THE NATURE AND DYNAMICS OF CHANGES IN LIPIDS AND FATTY ACIDS DURING POSTHARVEST NEEDLE ABSCISSION, THEIR ROLE IN COLD ACCLIMATION, ULTRA-STRUCTURAL CHANGES, AND NEEDLE ABSCISSION RESISTANCE IN BALSAM FIR, *ABIES BALSAMEA*, L.

by

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DEDICATION PAGE

To my grandchildren who make my heart smile, my students at Shenyang No. 2 High School, Shenyang, China who have given me inspiration, and my best supporter and soulmate, Dave, who has made me believe in myself

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ABSTRACT

Balsam fir is an important species of Atlantic Canada's Christmas tree and greenery industries that is suffering due to excessive needle loss postharvest. A series of investigations were carried out to: (i) characterize and establish links between polar lipid and fatty acids (FA) and postharvest needle loss; (ii) uncover the differences in the nature and quantity of polar lipid and fatty acids in contrasting genotypes with high and low needle retention duration (NRD); (iii) explore temporal changes in polar lipids and fatty acids; (iv) establish the genotypic differences in polar lipids and FA during temporal changes, and (v) confirm the ultrastructural changes of cells of balsam fir postharvest using scanning and transmission electron microscopy. Polar lipids decreased significantly postharvest in all studies ($p < 0.001$). The mean percentage of monogalactosyldiacylglycerol (MGDG) decreased significantly postharvest ($p < 0.0001$), caused mostly by a decrease in MGDG 36:6 and 36:7. There was also a significant decrease in the MGDG: DGDG (digalactosyldiacylglycerol) ratio in all studies ($p < 0.05$), earlier in the poorer needle retaining clone. During cold acclimation, Clone 506 was identified as a high needle abscission resistant (NAR) clone, while Clones 9, 37, and 566 all proved to be lower. MGDG decreased during the fall and early winter in all clones, with a subsequent increase in DGDG and a significant decrease in the MGDG: DGDG ratio in all clones tested ($p < 0.001$). There was a significant decrease also in the GL: PL ratio ($p < 0.001$). There was significantly less DGDG in Clone 506 than in Clone 9 ($p < 0.05$). LPG 16:1 was eight times higher in Clone 506. In balsam fir, there was a significant reduction ($p < 0.001$) in α – linolenic acid in all clones postharvest. During cold acclimation, there was an increase in α – linolenic acid in one of the lower clones, Clone 566, but an increase in Δ5-UPIFA, 18:3 (pinolenic acid) in the other clones, suggesting different metabolism ($p < 0.001$). Chloroplast disorganization was confirmed. In addition, most of all stoma were closed in both clones, however, there was more fungal hyphae associated with the poorer NRD clone.

LIST OF ABBREVIATIONS AND SYMBOLS USED

ABA	abscisic acid
ACL	average chain length
ANOVA	analysis of variance
AZ	abscission zone
BHT	butylated hydroxytoluene
CDD	cold degree days
CK	cytokinin
CoV	coefficient of variation
DAG	diacylglycerol
DBI	double bond index
DGK	diacylglycerol kinase
DGPP	diacylglycerol pyrophosphate
DGDG	digalactosyldiacylglycerol
FA	fatty acid
FAME	fatty acid methyl ester
FFA	free fatty acid
FID	flame ionizer detector
GL	galactolipid
IAA	indoleacetic acid
IP_3	inositol triphosphate
JA	jasmonic acid
LOX	lipoxygenase
lysoPA	lysophosphatidic acid
lysoPL	lysophospholipid
MII	membrane injury index
MGDG	monogalactosyldiacylglycerol
MUFA	monounsaturated fatty acids
NAR	needle abscission resistance

NRD	needle retention duration
NADPH	nicotinamide adenine dinucleotide phosphate hydrogen
NL	needle loss
NRD	needle retention duration
PA	phosphatidic acid
PC	phosphatidylcholine
PCD	programmed cell death
PE	phosphatidylethanolamine
PI	phosphatidylinositol
PIP_2	phosphatidylinositol bisphosphate
PS	phosphatidylserine
PLA	phospholipase A
PLC	phospholipase C
PLD	phospholipase D
PL	phospholipid
PUFA	polyunsaturated fatty acid
QC	quality control
ROS	reactive oxygen species
RRT	relative retention time
SFA	saturated fatty acid
TAG	triacylglycerol
TEM	transmission electron microscope
USFA	unsaturated fatty acids
UI	unsaturation index
16:0	palmitic acid
16:3n3	hexadecatrienoic acid
18:1n9	oleic acid
18:2Δ5,9	taxoleic acid
18:2:n6	linoleic acid
18:3n3	α-linolenic acid
18:3Δ5,9,12	pinolenic acid

20:3Δ5,11,14	sciadonic acid
18:4Δ5,9,12,15	coniferonic acid
20:4Δ5,11,14,17	juniperonic acid
Δ5UPIFA	Δ5-polyunsaturated methylene-interrupted fatty acids.

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CHAPTER 1 – INTRODUCTION

Balsam fir (*Abies balsamea* L.) is the major horticultural Christmas tree and greenery crop in Atlantic Canada and the eastern United States (Burns and Honkala, 1990). Balsam fir Christmas trees are enjoyed for their unique aroma, architecture and dark green color (Burns and Honkala, 1990). Canada exports close to 2 million Christmas trees per year, primarily balsam fir. The value of farm cash receipts for Christmas trees in Canada in 2016 was 77.6 million dollars, with a net revenue of 43.1 million for the export market (Statistics Canada, 2017). One third of all trees exported from Canada to other parts of the world are provided by Atlantic Canada growers (Statistics Canada, 2017). The industry is now in jeopardy due to excessive needle abscission, postharvest.

Balsam fir has been ranked one of the top postharvest needle retaining Christmas tree species (Chatagner and Riley, 2007). Despite this fact, postharvest abscission challenges have become more predominant for this species in the past 12 years. In 2008, it was estimated that under normal storage and shipping conditions, one in three fir trees shed all their needles in less that three weeks (MacDonald et al., 2010). This problem is largely due to pressure for earlier harvesting for the local and export market, and warmer autumn temperatures (Chastagner and Riley, 2007). In addition, the popularity of the artificial tree has become difficult to compete with, as this product comes in many shapes, colors, forms, and does not shed needles. Between 2012 and 2016, the value of artificial trees imported into Canada exceeded the value of real trees exported from Canada (Fig. 1). The Canadian domestic market has also declined (Statistics Canada, 2017). A full understanding of balsam fir physiology could help mitigate postharvest needle loss and change the trend from artificial back to use of real trees at Christmas.

Postharvest needle abscission in balsam fir has been attributed to several factors, such as the environment, genetic variations, rough postharvest handling and nutrition (Mitcham-Butler et al., 1987; Hatcher, 1990; Thiagarajan and Lada, 2010; MacDonald et al., 2014a; Georgeson, 2013). Drought stress caused by dehydration was hypothesized to be the most relevant factor accelerating abscission due to the fact that the trees exist as a root-detached system for varied periods of time (Chastagner, 1986; MacDonald and Lada, 2014). It is

well documented that dehydration can cause premature senescence (yellowing) and abscission in various Christmas tree species (Chastagner, 1986). Links have been established between decline in water status and dehydration in balsam fir and decreased needle retention postharvest. However, it is thought that dehydration alone cannot be the only biophysical trigger for needle drop (Lada and MacDonald, 2015). According to Lada and MacDonald, 2015, there needs to be a signal for abscission when there is water stress, as well as a signal for abscission when there is no water stress. In an effort to find the signaling pathways related to postharvest abscission studies have been carried out at the Christmas Tree Research Center in Truro, Nova Scotia. It has been found that the plant growth regulator (PGR), ethylene, induces abscission postharvest, and is probable that dehydration, as well as rough handling, may be stressors that trigger its release (MacDonald, 2010). Many other PGR and volatile terpene compounds have been studied, but no definite causation has been found to completely explain or reveal the signaling pathways involved in the physiology behind postharvest needle abscission in balsam fir (Thiagarajan, 2012; Georgeson, 2013; Korankye, 2013). However, links have been made, it has been found that abscisic acid (ABA) increased by 32-fold and trans-zeatin riboside increased by fourfold during peak abscission postharvest. Other cytokinins (CK) all doubled during abscission. In addition, there was a 95 % decrease in auxin indole-3-acetic acid (IAA); MacDonald and Lada, 2014). It has also been established that certain nutrient elements including nitrogen negatively affect abscission postharvest in balsam fir (Georgeson, 2013).

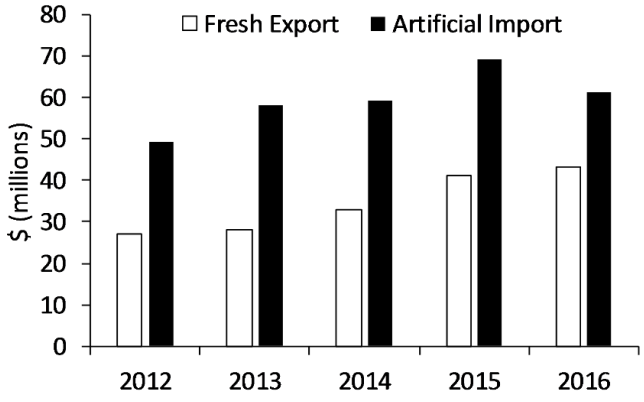

Figure 1. A comparison of Christmas tree fresh export market to the artificial import market (Statistics Canada, 2017).

Strategies to increase needle retention in postharvest balsam fir have also been investigated. Two main factors found to increase postharvest needle abscission resistance (NAR) are cold acclimation and high humidity. Early harvest before cold acclimation, has been linked to premature needle abscission (Mitcham-Butler et al., 1987; Chastagner and Riley, 2003; Rajasekaran and Thiagarajan, 2006; MacDonald and Rajasekaran, 2008; Thiagarajan and Lada, 2010). Humidity levels during postharvest storage or transport are directly related to longer needle retention (MacDonald et al., 2012). Despite discovering factors that delay abscission in postharvest balsam fir, research is still required to understand these mechanisms, and the underlying physiological changes.

One area that has never been explored with regard to abscission in balsam fir postharvest is lipid and fatty acids (FA). Lipids, and more recently FA, have been found to serve a variety of roles in signal transduction in plant cells. They act as ligands that activate signal transduction pathways and are mediators of signaling pathways. It is possible that lipids may act as signals for needle drop and be activated when there is water stress or dehydration, as is known that lipid- mediated signaling occurs in response to environmental stresses such as drought (Okazaki and Saito, 2014). It has been found in some higher plants that a reduction of structural phospholipids (PL) with a subsequent increase in signaling PL postharvest could indicate that there is cell signaling going on. Polar lipid clone analysis could reveal whether there are signaling lipids increasing, leading up to peak abscission. For instance, an increase in phosphatidic acid (PA), one of the main signaling PL, has been linked to membrane breakdown, which has been documented during abscission in balsam fir (Welti et al., 2002; Hong et al., 2009; MacDonald et al., 2014b). Could PA be the signal for membrane breakdown? PA has also been implicated in signaling pathways related to ABA and ethylene in other plants, and has been suggested as a potential link between the two (Sun et al., 2006; Okazaki and Saito, 2014). I postulate that lipid and FA analysis could reveal increases in PA and other signaling lipids, which will help in our understanding of the linkages between lipids and FA and their role in postharvest needle abscission.

Another important reason for studying polar lipids and FA is to gain understanding about the changes that occur to membranes and their integrity, and the ultrastructural changes that take place in the cells postharvest (Partelli et al., 2009). We know there is documented loss of membrane integrity in postharvest studies of balsam fir and the membranes of the cell are the source of signaling lipids and FA (Eyster, 2007; Lada and MacDonald, 2015). In excised leaves from *Catharanthus roseus*, it was determined that polar lipids, both PL and glycolipids, were substantially reduced and reorganized causing lipid phase changes leading to abscission. This was suggestive of substantial changes in fluidity and permeability of the membrane, in addition to lipid functional changes and possible ultrastructural changes (Mishra et al., 2006). I postulate that there will be a reduction of lipids and/or FA postharvest along with decline in membrane integrity and chlorophyll fluorescence in balsam fir leading up to abscission.

There is additional evidence during postharvest balsam fir studies that there could be disruption in intracellular membranes, particularly chloroplast membranes. Fluorescence decreased as needle loss increased in balsam fir postharvest showing a significant negative relationship between fluorescence or stress index and postharvest abscission in a past study ($R^2 > 0.90$; MacDonald et al., 2015). A decrease in fluorescence has also been recorded in other studies (MacDonald and Lada, 2014). I postulate that chloroplast membrane integrity is compromised postharvest and breakdown of lipids could supply a source of signaling lipids. Connections have also been made between lipid and FA profile changes postharvest and membrane stability and chloroplast function in relation to postharvest abscission in other plant species, suggesting there could be a relationship among lipids and FA changes, signaling and needle drop in balsam fir postharvest (Mishra et al., 2006; Partelli et al., 2009). Polar lipid and FA studies could reveal which organelle membranes are affected postharvest by looking at the lipid classes that are involved, and reveal some evidence of cell signalling and the origin by finding out the order in which lipids and FA change occurs, postharvest.

Improvements in needle retention provide clues to biochemical signals and processes. One of beneficial factors that was found to increase postharvest needle abscission duration

(NRD) was cold acclimation. Cold acclimation significantly improves postharvest needle abscission in some clones of balsam fir (MacDonald and Lada, 2008). There are biochemical alterations to curb lipid phase changes in cold temperatures to prevent membrane leakage in balsam fir in the winter. Early harvest before cold acclimation has been linked to premature postharvest needle abscission (Hatcher, 1990; Mitcham-Butler et al., 1987; Chastagner and Riley, 2003; Rajasekaran and Thiagarajan, 2006; MacDonald et al., 2014b; Thiagarajan and Lada, 2010), but it seems low needle retaining genotypes benefit more from cold acclimation than high needle retaining genotypes (MacDonald et al., 2014). Therefore, I hypothesize that lipid profile differences between various clones will reveal the reasons why some clones retain needles better than others. This is a first of its kind study, extracting lipids and FA from balsam fir needles postharvest. Also, there have been no reports on anyone studying the ultrastructure of balsam fir needles postharvest to see changes that take place in the needles.

The overall objective of this research is to uncover the nature, dynamics of change, clonal variations in polar lipids and FA after root detachment in balsam fir and during cold acclimation. In addition, the goal is to study ultrastructural changes in balsam fir needles postharvest to visualize how polar lipid changes are affecting intracellular and surface needle structure. The diagram (Fig.2) below summarizes the information that will be revealed during this research, and the detailed hypotheses and specific objectives are found in chapter three.

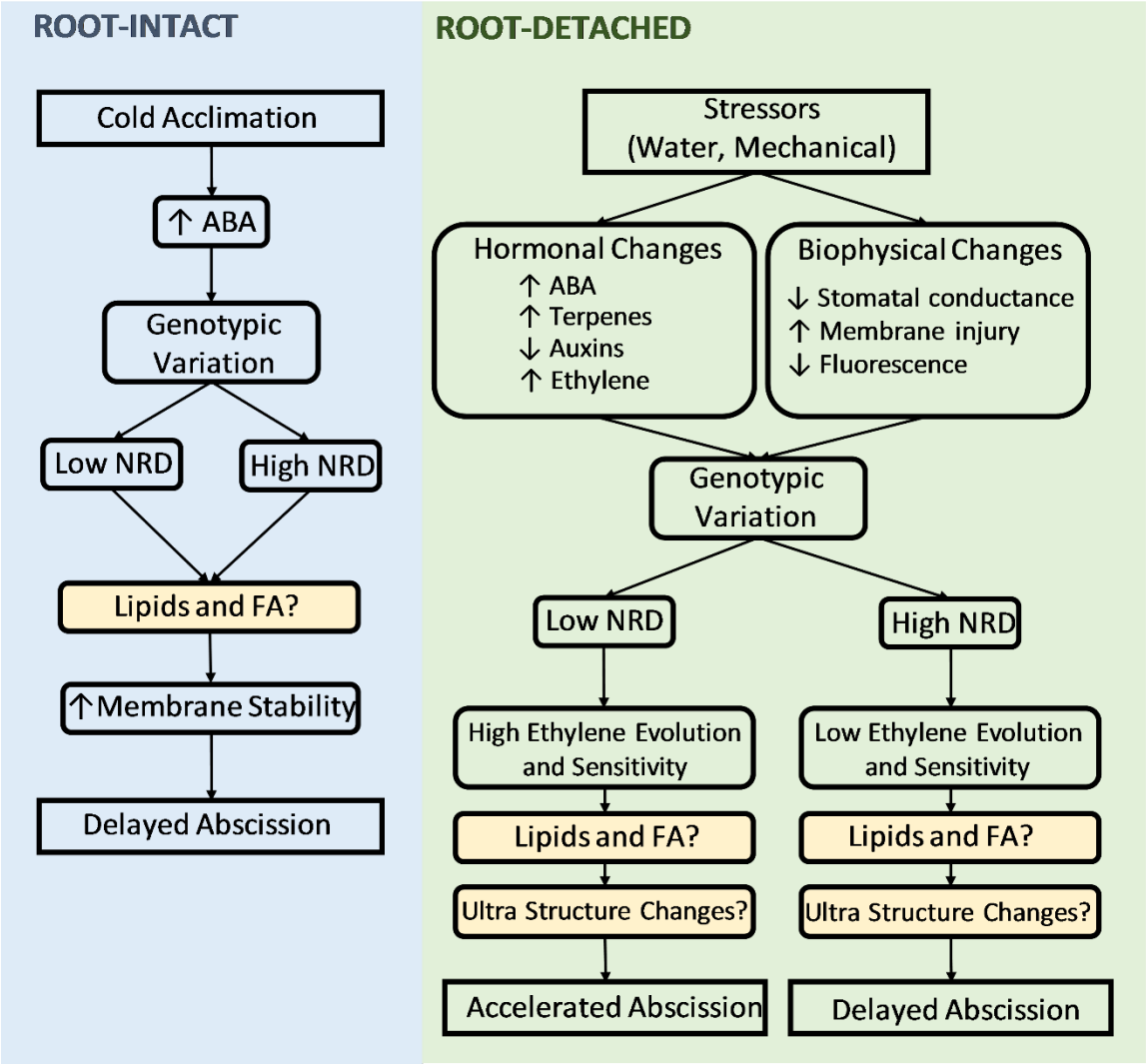

Figure 2. Schematic representation of the role of lipids and FA to needle abscission physiology.

CHAPTER 2 – LITERATURE REVIEW

2.1 PREFACE

This literature review was first published as a review paper for the American Journal of Plant Sciences. The full reference is included below:

MacDonald GE, Lada RR, Caldwell CD, Udenigwe C, MacDonald MT. 2019. Potential roles of FA and lipids in postharvest needle abscission physiology. American Journal of Plant Sciences, 2019, 10, 1069-1089.

2.2 INTRODUCTION

This chapter is divided into six major sections. The first section gives the background on abscission. The second section provides information regarding the structure and nomenclature of lipids and FA. Section three discusses briefly the physiological, biophysical and biochemical changes that are known to occur in balsam fir postharvest. Section four relates the roles of glycerolipids and FA in the stress response in plants. The fifth section is on signaling lipids and roles they may play in plants. It is my intention that this chapter will give the reader the necessary background to understand this research.

2.3 ABSCISSION IN PLANTS

Balsam fir Christmas trees are harvested by detaching them from their roots, depriving the stems and needles from root-derived factors. This puts the tree under stress and it has been found that abscission of needles is a problem postharvest. Initially it is the wounding, but the most predominant stress is water deficit or drought, even when the root-detached trees are rehydrated (Zhao et al., 2010; Lee et al., 1998; MacDonald and Lada, 2014). Survival of cells postharvest depends on level of drought tolerance, and the ability to maintain membrane homeostasis while adapting to stress (Thompson et al., 1982; Sexton and Orcutt, 1996; Rawyler et al., 2002). Senescence is often linked to abscission (Cothren et al., 2001), a stress response that has becomes problematic in root-detached balsam fir.

Abscission is a physiological plant process, which results in release of entire organs, such as leaves or needles, flowers, seeds and fruit from the main body of the plant. This is an adaptation to shed non-functioning, damaged or infected organs, or to make possible the fall of fruit and dispersion of seeds (Lewis et al., 2006; Cho et al., 2008; Estornell et al., 2013). However, in many agricultural species low yield or damaged products can be attributed to premature abscission, which is the case in Christmas trees **(**Patterson**,** 2001). The physiological mechanisms that control abscission are poorly understood and species specific (Hinesley and Snelling, 1997; Bates et al., 2004; Lewis et al., 2006; Vashisth and Malladi, 2013).

Abscission usually occurs in a band of densely packed cells referred to as the abscission zone (AZ), varying from a few to many cells thick at the junction between the organ and the main plant (Sexton and Roberts, 1982; Taylor and Whitelaw, 2001). These cells are supposedly predetermined at an early age (Patterson, 2001). For a brief period of time before abscission, these cells are able to respond to chemical signals that trigger abscission. Abscission is usually preceded by cell enlargement in the AZ. During abscission, the middle lamella of the cell wall is dissolved by wall degrading enzymes, such as cellulase and pectinase, forming a fracture plane. These diverse hydrolytic enzymes orchestrate abscission and seal the AZ cell walls on the remaining part of the plant (Addicott, 1982). In balsam fir, it has been found that prolonged exposure to ethylene activates the hydrolytic enzyme cellulase, which weakens cell walls and causes abscission. We still do not know the full underlying mechanisms that promote abscission and whether FA signaling could be involved. The lipid cutin is embedded in the cell wall, but changes in this lipid in the needle cells is unknown to date.

Abscission, in general, is regulated by environmental as well as developmental cues. The cells of the AZ are able to respond to certain signals and abscission may be activated in times of stress mediated by hormones. To date the only hormone conclusively found to induce abscission is ethylene. Other growth regulators that may be related to abscission are ABA; auxins; jasmonic acid (JA); polyamines; brassinosteroids; CK; and gibberellins. Signals from hormones potentially lead to the activation of genes promoting AZ separation,

but it may be possible that FA and lipids are involved either before or after hormone production (Kubigsteltig et al., 1999; Estornell et al., 2013; MacDonald, 2012).

2.4 Polar lipids and fatty Acids

2.4.1 General Background

The main components of biological membranes are glyceroplipids, with up to 1000 diverse lipid species in one cell (Welti et al., 2002). Each lipid has a specific FA composition highly preserved in the plant kingdom. Compositional changes in glycerolipids and FA occur in response to stress, and membrane composition may determine stress resistance. In addition, glycerolipids and FA have more recently been found to be signaling molecules and/or precursors giving rise to signaling molecules related to abscission and/or senescence (Lessire et al., 2009). There has been very little research on lipids and FA in balsam fir, and in addition, conifers have not been studied extensively in that regard. The following review describes the potential lipids that will be found in balsam fir both pre-harvest and postharvest, as these are typical in higher other plants.

2.4.2 Fatty Acids

Cells of plants typically contain 5-10% lipids, primarily in the cellular membranes, with 40-50% of these found in the chloroplast (Ohlrogge and Browse, 1995). Lipids are FA and their derivatives, FA being organic compounds synthesized by the condensation of malonyl coenzyme-A units by FA synthase in the plastids. FA make up the acyl chains or "tails" of all glycerolipids, and are either saturated (SFA) or unsaturated (USFA), having no double bonds or at least one double bond, respectively (Fig. 3). FA are dynamic molecules, can vary in the degree of unsaturation, and SFA can become unsaturated through the action of desaturase enzymes controlled by FA desaturase genes (Makarenko et al., 2014). If lipids contain more than one double bond, they are called polyunsaturated FA or PUFA. Those with one double bond are monounsaturated fatty acids (MUFA) Five FA make up over 90% of the acyl chains in plant glycerolipids: 18:1n9, oleic acid; 18:2n6; linoleic acid; 18:3n3, α-linolenic acid; 16:0, palmitic; and in some species 16:3n3; hexadecatrienoic acid. Under certain conditions the majority of all FA are made up of PUFA, 18:3n3 (Buchanan

et al., 2000). FA structure is known to change with varying environmental conditions, and composition is important in determining the ability of plants to respond to stress (Nishida and Murata, 1996; Buchanan et al., 2000). Free FA (FFA), are found in very small amounts in plant cells, but can be hydrolyzed from lipids in all membranes (Ohlrogge and Browse, 1995).

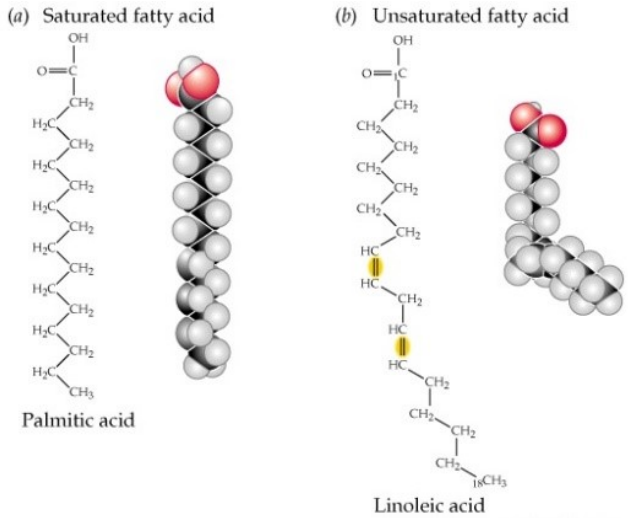

Figure 3. SFA, palmitic with 0 double bonds versus USFA, 18:2 *n6*, with two double bonds (Buchanan et al., 2000).

2.4.3 Types of Lipids

In plants, there are 3 main classes of membrane lipids; glycerolipids, sphingolipids, and sterols (Buchanan et al, 2000; Fahy et al., 2009; Furt et al., 2011). Glycerolipids consist of 4 main groups: galactolipids (GL), PL; triacylglycerols (TAG); and sulfolipids (Buchanan et al., 2000). In leaves, the glycerolipids studied in relation to membrane damage are primarily GL and PL, therefore, they are the focus of this research. It should be noted, however that knowledge is increasing in the area of cuticular lipids and sphingolipids and their role in adaptation to stress, but they are not included in this review (Benning et al., 2003). GL, are associated mostly with the chloroplast membranes, and PL compose primarily other membranes of the cell, particularly the plasma membrane (Lee, 2000; Dörmann and Benning, 2002**).**

Lipid nomenclature has been reviewed and universally accepted in 2005, updated once more in 2009, to meet the growing field of lipidomics (Fahy et al., 2009). To understand composition and signaling involving glycerolipids, there have been notable changes to the naming an abbreviating of lipid classes and species, of which there are 3009 categories listed in the LIPID MAPS database (Fahy et al., 2009). This review will be using the updated names and abbreviations.

2.4.4. Chloroplast Lipids

GL, monogalactosyldiacylglycerol (MGDG), and digalactosyldiacylglycerol (DGDG; Fig. 4), found in the chloroplast membranes are the most abundant glycerolipids in leaves, and major sources of PUFA (Dörmann and Benning, 2002; Kaniuga, 2008). These lipids have one or two galactosyl head groups, and contain no phosphate (Buchanan et al., 2000; Hölzl and Dörmann, 2007). MGDG is abundant in the thylakoids and the inner chloroplast membrane, whereas DGDG is more abundant in the outer membrane. The lipid composition of the chloroplast is shown in Figure 4, including the other glycerolipids found there. MGDG is confined to the chloroplast, whereas DGDG is sometimes found in the plasma membrane, and tends to replace PL in times of phosphate starvation (Anderson et al., 2005). GL are thought to be important for photosynthesis, and often they are one of the first organelles to be damaged in times of stress resulting in photoinactivation. Chloroplast remodeling occurs in stressful situations such as water deprivation (Block et al., 2007).

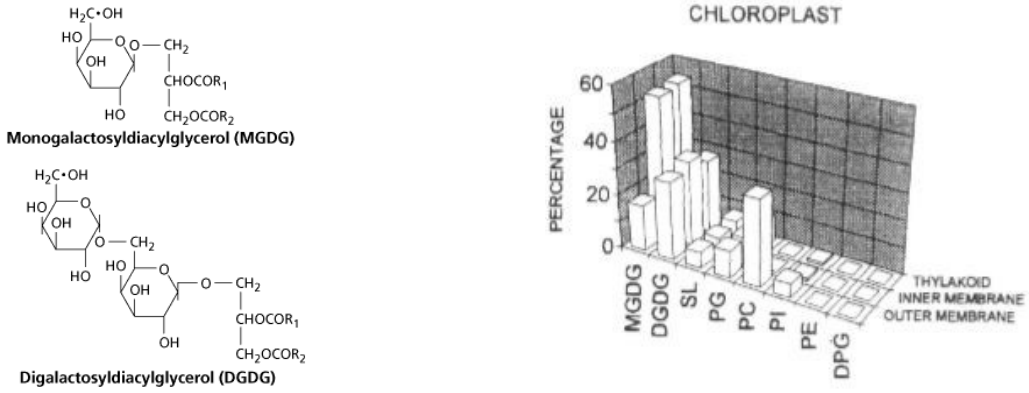

Figure 4. (left) Structure of the common GL, MGDG, and DGDG (Dörmann, 2013); (right) Lipid composition of the chloroplast membranes (Marechal et al., 1997).

