Cytokinins are a group of phytohormones active in cell division, growth, and differentiation in the presence of auxins (Astot et al. 2000). The most widely distributed and biologically active cytokinin in plants is trans-zeatin (Srivastava 2002). In contrast, cis-zeatin has much less activity, and has been recently thought to be present mainly in tRNA as a ribosylated derivative, and there were only sporadic reports of its occurrence as a free cytokinin (Srivastava 2002).

Efforts to elucidate the biosynthetic origin of cytokinins in plants have been inconclusive. Early suggestions that tRNA degradation could be the major source of free, active cytokinins were disproved when calculations of tRNA turnover rates showed that a tRNA-independent de novo biosynthetic pathway also must be present in plants (Haberer and Kieber 2002). A major breakthrough was the discovery of a cytokinin biosynthetic enzyme in a slime mold (Taya et al. 1978). Cell-free extracts from slime mold can convert adenosine monophosphate (AMP) and dimethylallyl-pyrophosphate (DMAPP) to the free cytokinin isopentenyladenosine-5-monophosphate (iPMP), and the corresponding nucleoside isopentenyladenosine (iPA). Thus, it has been proposed that iPMP is also the primary cytokinin intermediate in plants, and zeatin cytokinins are formed by hydroxylation of iPMP and its derivatives (Fig. 4) (Letham and Palni 1983).

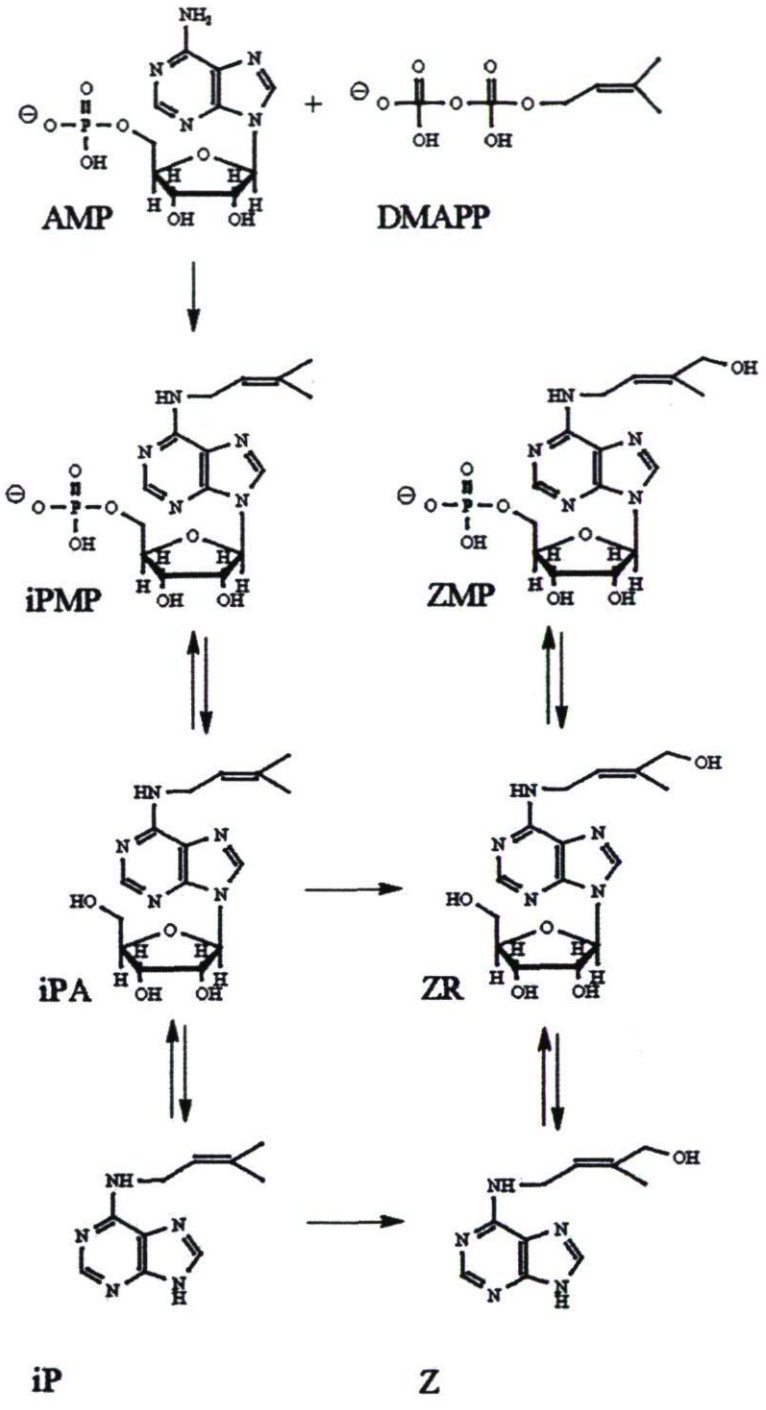


Figure 4: Biosynthesis of cytokinins. AMP = adenosine monophosphate; DMAPP = dimethylallyl-pyrophosphate; iPMP = isopentenyladenosine-5-monophosphate; iPA = isopentenyladenosine; iP = isopentenyladenine; ZMP = zeatinriboside-5'-monophospate; ZR = zeatinriboside; Z = zeatine (Astot et al. 2000)

In terms on senescence and abscission, cytokinins are believed to play an inhibitory role. As plants begin to senesce, cytokinin concentrations in the leaves and sap show a marked decrease (Nooden et al. 1990; Singh et al. 2006). In fact, Even-Chen et al. (1978) were able to detect significant decreases in cytokinins before any physical characteristics were observed to indicate senescence was occurring. Such results suggest that decreases in cytokinins could be a trigger in inducing senescence. Exogenous application of cytokinins on senescing tissue has demonstrated the ability to delay or reverse the process in some species, though the effect is less pronounced in a natural system (Adedipe and Fletcher 1971).

Roots are believed to be the major source of cytokinin synthesis (Chen et al. 1985). Treatments that injure or impair root functions, such as derooting, water deficit, or mineral deficiency promotes leaf senescence and abscission, possibly through decreases in cytokinins (Van Staden et al. 1988). As such, cytokinins are of particular importance when considering problems with needle senescence and abscission in the Christmas tree industry since part of the harvesting procedure involves removal of the roots. It is speculated that derooting of balsam fir trees could affect the translocation of cytokinins from the roots to the leaves similar to decreases in leaf cytokinin concentration when exposed to drought stress (Bano et al. 1993). Such a relationship could allow for exogenous cytokinin application to inhibit loss of chlorophyll and protein from senescing needles while stimulating chlorophyll production, chloroplast differentiation, and synthesis of chloroplast proteins (Fletcher et al. 1973; Axelos et al. 1984).

2.3.3 Auxins

According to Srivastava (2002), auxin is involved in nearly all aspect of plant growth and development, from embryo to adult reproductive plant. The processes that are regulated include pattern formation in embryo development, induction of cell division, stem and coleoptile elongation, apical dominance, induction of rooting, vascular tissue differentiation, fruit development, and tropic movements such as bending of shoots towards light or of roots toward gravity. Becker and Hedrich (2002) state that growth promotion by auxins is thought to take place by two mechanisms: 1) by promoting the

transport of H+ ions across cell walls, acidifying the membranes and causing pH-dependent enzymes to break the bonds between cellulose and microfibrils increasing their extensibility, and 2) by inducing transcription of specific mRNAs necessary for sustained growth.

Indole-3-acetic acid (IAA) is considered the principal auxin in plants, though several other naturally occurring indole derivatives are known to express auxin activity. (Srivastava 2002). Salisbury and Ross (1985) state that IAA is chemically similar to the amino acid tryptophan and it has since been established IAA is derived from tryptophan. There are two mechanisms known for the synthesis of IAA, both involve the removal of the amino group and the terminal carboxyl group from the side-chain of tryptophan (Sembdner et al. 1980). According to Salisbury and Ross (1985), the preferred pathway for most plant species involves donation of the amino group to another α-keto acid by transamination reaction to form indolepyruvic acid, and then decarboxylation of indolepyruvate to form indoleacetaldehyde. Finally, indoleacetaldehyde is oxidized to IAA (Fig. 5).

Our understanding of the role of auxins in senescence and abscission is conflicted. Some reports have suggested that auxins may be able to delay senescence. Both Sacher (1959) and Osborne and Hallaway (1969) found that exogenously applied auxins inhibited leaf protein degradation and, subsequently, senescence and abscission. Thus, it has been hypothesized that continuous auxin production is necessary to prevent leaf abscission (Osborne 1989). However, there are numerous cases in which auxins are not very powerful in their effects on senescence, and high concentrations have to be used compared with cytokinins. For instance, in oat leaves, IAA only retarded senescence at a concentration about 300 times greater than that of kinetin (Shibaoka and Thimann 1970). In some cases, auxins do not delay senescence and may even promote the process (Mishra and Gaur 1980). Further complications arise as auxin stimulates the production of ethylene when its concentration is only slightly above the physiological level, which can trigger senescence and abscission in some species (Thimann 1980). Endogenous

auxin content does not show a consistent pattern in the senescence of different species, so it is difficult to generalize about the role of auxin in senescence (Nooden 1988).

An interesting role for auxin in abscission may be found in its ability to modify ethylene sensitivity of a plant. In one study, IAA reduced pedicel abscission in soybean flowers, an effect which could not be overcome with a treatment with ethephon, an ethylene releasing compound (Oberholster et al. 1991). In a second study, treatment with auxin reduced abscission in rose pedicels and reversed ACC and ethylene induced abscission (Goszczynska and Zieslin 1993). Both these results indicate that auxin is strongly able to decrease the sensitivity of abscission zone cells to ethylene.

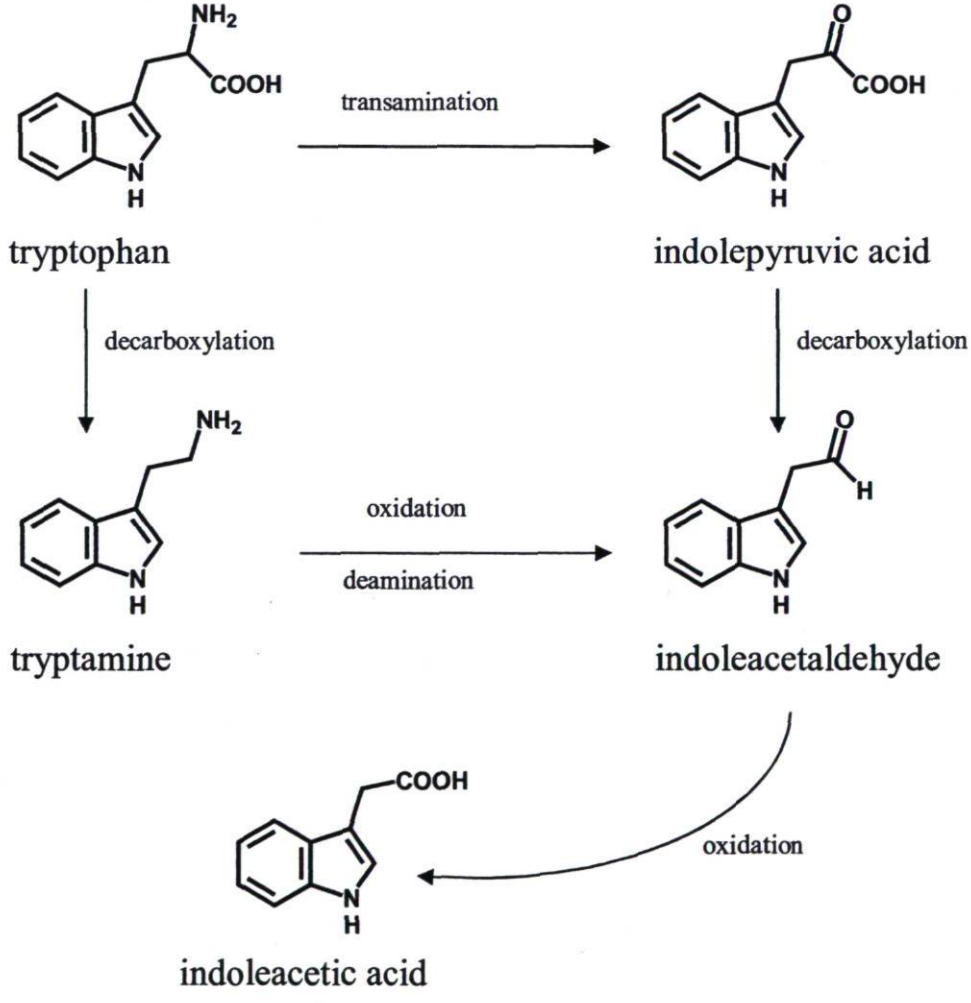


Figure 5: Biosynthesis of indoleacetic acid from tryptophan through two potential pathways

2.3.4 Jasmonic acid

Jasmonic acid (JA) and its derivatives are found to naturally occur in a wide range of higher plants and are believed to be derived from fatty acids (Meyer et al. 1984; Creelman and Mullet 1997). The biosynthesis of JA begins with linolenic acid. This fatty acid is converted to 13-hydroperoxylinolenic acid by lipoxygenase. 13-hydroperoxylinolenic acid is a substrate for allene oxide synthase and allene oxide cyclase resulting in the formation of 12-oxo-phytodienoic acid. Following reduction and three steps of oxidation, JA is formed (Fig. 6). JA can be catabolized to form MeJA and numerous conjugates and catabolites that may have biological activity (Hamberg and Gardner 1992). The accumulation of JA in plants in response to wounding, or treatment with elicitors and systemin, can be blocked using inhibitors of lipoxygenase (Doares et al. 1995). Therefore, increases in JA level mediated by these inducers results from *de novo* synthesis rather than release from JA conjugates.

JA is known to regulate a variety of processes, including root growth, tendril coiling, and plant defense from insects and pathogens (Andresen et al. 1992). However, JA is also involved in senescence. Both oat and barley leaves demonstrated accelerated senescence after treatment of 45μM JA (Weidhase et al. 1987). It was also observed that treatment with JA resulted in the synthesis of new proteins. Muller-Uri et al. (1988) proposed that the newly synthesized proteins triggered by JA were directly involved in triggering senescence. However, it seems unlikely that this is the case, as treatment with cytokinins can reverse JA-induced senescence despite continued synthesis of those proteins (Weidhase et al. 1987). The current view is that JA is part of the signal transduction pathway, but is not a direct cause of senescence or abscission (Sembdner and Parthier 1993).

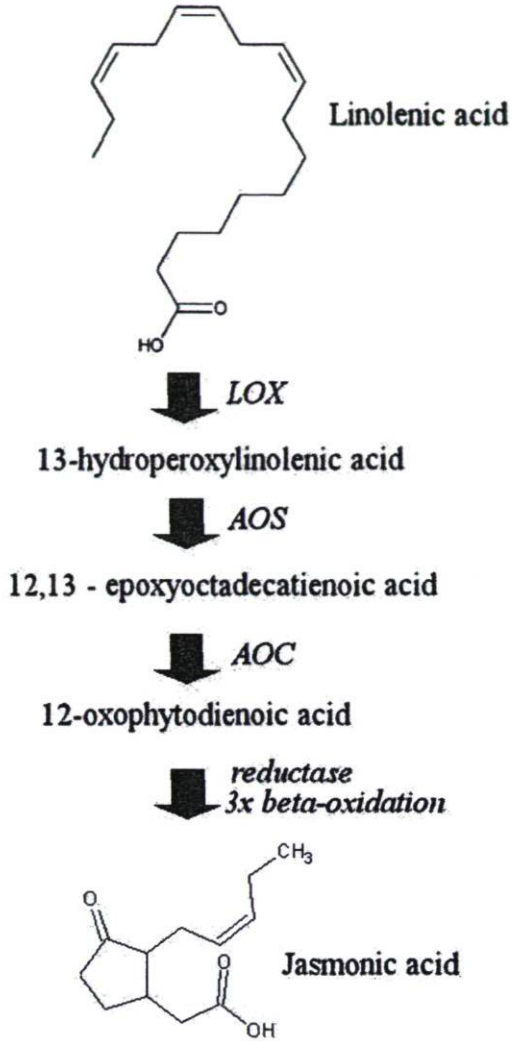


Figure 6: Biosynthesis of jasmonic acid from linolenic acid modified from Creelman and Mullet (1997). LOX: lipoxygenase, AOS: allene oxide synthase, AOC: allene oxide cyclase

2.3.5 Ethylene

Ethylene, the simplest unsaturated hydrocarbon, regulates many diverse metabolic and developmental processes in plants. The history of its discovery as a signal molecule or plant hormone was described by Abeles et al. (1992). In the nineteenth century, several articles appeared reporting that leaks in illuminating gas caused premature senescence and defoliation of plants in greenhouses and of trees near gas lines. In 1901, the Russian plant physiologist Neljubov proved that the active principle in illuminating gas was ethylene and was credited with the discovery that ethylene is a biologically active gas. Chemical proof that plants produce ethylene was later provided by Gane (1934), who

analyzed the gases released by 60 lbs of ripening apples. Thus, the stage was set to investigate the synthesis and function of ethylene as an endogenous signal molecule in plants.

The ethylene biosynthetic pathway was elucidated primarily by Yang and Hoffman (1984). Ethylene is derived from the amino acid, methionine, which is converted to S-adenosyl-methionine (AdoMet) by AdoMet synthase. AdoMet serves as an intermediate in a variety of synthetic pathways, including polyamines. AdoMet can be converted into 1-aminicyclopropane-1-carboxylic acid (ACC) through ACC synthase, and is the first committed step in the production of ethylene (Adams and Yang 1979). The final step converts ACC to ethylene with the enzyme ACC oxidase in the presence of oxygen (Fig. 7). Notice that the product of ACC synthase, besides ACC, is the formation of 5-methylthioadenosine (MTA), which preserves the methylthio group for production of new methionine. Thus, high rates of ethylene biosynthesis can be maintained even when the supply of methionine is low (Miyazaki and Yang 1987).

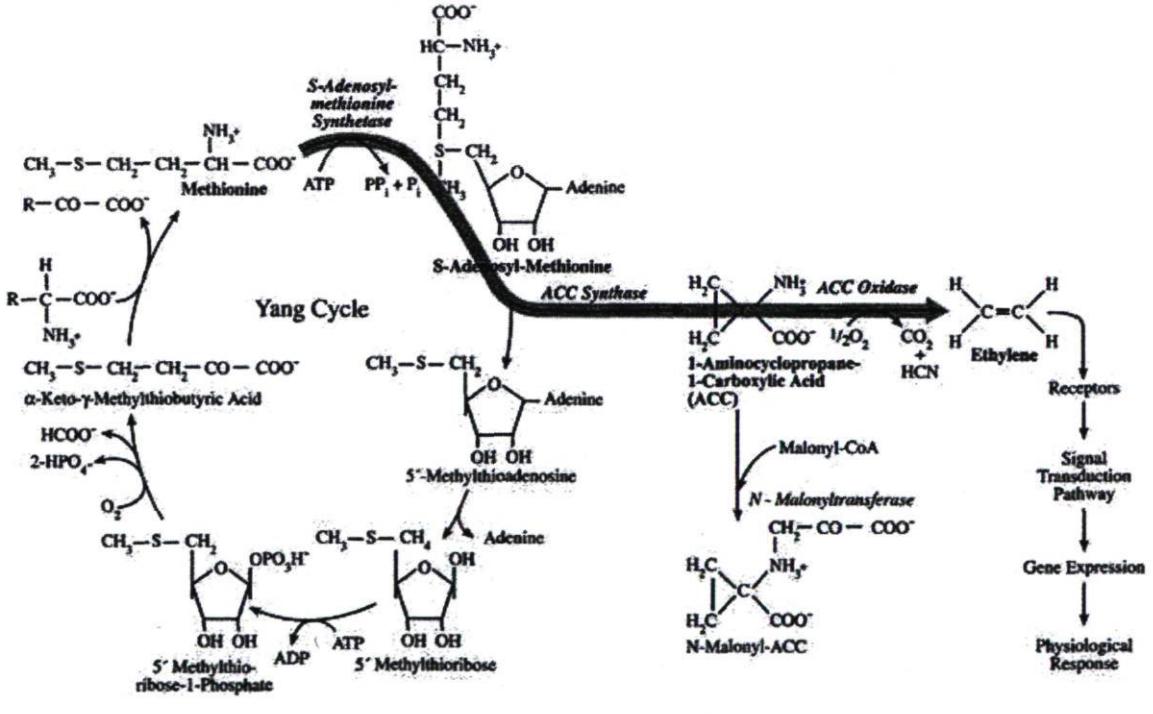


Figure 7: The Yang cycle and biosynthesis of ethylene from methionine (highlighted pathway). Modified from Yang and Hoffman (1984) and Schaller and Kieber (2002).

Almost all biotic and abiotic stresses seem to elucidate some ethylene response. However, the role of ethylene in plant stress is still in development (McMichael et al. 1972; Munne-Bosch et al. 2004, McManus 2008). For example, it has been shown that drought increases ethylene production by 120-300% in jack pines and 110% in white pines (Rajasekaran and Blake 1999; Islam et al. 2003). Similar results were observed from biotic stresses in the forms of pathogens, where Fuhrer (1985) observed increased levels of ethylene production. In several instances, stress symptoms have been enhanced, reduced, or not affected by ethylene. In other words, ethylene seems to mediate defense in some situations and suppress it in others (Bleecker and Kende 2000). Beltrano et al. (1997) suggests that an increase in ethylene production due to stress makes logical sense, as free radicals associated with stress accelerate the conversion of ACC to ethylene. In addition, it has been clearly elucidated that ethylene causes membrane damage by lowering antioxidant concentrations and shifting the oxidation balance (Munne-Bosch et al. 2004).

Ethylene has a profound effect on plant growth and development, though is usually associated with fruit ripening. Fruit ripening is a sequence of biochemical events resulting in loss of chlorophyll, formation of pigments, flavors, and aromas, softening of the flesh, and eventual abscission of the fruit. Other senescence processes regulated by ethylene are fading of flowers and abscission of petals. Fading of flowers shares many similarities with ripening of climacteric fruits. Both fruit ripening and flower fading can now be delayed by reducing synthesis or sensitivity to ethylene with biotechnological methods (Bleecker and Kende 2000).

Ethylene also has a prominent role in the senescence and abscission of leaves. For example, McGlasson et al. (1975) have shown that, in tomato leaf segments, ethylene production and respiration decrease and then increase as in the climacteric in flowers and fruit. More detailed studies have revealed that a surge of both ethylene production and respiration either coincided with or preceded the rapid phase of chlorophyll degradation in bean, tobacco, and sugar beet leaf discs (Aharoni et al. 1979; Gepstein and Thimann 1981). Several approaches have helped to confirm the effect of ethylene. Use of ethylene

inhibitors, such as silver ions or AVG has proven to delay senescence and reduce abscission by 40% (Beyer 1976; Rath et al. 2006). In addition, studies with mutants lacking enzymes to synthesize ethylene or lacking ethylene receptors have shown delayed senescence when compared to wild type plants (Picton et al. 1993; Bleecker et al. 1988).

More recent studies of genetic engineering have added strength to the involvement of ethylene in senescence. Two strategies have been used: genetic modification to inhibit ethylene synthesis and modification to reduce ethylene sensitivity. In tomatoes, an antisense construct of cDNA encoding ACC oxidase was used to reduce ethylene formation (Hamilton et al. 1990). In this case, ripening of the transgenic fruits was retarded, and storage life was extended. A similar approach was followed by Oeller et al (1991), who transformed tomato plants with the antisense construct of a cDNA coding for ACC synthase. Ethylene production in transgenic fruits was inhibited by 99.5%, and ripening was suppressed. Application of ethylene restored normal ripening. Ethylene synthesis and ripening could also be inhibited by removing ACC from the precursor pool. This was achieved by expressing a bacterial ACC deaminase gene in tomatoes (Klee et al 1991). In addition, it is known that the etr1 mutation confers ethylene insensitivity in Arabidopsis. When expressed in tomato and petunia, etr1 causes significant delays in fruit ripening, flower fading, and flower abscission (Wilkinson et al 1997).

From what little work has been done regarding the role of ethylene in needle retention in conifers, it appears that ethylene induces needle abscission in much the same manner it induces fruit, petal, and leaf abscission in other species. ACC and ethylene increase linearly with increasing needle damage in Norway spruce and silver fir (Wilksch et al. 1998) in a natural system. Such a relationship suggests that ethylene may have a direct role in needle loss and could be used as a bioindicator of needle health. In addition, it was shown that increases in ethylene production in silver fir trees was associated with chlorophyll degradation, followed by premature abscission of needles (Fuhrer 1985).

The mode of action of ethylene has been well elucidated in literature. Ethylene has been shown to act directly on the abscission zone by promoting the production of cell wall

hydrolytic enzymes, such as cellulase (Sexton and Roberts 1982; Tucker et al. 1988). One such enzyme was determined to have an isoelectric point of 9.5. The mRNA of the 9.5 cellulase was found to accumulate with exposure to ethylene and to decrease when ethylene was removed or inhibited (Tucker et al. 1988). Similarly, ethylene induced abscission was shown to coincide with polygalacturonase (PG) activity (Taylor et al. 1990). Ethylene has also been shown to enhance growth in abscission zone cells, but not neighboring cells implying that ethylene may have a direct role in weakening of the cells walls and the mechanical forces required to facilitate abscission (Wright and Osborne 1974).

Of interest is the antagonistic relationship between ethylene and auxin; IAA is considered to inhibit abscission while ethylene is thought to promote it. The general rule portrays that provided the flux of IAA to the abscission zone is maintained, then cell separation is inhibited and abscission does not occur (Addicott 1982; Sexton et al. 1985). Thus, anything that can affect auxin transport can, in turn, affect ethylene sensitivity and abscission. However, ethylene itself is a potent inhibitor of auxin transport and may modify sensitivity to ethylene (Beyer and Morgan 1971). Furthermore, excessive concentrations of auxin can stimulate ethylene production and thus accelerate abscission (Sexton and Roberts 1982).

The interaction between auxin and ethylene has allowed for the construction of a three stage model to describe abscission (Fig. 8). During stage I, abscission is inhibited by auxins and ethylene treatment may fail to induce abscission. As auxin flux to the leaf declines, its sensitivity to ethylene increases. When the abscission zone reaches stage II, the abscission zone cells will be sensitive to ethylene. Exposure to ethylene at this point marks the start of stage III, where ethylene can trigger production of cellulase and other cell wall hydrolytic enzymes, turgor expansion of abscission zone cells, and, ultimately, abscission (Brown 1997). Ethylene is believed to accelerate the process by decreasing the time spent in stage I through disruption of auxin transport (Beyer 1973). A similar effect could be observed from water stress, mechanical stress, or derooting (Brown 1997).

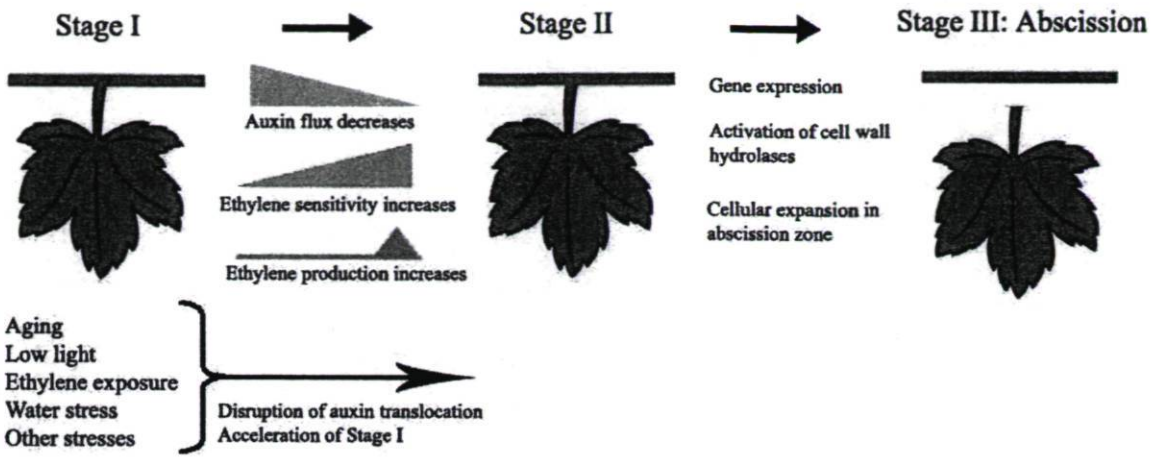


Figure 8: Model of auxin and ethylene interaction to stimulate abscission. As auxin flux decreases, ethylene sensitivity increases, resulting in abscission. Stresses or ethylene treatment can accelerate the process through disruption of auxin transport (modified from Brown 1997).

2.3.6 Reactive oxygen species

For both plants and animals, the development of free radicals, especially under stress conditions, is an unavoidable aspect of life (Romero et al. 2004). A free radical is a highly reactive compound with an unpaired electron in its outermost shell. Free radicals will quickly react with nearby compounds or molecules to achieve a stable configuration. Often the molecule that reacted with the free radical becomes a radical itself, beginning a cascade of reactions. Thus, the chain reaction continues and can be thousands of events long (Goldfarb 1999).

The term "reactive oxygen species" (ROS) has been introduced to describe radicals, such as the superoxide anion ($O_2^{\bullet}$) and the hydroxyl radical ($OH^{\bullet}$), as well as potential sources of radicals, such as singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2) (Mittler 2002). One source of ROS is from normal metabolic processes, such as photosynthesis or respiration, while another can be from external factors like drought or osmotic stress due to the onset of NADPH oxidases (Dizdaroglu et al. 2002; Mittler 2002). Normally, the production of ROS in cells is low at approximately 240 $\mu M\ s^{-1}$ $O_2^{\bullet}$ and a steady state of 0.5 μM H_2O_2 in chloroplasts. However, stresses such as drought, salinity, temperature, heavy metals, ultraviolet radiation, and ozone that disrupt the cellular homeostasis of

cells can raise production of ROS to 720 μM s^{-1} $O_2^{\bullet}$ with a steady state of 5 to 15 μM H_2O_2 in chloroplasts (Mittler 2002).

Due to the prevalence of ROS during stress-induced abscission, it has been speculated that they are a signal or mediator for abscission (Sakamoto et al. 2008b). In an experiment with petioles from pepper were separated at the abscission zone, it was found that application of H_2O_2 enhanced cellulase expression and abscission. Conversely, application of ROS inhibitors prevented cellulase expression and delayed abscission (Sakamoto et al. 2008a). Other experiments found that certain synthetic and natural antioxidants delayed senescence and abscission in tomato plants as a seed preconditioning treatment, though the delay was not confirmed to be through sequestering of ROS (MacDonald and Lada 2008; MacDonald et al. 2009a; 2009b).

2.4 Mitigating senescence and abscission

2.4.1 Antioxidants

The potential for ROS to be generated during abscission prompted investigation into the role of certain antioxidants in mitigating senescence. One recent study in tomato plants screened various antioxidants (MacDonald et al. 2009b). They found that Ambiol, β-carotene, and ascorbic acid were very effective at maintaining normal physiological function under severe water deficit.

β-carotene (Fig. 9) is the precursor of vitamin A, and is a major carotenoid present in sweet potatoes, carrots, and mango (Ben-Amotz and Fishler 1998; Pott et al. 2003). The addition of β-rings decreases pigmentation, giving β-carotene its characteristic orange pigmentation. Of the carotenoids, β-carotene is the most well known and has been well established to have antioxidant properties. However, Burton and Ingold (1984) caution that the antioxidant properties of β-carotene only exist at the partial pressure of oxygen in air (and most physiological systems). At higher oxygen concentrations it tends to take on an autocatalytic pro-oxidation effect.

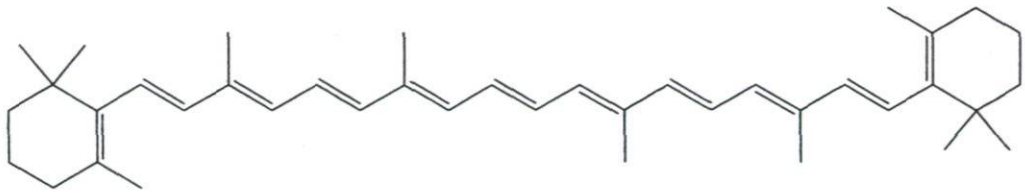


Figure 9: Chemical structure of β-carotene as shown in Howitt and Pogson (2006)

Ascorbic acid (Fig. 10) is a natural antioxidant synthesized in all plants and plant cell types, functioning as a primary antioxidant by scavenging hydrogen peroxide (H_2O_2), as well as a secondary antioxidant by maintaining the α-tocopherol pool scavenging radicals on the inner regions of cellular membranes (Wheeler et al. 1998; Alscher and Hess 1993). Though often only considered within the framework of antioxidant defenses, ascorbic acid has also been linked to cell growth, cell division, as well as acting as a cofactor for many enzymatic processes (Smirnoff 1996).

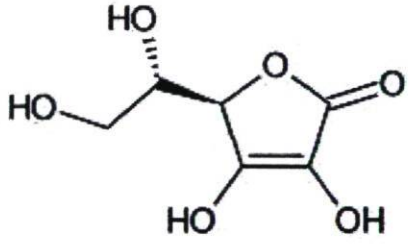


Figure 10: Chemical structure of ascorbic acid, as shown in Wheeler et al. (1998)

Ambiol (Fig. 11), or 2-methyl-4-[dimethylaminomethyl]-5-hydroxy-benzimidazole, was derived from 5-hydroxybenzimidazole using electrophilic substitution in Russia in the early 1980s (Smirnov et al. 1985). Ambiol was of initial interest due to its ability to promote growth in a variety of species, but has since been widely established to help plants survive stress induced senescence (Fig. 11) (Vichnevetskaia 1999; Darlington et al. 1996; Rajasekaran and Blake 2002; Rajasekaran et al. 2004; Rajasekaran et al. 2005b). The most recent work has been on drougt-induced senescence in tomatoes (MacDonald et al. 2008; 2009a; 2009b; 2010a).

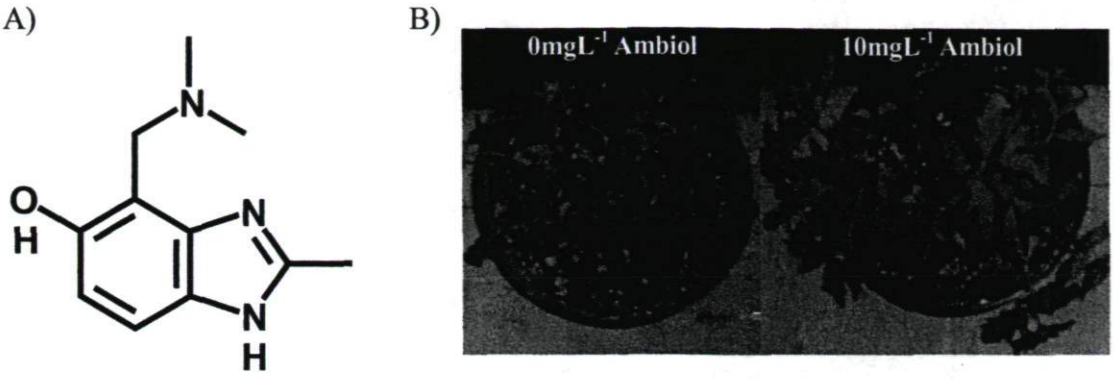


Figure 11: (A) Chemical structure of Ambiol, a synthetic antioxidant with antistress and plant growth regulating properties; (B) Ambiol mitigating effect on drought-induced senescence (MacDonald 2006)

To date, none of the aforementioned antioxidants are known to have been tested directly on needle abscission of balsam fir. It seems plausible that those antioxidants could provide a natural solution to needle abscission and be of interest to industry and consumers. However, from a physiological stand point, there are severe limitations to using antioxidants to study needle abscission. The most prominent limitation is that it is not known exactly how these antioxidants delay senescence and abscission. For example, Rajasekaran and Blake (1999) found that Ambiol inhibited ethylene evolution in Jack pine, but it is unknown whether Ambiol inhibited ethylene synthesis directly or indirectly. Other technologies to delay abscission must be considered.

2.4.2 Aminoethoxyvinyglycine

As ethylene has been shown to induce abscission in a number of species, there are many abscission mitigating technologies available which reduce or eliminate ethylene activity (Khan 2006). Perhaps the most common is silver thiosulfate, which was first used in cut flowers by Veen and van de Geijn (1978). Regrettably, silver is a heavy metal that persists in soil, may be a hazardous water pollutant, and is potentially harmful to humans (Khan 2006). Such environmental concerns regarding use of a heavy metal in horticulture have prompted research into alternative means of ethylene control.

Several non-toxic ethylene inhibitors have been discovered, though one of the most widely used is an organic molecule, AVG (Fig. 12). AVG is a derivative of the antibiotic rhizobitoxine and strongly inhibits the production of ethylene in various plant tissues (Lieberman 1975). For example, AVG has been shown to delay ripening in pears and apples (Ness and Romani 1980; Capitani et al. 2002). AVG has also demonstrated the ability to delay abscission of various plant organs (Rath et al. 2006).

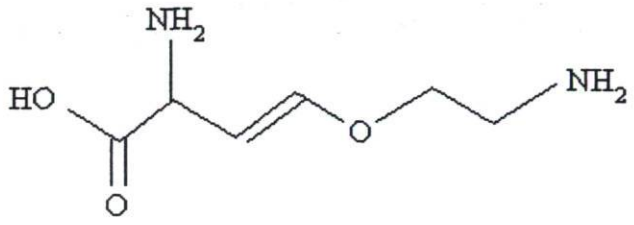


Figure 12: Chemical structure of the ethylene inhibitor aminoethoxyvinylglycine.

AVG is so widely used for physiological and horticultural properties because its mode of action is so well known. Under normal circumstances the biosynthetic precursor of ethylene, ACC, is produced by a pyridoxal phosphate-dependent enzyme, ACC synthase. ACC synthase catalyzes the conversion of AdoMet to ACC, which is the rate limiting step (Yang and Hoffman 1984; Adams and Yang 1979). AVG inhibits ACC synthase, thereby negating ethylene synthesis (Adams and Yang 1979; Huai et al. 2001; Capitani et al. 2002). There is also emerging evidence that some of the physiological effect of AVG may also be due to its ability to alter protein synthesis (Saltveit 2004).

Extensive knowledge of how AVG interrupts ethylene synthesis has allowed AVG to be used to study the participation of ethylene in bud break (Pereira-Netto, 2001), dry matter partitioning in rice (Mohapatra et al., 2000), fruit ripening (Clayton et al., 2000 and Wang and Dilley, 2001), fungal pathogenesis (Robison et al., 2001), nodulation in legumes (Mann et al., 2001 and van Spronsen et al., 2001) and response to chilling stress (Hong and Gross, 2000). Ideally this same knowledge will allow AVG to be useful to study the role of ethylene in needle abscission of balsam fir.

2.4.3 1-methylcyclopropene

Several organic compounds have been discovered relatively recently which will competitively bind to an ethylene receptor and, consequently, negate abscission. One of the earliest organic compounds discovered was diazocyclopentadiene (DACP) (Sisler and Blankenship 1993a). DACP alone is a weak inhibitor of ethylene responses, but upon irradiation with visible light several much more active compounds are released which inhibit ethylene effects in several species (Sisler and Blankenship 1993b). One of those products is 1-MCP.

1-MCP is a small planar molecule with a methyl group attached to the double bond (Fig. 13). Prolonged exposure to 1-MCP has been shown to delay abscission in a variety of flowers, even when exposed to high concentrations of exogenous ethylene (Serek et al. 1994a; Serek et al. 1996). In addition, 1-MCP reduces several other effects associated with ethylene, such as electrolyte leakage (Serek et al. 1995), xylanase production (Anderson et al. 1996), ACC oxidase transcripts (Leliévre 1997), and leaf yellowing (Müller et al. 1997). Numerous other studies are available to document the effects of 1-MCP on any number of species.

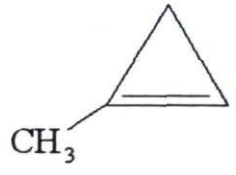


Figure 13: Chemical structure of the ethylene competitor 1-methylcyclopropene.

It has been well established that 1-MCP functions as a competitor for ethylene receptors, which prevents ethylene from binding to induce abscission. Lineweaver-Burk plots have been used to demonstrate the competitive action in banana and tomato ripening as well as flower abscission (Serek et al 1994b). Both ethylene and 1-MCP should bind to a receptor and withdraw electrons causing a ligand substitution to induce an action response (Sisler and Goren 1981; Sisler 1991). However, it has been proposed that after

substitution, ethylene can leave the receptor and would not be part of the active complex. Due to the high degree of ring strain, the binding of 1-MCP to the receptor would be so much stronger, that it likely could not leave the receptor. Thus, an active complex could not form (Sisler and Serek 1997). The strong evidence for 1-MCP as a receptor blocker makes it a prime candidate to study abscission in balsam fir.

3.0 OBJECTIVES

The overall goal of this research is to determine the role ethylene plays in needle abscission and to understand the physiological events occurring in balsam fir from harvest to abscission. The developed hypotheses focus on five major areas of needle abscission: (1) the effect of ethylene on needle abscission, (2) the effect of ethylene inhibition or receptor blockage on abscission, (3) enzyme activity associated with ethylene, (4) the effect of ethylene on different balsam fir genotypes, and (5) dehydration as a trigger for ethylene evolution. Specific objectives related to the aforementioned research areas are as follows:

1.a) To determine exogenous ethylene concentration and exposure time required to induce abscission in balsam fir.

1.b) To determine whether balsam fir branches synthesize endogenous ethylene prior to abscission and in sufficient quantities to induce abscission.

2.a) To determine the effect of ethylene inhibition with aminoethoxyvinylglycine on balsam fir branches in the presence and absence of exogenous ethylene

2.b) To determine the effect of ethylene receptor blockage with 1-methylcyclopropene on balsam fir branches in the presence and absence of exogenous ethylene

3.a) To develop a protocol to identify presence of cellulase in plant tissue and to quantify ethylene-induced cellulase activity in needle tissue

4.a) To identify the needle retention duration characteristics of genotypes available at the Debert Tree Breeding Center.

4.b) To determine whether genotypic differences in needle retention may be attributed to differences in ethylene evolution.

4.c) To determine whether genotypic differences in needle retention may be attributed to differences in ethylene sensitivity.

5.a) To determine the effect of exogenous ethylene on water use and xylem pressure potential in a branch.

5.b) To determine the effect of low temperatures or high humidity on branch water relations and abscission.

4.0 GENERAL METHODOLOGY

4.1 Sample collection

Samples were collected from a balsam fir tree stand at the Tree Breeding Centre, Department of Natural Resources, Debert, NS (45° 25' N, 63° 28' W). The Department of Natural Resource originally collected approximately 200 genotypes from across Nova Scotia based on preferred needle characteristics, such as color, orientation, and retention. Each genotype was grafted to existing root-stock. All genotypes were grafted and transplanted at the same time, thus the entire orchard is 15 years old.

Two preliminary experiments allowed for classification of genotypes based on needle retention characteristics of available balsam fir. A branch was taken from each genotype in autumn 2007, winter 2008, autumn 2008, and winter 2009 and screened for needle retention duration (MacDonald and Lada 2008; Veitch 2009, unpublished results). From these studies, genotypes were classified as low (0-20 days), moderate (21-40 days), or high (41-60 days) NRD.

The orchard is approximately 4 ha and consists of 21 rows, each with 75 trees. Each row is spaced 3 m apart and trees were spaced 2 m apart within a row. A branch cutting from 2 year growth, collected from the south eastern side of the tree from a height of 1.5 m served as a sample. Samples were immediately placed in a container with distilled water for transport to a growth chamber.

4.2 Growth chamber conditions

The protocol for growth chamber conditions and experimental set-up were similar to Rajasekaran and Thiagarajan (2006). Unless otherwise specified, all work was conducted in an environmentally controlled growth chamber at a 22°C-15°C day-night (16h-8h) temperature regime, 50% relative humidity, and 80 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light intensity from incandescent and fluorescent lights. Branches were initially weighed, and then placed in a 250 mL flask filled with 200 mL of distilled water, which was sufficient for an entire experiment. The neck of each flask was sealed with cotton gauze to prevent evaporation

and provide added stability to a branch. Finally, the entire apparatus was weighed and placed in an ethylene incubation chamber (Appendix I)

4.3 Response variables

4.3.1 Needle retention duration (NRD)

Each day, all fallen needles were collected and weighed. The primary measurement of abscission was NRD, which was initially defined as the number of days required for complete needle abscission. Previous experiments determined that the mass of needles on a 2-year old branch account for approximately 50% of the total fresh mass (MacDonald and Lada 2008; MacDonald et al. 2009c; MacDonald et al. 2010b). Thus, complete needle abscission was defined as having lost 50% of the initial fresh mass via abscission.

4.3.2 Average water use (AWU)

Average daily water use ($mL \cdot g^{-1} \cdot d^{-1}$) was calculated as the sum of the change in mass of an apparatus (excluding mass loss to due abscission) per unit fresh weight over the duration of the experiment or until a branch had lost all needles:

$$AWU = \frac{(InitialMass - FinalMass) + NeedleMass}{Time}$$

4.3.3 Xylem pressure potential (XPP)

The XPP of branches was measured using a Plant Moisture System Pressure Bomb (PMS Instrument Co., Corvallis, USA). A clipping from the primary branch was mounted upside down inside a pressure chamber and pressure was increased gradually at 0.01MPa per minute, until the water droplets appeared on the cut surface. The pressure required to release a water droplet was recorded. To minimize disturbance to a branch, XPP was recorded at the end of each experiment (unless noted otherwise).

4.3.4 Ethylene evolution

Ethylene evolution was determined by transferring each branch to a sealed 80 L container for 3 hours to allow ethylene to increase to a detectable concentration, which began each

day at 8:00 am. Ethylene concentration was measured with a portable ethylene analyzer (Levitt-Safety, Moncton, NB), sensitive to 0.1 $\mu L \cdot L^{-1}$. The ethylene analyzer was determined to be an appropriate method of analysis because it was portable, allowed for direct measurement from EIC, allowed for quick analysis of large number of samples, and was very reliable in the range of 0 ppm to 100 ppm ethylene (standardization of the portable ethylene analyzer is available in Appendix II). In addition, the ethylene analyzer is very specific to ethylene as the majority of interfering gases must be at extremely high concentrations (i.e. 10^5 ppm) to a 1 ppm ethylene detection error or exist as liquids in growth chamber conditions.

Ethylene evolution rates from a branch could be calculated by the following equation:

$$Ethylene\ evolution = \frac{Concentration \times 80L}{3h \times Mass}$$

Where ethylene evolution is reported in $\mu L \cdot g^{-1} \cdot h^{-1}$, concentration is $\mu L \cdot L^{-1}$, and mass is the fresh weight (g) of a branch. In each case, the day of peak ethylene evolution (DPE) was also recorded.

5.0 EFFECT OF ETHYLENE ON ABSCISSION IN BALSAM FIR

The following publications have been generated from content of Chapter 5:

MacDonald MT, Lada RR, Martynenko AI, Dorais M, Pepin S, Desjardins Y. 2009. Ethylene modulates needle abscission in root-detached balsam fir. HortScience. 44: 1142 (abstract).

MacDonald MT, Lada RR, Martynenko AI, Dorais M, Pepin S, Desjardins Y. 2010. Ethylene triggers abscission in root-detached balsam fir. Trees. 24: 879-886.

MacDonald MT, Lada RR, Martynenko AI, Dorais M, Pepin S, Desjardins Y. 2010. Ethylene exposure duration affects postharvest needle abscission in balsam fir (*Abies balsamea* L.). HortScience. IN PRESS.

5.1 Introduction

Little is known about needle abscission in balsam fir. It has been speculated that earlier harvest dates and warmer autumns result in accelerated post-harvest needle abscission (Mitcham-Butler et al. 1988; Chastagner and Riley 2003). MacDonald and Lada (2008) suggested that cold acclimation is necessary for superior needle retention in balsam fir, which likely explains why earlier harvest dates and warm temperatures adversely affect needle retention. However, there are no known studies that satisfactorily explain why needles should be shed after harvest or offer a potential mechanism for needle abscission in balsam fir. Hypotheses must be drawn from other species.

Likely the most studied cause of abscission is the phytohormone ethylene, ultimately derived from methionine (Met) (Yang and Hoffman 1984). Under normal circumstances, most Met is used for transmethylation, leading to nucleic acid, protein, lipid, and other metabolite modifications (Giovanelli et al. 1985). Met is also converted into S-adenosylmethionine (AdoMet), which serves as an intermediate in the biosynthesis of polyamines and is implicated in plant growth (Ravanel et al. 1998). Utilization of the methyl group of AdoMet in transmethylation is accompanied by recycling and regeneration of Met, a set of reactions designated as the activated methyl cycle. (Miyazaki and Yang 1987; Ravanel et al. 2004). However, Met will undergo a different path when plants are exposed to most stress conditions, such as water stress (e.g. Chen et

al. 2002), salt stress (e.g. Gomez-Cadenas et al. 1998), mechanical stress (e.g. Hiraki and Ota 1975), biotic stress (e.g. Fuhrer 1985), ozone stress (e.g. Sharma and Davis 1994), or warming after chilling (e.g. Lara and Vendrell 2003). In instances of stress, AdoMet is converted to 1-aminocyclopropane-1-carboxylic acid (ACC) which is subsequently oxidized to ethylene via the Yang cycle (Yang and Hoffman 1984). For example, drought stress increased ethylene production by 120 to 300% in jack pine and 110% in white pine (Rajasekaran and Blake 1999; Islam et al. 2003)

Although most commonly associated with ripening, ethylene plays a role throughout the entire life of a plant. Ethylene is a regulator of seed germination, seedling growth, leaf and petal abscission, organ senescence, and stress response (Abeles et al. 1992). In the broadest of terms, ethylene triggers senescence of plant organs, influences plant growth and morphology, and acts as a stress hormone (Bleecker and Kende 2000). Accelerated abscission due to ethylene has been shown in several species, including tomato, tobacco, and many flowers (Aharoni et al. 1979; Gepstein and Thimann 1981; Bleecker and Kende 2000). Although the exact effect of ethylene on senescence in under constant study, it is believed that ethylene is responsible for the increased production of several hydrolytic enzymes, such as cellulase or polygalacturonase, which weaken the cell walls of abscission zone cells (Sexton and Roberts 1982; Tucker et al. 1988). Ethylene has also been shown to accelerate senescence and abscission by decreasing auxin transport, thereby increasing the sensitivity of a plant to ethylene, and by inducing the mechanical forces necessary to facilitate abscission (Beyer and Morgan 1971; Wright and Osborne 1974).

Far fewer studies have been completed regarding the role of ethylene in needle abscission of conifers, though it is speculated to work in a similar manner as other plants. Wilksch et al. (1998) has shown that ethylene increases linearly with increased needle damage and abscission in Norway spruce. Fuhrer (1985) has also shown that increases in ethylene production in silver fir trees were correlated with chlorophyll degradation, followed promptly by premature abscission of needles. Whether this is the case in balsam fir remains to be seen, but is an issue of the utmost importance. If ethylene can be

demonstrated to be involved in post-harvest needle abscission in balsam fir, ethylene mitigating technologies such as aminoethoxyvinylglycine (Rath et al. 2006) and 1-methylcyclopropene (Golding et al. 1998) may be available for practical use in Christmas tree and greenery industries.

Several objectives must be met to understand the role of ethylene in abscission. First, it must be determined whether exogenous ethylene can trigger or accelerate abscission. With literally no studies regarding ethylene and balsam fir, determining the effect of exogenous ethylene may prove challenging. In many herbaceous species, an exposure to relatively low concentrations of ethylene for up to one day is enough to trigger abscission, often in a manner of hours (Suttle and Hultstrand 1991; Brown 1997). There has been little work on effect of ethylene on conifers so it is unknown whether balsam fir will have a similar response. In addition, it must be determined whether endogenous ethylene is involved in abscission.

5.2 Effect of ethylene exposure duration on needle abscission in balsam fir

5.2.1 Objective

To determine exogenous ethylene concentration and exposure time required to induce needle abscission in balsam fir.

5.2.2 Methods

Two experiments were conducted to determine the effects of short-term ethylene exposure on needle abscission. One branch was collected from each randomly selected tree, sampled from a population of moderate NRD genotypes on March 10, 2008 for experiment 1 and again on May 15, 2008 for the experiment 2. In each experiment, branches were exposed to 5 concentrations of exogenous ethylene (0, 10, 100, 500, and 1000 ppm) for 24 h. Each treatment had four replications, where a single branch represented a replicate. Ethylene was injected into each EIC with a syringe and monitored using a portable ethylene analyzer (Levitt-Safety, Moncton, NB) to confirm treatment concentrations were maintained over 24 hours. Samples were then removed from the EIC

and randomly positioned in the growth chamber and monitored for the remainder of the experiment.

The response variables in the first experiment were percentage needle loss and average daily water use (AWU). The response variables in the second experiment were needle retention duration (NRD), AWU, XPP, and chlorophyll index. Chlorophyll index of needles was measured with a Minolta SPAD 504 meter (Minolta, USA) on needles that were lost. Several needles were used to cover the sensor and the instrument measured the transmittance by needles at two wavelengths (650 nm and 940 nm) that are differentially absorbed by chlorophyll (Martinez and Guiamet 2004). Results were averaged from 5 readings of different needle samples from each replication.

Two other experiments were conducted to determine the effects of long-term ethylene exposure on abscission. In the first experiment, branches were continuously exposed to 0, 10, 100, 500, or 1000 ppm in an EIC. There were four replications. After 24 h, branches were removed to determine needle loss and water use, replaced, and ethylene was reintroduced to the chamber. This process continued until abscission was complete (approximately 14 days). The response measurements were average daily water use and NRD.

The second experiment used the same continuous exposure regime described above. However, the only treatments were 0 ppm and 1000 ppm ethylene, each replicated 10 times. The response measurements were average daily water use, NRD, XPP, and chlorophyll index.

Regression analysis using Minitab 15 (Minitab 15, Minitab Inc., PA, USA) was used to analyze percentage needle loss using time (days) as the explanatory variable. NRD, XPP, AWU, and chlorophyll index in the short-term ethylene exposure experiment and NRD in the long-term ethylene exposure experiment were submitted to an analysis of variance (ANOVA) using SAS 9.1 (SAS Institute, NC, USA). Significant differences were then analyzed with least significant difference (LSD) multiple means comparison using a 5%

significance level. A 2-sample t-test was used to detect significant differences in NRD, XPP, AWU, and chlorophyll index in the final experiment, which compared effects from long-term exposure to 1000 ppm ethylene to a 0 ppm control.

5.2.3 Results

Short-term exposure (24-h) to ethylene delayed abscission in balsam fir branches. When comparing branches for percentage needle loss after 70 days, a clear trend was observed (Fig.14). Those branches exposed to 500 ppm or 1000 ppm ethylene for 24 h had significantly less needle loss (39.3% and 13.6%, respectively) than the control (70.3%). Regression analysis confirmed a significant relationship between concentration and percentage needle loss (Fig. 15).