2.4.5 Phospholipids

PL (Fig. 5) are synthesized by esterification of FA to two hydroxyl groups of sn-glycerol-3 phosphate to produce PA. All other PL are created by esterification of various head groups. FA are attached to the stereospecific or sn-1 and 2 positions. Fig. 5 also shows the three "sn" positions in a PL molecule. Plant structural PL include the lipid classes, in order of abundance, 1) phosphatidylcholine (PC), 2) phoshatidylethanolamine (PE) 3) phosphatidylglycerol (PG), and 4) phosphatidylserine (PS; Mosblech et al., 2008; Furt et al., 2011). Signaling PL have been identified as, 1) PA, 2) diacylglycerolpyrrophosphate, 3) lysophophatidic acid, and 4) phosphoinositol (PI) including phosphoinositol polyphosphates (phosphorylated derivatives of PI). The latter are named according to number and position of phosphate groups, such as phosphatidylinositol monophosphate (PIP), phosphatidylinositol bisphosphate (PIP_2), and inositol triphosphate (IP_3); Kooijman et al., 2005; Bargmann and Munnik, 2006; Mosblech et al., 2008; Wang and Chapman, 2013). Classes are determined by the head group of the PL, but species are determined by the FA composing the tails of the particular class of PL. The composition of the tail groups is specific to various organelles and thought to be related to function (Meijer and Munnik, 2003; Furt et al., 2011; Tayeh et al., 2013).

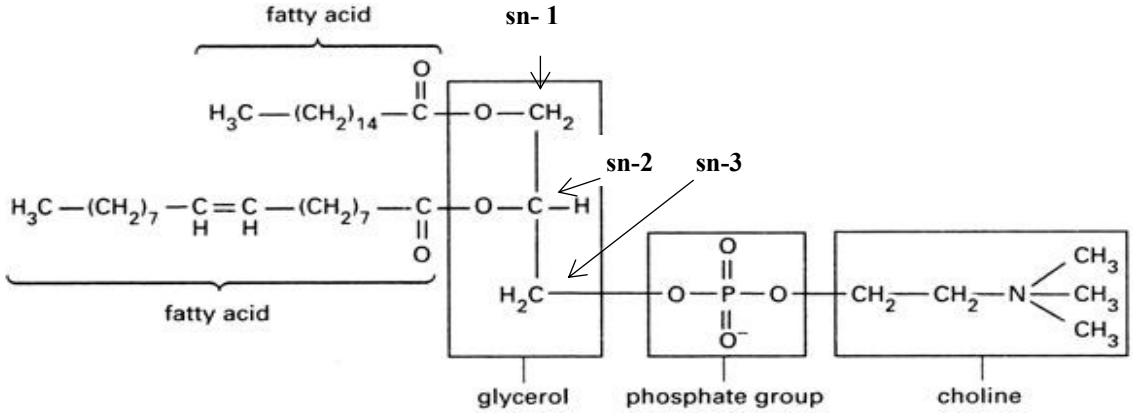

Figure 5. PL showing PC structure. All PL have the same basic structure with different head groups instead of choline and varied FA. Sn 1, 2, and 3 positions are marked (Oxford Dictionary of Chemistry, 2014).

2.4.6 Fatty Acid and Lipid Synthesis

FA are necessary for production of cellular lipids. FA synthesis starts with acetyl Co-enzyme A, a product of the citric acid cycle. It is the direct precursor for the methyl end of the FA. Synthesis starts with the carboxylation of acetyl coenzyme-A into malonyl coenzyme - A utilizing acetyl coenzyme-A carboxylase. Malonyl coenzyme-A is, in turn, acted on to produce SFA, palmitate and stearate, the latter being more common in plants (Harwood, 2018). These SFA can be further elongated and desaturated to form specific MUFA and PUFA (Fig. 6).

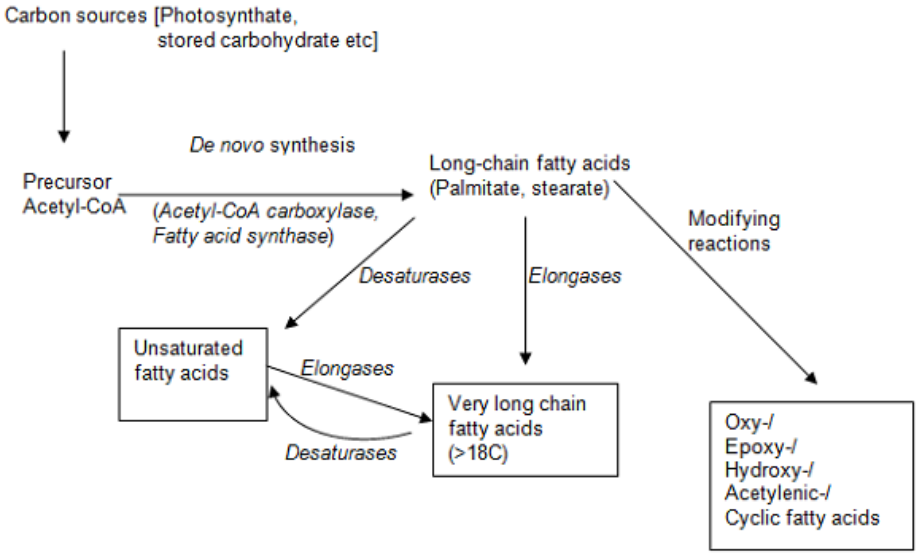

Figure 6. Summary of FA synthesis resulting in unsaturated, saturated and unusual FA (coenzyme A = CoA).

Lipids are synthesized in the chloroplast and endoplasmic reticulum *via* prokaryotic and eukaryotic pathways, respectively (Maréchal et al., 1997; Block et al., 2007). The prokaryotic pathway results in16-C FA in the sn-2 position, whereas the prokaryotic pathway results in a lipid containing an 18-carbon FA in the sn-2 position. It is not known which pathway the balsam fir uses. However, in all cells, lipid trafficking is required to distribute lipids to all parts of the cell, including the cuticle. A disruption in any or all functional membranes can interfere with synthesis and distribution of lipids (Block et al., 2007; Haucke and Paolo, 2007; Hurlock et al., 2014). Maintaining healthy chloroplast membranes is important for balsam fir postharvest for both photosynthesis and lipid synthesis (Block et al., 2007). Lipid synthesis begins in the chloroplast: firstly, incorporating FA into GL, MGDG and DGDG, and PG, the main PL in the thylakoid and

inner chloroplast membrane (Joyard et al., 2010). In Figure 7, you can see the importance of 18:3 in chloroplast membrane lipids, almost always in the sn-1 position. The FA in the sn-2 position varies depending on the pathway utilized. In addition to chloroplast lipid biosynthesis, there is a huge flux of FA to the endoplasmic reticulum for processing into other PL, which in turn are transported to the plasma membrane and other intracellular organelle membranes.

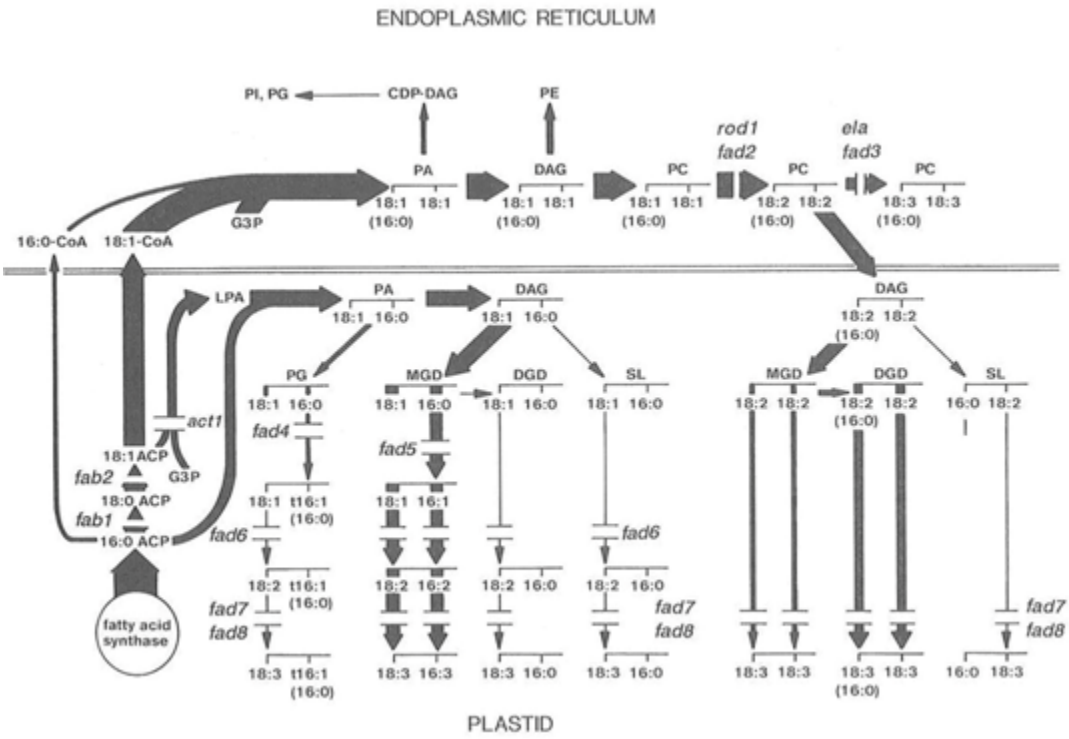

Figure 7. The two-pathway scheme for membrane glycerolipid synthesis in the leaves of *Arabidopsis* (Wallace and Browse, 2002).

2.5 PHYSIOLOGICAL/BIOCHEMICAL CHANGES DURING ABSCISSION POSTHARVEST

Biophysical and hormonal changes in postharvest balsam fir are not fully understood. In MacDonald and Lada (2014), many physical and phytohormonal changes were monitored in balsam fir branches postharvest. There was a decrease in water usage, xylem pressure potential, relative water content, capacitance, needle break strength, chlorophyll index, and IAA postharvest. On the other hand, there was an increase in needle loss, membrane injury index (MII), ABA, and CK. Decreases in water use, xylem pressure potential, relative

water content, and capacitance combined with increases in MII and ABA found in this study are consistent with water deficit stress or dehydration. Decrease in IAA is related to increasing sensitivity to ethylene, whereas the decrease in break strength is linked to cell wall breakdown and abscission (MacDonald et al., 2010; Basu et al., 2013). Prior research has identified prolonged exposure to ethylene as a mediator of abscission in balsam fir postharvest (MacDonald et al., 2011b), while MacDonald and Lada (2014) additionally suggest key roles for ABA, IAA, and CK, in postharvest needle abscission.

Another biophysical change of great interest is needle retention related to clonal variations and cold acclimation. Cold acclimation data is important to Christmas tree producers in planning their harvest date. In addition, earlier harvest is becoming necessary for timely export to foreign countries, and in some cases is resulting in poorer needle retention postharvest. MacDonald et al. (2014) investigated the effect of cold acclimation on balsam fir. It was found that high-NRD, needle abscission duration, trees changed very little from September to January in needle retention, whereas low-NRD trees increased significantly in needle retention. Many studies have been published on cold acclimation for other plant species, and it has been shown that lipid and FA composition has a great impact on the ability to survive at cold temperatures (Scotti-Campos et al., 2013; Bohn et al., 2007; Partelli et al., 2011). The degree of unsaturation of the needles also could be related to needle retention postharvest in balsam fir.

2.6 Roles of glycerolipids and Fatty Acids in stress responses

Plants need mechanisms to allow them to adapt to an ever-changing environment. Unlike animals, plants cannot move away from adverse environments, nor do they have sense organs such as eyes and ears. Plants can, however, sense stimuli from their environment through disturbances or changes in membranes (Barkla and Pantoja, 2011; Furt et al., 2011). Some changes are adaptive, whereas others may be symptoms of stress injury. Biotic and abiotic stressors can interfere with membrane integrity, depending on the source and degree of stress (Barkla and Pantoja, 2011). Stress is thought mostly to be due to dehydration in balsam fir postharvest, so the following reviews what could happen in cell and chloroplast due to stress (MacDonald et al., 2015).

GL and PL play essential roles as mitigators of stress. As stated previously, membrane integrity is important to the survival of the cell. Membranes are very dynamic, and can change due to external cues such as abiotic stresses. In addition, they can be reservoirs for biologically active lipids (Shah et al., 2005). Photosynthetic membranes are particularly sensitive to environmental cues, and have a high degree of plasticity to prevent photo inactivation, which can lead to cell damage (Buchanan et al., 2000). Reactive oxygen species (ROS) or intermediates, such as hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\bullet}OH$), and superoxide ($O_2^{\bullet-}$), are now thought to be the primary cause of cell damage during stress, and could be related to balsam fir needle abscission and/or senescence postharvest (Sun et al., 2011). Normal aerobic processes in the cell generate ROS, *via* the enzyme nicotinamide adenine dinucleotide phosphate-oxidase, whereas antioxidant defense systems scavenge ROS and maintain them at non- damaging levels (Bartoli et al., 1996; Laloi, 2004). Small amounts of ROS serve as signal molecules and will be discussed in an upcoming section (Song et al., 2013). Successful activation of antioxidant enzymes, such as catalase and superoxide dismutase, will produce enough antioxidants to keep ROS in check. However, there can be an oxidative burst caused by abundance of respiratory burst homologs, nicotinamide adenine dinucleotide phosphate-oxidases, related to a change to beta oxidation in the cell during stress. This upsets the delicate ROS/antioxidant balance creating oxidative stress, resulting in lipid degradation, and damaging the cellular membranes (Tintinger et al., 2008). This is linked to loss of membrane integrity which has been found to correlate with abscission in and senescence in balsam postharvest (Kar, 2011; MacDonald and Lada, 2014). It is possible that certain clones of balsam fir postharvest can more efficiently scavenge ROS due to their membrane FA composition and structure than others.

FA are critical in preventing damage by ROS, thus preventing membrane leakage leading to cell senescence, and possibly abscission. As previously stated, FA determine the species of each lipid class. For example, lipid species are shown here with the FA composing them in brackets: 34:2 (16:0–18:2), 34:3 (16:0–18:3), 36:4 (18:2–18:2), 36:5 (18:2–18:3), and 36:6 (18:3–18:3). These are the most common arrangement found in GL and PL (Vu et al.,

2014). Increased fluidity of membranes is related to increased desaturation of membrane lipids, and often a desaturase enzyme will be activated in times of stress to preserve fluidity (Wang et al., 2006).

One of the best known examples of compositional changes in FA and lipids is the unsaturation of glycerolipid FA seen during cold acclimation, particularly in the chloroplast membranes (Bohn et al., 2007). Increases of USFA in cellular membranes, both at the beginning and along the acclimation period are important for increased cold sensitivity (Partelli et al., 2011). This maintains proper membrane fluidity, stability of the chloroplast membranes, and ROS scavenging ability. There is an increase in USFA versus SFA during cold acclimation. It is thought that CK may play a role in increasing membrane desaturation and the ability to deal with drought stress (Ivanova et al., 1998; Kull et al., 1978).

It is also thought that CK triggers more DGDG production, decreasing the MGDG: DGDG ratio in the cell. Under non-stressful conditions, MGDG makes up approximately 50% of chloroplast membrane lipids, as opposed to 20% by DGDG under non-stressful conditions. MGDG does not form bilayers and forms hexagonal-II phases in water, whereas DGDG is able to form bilayers. MGDG is converted into DGDG during low temperature stress or drought stress, which results in more bilayer lipids in the chloroplast and greater stability (Ivanova et al., 1998; Dörmann, 2013).

The fact that CK is thought to trigger these adaptations during stress, does not explain the doubling of CK seen during abscission in balsam fir (MacDonald and Lada, 2014).This finding in balsam fir contradicts previous studies in which CK are thought to be able to negate the effects of water stress since they are thought to be antagonistic of ABA (Pospíšilová et al., 2000). However, various CK may have varying effects on the cell, and sometimes exogenous applications, used in most studies, may produce a different result than endogenous formation. In he case of balsam fir postharvest, it would be interesting to test CK in different parts of the branch versus the whole branch, as CK have been found to

have hierarchal distribution and local effect in other conifer species with terminal parts of a branch having twice as much as lateral parts of a branch (Chen et al., 1996)

Cold acclimation changes in other plants can possibly shed light on clonal differences previously mentioned in postharvest balsam fir in high and low NRD clones. Prior to cold acclimation, high NRD clones may have more USFA in their membranes than low NRD clones, making them a better choice for earlier harvest. Their membranes are more fluid and they can effectively scavenge ROS for longer. In addition, these high NRD clones, if indeed this is true, would have a more plentiful source of precursor molecules for jasmonic acid (JA), production, which is known to enhance stress tolerance (Sun et al., 2006; Gfeller et al., 2010). If stress has a long duration, lipid biosynthesis may not be able to keep up with replacing the degraded membrane lipids or the products of lipid degradation or ROS may become cytotoxic, and finally the branches succumb to the stress by abscising needles. High NRD clones may not change much during cold acclimation, whereas low NRD clones could last longer after acclimation due to greater desaturation of their membrane lipids than previously, now having the benefits that the high NRD clones had earlier in the season. If this is so, there should be a greater increase in unsaturation before and after cold acclimation in low NRD clones than in high NRD clones.

2.7 PHYSIOLOGICAL/BIOCHEMICAL CHANGES RELATED TO LIPID SIGNALING

2.7.1 Signal Transduction and Phospholipases

Plasma membrane lipids are the first to receive environmental stimuli or stimuli from a neighboring cell (Burnette et al, 2003; Browse, 2005). Hormones react with receptors on the surface of the cell, initiating a signaling cascade, often referred to as a signal transduction pathway that will produce precursor lipids, their effector enzymes, as well as the resulting breakdown products (Buchanan et al, 2000; Meijer and Munnik, 2003; Mayr et al., 2006; Zytkowiak et al., 2005; Kachroo and Kachroo, 2009). These messengers stimulate a response or amplify the signal to help increase stress tolerance, or help the plant adapt to their new environment. Signaling involves interactions and crosstalk between lipids and phytohormones, ROS, Ca^{2+}, enzymes, and transcription factors (Lewis et al.,

2006; Peterson et al., 2010; Gil-Amado and Gomez-Jimenez, 2013). Therefore, when a fir tree is cut, it initially responds to wounding stress and later on to drought stress. These two stimuli may bring about different responses, and in addition responses will vary due to the speed of imposition, degree of stress, and interaction with other chemicals (Mazliak, 1983; Kar, 2011; Okazaki and Saito, 2014). Inherently there will be responses at the cellular level related to survival, which may inhibit growth, initiate abscission of some plant parts to preserve the rest, or result in cell death (Okazaki and Saito, 2014).

Lipid signaling pathways are complex, interrelated, and involve numerous enzymes. Phospholipases are key ubiquitous enzymes related to stress response, and lipid and FA signaling (Li et al., 2009). Three main types of this enzyme class cleave PL in different locations (Fig. 8). The major phospholipases are phospholipase A, C, and D; PLA, PLC, and PLD, respectively. PLA hydrolyzes membrane PL into corresponding lysoPLs, such as lysoPC, and FFA (Alferez et al., 2005). These FA can act as precursors for oxylipin biosynthesis, or FA can be signaling molecules in their own right. For instance, oleic acid has been known to stimulate phospholipase enzymes that cleave PL during stress. The second messengers, lysoPL, can activate H-ATPase in the tonoplast, causing acidification of the cytoplasm, a known stress response. PLC activates the IP_3 pathway, and collaborates with another enzyme, diglycerol kinase (DGK), to activate the diacylglycerol (DAG) pathway. DAG is further transformed into another second messenger, PA. PLD hydrolyzes PL resulting directly in the formation of PA *via* a different pathway (Munnik, 2001; Testerink and Munnik, 2011). Phosholipase in their role during stress are summarized in Figure 9. There is very little research done on gymnosperm lipid signaling, but due to the ubiquitous nature of these phospholipases, it is likely they are involved in balsam fir signaling postharvest.

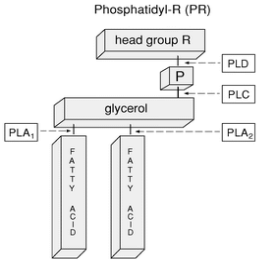

Figure 8. Hydrolysis, and abbreviations of the common phospholipases (Tayeh et al., 2013).

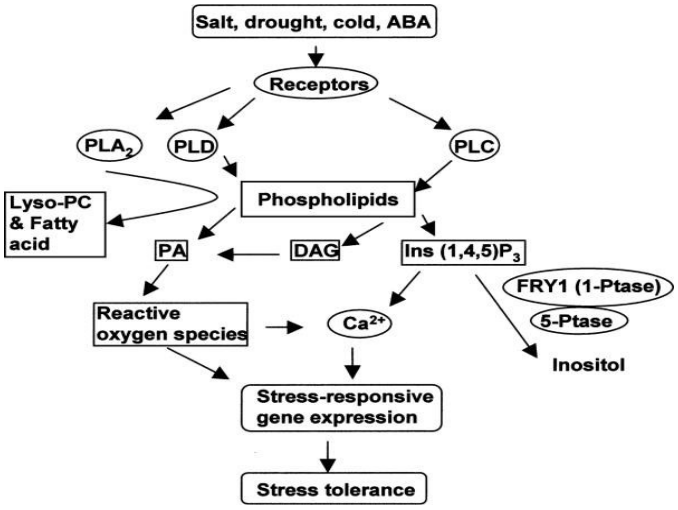

Figure 9. Some roles of PLA, PLC, and PLD in the cell related to stress tolerance (Meijer and Munnik, 2003).

2.7.2 Inositol Polyphosphates and Signaling

PI and its many phosphorylated isomers have the ability to permeate both hydrophobic and hydrophilic environments, making them perfect signaling molecules (Laxault and Munnik, 2002; Haucke and Paolo, 2007). The IP_3 and DAG signal transduction pathways are most well-known (Fig. 10). A transient increase in PIP_2, the initiator of this pathway in the cell membrane in response to stress has been well documented (Buchanan et al, 2000). PIP_2 is the precursor molecule for both IP_3 and DAG, which are both second messengers in the cell. A signaling molecule, potentially a hormone, binds to the receptor in the plasma membrane. These receptors have a wide array of ligands and are associated with g-proteins. An activated g-protein in turn activates PLC. PLC cleaves PIP_2, which is in the plasma membrane, into IP_3 and DAG. After this cleavage, two distinct downstream pathways are activated, referred to as the IP_3 pathway and the DAG pathway (Buchanan et al., 2000). IP_3 binds to receptors on the endoplasmic reticulum (ER) of the cell and mobilizes Ca^{+2}, calcium, from the ER into the cytosol. Ca^{+2} is also considered a second messenger. IP_3 has been implicated in ABA expression and stomatal closure, which we know occurs in balsam fir postharvest (Burnette et al., 2003; Thiagarajan, 2012).

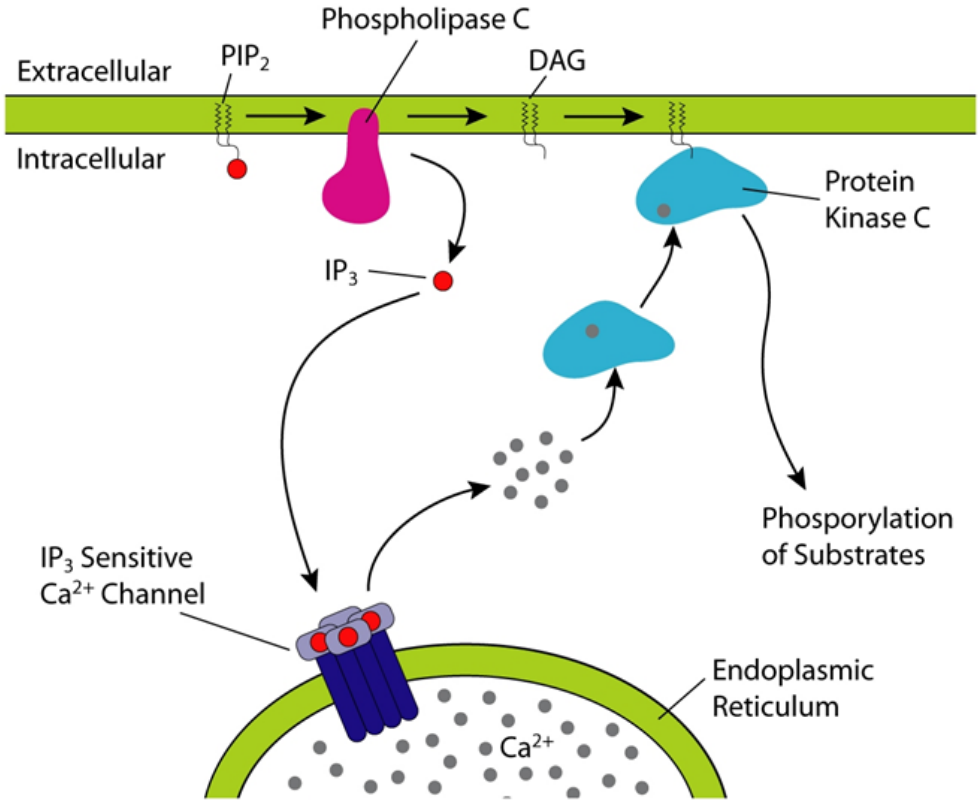

Figure 10. IP_3 and DAG pathways activated by PIP_2. (Creative Commons)

2.7.3 Phosphatidic Acid and Signaling

PA is emerging as a very versatile second messenger in the cell (Laxault et al., 2002; Munnik and Testerink, 2009; Furt et al., 2011; Tayeh et al., 2013). PA is a stress signaling molecule in plants playing a role in degradation, signaling, and lipid turnover. PA (Fig. 11) is produced 1) *via de novo* PL bio-acylation of lysoPA, 2) PLD hydrolysis of PL, and 3) the DAKP pathway. Using the latter pathway, PIP_2 is a critical cofactor for PLD, and profoundly affects the activity of PLD (Oude Weernink et al., 2007). PLD and PA are linked to wounding and drought stress, the two identified stresses that postharvest balsam fir have to deal with. There have been many links made between PA and/or its catalyzing enzymes to biophysical and phytohormonal changes in the cell (Bargmann and Munnik, 2006).

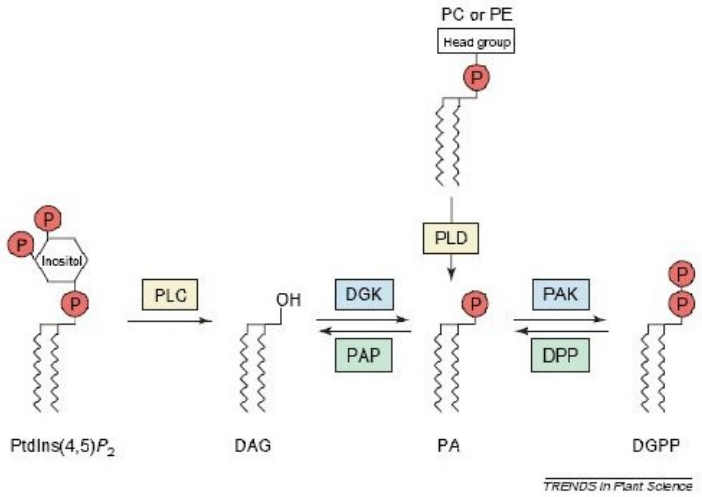

Figure 11. Formation and attenuation of PA (Tayeh et al., 2013).

2.7.4 Oxylipins and Signaling

The most commonly known oxylipin is JA and its derivatives. JA is rapidly emerging as an important lipid-based signaling molecule in plants, related to many stress responses (Howe, 2004). JA synthesis originates from PUFA, 18:3n3 and 16:3n3, primarily found in the chloroplast membranes, replenished as needed (Fig. 12). When stress is detected in the cell, these FA are converted into 13-hydroperoxylinolenic acid by lipoxygenase (LOX). This, in turn, is a substrate for allene oxide synthase and allene oxide cyclase resulting in the formation of 12-oxo-phytodienoic acid. JA is formed after reduction and 3 steps of oxidation (Fig. 12). JA can be catabolized to form methyl jasmonate, and numerous conjugates and metabolites (Sembner and Parthier, 1993; Howe, 2004; Browse, 2005; Reinbothe et al., 2009. JA has been found to be related to ethylene synthesis, which induces abscission in balsam fir postharvest (Mosblech et al., 2008; Schaller and Stintzi, 2009; Engleberth, 2011). In *Zea mays*, induced JA levels resulted in maximal ethylene levels 8 – 16 hours later (Schmelz et al., 2003). In conifers, insect attack and mechanical wounding, known stimuli that produce JA, and treatment with methyl jasmonate, increased ethylene levels. In addition, JA and ethylene were found to work together to elicit a stress response in tobacco plants (Onkokesung et al., 2010).