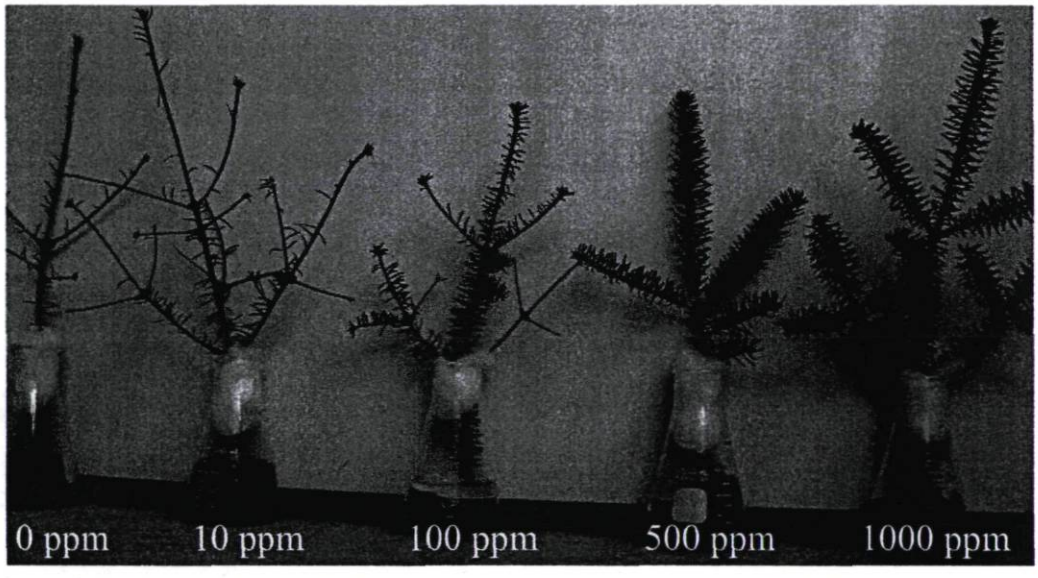


Figure 14: Needle loss at day 70 of balsam fir branches exposed to ethylene for 24 hours.

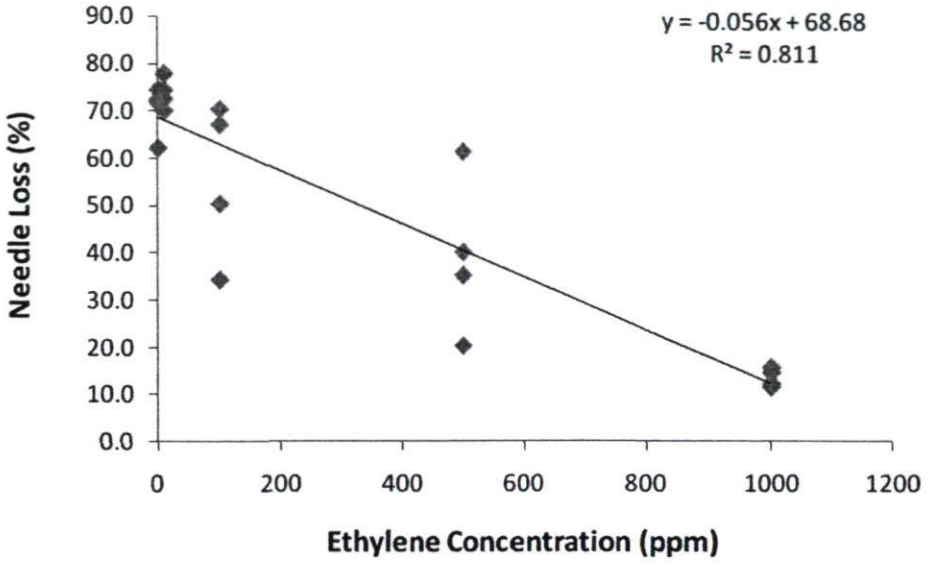


Figure 15: Significant ($p < 0.001$) linear relationship between ethylene 24-hour exposure concentrations and percentage needle loss in balsam fir at day 70 ($n = 20$). The relationship is described by Needle Loss = -0.056*Concentration+68.68. Needle loss in expressed as a percentage of initial fresh weight.

When NRD was used as an abscission response in a second experiment, those branches exposed to any concentration of ethylene for 24 h performed significantly better than the control (Fig. 16). There were no significant differences in average daily water use (0.05 ± 0.003 $mL \cdot g^{-1} \cdot d^{-1}$), chlorophyll index (12.07 ± 1.23), or XPP (-0.33 ± 0.03 MPa) in response to ethylene.

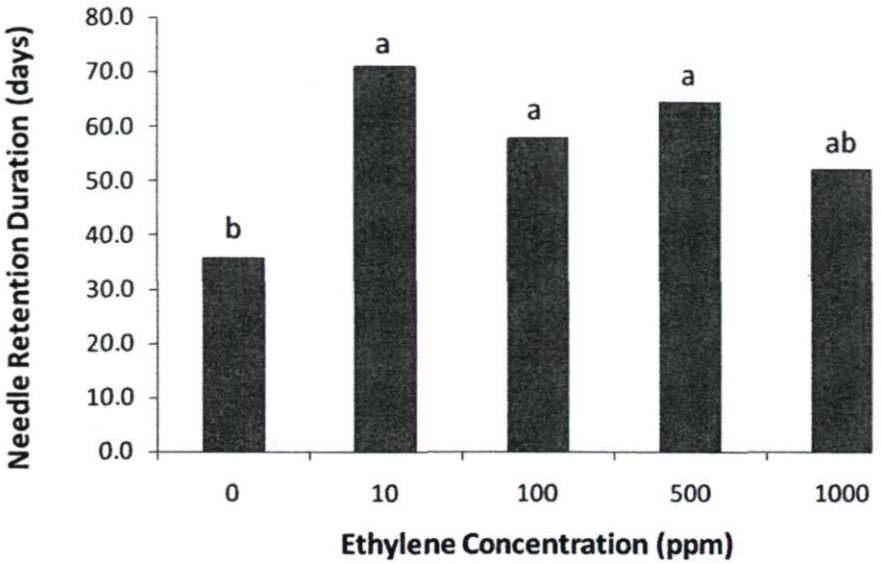


Figure 16: Differences in NRD of balsam fir branches exposed to ethylene for 24 hours determined from 4 replicates. Letter groupings indicate significant differences at $\alpha = 0.05$ using LSD multiple means comparison.

Long-term exposure to ethylene induced abscission in balsam fir branches (Fig. 17). All concentrations significantly decreased NRD, but 1000 ppm ethylene had the most profound effect reducing NRD by 60%. Daily exposure to 1000 ppm ethylene caused abscission to begin at day 5 and increase exponentially afterwards (Fig. 18, Fig. 19).

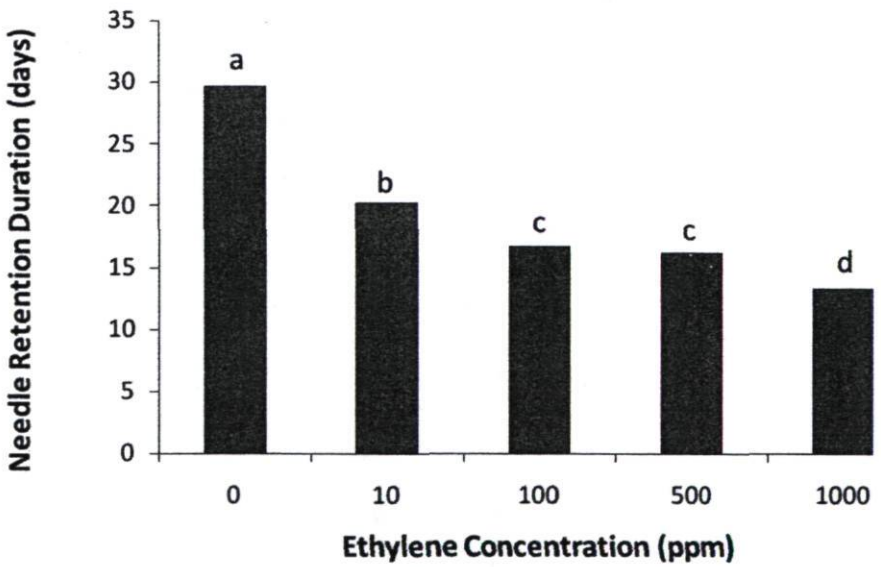


Figure 17: Differences in NRD of balsam fir branches exposed to ethylene daily determined from 4 replicates (n=4). Letter groupings indicate significant differences at $\alpha = 0.05$ using LSD multiple means comparison.

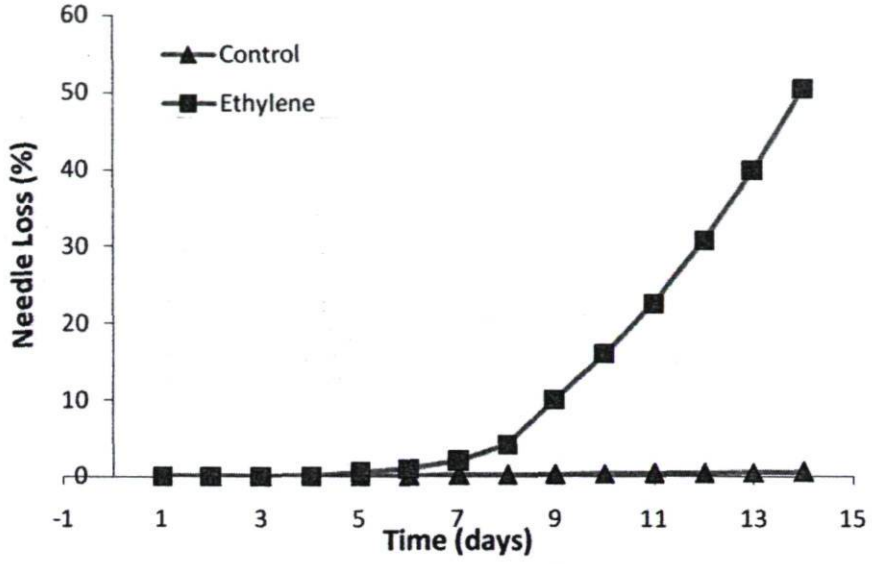


Figure 18: Progressive needle loss due to long-term exposure to 0 ppm ethylene (control) and 1000 ppm ethylene in balsam fir branches. Percentage needle loss is determined as mass of needles lost compared to total mass of branch.

Figure 19: Progressive needle loss (calculated using fresh weight) in a balsam fir branch after daily exposure to 1000 ppm ethylene after: A) 0 days; B) 8 days (2.2% needle loss); C) 11 days (16.8% needle loss); D) 13 days (33.3% needle loss).

Daily exposure to 1000 ppm ethylene was used in a second experiment to investigate effects of ethylene on NRD, average daily water use, XPP, and chlorophyll index. Exposure to 1000 ppm ethylene significantly decreased NRD from 35 days to 14 days and XPP from -0.16 ± 0.02 MPa to -0.42 ± 0.07 MPa. Exposure to 1000 ppm ethylene significantly increased average daily water use from 0.05 ± 0.007 $mL \cdot g^{-1} \cdot d^{-1}$ to 0.09 ± 0.008 $mL \cdot g^{-1} \cdot d^{-1}$. There was no significant influence on chlorophyll index (19.94 ± 2.50).

5.3 Dynamics of ethylene evolution and link with needle abscission

5.3.1 Objective

To determine the dynamics of ethylene evolution prior to needle abscission.

5.3.2 Methods

Branches were collected from 70 balsam fir trees (all genotypes were included in the sampling frame) randomly sampled from a clonal orchard on September 15, 2009. Branches were immediately placed in distilled water and transported to an environmental controlled growth room as described in Chapter 4. In addition, 10 branches were cut from one randomly selected tree. All samples were monitored for NRD, DPE, and ethylene evolution. Correlation and regression analysis was conducted using Minitab 15 for the three variables.

5.3.3 Results

Peak ethylene evolution rates were detected in the range of 10.5 to 20.0 $\mu L \cdot g^{-1} \cdot h^{-1}$, but there was no significant correlation between peak ethylene evolution and NRD or DPE. There was, however, a significant relationship between NRD and DPE ($p < 0.001$). Regression analysis suggests a highly significant ($p < 0.001$), strong, positive relationship between DPE and NRD ($r^2 = 0.9899$) (Fig. 20). In general, peak ethylene evolution rates were observed 1 to 2 days prior to needle abscission.

There was a distinct pattern when comparing needle abscission to ethylene evolution over time (Fig. 21) where ethylene evolution preceded the onset of abscission by 3 to 4 days. The increase in ethylene evolution coincided with an increase in needle loss, culminating in complete needle shed shortly after peak ethylene evolution rates were observed.

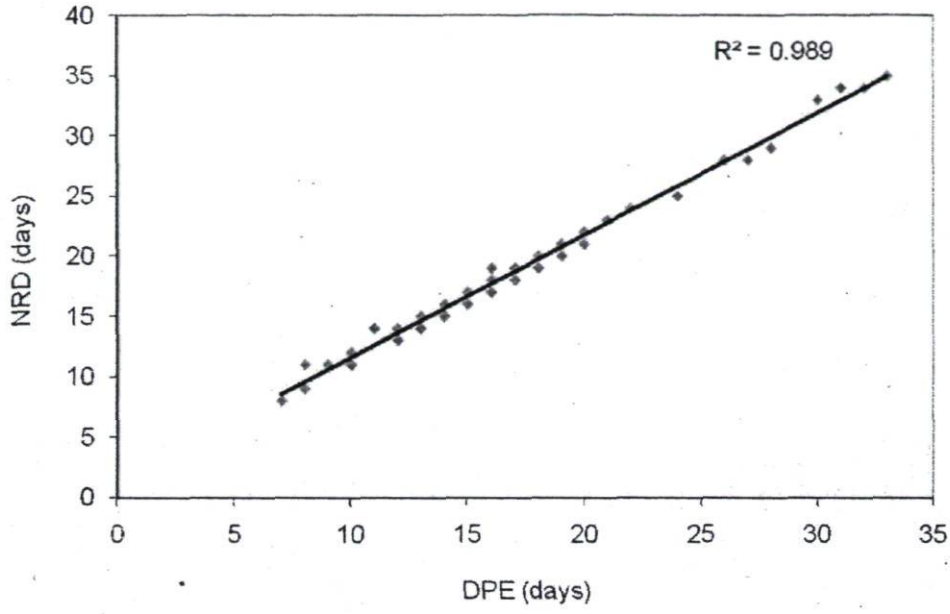


Figure 20: Significant relationship ($p < 0.001$) between day of peak ethylene evolution and needle retention duration, where peak ethylene was observed 1 to 2 days before needle shed ($n = 70$). The relationship can be described by NRD = 1.02(DPE) + 1.45.

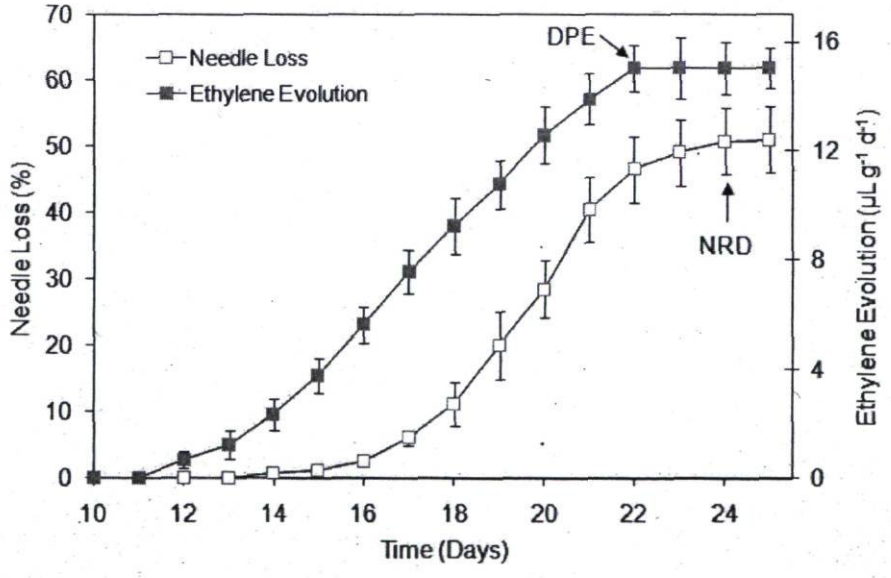


Figure 21: The progression of needle loss (as a percentage of branch fresh mass) and ethylene evolution in a balsam fir genotype with a DPE of 22 days and NRD of 24 days (n=10). The trends observed comparing needle loss to ethylene evolution in this genotype were consistent with other genotypes. Needle loss is expressed as a percentage of the fresh weight of a branch.

5.4 Discussion

Ethylene exposure duration is an important component modifying abscission responses. A 24 h exposure period increased NRD by approximately 50 to 100%, while daily ethylene exposure decreased NRD by approximately 30 to 70%. Due to the fact that many papers have found that ethylene induces abscission (Aharoni et al. 1979; Gepstein and Thimann 1981; Bleecker and Kende 2000), it was not unexpected that prolonged exposure to ethylene would also induce abscission in balsam fir. However, in many other species the ethylene concentration and exposure duration to induce abscission is much lower (i.e. < 10 ppm for 24 hours) (Van Doorn 2002).

There are no known reports of ethylene delaying abscission, at short-term exposure or lower concentrations, as was the case in short-term exposure in balsam fir. There have been cases where ethylene was found to have no effect of abscission. Abeles et al. (1992) reported that ethylene was ineffective at inducing abscission in as many as 60% of species. However, it should be noted that many of the studies reported by Abeles et al. (1992) were in root-intact systems, where it may be difficult to detect a delay in leaf abscission. In a root-detached system, such as balsam fir, MacDonald and Lada (2008) demonstrated that abscission will occur between 6 and 60 days, regardless of exposure to exogenous ethylene. In this case, it would be possible to report accelerated abscission, no change, or delayed abscission. In root-intact systems, abscission primarily occurs as a response to disease or to shed organs that no longer provide essential functions to a plant (Patterson and Bleecker 2004). If neither condition exists, then ethylene would either induce abscission or there would be no observed effect. A delay in abscission would not be possible to detect and may be confounded with "no observed effect".

One possible explanation to the beneficial effects of ethylene may be found in the activation of certain defense related proteins. For example, ethylene quickly triggers proteinase inhibitor genes in tomato (Ryan 1990), pathogenesis related proteins in tobacco (Boller 1991), and defensin and thionin genes in Arabidopsis (Eppel et al. 1997). These activated proteins are generally considered to be required for wound healing or

pathogen defense, but it is not known what effect they may have on needle abscission when no actual threat is present and ethylene is removed.

The opposite effect of the short-term and long term effect of ethylene on balsam fir needle abscission observed in our experiments support the synthesis-degradation balance theory suggested by Brown (1997), where ethylene may influence both cell wall synthesis and degradation at the abscission zone. The synthesis-degradation balance shifts to cell wall synthesis if ethylene concentration or sensitivity is low; while the balance shifts to cell wall degradation if concentration or sensitivity is high. A previous study showed that ethylene induced transcripts disappeared in bean leaf abscission zones when the partial pressure of ethylene was reduced using hypobaric chambers (Sexton et al. 1985). The abscission process not only halts if ethylene declines before a certain point, but is reversed and the force required to abscise is increased (Abeles et al. 1992; Biggs 1971).

Both short-term and long-term ethylene exposure results could have practical significance for the Christmas tree industry. First, if producers were to expose their stands to certain concentrations of ethylene for 24 hours, abscission may be delayed. Needle shed is thought to be the primary reason that artificial trees are gaining favour over natural trees, so any delay in abscission is of paramount importance to producers. Second, the manner in which trees are handled and transported would impose a mechanical stress, which has been found to induce ethylene production (Emery et al. 1994; Morgan and Drew 1997). It is speculated that there is very little airflow when trees are packed for transport and covered, which may cause an ethylene build-up and result in long-term ethylene exposure. It is possible that long-term ethylene exposure during transport is accelerating post-harvest needle abscission.

There was a very strong relationship observed between ethylene evolution and needle abscission. In most branches, ethylene was detected shortly before the onset of abscission. In addition, peak ethylene evolution was detected only 1-2 days before complete needle abscission. This pattern of ethylene evolution over the course of

abscission is typical of many species (Ben-Yehoshua and Aloni 1974; Jackson and Osborne 1970).

The peak ethylene evolution rates of balsam fir were determined to be between 10.5 and 20.0 $\mu L \cdot g^{-1} \cdot h^{-1}$, which easily reaches concentrations necessary for abscission (MacDonald et al. 2009c; MacDonald et al. 2010b). Ethylene evolution was much higher in balsam fir than many other species. For example, the peak ethylene evolution rate was only 0.015 $\mu L \cdot g^{-1} \cdot h^{-1}$ for Douglas fir (Hudgins and Franceschi 2004), 0.020 $\mu L \cdot g^{-1} \cdot h^{-1}$ for orange explants (Ben-Yehoshua and Aloni 1974), and 3.0 $\mu L \cdot g^{-1} \cdot h^{-1}$ for bean explants (Rubinstein and Abeles 1965). In the above cases, ethylene evolution was thought to be triggered by mechanical wounding and was observed much earlier than those evolution rates of balsam fir. In balsam fir, the initial harvest would be considered the mechanical stressor. However, peak ethylene evolution was often not observed until several weeks after harvest, which suggests that mechanical stress is not the primary trigger for needle abscission in balsam fir. Perhaps a difference in signals to begin ethylene evolution, such as mechanical stress versus water deficit, may also explain the differences in ethylene evolution rates.

5.5 Conclusions

It was concluded that prolonged exposure to exogenous ethylene is required to induce abscission. Though concentrations as low as 10 ppm of ethylene were found to significantly accelerate abscission, 1000 ppm was the most effective concentration. It is recommended that future experiments requiring exogenous ethylene use daily injections of 1000 ppm ethylene. In addition, short-term exposure to ethylene will delay abscission, though its mode of action is unknown.

Studies on endogenous ethylene evolution confirm previous studies using exogenous ethylene. Ethylene evolution increases slightly before the onset of abscission and ethylene evolution continues to increase throughout the process of abscission. Ethylene is strongly implicated as the physiological signal to begin abscission. The use of ethylene

inhibitors should be considered to confirm the role of ethylene in needle abscission of root-detached balsam fir.

6.0 THE EFFECT OF ETHYLENE ACTION INHIBITORS ON ABSCISSION

Results from Chapter 6 have been published in:

MacDonald MT, Lada RR, Martynenko AI, Dorais M, Pepin S, Desjardins Y. 2010. Ethylene triggers abscission in root-detached balsam fir. Trees. 24: 879-886

6.1 Introduction

Post-harvest needle abscission is a major challenge and is influenced by many factors. Ethylene is considered one of the most important abscission inducing factors in angiosperms effectively inducing abscission in several species, including tomato (Aharoni et al. 1979), tobacco (Gepstein and Thimann 1981), and many cut-flowers (Bleecker and Kende 2000). It is believed that ethylene is responsible for the increased production of several hydrolytic enzymes, such as cellulase or polygalacturonase, which weaken the cell walls of abscission zone cells to facilitate leaf abscission (Sexton and Roberts 1982; Tucker et al. 1988). Ethylene has also been shown to accelerate senescence and abscission by decreasing auxin transport, thereby increasing the sensitivity of the abscission zone to ethylene, and by inducing the mechanical forces necessary to allow abscission (Beyer and Morgan 1971; Wright and Osborne 1974). Far fewer studies are available regarding ethylene-induced post-harvest needle abscission in the gymnosperms such as conifers, though it has been shown that ethylene increases linearly with increased needle damage and abscission in natural stands of Norway spruce (Wilksch et al. 1998) and silver fir (Fuhrer 1985). Despite substantial evidence for ethylene as an abscission signal in many angiosperms, there is currently little information available on the role of ethylene in post-harvest needle abscission of balsam fir.

Some initial studies have been conducted in balsam fir to determine if ethylene could induce abscission. In absence of any external treatments, abscission in a root-detached balsam fir branches occurred in approximately 35 days (MacDonald and Lada 2008). When balsam fir branches were exposed to a daily dose of exogenous ethylene, the length of time required for abscission was reduced to 14 days (MacDonald et al. 2009c). In addition, it was found that endogenous ethylene increases immediately preceding

abscission between 10.5 and 20 $\mu L \cdot g^{-1} \cdot h^{-1}$, which corresponds to approximately 12 to 23 ppm in an 80 L chamber after 3 hours. Prolonged exposure to exogenous ethylene at 10 ppm was sufficient to induce abscission (Chapter 5).

If ethylene is a key signal for abscission in root-detached balsam fir, then inhibition of ethylene action should delay abscission. Though several effective ethylene inhibitors exist, the most popular are aminoethoxyvinylglycine (AVG) and 1-methylcyclopropene (1-MCP). AVG is known to inhibit biosynthesis of ethylene by inhibiting conversion of S-adenosyl-L-methionine (AdoMet) to 1-aminocyclopropane-1-carboxylic acid (ACC) (Boller et al. 1979; Yu et al. 1979). In contrast, 1-MCP has been shown to delay abscission in several flower species (Serek et al. 1995; Porat et al. 1995) by competitively binding to ethylene receptors (Sisler et al. 1996; Serek et al. 1994b). If ethylene is, in fact, required for abscission, a 1-MCP or AVG treatment should delay or negate post-harvest needle abscission. Thus, the objective of this experiment was to confirm the role of ethylene as a signal molecule for post-harvest needle abscission in root-detached balsam fir. This objective was achieved by determining the effect of various concentrations of 1-MCP and AVG on ethylene evolution and needle abscission in balsam fir.

6.2 Inhibition of ethylene synthesis with aminoethoxyvinylglycine

6.2.1 Objective

To determine whether inhibiting ethylene synthesis with AVG will increase NRD in balsam fir in the presence and absence of exogenous ethylene.

6.2.2 Methods

A random sample of 48 branches was collected on July 17, 2009 from a clonal orchard (described in Chapter 4). Only trees with a moderate NRD were included in the random sample. A single branch was sampled from each tree, and then transported, and stored as described in Chapter 4. The experiment was designed as a 2 x 6 factorial. The first factor was ethylene concentration (0 ppm, 1000 ppm) and the second factor was AVG (Sigma-

Aldrich, Oakville, ON, Canada) concentration (0, 1, 10, 100, 500 or 1000 ppm). The experiment was replicated 4 times, where a branch represented a replicate. AVG was applied to each branch by dissolving a known concentration into the water supply. In this manner, each branch was provided AVG for the entire duration (90 days) of the experiment.

In cases where a sample was to be exposed to 1000 ppm exogenous ethylene, the incubation chamber was injected with 80 mL ethylene daily to maintain a concentration of 1000 ppm. A concentration of 1000 ppm ethylene was previously determined to accelerate abscission (MacDonald et al. 2009c). To prevent a build up of CO_2, each EIC was aerated and re-injected with ethylene each day. The control was 0 ppm exogenous ethylene, which was also placed in an EIC. Each EIC received one branch.

Response variables were NRD, DPE, AWU, and ethylene evolution, which are described in detailed in Chapter 4. Regression analysis was conducted with Minitab 15 (Minitab Inc., State College, PA) to determine the relationship of AVG with NRD, DPE, and AWU. All statistical assumptions, such as normal distribution, homogeneity, and independence were confirmed.

6.2.3 Results

AVG inhibited endogenous ethylene evolution in balsam fir. Those branches exposed to 0 ppm AVG released ethylene at a peak rate of 12.5 $\mu L \cdot g^{-1} \cdot h$. However ethylene evolution from branches provided AVG as low as 100 ppm was below the detectable limit. In the absence of exogenous ethylene, AVG was also effective at delaying abscission. AVG concentrations between 100 ppm and 1000 ppm all significantly ($p < 0.001$) increased NRD. A concentration of 1000 ppm AVG was the most effective, increasing NRD by 113% (Fig. 22). There was no effect between AVG and NRD in the presence of exogenous ethylene.