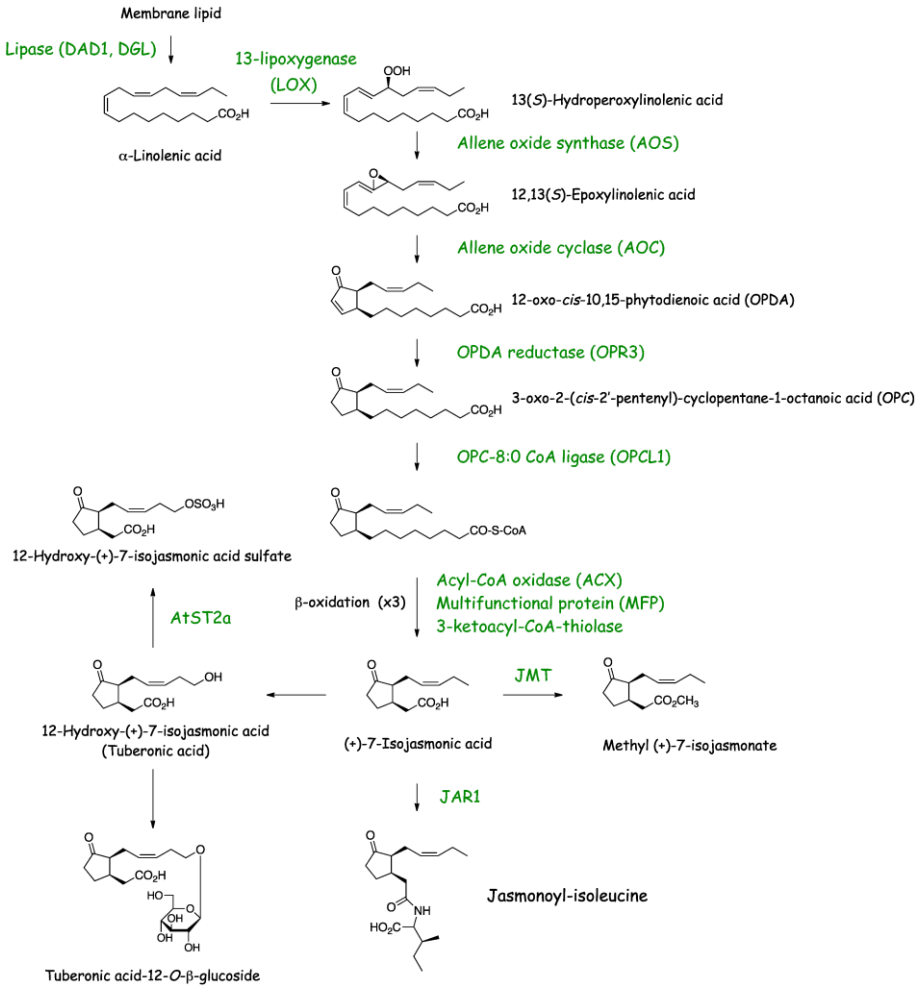

Figure 12. Synthesis of jasmonate and derivatives *via* the octadecanoic pathway (Riken, n. d.).

2.8 POSSIBLE INTERACTIONS - SIGNALING MOLECULES AND OTHER CELL MOLECULES

Previously some molecules have been identified as being linked to abscission postharvest in balsam fir. There are some possible interactions that could occur in the needle cells, as some of these relationships have been identified in other plants. There is enough information below to justify sampling needles, both pre-harvest and postharvest. There is a multitude of findings about signaling lipids. ROS, Ca^{+2}, and phytohormones and their role during stress, but not many researchers look at all the components in one study. Consistently, ethylene and ABA have been identified as phytohormones related to

abscission in balsam fir postharvest, as well as other plants (Daszkowski-Golec and Szarejko, 2013a, b; MacDonald, 2010; MacDonald and Lada, 2014). A low concentration of ethylene has been found to delay abscission, whereas a high concentration induces abscission (MacDonald et al., 2011b). Interestingly, the same relationship exists between ABA and abscission, suggesting a linkage between the two (Thiagarajan, 2012). These results concur with research by Chen and Wang (2002) on abscission and senescence in poplar trees. In both, ABA delayed abscission in drought resistant genotypes, and promoted abscission in drought sensitive types, which could shed some more light on clonal abscission variations in balsam fir, postharvest (Thiagarajan, 2012). PA has been implicated in signaling pathways related to ABA and ethylene, and can be suggested as a potential link between the two (Sun et al., 2006; Okazaki and Saito, 2014)

ABA stimulates PLDα1, which in turn stimulates PA production from PL, and subsequently this leads to endogenous ethylene production, making a connection between ABA, PA, and ethylene production (Sharp and leNoble, 2002; Gómez-Cadenas et al., 1996; Miao et al., 2000). To further substantiate this, Ritchie and Gilroy (1998) suggested that ABA activates the enzyme PLD to produce PA that is involved in triggering the subsequent ABA responses, one of which could be ethylene production. In addition, an increase in ABA in water-stressed plants promoted the synthesis of the precursor for ethylene, 1-amino cyclopropane -1-carboxylic acid (ACC; Gómez-Cadenas et al., 1996). In regards to senescence, independent action of ABA and ethylene has been suggested (Zacarias and Reid, 1990), but the relationship between these two hormones leading up to abscission in balsam fir postharvest is unknown. We do know they both increase postharvest in balsam fir. Thiagarajan (2012) hypothesized that high concentrations of ABA might hold an upstream pathway and synergistic role with ethylene to induce abscission in root-detached balsam fir. It can now be suggested that PA may be a link in that pathway, and ROS have also be suggested as playing a part.

Recent studies have shown that drought related stress responses are mediated by ROS, in particular, hydrogen peroxide (Bailey-Serres and Mittler, 2006; Anjum et al., 2014). Protein kinase C activated by Ca^{2+} and DAG facilitates assembly and activation of NADPH

oxidase on the outer membrane, which generates ROS (Tintinger et al., 2008). Droillard et al. (1989) found that ROS resulted in a burst of ethylene with a subsequent decrease in catalase and superoxide dismutase, ROS scavengers. It has also been shown that PLDα1 and PA are involved in ROS production. Depletion of PLDα1 in *Arabidopsis* lessens the ROS production, while the addition of PA restores it (Song et al., 2013). It has been suggested that un-scavenged ROS stimulates drought-induced ABA synthesis placing ROS upstream of ABA. In addition, there is a demonstrated relationship between ROS, ABA, and ethylene during drought stress (Kar, 2011). **I**f this is the case IP_3 could be a lipid second messenger as well in this scenario. ABA regulates stomatal closure, which occurs upon activation of drought sensors in the outer plasma membrane. One key component in this mechanism is an initial increase in cytosolic Ca^{+2} concentrations that occurs within minutes of ABA exposure in *Arabidopsis* (McAnish et al., 2000; Staxen et al., 1999; Fan et al., 2001; Webb et al., 2001; Daszkowska-Golec and Szarejko, 2013a, b). This rapid increase in Ca^{2+} is preceded by an increase in IP_3 and is dependent on increased PLC activity (Lee et al., 1998; Staxen et al., 1999; Song et al., 2013). ABA could be the hormone that starts the IP_3 and DAG pathways. Lee et al. (1996) found that ABA induced PI turnover in guard cells.

Alternate studies have named ethylene as the mediator of PLD synthesis, further confirming that there is more than one pathway. In glucose-starved carrots ethylene mediated a PLD catabolic pathway leading to PA production (Lee et al., 1998). Other sources say that ethylene uploads the PLDα gene (Fan et al., 1997). These studies place PA downstream of ethylene, and remember that PA has been found to relate to H_2O_2 production. A very interesting study by Sakamoto et al., (2008) has proposed the pathway for petiole abscission in detached *Capsicum* leaves, and has placed H_2O_2 downstream of ethylene. Unfortunately, PLD, PA, and ABA were not included in this study. The *Capsicum* leaves were root-detached and hydrated and placed in an in vitro system to study stress-induced abscission in the abscission zone under various conditions. Firstly, similar to what we have observed in the balsam fir during postharvest abscission, IAA was depleted, and the addition of IAA inhibited abscission. Low levels of IAA have been linked to greater ethylene sensitivity and more sensitivity to H_2O_2, thus mitigating stress (Scherer,

1996; Morgan and Drew, 1997; Gangwar et al., 2012). Abscission was found to be induced by ethylene in *Capsicum* leaves, as we know happens in balsam fir postharvest. However, a continuous production of H_2O_2 by AZ cells was also recorded under stressful conditions prior to abscission, and this was related to cellulase production, an enzyme known to be involved in cell wall breakdown in plants, including balsam fir postharvest (Patterson, 2001; MacDonald et al., 2009). Abscission was inhibited by addition of catalase, a ROS scavenging enzyme, making a direct connection between ROS and abscission. H_2O_2 inhibitors reduced abscission in the presence of ethylene stimulants, proving that H_2O_2 was working downstream of ethylene in *Capsicum*, and in turn upregulates the expression of cellulase genes. If this is the case, ethylene could stimulate the activity of PLD to produce PA, which in turn caused the production of H_2O_2 and cellulase production – and ultimately abscission. This may happen in balsam fir postharvest.

Conflicting reports as to the role and identity of PLD and PA suggests multiple PLD and PA pathways in plants a multiplicity of responses. Plants have 12 genes controlling 12 different PLD species compared to animals that have only 2, so there should many more biochemical pathways involving these isomers in plants than animals (Xiong and Zhu, 2001). The PLD isomers shown on the left in Figure 13 show different pathways, one involving the ABA response and another involving the ROS response. If this is so, could there be some crosstalk between the two. Notice that here PLDα1 stimulates PA and then ABA, contradicting research mentioned earlier. It seems there is still more research to be done to find all the answers. It could be that some the isomers of PLD work cooperatively to achieve drought tolerance.

Further research about PLD shows that there is an increase in PLDα and subsequent PA prior to JA production as well. It is thought that PA begins mitogen-activated protein kinase (MAPK) signaling into the nucleus affecting gene expression of CYP94B1 which is involved in JA-Ile turnover and attenuation of JA responses (Koo et al., 2006). JA is known to increase one hundred fold when a plant is under stress. JA is now thought to be involved in stomatal closure during drought stress. Recently researchers have gone so far to say that a burst of JA is needed for production of ABA (Engleberth et al., 2011). In postharvest

balsam fir, JA has not been tested, but this phytohormone is associated with wounding, which occurs when the tree is cut. ABA increases after the initial wounding, but more research is needed in postharvest balsam fir to determine how quickly it accumulates and the relationship it has to JA (MacDonald and Lada, 2014).

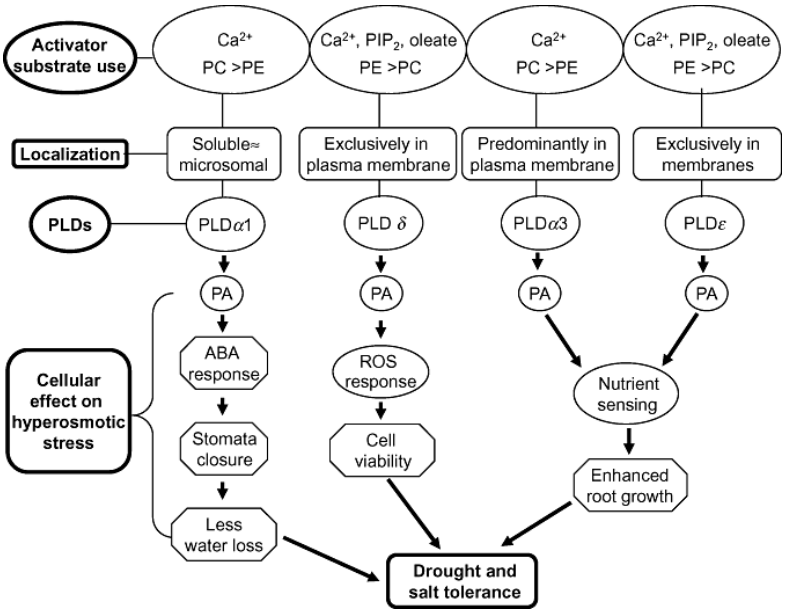

Figure 13. Multiple pathways for carious PLD enzymes that occur during drought stress and other stresses in plants (Hong et al., 2010).

2.9 SUMMARY

In this review various reports and papers describing the roles of plant lipids as mitigators of stress and as signaling molecules have been reported. Studies increasingly indicate that FA and glycerolipids crosstalk with phytohormones, enzymes, Ca^{+2}, and ROS to achieve responses to water deficits and other stresses that occur when a tree is detached from its roots, even when water is provided, as in the case of a Christmas tree. In addition, cellular membranes are also the target of damage leading to membrane leakage in most of these reports, and decrease in membrane integrity has been associated with senescence and abscission in postharvest balsam fir. This research in necessary to gain a better understanding of membrane structural and compositional changes in the needle of balsam fir postharvest. In addition, identification of signaling molecules and has the potential to connect the stimulus perception at the cell membrane to intracellular actions and

physiological responses to stress in balsam fir postharvest and allow us to build on this knowledge to develop new technologies to protect membranes and increase resistance to needle abscission in root-detached balsam fir. This background information leads us to the objectives and hypothesis found in the next chapter.

CHAPTER 3 – OBJECTIVES AND HYPOTHESES

3.1 HYPOTHESES

Six individual hypotheses were created, which correspond to the specific objectives in various chapters of this thesis.

1. Balsam fir will suffer more needle loss postharvest in response to a decline in polar lipids and breakdown of the FA associated with these lipids (linked to objectives in chapters five and six).

2. In low NRD clones the decline in lipids and breakdown of FA will occur earlier postharvest than in high NRD clones (linked to the objectives in chapter six).

3. Lower temperatures and reduced photoperiods that occur during cold acclimation will result in a decline in monolayer lipids and SFA in favor of bilayer lipids and USFA (linked to the objectives in chapters seven and eight).

4. Lower temperatures and reduced photoperiod will cause different changes in low and high NRD clones (linked to the objectives in chapters seven and eight).

5. The Scanning Electron Microscope (SEM) and Transmission Electron Microscope (TEM) will reveal surface and intracellular changes postharvest in membrane ultrastructure such as the cell membrane and chloroplast membranes to confirm findings about polar lipid changes (linked to the objectives in chapter nine).

6. Changes observed with the SEM and TEM will reveal genotypic differences (linked to the objectives in chapter nine).

3.2 OBJECTIVES

The specific objectives of this research were to:

1. Characterize the nature of lipids and FA in balsam fir, and determine links between polar lipid and FA changes in balsam fir postharvest and needle loss (linked to hypothesis in chapter five and number one above).

2. Determine the differences in polar lipid and FA in contrasting genotypes (high and low NRD) and the dynamics of change postharvest in balsam fir trees (linked to hypothesis in chapter six, and number two above).

3. Determine changes in polar lipids and FA profiles in needles sampled monthly onsite from September through to February in Nova Scotia (linked to chapter seven and eight, and number three above).

4. Identify genotypic differences in polar lipids and FA, if any, onsite at each sampling period during the process of cold acclimation, to see if there are any clonal variations that would help to explain the reason why low NRD balsam fir benefit more from cold acclimation than high NRD clones. This should provide a clue to why various balsam fir retain needles for varying lengths of time (linked to hypotheses in chapter 7 and 8, and number four above).

5. To examine the ultrastructure of membranes in a low and high NRD clone of balsam fir postharvest. The intention is to use SEM and TEM technologies to visualize intracellular changes to chloroplasts and other membranes, and surface changes, in order to substantiate, or understand, polar lipid changes found in subsequent research (linked to hypothesis in chapter nine, and number five and six above).

CHAPTER 4 – GENERAL METHODOLOGY

4.1 POSTHARVEST HANDLING

4.2.1 Collecting and Setting Up Branches

The protocol for sampling and postharvest set-up was based on previous studies by MacDonald et al. (2011b). Samples were taken from a 16-year-old balsam fir orchard at the Tree Breeding Center, Department of Natural Resources, Debert, Nova Scotia, Canada (lat. 45°25′ N, long. 63°28′ W). Each branch was cut from 2-year growth at 1.5 m aboveground. Samples, branches, for all experiments were taken from the balsam fir, *Abies balsamea, L*. Cut ends of the branches were submerged in deionized water in a clean bucket for transfer to the laboratory and acclimated to room temperature for 24 hours. All branches were given a fresh cut 2.5 cm above the previous cut while stem ends were held under the surface of the water to reduce risk of cavitation, weighed, and then placed in a 100-mL amber bottle filled with 100 mL of distilled water. The neck of each flask was plugged with cotton wool to reduce direct water evaporation and provide added stability to a branch (Figures 14 and 15). The branch and entire apparatus was weighed, which allowed for quantification of water uptake throughout the experiment. Branches were provided water throughout the experiment and the growth room was maintained at conditions similar to typical display conditions for balsam fir (i.e. 22°C constant temperature, 25% relative humidity, and a light intensity of 90 μmol m^{-2} s^{-1}).

Figure 14. Wrapping a balsam fir branch with cotton.

Figure 15. Experimental set up of balsam fir branch, wrapped with gauze, and placed in amber bottle.

Five clones were used for this research. Clone 706 was used in the first experiment (Chapter 5). In Chapters 6, a low NRD genotype, Clone 9, was used in addition to a high NRD clone, 506. In Chapters 7 and 8, two low NRD clones were used, 9 and 566, along with two high NRD clones, 506 and 37. High NRD clones are expected to shed all their needles in 33 to 43 days, whereas low NRD clones are expected to shed their needles in less than 20 days, usually 7 – 19 (Adams and Lada, 2011).

4.2.2 Per Cent Needle Loss (PNL)

Needle loss was calculated as a percentage of the fresh weight of abscised needles to the fresh weight of initial branch weight (MacDonald and Lada, 2012). Each day the branches used to determine needle loss were subjected to a "finger run" test, and this dislodged loose needles from the branch (MacDonald, 2010). To perform the "finger run" test, each branch was gently passed three times through the index and middle fingers always using the same operator for consistency, and the fallen needles were quantified gravimetrically and recorded to arrive at a fresh needle mass lost (g). The experiment was run until all branches

reached peak abscission. Typical postharvest needle abscission follows a sigmoidal curve, and two time points were used to compare needle abscission patterns: needle abscission commencement (NAC), PNA. NAC was defined as the point where postharvest abscission began (1% needle loss), and PNA was defined as the day which had the highest rate of needle abscission.

4.2.3 Stress Index (Fluorescence)

This procedure was used in all experiments except the first (Chapter 5). Needles from branches in the field were tested immediately when returning from the laboratory, in addition to weekly testing postharvest of needles that were still on the intact branch just before it was sacrificed for lipid and FA analysis. This maximum photochemical efficiency or optimum quantum yield of photosystem II (Fv/Fm), was fluorometrically determined using a MINI-PAM Photosynthesis Yield Analyzer (Walz, http://www.walz.com). Four to five attached needles were randomly selected from one branch and mounted on a piece of tape very close together with the top side of the needles facing up. These were placed in a leaf clip and dark-adapted for 15 min before Fv/Fm measurements with illumination by application of a saturation flash. Three random replicates for each experimental unit were done, at the time of sampling.

4.2.4 Water Uptake

Water uptake was estimated by weighing each bottle at the beginning of the experiment after being filled with water and then, weekly, each branch was removed from its flask and the flask was weighed again. The difference in mass was estimated as water uptake and the rate of water uptake was calculated as water uptake (mL g^{-1} d^{-1}):

$$Water\ Uptake = \frac{M_n - M_{n+1}}{7 \cdot M_b}$$

M_n is the mass of the flask on any given week while M_{n+1} is the mass of the flask on the subsequent week. M_b is fresh weight of the branch. The critical value for water uptake is

0.05 mL g^{-1} d^{-1}, below which is the point at which a branch will commit to abscission (Lada et al., 2016)

4.2.5 Membrane Injury Index (MII)

MII uses the percentage of electrolyte leaked into solution to quantify membrane integrity (Thiagarajan, 2012). Centrifuge tubes were filled with 30 mL of distilled water and were adjusted to room temperature (22-24 °C). The electrical conductivity of the distilled water (EC_w) alone was measured using a CDM 2e Conductivity Meter (Bach-Simpson, London, ON). Afterward, 0.4 g of needles were removed from each branch and completely submerged in the distilled water. The tubes were sealed and left at room temperature for 24 h. Initial conductivity (EC_0) was measured to determine the amount of electrolytes leached into solution. Sealed tubes were then placed in a forced-air oven for at least 4 h at 90 °C to kill tissues and then cooled to room temperature. Final conductivity measurements (EC_f) were taken after equilibrating to 25 °C to determine maximum leakage from the dead cells. MII was then calculated using the following formula:

$$MII = \frac{EC_o - EC_w}{EC_f - EC_w} x100$$

4.3 LIPIDS AND FA

4.3.1 Onsite Sampling

The goal was to observe lipid changes in needles remaining attached to the branch during various stages of abscission, so whole needles that had not yet undergone abscission were used for lipid extraction and polar lipid and FA analysis. Liquid nitrogen obtained from Praxair, Truro, NS was carried to the balsam fir orchard in Debert, NS in a 4.5 L liquid nitrogen dewar with a secure screw on lid. A 2 L stainless steel bowl was used for onsite freezing of branches. With gloved hands, liquid nitrogen was carefully poured into the bowl until the bowl was approximately one third full. A branch was dipped into the bowl using stainless steel tongs for approximately five seconds and removed. At that time needles were able to be knocked off the branch easily and stored in a 100 mL centrifuge

tube. Samples were carried back to the laboratory in a Styrofoam cooler containing liquid nitrogen, taken back to the laboratory for storage in a -80°C freezer to halt metabolic processes and to store until lipid extraction.

4.3.2 Lipid Extraction

For experiment one, lipids were extracted at the Kansas Lipidomic Research Center (Manhattan, KS, USA). Needles were quantified and sent on dry ice in a Styrofoam container. For subsequent experiments, 175 total branches of needles were extracted at the Dalhousie Agricultural Campus and extracts shipped to Kansas. All extractions were done using an extraction protocol adapted from *Arabidopsis* leaf tissue (Bligh and Dwyer, 1959). Needle samples were brought to the laboratory in a Styrofoam container of liquid nitrogen to keep frozen. For extraction, 1 g of frozen needles were pulverized into smaller pieces using a mortar and pestle and incubated in 1 mL of isopropanol with 0.01% butylated hydroxytoluene (BHT) at 75 °C for 15 min. Afterwards, 1.5 mL of chloroform and 0.6 mL of water were added. After shaking for 1 h at room temperature, the solvent was transferred to a new glass tube with a Teflon-lined screw-cap using a Pasteur pipette, and total 0.7 mL chloroform: methanol (2:1) was added and shaken for 30 min. The extraction was completed by adding 4 mL of chloroform: methanol (2:1) ten times and collecting the solvent. The solvent extracts were washed once with 1 mL KCl (1.0 M) and once with 0.66 mL water. The solvent was evaporated under nitrogen and the lipid extract was dissolved in 1 mL chloroform and stored in a -20°C freezer until time for shipping. Prior to shipping, 2 mL clear glass vials with PTFE-lined solid screw cap lids were quantified using an electronic scale accurate to three decimal points. Styrofoam container containing dry ice. Samples were transferred using a pipette to the new pre-weighed 2 mL vials, evaporated under nitrogen again and quantified. Dry samples were shipped to Kansas in a styrofoam container containing dry ice by courier.

4.3.3 Fatty Acid Analysis

FAME were prepared using methanolic hydrochloric acid according to Christie (1982). FAME were dissolved in 100–200 µL of hexane and placed in 2 mL gas chromatography

(GC) vials with inserts. FAME were quantified using gas chromatography GC-FID analysis performed at the Kansas Lipdomic Center, Kansas State University, on an Agilent Technologies 6890N Network GC system equipped with a HP-88 capillary column (100 m × 0.25 mm I.D., 0.20 µm film thickness) coupled with a FID. Injector and FID temperatures were set at 275 and 260°C, respectively. The carrier gas (Helium) pressure was 51.61 psi with a flow rate of 1.6 $mLmin^{-1}$min with continuous flow. For the detector, hydrogen flow rate was 30.0 $mLmin^{-1}$, air flow rate was 400 mL/min, and makeup flow rate (Helium) was 25 $mLmin^{-1}$. The GC oven temperature ramp was operated as follows, initial temperature of 150 °C held for 1min, increased at 10 °C/min to 175 °C, held 10 min then increased at 5 °C/min to 210°C, and held for 5 min. At last, temperature was increased at 5 °$Cmin^{-1}$to a final temperature of 230 °C, held 15 min. Total run time was 44.5 min. The sampling rate of the FID was 20 Hz. The FAME solutions were injected in the volume of 1 µL using an autosampler Agilent Technologies 7683 Series Injector in the splitless mode. The chromatograph worked under Agilent Technologies Enhanced Chemstation software. The FA were identified by comparison of RRT of the compounds in the sample with RRT of Supelco 37 component FA methyl ester mix standards. The moles of FA were calculated by the moles of internal standard (pentadecanoic acid, 15:0) and peaks' area.

4.3.4 Lipid Class and Species Analysis

Lipid extracts from 100 samples were analyzed on a triple quadrupole mass spectrometer equipped for electrospray ionization (ESI–MS/MS; Applied Biosystems API 4000) at the Kansas Lipdomic Center, Kansas State University, US. ESI-MS/MS analysis parameters and acquisition parameters are given in Table 1 and 2, respectively. The lipids in each class were quantified in comparison to two internal standards of the class. Lipid species within each head group were identified by total carbon number and total double bonds. Molecular species of each head class were quantified by comparing with the signals of the internal standards (Welti et al., 2002). A list of lipids able to be quantified from this procedure is shown in Table 3.

4.3.5 Q-test

All samples, with exception of the first 15, were subjected to quality control tests. Average coefficient of variation (CoV) for lipid analytes is a function of corrections used in data processing; CoV is equal to the standard deviation of the measurements for each analyte divided by the average. CoV was calculated from the QC samples, without any correction, using only the linear trend correction within each day's sample set, only the correction to the overall average across sample sets, or both corrections (as done on the experimental data).

Table 1. ESI-MS/MS analysis parameters (using Applied Biosystems API 4000) for plant lipids.

Class	Ion Analyzed	Positive Ion Scan Mode	m/z range	Reference
PA	$(M + NH_4)^-$	NL of 115.00	500 – 850	Shiva et al. 2013
PC/LPC	$(M + H)^-$	Pre of *m/z* 184.07	450 – 960	Brügger et al. 1997
PE/LPE	$(M + H)^-$	NL of 141.02	420 – 920	Brügger et al. 1997
PG	$(M + NH_4)^-$	NL of 189.04	650 – 1,000	Taguchi et al. 2005
LPG	$(M-H)^-$	Prec 153	-	Welti et al. 2002
PI	$(M + NH_4)^-$	NL of 277.06	790 – 950	Taguchi et al. 2005
PS	$(M + H)^-$	NL of 185.01	600 – 920	Brügger et al. 1997
DGDG	$(M + NH_4)^-$	NL of 341.13	890 – 1,050	Isaac et al. 2007
MGDG	$(M + NH_4)^-$	NL of 179.08	700 – 900	Isaac et al. 2007

DGDG, digalactosyldiacylglycerol; ESI-MS/MS, electrospray ionization tandem mass spectrometry; MGDG, monogalactosyldiacylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; LPC, lyso-phosphatidylcholine; PE, phosphatidylethanolamine; LPE, lysophosphatidylethanolamine; PG, phosphatidylglycerol; LPG, lysophosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine.

Table 2. Acquisition parameters of lipid classes using Applied Biosystems API 4000, triple quadrupole mass spectrometer

Parameter	PA	PC/LPC	PE/LPE	PG	PI	PS	DGDG	MGDG
Typical Scan Time (min)	3.51	1.28	3.34	3.21	4.00	4.01	1.67	1.67
Depolarization Potential (V)	100	100	100	100	100	100	90	90
Exit Potential (V)	14	14	14	14	14	14	10	10
Collision Energy (V)	25	40	28	20	25	26	24	21
Collision Exit Potential (V)	14	14	14	14	14	14	23	23

Table 3. Plant membrane lipids determined by the procedure described herein.

Class	Lipid Species
LPC	16:0, 16:1, 18:0, 18:1, 18:2, 18:3
LPE	16:0, 16:1, 18:1, 18:2, 18:3
LPG	16:0, 16:1, 18:1, 18:2, 18:3
PA	32:0, 34:6, 34:5, 34:4, 34:3, 34:2, 34:1, 35:4?, 35:3?, 35:2?, 35:1?, 36:7, 36:6, 36:5, 36:4, 36:3, 36:2, 37:4?, 37:3?, 38:7, 38:6?, 38:5?, 38:4?
PC	30:1, 32:0, 33:3: 33:2, 34:4, 34:3, 34:2, 34:1, 35:4, 35:3, 35:2, 35:2, 35:0, 36:6, 36:5, 36:4, 36:3, 36:2, 36:1, 37:5, 37:4, 37:3, 38:6, 38:5, 38:4, 38:3, 38:2, 40:5, 40:4, 40:3, 40:2, 40:1, 42:4?, 42:3?, 43:2?, 43:3?
PE	32:3, 32:2, 32:1, 32:0, 34:4, 34:3, 34:2, 34:1, 35:4?, 35:3?, 35:2?, 35:1?, 36:6, 36:5, 36:4, 36:3, 36:2, 36:1, 37:4?, 37:3?, 38:6, 38:5, 38:4, 38:3, 39:4, 39:3, 39:2, 40:7., 40:3, 40:2, 42:4, 42:3, 42:2, 42:1?, 44:4?, 44:3?, 44:2?
PG	32:1?, 32:0?, 34:4, 34:3, 34:2, 34:1, 34:0, 35:4?, 35:3?, 35:2?, 35:1?, 36:7, 36:6, 36:5, 36:4, 36:3, 36:2, 36:1
PI	30:4?, 30:3?, 32:4?, 32:3, 32:2, 32:1, 32:0, 34:4, 34:3, 34:2, 34:1, 35:4?, 35:3?, 35:2?, 35:1?, 36:7, 36:6, 36:5, 36:4, 36:3, 36:2, 36:1, 37:4?, 37:3?
PS	34:4, 34:3, 34:2, 34:1, 35:3?, 36:6, 36:5, 36:4, 36:3, 36:2, 36:1, 38:6, 38:5, 38:4, 38:3, 38:2, 38:1, 40:4, 40:3, 40:2, 40:1, 42:4?, 42:3, 42:2, 42:1, 44:3
DGDG	34:6, 34:5, 34:4, 34:3, 34:2, 34:1, 35:3?, 35:2?, 36:7, 36:6, 36:5, 36:4, 36:3, 36:2, 36:1, 37:3?, 38:7, 38:6, 38:5, 38:4, 38:3
MGDG	34:6, 34:5, 34:4, 34:3, 34:2, 34:1, 36:6, 36:7, 36:5, 36:4, 36:3, 36:2, 36:1, 38:6, 38:5, 38:4, 38:3

DGDG, digalactosyldiacylglycerol, MGDG, monogalactosyldiacylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; LPC, lyso- phosphatidylcholine; PE, phosphatidylethanolamine; LPE, lysophosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine. Where there are question marks, this indicates that it is possible, based on the FA present, that it is a lipid species.

CHAPTER 5 – LIPID AND FA CHANGES LINKED TO POST-HARVEST NEEDLE ABCISSION IN BALSAM FIR

5.1 PREFACE

This work has been submitted for publication in the journal, Trees:

MacDonald GE, Lada RR, Caldwell CD, Udenigwe C, MacDonald M. 2019. Lipid and FA changes linked to post-harvest needle abscission in balsam fir, *Abies balsamea*. Trees. In Press.

This work was presented at the Gordon Research Conference: Integration of Lipid Metabolism in Galveston, Texas (2015).

5.2 INTRODUCTION

Balsam fir, *Abies balsamea* L., is the primary species of Christmas tree grown in Canada, grossing approximately $78 million in 2016 (Statistics Canada, 2017). It is the preferred species for Christmas trees and greenery due to its gratifying fragrance and rich green color (Burns and Honkala, 1990). Due to the potential for excessive needle loss, there has been a trend towards consumer preference for a "no-mess" Christmas tree, thus increasing the sales for artificial trees (Statistics Canada, 2017). More research is necessary to advance our current knowledge of the physiological mechanisms causing needle abscission postharvest.

Root detachment cuts needles off from root derived factors, but the stem is still present. Root cutting results in initial wounding, but water deficit causing dehydration has been found to be the most critical postharvest concern in the balsam fir (Testerink and Munnik, 2011; Lada and MacDonald, 2015; MacInnes, 2015). Even though trees are given a fresh cut and placed in water, there is variability in their ability to remain hydrated. The cause of water deficit stress is not known, but some possibilities are cavitation, embolism, stomatal dysfunction, bacterial contamination, or blockage of xylem vessels (Lada and MacDonald, 2015; Lada et al., 2016). Whatever the cause may be, water deficit stress negatively affects

membrane integrity (MacDonald and Lada, 2014). Acyl lipids are one of the main components of membranes. Glycerophospholipids are found in the lipid bilayer of the cell and most intracellular membranes. The chloroplast is composed of primarily GL, MGDG, and DGDG. In previous studies of balsam fir postharvest, both cell and chloroplast membranes have been found have been damaged based on membrane injury and fluorescence studies (Lada and MacDonald, 2015). Stress triggers the formation of free radicals and ROS, which in turn triggers lipid peroxidation (Bailey-Serres and Mittler, 2006). This can result in severe membrane perturbation, structural changes in membrane lipids, increased membrane leakage, and eventually cell death (Nilsen and Orcutt, 1996). Membrane changes may also trigger abscission or needle loss caused by the loosening and separation of the cells in the abscission zone primarily due to the breakdown of the cell wall by enzymes such as cellulase (MacDonald et al., 2011a). Abscission can occur as the final stage of senescence, or it may happen independently of senescence in balsam fir (MacDonald and Lada, 2014). Ethylene production has been found to promote abscission in postharvest balsam fir and lipid peroxidation is thought to lead to ethylene synthesis (MacDonald, 2010). Therefore, it is logical to suggest that changes in lipids and FA changes occur postharvest, and that these changes could be linked to postharvest abscission. The objective of this research is to identify the changes that occur in lipids postharvest in balsam fir and to determine if these changes are linked to abscission.

5.3 Materials and Methods

5.3.1 Sample Collection

A total of 84 branches from Clone 706 were collected from a 16-year-old balsam fir orchard at the Tree Breeding Center, Department of Natural Resources, Debert, Nova Scotia, Canada (lat. 45°25′ N, long. 63°28′ W). Each branch was cut from 2-year growth at 1.5 m aboveground. Needles from 6 randomly chosen branches were checked for membrane injury (MII), frozen in liquid nitrogen onsite, and stored in a -80°C freezer to store for lipid analysis. The other branches were immediately placed in a container with distilled water to equilibrate water status and transported to the laboratory.

5.3.2 Experimental Setup and Design

Once in the laboratory, all branches were given a fresh cut 2.5 cm above the previous cut while stem ends were put in water to reduce risk of cavitation, weighed, and then placed in a 100-mL amber bottle filled with 100 mL of distilled water. The neck of each flask was plugged with cotton wool to reduce direct water evaporation and provide added stability to a branch. The branch and entire apparatus was weighed, which allowed for quantification of water uptake throughout the experiment. Branches were provided water throughout the experiment and the growth room was maintained at conditions similar to typical display conditions for balsam fir (i.e. 22°C constant temperature, 25% relative humidity, and a light intensity of 90 μmol m^{-2} s^{-1}).

Two experiments were run simultaneously. In one experiment, six branches of those collected were kept in water in the laboratory for 12 weeks to determine mean needle loss and water uptake. This experiment was set up as a completely randomized design where the factor of interest was time. In a side experiment, 78 branches were kept to sacrifice weekly for MII and to freeze for subsequent lipid analysis.

5.3.3 Per Cent Needle Loss (PNL)

Needle loss was calculated as a percentage of the fresh weight of abscised needles to the fresh weight of initial branch weight (MacDonald and Lada, 2012). Each day the branches used to determine needle loss were subjected to a "finger run" test, and this dislodged loose needles from the branch (MacDonald, 2010). To perform the "finger run" test, each branch was gently passed three times through the index and middle fingers, and the fallen needles were quantified gravimetrically and recorded to arrive at a fresh needle mass lost (g).

5.3.4 Water Uptake

Water uptake was estimated by weighing each bottle at the beginning of the experiment after being filled with water and then, weekly, each branch was removed from its flask and the flask was weighed again. The difference in mass was estimated as water uptake and the rate of water uptake was calculated as water uptake (mL g^{-1} d^{-1}):

$$Water\ Uptake = \frac{M_n - M_{n+1}}{7 \cdot M_b}$$

M_n is the mass of the flask on any given week while M_{n+1} is the mass of the flask on the subsequent week. M_b is fresh weight of the branch. The critical value for water uptake is 0.05 mL g^{-1} d^{-1}, below which is the point at which a branch will commit to abscission (Lada et al., 2016).

5.3.5 Membrane Injury Index (MII)

MII uses the percentage of electrolyte leaked into solution to quantify membrane integrity (Thiagarajan, 2012). Centrifuge tubes were filled with 30 mL of distilled water and were adjusted to room temperature (22-24 °C). The electrical conductivity of the distilled water (EC_w) alone was measured using a CDM 2e Conductivity Meter (Bach-Simpson, London, ON). Afterward, 0.4 g of needles were removed from each branch and completely submerged in the distilled water. The tubes were sealed and left at room temperature for 24 h. Initial conductivity (EC_0) was measured to determine the amount of electrolytes leached into solution. Sealed tubes were then placed in a forced-air oven for at least 4 h at 90 °C to kill tissues and then cooled to room temperature. Final conductivity measurements (EC_f) were taken after equilibrating to 25°C to determine maximum leakage from the dead cells. MII was then calculated using the following formula:

$$MII = \frac{EC_o - EC_w}{EC_f - EC_w} x100$$

5.3.6 Lipid Extraction

The goal was to observe lipid changes in needles remaining attached to the branch during various stages of abscission, so whole needles that had not yet undergone abscission were used for lipid extraction and FA analysis. Those needles used for analysis were frozen in liquid N to halt metabolic processes and then stored at -80°C until analysis. Percentage needle loss data were used to determine the time points at which to analyze needles for

polar lipids and FA. Samples from December 21were the fresh samples. Samples from Feb. 2 (day 42) were chosen as it was just prior to commencement of abscission. Finally, samples from Mar. 15 (day 84) were chosen as branches were at their peak abscission rate.

All FA were extracted at the Kansas Lipidomic Research Center (Manhattan, KS, USA) using an extraction protocol for *Arabidopsis* leaf tissue adapted from Bligh and Dwyer, 1959. For extraction, 1 g of frozen needles were cut into smaller pieces and incubated in 1 mL of isopropanol with 0.01% BHT at 75 °C for 15 min. Afterwards, 1.5 mL of chloroform and 0.6 mL of water were added. After shaking for 1 h at room temperature, the solvent was transferred to a new glass tube with a Teflon-lined screw-cap using a Pasteur pipette, and total 0.7 mL chloroform: methanol (2:1) was added and shaken for 30 min. The extraction was completed by adding 4 mL of chloroform: methanol (2:1) ten times and collecting the solvent. The solvent extracts were washed once with 1 mL KCl (1.0 M) and once with 0.66 mL water. The solvent was evaporated under nitrogen and the lipid extract was quantified and dissolved in 1 mL chloroform. Lipid samples were analyzed on a triple quadrupole mass spectrometer equipped for electrospray ionization (ESI). The lipids in each class were quantified in comparison to two internal standards of the class. Five replicates of each treatment for each genotype were analyzed. The tissues after lipid extraction were dried in an oven at 105°C and dry weights were determined (3 –20 mg).

5.3.7 Fatty Acid Analysis

FAME were prepared using methanolic hydrochloric acid according to Christie (1982). FAME were dissolved in 100–200 µL of hexane and placed in 2 mL gas chromatography (GC) vials with inserts. FAME were quantified using gas chromatography GC-FID analysis performed at the Kansas Lipdomic Center, Kansas State University, on an Agilent Technologies 6890N Network GC system equipped with a HP-88 capillary column (100 m × 0.25 mm I.D., 0.20 µm film thickness) coupled with a FID. Injector and FID temperatures were set at 275 and 260°C, respectively. The carrier gas (Helium) pressure was 51.61 psi with a flow rate of 1.6 $mLmin^{-1}$min with continuous flow. For the detector, hydrogen flow rate was 30.0 $mLmin^{-1}$, air flow rate was 400 mL/min, and makeup flow rate (Helium) was 25 $mLmin^{-1}$. The GC oven temperature ramp was operated as follows,

initial temperature of 150 °C held for 1min, increased at 10 °C/min to 175 °C, held 10 min then increased at 5 °C/min to 210°C, and held for 5 min. At last, temperature was increased at 5 °C$min^{-1}$to a final temperature of 230 °C, held 15 min. Total run time was 44.5 min. The sampling rate of the FID was 20 Hz. The FAME solutions were injected in the volume of 1 µL using an autosampler Agilent Technologies 7683 Series Injector in the splitless mode. The chromatograph worked under Agilent Technologies Enhanced Chemstation software. The FA were identified by comparison of RRT of the compounds in the sample with RRT of Supelco 37 component FA methyl ester mix standards. The moles of FA were calculated by the moles of internal standard (pentadecanoic acid, 15:0) and peaks' area.

5.3.8 Unsaturation Index (UI)

The degree of unsaturation in the balsam fir needle lipids was evaluated by the UI (Makarenko et al. 2014);

$$UI = \sum p_j /100$$

where p_j is the content (wt %) of USFA multiplied by the number of double bonds in each acid.

5.3.9 Statistical Analysis

Needle loss and water uptake data were submitted to repeated measures analysis using PROC MIXED in SAS 9.3. All other data were submitted to analysis of variance (ANOVA), using Minitab® 17 to determine effects of treatment significant at $\alpha = 0.05$. In all analyses, the fixed independent variable was sampling date. Assumptions of normality, homogeneity, and independence were confirmed for all analyses. Significant effects were separated using Tukey's multiple means comparison.

5.4 RESULTS

Physiological processes such as needle abscission, water uptake, and MII followed their traditional progression. Needle loss followed an approximate logistic curve over the 12 weeks of study (Fig. 16). After the first week, needles had lost approximately 0.5% of their fresh weight in needles. Needle loss continued to increase slowly, remaining under 2% for the first 7 weeks, and then increased exponentially for the remainder of the experiment. Water uptake was high initially but decreased by 58% after the first week and then gradually decreased to approximately 0.05 mL g^{-1} d^{-1} by the end of the experiment (Fig. 17). Membrane injury was a little more erratic than needle abscission and water uptake, with several peaks observed in the temporal curve throughout the experiment (Fig. 18). However, membrane injury was significantly higher at weeks 11 and 12 than initial values, with a 7-fold and 5-fold increase in membrane injury, respectively.

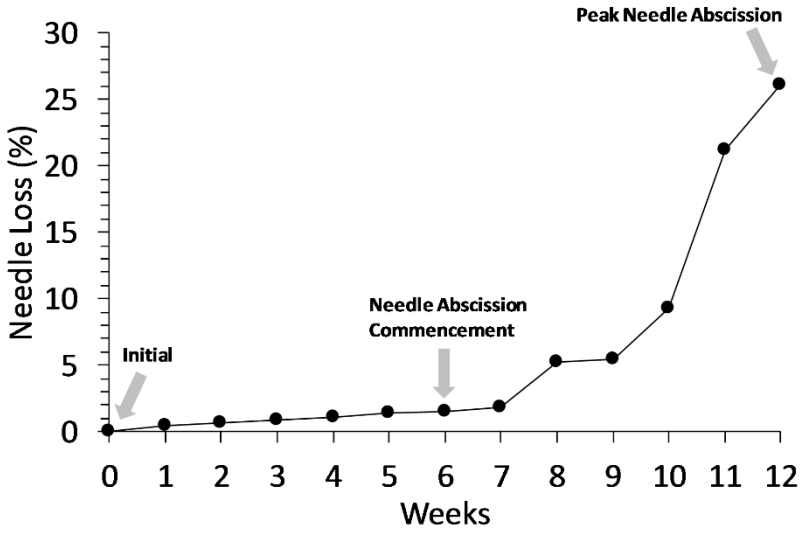

Figure 16. Percentage needle loss by fresh weight of balsam fir branches. Each point represents the average of 6 replicates, where letter groupings denote significant difference at 5% significance as determined by Tukey's multiple means comparison. Arrows indicate sampling times for lipid analysis.

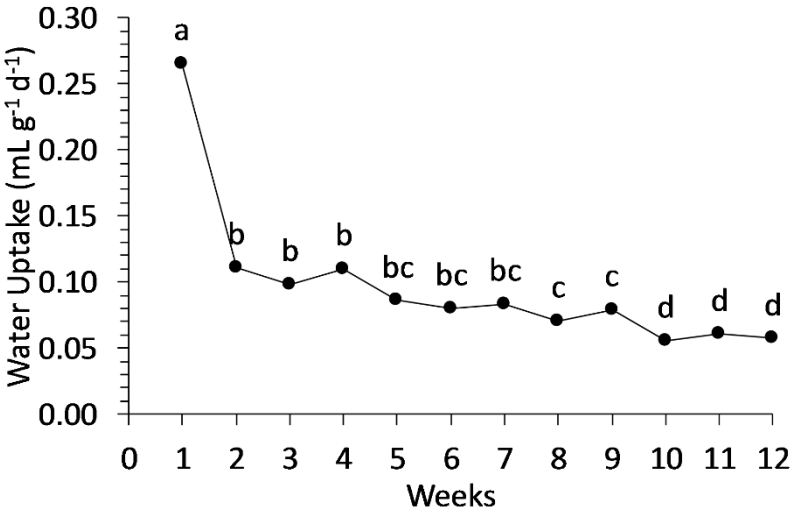

Figure 17. Water uptake of balsam fir branches. Each point represents the average of 6 replicates, where letter groupings denote significant difference at 5% significance as determined by Tukey's multiple means comparison. Arrows indicate sampling times for lipid analysis.

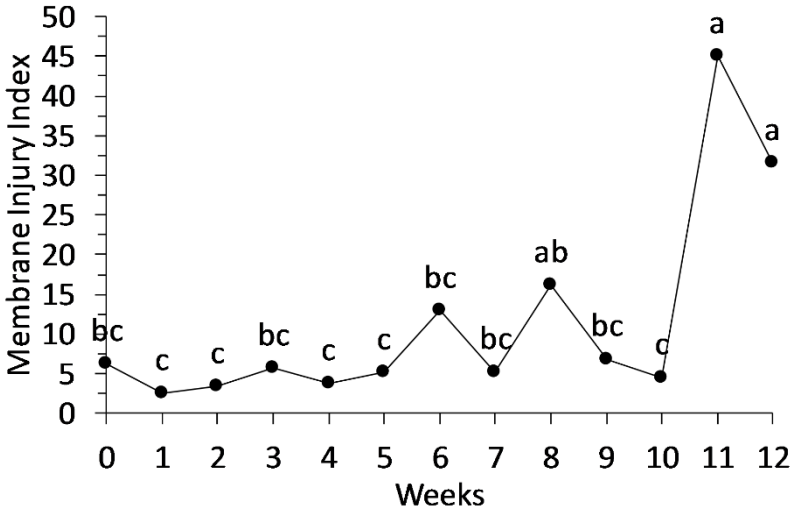

Figure 18. Membrane injury index of balsam fir branches, where a higher value denotes more membrane injury. Each point represents the average of 6 replicates, where letter groupings denote significant difference at 5% significance as determined by Tukey's multiple means comparison.

Total polar lipids (nmol mg^{-1} DW) were analyzed at 3 time points (identified in Fig. 16). Total polar lipids decreased significantly at each of the three time periods ($p < 0.001$). A 50% polar lipid decrease (Fig. 19) occurred by week 6 postharvest, just prior to the commencement of abscission. The total decrease by the time of peak abscission was 88% compared to initial values. Furthermore, there were significant changes in the distribution

of polar lipids over time postharvest (Fig. 19). The most plentiful lipid classes in fresh needles were MGDG (33%) and DGDG (28%), but after 6 weeks the relative percentages of MGDG and DGDG were significantly lower (Fig. 19; $p < 0.001$). By the time of peak abscission, MGDG and DGDG were only 11.5% and 13%, respectively, of the total polar lipids while PC comprised the largest percentage of polar lipids by peak abscission. There was a significant relationship between each lipid and percentage needle loss, with the exception of PC (Table 4). Lipids DGDG and MGDG were negatively associated with needle abscission, while the others were positively associated with needle abscission. The strongest relationship was a logarithmic relationship between DGDG and needle abscission that explained 87.3% of the variation (Fig. 20).

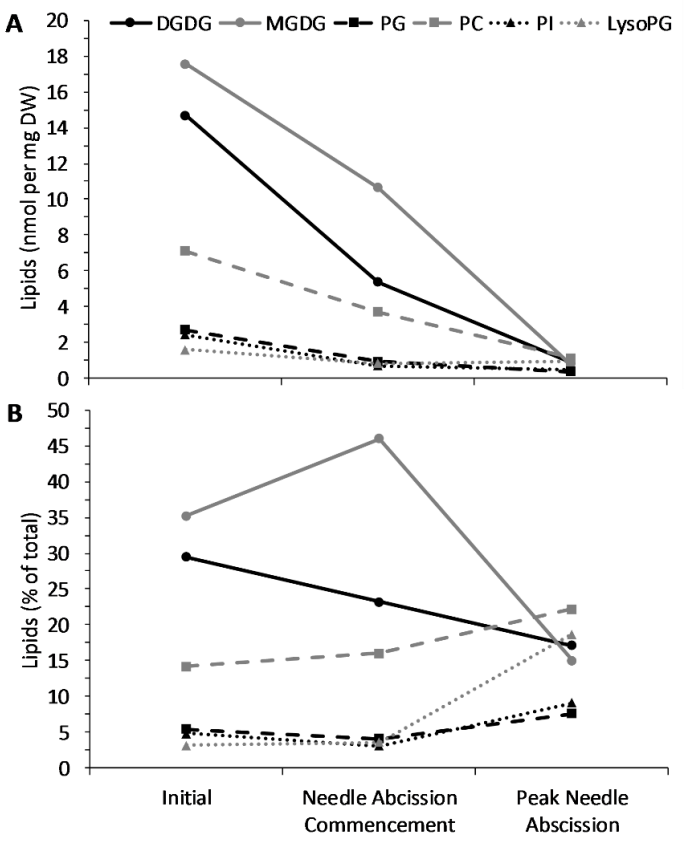

Figure 19. Concentration of lipids in balsam fir branches as postharvest abscission progresses A) expressed by concentration and B) expressed by percentage contribution to total amount of lipids. Each point is the average of 6 replicates. DGDG = digalactosyldiacylglycerols; MGDG = monogalactosyldiacylglycerols; PG = phosphotidylglycerol; PC = phosphotidylcholines; PI = phosphotidylinositols;.lyso PG = lysophosphotidylglycerol.

Table 4. The correlation, coefficient of determination, and *p*-value of classes of lipids and FA when compared to percentage needle abscission. N = 15 in each.

Parameter	r	R^2	*p*-value
Lipids			
DGDG*	- 0.642	0.412	= 0.010
MGDG	- 0.772	0.596	= 0.001
PG	+ 0.657	0.432	= 0.008
PC	+ 0.293	0.086	= 0.290
PI	+ 0.722	0.521	= 0.002
LysoPG	+ 0.785	0.616	= 0.001
FA			
SFA	- 0.469	0.220	= 0.078
USFA	- 0.735	0.540	= 0.002
SFA:USFA	+ 0.833	0.694	< 0.001

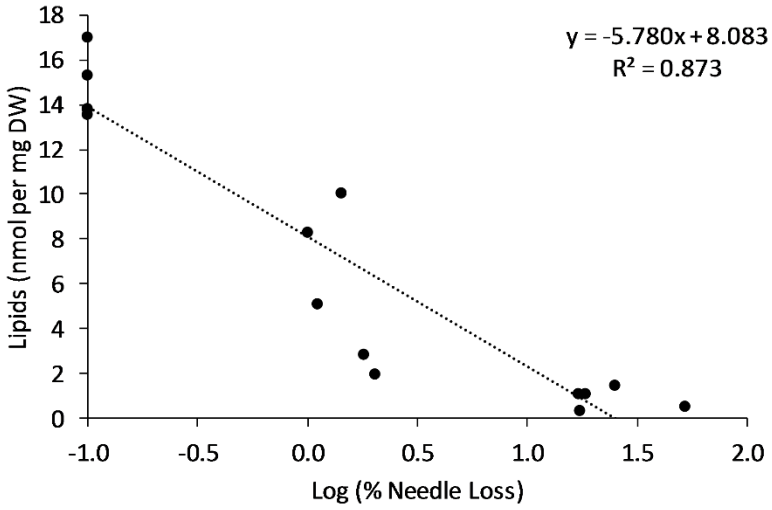

Figure 20. The logarithmic relationship between percentage needle abscission and concentration of DGDG. A logarithmic transformation was applied to percentage needle loss, so values to the far left correspond to very low levels of abscission (i.e. 0.1%). The variation in $\log_{10}$ (needle abscission) explains 87.3% of the variation in DGDG. N = 15.

Significant changes during this study occurred in four main lipid species: MGDG 36:5, 36:6, 36:7, and DGDG 36:6 (Fig. 21). MGDG species seem to decay later than the DGDG lipid species. Another point of interest is that three species were identified in the balsam fir that have not previously been reported in plants; DGDG 36:7, DGDG 38:7, and MGDG

36:7 (Table 5). The first two compose < 1% of total lipids, whereas the last one composes approximately 3% of total lipids.

There was a decline in mean mass of FA (nmol mg^{-1} DW), a four-fold drop by the time of peak abscission. The decrease of USFA was most pronounced. The FA % composition of needle lipids changed throughout the duration of the experiment (Table 6). Among the SFA, 16:0 predominated, doubling in % composition from harvest to peak abscission. All other FA significantly decreased in % composition. Among the unsaturated acids, 18:3n3 predominates (Fig. 22), although its relative content was significantly lower ($p = 0.009$) as abscission progressed. 18:2 cis also shows significant decrease. The ratio of USFA: SFA increased from 0.25 to 1 during the experiment. The UI varied decreased significantly by the time of peak abscission ($p < 0.001$).

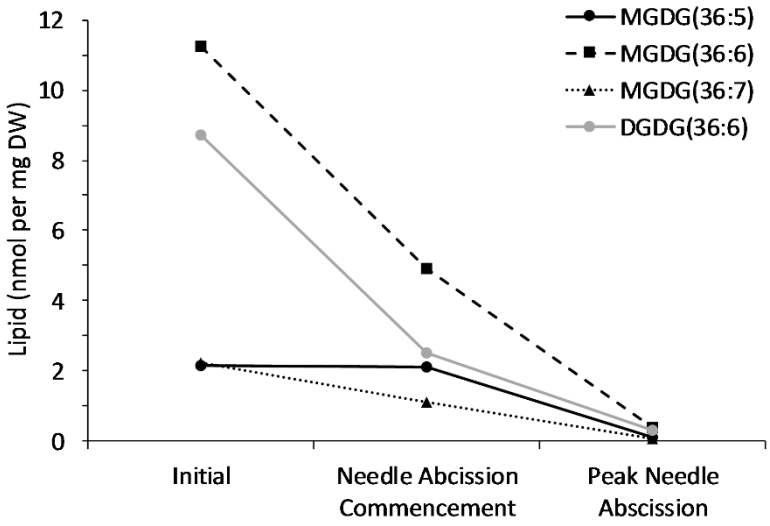

Figure 21. Concentration (nmol per mg dry weight) of lipid species, MGDG 36:5, 36:6, 36:7, and DGDG 36:6 in balsam fir needles as abscission progresses. Each point represents the average of 6 replicates, where letter groupings denote significant difference at 5% significance as determined by Tukey's multiple means comparison. Initially (December 21, 2012), at needle abscission commencement (NAC – February 2, 2013), and peak needle abscission (PNA - March 15, 2013)

Table 5. The concentration of three lipid species detected in balsam fir trees initially (December 21, 2012), at needle abscission commencement (NAC – February 2, 2013), and peak needle abscission (PNA - March 15, 2013).

Lipid	Initial (ng/g)	NAC (ng/g)	PNA (ng/g)
MGDG (36:7)	2.22	1.13	0.09
DGDG (36:7)	0.28	0.05	0.01
DGDG (38:7)	0.16	0.06	0.01

Table 6. Mean % FA in needles sampled initially (December 21, 2012), at needle abscission commencement (NAC - February 2, 2013), and during peak needle abscission (PNA – March 15, 2013). The mean % s followed by the same letters horizontally are not significantly different (α=.005).