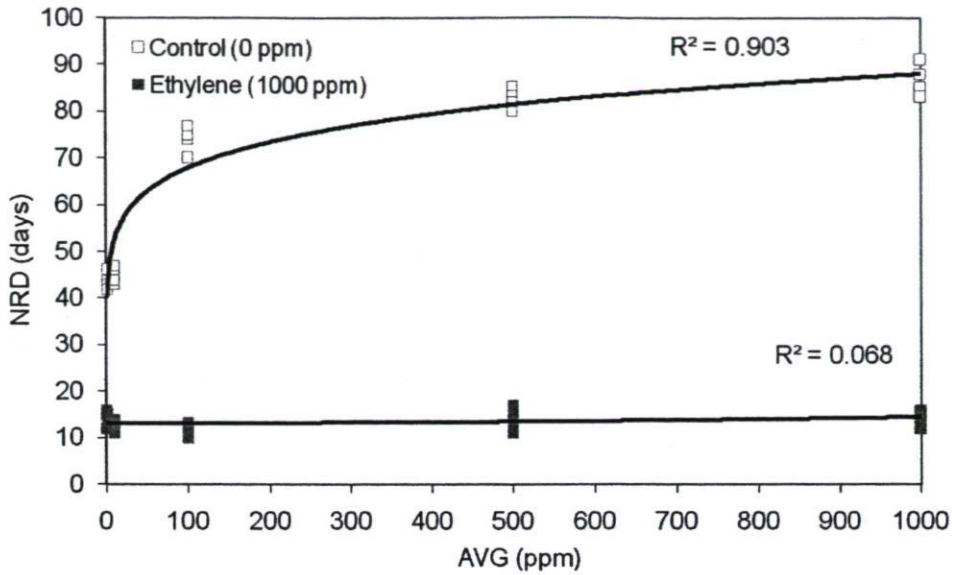


Figure 22: Relationship between AVG concentration and needle retention duration (NRD) in branches exposed to 0 ppm ethylene and 1000 ppm ethylene. A significant hyperbolic relationship ($p < 0.001$) was found when there was 0 ppm ethylene as described by $NRD = 40.5(AVG)^{0.112}$. There was no relationship in the presence of 1000 ppm ethylene. In each case $n = 24$.

There was a relationship between AVG and AWU with ethylene ($p=0.019$) and without ethylene ($p=0.035$), where those branches treated with any concentration of AVG consumed less water than their respective controls (Fig. 23). The effect was more noticeable in the presence of ethylene, where branches exposed to 1000 ppm AVG consumed 23% less water than their respective controls. In addition, those branches exposed to ethylene consume, on average, 4 times more water than control branches.

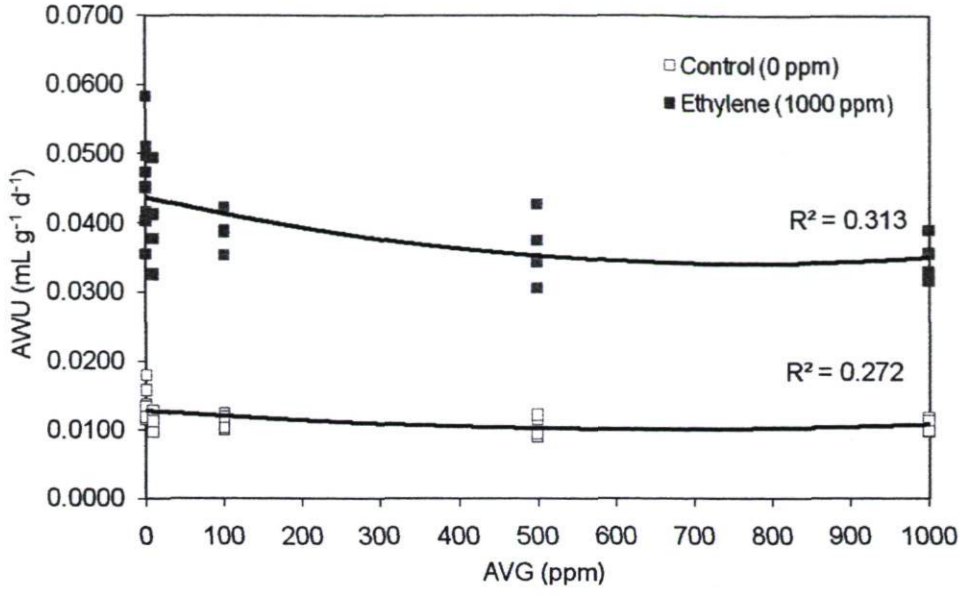


Figure 23: Relationship between AVG concentration and average daily water use (AWU) in branches exposed to 0 ppm ethylene and 1000 ppm ethylene. A significant quadratic relationship ($p = 0.035$ in control, $p=0.019$ in ethylene) was found in each case. $n = 24$. In the absence of exogenous ethylene, the relationship was described by $AWU = 6x10^{-9}(AVG)^2 - 8x10^{-6}(AVG) + 0.012$. In the prescence of exogenous ethylene, the relationship was described by $AWU = 2x10^{-8}(AVG)^2 - 3x10^{-5}(AVG) + 0.043$.

6.3 Blocking ethylene receptors with 1-methylcyclopropene

6.3.1 Objective

To determine whether blocking ethylene receptors using 1-methylcyclopropene would increase NRD in the presence and absence of exogenous ethylene.

6.3.2 Methods

A random sample of 40 branches was collected on April 14, 2009 from a clonal orchard. Only trees with a moderate NRD were included in the random sample. A single branch was sampled from each tree, and then transported, and stored as described in Chapter 4. The experiment was designed as a 2 x 5 factorial with 4 replicates, where a single branch was treated as a replicate. The first factor was ethylene concentration (0 ppm, 1000 ppm) and the second factor was 1-MCP (0, 2.5, 5.0, 7.5, or 10.0 g).

When EthylBloc™ is dissolved in water, 1-MCP is produced as a gas. Thus, the 1-MCP treatments were applied by dissolving a known mass of EthylBloc™ (Floralife Inc,

Walterboro, SC, USA) in 500 mL distilled water. Care was taken to place flasks containing EthylBloc™ into an EIC prior to adding water, so that the EIC could be immediately sealed to limit loss of 1-MCP gas. Each EthylBloc™ solution was placed in an EIC along with balsam fir branch for a 24 h exposure period. After 24 hours, EthylBloc™ was removed from all EICs and branches were ready for exposure to exogenous ethylene. In cases where a sample was to be exposed to exogenous ethylene, the EIC was injected with 80 mL ethylene daily to maintain a concentration of 1000 ppm. A concentration of 1000 ppm ethylene was previously determined to accelerate abscission (MacDonald et al. 2009c). The control was 0 ppm exogenous ethylene.

Response variables were NRD, DPE, AWU, and ethylene evolution, which are described in detailed in Chapter 4. Regression analysis was conducted using Minitab 15 (Minitab Inc., State College, PA) to determine the relationship between 1-MCP with NRD and DPE. All statistical assumptions, such as normal distribution, homogeneity, and independence were confirmed.

6.3.3 Results

The quantity of 1-MCP applied was strongly related to several response variables. There was a significant relationship ($p < 0.001$) between 1-MCP mass and NRD both with and without exogenous ethylene (Fig. 24). In the absence of 1-MCP, ethylene decreased NRD by 63%. However, the presence of 1-MCP was able to increase NRD in all treatments. When no exogenous ethylene was present, 10 g of 1-MCP had the most significant effect, effectively increasing NRD by 73% when compared to the 0 g 1-MCP control. When exposed to ethylene, 5 g of 1-MCP had the most significant effect, increasing NRD by 147% when compared to the 0 g 1-MCP control.

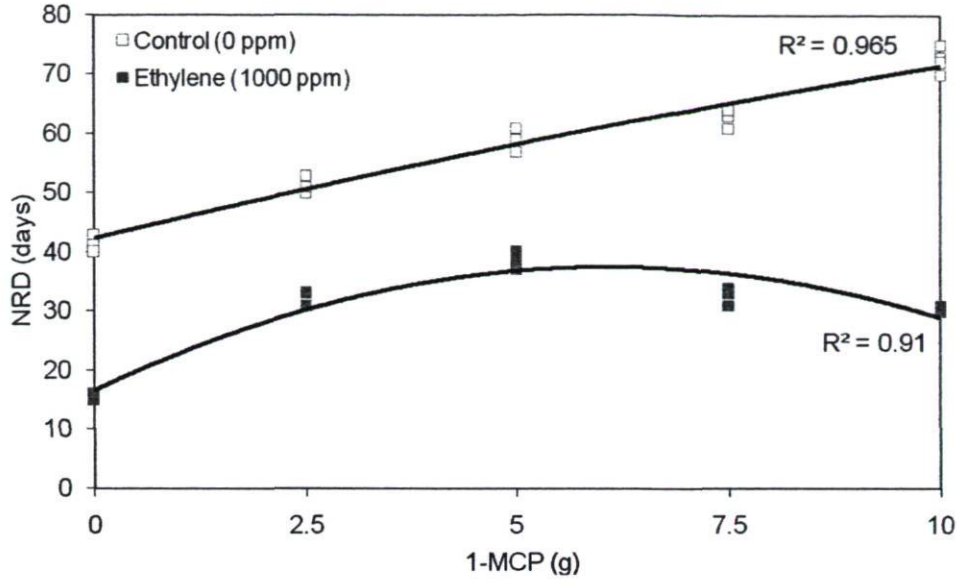


Figure 24: Relationship between mass of 1-MCP and needle retention duration (NRD) in branches exposed to 0 ppm ethylene and 1000 ppm ethylene. A significant quadratic relationship ($p < 0.001$) was found in each case ($n = 20$). In the absence of exogenous ethylene, the relationship can be described by $NRD = -0.05(1\text{-MCP})^2 + 3.41(1\text{-MCP}) + 42.45$. In the presence of exogenous ethylene, the relationship can be described by $NRD = -0.56(1\text{-MCP})^2 + 6.86(1\text{-MCP}) + 16.61$.

There was also a significant relationship ($p < 0.001$) between 1-MCP quantity and AWU with and without exogenous ethylene (Fig. 25). In the absence of 1-MCP, ethylene increased AWU by 118%. The presence of 1-MCP was able to maintain low AWU in all treatments. In the absence of ethylene, 10 g of 1-MCP had the most significant effect, effectively decreasing AWU by 45% compared to the 0 g 1-MCP control. In presence of exogenous ethylene, 5.0 g and 7.5 g of 1-MCP effectively reduced AWU by 53% and 54%, respectively, compared to the 0 g 1-MCP control.

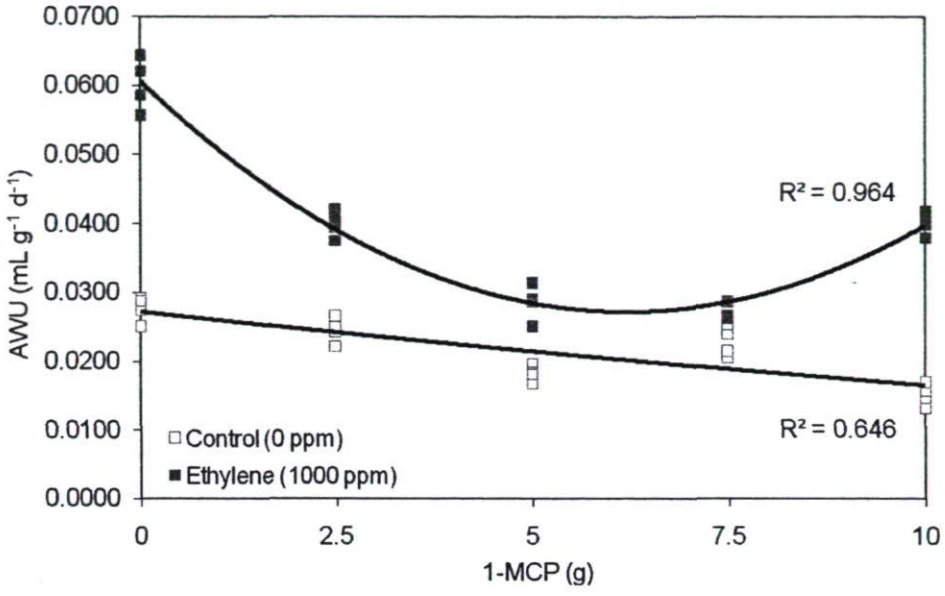


Figure 25: Relationship between mass of 1-MCP and average daily water use (AWU) in branches exposed to 0 ppm ethylene and 1000 ppm ethylene. A significant quadratic regression ($p < 0.001$) was found in each case ($n = 20$). In the absence of exogenous ethylene, the relationship can be described by AWU = -0.001(1-MCP) + 0.027. In the presence of exogenous ethylene, the relationship can be described by AWU = -0.0001(1-MCP)^2 – 0.01(1-MCP) + 0.06.

There was no significant difference in ethylene evolution rate or DPE due to 1-MCP. The average peak ethylene evolution was 14.5 ± 0.4 $\mu L \cdot g^{-1} \cdot d^{-1}$ in all balsam fir branches.

6.4 Discussion

AVG is known to inhibit the conversion of AdoMet to ACC in other species (Boller et al. 1979; Yu et al. 1979). In all branches treated with the ethylene synthesis inhibitor, AVG, ethylene was below the detectable limit of 0.1 ppm, which confirms that AVG can inhibit ethylene synthesis in balsam fir. Once again, ethylene is indicated as the signal molecule triggering needle abscission. NRD more than doubled in AVG treated branches unexposed to exogenous ethylene, however, exposure to exogenous ethylene offset any benefit of AVG; this again suggests that ethylene is a signal molecule for abscission in balsam fir. Still, abscission did eventually occur in AVG-treated branches without any exogenous ethylene. It is possible that even endogenous levels below 0.1 ppm can induce abscission.

Pre-exposing balsam fir branches to the ethylene receptor blocker, 1-MCP, delayed needle abscission in branches with and without exogenous ethylene present. The fact that 1-MCP can delay abscission is hardly new information, as there are literally hundreds of papers available on the topic which demonstrate delayed abscission in the presence and absence of ethylene (Sisler et al. 1996; Serek et al. 1994a). However, 1-MCP has never been used in post-harvest balsam fir and the effectiveness of 1-MCP at delaying abscission in control branches is very strong evidence for ethylene being a signal molecule of abscission in the species. Interestingly, all branches incubated with 1-MCP eventually shed all needles, albeit 30 days later than the control, but the cause is unknown. The persistence of 1-MCP has proven difficult to measure and positive effects last for varying durations in different species (Sisler and Serek 1997). It is possible that the effect of 1-MCP dampens over time, allowing ethylene to finally induce abscission in a synchronous manner.

It appears that ethylene does, in fact, induce abscission in balsam fir. However the physiological mechanism of ethylene in balsam fir remains unknown, though it has been well elucidated in other species. Ethylene has been shown to act directly on the abscission zone by promoting the production of cell wall hydrolytic enzymes, such as cellulase (Sexton and Roberts 1982; Tucker et al. 1988) and polygalacturonase activities (Taylor et al. 1990). In addition, ethylene inhibits auxin transport, which may influence the sensitivity of ethylene responses (Beyer 1973; Sexton et al. 1985). Further studies are necessary to determine if ethylene stimulates production of any degradative enzymes in balsam fir and/or influences the flux of auxins.

The discoveries of ethylene evolution in root-detached balsam fir and the effects of known inhibitors could have a significant practical application in the Christmas tree and greenery industries. Both AVG and 1-MCP were capable of essentially doubling the lifespan of balsam fir branches (Fig. 26). Of particular interest is the fact that a convenient way to sell, store, transport, and use 1-MCP has already been developed (Daly and Kourelis 2001). In fact, 1-MCP is already commonly used in the ornamental horticulture and apple storage industries (Read and Staby 2008). Though the

aforementioned methods were not originally designed for balsam fir, they could be easily modified. Also, it is a relatively simple task to introduce AVG to water supplied to Christmas trees by producers and/or consumers. Further studies may be required to develop these technologies and identify optimum concentrations for an entire root-detached tree rather than smaller branches.

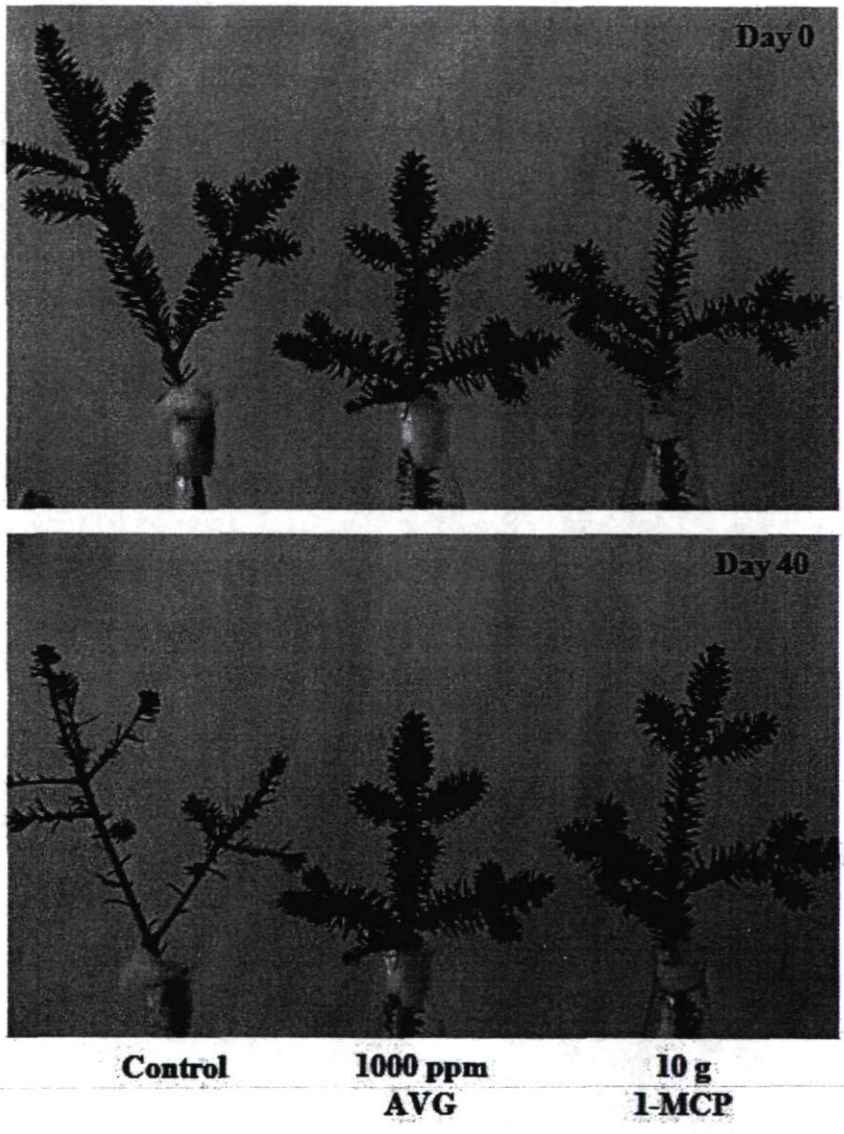


Figure 26: Root-detached balsam fir branches on day 0 and day 40 in the absence of exogenous ethylene. Abscission is easily visible on the control (no AVG, no 1-MCP) while there is virtually no abscission occurring in 1000 ppm AVG and 10 g 1-MCP treatments after 40 days.

6.5 Conclusions

Prior to this study, it was established that ethylene evolution increased prior to abscission and exogenous ethylene could accelerate abscission. From this study it can be concluded that blocking ethylene synthesis or ethylene receptors delays abscission in balsam fir. Ultimately, this study confirms the role of ethylene as a signal for needle abscission. However, the mechanism through which ethylene induces abscission remains unclear. A better understanding of ethylene-induced proteins may uncover further details in post-harvest needle abscission.

7.0 ENZYMOLOGICAL EVIDENCE OF ETHYLENE-INDUCED ABSCISSION

Results from Chapter 7 are prepared as:

MacDonald MT, Lada RR, Martynenko AI, Dorais M, Pepin S, Desjardins Y. 2010. Ethylene-induced cellulase activity in post-harvest balsam fir. Trees. UNDER REVIEW

7.1 Introduction

Ethylene has been well established to promote abscission in many different species (Brown 1997; Sexton et al. 1985), but little was known about the potential role for ethylene in needle abscission of root-detached balsam fir. Recent work has investigated ethylene as a signal for abscission in balsam fir and has uncovered part of the physiological mode of action for abscission (MacDonald et al. 2009c; MacDonald et al. 2010b; Chapter 5; Chapter 6). Sometime after harvest (often several weeks, though it varies among trees), a currently unidentified signal begins synthesis of endogenous ethylene. Ethylene evolution increases over several days before reaching a peak, which coincides with complete needle abscission (MacDonald et al. 2010b). Complimentary studies have also shown exogenous ethylene to accelerate abscission and ethylene inhibitors to delay abscission by 80 to 140% (MacDonald et al. 2009; MacDonald et al. 2010b). Although, these studies uncovered ethylene as the signal triggering post-harvest needle abscission in root-detached balsam fir, the enzymological reasoning through which ethylene facilitates abscission remains unknown in balsam fir.

Studies in other species have suggested that ethylene activates certain genes which code for a suite of hydrolytic enzymes that dissolve cell walls during abscission (Bleecker and Patterson 1997; Deikman 1997). Two major enzyme groups are thought to be associated with abscission. The first are pectinases, which were thought to be required for abscission due to the dissolution of the middle lamella and increase in soluble pectin fractions (Addicott 1982; Moore 1968). However, there was no increase in pectinase activity in bean plants prior to abscission (Berger and Reid 1979) and only an increase after abscission in tomato flower pedicels (Tucker et al. 1984). More likely is the second group of enzymes, known as cellulases, which have consistently been associated with

abscission (Abeles et al. 1992). One isozyme of cellulose was found to increase during abscission in bean leaves (Durbin et al. 1981) and antibodies specific to cellulase were used to demonstrate that cellulase was undetectable before abscission, was localized to the abscission zone, and coincided with a decrease in break strength at the abscission zone (de Campillo and Bennett 1996; Sexton et al. 1980; Durbin et al. 1981).

If ethylene is considered the signal for abscission in balsam fir, then it is likely that cellulase activity increases prior to abscission. The objective of these experiments is to determine whether there is an increase in cellulase activity due to elevated concentrations of endogenous and exogenous ethylene. Since cellulases are considered difficult to isolate and characterize (Imam et al. 1993), zymographic techniques to analyze cellulase in balsam fir must also be developed.

7.1 Objective

To develop a protocol to identify presence of cellulase in plant tissue and to quantify ethylene-induced cellulase activity in needle tissue.

7.2 Methods

7.2.1 Sample collection and treatments

Fifteen balsam fir branches were collected from a clonal orchard on February 11, 2010 from moderate NRD genotypes. A single branch was cut from each tree, transported, and prepared as described in Chapter 4. Five branches were randomly selected to be continuously exposed to 1000 ppm exogenous ethylene for 14 days. The remaining branches were not exposed to exogenous ethylene and would later be subdivided into a control (showing no signs of abscission) group and an endogenous ethylene group. The control consisted of 5 branches sampled on day 14, the endogenous ethylene treatment consisted of 5 branches synthesizing ethylene at their peak rate.

A previous study by MacDonald et al. (2009c) identified that endogenous ethylene peaked 1 to 3 days before complete abscission. Ethylene evolution was measured daily

using a portable ethylene analyzer and peak ethylene evolution was determined to have occurred after measuring consistent ethylene evolution rates. When peak ethylene evolution was measured, the corresponding needles were frozen in liquid nitrogen and then stored at -80 °C until enzyme extraction. It is important to note that branches exposed to exogenous ethylene would shed needles much earlier than the control. Thus, the 5 control branches were frozen at the same time as the exogenous ethylene treated branches. Those 5 control branches were showing no signs of abscission. The remaining 5 control branches were frozen during peak ethylene to represent a response to endogenous ethylene.

Response variables for each treatment were NRD, DPE, XPP, AWU, and ethylene evolution, which are described in detail in Chapter 4. Data were submitted to an analysis of variance and least significant difference mean separations at $\alpha = 0.05$.

7.2.2 Optimization of cellulase digestion

Optimum pH was determined by spotting cellulase onto agar plates infused with cellulose. Each plate contained 3% agar and 0.2% carboxymethylcellulose (CMC) (Sigma-Aldrich, Oakville, ON, Canada) made to a volume of 20 mL in buffer. Three different buffers were selected to include a wide range of pH: 0.1 M sodium acetate (pH = 5.0), phosphate buffer solution (PBS) (pH = 7.0), and 0.1 M tris(hydroxymethyl)aminomethane (TRIS) (pH = 8.8).

Cellulase (Sigma-Aldrich, Oakville, ON, Canada), isolated from *Aspergillus niger*, was diluted in 200 µL of deionized water. The aqueous cellulase contained 50 units of activity, where 1 unit of cellulase activity would liberate 1.0 mole of cellulose from cellulose in 1 hr at pH 5 and 37°C. Each plate was spotted with 4 µL, 20 µL, and 40 µL cellulase, which corresponded with 1, 5, and 10 units of activity, respectively. Each plate was incubated for 1 hour at 37 °C. After incubation, each plate was stained with 0.1% aqueous Congo Red stain for 20 minutes and then destained with 1 M NaCl for 2 hours (Xue et al. 1999).

A limitation of cellulase spotting was that the aqueous cellulase was able to spread over the agar-CMC surface to varying degrees, which may cause a dilution of surface CMC digestion. To correct this problem the above procedure was repeated with the exception that surface spotting was replaced with 1 cm diameter wells capable with a capacity of 200 µL. In this case, each aliquot of cellulase was diluted to 100 µL. In addition, a blank of 100 µL buffer was used to represent 0 units of activity. Cellulase standards were replicated 5 times.

7.2.3 Cellulase extraction

The cellulase extraction was modified from a method described by Feiz et al. (2006). Approximately 30 g of needle tissue (fresh weight) was frozen in liquid nitrogen and homogenized in 30 mL of deionized water using a mortar and pestle. The homogenate was layered onto a 10% glycerol solution and let to sediment for several hours. The cell wall pellet was suspended in deionized water and washed 3 times by repeated centrifugation. The pellet was then suspended in 10 mL of 0.2 M $CaCl_2$ and centrifuged. The supernatant was collected, dried down, and resuspended in 1 mL of a PBS buffer (pH = 7.0). This extraction was repeated for each replicate, resulting in 15 individual extractions or 5 extractions per treatment.

7.2.4 Analysis of cellulase activity

Two different assays were conducted to determine cellulase activity. The first was a CMC digestion assay, which is a simplification of a zymogram overlay described by Xue et al. (1999). A mixture of 3% agar and 0.2% CMC was made in PBS buffer. Twelve wells were made as the solution set. Three wells were filled with 200 µL cellulase standards representing 0, 5, and 10 units of cellulase activity. The remaining nine wells were filled with 200 µL of extract from the no abscission control, endogenous ethylene-induced abscission, and exogenous ethylene-induced abscission. Only 3 of 5 replicates from each treatment were used for CMC digestion; the remaining replicates were saved for the second assay. Since the digestion area was kept consistent in each well, image analysis could be used to quantify cellulase activity. A standard curve was generated by measuring the ratio of red: green using CIAS 2.0 Image Measurement Software (Jandel

Scientific, San Rafael, CA) and plotting against cellulase activity of the cellulase standards.