FA	Initial (%)	NAC (%)	PNA (%)	P-value
16:0	10.0 b	10.0 b	20.0 a	= 0.004
18:1	14.0 a	10.5 b	9.0 c	< 0.001
18:2	17.7 a	17.7 a	16.9 a	= 0.383
18:3n3	27.0 a	20.0 a	11.6 b	= 0.009
18:3n6	8.2 a	7.7 ab	7.0 b	< 0.001
20:0	1.5 a	0.8 b	0.7 b	< 0.001
20:2	3.0 a	1.9 b	1.6 c	< 0.001
UI	1.9 a	1.8 a	1.4 b	< 0.001

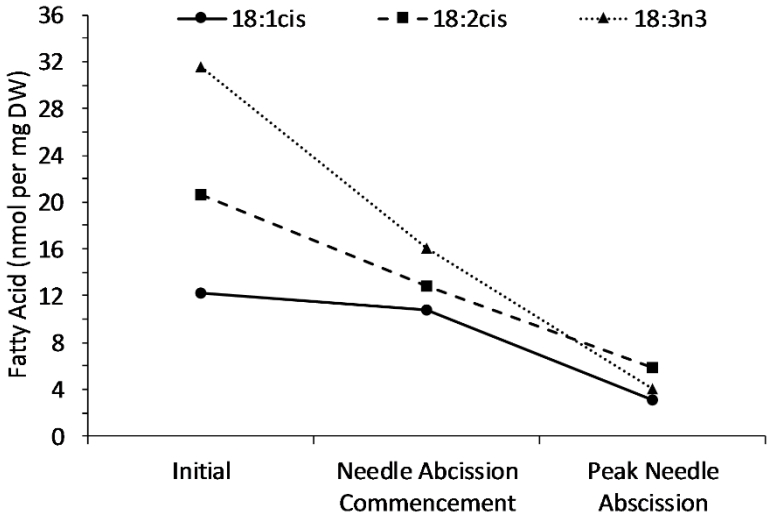

Figure 22. Concentration (nmol per mg dry weight) of three FA, 18:1, 18:2cis, 183n3 in balsam fir needles as abscission progresses. Each point represents the average of 6 replicates, where letter groupings denote significant difference at 5% significance as determined by Tukey's multiple means comparison. initially (December 21, 2012), at needle abscission commencement (NAC – February 2, 2013), and peak needle abscission (PNA - March 15, 2013)

Most FA in fresh balsam fir needles were polyunsaturated FA (PUFA), which were 3 times higher than SFA (SFA; Fig. 23). All FA decreased as abscission progressed, though SFA decreased the least over time. The concentration of PUFA decreased by 78%, MUFA decreased by 74%, and SFA decreased by only 46%. Both SFA and USFA were correlated with needle abscission, however the ratio of SFA: USFA was much stronger (Table 4). SFA: USFA was positively correlated with needle abscission, which means that the concentration of SFA relative to USFA increased as abscission progressed.

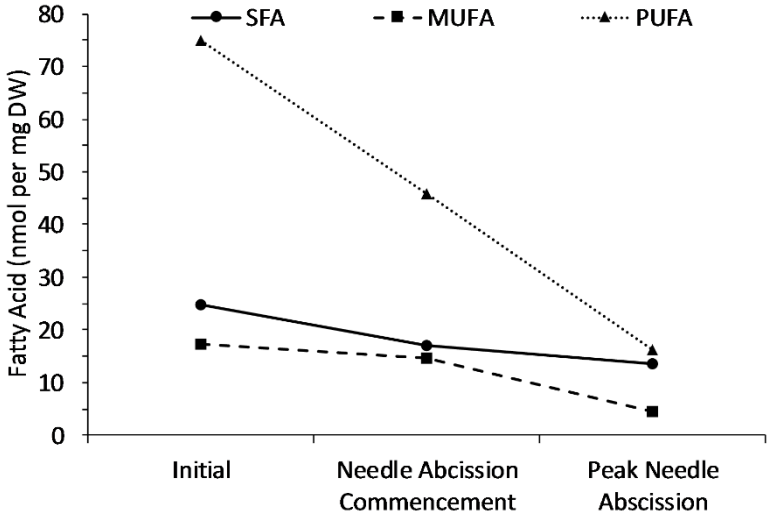

Figure 23. Concentration (nmol per mg dry weight) of SFA, MUFA, and PUFA in balsam fir needles as abscission progresses. Each point represents the average of 6 replicates, where letter groupings denote significant difference at 5% significance as determined by Tukey's multiple means comparison.

5.5 Discussion

The postharvest abscission curve in our experiment follows a typical sigmoid curve, with needle loss commencing between weeks 6 and 7, and peaking at week 12. There was a short pause in abscission between weeks 8 and 9, which does not always occur but has been noted in other studies (Lada and MacDonald, 2015). The time between commencement and peak abscission varies greatly among genotypes and based on time of harvest, so it can be difficult to compare (Lada and MacDonald, 2015).

Past studies of postharvest balsam fir have shown an initial water uptake between 0.15 – 0.20 $mLg^{-1}d^{-1}$, with a significant decrease in the first two weeks (Lada and MacDonald, 2015). In our experiment (Fig. 17), mean water consumption in the freshly cut balsam fir was a relatively high 0.27 $mLg^{-1}d^{-1}$, and by week two the mean was 0.11 $mLg^{-1}d^{-1}$. The critical value is thought to be 0.05 $mLg^{-1}d^{-1}$, after which abscission occurs (Lada and MacDonald, 2015). Water uptake was at that critical value, at 0.05 mL g^{-1} d^{-1}, by the end of the experiment. It is possible that the reduction in water uptake by week 2 postharvest could be a reduction in stem conductivity. This happens often in cut flowers due to stem occlusion from microbial or depositing of organic substances in the xylem vessels (Burge et al., 1996). Studies on conductivity in balsam fir have shown that there was a decline in water use and water pressure 10 days after harvest. This was hypothesized as ABA induced stomatal closure (MacInnes, 2015). ABA has been found to promote abscission and has been also been linked to PLD activation (Thiagarajan, 2012). PLD cleaves FA from lipids during the stress response. These FA have been found to be signaling molecules in *Arabidopsis*, but very little research has been done on gymnosperm lipid signaling (Lessire et al., 2009). It is possible they are involved in balsam fir signaling postharvest.

Loss of acyl lipids has been known to occur during water-deficit stress, but it is impossible to say that such changes are reflective of progressive dehydration or water deficit. This could be linked to ABA, phospholipase D production and lipolysis (Kar, 2011). Hanrouni et al., (2001) studied water-deficit stress in safflower aerial parts and found that severe stress caused a sharp decrease in polar lipids. Studies prior to that showed comparable results (Svenningson and Liljenberg, 1986; Hubac et al., 1989). More recently, Martins Junior et al., (2008) found that there was a gradual loss of polar lipids during water-deficit stress. Loss of lipids may be due to several reasons including lipid utilization in the octadecanoic pathway forming JA or derivatives, which are believed to work with ABA to regulate the response to water stress, enzymatic or non-enzymatic lipid degradation, membrane remodeling, and/or inhibition of FA and lipid biosynthesis (Bailey-Serres and Mittler, 2010; deOllas et al., 2015). Lipid compositional changes are also common, as is loss of membrane integrity (Narayanan et al. 2016). There is evidence of membrane breakdown in this research, especially in the last two weeks causing ion leakage

Judging from the evident deterioration and lowering percentage of lipid classes DGDG and MGDG, especially noted in species, DGDG 36:6, MGDG 36:7, 36:6, and 36:5, this would suggest release of 18:4, 18:3, and 18:2 as free FA from the chloroplast membranes. Free FFA are linked to membrane deterioration (Lennen et al., 2011). FFA are non-esterified FA and often accumulate in plants membranes due to desiccation and activation of phospholipases. An increase of levels of "free" long chain FA make the membranes susceptible to degradation (Maranushi et al., 1981). There is evidence that FA, linolenic, and linoleic acids, are the major PUFA found in plants, and are common substrates for LOX and dioxygenases. These FA and are easily converted to highly reactive hydroperoxide and other ROS causing oxidative stress (Bargmann and Munnik. 2006). There is also increasing evidence that FFA are related to other cellular hormones and may act as signals in the plant cell. FFA, 18:3n3 and 18:2 cis, are precursors for oxygenated compounds such as JA formed during stress (Ryu and Wang, 1998). 18:4 has not been studied as much, possibly because it is likely coniferonic acid (18:4 Delta (5,9,12,15), found mostly in conifers (Makarenko et al., 2014).

Three lipid species were identified in the balsam fir that have not previously been classified as plant lipids: DGDG 36:7, DGDG 38:7, and MGDG 36:7 (Kansas Lipdomic Research Center). This is likely because there are Δ5-polyunsaturated methylene-interrupted FA (Δ5-UPIFA), FA found in conifers that are rarely found elsewhere. The main USFA are represented by oleic (18:1), linoleic (18:2*cis*), and linolenic (18:3n3) acids. However, in pine trees the following Δ^5-acids have been found: taxoleic (C18:2Δ5,9), pinolenic (C18:3Δ5,9,12), skiadonic (C20:3Δ5,11,14), coniferonic acid (C18:4 (Δ5, 9,12,15)) and juniperonic (C20:4 (Δ5,11,14,17) acids (Wolff et al., 1998). It appears that in the future it would be valuable to be able to quantify these in balsam fir, as oleic, linoleic, and linolenic acids are thought to be precursors for the Δ5-acids, so the balance between these could be important for needle retention (Knutson et al., 1998).

5.6 Conclusion

Several lipid and FA changes were found during postharvest abscission of balsam fir. There was a decrease in water use and an increase in needle abscission and MII, which are consistent with water deficit stress. There was a decrease in total lipids, total FA, and total PUFA postharvest. These changes point toward loss of lipids and subsequently FA leading up to postharvest abscission in balsam fir. The most probable cause of lipid and FA loss may be non-enzymatic degradation of lipids by ROS, reactive oxygen species, or enzymatic degradation by LOX. MGDG and DGDG decreased more than the other lipid classes indicating initial chloroplast membrane breakdown. A contributing factor to decline of lipids and FA could be a compromised ability of the chloroplasts to synthesize FA and lipids due to too much damage. There was a significant decrease particularly in 18:3n3. This is found in the greatest abundance in MGDG and DGDG. 18:3n3 correlated strongly with abscission as did 18:2 *cis*. USFA: SFA ratios changed significantly from 4:1 to 1:1 from initial sampling to peak abscission significantly lowering the UI suggesting more SFA and fewer unsaturated. There was a high negative correlation between the ratio of unsaturated: SFA and abscission further suggesting a decline in unsaturated. There is indication that FA and lipids are linked to postharvest needle loss, but it remains to be determined whether there is a direct cause and effect relationship.

CHAPTER 6 – LIPID AND FATTY ACID CHANGES IN TWO CONTRASTING GENOTYPES

6.1 INTRODUCTION

The balsam fir, *Abies balsamea* L., is the primary species of Christmas tree grown in Atlantic Canada, grossing approximately $78 million each year (Statistics Canada, 2017). Postharvest needle loss has been identified as a major challenge by the industry, and it has been found that genotype has significant influence on postharvest needle retention (MacDonald and Lada, 2008). In a past study by MacDonald et al. 2015, it was found that there was a decrease in all polar lipids and FA, postharvest. The saturated to unsaturated ratio correlated strongly and positively with needle loss. In addition, there was a decrease in the percentage of GL, MGDG, and DGDG indicating possible chloroplast breakdown in the needle cells. In this earlier study, however, only one genotype (Clone 706) was tested, therefore, to test more genotypes for changes in lipids and FA postharvest in balsam fir from the same orchard could help support the results that occurred in this past study and help us postulate the differences between a high and a low NRD clone and the underlying relationship with lipids and FA.

FA are components of cell and intracellular membrane lipids, and the survival of needle cells postharvest depends on the plant's ability to adapt to stress, which involves maintaining membrane homeostasis. Christmas trees are detached from the root, and this is the initial stimulus for abscission. Balsam fir lose needles postharvest that they would not have lost if the roots were intact (Lada and MacDonald, 2016). The primary stress to membranes postharvest is thought to be dehydration, which could be caused postharvest by cavitation, embolism, xylem blockage, stomatal dysfunction, and/or bacterial infection (MacDonald and Lada, 2014). It has been found that loss of root pressure has recently been shown to be directly correlated with needle loss postharvest in balsam fir (McInnnes, 2015). Lipids and subsequent FA are known to rearrange themselves to preserve compartmentalization during drought stress, for example increasing unsaturation of FA in an attempt to stabilize membranes, but also increasing the number of FA that are susceptible to oxidation. When oxidation of lipids and FA occurs there is unrepairable damage to the cells (Scotti-Campos and Pham-Thi, 2016)

The chloroplasts of the cell are essential for survival. Breakdown of MGDG and DGDG postharvest indicates thylakoid and chloroplast envelope may have been damaged. GL decline was seen in MacDonald et al. 2015 for Clone 706 from the Debert, NS orchard collection. Possibly chloroplast breakdown signals subsequent needle abscission in an attempt to preserve unaffected needles. In other studies on balsam fir postharvest abscission, the chlorophyll index of postharvest balsam fir begins relatively high, but gradually decreases until abscission (MacDonald and Lada, 2014).

The hypotheses that this experiment tested were that: balsam fir needles would suffer more needle loss with a decrease in the amount of polar lipids and FA. The loss of polar lipids would be more noticeable in the chloroplast lipids, MGDG and DGDG. The loss of lipids and FA would occur earlier in the low NRD clone. In addition, there would be a significant decrease in USFA and an increase in SFA postharvest linked to lipid and FA changes. The objective of this experiment was to confirm changes that in occur in balsam fir postharvest by comparing changes in this study with previously recorded changes, and to determine some differences between a high and low NRD clones in lipid and FA metabolism. Also the objective was to identify these differences to see if any polar lipid or FA changes or profile differences could help explain why these two genotypes undergo abscission at different rates once the root is detached.

6.2 Materials and Methods

6.2.1 Sample Collection

A total of 90 branches from Clones 506 and 9 were collected from a 16-year-old balsam fir orchard at the Tree Breeding Center, Department of Natural Resources, Debert, Nova Scotia, Canada (lat. 45°25′ N, long. 63°28′ W) on October 1, 2014. Each branch was cut from 2-year growth at 1.5 m aboveground. Needles from 5 randomly chosen branches of each clone were frozen in liquid nitrogen onsite, and stored in a -80°C freezer to store until lipid analysis. In addition, these branches (needles) were subjected to testing for membrane injury, fluorescence and capacitance. The other branches were immediately placed in a

container with distilled water to equilibrate water status and were transported to the laboratory.

6.2.2 Experimental Setup and Design

Once the branches are received in the laboratory, they were acclimated to the new setting. All branches were then given a fresh cut 2.5 cm above the previous cut while stem ends were held under water to reduce risk of cavitation, weighed, and then placed in a 100-mL amber bottle filled with distilled water. The neck of each flask was plugged with cotton wool to reduce direct water evaporation (making it almost negligible) and provide added stability to a branch. The branch and entire apparatus was weighed, which allowed for quantification of water uptake throughout the experiment.

Five branches of those collected from each of Clones 506 and 9 were kept in water in the laboratory for 10 weeks to monitor a needle loss and water uptake. From this, mean dates were established for NAC and PNA and cumulative per cent needle loss was calculated. In a parallel experiment, branches were sacrificed for polar lipid, FA, and MII analyses. For branches 506, needles were collected for ten weeks postharvest. Due to unforeseen loss of branches, two weeks of needles collection were skipped, week 5 and 6 (resumed prior to NAC). For Clone 9, needles were collected each week for four weeks and again week 9. Samples were taken up to just before needle abscission commencement and again at peak abscission. Due to the varying lengths of these experiments, they were analyzed as two separate completely randomized designs. In addition, the first five time point were compared using ANOVA, but week nine was compared by two sample *t*- test.

6.2.3 Per Cent Needle Loss (PNL)

Needle loss was calculated as a percentage of the fresh and dry weight of abscised needles to the fresh weight of initial branch weight and total dry weight of needles respectively (MacDonald and Lada, 2012). Each day the branches used to determine needle loss were subjected to a “finger run” test using the same operator, and this dislodged loose needles from the branch (MacDonald, 2010). To perform the “finger run” test, each branch was

gently passed three times through the index and middle fingers, and the fallen needles were quantified gravimetrically and recorded to arrive at a fresh needle mass lost (g). Dry needle loss was determined by drying needles for 24 h in an oven at 90°C and quantifying again. This was determined as a dry weight of all needles.

6.2.4 Water Uptake

Water uptake was estimated by weighing each bottle at the beginning of the experiment after being filled with water and then, weekly, each branch was removed from its flask and the flask was weighed again. The difference in mass was estimated as water uptake and the rate of water uptake was calculated as water uptake (mL g^{-1} d^{-1}):

$$Water\ Uptake = \frac{M_n - M_{n+1}}{7 \cdot M_b}$$

M_n is the mass of the flask on any given week while M_{n+1} is the mass of the flask on the subsequent week. M_b is fresh weight of the branch. The critical value for water uptake is 0.05 mL g^{--1} $d\text{-}^{-1}$, below which is the point at which a branch will commit to abscission (Lada et al., 2016).

6.2.5 Membrane Injury Index (MII)

MII uses the percentage of electrolyte leaked into solution to quantify membrane integrity (Thiagarajan, 2012). Centrifuge tubes were filled with 30 mL of distilled water and were adjusted to room temperature (22-24 °C). The electrical conductivity of the distilled water *(EC_w)* alone was measured using a CDM 2e Conductivity Meter (Bach-Simpson, London, ON). Afterwards, 0.4 g of needles were removed from each branch and completely submerged in the distilled water. The tubes were sealed and left at room temperature for 24 h. Initial conductivity (EC_0) was measured to determine the amount of electrolytes leached into solution. Sealed tubes were then placed in a forced-air oven for at least 4 h at 90 °C to kill tissues and then cooled to room temperature. Final conductivity measurements

(EC_f) were taken after equilibrating to 25 °C to determine maximum leakage from the dead cells. MII was then calculated using the following formula:

$$MII = \frac{EC_o - EC_w}{EC_f - EC_w}$$

6.2.6 Capacitance

Capacitance (pF) is used to monitor the water status of the branch. Capacitance was measured on the stem of each branch in three random locations when the branches were sacrificed for polar lipid and FA. This was achieved using a plant tissue BK Precision Capacitance Meter (P&K Maxtee Inc., Chicago, IL, USA). Two prongs (electrodes) at a set distance of 1 cm were pushed into the stem to a depth of 0.5 cm. The potential charge was measured between the two prongs and recorded. This gives a reading in pF. Less than 5 pF indicates poor moisture status.

6.2.7 Lipid Extraction

The goal was to observe lipid changes in needles remaining attached to the branch weekly postharvest, so whole needles that had not yet undergone abscission were used for lipid extraction and FA analysis. Those needles used for analysis were frozen in liquid N to halt metabolic processes and then stored at -80°C until analysis. All FA were extracted at the Dalhousie Agricultural Campus Chemistry Department using an extraction protocol for *Arabidopsis* leaf tissue adapted from Bligh and Dwyer, 1959. For extraction, 1 g of frozen needles was broken into smaller pieces while frozen in a mortar and pestle and incubated in 1 mL of isopropanol with 0.01% BHT at 75 °C for 15 min. Afterwards, 1.5 mL of chloroform and 0.6 mL of water were added. After shaking for 1 h at room temperature, the solvent was transferred to a new glass tube with a Teflon-lined screw-cap using a Pasteur pipette, and total 0.7 mL chloroform: methanol (2:1) was added and shaken for 30 min. The extraction was completed by adding 4 mL of chloroform: methanol (2:1) ten times and collecting the solvent. The solvent extracts were washed once with 1 mL KCl (1.0 M) and once with 0.66 mL water. The solvent was evaporated under nitrogen and the lipid extract was quantified and dissolved in 1 mL chloroform.

6.2.8 Lipid Class and Species Analysis

Lipid extracts from 100 samples were analyzed on a triple quadrupole mass spectrometer equipped for electrospray ionization (ESI–MS/MS; Applied Biosystems API 4000) at the Kansas Lipidomic Center, Kansas State University, US. ESI-MS/MS analysis parameters and acquisition parameters are given in Tables 7 and 8 respectively. The lipids in each class were quantified in comparison to two internal standards of the class. Lipid species within each head group were identified by total carbon number and total double bonds. Molecular species of each head class were quantified by comparing with the signals of the internal standards (Welti et al., 2002). A list of lipids able to be quantified from this procedure is shown in Table 9.

Table 7. ESI-MS/MS analysis parameters (using Applied Biosystems API 4000) for plant lipids. DGDG, digalactosyldiacylglycerol; ESI-MS/MS, electrospray ionization tandem mass spectrometry; MGDG, monogalactosyldiacylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; LPC, lyso-phosphatidylcholine; PE, phosphatidylethanolamine; LPE, lysophosphatidylethanolamine; PG, phosphatidylglycerol; LPG, lysophosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine.

Class	Ion Analyzed	Positive Ion Scan Mode	m/z range	Reference
PA	$(M + NH_4)^-$	NL of 115.00	500 – 850	Shiva et al., 2013
PC/LPC	$(M + H)^-$	Pre of *m/z* 184.07	450 – 960	Brügger et al., 1997
PE/LPE	$(M + H)^-$	NL of 141.02	420 – 920	Brügger et al., 1997
PG	$(M + NH_4)^-$	NL of 189.04	650 – 1,000	Taguchi et al,. 2005
LPG	$(M-H)^-$	Prec 153	-	Welti et al.. 2002
PI	$(M + NH_4)^-$	NL of 277.06	790 – 950	Taguchi et al., 2005
PS	$(M + H)^-$	NL of 185.01	600 – 920	Brügger et al., 1997
DGDG	$(M + NH_4)^-$	NL of 341.13	890 – 1,050	Isaac et al., 2007
MGDG	$(M + NH_4)^-$	NL of 179.08	700 – 900	Isaac et al., 2007

Table 8. Acquisition parameters of lipid classes using Applied Biosystems API 4000, triple quadrupole mass spectrometer.

Parameter	PA	PC/LPC	PE/LPE	PG	PI	PS	DGDG	MGDG
Typical Scan Time (min)	3.51	1.28	3.34	3.21	4.00	4.01	1.67	1.67
Depolarization Potential (V)	100	100	100	100	100	100	90	90
Exit Potential (V)	14	14	14	14	14	14	10	10
Collision Energy (V)	25	40	28	20	25	26	24	21
Collision Exit Potential (V)	14	14	14	14	14	14	23	23

Table 9. Plant membrane lipids determined by the procedure described herein DGDG, digalactosyldiacylglycerol, MGDG, monogalactosyldiacylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; LPC, lyso- phosphatidylcholine; PE, phosphatidylethanolamine; LPE, lysophosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine. Where there are question marks, this indicates that it is possible, based on the FA present, that it is a lipid species.

Class	Lipid Species
LPC	16:0, 16:1, 18:0, 18:1, 18:2, 18:3
LPE	16:0, 16:1, 18:1, 18:2, 18:3
LPG	16:0, 16:1, 18:1, 18:2, 18:3
PA	32:0, 34:6, 34:5, 34:4, 34:3, 34:2, 34:1, 35:4?, 35:3?, 35:2?, 35:1?, 36:7, 36:6, 36:5, 36:4, 36:3, 36:2, 37:4?, 37:3?, 38:7, 38:6?, 38:5?, 38:4?
PC	30:1, 32:0, 33:3: 33:2, 34:4, 34:3, 34:2, 34:1, 35:4, 35:3, 35:2, 35:2, 35:0, 36:6, 36:5, 36:4, 36:3, 36:2, 36:1, 37:5, 37:4, 37:3, 38:6, 38:5, 38:4, 38:3, 38:2, 40:5, 40:4, 40:3, 40:2, 40:1, 42:4?, 42:3?, 43:2?, 43:3?
PE	32:3, 32:2, 32:1, 32:0, 34:4, 34:3, 34:2, 34:1, 35:4?, 35:3?, 35:2?, 35:1?, 36:6, 36:5, 36:4, 36:3, 36:2, 36:1, 37:4?, 37:3?, 38:6, 38:5, 38:4, 38:3, 39:4, 39:3, 39:2, 40:7., 40:3, 40:2, 42:4, 42:3, 42:2, 42:1?, 44:4?, 44:3?, 44:2?
PG	32:1?, 32:0?, 34:4, 34:3, 34:2, 34:1, 34:0, 35:4?, 35:3?, 35:2?, 35:1?, 36:7, 36:6, 36:5, 36:4, 36:3, 36:2, 36:1
PI	30:4?, 30:3?, 32:4?, 32:3, 32:2, 32:1, 32:0, 34:4, 34:3, 34:2, 34:1, 35:4?, 35:3?, 35:2?, 35:1?, 36:7, 36:6, 36:5, 36:4, 36:3, 36:2, 36:1, 37:4?, 37:3?
PS	34:4, 34:3, 34:2, 34:1, 35:3?, 36:6, 36:5, 36:4, 36:3, 36:2, 36:1, 38:6, 38:5, 38:4, 38:3, 38:2, 38:1, 40:4, 40:3, 40:2, 40:1, 42:4?, 42:3, 42:2, 42:1, 44:3
DGDG	34:6, 34:5, 34:4, 34:3, 34:2, 34:1, 35:3?, 35:2?, 36:7, 36:6, 36:5, 36:4, 36:3, 36:2, 36:1, 37:3?, 38:7, 38:6, 38:5, 38:4, 38:3
MGDG	34:6, 34:5, 34:4, 34:3, 34:2, 34:1, 36:6, 36:7, 36:5, 36:4, 36:3, 36:2, 36:1, 38:6, 38:5, 38:4, 38:3

6.2.9 Fatty Acid Analysis

FAME were prepared using methanolic hydrochloric acid according to Christie (1982). FAME were dissolved in 100–200 µL of hexane and placed in 2 mL gas chromatography (GC) vials with inserts. FAME were quantified using gas chromatography GC-FID analysis performed at the Kansas Lipdomic Center, Kansas State University, on an Agilent Technologies 6890N Network GC system equipped with a HP-88 capillary column (100 m × 0.25 mm I.D., 0.20 µm film thickness) coupled with a FID. Injector and FID temperatures were set at 275 and 260°C, respectively. The carrier gas (Helium) pressure was 51.61 psi with a flow rate of 1.6 $mLmin^{-1}$min with continuous flow. For the detector, hydrogen flow rate was 30.0 $mLmin^{-1}$, air flow rate was 400 mL/min, and makeup flow rate (Helium) was 25 $mLmin^{-1}$. The GC oven temperature ramp was operated as follows, initial temperature of 150 °C held for 1min, increased at 10 °C/min to 175 °C, held 10 min then increased at 5 °C/min to 210°C, and held for 5 min. At last, temperature was increased at 5 °$Cmin^{-1}$to a final temperature of 230 °C, held 15 min. Total run time was 44.5 min. The sampling rate of the FID was 20 Hz. The FAME solutions were injected in the volume of 1 µL using an autosampler Agilent Technologies 7683 Series Injector in the splitless mode. The chromatograph worked under Agilent Technologies Enhanced Chemstation software. The FA were identified by comparison of RRT of the compounds in the sample with RRT of Supelco 37 component FA methyl ester mix standards. The moles of FA were calculated by the moles of internal standard (pentadecanoic acid, 15:0) and peaks' area.

6.2.10 Q-test

Average CoV for lipid analytes is a function of corrections used in data processing; CoV is equal to the standard deviation of the measurements for each analyte divided by the average. CoV was calculated from the QC samples, without any correction, using only the linear trend correction within each day's sample set, only the correction to the overall average across sample sets, or both corrections (as done on the experimental data).

6.2.11 Fatty Acid Calculations

The average chain length (ACL) was calculated as ACL = [Σ % Total14 × 14) + … + (Σ % Total n × n)] / 100 (n = carbon atom number). The double bond index was calculated as DBI = Σ mol % of USFA × number of double bonds of each USFA. The peroxidizability index was calculated as PI = [(%Monoenoic × 0.025) + (%Dienoic × 1) + (%Trienoic × 2) + (%Tetraenoic × 4) + (%Pentaenoic × 6) + (%Hexaenoic × 8)]. SFA were calculated as SFA = Σ % (14:0 +16:0 +…24:0). USFA were calculated as USFA = Σ % (MUFA+PUFA). The degree of unsaturation in the balsam fir needle lipids was evaluated by the unsaturation index as in Makarenko et al. 2014, $UI = \sum p_j /100$ where p_j is the content (wt %) of USFA multiplied by the number of double bonds in each acid. The oleic desaturation ratio (ODR) and the linolenic desaturation ratio (LDR) were used to determine the efficiency of the desaturation of oleic to linoleic acid (ODR) and linoleic acid to α – linolenic acid. (LDR) = C18:2cis + C18:3n3/ C18:1 + C18:2cis + C18:3n. LDR = C18:3n3/ C18:2cis + C18:3n3 (Pleines and Friedt 1988). The degree of unsaturation in the balsam fir needle lipids was evaluated by the UI (Makarenko et al., 2014);

$$UI = \sum p_j /100$$

where p_j is the content (wt %) of USFA multiplied by the number of double bonds in each acid.

6.2.12 Statistical Analyses

Needle loss and water uptake data were submitted to repeated measures analysis using PROC MIXED in SAS© 9.3. All other data were submitted to analysis of variance (ANOVA), using Minitab® 17 to determine effects of treatment significant at α = 0.05. In all analyses, the fixed independent variable was sampling date. Assumptions of normality, homogeneity, and independence were confirmed for all analyses. Significant effects were separated using Tukey's multiple means comparison. A two-sample t-test was used to determine the difference between Clone 506 and Clone 9 at peak abscission. Correlation analysis using Minitab® 17 was used to determine the relationship between needle loss and polar lipid classes and FA.

6.3 RESULTS

6.3.1 Establishing Abscission Differences between Two Balsam Fir Genotypes

Physiological processes such as needle abscission, water intake, and MII followed their traditional progression for Clone 506. Needle loss Clone 506 followed an approximate logistic curve over the 10 weeks of study (Fig. 24). After the first week, needles had lost approximately 0.4 % of their fresh weight in needles. Needle loss continued to increase slowly, remaining under 2% for the first 7 weeks, and then increased rapidly for the remainder of the experiment. Ultimately, branches in Clone 506 lost 5.9% of their dry mass in needles by week 10. Clone 9 has a similar pattern of abscission, but at almost double the abscission at 11.5%. Clone 9 also had several replicates that went through needle mummification, where needles dried out rapidly but remained intact without the ability to abscise. These branches were removed from the study, but one consequence was having limited branches available for other measurements. Through this experiment, Clone 9 was the low NAR clone and Clone 506 was the high NAR clone.

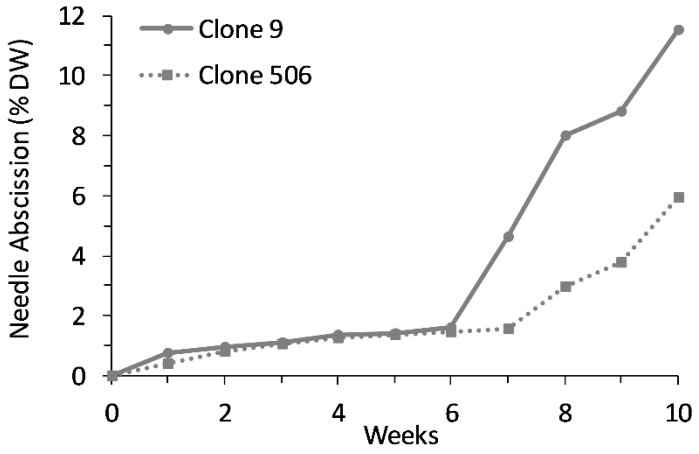

Figure 24. Needle abscission (% DW) in Clone 9 and Clone 506 over a ten-week timespan postharvest.

Chlorophyll fluorescence decreased in both clones over the 10 weeks (Fig. 25). There were no differences in fluorescence in the first 4 weeks and then there were many missing data points due to replicates being removed. However, by week 10, it was determined that Clone 9 had significantly ($p < 0.001$) lower fluorescence than Clone 506. Chlorophyll fluorescence in Clone 9 was 42% lower than Clone 506 by week 10.

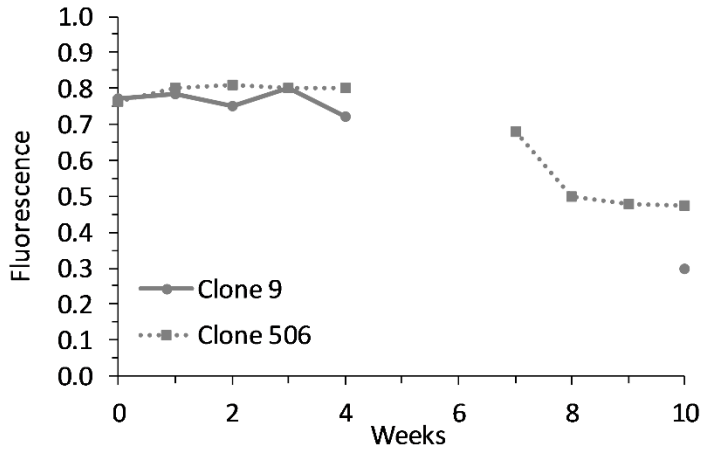

Figure 25. Chlorophyll fluorescence (F_v/F_m) of Clone 506 and Clone 9 needles over a ten-week time span.

Membrane injury index increased over 10 weeks (Fig. 26). Membrane injury index was significantly higher in Clone 9 from 2 – 4 weeks, but several missing data points made it impossible to determine what happened between weeks 5 and 9. By the 10th week, there was no longer any significant difference in MII between clones.

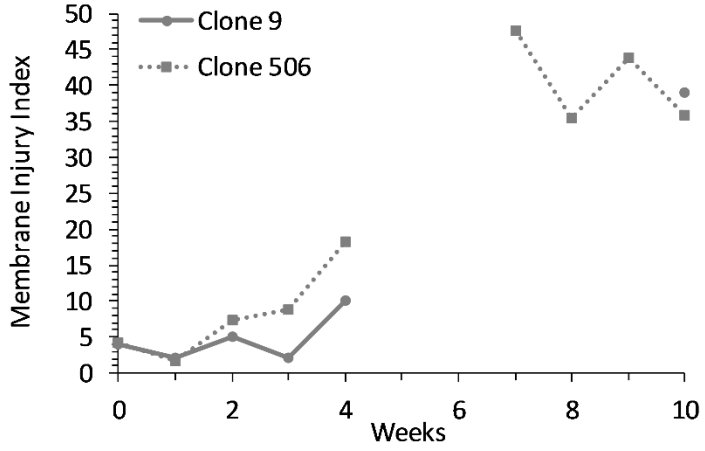

Figure 26. Membrane injury of Clone 506 and Clone 9 needles taken weekly over a ten-week time span.

Stem capacitance was significantly higher in the high NRD Clone (506) than in the lower NRD clone onsite (Fig. 27). In Clone 506, the capacitance decreased by half in the first week, and continued to decrease postharvest to 8.6 pF on week ten. In Clone 9, the

capacitance started at 38 pF, there was some fluctuation due sacrificing randomly chosen branches weekly, and it ended up at 9.3 pF on week ten (Fig. 27).

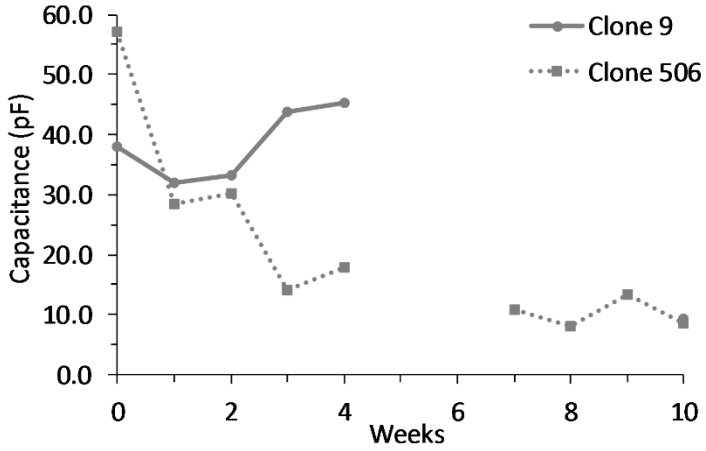

Figure 27. Stem capacitance in Clone 506 and Clone 9 taken weekly over a ten-week time span postharvest.

Water uptake was high for the first week, but this dropped drastically by week two. It was at the critical level of 0.05 mL g^{-1} d^{-1} by week five of the experiment (Fig. 28).

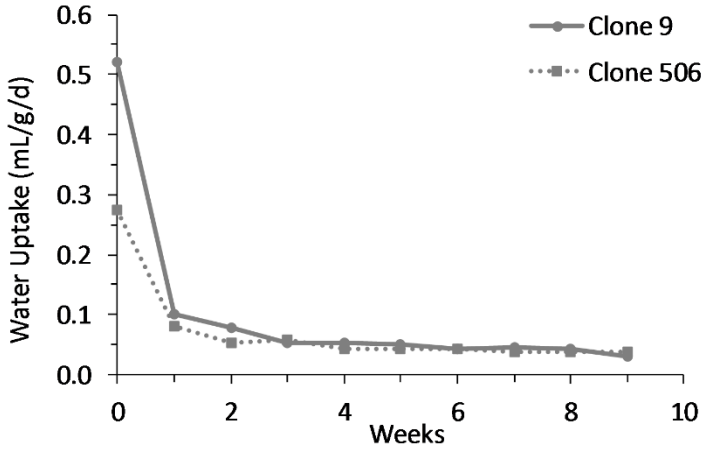

Figure 28. Water uptake take in Clone 506 and Clone 9 over a ten-week time span postharvest,

6.3.2 Polar Lipids

Both Clones 506 and 9 contained the same lipid classes (Table 10). There were no notable differences between the percentages of polar lipids when sampled onsite in early October. Total polar lipids (nmol. mg^{-1}.DW) were analyzed at 10 time points for Clone 506 and five

time points for Clone 9. Total polar lipids decreased significantly by the seventh week postharvest for Clone 506 ($p < 0.001$), showing almost a 45% polar lipid decrease. The total decrease by the tenth week postharvest was 88%, compared to initial values. In Clone 9, there was no significant difference in total polar lipids in the first four weeks. Total polar lipids by week 1 showed a 60% decrease ($p < 0.001$).

6.3.3 Lipids Clone 506

There are significant changes in the distribution of polar lipids over time postharvest (Table 10). The most plentiful lipid class in fresh needles was MGDG (36%). By the end of the ten weeks MGDG comprised only 25 % of the polar lipids. Changes were clearly seen in a few lipid species, MGDG 36:6, MGDG 36:7 and MGDG 36:5. The percentage of MGDG 36:6 decreased significantly postharvest ($p < 0.001$). It originally comprised 26% of total lipids, was reduced to 15% by week five. MGDG 36:7 was also significantly reduced ($p = 0.001$), from 4% to 2% but it decayed later than the MGDG 36:6. There was a significant increase in MGDG 36:5 ($p < 0.001$). It increased by 2.5 times by week 4.

DGDG was the second most abundant lipid class (28%). There was no significant change in the percentage of this lipid class, however there was a significant change in the MGDG/DGDG ratio. The ratio decreased from approximately 1.28 to 0.89 by week 8. In addition to this, there was one species, DGDG 36:6 that decreased significantly. PA also increased significantly from week one to week ten ($p < 0.001$), almost four-fold by week by week ten.

6.3.4 Lipid Classes, Clone 506 versus Clone 9, for the First Five Time Points and Week Ten

Results of lipid analysis, based on the first five sampling periods (Table 10), showed many signs of degradation of chloroplast lipids starting in the low NRD clone, Clone 9, but not in the high NRD clone, Clone 506. Although there was no overall decline in mean DGDG, there was a significant interaction effect of week and clone ($p = 0.025$) on mean DGDG 36:6. The lipid species DGDG 36:6 decreased significantly by four weeks postharvest in

Clone 9 but not in Clone 506. There was also a significant interaction effect on mean MGDG (p = 0.003). The change in MGDG was irregular possibly due to the changes occurring at different rates in the various MGDG species. Between the second and third week postharvest there was a significant ($p < 0.001$) overall increase in MGDG 35:5. However, due to the interaction effect of week and clone on both mean MGDG 36:6 and MGDG 36:7 ($p < 0.05$), there was a significant decrease in these lipid species in Clone 9, by week four. By the fourth week postharvest, in mean MGDG 36:6, there was an 80% mean decrease in this lipid species in Clone 9 only. In addition, there was a 35% mean decrease in in Clone 9 in mean MGDG 36:7.

Also there were changes in mean percentage of PL. PC increased as a result of a decrease in GL. The only clone in which a significant increase occurred in mean PC was Clone 9 (interaction effect $p < 0.001$). Mean PC increased in Clone 9 between week three and four by 20%. Another notable change was in phosphatidic acid (PA). Mean PA generally increases as a result of degradation. There was a borderline interaction effect (p = 0.049) of week and clone on production of PA. There was a significant five times increase in mean PA in Clone 9 from week one to week two, postharvest.

A comparison using a two sample t-test was made at peak abscission for Clone 506 and Clone 9. There were some differences. There was significantly more mean 36:6 in Clone 9 and significantly more mean PA in Clone 506 (p = 0.006 and 0.021, respectively) at peak abscission. Clone 9 also had significantly more PC ($p < 0.001$).

Table 10: Comparison of lipid classes by percentage of total lipids for postharvest balsam fir branches sampled over a ten week period. Branch samples were comprised of 5 randomly chosen branches from Clones 506 and 9 each week. Those in bold show a significant difference.

Lipid Class	Weeks								
	Initial	1	2	3	4	7	8	9	10
DGDG									
9	28.7	27.6	27.4	28.2	27.7	-	-	-	25.5
506	28.8	28.0	27.2	29.7	28.4	28.9	28.1	27.3	25.5
MGDG									
9	39.0	30.9	41.5	38.4	33.9	-	-	-	29.1
506	36.1	39.2	37.1	33.3	42.1	31.7	26.2	28.0	25.6
PG									
9	4.4	4.1	4.4	4.6	4.7	-	-	-	5.1
506	4.4	4.8	4.5	4.7	3.6	4.4	4.9	4.5	5.6
PC									
9	18.9	17.9	15.9	17.9	**22.0**	-	-	-	**26.5**
506	19.7	17.6	18.8	18.8	**14.6**	17.8	23.7	24.6	**20.2**
PE									
9	3.2	3.6	2.3	2.5	3.1	-	-	-	4.1
506	2.7	2.5	2.6	2.7	2.3	2.7	3.5	3.6	3.5
PI									
9	4.5	**9.5**	4.8	5.0	5.7	-	-	-	6.3
506	4.9	**4.5**	5.4	6.1	4.5	7.3	7.1	6.8	8.5
PS									
9	0.0	0.5	0.0	0.0	0.0	-	-	-	0
506	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.1
PA									
9	**0.6**	3.1	2.4	2.6	1.9	-	-	-	**2.0**
506	**1.3**	1.5	3.1	2.4	2.4	3.7	3.4	2.3	**4.9**
LPG									
9	**0.4**	1.0	1.1	0.4	0.6	-	-	-	**0.7**
506	**1.9**	1.6	1.2	1.9	1.8	2.9	2.4	2.3	**4.0**
LPC									
9	0.1	1.7	0.1	0.2	0.2	-	-	-	**0.4**
506	0.1	0.1	0.1	0.3	0.2	0.5	0.4	0.4	**1.6**
LPE									
9	0.1	0.1	0.1	0.2	0.1	-	-	-	0.1
506	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.2	0.6

6.3.5 Δ5-UPIFA

There are Δ5-polyunsaturated methylene-interrupted FA (Δ5-UPIFA) found in balsam fir needles. The following Δ^5-acids have been found: taxoleic (C18:2Δ5,9), pinolenic

(C18:3Δ5,9,12), sciadonic (C20:3Δ5,11,14), coniferonic acid (C18:4 (Δ5, 9,12,15)) and juniperonic (C20:4 (Δ5,11,14,17) acids. These FA compose 17 - 18% of all FA in balsam fir needles.

6.3.6 Fatty Acid Clone 506

In Clone 506 (Tables 11 and 12), there is a decrease over time in mean percentage of USFA 5,9,12 15-18:4, 18:3n3, and 7-16:1 ($p < .004$). In 5,9,12 15-18:4, there is no significant difference ($p < 0.001$) until week eight and ten. By week 10, there is an approximately 38% decrease. In 18:3n3, there is a significant decrease in the samples randomly selected in week three compared to those collected onsite and after one week postharvest. However, week four did not show a similar decline, so the week three drop could have been coincidental due to random sampling of different branches every week. Samples collected on week seven – ten postharvest all showed a significant decrease in 18:3n3. By week ten there was an approximately 37% decrease from the initial values. FA 7-16:1 decreased by 41% by week two and continued to decrease by over 50% by week ten postharvest. Other FA showed a significant increase. ($p < 0.004$): SFA 22:0 and 20:0, and PUFA 5,9 18:2, and cis18:2.

6.3.7 Fatty Acid Clone 506 versus Clone 9

Tables 11 and 12 show mean FA for both clones. ANOVA was performed for the first five time points and a two sample t-test for the final values. There was a significant decline in both clones in mean 18:3n3 over time ($p < 0.000$). By week three and four, a decline is seen. In the first five data points, there was a marginally significant interaction between clone and week ($p = 0.099$) for 18:4. However, there was no decline in mean 18:4 in either clone in the first four weeks. Similar results were found for 18:2 ($p = 0.049$). Another interaction existed between clone and week for 14-methyl 16:0. Clone 9 was significantly higher for the first and last two time points, but on week two the mean percentage of 14-methyl 16:0 was the same as in Clone 506. 7-16:1 in both clones dropped approximately 40% in mean 14-methyl 16:0 in the first week postharvest.

There were several clonal differences noted: Clone 506 was contained significantly more mean SFA 14:0, 16:0, and 23:0, in addition to more 20:3n3. Clone 9 contained significantly more of the Δ5-UPIFA 20:3 and 20:4.

In both clones, NL was inversely related to decreasing peroxidation index (PI) and DBI. It also was related to decreasing to linoleoyl-desaturase ratio (LDR) in Clone 506 (R^2 = 62%, 62%, and 58%, respectively.

6.4 Discussion

6.4.1 Needle Loss, MII, Fluorescence, Water Intake, and Capacitance

The postharvest abscission curves for Clones 506 and 9 in this experiment seemed to follow the same beginning of the typical sigmoid curve but more time was required to verify this (Lada and MacDonald, 2015). NAC was between week six and seven in Clone 9, the low NRD clone, and between week seven and eight in Clone 506, the high NRD clone, with more rapid needle loss happening in Clone 9. Both of these clones lasted longer postharvest than predicted in earlier studies. Clone 9 peaked in 17 days pre- cold acclimation and Clone 506 peaked in 47 days in previous studies (MacDonald and Lada, 2008). This shows that it is hard to compare year to year, as environmental conditions can vary so much preharvest, and this has an effect on postharvest abscission (Lada and MacDonald, 2015).

Membrane injury is apparent in chloroplasts of balsam fir. The chlorophyll index of postharvest balsam fir begins relatively high, but gradually decreases in both clones until abscission (MacDonald and Lada, 2014). Chlorophyll fluorescence is one tool to assess photosynthetic activity in a plant, chloroplast stability, and general physiological status of a plant (Ball et al., 1995). There is a distinct relationship between fluorescence and needle abscission in balsam fir (MacDonald et al, 2015). Fluorescence gradually decreased postharvest until abscission. In addition, there is a very strong negative relationship (R^2 > 90%) between fluorescence and needle abscission, which suggests at the very least that fluorescence and chloroplast integrity are associated with postharvest needle abscission (MacDonald and Lada, 2015). This can be tied in to changes in MGDG and DGDG that

are degrading postharvest in past studies and discussed earlier in this thesis (MacDonald et al., 2015). There is also an increase in MII suggesting ion leakage through the cell membrane but this occurs later than chloroplasts changes (Lada and MacDonald, 2015).

Table 11. Comparison of FA below 20:0 by percentage for postharvest balsam fir branches sampled over a ten-week period. Branch samples were comprised of five randomly chosen branches from Clones 506 and 9 each week.

FA	Weeks								
	Initial	1	2	3	4	7	8	9	10
14:0									
9	2.0	1.6	1.7	1.9	1.6	-	-	-	2.1
506	2.3	2.0	1.6	2.9	2.5	2.4	3.0	2.4	3.2
16:0									
9	11.3	11.0	11.3	11.9	11.1	-	-	-	11.8
506	11.9	13.0	12.3	12.3	12.0	11.3	13.5	12.1	13.7
7-16:1									
9	0.8	0.5	0.5	0.4	0.4	-	-	-	0.4
506	0.8	0.6	0.5	0.4	0.4	0.3	0.4	0.4	0.4
14methyl-16:0									
9	**5.4**	**5.8**	**5.1**	**6.3**	**6.4**	-	-	-	5.6
506	**5.0**	**4.9**	**5.4**	**5.4**	**5.2**	5.3	5.3	5.5	6.0
17:0									
9	0.4	0.4	0.4	0.5	0.4	-	-	-	0.5
506	0.4	0.5	0.4	0.5	0.5	0.5	0.5	0.5	0.5
18:0									
9	3.7	4.1	4.6	4.5	4.6	-	-	-	5.1
506	3.8	4.3	3.9	5.4	4.1	4.4	4.8	5.3	5.1
18:1cis(9-18:1)									
9	12.5	13.4	12.6	14.5	14.9	-	-	-	13.8
506	12.6	12.4	13.5	12.8	13.2	14.3	12.5	13.8	13.2
5,9-18:2									
9	3.3	3.6	3.4	3.4	3.6	-	-	-	4.7
506	3.6	3.4	3.5	3.9	3.7	4.6	4.5	4.8	4.8
18:2cis (9,12-18:2)									
9	16.5	17.3	15.6	17.0	18.5	-	-	-	19.6
506	16.4	14.9	16.8	16.7	16.2	17.9	18.1	18.5	17.8
5,9,12-18:3									
9	5.0	5.6	5.1	5.3	5.8	-	-	-	5.0
506	5.2	4.7	5.2	5.6	5.2	6.0	5.3	5.4	5.5
18:3n3 (9,12,15-18:3)									
9	25.2	21.1	24.1	20.0	17.8	-	-	-	16.9
506	24.5	25.1	22.5	19.0	22.4	18.0	16.6	16.7	15.4
5,9,12,15-18:4									
9	4.4	4.5	4.3	3.7	3.7	-	-	-	2.8
506	4.1	4.5	4.0	4.2	4.2	3.5	3.0	3.4	2.8

Table 12. Comparison of FA above 20:0 by percentage for postharvest balsam fir branches sampled over a ten-week period. Branch samples were comprised of 5 randomly chosen branches from Clones 506 and 9 each week.

FA	Weeks								
	Initial	1	2	3	4	7	8	9	10
20:0									
9	0.9	1.2	1.0	1.1	1.0	-	-	-	1.4
506	0.9	1.0	1.1	1.1	1.0	1.3	1.4	1.4	1.4
20:2									
9	0.4	0.4	0.4	0.3	0.4	-	-	-	0.1
506	0.3	0.3	0.3	0.3	0.3	0.3	0.1	0.2	0.2
5,11,14-20:3									
9	3.4	**4.0**	3.7	4.1	4.4	-	-	-	4.1
506	3.3	**3.3**	3.5	4.0	3.9	3.9	4.0	3.7	3.7
22:0									
9	1.6	1.9	1.6	1.7	1.9	-	-	-	2.5
506	1.6	1.7	1.8	1.8	1.7	2.1	2.4	2.2	2.4
20:3n3(11,14,17-20:3)									
9	0.2	0.2	0.2	0.2	0.1	-	-	-	0.1
506	0.2	0.2	0.3	0.2	0.2	0.2	0.2	0.1	0.2
5,11,14,17-20:4									
9	1.5	1.6	2.0	1.5	1.6	-	-	-	1.6
506	1.4	1.5	1.6	1.4	1.5	1.5	1.8	1.4	1.4
23:0									
9	0.7	0.8	1.0	0.6	0.6	-	-	-	0.7
506	0.7	0.8	0.8	0.8	0.7	0.7	1.0	0.6	0.7
24:0									
9	0.9	1.1	1.3	1.1	1.2	-	-	-	1.5
506	1.0	1.0	1.1	1.1	1.0	1.4	1.7	1.5	1.7

Past studies of postharvest balsam fir have shown an initial water uptake between 0.15 – 0.20 $mL \cdot g^{-1} \cdot d^{-1}$, with a significant decrease in the first two weeks (Lada and MacDonald, 2015). In our experiment, mean water consumption in the freshly cut balsam fir was a relatively high 0.27 $mL \cdot g^{-1} \cdot d^{-1}$ and 0.52 for Clones 506 and 9, respectively, and by week two the means were 0.08 and 0.1 $mL \cdot g^{-1} \cdot d^{-1}$. It seemed that the low NRD clone took in more water in the first week, possibly due to the fact that the high NRD clone reacted more quickly to the water deficit stress by stomatal closure. The critical value is thought to be 0.05 $mL \cdot g^{-1} \cdot d^{-1}$, after which abscission occurs (Lada and MacDonald, 2015). Water uptake was at that critical value, at 0.05$mL \cdot g^{-1} \cdot d^{-1}$, by the end of week five. There has been consistently a change in water status that has been linked to postharvest abscission in balsam fir (MacDonald and Lada, 2014; Lada and MacDonald, 2015). Stem capacitance was taken in this study as a measure of water status. Stem capacitance has been found to be a good measure of membrane function and stability in the stem and is highly coordinated

to leaf hydraulic ability, with the leaf always being more vulnerable than the stem in loss of this ability (Blackmann and Brodribb, 2011; Nolfe et al., 2015). This current study shows that the stem capacitance was significantly higher in the high NRD clone than in the low NRD clone initially in the field. However, the water status dropped quickly, after one week in the high NRD clone to half and more slowly in the low NRD clone. Lowering capacitance during this study confirms a drop in water status related to needle abscission.

6.4.2 Polar Lipids

Membrane lipids are important in maintaining cell integrity and ensuring the activity of proteins that are related to membranes, including essential chloroplast proteins (Leshem, 1992). Loss of acyl lipids has been known to occur during water-deficit stress, and this is not unusual since in times of stress, plants try to adapt by modifying lipid structure. If stress continues there can be subsequent irreversible cell injury and possibly cell death (Harwood, 1998). It has been confirmed in section 6.4.1 that there is stress due to water deficit, not only in the current study, but also in past studies on balsam fir postharvest (Lada and MacDonald, 2015) by other scientists. Polar lipids have decreased during water-deficit stress in other studies as well involving different plants (Svenningson and Liljenberg, 1986; Hubac et al., 1989; Hanrouni et al., 2001). Both clones in this study showed a sharp decrease in acyl polar lipids, as did Clone 706 from the same orchard in a previous study. This decrease also could be linked to ABA increase postharvest, phospholipase D production and subsequent lipolysis (Bargmann and Munnik, 2006; Kar, 2011; Thiagarajan et al., 2016). There are also suggested links between ABA production and ethylene production, and ethylene production has been linked to postharvest needle loss, so it is possible that ethylene is the trigger for lipid breakdown (Thiagarajan, 2012).

MGDG seems to be the major chloroplast membrane to be affected postharvest in both clones, as well as in Clone 706 of a previous study (MacDonald et al., 2015). Looking at chloroplast structure, MGDG is the main polar lipid to comprise the thylakoid membranes. MGDG, DGDG, and PG are the nonpolar lipid constituents of the thylakoid membrane of higher plant chloroplasts. MGDG and DGDG are present in the membrane at 56% and 29%, respectively, of the total lipid content. MGDG is much higher than Clone 706 from

a previous study, but in that study branches were cut after cold acclimation (MacDonald et al., 2015). DGDG is a bilayer-forming lipid, while MGDG alone will only form hexagonal-II structures. MGDG is the most abundant lipid in the thylakoids and the inner membrane, whereas DGDG is more abundant in the outer envelope (Boudière et al., 2014). It is important to keep a constant MGDG/DGDG ratio for cell homeostasis, but this could change due to environmental stresses and have an effect on photosynthetic efficiency. In the case of water deficit studies, MGDG is the most susceptible lipid to damage, which accounts for why there was a reduction in the percentage of MGDG in this current and previous studies (Scotti-Campos and Pham-Thi, 2016; MacDonald et al., 2015). The loss of MGDG decreases the ratio of MGDG/DGDG, and upsets homeostasis and this can affect integrity of the chloroplast membranes and photosynthesis. The same decline is not seen in DGDG percentage in this study, but interestingly, during stress, more DGDG can be produced in an attempt to stabilize the membranes (Boudière et al., 2014). In the previous study with Clone 506, there was a significant decrease in the percentage of DGDG. However, since this study was performed in December in Nova Scotia, cold acclimation could have had an effect on those results.

Interestingly, the lipid species that deteriorated in this current study were MGDG 36:7, MGDG 36:6, and DGDG 36:6. In Vigna, it has been found that the long chained highly USFA are the main target for water-deficit stress-related lipid peroxidation. MGDG is known to contain 90% of 18:3n3 in its structure. This is good for membrane fluidity, but creates a target in stressful situations. In stressful times such as water deficit, the chloroplasts of a cell can become damaged and it is thought that ROS are formed (Bailey-Serres and Mittler, 2006). It is also thought that these damaged cells result in the formation of an enzyme, ubiquitin ligase that acts as a signal to degrade ROS producing chloroplasts (Woodson et al., 2015; Lavelle, 2019). It is possible that the chloroplasts are breaking down postharvest in balsam fir. The study by Woodson et al, 2015 was accompanied by TEM microphotographs that showed chloroplast breakdown. This could be the only way to tell for sure what is happening to the chloroplasts in balsam fir postharvest.

PA increase postharvest could be indicative of PLD activity due to stress. PA has fairly recently been identified as a signaling molecule in plants. Sometime it will increase 20 times in plant cells in times of stress. The increase seen here is not that great, but could be important, as PA is hard to quantify due to its transient nature. The downstream affect has not been clearly identified, but it could be related to ethylene or ABA production (Testerink and Munnik, 2011).

6.4.3 Δ5-UPIFA

Δ5-UPIFA have not been studied thoroughly as they only occur in conifers and a few other species, and, in conifers, most studies have been on seeds (Knutzon et al., 1998). The main difference noted between these needle Δ5-UPIFA and a previous study done by Wolff et al., 1997 on conifer seeds, was the lower overall total of approximately 18% in needles compared to 30% in seeds. Also there is more coniferonic acid, found to be less than 0.2% in all seeds, but present in approximately 3% in balsam fir needles. There is no significant difference in the number of these FA in initial samples of balsam fir needles. However, there is more overall accumulation during the course of this experiment of mean coniferonic acid (C18:4 (Δ5, 9, 12, 15) and juniperonic acid (C20:4 (Δ5, 11, 14, 17) in the lower NRD clone, so there could be some involvement of these FA in postharvest needle retention (Wolff et al., 1998).