The second assay was a method of zymography based on methods described by Schwartz (1987). First, a cellulase standard and extracts from control, endogenous ethylene, and exogenous ethylene were separated using SDS-PAGE by the method of Laemmli (1970). Gels were then layered onto a plate containing 3% agar and 0.2% CMC (Xue et al. 1999). This procedure was performed twice with replicates remaining after CMC digestion. Zymography did not allow for quantification of cellulase activity, but it was able to qualitatively identify cellulase-like activity, determine the number of isoenzymes present, and estimate the molecular weigth of each isoenzyme.

In both assays, gels were allocated 1 h at 37°C for incubation. After incubation, gels were stained with 0.1% Congo Red for 20 minutes, and then destained with 1 M of aqueous NaCl for 2 h. In the well assay, cellulase activity was observed as semi-transparent coloring at the bottom of a well. In zymography, cellulase was initially observed as semi-transparent coloring. However, in storage, the areas of cellulase activity eventually appeared as a darker area. The zymography became easier to interpret when photographs were adjusted to grayscale.

7.3 Results

Cellulase activity was visible as an area of lighter staining in all buffers after cellulase spotting (Fig. 27). The best buffer was PBS, which clearly showed areas of CMC digestion even at 1 unit of activity. Both TRIS and sodium acetate were relatively poor buffers, though CMC digestion was observed as low as 1 unit of activity in both. However, the outline and contrast between digestion zones were not nearly as crisp as that of the PBS buffer.

When cellulase activity was observed in plate wells, CMC digestion was visible as a brighter area at the bottom of each well. PBS was the best buffer once again (Fig. 28). In PBS, the wells containing both 5 and 10 units of activity were almost transparent. Sodium

acetate could also detect 5 and 10 units of cellulase activity, though CMC digestion was less obvious. TRIS was not a suitable buffer for the well assay. Unfortunately, all buffers had reduced sensitivity, where 1 unit of cellulase activity was difficult to detect visually.

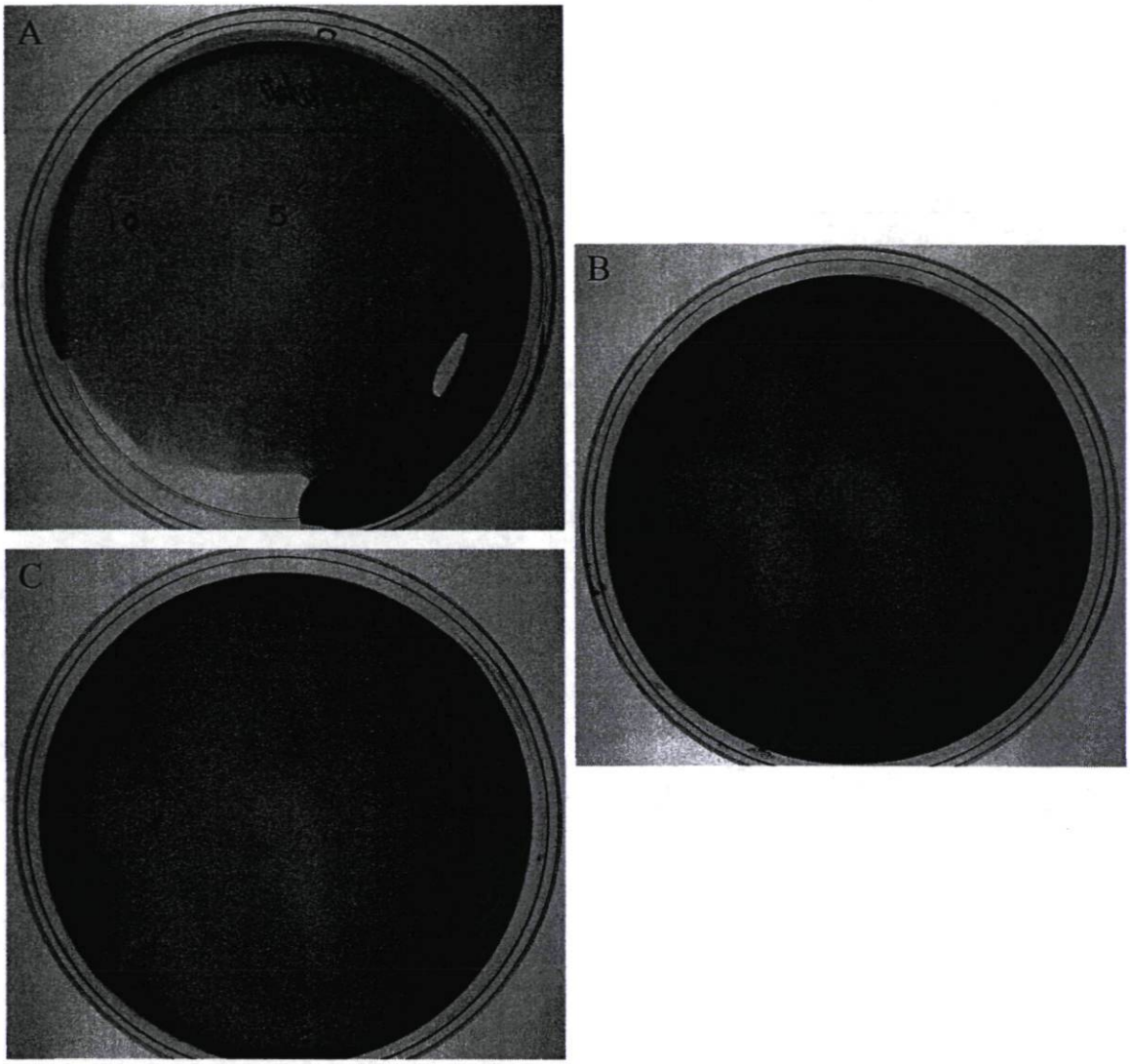


Figure 27: Cellulase activity on 3% agar/0.2% carboxymethylcellulse plates made of three different buffers: A) sodium acetate, B) PBS, and C) TRIS. On each plate, digestion zones represent 10, 5, and 1 units of cellulase activity moving from left to right.

However, image analysis was able to detect all levels of cellulase activity by measuring the ratio of red: green intensity. A linear relationship was detected from 0 to 5 units of activity (Fig. 29), though 10 units of cellulase activity had the same ratio as 5 units of cellulase activity.

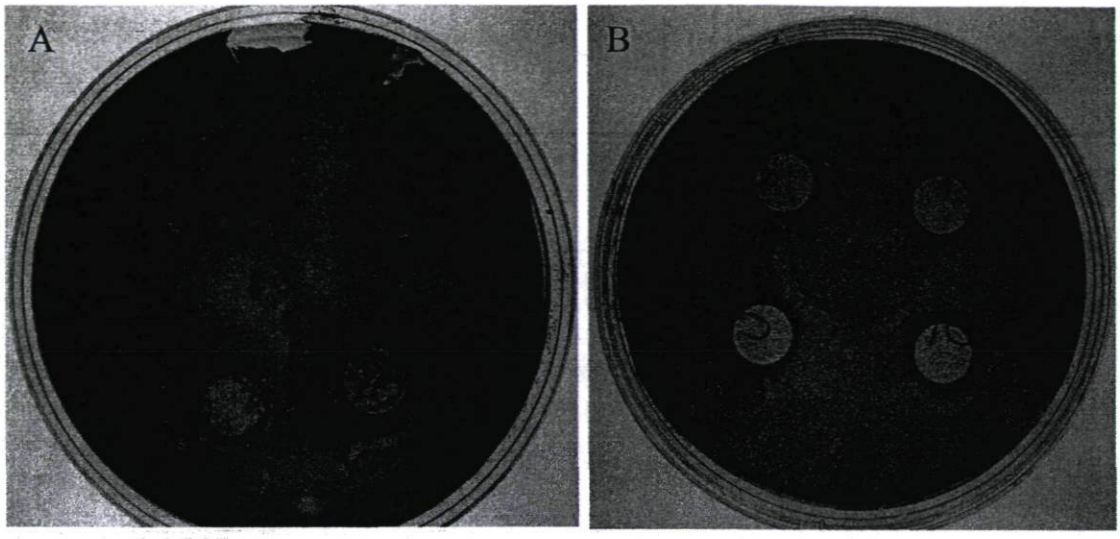


Figure 28: Cellulase activity in wells made in 3% agar/0.2% carboxymethylcellulse plates made of three different buffers: A) sodium acetate, B) PBS. Top left well is 0 units activity, top right is 1 unit activity, bottom left is 5 units activity, and bottom right is 10 units activity.

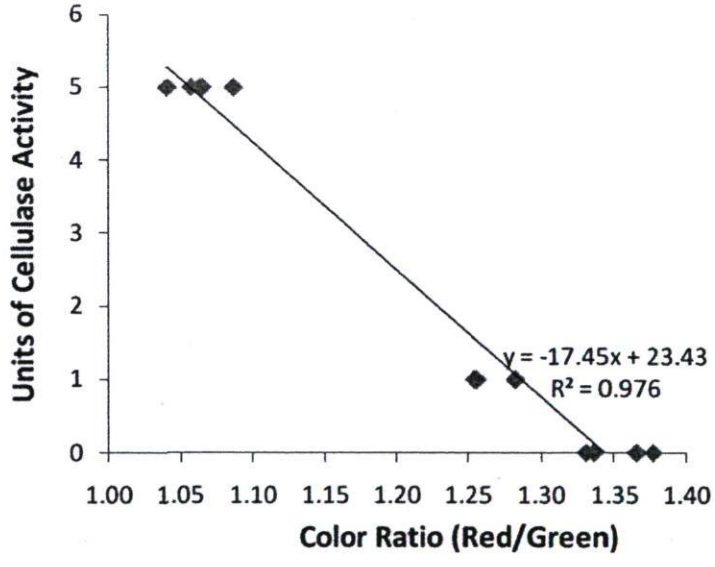


Figure 29: Standard curve of cellulase activity by comparing the ratio of red: green in CMC digestion zones. Each level of cellulase activity was replicated 5 times. The relationship using color ratio to predict enzyme activity is best described as Cellulase Activity = -17.5(Color Ratio) + 23.43.

Despite reduced visual sensitivity, the well assay was determined to be the preferred assay. In the well assay, volume of sample could be consistent with each sample while maintaining a controlled surface area. In the spotting assay, it was difficult to control the area a sample may spread, which could potentially result in overlapping digestion zones. In addition, image analysis would allow for an estimation of cellulase activity.

As observed previously (MacDonald et al. 2009c; MacDonald et al. 2010b; Chapter 5; Chapter 6), continuous exposure to exogenous ethylene successfully induced abscission (Fig. 30). Branches continuously exposed to ethylene had a NRD of 14.2 d, compared to 35.4 d in the absence of exogenous ethylene. Peak ethylene evolution was observed an average of 45 hours before complete abscission in the presence of exogenous ethylene and 38 hours before abscission in the absence of exogenous ethylene (Table 2). In the absence of abscission, there were no visible signs of abscission after 14 days and no endogenous ethylene could be detected.

Figure 30: Comparison of a branch continuously exposed to ethylene (left) to a branch in the absence of ethylene after 15 days.

Table 2: Summary of abscission related parameters in balsam fir branches with no signs of abscission (control), endogenous ethylene-induced abscission, and exogenous ethylene-induced abscission. Significant differences were determined using LSD multiple means comparison at $\alpha = 0.05$. DPE = day of peak ethylene evolution; NRD = needle retention duration; XPP = xylem pressure potential (n=5).

Treatment	Data Collection (days)	DPE (days)	Peak C_2H_4 ($\mu L \cdot g^{-1} \cdot d^{-1}$)	NRD (days)	XPP (MPa)
Control	14.2^a	N/A	N/A	N/A	-0.10^a
Exo. ethylene	14.2^a	12.4^a	15.4^a	14.2^a	-0.55^b
Endo. ethylene	35.4^b	33.8^b	16.7^a	35.4^a	-0.65^b

In the well assay, CMC digestion zones were not visible in extracts from balsam fir needles which had no signs of abscission. However, there was obvious CMC digestion in branches experiencing abscission due to endogenous and exogenous ethylene. When quantified using the standard curve (Fig. 29) the needles from control branches were found to have 0.39 units of cellulase activity. Cellulase activity was significantly ($p < 0.001$) increased in needles experiencing abscission. Branches with endogenous ethylene-induced abscission had 3.07 units of cellulase activity while branches with exogenous ethylene-induced abscission had 4.58 units of cellulase activity, which was 11.8 times and 7.9 times greater than the control, respectively (Fig. 31).

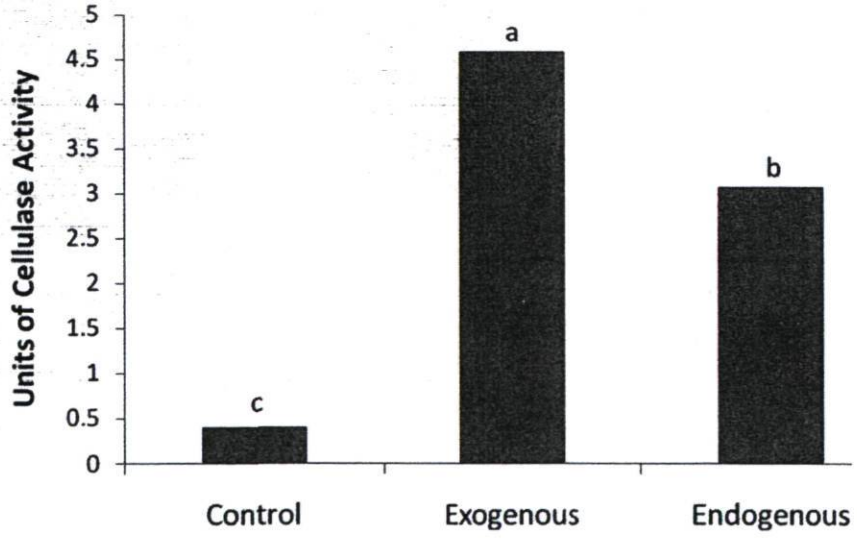


Figure 31: Cellulase activity of needles from branches with no abscission (control), endogenous ethylene-induced abscission, and exogenous ethylene-induced abscission. Significant differences were detected using LSD comparison from cellulase extraction of 3 balsam fir branches at $\alpha = 0.05$.

Zymography confirmed the results from the well assay (Fig. 32). The cellulase standard had three distinct isozymes of cellulase present. Two isozymes had a relatively high molecular weight estimated at 125 kDa and 75 kDa, while one had a low molecular weight estimated at 25 kDa. The 25 kDa isozyme did not appear in any treatments. However the 75 kDa and 125 kDa isozyme appeared in both endogenous and exogenous ethylene-induced abscission, though the 125 kDa isozyme was greatly diminished in both. There was no evidence of 25 kDa or 125 kDa isozymes in the control, but there was very light staining at 75 kDa, which would suggest a small amount of cellulase activity.

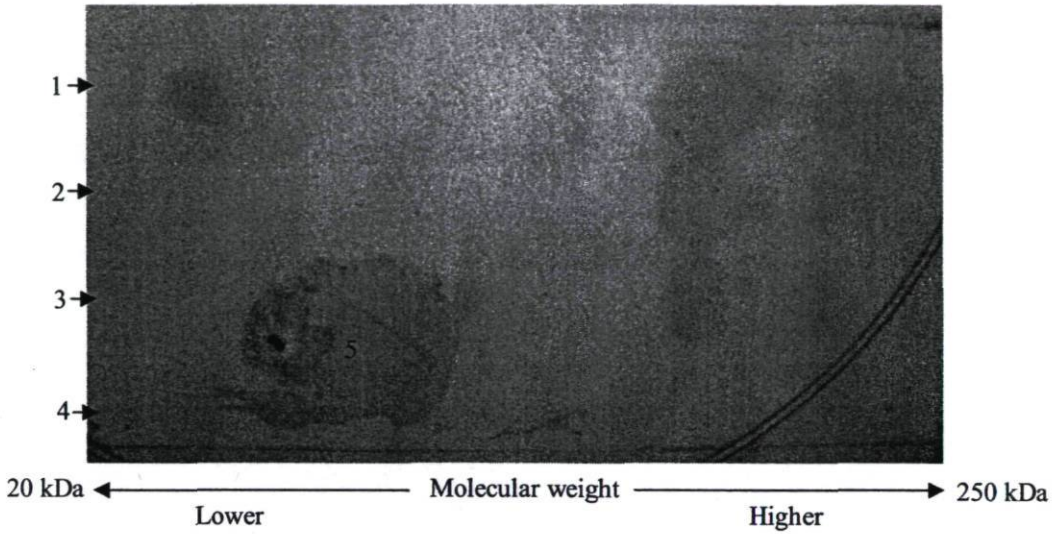


Figure 32: Zymogram of cellulase activity in balsam fir needles under various treatments. Treatment 1 is the cellulase standard, which three different isozymes can be seen. Treatment 2 is after 14 days of exposure to 1000 ppm ethylene. Treatment 3 is after 35 days, where endogenous levels of ethylene were allowed to increase prior to abscission. Treatment 4 is an untreated branch after 14 days, where there were no detectable levels of ethylene. The large spot at label 5 should be disregarded. It is an area where the gel was injured during storage.

7.4 Discussion

Many trends in ethylene and balsam fir abscission were consistent with other studies. Exogenous ethylene greatly reduced NRD, as shown in MacDonald et al. (2009c) and MacDonald et al. (2010b). In addition, ethylene evolution rates, timing, and XPP were all consistent with those presented in Chapter 4. These results provide further evidence to support the role of ethylene in abscission.

Interestingly, cellulase activity in needles of branches undergoing endogenous ethylene-induced abscission increased by approximately 8-fold compared to a control, while it increased by approximately 12-fold for needles of branches undergoing exogenous ethylene-induced abscission. Cellulase has been associated with abscission in fruits and/or leaves in a variety of species, such as bean (Lewis and Varner 1970), tomato (Taylor et al. 1990), peach (Bonghi et al. 1992) and citrus (Goren et al. 1973). However, there have been no previous studies on cellulase activity of balsam fir or any other Christmas tree variety.

The exact role of cellulase in abscission has been difficult to determine, partially because there are different forms of cellulase present (De Campillo 1988; Dows and Brady 1991), not all of which are actually involved in abscission (Brown 1997). For example, Durbin et al. (1981) found several different cellulases present in bean leaves, but only the form with an isoelectric point of 9.5 increased during abscission. Certainly evidence from balsam fir would support the form of two different cellulases, though in this case both forms increased during abscission.

Several hypotheses have been presented to explain the role of cellulase in organ abscission. The most frequent one is that cellulases degrade and weaken the cell wall to allow abscission (Sexton et al. 1985; Brown 1997). However, anatomical studies often show swelling rather than degradation of the primary wall (Brown 1997). It has also been proposed that cellulase is not involved in the actual separation, but is preparing the plant for wound healing (Addicott 1982). More consistent with the swelling shown in anatomical studies is the possibility that cellulase is involved in the turnover of hemicelluloses, which results in cellular expansion capable of generating forces to induce mechanical abscission (Sexton 1976; Sexton and Redshaw 1981). In addition, cellulases purified from plant extracts demonstrate differential activities against various xylogucans, which may result in cell separation (Brummell et al. 1984). Regardless of mechanism, cellulase activity is consistently associated with abscission in many species (Abeles et al. 1992), now including balsam fir.

7.5 Conclusions

Two complementary methods were used to detect cellulase activity. Image analysis of a cellulase standard on the digestion well assay allowed for quantification of cellulase activity, while zymography was able to identify presence of different cellulase isozymes.

Both methods were able to detect cellulase activity from needle extracts of balsam fir experiencing abscission. In general, the cellulase activity of balsam fir experiencing abscission is approximately 10 times greater than a control showing no signs of abscission.

8.0 THE RELATIONSHIP BETWEEN ETHYLENE SENSITIVITY AND NEEDLE ABSCISSION RESISTANCE IN GENOTYPES

The following scientific contributions have been generated from content of Chapter 8:

MacDonald and Lada RR. 2008. Cold acclimation can benefit only the clones with poor needle retention duration (NRD) in balsam fir. HortScience. 43: 1273 (abstract).

MacDonald MT, Lada RR, Martynenko AI, Dorais M, Pepin S, Desjardins Y. 2010. Is there a relationship between ethylene evolution, ethylene sensitivity, and needle abscission in root-detached balsam fir? 28th International Horticultural Congress, Aug. 22-27.

MacDonald MT, Lada RR, Martynenko AI, Dorais M, Pepin S, Desjardins Y. 2010. Is there a relationship between ethylene evolution, ethylene sensitivity, and needle abscission in root-detached balsam fir? HortScience. IN PRESS.

8.1 Introduction

Several recent studies have attempted to identify ethylene as the physiological trigger to induce needle abscission in balsam fir. When detached balsam fir branches were exposed to exogenous ethylene, the length of time required for abscission was decreased by 60-70% (MacDonald et al. 2009c; MacDonald et al. 2010b). In contrast, blocking ethylene receptors with 1-methylcyclopropene negated the abscission due to exogenous and endogenous ethylene while inhibition of ethylene synthesis using aminoethoxyvinyglycine negated the abscission due to endogenous ethylene (MacDonald et al. 2010b). In both studies it was concluded that ethylene is the signal required for abscission in balsam fir, though it remains unclear what factor(s) may induce ethylene evolution and, subsequently, abscission in balsam fir.

Factors influencing needle abscission in several species are well known, such as hydration (Hinesley and Snelling 1997), nutrition (Balster and Marshall 2000), cold acclimation (Mitcham-Butler et al. 1987a) or pollution (Glutch 1988). However, there is limited information available specific to balsam fir. One potential factor is harvest date. A study conducted with Fraser fir, a species closely related to balsam fir, concluded that a delay in harvest date would significantly improve post-harvest needle retention (Mitcham-Butler et al. 1988). A similar conclusion was made from a study on balsam fir,

which found that an earlier harvest could reduce needle retention by approximately 1 week (MacDonald and Lada 2008).

It has been shown that there is a great deal of genotypic variation in needle abscission characteristics of fir trees. In an experiment with Noble and Nordmann fir trees, significant differences were found in abscission of needles from trees growing adjacent to one another (Chastagner and Riley 2003). A separate study determined that needle abscission characteristics were highly heritable among Nordmann fir trees (Nielsen and Chastagner 2005). Similar results have been reported in balsam fir. When 195 different genotypes were tested, some genotypes shed all needles as early as 6 days after harvest while others did not shed needles until after 60 days (MacDonald and Lada 2008).

How might the genotypic variation in balsam fir be related to other key abscission related factors? Perhaps certain genotypes are less affected by an early harvest or are less sensitive to the effects of ethylene. Many species have identified ethylene-insensitive or ethylene-overproducing varieties (Lanahan et al. 1994; Guzman and Ecker 1990; Knoester et al. 1997), where effects can be observed in typical ethylene responses, such as ripening or abscission. Thus, the objective of this research was to determine the relationship between genotypic variation, harvest time, and ethylene evolution. More specifically, this research attempts to answer: (1) whether there is any genotypic difference in needle abscission of early and late harvested balsam fir, (2) whether genotypic differences in abscission may be affected by increased sensitivity to ethylene, and (3) whether different genotypes can be identified by their ability to evolve ethylene.

8.2 Classification of genotypes according to needle retention characteristics

8.2.1 Objective

To identify the needle retention duration characteristics of all genotypes available at the Debert Tree Breeding Center.

8.2.2 Methods

Samples were collected over 2 years. Early harvest samples were collected on October 15, 2006 and 2007. Late harvest samples were collected on January 26, 2007 and 2008. In the first year, 114 branches were collected each harvest and 169 branches were collected in the second year, where each branch represented a different genotype. It is acknowledged that the late harvest samples were collected later than any Christmas tree harvest dates in Nova Scotia, but a severe winter storm made the trail inaccessible until that date. Air temperature in Debert was recorded hourly at the Debert weather station. Growing degree days were calculated with the following equation from Hassan and Bourque (2009):

$$\text{Growing degree days} = \sum_{i=1}^{i=n} \overline{T}_a - T_{base} \text{ ; when } \overline{T}_a - T_{base} > 0$$

In the above equation, $\overline{T}_a$ is the daily mean air temperature as determined from the average of daily minimum and maximum temperatures, T_{base} is the threshold temperature as determined by the temperature below which plant growth ceases (approximately 5°C for balsam fir (Hassan et al. 2007)), i = 1 represents the start of a month, and i = n represents the last day of a month.

The primary response variable was needle retention duration (NRD). With respect to the experiment on harvest date, NRD was subdivided into initial NRD (NRD_0) for early harvest and final NRD (NRD_f) for late harvest. The difference between NRD_0 and NRD_f (calculated as $NRD_f - NRD_0$) was reported as ΔNRD. In addition, several physical characteristics were collected to be evaluated as potential predictors for needle retention, including needle length, branch length, number of buds, color, and softness. Needle length was calculated as the average of 10 randomly collected needles. Branch length was calculated as the distance from the fresh cut to the end of the primary branch. A scale from 1-6 was developed for color and softness. To reduce variability in using each scale, the same technician evaluated all branches.

8.2.3 Results

In general, temperatures in 2007 and 2008 were both relatively consistent with normal temperatures for the area (Table 3). However, it should be noted that the mean temperature and number of growing degree days was lower in October 2008 than October 2007. When the two week period prior to sample collection was analyzed for each year, the differences became much more pronounced. From Oct 1 – Oct 14, 2007 the average temperature was 9.8 °C with 72.6 growing degree days and 2 instances of frost. In the same span of time for 2008 the average temperature was 8.7 °C with 52.2 growing degree days and 6 instances of frost. The opportunity for some degree of cold acclimation was greater for the early harvest in 2008 than in 2007.

Table 3: Average monthly temperature and growing degree days in Debert for both seasons of balsam fir sampling gathered from a Debert weather station. Normal temperature and growing degree days for Debert were obtained from Environment Canada (2010).

Year	2007	2008	Normal
Mean Temperature (°C)			
September	13.7	13.9	13.7
October	8.4	7.8	8.0
November	2.5	2.9	3.0
December	-6.8	-2.3	-3.6
January	-6.0	-10.2	-6.6
Min Temperature (°C)			
September	7.1	8.3	7.9
October	2.2	2.4	3.0
November	-3.4	-1.7	-1.0
December	-12.6	-8.5	-8.2
January	-11.4	-16.8	-11.7
Max Temperature (°C)			
September	20.1	19.4	19.5
October	14.6	13.3	12.9
November	8.4	7.6	6.9
December	-1.1	3.8	1.0
January	-0.5	-3.5	-1.5
Growing Degree Days			
September	259.5	267.2	262.4
October	116.1	95.5	105.1
November	31.6	57.6	32.7
December	0.0	11.2	4.1
January	0.6	0.0	1.3

Harvest date had a significant impact on NRD in 2007, when branches harvested earlier would experience complete abscission approximately 6 days earlier than the late harvest (Fig. 33). There was no significant difference observed in 2008. However, in both years NRD_0 was a significant predictor for NRD_f, though the coefficient of determination was quite low (in 2007, $R^2 = 22.8\%$; in 2008, $R^2 = 15.2\%$). It was much more reliable to use NRD_0 to predict ΔNRD (in 2007, $R^2 = 77.7\%$; in 2008, $R^2 = 53.1\%$). It can be seen in Fig. 34 that those branches with a high NRD_0 were less affected by harvest date. No physical characteristics were useful in predicting NRD (Table 4).

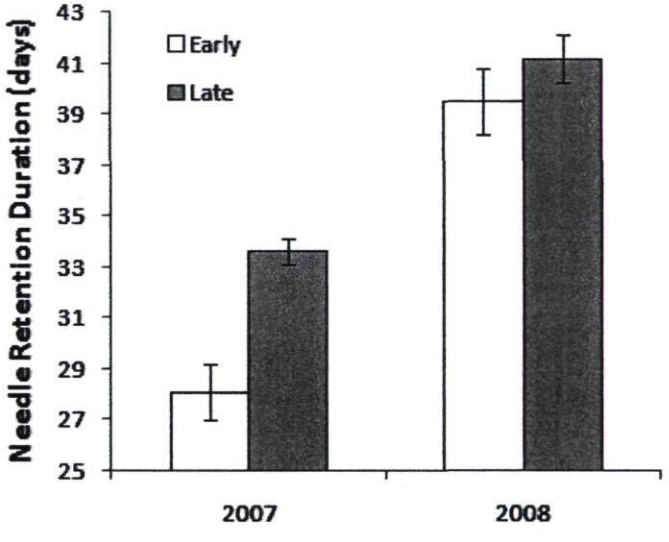


Figure 33: Comparison of needle retention duration of early and late harvest dates in 2007 (n = 114) and 2008 (n = 169). There was a significant difference in 2007 ($p < 0.001$) but no difference in 2008 ($p = 0.274$).

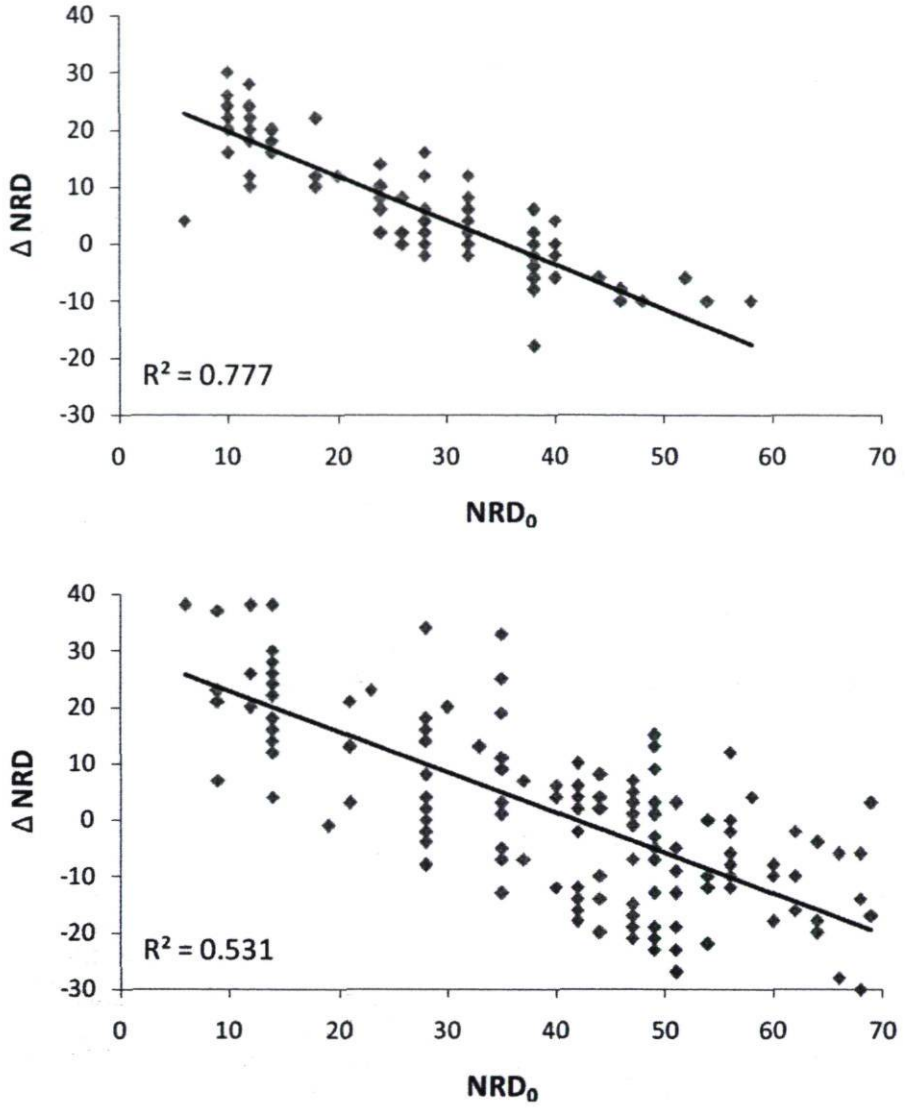


Figure 34: Significant ($p < 0.001$) linear regression of NRD_0 with the ΔNRD as a result of later harvest date in: A) 2007 ($n = 114$), which is described by $\Delta NRD = -0.774NRD_0 + 27.31$; B) 2008 ($n = 169$), which is described by $-0.715NRD_0 + 29.93$. For reference, $\Delta NRD = NRD_f - NRD_0$.

Table 4: Correlation of certain physical parameters with needle retention duration of balsam fir trees sampled in 2006. No parameters were significant at $\alpha = 0.05$.

Parameter	Correlation Coefficient	p-value
Needle Length	- 0.096	0.184
Branch Length	- 0.038	0.621
Bud Count	+ 0.044	0.567
Softness	+ 0.022	0.793
Color	+ 0.013	0.863

8.3 Genotypic differences in needle abscission in response to exogenous ethylene

8.3.1 Objective

To determine whether genotypic differences in needle retention may be attributed to differences in sensitivity to ethylene.

8.3.2 Methods

It was previously established that prolonged exposure to exogenous ethylene could induce premature abscission in balsam fir (MacDonald et al. 2009c; 2010b). This portion of the study was conducted to determine the response of different genotypes to ethylene. Branches were collected from a clonal orchard on Jan 29, 2009 as described in Chapter 4. This experiment was designed as a 3 x 2 factorial. The first factor was genotype. Based on work by MacDonald and Lada (2008), needle retention characteristics of available balsam fir genotypes were classified as low (0-20 days), moderate (21-40 days), or high (41-60 days). The second factor was ethylene concentration, which was either 0 ppm (control) or 1000 ppm. Each treatment was replicated 5 times.

For balsam fir, ethylene exposure must be prolonged in order to induce abscission (MacDonald et al. 2009). After each branch was placed in an incubation chamber, branches randomly assigned to the ethylene treatment were injected with 1000 ppm ethylene. After 24 hours all branches were removed, weighed, and inspected for needle abscission by running fingers across a branch. Each branch was then replaced in its assigned incubation chamber and appropriate treatments were exposed to 1000 ppm ethylene once again. The procedure continued until complete abscission had occurred. Response variables at the end of the experiment were NRD, xylem pressure potential (XPP), average daily water use (AWU), and chlorophyll index. The details of all response variables are described below.

8.3.3 Results

There was a significant ($p < 0.001$) interactive effect on NRD for genotype and ethylene. The effect of ethylene on NRD was relatively consistent, where ethylene decreased NRD

by between 60-70% in all genotypes (Fig.35). In the control treatment, genotypic differences were as expected, where branches categorized as having high needle retention characteristics had the highest NRD and low needle retention characteristics had the lowest NRD. The same trend occurred in the ethylene treatment with the exception that there was no significant difference between moderate and high needle retention characteristics (Fig. 36).

Figure 35: Three genotypes classified as having low, moderate, and high NRD characteristics in the absence of ethylene (left) and continuously exposed to ethylene (right) after 15 days.

Ethylene had no effect on AWU, though there was a significant ($p = 0.012$) effect from genotype (Fig. 36). The low NRD genotype that was not exposed to ethylene had the highest AWU; the high NRD genotype used significant less water than other genotypes. On the other hand, ethylene was the only factor to have a significant ($p < 0.001$) effect on XPP (Fig. 36). In all tested genotypes, prolonged exposure to ethylene decreased XPP by 140-170%.

There were no significant effects on chlorophyll from any factor. Regardless of genotype or ethylene, the average chlorophyll index for balsam fir branches is reported as 18.6. There were no visible symptoms of senescence before abscission in balsam fir.

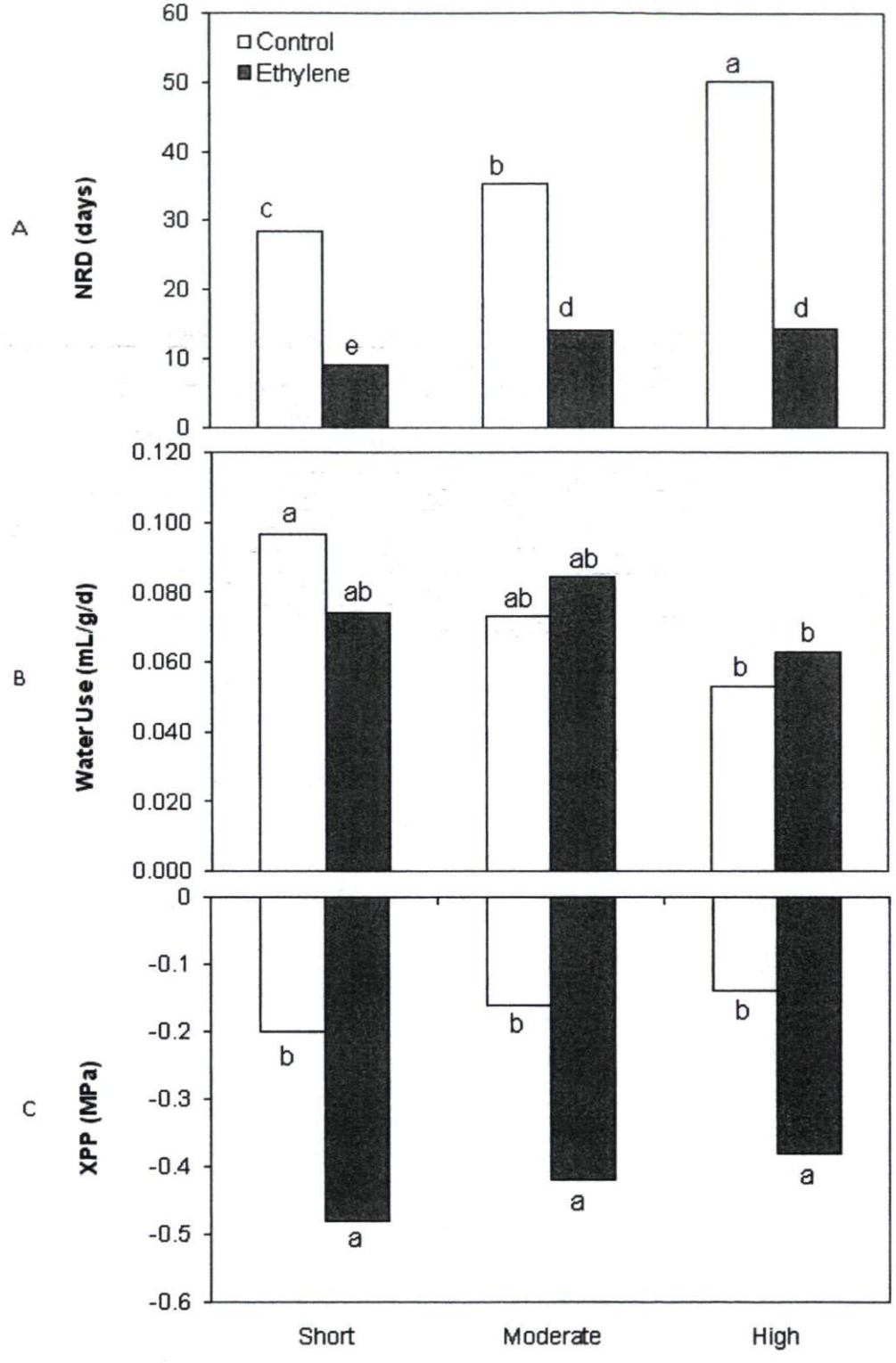


Figure 36: The effect of exogenous ethylene on A) needle retention, B) average daily water use, and C) xylem pressure potential of detached balsam fir branches which were classified by needle retention characteristics. Each treatment had 5 replications. Letter groupings indicate a significant differences using least squares means comparison at 5% significance level.

Both AWU and XPP had a significant relationship with NRD in balsam fir (Fig. 37), though the relationship between XPP and NRD was much stronger. When stepwise regression was performed, XPP and AWU were each identified as significant explanatory variables for multiple linear regression. The model was NRD = 61.2 + 6.9XPP - 200AWU with an adjusted R^2 = 68.9%. In general, the relationship suggests that needle retention decreases as when water use increases and/or XPP decreases.

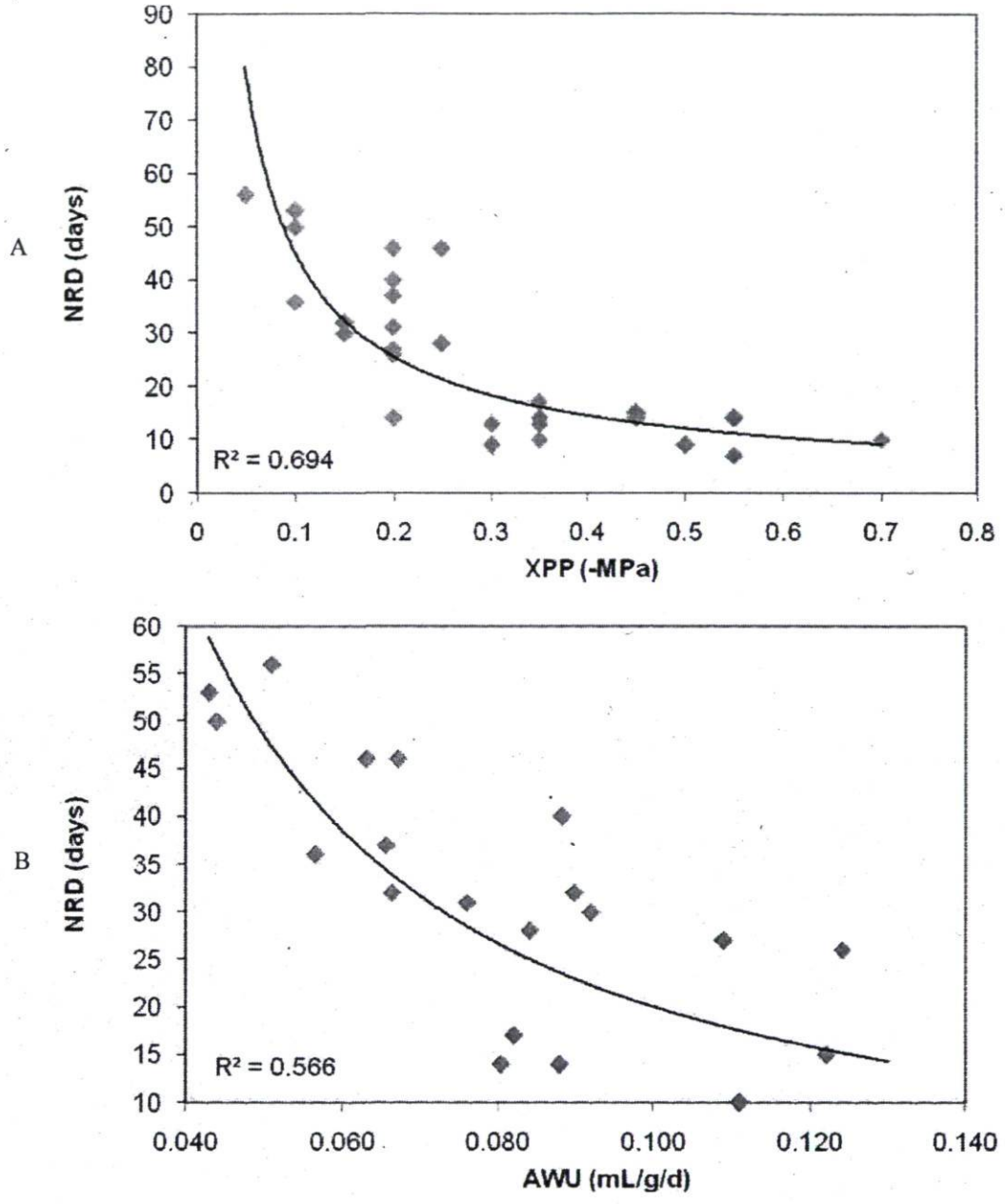


Figure 37: A) Significant relationship between xylem pressure potential and needle retention as described by NRD = $6.79XPP^{-0.82}$. It should be noted that XPP was expressed as a positive number because a power function best describe the relationship best, but would not be possible with negative values; B) significant relationship between average daily water use and needle retention as described by NRD = $1.071AWU^{-1.27}$.

8.4 Genotypic differences in endogenous ethylene evolution and sensitivity

8.4.1 Objective

To determine whether genotypic differences in needle retention may be attributed to differences in ethylene evolution.

8.4.2 Methods

This experiment was designed to determine if observed genotypic differences in NRD could be explained by sensitivity or evolution of ethylene. Branches were randomly selected from genotypes classified as having low NRD and high NRD characteristics (35 branches from each genotype) as described in Chapter 4. Each branch was placed in an incubation chamber and exposed daily to 0, 10, 50, 100, 250, 500, or 1000 ppm exogenous ethylene. This was replicated 5 times. After 24 hours, each sample was removed, inspected for needle loss, and then placed in an empty to determine ethylene evolution (as described in Chapter 4). Response variables were NRD and ethylene evolution, which are described in more detail below.

8.4.3 Results

There were significant ($p < 0.001$) linear relationships between ethylene concentration and needle retention in low and high NRD genotypes (Fig. 38). It was also shown that the slope in a low NRD genotype was significantly ($p = 0.0379$) steeper than that of high a NRD genotype. A comparison of both equations would suggest that low NRD genotypes are influenced more by ethylene concentration.

Ethylene evolution rates were not affected by exogenous ethylene, but there was a significant ($p < 0.001$) difference between genotypes. The high NRD genotype released ethylene at a rate of 9.9 $\mu L \cdot g^{-1} \cdot h^{-1}$. The low NRD genotype released ethylene 47% faster, at a rate of 14.6 $\mu L \cdot g^{-1} \cdot h^{-1}$.

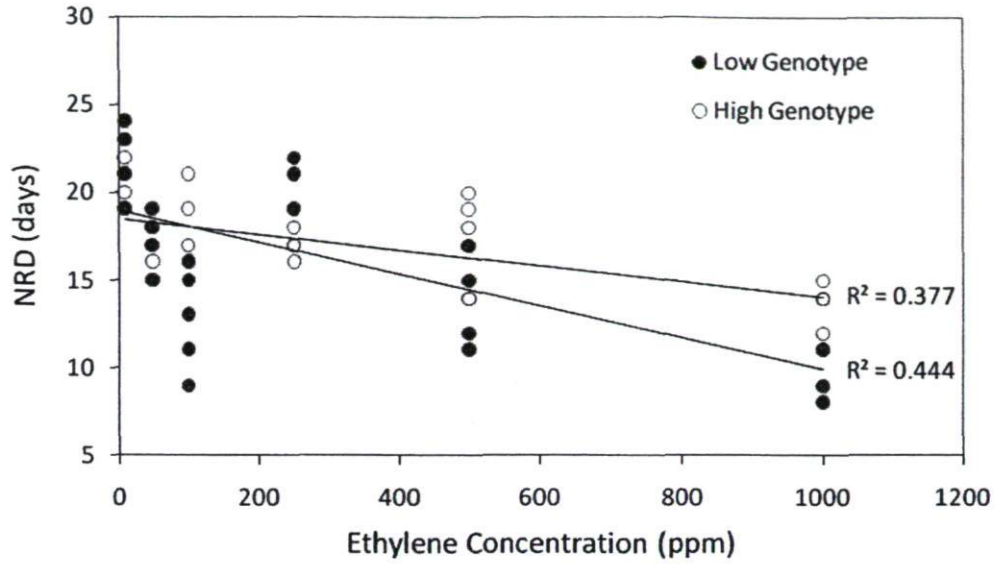


Figure 38: Linear relationships between ethylene concentration and NRD in balsam fir categorized as having low and high needle retention characteristics. In high NRD genotypes, the relationship is described as NRD = -0.004Concentration + 18.46. In low NRD genotypes, the relationship is described as NRD = -0.009Concentration + 18.97. There is a significant ($p = 0.0379$) difference between the slopes of each equation. N=35.

8.5 Discussion

There were conflicting results regarding the effect of harvest date on needle retention. In 2007 early harvest decreased average needle retention by approximately 1 week, which is similar to the effect of early harvest on other conifer species (Chastagner and Riley 2003; Mitcham-Butler et al. 1988). However, in 2008, early harvest decreased average needle retention by 2 days, which was not a significant difference. From this experiment we cannot be certain of the reason for such different results between 2007 and 2008, but it is speculated that the weather in weeks prior to sample collection may contribute. Cold temperatures have been shown to have a profound effect on plant physiology including accumulation of sugars (Beck et al. 2004), down regulation of photosynthesis (Oquist and Huner 2003; Allen and Ort 2001), reduction of stomatal conductance (Delucia 1986), and hardening of plasma membranes (Uemura et al. 1995; Steponkus 1984). Though the effects of specific cold-induced changes in physiology on needle retention have not been studied, it has been demonstrated that a period of cold acclimation is beneficial for conifers (Mitcham-Butler et al. 1987a; McGuire et al. 1962). With cooler weather, determined by lower average temperature, lower growing degree days, and more

instances of frost, the opportunity for cold acclimation would be greater in October 2008 than October 2007.

Perhaps more interesting is the relationship between initial needle retention and the change experienced from a delay in harvest. In both years of data collection it is shown that genotypes with poor initial needle retention receive more benefit from a late harvest and *vice versa*. In addition, genotypes were identified where harvest time had little to no influence on needle retention. Such a finding could have a practical impact for producers. Perhaps genotypes resistant to detriments from early harvest could be grafted onto existing root stock, similar to practices of grafting certain disease resistant trees to root stock (Thompkins 1989). In this manner, Christmas tree producers could develop a plantation of trees specifically for early harvest.

Daily exposure to high concentrations (1000 ppm) of exogenous ethylene induced premature abscission in balsam fir, with a similar response across all tested genotypes. The response to 1000 ppm ethylene was consistent with several prior studies (MacDonald et al. 2009c; MacDonald et al. 2010b). One such study used a combination of endogenous ethylene, exogenous ethylene, receptor blockers, and ethylene inhibitors to conclude that ethylene was the signal required for abscission in root-detached balsam fir (MacDonald et al. 2010b). Our study adds to the current knowledge of ethylene in balsam fir by identifying that genotypes with low needle retention are more sensitive to ethylene. When the relationship of low and high NRD genotypes was plotted against ethylene, the rate of change for NRD in low NRD genotypes was more than double that of high NRD genotypes. Furthermore, endogenous ethylene evolution rates were almost 50% higher in low NRD genotypes. Thus, it appears low NRD genotypes are more sensitive to and over-express the signal responsible for abscission.

Only a small piece of the abscission pathway in balsam fir has been uncovered. In essence it has been established that trees begin to synthesize ethylene after harvest, which results in premature abscission (MacDonald et al. 2010b). In addition, we now know that the severity in response is different for different genotypes. It is expected that ethylene

likely invokes certain degradative enzymes, such as cellulases, polygalacturonases, and pectinases to weaken cell walls in the abscission zone in a similar manner to that observed in other species (Chapter 7; Sexton and Roberts 1982; Tucker et al. 1988).

There has also been no conclusive evidence as to why ethylene synthesis should begin, though there is some interesting evidence provided in this research. When NRD was compared to AWU and XPP, there was a highly significant relationship. It was shown that NRD decreased as AWU increased, which supported previous findings by MacDonald et al. (2010). It was also shown that NRD decreased as XPP decreased which, though not reported in balsam fir, had been suggested in other species (Hinesley 1984; Chastagner and Riley 2003). However, these relationships also suggest that XPP becomes more negative when water use increases, which is counterintuitive. It is likely that this study has suggested a weakness in using AWU as a response variable. Over time, water use diminishes in all branches, but AWU is determined from the length of time until complete abscission. In genotypes with high NRD, the length of time until abscission is much longer, thus AWU appears much shorter. It appears AWU, as it is currently calculated, will be a poor indicator of water use in branches with vastly different NRD unless measured at the same day for all branches. Regardless, a decrease in XPP still suggests a change in water relations after harvest could trigger ethylene synthesis to begin then culminate in complete needle abscission. Further research is needed to investigate that hypothesis.

8.6 Conclusions

In conclusion, there is some evidence to suggest that earlier harvest may be detrimental to needle retention in balsam fir, though complications due to environmental conditions between seasons may have influenced these results. More specifically, genotypes with poor needle retention characteristics have significantly reduced needle retention and display increased sensitivity and ethylene evolution rates. Meanwhile those genotypes with high needle retention are less affected by early harvest and appear less affected by ethylene. Identification of genotypes resistant to harvest time and ethylene may be of practical interest to producers.

9.0 THE RELATIONSHIP BETWEEN WATER RELATIONS AND ETHYLENE-INDUCED ABSCISSION

Results from Chapter 9 are prepared as:

MacDonald MT, Lada RR, Martynenko AI, Dorais M, Pepin S, Desjardins Y. 2010. The effect of managing water relations on ethylene evolution and needle abscission in post-harvest balsam fir. HortScience. UNDER REVIEW

9.1 Introduction

Ethylene is the simplest unsaturated hydrocarbon, which regulates many diverse metabolic and developmental processes in plants. The most studied process related to ethylene is its role in senescence and abscission on various plant tissues (Brown 1997). Typically, ethylene is thought to increase prior to abscission and stimulate activity of several enzymes, such as cellulase and pectinase (Sexton and Roberts 1982; Taylor et al. 1990). The enzymes have a variety of roles, which include weakening of cell walls, dissolution of middle lamella, and swelling of cells in the abscission zone (Sexton et al. 1985; Addicott 1982; Wright and Osborne 1974).

Recently, ethylene has been strongly implicated as the signal responsible for abscission in balsam fir through several different experiments (Chapters 4 to 8). One such experiment measured the increase of endogenous ethylene in root-detached balsam fir and found that it coincided with needle abscission (MacDonald et al. 2010b). When balsam fir branches were exposed to a continuous source of ethylene, abscission was greatly accelerated. However, when synthesis or perception of ethylene was inhibited, abscission was greatly delayed (MacDonald et al. 2009c; 2010b). Endogenous and exogenous ethylene was also found to stimulate cellulase activity immediately preceding abscission (Chapter 6).

An initial report by Lada and Thiagarajan (2005) stated that balsam fir branches which consumed more water would tend to lose needles more quickly that other branches. This finding prompted water use and xylem pressure potential to be monitored in several future studies. MacDonald et al. (2010b) found a significant relationship to suggest that branches which consume more water lose needles faster. It was also found that

endogenous or exogenous sources of ethylene decreased XPP (Chapter 6) and genotypes with poor needle retention characteristics also had a lower XPP (Chapter 7). Originally it was thought that decreased water status may trigger ethylene, but the fact that exogenous ethylene decreased XPP in a much shorter timeframe may suggest that ethylene somehow modifies water uptake or transpiration.

Studies on the influence of ethylene on stomata have had conflicting results. Aharoni (1978) modified ethylene production using AVG as well as maintaining leaves under subatmospheric pressures. In each case, ethylene production was significantly decreased with no change in water status deficit. Similar results were found when 10 μM and 100 μM of AVG were applied to *Vicia faba* and *Gycline max* (Tissera and Ayres 1986; Taylor et al. 1988). Futhermore, aerial fumigation with ethylene did not affect stomatal conductance in *Lycopersicon esculentum* or *Pisum sativum* (Taylor and Gunderson 1986).