6.4.4 Fatty Acids

Cis-7-hexadecenoic (7-16:1) is an unusual FA found rarely in plants. *Cis* FA are valuable for membrane stability having a 30 ° bend in their structure where the bond exists helping to keep the FA away from each other and provides more fluidity to membranes (Aid, 2019). It is thought, however, that this FA 7-16:1 is associated with neutral lipids, such as triacylglycerols (TAG), as opposed to membrane structure (Guiias et al., 2016). Interestingly, there was less than 0.1% of this FA, but a sharp decrease in 7-16:1 in both clones postharvest. TAG synthesis is not common in vegetative parts of plants, particularly leaves, as leaves are primarily for starch production. If TAG exists in leaves it is often a very low percentage. Therefore, most FA in leaves are used for building photosynthetic

membranes (Changcheng and Shanklin, 2016). However, it is possible that there are oil droplets or lipoproteins, plastoglobuli, in the needles of conifers, and that these are related to postharvest needle abscission. Possibly these are used in times of stress. There is limited knowledge of this, but this could be revealed by TEM studies and may help explain postharvest lipid physiology in the balsam fir. It is typical for these TAG to be hydrolyzed under conditions of carbon and energy deficiency, which could account for the loss of these lipids postharvest.

Alpha linolenic acid (18:3n3) deteriorated in both clones of balsam fir needles in this study. Postharvest deterioration of this FA was found in one other clone of balsam fir (Clone 706) postharvest as well (MacDonald et al., 2015). If you look at one of the polar lipids that are declining most rapidly, MGDG 36:6, this definitely has 18:3 in its structure, however there is no way to confirm it is α-linolenic acid as opposed to pinolenic acid (5, 9, 12 – 18:3). Due to the fact that there is five times more linolenic than pinolenic acids leads us to believe that at least one of the FA in MGDG 36:6 would be linolenic and related to the breakdown of this FA. It is thought that in plants 18:3n3 is in the sn-2 position when they are produced *via* the eukaryotic pathway.

Coniferonic acid (C18:4 (Δ5, 9, 12, 15) did not show a decline until the eighth week postharvest, but this occurred in both clones. There is evidence in microalgae that coniferonic acid is the product of the α-linolenic acid, by Δ^5-desaturase activity (Kajikawa et al. 2006). Both coniferonic and α-linolenic seem to be the target of lipid degradation postharvest in balsam fir needles, first α-linolenic and later coniferonic. Possibly lipid degradation is related to postharvest abscission. These are also related to the subsequent loss of MGDG 36:6 and 36:7, chloroplast lipids, so it seems likely that coniferonic acid and α-linolenic acids could be part of the thylakoid membrane lipid structure, and possibly chloroplast degradation is related to postharvest abscission. In a study on cucumber thylakoids, α-linolenic acid was listed as the top contributor to thylakoid structure, however, cucumbers do not have coniferonic acid (Shu et al., 2015). In a study on FA composition of lipids in chloroplast membranes of tobacco plants, α-linolenic acid was also listed as one of the primary components of the thylakoids (Popov et al., 2017). The

chloroplast envelopes are also involved in chloroplast stability and may also contain this FA.

6.5 Conclusions

There was a decrease in polar lipids in balsam fir over time postharvest suggesting a degradation of cellular lipids. There was a major decline in MGDG suggesting chloroplast breakdown. Changes were clearly seen in a few lipid species, a decline in MGDG 36:6, MGDG 36:7, DGDG 36;6, and an increase in MGDG 36:5. The lipid species declined in week four in the low NRD clone and in week seven in the high NRD clone. PA also increased significantly by week ten. There was a decrease over time in mean percentage of USFA 5,9,12 15-18:4, 18:3n3, and 7-16:1. This decline could be related to enzymatic or non-enzymatic lipid degradation, which may result or produce a signal for postharvest abscission. SFA 22:0 and 20:0, and PUFA 5,9 18:2, and cis18:2, showed a significant increase.

CHAPTER 7 – SEASONAL CHANGES IN POLAR LIPIDS LINKED TO POSTHARVEST NEEDLE LOSS IN FOUR GENOTYPES OF BALSAM FIR

7.1 PREFACE

This work is currently under review with Journal of Plant Growth and Regulation:

MacDonald GE, Lada RR, Caldwell CD, Udenigwe C, MacDonald M. 2019. Seasonal changes in polar lipids linked to postharvest needle loss in four genotypes of balsam fir, *Abies balsamea,L.*

This work was also presented at the Annual Conference of the American Society for Horticultural Science in Washington, DC (2018).

7.2 INTRODUCTION

In Nova Scotia, Canada, balsam fir Christmas trees are being harvested earlier each autumn to meet an increasing export market. There is also evidence that fall temperatures are rising in Nova Scotia (Statistics Canada, 2017). These two factors combined are believed to cause premature postharvest needle abscission in balsam fir due to incomplete cold hardening (Gusta et al., 2005; Thiagarajan and Lada, 2010; MacDonald et al., 2014b). In nature, low temperatures and shorter photoperiods in autumn and early winter induce cold acclimation, which creates a disadvantage for early-harvested trees (Odlum and Blake 1996, Lizotte, 2015, Greer et al., 2000). Multiple studies have confirmed that needle abscission is significantly delayed if trees are harvested in November or December, as compared to the October harvest commonly used in industry (Thiagarajan and Lada, 2010; Thiagarajan, 2012; MacDonald et al., 2014b).

Many cellular processes are affected by cold acclimation, including gene expression, membrane lipid composition, increases in ABA, accumulation of soluble sugars and other osmolytes, and increased levels of antioxidants to protect from ROS damage (Xin and Browse 2000; Thiagarajan and Lada 2010). Changes in cellular membranes are often discussed with relation to cold acclimation as a reorganization of polar lipids and FA that

occur to keep membranes fluid in cold conditions. One example of lipid reorganization is the decrease of a monolayer lipid, such as MGDG, and an increase of a more stable lipid bilayer, such as DGDG; Dörmann, 2013). Other changes such as desaturation of FA in structural PL or an increase in PL that contain USFA in plants may occur (Partelli et al. 2011). Structural PL include PC, PE, PG, PS, and PI. Phospholipids PC and PE contribute up to 68 – 80% of the aforementioned structural PL. Other PL, such as PA, LysoPL, and derivatives of PI are thought to have roles in signaling (Buchanan et al. 2000). These polar lipids have never been tested during cold acclimation in balsam fir but have been found to be related to other changes in plants such as increased ABA and ROS (Kar, 2011; Okazaki and Saito, 2014).

Balsam fir is a very cold hardy conifer, able to withstand temperatures of -198°C once frozen (Salkai and Weiser, 1973), indicating that this tree must have physiological mechanisms in place to withstand very low temperatures. Low temperatures, in turn, seem to influence the postharvest quality of balsam fir. However, variability exists among various balsam fir genotypes. Some genotypes tend to retain their needles longer regardless of harvest date, while others with poor needle retention seem to require cold acclimation (MacDonald and Lada, 2008; MacDonald et al., 2014b). The established guideline for the NAR of the balsam fir genotypes is that high NAR clones should retain its needles over 40 days postharvest, a moderate NAR clone should retain its needles between 20 and 40 days, and a poor NAR clone would retain its needles for less than 20 days (MacDonald and Lada, 2008). It may be that postharvest needle retention differences between genotypes may be linked to their ability to acclimate to cold. The objectives of this research were to identify changes that occur to polar lipids during cold acclimation and to define any relationship those changes may have to genotypic variability in needle abscission resistance. This will improve our understanding of varying lengths of postharvest needle retention in balsam fir clones and why low NAR clones benefit more than high NAR clones.

7.3 Materials and Methods

7.3.1 Sampling and Experimental Design

The plant material for this investigation was collected from the Debert clonal tree orchard (45 44’ N, -63 50’ W) located in Debert, NS, Canada from September, 2013 to February, 2014. The Christmas tree germplasm collection has over 220 genotypes of balsam fir. Four genotypes, identified as Clones 9, 37, 506, and 566, were chosen for this study. In previous studies, the first two of these genotypes exhibited a high NAR when harvested in October and kept in unhydrated conditions, whereas the latter two exhibited low NAR (MacDonald and Lada, 2008). The experiment was set up as a 4 x 5 factorial. Four clones were sampled with five replicates on September 18, 2013, October 28, 2013, Nov. 25, 2013, Dec. 30, 2013, and Feb. 5, 2014, which would represent autumn and the beginning of winter in Nova Scotia. Branches with 2 years of growth were used for this study and were cut from approximately 20-year old trees at an elevation of approximately 1 m from the ground level on the south facing side of the trees. As sampled, needles were immediately immersed in liquid nitrogen and stored at –80 °C until lipid extraction. Fresh needles were collected in the field, added to prepared centrifuge tubes, initial conductivity measurements taken, and then transported to the laboratory. Fluorescence was measured immediately when returning to the laboratory. The opposite branches of each sample (branch immediately opposite on the stem) were cut, hydrated and taken to the laboratory to keep in bottles until the commencement of abscission. Once in the laboratory, branches were given a fresh aseptic cut 2.5 cm above the previous cut while submerged in water to reduce risk of cavitation, weighed, and then placed in a 100 mL amber bottle filled with 100 mL of distilled water. The neck of each flask was plugged with cotton wool to reduce direct water evaporation and provide added stability to a branch. Afterward, the entire apparatus was weighed, which allowed for quantification of needle loss throughout the experiment. Each branch was placed on a light rack and maintained at an average temperature of 22 °C with a light intensity of 85 - 95 $\mu mol \cdot m^{-2} \cdot s^{-1}$ supplied by 440 nm fluorescent lights.

7.3.2 Weather Parameters

Temperatures and photoperiod from 30 days prior to the start of the experiment were recorded from the Debert Weather Station, which is within 0.5 km of the orchard. Photoperiod was taken on the days of sampling only. However, temperatures leading up to the sampling days were used to calculate minimum temperature (T_{min}), maximum temperature (T_{max}), days exposure to freezing temperatures, and cold degree days (CDD), which are all shown in Table 13. CDD was determined by using the following formula:

$$Cold\ Degree\ Days = \sum_{i=1}^{i=n} T_{base} - T_a, \text{ when } T_{base} - T_a > 0; \text{ otherwise } 0$$

In the above equation, T_a is the daily mean air temperature calculated from the daily minimum and maximum temperatures. T_{base} is the threshold temperature of 5°C determined for balsam fir as the time, which cold plant growth no longer occurs in the fall (Hassan et al. 2007), and *i* is day of the period prior to sampling, with 1 being the first day and n being the last day.

Table 13. Temperature parameters 30 days prior to first sampling periods and between each sampling time.

Sampling Date	CDD (days)	T_{min} (°C)	T_{max} (°C)ma	CD Below 0°C	Photoperiod (h)	T_{max}
Sept. 18	0	5	21	0	12.4	19
Oct. 28	9	-4	18	7	10.3	13
Nov. 25	102	-8	17	28	9.2	7
Dec. 30	354	-23	8.3	60	8.8	1
Feb. 5	417	-29	9.4	95	9.9	-1

Parameters are CDD (cold degree days), T_{min} (minimum temperature in the days prior to testing), T_{max} (highest temperature experienced in the month prior and since the last sampling date), CD below 0°C (cumulative days). All temperatures are in °C. Photoperiod represents the daylight hours on the day of sampling.

7.3.3 Needle Abscission

Needle abscission was measured by collecting the number of needles that would fall after a 'finger run' test each day (MacDonald et al., 2014a). The mass of those needles was measured fresh and after oven drying. The experiment was run until all branches reached peak abscission. Typical postharvest needle abscission follows a sigmoidal curve, and two time points, NAC and PNA were used to compare needle abscission patterns. NAC was defined as the point where postharvest abscission began (1% needle loss), and PNA was defined as the day which had the highest rate of needle abscission (Lada and MacDonald, 2015).

7.3.4 Chlorophyll Fluorescence

Maximum photochemical efficiency or optimum quantum yield of photosystem II (Fv/Fm), was fluorometrically determined using a MINI-PAM Photosynthesis Yield Analyzer (Heinz Walz, Effeltrich, Germany). Plants were dark-adapted for 15 min before Fv/Fm measurements with illumination by application of a saturation flash. Three random replicates for each experimental unit were done, at the time of sampling.

7.3.5 Membrane Injury (MII)

MII uses the percentage of electrolytes leaked into solution to quantify membrane integrity. Test tubes were filled with 30 mL of distilled water and were allowed to adjust to room temperature (24 °C). The electrical conductivity of the distilled water (EC_w) alone was measured using a CDM 2e Conductivity Meter (Bach-Simpson, London, ON). Afterward, approximately 0.4 g of needles were removed from each branch and completely submerged in a centrifuge tube. The tubes were sealed and left at room temperature for 24 h. Initial conductivity (EC_0) was measured to determine the amount of electrolytes leached into solution. Sealed tubes were then placed in a forced-air oven for 4 h at 90 °C to kill tissues and then cooled to room temperature. Final conductivity measurements (E_f) were taken after equilibrating to 25 °C to determine maximum leakage. MII was then calculated using the following equation (Odlum and Blake, 1996):

$$MII = \frac{EC_o - EC_w}{EC_f - EC_w} x100$$

7.3.6 Lipid Extraction

The goal was to observe lipid changes in needles remaining attached to the branch each month, so whole needles that had not yet undergone abscission were frozen in liquid N, stored at -80°C, and then used for lipid extraction. All lipids were extracted at the Kansas Lipidomic Research Center (Manhattan, KS, USA) using an extraction protocol for *Arabidopsis* leaf tissue adapted from Bligh and Dwyer (1959). Approximately 1 g of frozen needles were cut into smaller pieces and incubated in 1 mL of isopropanol with BHT at 75 °C for 15 min for. Afterwards, 1.5 mL of chloroform and 0.6 mL of water were added to solution. The solvent was shaken at room temperature for 1 hour and then transferred to a new glass tube with a Teflon-lined screw-cap using a Pasteur pipette. A total of 0.7 mL chloroform:methanol (2:1) was added, shaken for 30 min. The extraction was completed by adding 4 mL of chloroform: methanol (2:1) ten times, shaking for 1 hour and collecting the solvent. The solvent extracts were washed once with 1 mL KCl (1.0 M) and once with 0.66 mL water. The solvent was evaporated under nitrogen and the lipid extract was quantified and dissolved in 1 mL chloroform. The tissues, after lipid extraction, were dried in an oven at 105°C and dry weights were determined (3 –20 mg).

7.3.7 Lipid Class and Species Analysis

Lipid extracts from 100 samples were analyzed on a triple quadrupole mass spectrometer equipped for electrospray ionization (ESI–MS/MS; Applied Biosystems API 4000) at the Kansas Lipdomic Center, Kansas State University, US. ESI-MS/MS analysis parameters and acquisition parameters are given in Tables 14 and 15. The lipids were quantified in comparison to two internal standards of the class. Lipid species within each head group were identified by total carbon number and total double bonds. Molecular species of each head class were quantified by comparing with the signals of the internal standards (Welti et al. 2002). A list of lipids able to be quantified from this procedure is shown in Table 16.

7.3.8 Q-test

Average CoV for lipid analytes is a function of corrections used in data processing; CoV is equal to the standard deviation of the measurements for each analyte divided by the average. CoV was calculated from the QC samples, without any correction, using only the linear trend correction within each day's sample set, only the correction to the overall average across sample sets, or both corrections (as done on the experimental data).

Table 14. ESI-MS/MS analysis parameters (using Applied Biosystems API 4000) for plant lipids.

Class	Ion Analyzed	Positive Ion Scan Mode	m/z range	Reference
PA	$(M + NH_4)^-$	NL of 115.00	500 – 850	Shiva et al. 2013
PC/LPC	$(M + H)^-$	Pre of *m/z* 184.07	450 – 960	Brügger et al. 1997
PE/LPE	$(M + H)^-$	NL of 141.02	420 – 920	Brügger et al. 1997
PG	$(M + NH_4)^-$	NL of 189.04	650 – 1,000	Taguchi et al. 2005
LPG	$(M-H)^-$	Prec 153	-	Welti et al. 2002
PI	$(M + NH_4)^-$	NL of 277.06	790 – 950	Taguchi et al. 2005
PS	$(M + H)^-$	NL of 185.01	600 – 920	Brügger et al. 1997
DGDG	$(M + NH_4)^-$	NL of 341.13	890 – 1,050	Isaac et al. 2007
MGDG	$(M + NH_4)^-$	NL of 179.08	700 – 900	Isaac et al. 2007

DGDG, digalactosyldiacylglycerol; ESI-MS/MS, electrospray ionization tandem mass spectrometry; MGDG, monogalactosyldiacylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; LPC, lyso-phosphatidylcholine; PE, phosphatidylethanolamine; LPE, lysophosphatidylethanolamine; PG, phosphatidylglycerol; LPG, lysophosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine.

Table 15. Acquisition parameters of lipid classes using Applied Biosystems API 4000, triple quadrupole mass spectrometer.

Parameter	PA	PC/LPC	PE/LPE	PG	PI	PS	DGDG	MGDG
Typical Scan Time (min)	3.51	1.28	3.34	3.21	4.00	4.01	1.67	1.67
Depolarization Potential (V)	100	100	100	100	100	100	90	90
Exit Potential (V)	14	14	14	14	14	14	10	10
Collision Energy (V)	25	40	28	20	25	26	24	21
Collision Exit Potential (V)	14	14	14	14	14	14	23	23

Table 16. Plant membrane lipids determined by the procedure in Chapter 6.3.7.

Class	Lipid Species
LPC	16:0, 16:1, 18:0, 18:1, 18:2, 18:3
LPE	16:0, 16:1, 18:1, 18:2, 18:3
LPG	16:0, 16:1, 18:1, 18:2, 18:3
PA	32:0, 34:6, 34:5, 34:4, 34:3, 34:2, 34:1, 35:4?, 35:3?, 35:2?, 35:1?, 36:7, 36:6, 36:5, 36:4, 36:3, 36:2, 37:4?, 37:3?, 38:7, 38:6?, 38:5?, 38:4?
PC	30:1, 32:0, 33:3: 33:2, 34:4, 34:3, 34:2, 34:1, 35:4, 35:3, 35:2, 35:2, 35:0, 36:6, 36:5, 36:4, 36:3, 36:2, 36:1, 37:5, 37:4, 37:3, 38:6, 38:5, 38:4, 38:3, 38:2, 40:5, 40:4, 40:3, 40:2, 40:1, 42:4?, 42:3?, 43:2?, 43:3?
PE	32:3, 32:2, 32:1, 32:0, 34:4, 34:3, 34:2, 34:1, 35:4?, 35:3?, 35:2?, 35:1?, 36:6, 36:5, 36:4, 36:3, 36:2, 36:1, 37:4?, 37:3?, 38:6, 38:5, 38:4, 38:3, 39:4, 39:3, 39:2, 40:7., 40:3, 40:2, 42:4, 42:3, 42:2, 42:1?, 44:4?, 44:3?, 44:2?
PG	32:1?, 32:0?, 34:4, 34:3, 34:2, 34:1, 34:0, 35:4?, 35:3?, 35:2?, 35:1?, 36:7, 36:6, 36:5, 36:4, 36:3, 36:2, 36:1
PI	30:4?, 30:3?, 32:4, 32:3, 32:2, 32:1, 32:0, 34:4, 34:3, 34:2, 34:1, 35:4?, 35:3?, 35:2?, 35:1?, 36:7, 36:6, 36:5, 36:4, 36:3, 36:2, 36:1, 37:4?, 37:3
PS	34:4, 34:3, 34:2, 34:1, 35:3?, 36:6, 36:5, 36:4, 36:3, 36:2, 36:1, 38:6, 38:5, 38:4, 38:3, 38:2, 38:1, 40:4, 40:3, 40:2, 40:1, 42:4?, 42:3, 42:2, 42:1, 44:3
DGDG	34:6, 34:5, 34:4, 34:3, 34:2, 34:1, 35:3?, 35:2?, 36:7, 36:6, 36:5, 36:4, 36:3, 36:2, 36:1, 37:3?, 38:7, 38:6, 38:5, 38:4, 38:3
MGDG	34:6, 34:5, 34:4, 34:3, 34:2, 34:1, 36:6, 36:7, 36:5, 36:4, 36:3, 36:2, 36:1, 38:6, 38:5, 38:4, 38:3

7.3.9 Statistical Analysis

All the experimental data was checked for normality, independence, and constant variance prior to statistical analyses using SAS. 9.4 (SAS Institute, Cary, NC). Five replicates were used for determining the polar lipids, FA, fluorescence, and MII. The experiment was designed as a 4 x 5 factorial with 4 (genotypes) x 5 (sampling times), but due to the repeated measuring, the independence assumption on the error terms required for analysis of variance of a factorial model was unlikely to be met. The appropriate assumptions on the error terms for these responses were normal distribution, constant variance, and a covariance structure of Є. The appropriate Akaike's information criterion (AIC) and Bayesian Criterion (BIC) were used to determine the best covariant structure, which was compound symmetry (Littell et al. 1998). The Mixed procedure in SAS software was used for monthly analysis. A software based macro was used to separated means upon significant differences at alpha = 0.05 and Tukey's procedure. NAC, PNA, and ADWU taken on the opposite samples in the laboratory were subjected to ANOVA. Scatterplots were examined of these parameters versus FA, fluorescence, NAC and PNA to identify relationships between them. If linearity was established, correlation analysis was performed using Minitab 18 Statistical Software.

7.4 RESULTS

7.4.1 Needle Abscission

There was no interaction effect between day sampled and genotype, but there was a significant difference between genotypes ($p < 0.001$) for both NAC and PNA in each sampling time. This suggests that there was a difference in abscission resistance in the four clones, but no clones benefited more from cold acclimation than the others. Clones 9, 37, 506, and 566 had mean NAC of 53, 42, 75, and 33 days and mean PNA of 58, 47, 80, and 36 days, respectively (Fig. 29). Clone 506 commenced needle abscission and reached peak abscission significantly later than the other 3 genotypes, identifying it as the highest NRD clone. All genotypes commenced needle abscission significantly earlier in the last sampling period in February, suggesting a response to temperatures above 9°C that occurred for 3

consecutive days in mid-January bringing the average maximum temperature that year to 4.4°C for January, where the thirty year average maximum temperature in Debert, NS is -1°C.

7.4.2 Confirming Cold Acclimation

Membrane injury was reported as MII and changed significantly due to sampling time ($p < 0.001$). There was however no effect on MII due to genotype or an interaction between sampling time and genotype. MII was relatively high in September and October with an average MII of 6.2, but decreased significantly in November to 3.7 (40% decrease), meaning that the membranes increased their stability. There was another significant decrease in MII on the February 5 sampling (Fig. 30 A).

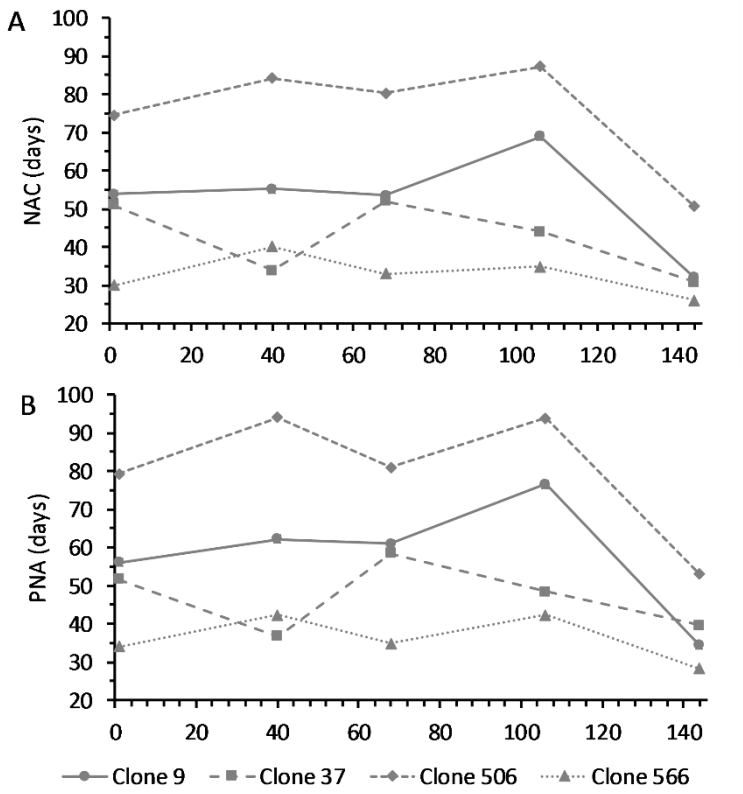

Figure 29. The length of time in days from harvest until A) needle abscission commencement (NAC) and B) peak needle abscission (PNA) for balsam fir branches. Mean values were calculated from 5 replicates and displayed for each of the sampling dates for each of the four genotypes used. Data points correspond to day 1 (Sept. 18), day 40 (Oct. 28), day 68 (Nov. 25), day 106 (Dec. 30), and day 144 (Feb. 4).

Chlorophyll fluorescence was reported as Fv/Fm and changed significantly due to sampling month ($p < 0.001$). There was no interaction effect on Fv/Fm due to genotype and sampling time. Overall, the trend in Fv/Fm coincided strongly with changes in photoperiod through the study ($R^2 = 0.52$) and thus, decreased from 0.686 in September down to 0.460 in December. The Fv/Fm significantly increased beginning in February (Fig. 30 B), suggesting the chloroplasts beginning to regain activity for spring.

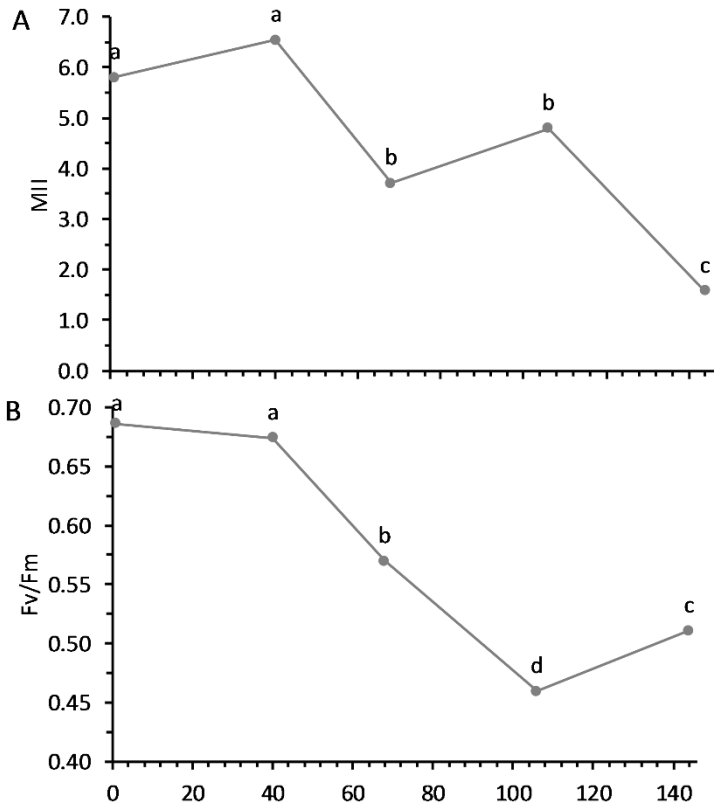

Figure 30. A) Membrane injury index (MII) and chlorophyll fluorescence (Fv/Fm) of freshly harvested balsam fir branches. Samples were pooled so that mean were calculated from 20 replicates due to no significant interaction effect with genotypes. Means with a different letter are significantly different as determined with Fisher's least significant difference mean comparison ($\alpha = 0.05$). Data points correspond to day 1 (Sept. 18), day 40 (Oct. 28), day 68 (Nov. 25), day 106 (Dec. 30), and day 144 (Feb. 4).

7.4.3 Lipid Classes and Species

There were approximately 200 lipid molecular species identified in the lipid classes. MGDG and DGDG, chloroplast GL, composed 65-70 % of all polar lipids, with PC contributing 15-20% (Table 17) GL. The major molecular species for PC are 34:3, 36:5, and 36:4. The major molecular species for MGDG are 36:5, 36:6 and 36:7. The major ones for DGDG are 35:3, 36:3, 35:5 and 36:6.

Table 17. Comparison of lipid classes by percentage of total lipids for balsam fir branches harvested at five different months. Branch samples were comprised of 4 different genotypes, but there was no interaction between harvest month and genotype so samples sizes could be pooled that n = 20. Values are expressed as mean ± standard error. The P-value denotes whether there was a significant difference in each class in at least one of the sampling dates.