More recent studies have found that ethylene may cause stomatal opening or, at the very least, delay or inhibit stomatal closure. Application of ethrel, an ethylene liberating agent, opened stomata in *Vicia faba* (Levitt et al. 1987). In another study, incubation of epidermal strips from *Vicia faba* with high concentrations of ACC resulted in a 13% increase in stomatal aperture (Merritt et al. 2001). Strong evidence for the role of ethylene comes from a study using ethylene over-producing mutants. In wild type Arabidopsis *in vitro*, application of ABA was able to induce stomatal closure. However, in ethylene over-producing mutants, the ABA-induced stomatal closure occurred to a lesser extent. In addition, ABA-induced stomatal closure in wild type could be overcome by exogenous ACC or ethylene application (Tanaka et al. 2005). Further investigations observed ethylene-induced effects *in planta*. Exogenous ethylene was able to inhibit stomatal closure in both ABA-irrigated and drought stressed conditions. In addition, the transpiration rate of all plants treated with ethylene was significantly higher (Tanaka et al 2005). Finally, Azuma et al. (2003) found a 60% increase in transpiration of rice seedlings exposed to ethylene when compared to a control.

Studies in balsam fir tend to support the concept that ethylene may impair stomatal function and leave them open, thus increasing water use and transpiration, which may lead to abscission. If that is true, branches stored in conditions to reduce transpiration may delay abscission. The objectives of this study were to determine the relationships between water use and needle abscission and determine if reducing transpiration will delay abscission in post-harvest balsam fir.

9.2 Water use in balsam fir in the presence and absence of exogenous ethylene

9.2.1 Objective

To determine the relationship between water use and needle abscission in various conditions known to modify NRD.

9.2.2 Methods

All branches were collected, transported, and stored as described in Chapter 4. Four different experiments were conducted to determine the relationship between AWU and NRD.

In the first experiment, 70 branches were randomly collected from the Tree Breeding Center and placed in a growth chamber and were not treated with ethylene. In the second experiment, continuous exogenous ethylene was introduced as a factor (0, 10, 100, 500, or 1000 ppm) with 4 replicates (i.e. 20 branches). The third experiment was designed as a 2 x 5 factorial where one factor was daily exogenous ethylene (0 ppm or 1000 ppm), the second factor was 1-MCP (0, 2.5, 5.0, 7.5, or 10 g), and there were 4 replicates (i.e. 40 branches). The final experiment was designed as a 2 x 6 factorial where one factor was daily exogenous ethylene and the other factor was AVG (0, 1, 10, 100, 500, or 1000 ppm) with 4 replicates (i.e. 48 branches). Application of 1-MCP and AVG was described in detail in Chapter 5.

Response variables were NRD, AWU, and daily water use. To calculate daily water use, each branch and flask unit was weighed daily. Any change in weight, once adjusted for needle loss, was assumed to be water use. Each experiment was submitted to regression

analysis using Minitab 15 using AWU as the explanatory variable and NRD as the response. In addition, analysis of the first experiment also used regression with day as the explanatory variable and AWU as the response.

9.2.3 Results

After the first day, AWU was relatively high at 0.41 $mL \cdot g^{-1} \cdot d^{-1}$ in untreated branches. However, AWU declined very rapidly. In only 5 days, AWU had been reduced to half its initial value. Within 2 weeks, AWU had stabilized at its minimum value of 0.04 $mL \cdot g^{-1} \cdot d^{-1}$ (Fig. 39). There was also a strong negative relationship between AWU and NRD in untreated branches (Fig. 40).

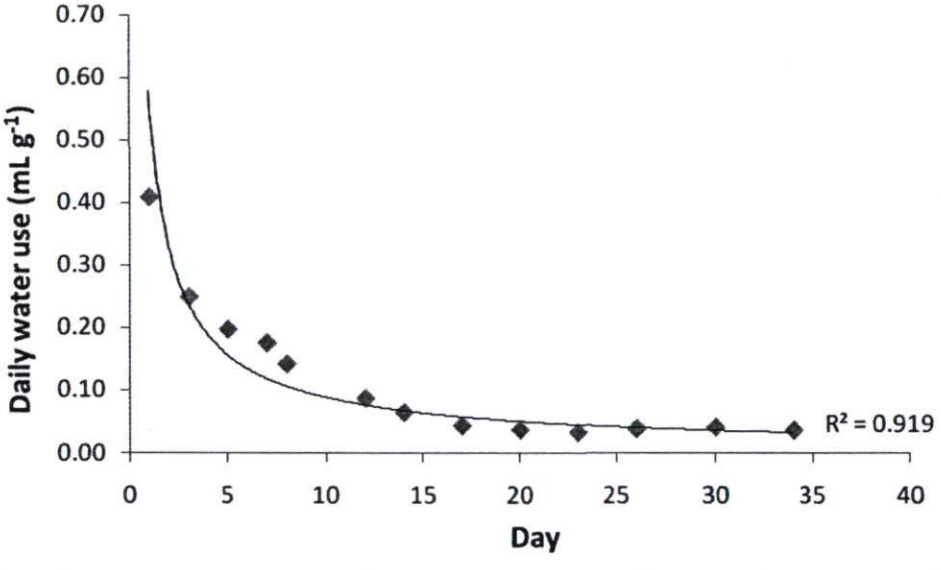


Figure 39: Declining water use of balsam fir after root-detachment taken from the average of 70 branches. The relationship is best described by $AWU = 0.58\ (Day)^{-0.81}$.

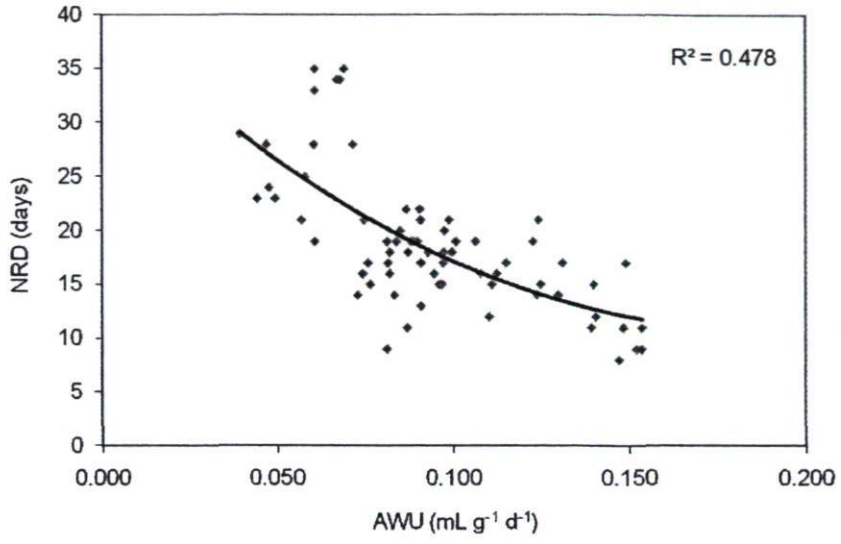


Figure 40: Significant ($p < 0.001$) relationship between AWU and NRD in branches in the absence of exogenous ethylene (n=70). The relationship was described by $NRD = 2.6(AWU)^{-0.75}$.

There was a significant negative relationship between AWU and NRD when branches were exposed to exogenous ethylene (Fig. 41). Similar relationships were observed when ethylene action was inhibited with either 1-MCP or AVG (Fig. 42). The negative relationships suggest that increased water consumption is associated with premature needle abscission.

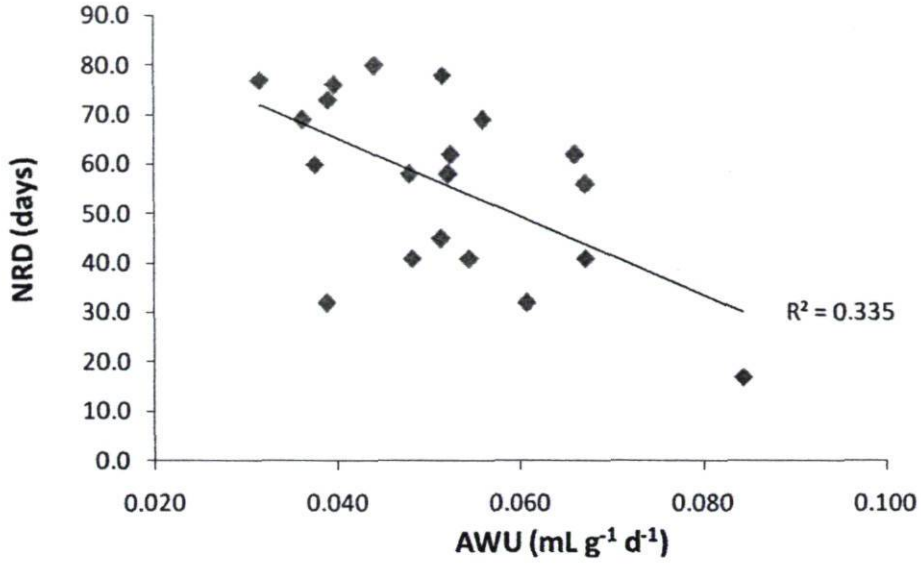
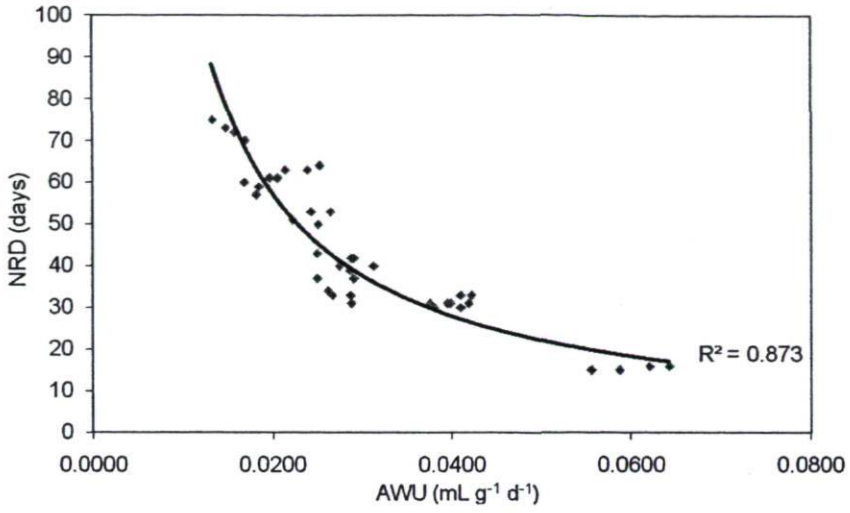


Figure 41: Significant ($p < 0.001$) relationship between AWU and NRD in branches after exposure to exogenous ethylene (n=20). The relationship is described by NRD = -792(AWU) + 97.

A



B

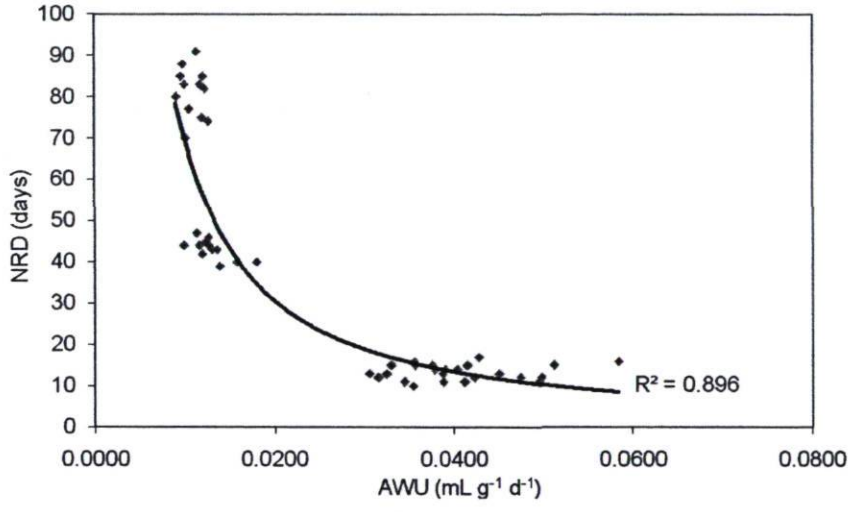


Figure 42: Significant ($p < 0.001$ in both) relationships between AWU and NRD in: A) branches exposed 1-MCP (n=40), which is described by $NRD = 1.0(AWU)^{-1.03}$, and B) branches exposed to AVG (n=48), which is described by $NRD = 0.3(AWU)^{-1.18}$. Each trend suggests that branches which consume more water will shed needles faster.

9.3 Effect of storage humidity on ethylene evolution and abscission in balsam fir

9.3.1 Objective

To determine the effects of high humidity on branch water relations and needle abscission.

9.3.2 Methods

Twenty-four branches were collected from the Tree Breeding Center on February 11, 2010 and transported as described in Chapter 4, but storage conditions were modified. The experiment was designed as a 2 x 3 factorial with 4 replicates, where a single branch served as a replicate. The first factor was continuous ethylene (0 or 1000 ppm) while the second factor was humidity (30, 60, or 90%). Humidity was maintained using misting fans with controlled water flow in the growth chamber and never changed by more than 1% of the assigned value. The chamber was kept at a day temperature of 22°C for 16 hours with a light intensity of 80 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and a night temperature of 15°C for 8 hours in darkness, which can be combined as an average temperature of 19.7°C. As a result, 30, 60, and 90% relative humidity corresponded with average vapour pressure deficits of approximately 1.59, 0.91, or 0.23 kPa.

Each branch was placed in an EIC and each EIC was placed in the growth chamber for 24 hours before the start of an experiment to allow EIC conditions to mimic those of the growth chamber. As noted in earlier experiments, the EIC was aerated daily to prevent CO_2 build up. This also allowed for humidity and temperature to be maintained at the same levels as the growth chamber.

Response variables were NRD, AWU, XPP, DPE, and ethylene evolution which are described in detail in Chapter 4. Analysis of variance was conducted using Minitab 15 to detect significant main and interaction effects. Effects deemed to be significant were separated using least squares means in SAS.

9.3.3 Results

There was a significant ($p<0.001$) interaction between ethylene concentration and humidity for both NRD and DPE. Humidity had no effect on needle retention in the presence of 1000 ppm ethylene, but higher humidity favoured needle retention in the absence of ethylene (Fig. 43). A 60% humidity increased NRD and DPE by approximately 10% when compared to 30% humidity. However, 90% humidity increased NRD and DPE by more than 3-fold when compared to 30% humidity (Table 5). There was a near perfect relationship between DPE and NRD (R^2=99.9%) where peak ethylene evolution was observed an average of 1.6 days before complete abscission (Fig. 44).

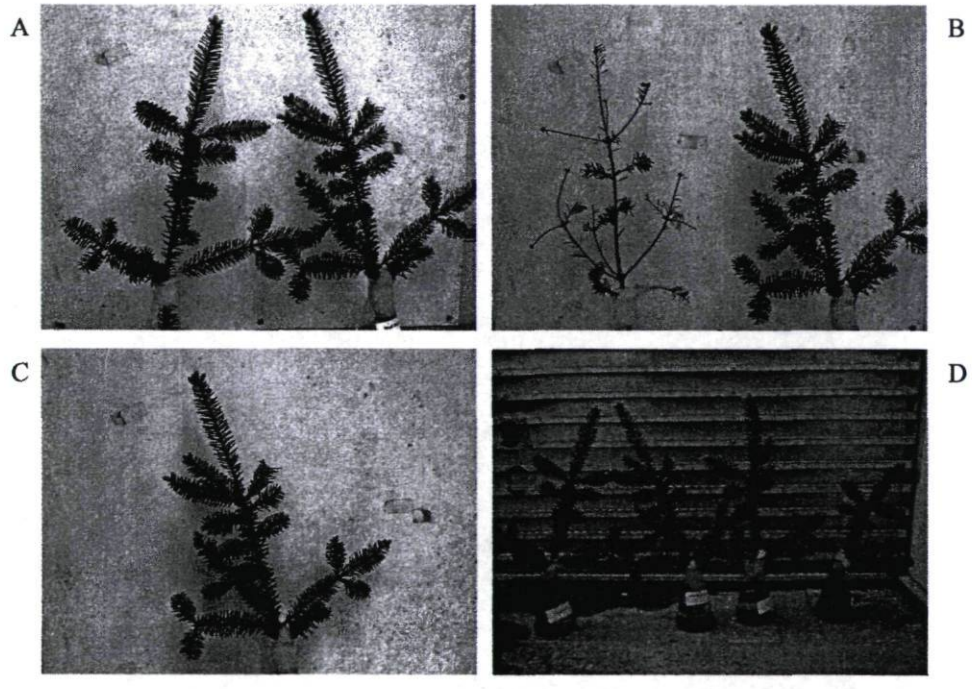


Figure 43: Comparison of branches stored at A) 30% (left) and 90% (right) humidity on day 1, B) 30% (left) and 90% (right) humidity on day 30, and C) 90% humidity on day 60 in the absence of exogenous ethylene. D) All replications stored at 90% humidity in the absence of exogenous ethylene are still vibrant and green at day 92.

Table 5: Effect of ethylene and humidity on root-detached balsam fir branches. Treatment means were calculated from 4 replications and separated using least squares means with $\alpha = 0.05$. NRD = needle retention duration; AWU = average daily water use; XPP = final xylem pressure potential; DPE = days until peak ethylene evolution.

Ethylene (ppm)	Humidity (%)	NRD (days)	AWU ($mL \cdot g^{-1} \cdot d^{-1}$)	XPP (MPa)	DPE (days)	C_2H_4 Peak Evolution ($\mu L \cdot g^{-1} \cdot h^{-1}$)
0	30	28.5^{c}	0.061^{a}	-0.73^{b}	27.0^{c}	7.5
	60	32.3^{b}	0.038^{b}	-0.40^{c}	30.5^{b}	6.4
	90	149.8^{a}	0.021^{c}	-0.09^{d}	145.8^{a}	6.1
1000	30	11.8^{d}	0.058^{a}	-0.94^{a}	10.3^{d}	5.8
	60	12.0^{d}	0.041^{b}	-0.84^{a}	10.3^{d}	5.4
	90	13.8^{d}	0.029^{bc}	-0.81^{a}	12.3^{d}	7.1

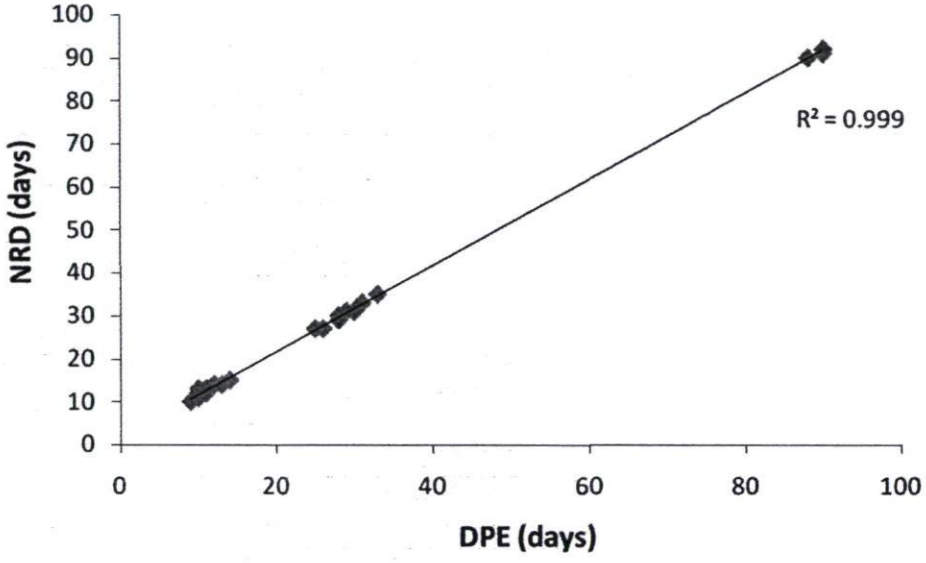


Figure 44: Significant ($p < 0.001$) and near perfect relationship between DPE and NRD. The relationship is best described by NRD = DPE + 1.6.

AWU was only affected by humidity. When branches were stored at 90% humidity instead of 30% in the presence of exogenous ethylene, AWU was reduced by 50%; when branches were stored at 90% humidity instead of 30% in the absence of exogenous ethylene, AWU was reduced by 67% (Table 5). XPP was generally lower at low humidity, regardless of exogenous ethylene. However, at 90% humidity, XPP was influenced by ethylene. At 90% humidity, XPP was four times lower in branches in the presence of exogenous ethylene when compared to branches stored in the absence of exogenous ethylene (Table 5). There was a significant ($p < 0.001$) relationship between AWU and XPP (Fig. 45)

Neither ethylene concentration nor humidity had any effect on the peak endogenous ethylene evolution rate, which ranged from 4.2 to 9.2 $\mu L \cdot g^{-1} \cdot h^{-1}$ when all replications were considered.

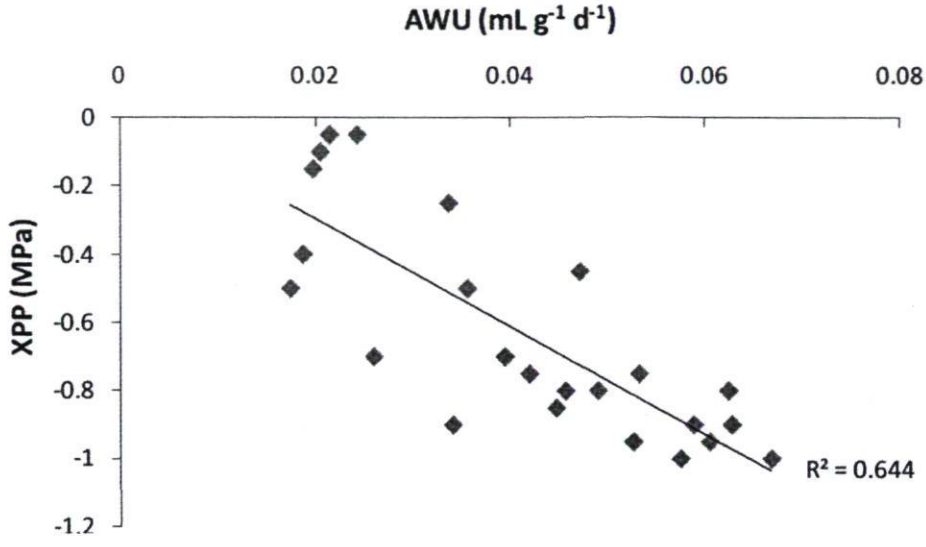


Figure 45: Significant ($p < 0.001$) linear relationship between AWU and XPP (n=24). The relationship is best described by XPP = -15.8(AWU) + 0.02.

9.4 Effect of storage temperature on ethylene evolution and abscission in balsam fir

9.4.1 Objective

To determine the effect of varying temperatures on branch water relations and abscission.

9.4.2 Methods

Twenty-four branches were collected from the Tree Breeding Center on March 16, 2010 and transported as described in Chapter 4, but storage conditions were modified. The experiment was designed as a 2 x 3 factorial. The first factor was continuous ethylene exposure (0 or 1000 ppm) while the second factor was temperature (5, 15, or 25 °C). The design had 4 replications, where a single branch served as a replicate. The chamber was kept at a humidity of 60%. In this experiment, treatments of 5, 15, and 25 °C corresponded with vapour pressure deficits of 0.35, 0.68, and 1.26 kPa.

Response variables were NRD, AWU, XPP, DPE, and ethylene evolution which are described in detail in Chapter 4. Analysis of variance was conducted using Minitab 15 to detect significant main and interaction effects. Effects deemed to be significant were separated using least squares means in SAS.

9.4.3 Results

NRD and DPE were significantly ($p < 0.001$) affected by both temperature and ethylene. Continuous exposure to exogenous ethylene reduced NRD by 47 to 65%, depending on temperature. However, NRD was significantly higher at 5°C in the presence and absence of ethylene (Table 6). NRD and DPE were very closely related (Fig. 46).

AWU and XPP were each influenced by temperature. In the absence of ethylene, AWU was 40% lower at 5°C than 25°C; XPP was 0.42 MPa higher at 5°C than 25°C. In the presence of ethylene, AWU was 51% lower at 5°C than 25°C; XPP was 0.39 MPa higher at 5°C than 25°C (Table 6). There was a significant relationship between AWU and XPP (Fig. 47).

The rate of endogenous ethylene evolution was significantly ($p < 0.001$) affected by temperature alone. There was no difference in ethylene evolution at 5°C and 15°C, which had a combined average evolution rate of 5.2 $\mu L \cdot g^{-1} \cdot h^{-1}$. However, there was a 51% increase in ethylene evolution at 25°C, resulting in a rate of 7.7 $\mu L \cdot g^{-1} \cdot h^{-1}$, when compared to 5°C.

Table 6: Effect of ethylene and temperature on root-detached balsam fir branches. Treatment means were calculated from 4 replication and separated using least squares differences with $\alpha = 0.05$. NRD = needle retention duration; AWU = average daily water use; XPP = xylem pressure potential; DPE = days until peak ethylene evolution.

Ethylene (ppm)	Temperature (°C)	NRD (days)	AWU ($mL \cdot g^{-1} \cdot d^{-1}$)	XPP (MPa)	DPE (days)	C_2H_4 Evolution ($\mu L \cdot g^{-1} \cdot h^{-1}$)
0	5	65.0^{a}	0.035^{c}	-0.34^{c}	61.5^{a}	4.9^{b}
	15	37.5^{b}	0.045^{b}	-0.64^{b}	34.5^{b}	4.7^{b}
	25	29.5^{c}	0.058^{a}	-0.76^{ab}	27.8^{c}	7.4^{a}
1000	5	20.8^{d}	0.032^{c}	-0.51^{b}	18.0^{d}	5.3^{b}
	15	13.3^{e}	0.042^{bc}	-0.60^{b}	11.5^{e}	5.8^{b}
	25	11.0^{e}	0.065^{a}	-0.90^{a}	9.5^{e}	8.0^{a}

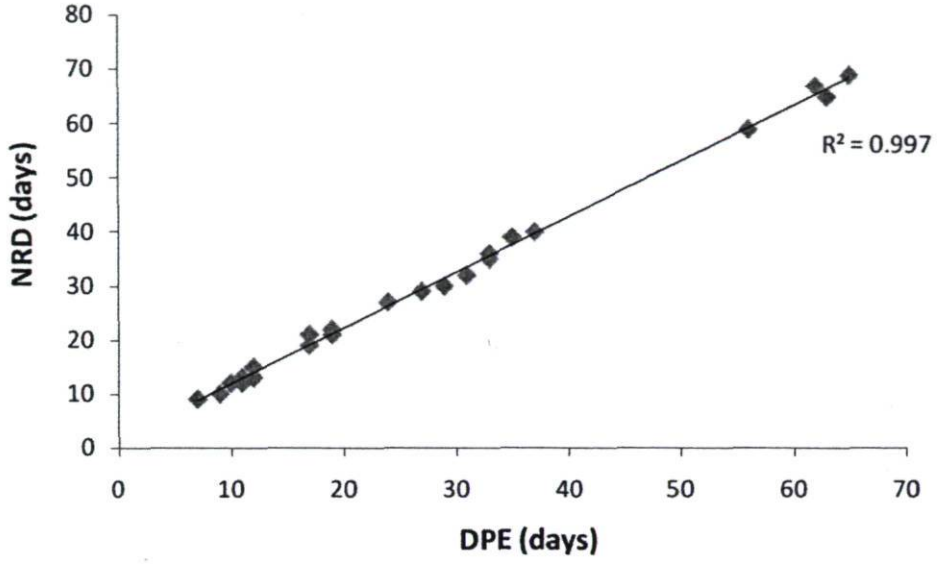


Figure 46: Significant ($p < 0.001$) and near perfect relationship between DPE and NRD (n=24). The relationship is best described by NRD = 1.03(DPE) + 1.5.

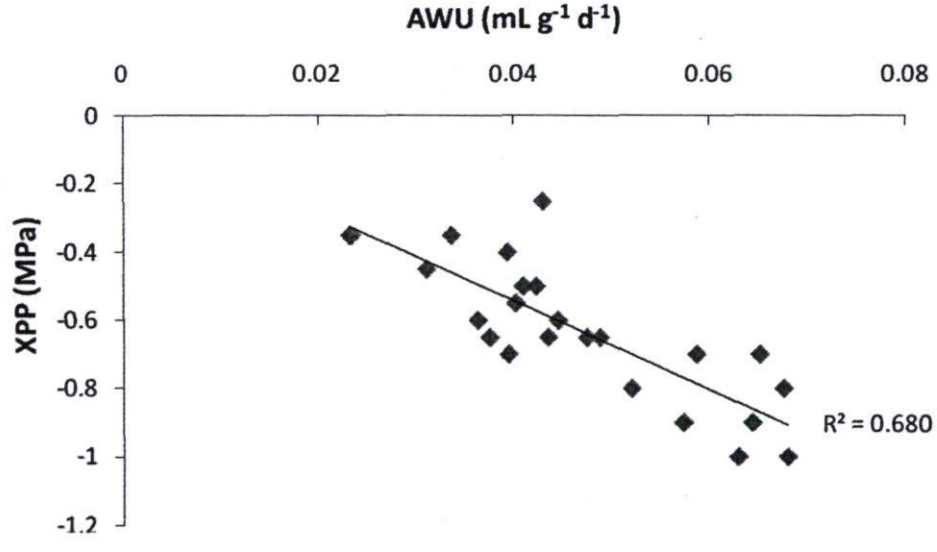


Figure 47: Significant ($p < 0.001$) linear relationship between AWU and XPP. The relationship is best described by XPP = -13AWU + 0.023.

9.5 Discussion

In all experiments, a branch with high AWU had a tendency to lose needles earlier than a branch with low AWU. These findings were consistent with Rajasekaran and Thiagarajan

(2005) and Chapter 7. Approximately 90% of all water uptake is lost by transpiration, thus differences in AWU likely represent differences in transpiration of root-detached balsam fir branches. It is possible that removal of roots limits hydraulic lift and would decrease water potential during periods of high transpiration. This theory would account for decreasing XPP observed in many branches before abscission. Indeed, when AWU was decreased using humidity or temperature, XPP was significantly higher.

In previous studies, it was found that continuous exposure to ethylene decreased XPP in balsam fir when compared to a control (Chapter 4; Chapter 6; Chapter 7). Several other studies suggested that ethylene might result in stomatal dysfunction, as determined through increases in transpiration, stomatal conductance, and stomatal aperture (Merritt et al. 2001; Azuma et al. 2003; Tanaka et al. 2005). From the aforementioned studies it was hypothesized that ethylene-induced stomatal dysfunction might result in premature abscission. However, when water loss was successfully mitigated using high humidity or low temperature, ethylene still induced abscission. These results do not necessarily dispute that continuous exposure to ethylene may result in some amount of stomatal dysfunction, but it is clearly not the major contributor to needle abscission.

Interestingly, when water loss was limited in the absence of ethylene, NRD was significantly increased. Branches stored at 5°C and 60% humidity had a 120% increase in NRD, while NRD was increased more than 3-fold at 90% humidity and 19.7°C. Mitcham-Butler et al. (1988) previously reported storage at lower temperature as an effective method of delaying abscission in Fraser fir, but to our knowledge this is the first mention of using high humidity to delay needle abscission in conifers. In addition, low temperatures and high humidity significantly delayed the evolution of ethylene and increased XPP. These results suggest that declining water status is the signal required to trigger ethylene evolution. Ethylene-induced abscission is a logical defense for a root-detached balsam fir branch. By shedding needles a branch is effectively reducing the effective surface area available for transpiration, thereby conserving water. In addition, it has been shown in other species that ethylene also triggers defense related proteins to heal exposed areas after abscission. Cellulase is one protein thought to play a role in

abscission wound response, which has also been discovered in balsam fir prior to abscission (Addicott 1982; Chapter 6).

There are some problems in suggesting that decreased water status triggers needle abscission in conifers. First, if we know that limiting water loss through humidity or temperature decreases abscission, we would expect antitranspirant compounds to have a similar effect. However, in most cases, there was no improvement noted (Chastagner and Riley 1991) or there was a reduction in transpiration, but not enough to be of any practical significance (Duck et al. 2003; Davis and Fretz 1972). Second, the lowest water potential observed in balsam fir leading to abscission was -1 MPa, which is considerably higher than threshold water potentials of -3 MPa to -4 MPa observed in closely related species such as Nordmann and Fraser fir (Chastagner and Riley 2005; Mitcham-Butler et al. 1988). It is proposed that the threshold water potential is not as relevant as the process of decreasing water potential in terms of ethylene evolution and abscission. Rather the threshold water potential is the point at which a branch could no longer recover, though further experimentation would be required to confirm.

9.6 Conclusions

Both the 90% humidity and 5 °C treatments were effective at delaying abscission in root-detached balsam fir in the absence of ethylene. The 90% humidity treatment was particularly effective with more than a 3-fold improvement in NRD. No treatments significantly improved NRD in the presence of exogenous ethylene. It was concluded that maintaining good water relations will delay abscission in root-detached balsam fir.

10.0 GENERAL DISCUSSION

Until recently, little was known about needle abscission in root detached balsam fir. It was understood that harvesting a tree would eventually result in abscission that would not have occurred if that tree was left intact. It was also understood that needle abscission was having a detrimental effect on the Christmas tree and greenery industries, supported by the fact that natural tree sales have remained static despite an increase in combined sales of natural and artificial trees (Koelling 1999; Davis 1996). Needle shedding was cited as a major source of consumer dissatisfaction. However, the work contained in this thesis has presented evidence to propose a series of physiological events that lead to abscission in balsam fir (Fig. 48), and propose solutions that are applicable to the Christmas tree industry.

When a branch is still attached to a tree, no remarkable physiology event are happening, with respect to abscission, provided there is no disease, shading, or other external factors that would cause senescence or abscission under natural conditions. The act of cutting, thereby separating a tree from its roots, must trigger abscission. One theory proposed that the mechanical stress associated with harvest would induce factors leading to abscission. However, this hypothesis seems unlikely as there is considerable lag of time between cutting and abscission. Depriving a tree of essential biological compounds from the root may also cause abscission. The initial trigger for abscission remains unknown, though there is no evidence from this thesis which would debunk this hypothesis.

After harvest, trees may be placed in water to reduce the risk of dehydration. Water consumption starts relative high, at a rate of approximately 0.2 $mL \cdot g^{-1} \cdot d^{-1}$ with a XPP of about -0.05 to -0.1 MPa. As shown in Chapter 8, water consumption and XPP experience an exponential decline over time. Within 2 weeks, daily water consumption declines to 0.05 $mL \cdot g^{-1} \cdot d^{-1}$ with a XPP of about -0.5 to -1.0 MPa. Many species are known to begin senescence beyond threshold water potentials, but those are typically much lower than that observed in balsam fir. For example, Nordmann and Fraser fir trees have a threshold water potential of -3 MPa and -4 MPa, respectively (Chastagner and Riley 2003;

Mitcham-Butler et al. 1988). However, when high XPP is maintained through high humidity or low temperatures, abscission is significantly delayed (Chapter 8).

After a certain length of time, which may vary considerably between genotypes (Chapter 7), endogenous ethylene is released. A portable ethylene analyzer sensitive to 0.1 ppm was typically able to detect ethylene approximately 1 week before complete needle loss, which was evolved at a rate < 1 $\mu L \cdot g^{-1} \cdot h^{-1}$ (Chapter 4). An experiment conducted in Chapter 5 demonstrated delayed needle abscission after short-term exposure to low concentrations of ethylene, thus it is speculated that certain defense related proteins may be the first ethylene-induced response in balsam fir, which actually benefit needle retention. The ethylene evolution rate continued to increase each day, where it could reach a rate of about 20 $\mu L \cdot g^{-1} \cdot h^{-1}$. As before, the peak rate of ethylene evolution may vary between genotypes (Chapter 7). Peak ethylene evolution was always observed 1 - 3 days before needle shed, which would suggest ethylene is the signal to begin abscission.

The role of ethylene was confirmed with several separate studies. If a balsam fir branch was continuously exposed to ethylene, even at relatively low concentrations of 10 ppm, the length of time required for abscission was significantly reduced. A concentration of 10 ppm decreased NRD by 32% while a higher concentration 1000 ppm reduced NRD by 56% in one experiment (Chapter 4). If ethylene action was inhibited by AVG or a receptor blocker such as 1-MCP, NRD increased by 113% and 73%, respectively, when compared to an untreated control (Chapter 5).

It is likely that ethylene stimulates cellulase activity in balsam fir needles. Untreated balsam fir branches which showed no signs of abscission had no detectable cellulase activity. When branches showing signs of abscission, both from endogenous and exogenous ethylene, were submitted to the same test, there was an approximate 10-fold increase in cellulase activity (Chapter 6). Many other reports have suggested that cellulase dissolves and weakens cell walls in the abscission zone of other species (Brown 1997). Thus, it is proposed that cellulase plays a similar role in balsam fir.

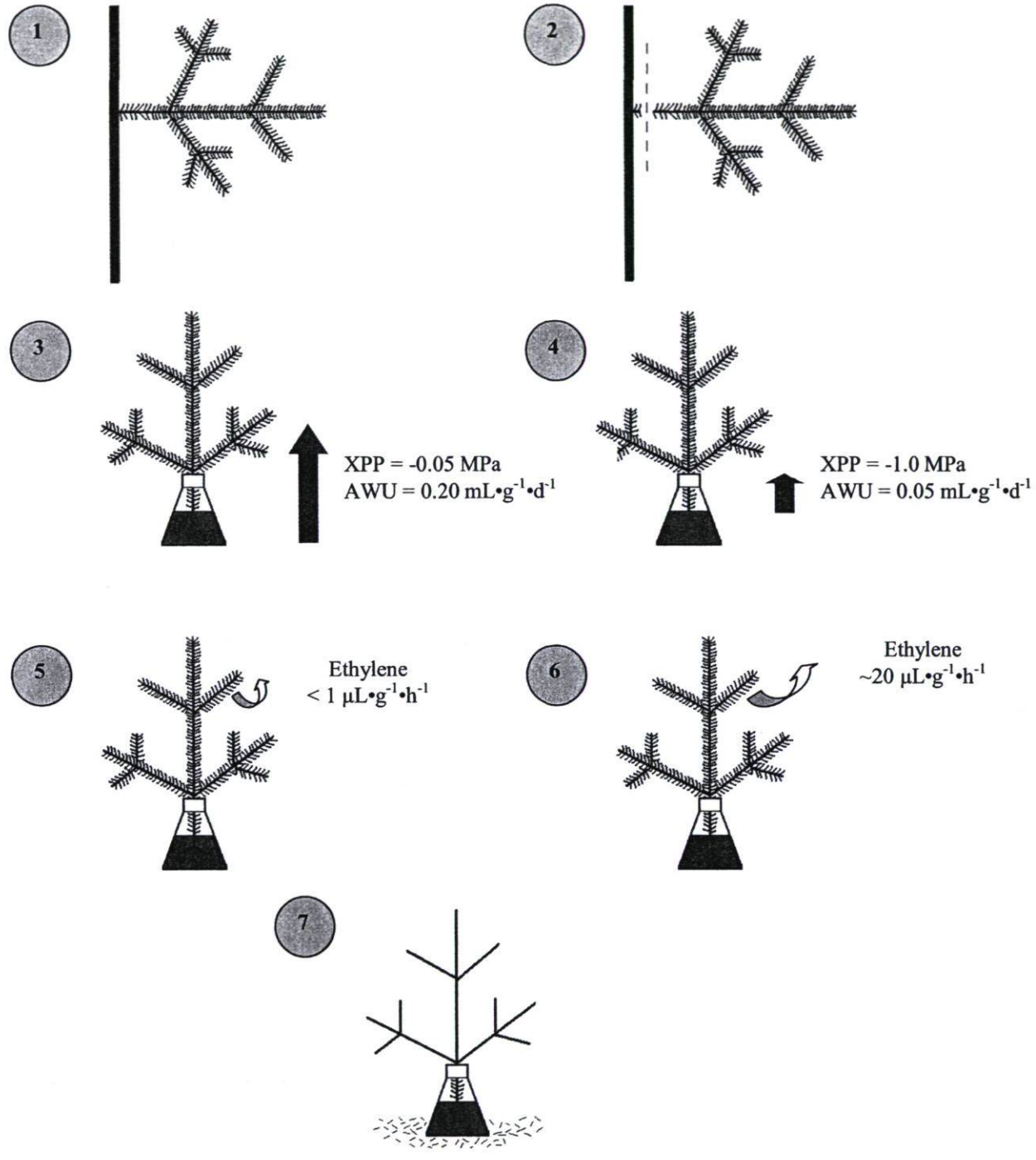


Figure 48: Schematic representation of key events leading to abscission in root-detached balsam fir. 1) Branch is still attached to tree, expecting normal physiological function. 2) Branch is cut from tree, thus "root-detached", which is the first step towards abscission of an otherwise healthy branch. 3) Water use and XPP are initially high, but 4) will rapidly diminish over time. 5) Ethylene evolution starts out low several days before abscission and then 6) peaks 1 – 3 days before abscission. 7) Increased ethylene evolution stimulates cellulase activity, which weaken cell walls, and 8) culminates in complete abscission.

One limitation of these results is they do not provide a satisfactory explanation for the trigger to begin an abscission sequence. The fact that ethylene is involved as a signal molecule has been thoroughly examined, but there is still no conclusive evidence for the trigger to begin ethylene synthesis. Recent work on *Capsicum* has found that certain reactive oxygen species (ROS) induce ethylene, cellulase activity, and abscission (Sakamoto et al. 2008a; 2008b). Supporting evidence is available in tomato and carrot species, where certain natural and synthetic antioxidants have inhibited ethylene and/or senescence and abscission (Rajasekaran and Blake 1999; MacDonald et al. 2008; 2009a; 2009b; 2010a). Presumably, these antioxidants are limiting the effect of ROS signalling, thus inhibiting the trigger for ethylene synthesis. No studies investigating the link of ROS to ethylene and abscission have been conducted in balsam fir or related species, but ROS are a plausible trigger that warrants further study.

An understanding of events leading to abscission is of practical importance to the Christmas tree and greenery industries and consumers. If any step leading to abscission is interrupted, it is expected that abscission could be stopped or, at the very least delayed. One example would be to commercialize non-toxic ethylene inhibitors, which should, in turn, negate cellulase activation and abscission. In the case of AVG and 1-MCP, a means of distribution have been established for many angiosperms and it is expected that these methods would be appropriate for gymnosperms as well. The only limitation would be to determine appropriate concentrations and exposure times for a whole tree, as opposed to an individual branch. Another example would be to take steps to maintain proper water status. While maintaining low temperatures of 5°C or 90% humidity in homes is likely not possible, perhaps transport and storage conditions of trees prior to sale could be improved. In addition, it would be strongly recommended that producers apply a misting spray of water to their trees periodically after harvest (it should be noted that some producers use this practice already).

Some new practices, which do not interrupt the abscission process, may also be of interest to the Christmas tree industry. The practice with perhaps the most potential

would be to graft trees to existing root stock. Most Christmas tree producers transplant seedlings into a field after they are a few years old. However, it was recommended by Chastagner and Benson (2000) that grafting existing trees known for pest resistance onto existing root stock would be a viable process for producers and should be incorporated into future industry practices. In our experiments, certain genotypes have been identified as having high NRD characteristics, which was shown to be linked to ethylene evolution and sensitivity (Chapter 7). It is proposed that genotypes could be selected based on NRD characteristics and grafted onto existing root stock to design a superior Christmas tree.

11.0 CONCLUSIONS

The overall goal of this research was to understand the events leading to abscission in root-detached balsam fir with a specific interest in the role of ethylene. This goal was achieved and described in detail in Chapter 10. The following briefly describes the key conclusions with respect to objectives presented in Chapter 3. For convenience, the original objectives are restated before each conclusion.

1.a) To determine exogenous ethylene concentration and exposure time required to induce abscission in balsam fir.

- It was determined that continuous exposure to ethylene from 10 to 1000 ppm will induce abscission in balsam fir, though 1000 ppm had the most profound effect. In addition, it was found that short term (24 h) exposure to the same concentrations would significantly delay abscission.

1.b) To determine whether balsam fir branches synthesize endogenous ethylene prior to abscission and in sufficient quantities to induce abscission.

- Ethylene evolution could be detected preceding abscission. Relatively low evolution rates could be detected several days before abscission, which would increase and peak 1 to 3 days before abscission. Typical peak ethylene evolution rates were found to vary between 10 $\mu L \cdot g^{-1} \cdot h^{-1}$ and 20 $\mu L \cdot g^{-1} \cdot h^{-1}$. At these rates, ethylene concentrations quickly increased to levels previously found to induce abscission.

2.a) To determine the effect of ethylene inhibition with aminoethoxyvinylglycine (AVG) on balsam fir branches in the presence and absence of exogenous ethylene.

- AVG delayed abscission by 113% in the absence of exogenous ethylene. However, AVG had no effect on abscission in the presence of exogenous

ethylene.

2.b) To determine the effect of ethylene receptor blockage with 1-methylcyclopropene on balsam fir branches in the presence and absence of exogenous ethylene.

- 1-MCP was effective at delaying abscission in the presence and absence of exogenous ethylene. Abscission was delayed by 73% in absence of and 147% in presence of exogenous ethylene, each compared to their respective controls.

3.a) To develop a protocol to identify presence of cellulase in plant tissue and to quantify ethylene-induced cellulase activity in needle tissue.

- A cellulose plate digestion method was developed. Needle extracts from branch showing no signs of abscission had very low cellulase activity, which could only be detected by software. Needle extracts from branches experiencing abscission from endogenous or exogenous ethylene both displayed cellulase activity about 10 times higher than the control.

4.a) To identify the needle retention duration of all genotypes available at the Debert Tree Breeding Center.

- NRD was highly variable in balsam fir genotypes, ranging from 6 to 60 days after root detachment. Classification of genotypes with low, moderate, and high NRD was achieved from two field seasons.

4.b) To determine whether genotypic differences in needle retention may be attributed to differences in ethylene evolution.

- Those genotypes classified as low NRD had significantly higher peak ethylene evolution than genotypes classified as high NRD.

4.c) To determine whether genotypic differences in needle retention may be attributed

to differences in ethylene sensitivity.

- All tested genotypes responded to an ethylene concentration as low as 10 ppm. However, it was determined that those genotypes classified as low NRD had a greater response to exogenous ethylene.

5.a) To determine the effect of exogenous ethylene on water use and xylem pressure potential in a branch.

- Water use and XPP both declined after a branch was cut. Branches exposed to exogenous ethylene used more water, but had a lower XPP. In most cases, water use and/or XPP was strongly related to NRD.

5.b) To determine the effect of low temperatures or high humidity on branch water relations and abscission.

- Exposure to 5 °C or 90% humidity reduced water use and greatly delayed abscission in the absence of ethylene. However, neither treatment had any significant effect on abscission in the presence of exogenous ethylene, despite reducing water use.

11.1 Future research

This research has generated a considerable amount of new knowledge regarding abscission in root-detached balsam fir, but there is still a great potential for future research. Future research areas may be considered as physiological, practical, and additional factors influencing abscission.

Physiological research should focus on further defining key physiological events leading to abscission. It was proposed that declining water potential triggers ethylene evolution, but the exact cause of declining water potential remains unknown. Is it simply the reduced hydraulic conductance associated with root detachment or, perhaps, changes in other phytohormones? It was also proposed that ethylene induces cellulase activity, but no work has been done on ethylene-induced gene expression or the possibility of other hydrolytic enzymes in balsam fir.

Practical research must focus on one important aspect, which is the commercialization of technologies for a whole tree. From this research, five potential methods to delay abscission have been identified: short-term exposure to ethylene, ethylene inhibition with AVG, blocking ethylene receptors with 1-MCP, storage at high humidity, and storage at low temperatures. The limiting factor is that all these potential technologies have been tested on branches. Ideally, the branches represent a whole tree response, but there may still be changes needed to optimum concentrations.

The last area for further study would be research into additional factors known to influence needle abscission. From this study, it has been shown that there is a high degree of variation between different genotypes, not only in abscission characteristics, but also in response so harvest date. It was speculated that a later harvest date allows more opportunity for cold acclimation, but how does cold acclimation delay abscission? Needle abscission in balsam fir is still in the early stages of research, which makes the possibility of futures studies that much more exciting.

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Appendix I: Design of ethylene incubation chamber

Many planned experiments required a method to incubate samples with ethylene gas. It was determined that an air tight chamber would need to be designed large enough to house a balsam fir seedling, allow light penetration, provide complete circulation, and monitor conditions such as temperature and humidity (Fig. 49).

The main part of the designed incubation chamber was an 80 L plastic cylindrical container, with a diameter of 48 cm. The lid was a clear Plexiglas designed by B&D Glass (Truro, NS). Rubber weather stripping was attached around the top rim of the container to provide an air tight seal between the Plexiglas lid and plastic container. A small 14 mm hole was drilled 5cm from the center of the Plexiglas lid to insert a rubber septum to allow injection or extraction of gases.

The inside of plastic bin had a hydrometer/thermometer (Nexxtech) attached to one side to monitor temperature and humidity. The other side had a 10 cm fan attached at a 45° angle facing toward the same side, ensuring circulation without the direct airflow causing dehydration of the sample.

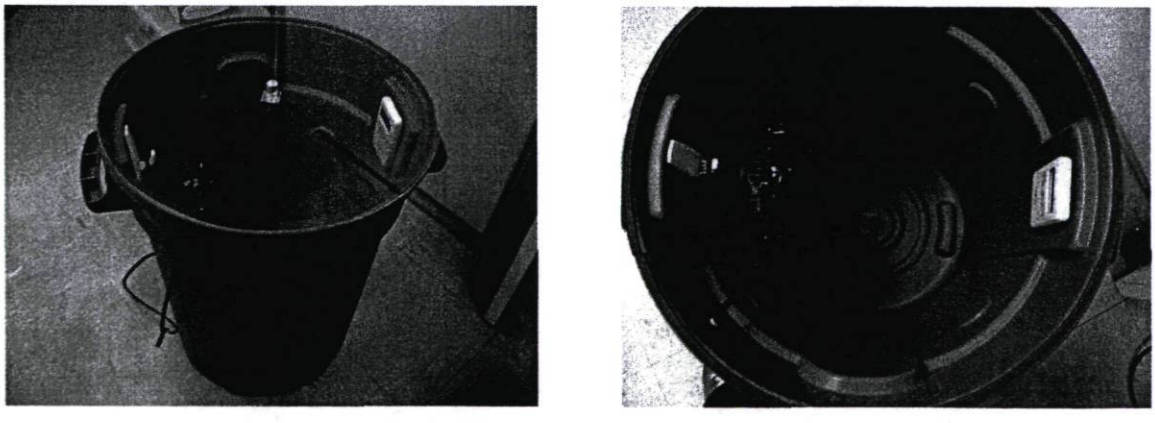

Figure 49: Air tight ethylene incubation chamber: (left) exterior; (right) interior

Appendix II: Standardization of portable ethylene analyzer

The portable ethylene analyzer was standardized by injecting known volumes of ethylene into an ethylene incubation chamber. Since the volume of each ethylene incubation chamber was known, the concentration of injected ethylene could be calculated and plotted against the concentration determined by the portable ethylene analyzer (Fig. 50).

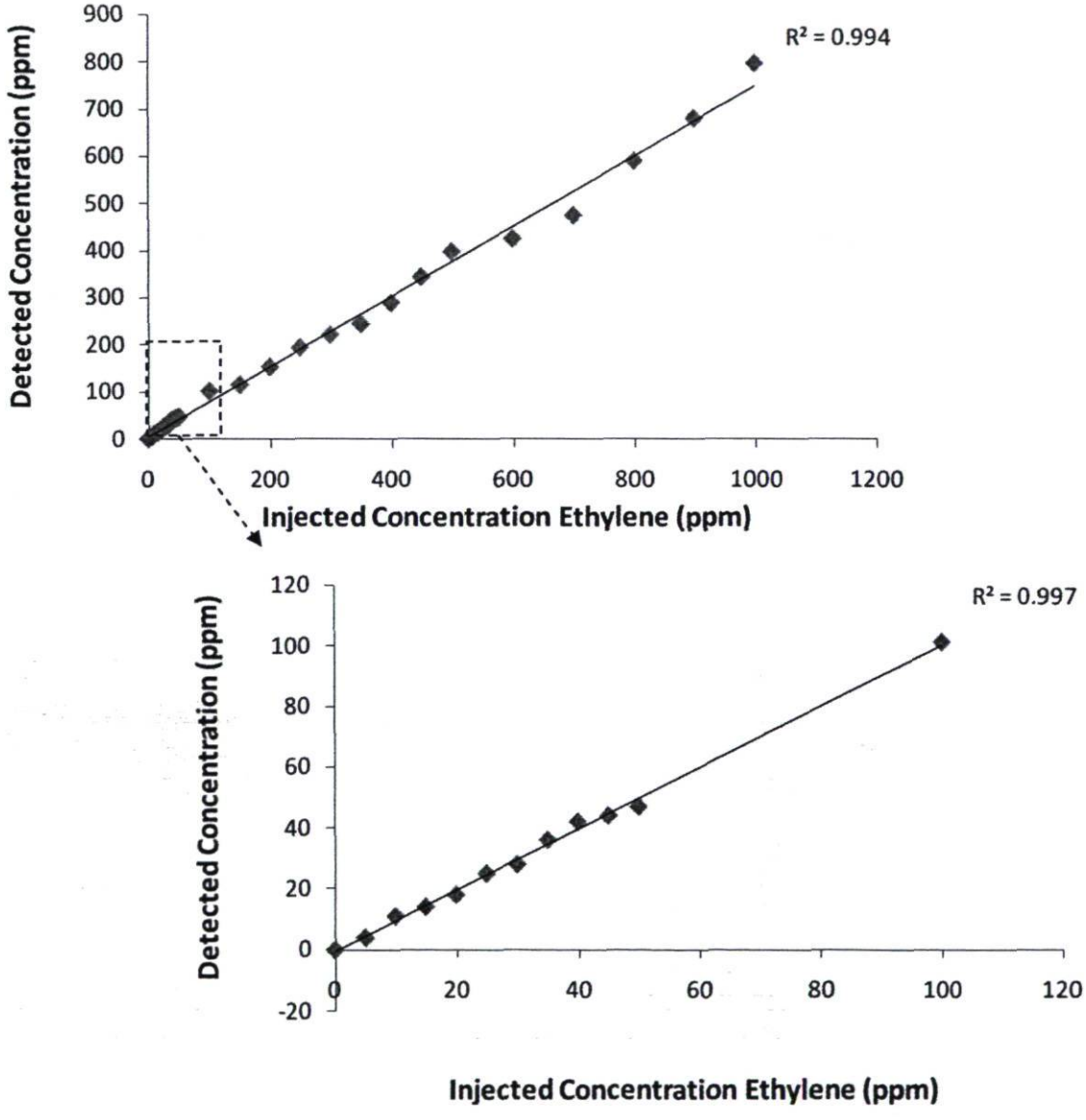

Figure 50: Standard curve for portable ethylene analyzer.

A linear relationship was found between 0 ppm and 1000 ppm ethylene. However, the detected concentrations begin to diverge from the injected concentrations after 100 ppm. For example, a concentration of 100 ppm was measured a 101 ppm by the portable ethylene analyzer, but an injected concentration of 150 ppm ethylene was measured as 115 ppm. The largest discrepancy was found at an injected concentration of 1000 ppm which was measured at only 797 ppm.

The portable ethylene analyzer performed excellent between 0 ppm and 100 ppm. The slope of the linear regression was determined to be 1.008 with $R^2 = 0.997$. In this case a perfect relationship would have a slope of exactly 1. The ethylene analyzer should be accurate at detecting ethylene released from balsam fir provided concentrations remain below 100 ppm.