Lipid Class	Day 1	Day 40	Day 68	Day 106	Day 144	P-value
DGDG	26.87 ± 0.36	27.10 ± 0.43	28.33 ± 0.38	30.04 ± 0.37	30.91 ± 0.35	< 0.001
MGDG	45.90 ± 0.87	40.98 ± 1.17	38.37 ± 0.81	33.86 ± 0.58	33.89 ± 0.52	< 0.001
PC	15.01 ± 0.39	18.12 ± 0.61	18.48 ± 0.44	19.56 ± 0.40	19.07 ± 0.46	< 0.001
PG	4.04 ± 0.20	3.89 ± 0.16	3.81 ± 0.15	5.11 ± 0.15	5.27 ± 0.21	< 0.001
PE	2.62 ± 0.17	3.74 ± 0.18	3.75 ± 0.21	4.76 ± 0.24	3.87 ± 0.22	< 0.001
PI	3.86 ± 0.15	3.99 ± 0.16	4.64 ± 0.17	4.75 ± 0.11	4.97 ± 0.22	< 0.001
PA	0.47 ± 0.06	0.93 ± 0.09	1.19 ± 0.34	0.66 ± 0.05	0.98 ± 0.14	= 0.049
LPC	0.05 ± 0.01	0.12 ± 0.02	0.14 ± 0.01	0.09 ± 0.01	0.08 ± 0.01	= 0.041
LPG	1.05 ± 0.19	0.96 ± 0.15	1.14 ± 0.15	1.00 ± 0.20	0.82 ± 0.18	= 0.344
LPE	0.11 ± 0.01	0.15 ± 0.01	0.14 ± 0.01	0.14 ± 0.01	0.12 ± 0.01	= 0.071

7.4.4 Seasonal Changes in Lipid Composition

There was no significant loss of mean total lipids during cold acclimation, but a redistribution of lipid classes was observed (Table 17). As for GL, DGDG significantly increased by 13% and MGDG decreased by 35%. There were significant changes ($p < 0.05$) in four lipid species causing the change in the ratio: DGDG 36:6, MGDG 36:5; MGDG 36:6, and MGDG 36:7, the first two significantly increasing and the latter two

decreasing. PC (27%), PE (45%) and PI (22%) significantly increased, and there were marginally significant increases in PA (52%) and LPC (44%).

A decrease in the MGDG:DGDG (M/D) and a decrease galactolipid:phospholipid (GL:PL) ratios are indicators of membrane stabilization (Fig. 31). There was a significant decrease in the mean M/D ratio, as well as a significant but more gradual decrease in the GL:PL ratio, suggesting increasing stability in the chloroplast with more cold acclimation. Both ratios levelled off in the December and February sampling periods. Mean M/D ratio show the highest significant correlation ($r \geq 0.8$) to CDD and T_{min}.

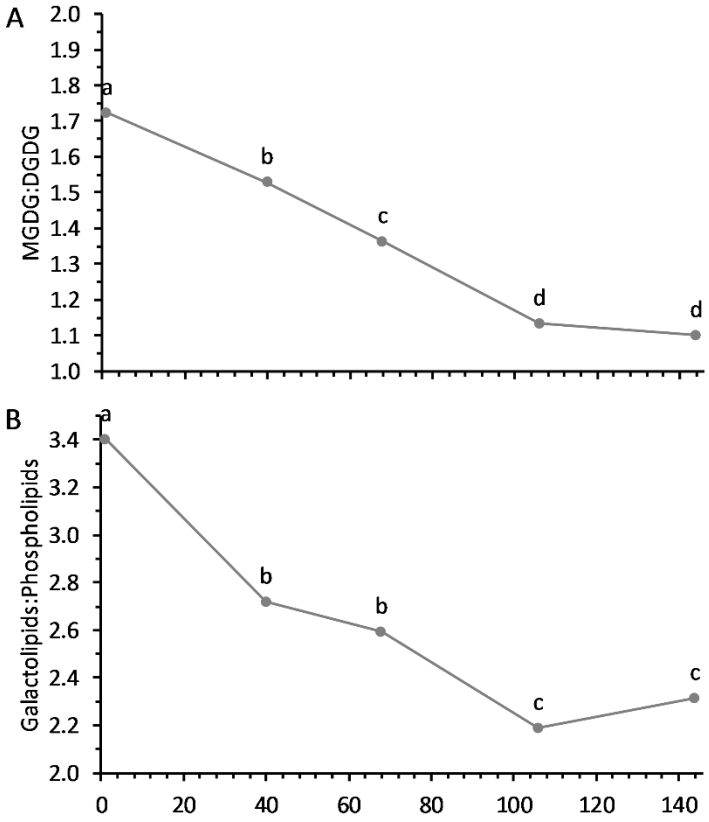

Figure 31. A comparison of the ratio of A) MGDG to DGDG and B) GL: PL in balsam for branches over five different harvest periods. Mean values were calculated from 20 replicates. Means with a different letter are significantly different as determined with Fisher's least significant difference mean comparison (α = 0.05). Data points correspond to day 1 (Sept. 18), day 40 (Oct. 28), day 68 (Nov. 25), day 106 (Dec. 30), and day 144 (Feb. 4).

7.4.5 Genotypic Changes in Lipid Composition

As previously stated, the needles on Clone 506 lasted longer postharvest, when harvested at each sampling period between September and February the next year. Therefore, any differences seen between Clone 506 and the other clones could provide information to explain why this clone has a higher NRD postharvest. Figure 32 shows four lipid classes that vary significantly between Clone 506 and some of the others with poorer NAC. There is significantly less DGDG in Clone 506 than in Clone 9. PC and PI are significantly less in Clone 506 than in Clones 9 and 37. LPG is significantly higher in 506 and 566. There is no one lipid class unique to just Clone 506. Lipid species that vary significantly from Clone 506 are shown in Table 18. PC 36:3, PC 37:3, and 38:5 are significantly less in Clone 506 than all other Clones. PG 35:3 and PG 35:2 are less in Clones 506 and 37 than 9 and 566. LPG 16:1 is eight times as much in Clone 506 than some of the poorer clones, although only slightly more than 566.

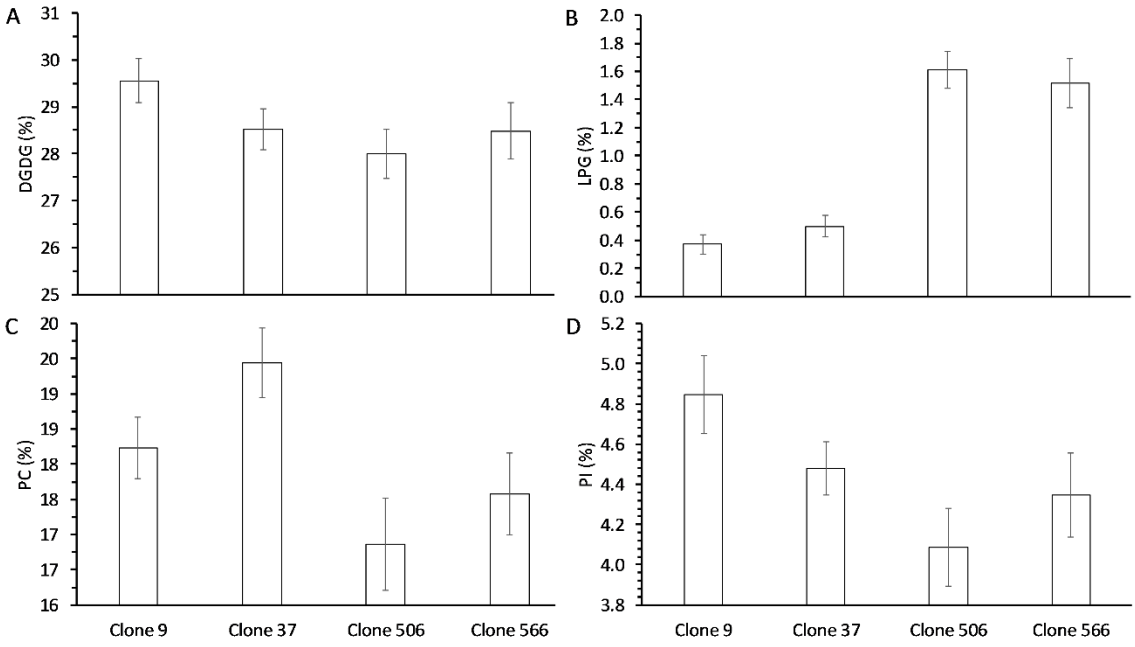

Figure 32. A comparison of the relative contribution of four different lipid classes to the overall lipid composition in four different balsam fir genotypes. Clone 506 was identified as having significantly higher needle retention. Means were calculated from 25 replicates and error bars represent standard error.

Table 18. Comparison of specific lipids (by percentage of total lipids) for balsam fir branches harvested from 4 different genotypes. The 6 lipids shown were chosen because they were the only lipids where the high needle retainer (Clone 506) had significantly different amounts ($P < 0.001$). Values are expressed as mean ± standard error.

Lipid	Clone 9	Clone 37	Clone 506	Clone 566
PC (36:3)	1.94 ± 0.07	2.10 ± 0.07	1.69 ± 0.07	1.89 ± 0.07
PC (37:3)	0.36 ± 0.01	0.40 ± 0.01	0.32 ± 0.01	0.41 ± 0.02
PC (38:5)	1.02 ± 0.03	1.16 ± 0.04	0.89 ± 0.03	0.98 ± 0.05
PG (35:3)	0.27 ± 0.02	0.25 ± 0.02	0.22 ± 0.02	0.27 ± 0.02
PG (35:2)	0.23 ± 0.02	0.20 ± 0.02	0.17 ± 0.02	0.24 ± 0.02
LPG (16:1)	0.18 ± 0.03	0.14 ± 0.03	1.25 ± 0.11	0.99 ± 0.11

7.5 Discussion

7.5.1 Needle Abscission and Cold Acclimation

There was no advantage of cold acclimation on postharvest needle retention; Clone 506 can be considered a high NAR clone and the others moderate. MacDonald et al., (2014b) found that high and moderate NAR clones do not necessarily benefit from cold acclimation, which may explain the lack of retention improvement in this study. Two clones were found to be low NAR varieties in other studies, but temperatures and moisture prior to harvest can alter needle retention (Thiagarajan and Lada, 2010, MacDonald and Lada, 2014; MacDonald et al. 2014a).

In February, all 4 clones showed a significant decrease in needle retention postharvest. In the month of January leading up to the sampling day in early February, there was an extended thaw from January 12th to 21st, some days reaching a high of 9.5°C. The reduced needle retention postharvest may be due to the broken winter dormancy negatively affecting the postharvest quality of the fir (Lizottte, 2015).

MII significantly decreased as a result of cold acclimation. Membrane injury was reduced by November, and decreased more between the end of December and the February sampling. Cold acclimation is known to reduce membrane leakage during cool temperatures (Thiagarajan et al., 2016), so this result was not surprising. However, the January thaw caused a significant decrease in NAR postharvest, so one might have suspected an increase in membrane injury after the time of the thaw. According to van Meer et al. (2008), phase transitions are very slow. It is possible that changes in membrane integrity had been initiated and signaling for abscission had occurred due to break in dormancy, and that is why NAR was significantly lower by the time of the February sampling.

Fluorescence also decreased significantly due to cold acclimation. This was noted on November 25th after >100 CDD and 28 days below 0°C. It is known that there is a photoperiod dependent photosynthetic reduction in conifers before the severe winter temperatures set in (Levitt, 1980; Thiagarajan and Lada, 2010; Sofronova et al., 2018). This is possibly due to down-regulating of photosynthesis in balsam fir during the winter months as this occurs in many conifers in the northern hemisphere. Due to increased environmental stress of overwintering of conifers, chlorophyll is reduced and carotenoids increase. The chlorophyll/carotenoid ratio decreases in the winter which may be an indicator of lowering photosynthetic activity. Carotenoids have anti-oxidative capacities that provide photoprotection for needles in the winter (Gamon et al., 2016). Chlorophyll fluorescence significantly decreased between October and November sampling dates, lending further support to cold acclimation of balsam fir. However, a strong link to photoperiod also exists which explains the increase between activity in the chloroplast between December 30th and February 5th.

7.5.2 Seasonal Changes in Lipid Composition

Seasonal changes in lipid profiles have been studied extensively in species such as *Arabidopsis*, but there have been few studies of lipids in conifers and no information on balsam fir. There are fewer major contributing species of polar lipids in balsam fir needles

than in *Arabidopsis* rosettes (MacDonald et al., 2015; Welti et al., 2002). GL are the most abundant lipid classes in balsam fir needles, similar to leaves in other species. It has been found that chloroplasts have abundant GL. It is not uncommon for chloroplast to have up to 80% GL (Dörmann and Benning, 2002). In this study, GL composed 65-70 per cent of all polar lipids. The other major contributor was PC and to a lesser extent PG. In a study done by MacDonald et al. (2017), one moderate NAR clone (706) was sampled for polar lipids from the Debert clonal orchard, sampling done in December. There was 33 per cent MGDG and 28 per cent DGDG in that study compared to December sampling in the current study of 33 to 30 resulting in a very similar M/D. This ratio is higher than reported in most plants, but that could be due to the fact that both of these were subjected to cold acclimation (Welti et al., 2002).

Few studies have elected to use lipidomics for cold acclimation analysis. Lipidomics provides greater detail, allowing identification of many minor lipid species, such as PA, PI, and lysophospholipids, which could increase during cooling and freezing temperatures if plants are not resistant due to PLD enzymatic activity (Welti et al., 2002). The marginally significant increase in PA and LPC is not enough to indicate a degradation problem. For instance, some studies with evidence of PLD activity have shown an increase from below 1 to 12 % in a short time, though this seldom occurs in plants adapted for cold climates (Welti et al., 2002). LPC can be a sign of degradation of PC but in our study, PC increases during cold acclimation. PI and its isomers are signaling lipids and the significant increase of PI could be important if there is a subsequent decrease in a structural lipid. The most researched pathway is the IP_3 (inositol triphosphate) signal transduction pathway, IP_3 has been implicated in stomatal closure and ABA expression (Burnette et al., 2003). There is evidence that ABA also plays a role in cold acclimation (Thiagarajan, 2012).

Studies have shown that MGDG, DGDG, sulfoquinovosyl diacylglycerol, and PG are all involved in maintaining the thylakoid structure and for the proper functioning of photosystem II and related proteins (Moellering et al., 2010). Therefore, changes in GL and PG are expected during cold acclimation. MGDG and DGDG are also known to stabilize the photosystem protein complexes in chloroplasts (Kobayashi 2016).

Maintaining a constant MGDG/DGDG ratio in thylakoid membranes, at least under standard growth conditions, seems crucial for the stability and functional integrity of photosynthetic membranes (Dörmann and Benning, 2002). An increase in the bilayer forming GL, DGDG, and a decrease in the monolayer forming MGDG during cold acclimation should help protect the chloroplast membranes from damage during the winter keeping the membrane more fluid. Due to the reduction of light and colder temperatures associated with winter, chloroplast membrane stabilization becomes ultimately important for survival. The ratio of MGDG to DGDG may vary according to the length of cold acclimation, as the biosynthesis of these compounds is tightly regulated to meet the needs of the cell under changing environmental conditions (Welti et al., 2002). MGDG content decreased more than DGDG in this study. This can be explained by the fact that it takes two MGDG to form one DGDG. The transition of MGDG to DGDG to maintain the appropriate ratio, which allows the reversible transition from the hexagonal II to lamellar α phase of the lipid bilayer, could be a very important factor in thylakoid biogenesis (Rocha et al., 2018).

The seasonal changes in balsam fir GL are consistent with those found by Öquist (1982) in *Pinus sylvestris*. MGDG decreased during the winter months and increased in the spring. DGDG increased during cold acclimation. Uremus and Stepkonpus (1997) performed a study by isolating the membranes of the chloroplast of rye leaves. They found that MGDG decreased as a result of cold acclimation, and that DGDG increased. This was to ensure better stability of the chloroplast to adapt to cold temperatures. Welti et al. (2002) found similar results, but there was a greater decrease in the MGDG:DGDG ratio as a result of freezing temperatures in PLD deficient *Arabidopsis*.

An increase in the per cent PC during cold acclimation has been reported in two pine species, *Pinus silvestris* and *Pinus nigra* (Öquist, 1992; Kojjma, 1990). As MGDG combines to form DGDG, the per cent of GL goes down, and PL goes up. Primarily, the PL increase is caused by PC percentage increase, but to a lesser extent PE. PI and PA have been identified to play a role in cell signaling (van Meer et al., 2006). PA is closely related to an increased activity of PLD. PA increase almost immediately in this cold acclimation

study but the amounts are not great. Some studies with evidence of PLD activity have shown an increase from below 1 to 12 % in PA in a short time (Welti et al., 2002). The increase in PA in this study is within the acceptable limits. PI, although significantly increased, it is found in very minute amounts so may not be important physiologically to explain either cold acclimation or cold acclimation induced needle retention.

7.5.3 Genotypic Changes in Lipid Composition

Clone 506, the top NAR clone postharvest, has significantly less DGDG and PC than at least one of the poorer NAR clones. It has been said that high NAR clones do not benefit as much from cold acclimation, so one could speculate that this could be related to this fact (MacDonald and Lada, 2008). DGDG is only one chloroplast membrane stabilizing molecules. There has been reference to MGDG/ DGDG + PG + SQDG in some studies, and in addition the amount of sterols can be important (Henrickson et al., 2006). It is possible that there is another mechanism at work in the high NAR clone for membrane stabilization that also benefits this clone postharvest.

A deficit in PC 36:3, PC 37:3, PC38:5, PG 35:3, PG 35:2 in the high NAR Clone 506 compared to the poorer clones could reveal a link or association to higher postharvest needle retention in Clone 506 balsam fir with more studies. LPG (16:1), however, is a signaling molecule, so the fact that it is significantly greater in Clone 506 could be important. Long term chilling in barley resulted in a two-folds increase in LPG with a decrease in LPG (16:1), so there is a possibility of some yet unidentified link between elevated LPG 16:1 and postharvest abscission in balsam fir (Margutti et al., 2019).

7.6 CONCLUSION

There is a redistribution of polar lipids during cold acclimation in all genotypes of balsam fir tested. The polar lipid MGDG decreased during the fall and early winter with a subsequent increase in DGDG and a significant decrease in the MGDG: DGDG ratio. There was a significant decrease also in the GL: PL ratio, influenced by the increase in PC, PE, and PG. There were some notable significant differences between the high NAR clone,

506, and the clones with poorer NAR. There is significantly less DGDG in Clone 506 than in Clone 9. PC and PI were significantly less in Clone 506 than in Clones 9 and 37. LPG was significantly higher in 506 and 566. There is no one lipid class unique to just clone 506. PC 36:3, PC 37:3, and 38:5 are significantly less in Clone 506 than all other clones. PG 35:3 and PG 35:2 are less in Clones 506 and 37 than 9 and 566. LPG 16:1 is eight times as much in Clone 506 than some of the poorer clones, although only slightly more than Clone 566. This data could potentially be linked to greater needle retention postharvest in high NAR clones. However, more polar lipid studies using many genotypes of various NAR clones and their performance postharvest are needed to verify a link between one of these differences and postharvest abscission in balsam fir.

CHAPTER 8 - LINKING CHANGES IN FATTY ACID COMPOSITION TO GENOTYPES DURING COLD ACCLIMATION IN BALSAM FIR NEEDLES

8.1 PREFACE

MacDonald GE, Lada RR, Caldwell CD, Udenigwe C, MacDonald M. 2019. Linking changes in fatty acid composition to genotypes during cold acclimation in balsam fir needles.

This work was presented at the Annual Conference of the American Society for Horticultural Science in Las Vegas, Nevada (2019).

8.2 INTRODUCTION

In Nova Scotia, balsam fir Christmas trees are being harvested earlier and earlier in the autumn to meet the increasing export market. There is also evidence that fall temperatures are rising in Nova Scotia (Statistics Canada, 2017). These two facts combined are causing premature postharvest needle abscission in Christmas trees. This could be due to incomplete cold hardening, one of the best known plant temperature responses to increasingly colder temperatures (Thiagarajan, 2012; MacDonald et al., 2014b). In nature, low temperatures in fall and early winter cause cold acclimation, the main trigger in plants being temperature, but also reduced light and shortening photoperiod play a role (Odlum and Blake, 1996, Greer et al., 2000). Benefits have been seen in the postharvest quality of balsam fir due to cold acclimation. When trees are harvested in October versus November and December in NS for export, they are not receiving the full benefit of cold acclimation and lose their needles more readily after cutting (Thiagarajan and Lada, 2010; Thiagarajan, 2012; MacDonald et al., 2014b).

Differences in needle retention after cold acclimation are not consistent with every genotype. It has been found that some trees with low needle retention duration (NRD) benefit greatly from cold acclimation postharvest, more so than those with high NRD (MacDonald and Lada, 2015). Most farmers in Nova Scotia have a mixture of genotypes

and would like all of these to be suitable for market in October. NRD is not a problem when a cool temperature and a low vapor pressure deficit can be maintained in the outdoor Christmas tree lot, the problem occurs during uncontrolled shipping to warmer climates and also when trees are brought indoors, as is the popular Christmas tradition (MacDonald and Lada, 2012, 2015). It is possible that when harvested early low NRD clones do not have good membrane integrity, but after cold acclimation these trees benefit from a greater unsaturation of FA in their cellular membranes which also helps to maintain integrity for some time after harvest.

In eukaryotes, FA are important part of cellular membranes in amphiphilic lipids and influence the integrity of all cellular membranes (Quehenberger et al., 2011; Harayama and Reizman, 2018). It has been established that there is a decrease in FA unsaturation postharvest, with low NRD clones showing decline in USFA and loss of membrane integrity sooner than high NRD clones (MacDonald et al., 2015; MacDonald et al., 2017). This could be related to the state of stability of the membranes prior to harvest or the action of a FA signaling molecule. In other frost resistant plants, it has been observed that during cold acclimation that USFA have been known to increase to help maintain membrane integrity and for proper protein function to survive the harsh winter. In balsam fir, this phenomenon could be related to genotypic differences in balsam fir clones. We know from the previous studies that cold acclimation leads to a redistribution of lipids (MacDonald et al., 2015), but subtle overall FA changes are not detectable. It is the objective of this research to establish an understanding of the FA profiles in four clones of balsam fir that differ in their NAR and correlate the changes in FA profiles with cold acclimation. Specifically the objective is to quantify all FA, both FFA that can be powerful signaling molecules, as well as FA present in complex lipids. This analysis will reveal chain length, number and position of double bonds, properties that influence membrane fluidity. In addition the purpose of this study is to identify FA changes linked to genotype or to sampling time. The hypothesis is that there will be an increase in FA desaturation during cold acclimation and genotypic differences as well.

8.3 Materials and Methods

8.3.1 Sampling and Experimental Set Up

The plant material for this investigation was collected from the Debert tree orchard (45 44' N, -63 50' W) located in Debert, NS, Canada from September to February. The Christmas tree germplasm collection has over 220 genotypes of balsam fir. For this study 4 genotypes, Clones 37, 506, 9 and 566, were chosen. In previous studies, the first two of these genotypes exhibited a long NRD when harvested in October and kept in unhydrated conditions, whereas the latter two exhibited short NRD. The weather parameters for this location were collected from the Environment Canada Weather Station located within a 2 km radius of the orchard. The monthly average photoperiod was also obtained from this website.

Shoots with 2 years of growth were used for this study. They were cut from approximately 20 year-old trees at an elevation of approximately 1 m from the ground level on the south facing side of the trees. Five trips were made to the Debert orchard between September and February, 2014. Sampling of all 4 genotypes took place roughly every 5-6 weeks from onsite. After each harvest, the branches (5 replicates per genotype per sampling period) were immediately frozen in liquid nitrogen, and stored at –80 °C until lipid extraction. In addition, samples taken onsite were subjected to testing for chlorophyll index (fluorescence), and membrane injury (MII) in the laboratory.

Opposite branches of each frozen sample were cut, hydrated and transported to the laboratory and kept hydrated in the laboratory in conditions. This was to determine NAC and PNA postharvest, with the assumption that they would exhibit needle loss similarly to the branches collected FA analysis. Once in the laboratory, all branches were given a fresh cut 2.5 cm above the previous cut while stem ends were under water to reduce risk of cavitation, weighed, and then placed in a 100 mL amber bottle filled with 100 mL of distilled water. The neck of each flask was plugged with cotton wool to prevent direct water evaporation and provide added stability to a branch. The branch and entire apparatus were weighed. The branches were kept at an average temperature of 20-24 °C with a light

intensity of 85 - 95 μmol·m^{-2}·s^{-1} supplied by fluorescent lights. The response variable measured every two days was NL, needle loss.

8.3.2 Needle Abscission

Needle abscission was measured by collecting the number of needles that would fall after a 'finger run' test each day (Mitcham-Butler et al., 1988; MacDonald et al., 2014a). The mass of those needles was measured fresh and after oven drying. The experiment was run until all branches reached peak abscission. Typical postharvest needle abscission follows a sigmoidal curve, and two time points were used to compare needle abscission patterns: NAC, PNA. NAC was defined as the point where postharvest abscission began (1% needle loss), and PNA was defined as the day which had the highest rate of needle abscission (Lada and MacDonald, 2015).

8.3.3 Fatty Acid Extraction

The goal was to observe FA changes in balsam fir needles in different genotypes throughout cold acclimation, whole needles attached to the branch when sampled were used for lipid extraction and FA analysis. Needles for analysis were frozen in liquid N to halt metabolic processes and then stored at -80°C until analysis. Percentage needle loss data were used to determine the time points at which to analyze needles for FA. Samples from December 21st were the fresh samples. Samples from Feb. 2nd (day 42) were chosen as it was just prior to commencement of abscission. Finally, samples from Mar. 15th (day 84) were chosen as branches were at their peak abscission rate.

All FA were extracted at the Dalhousie University Agricultural Campus using an protocol adapted from *Arabidopsis* leaf tissue (Bligh and Dwyer, 1959). For extraction, 1 g of frozen needle were cut into smaller pieces and incubated in 1 mL of isopropanol with 0.01% BHT at 75 °C for 15 min. Next, 1.5 mL of chloroform and 0.6 mL of water were added. After shaking for 1 h at room temperature, the solvent was transferred to a new glass tube with a Teflon-lined screw-cap using a Pasteur pipette, and total 0.7 mL chloroform: methanol (2:1) was added and shaken for 30 min. The extraction was completed by adding 4 mL of

chloroform: methanol (2:1) ten times and collecting the solvent. The solvent extracts were washed once with 1 mL KCl (1.0 M) and once with 0.66 mL water. The solvent was evaporated under nitrogen and the lipid extract was quantified and dissolved in 1 mL chloroform. The tissues after lipid extraction were dried in an oven at 105°C and dry weights were determined (3–20 mg).

8.3.4 Fatty Acid Analysis

FAME were prepared using methanolic hydrochloric acid according to Christie (1982). FAME were dissolved in 100–200 µL of hexane and placed in 2 mL gas chromatography (GC) vials with inserts. FAME were quantified using gas chromatography GC-FID analysis performed at the Kansas Lipdomic Center, Kansas State University, on an Agilent Technologies 6890N Network GC system equipped with a HP-88 capillary column (100 m × 0.25 mm I.D., 0.20 µm film thickness) coupled with a FID. Injector and FID temperatures were set at 275 and 260°C, respectively. The carrier gas (Helium) pressure was 51.61 psi with a flow rate of 1.6 mLmin^{-1}min with continuous flow. For the detector, hydrogen flow rate was 30.0 mL.min^{-1}, air flow rate was 400 mL/min, and makeup flow rate (Helium) was 25 mL.min^{-1}. The GC oven temperature ramp was operated as follows, initial temperature of 150 °C held for 1min, increased at 10 °C/min to 175 °C, held 10 min then increased at 5 °C/min to 210°C, and held for 5 min. At last, temperature was increased at 5 °C$min^{-1}$to a final temperature of 230 °C, held 15 min. Total run time was 44.5 min. The sampling rate of the FID was 20 Hz. The FAME solutions were injected in the volume of 1 µL using an autosampler Agilent Technologies 7683 Series Injector in the splitless mode. The chromatograph worked under Agilent Technologies Enhanced Chemstation software. The FA were identified by comparison of RRT of the compounds in the sample with RRT of Supelco 37 component FA methyl ester mix standards. The moles of FA were calculated by the moles of internal standard (pentadecanoic acid, 15:0) and peaks' area.

8.3.5 Q-test

Average CoV for lipid analytes is a function of corrections used in data processing; CoV is equal to the standard deviation of the measurements for each analyte divided by the average. CoV was calculated from the QC samples, without any correction, using only the linear trend correction within each day's sample set, only the correction to the overall average across sample sets, or both corrections (as done on the experimental data).

8.3.6 Statistical Analysis

All the experimental data was checked for normality, independence, and constant variance prior to statistical analyses using SAS® (SAS Institute). Five replicates were used for determining the parameters of onsite samples: FA, fluorescence, and MII. The experiment was designed as a 4 x 5 factorial with 4 (genotypes) x 5 (sampling times), but due to the repeated measuring, the independence assumption on the error terms required for analysis of variance of a factorial model was unlikely to be met. The appropriate assumptions on the error terms for these responses were normal distribution, constant variance, and a covariance structure of compound symmetry; as determined by the appropriate Akaike's information criterion (AIC) and Bayesian Criterion (BIC). The Mixed procedure in SAS software was used for monthly analysis. A software based macro was used to separate means upon significant differences at alpha = 0.05 and Tukey's procedure.

8.4 RESULTS

8.4.1 FA Profiles in September

The clone with significantly superior NRD ($p < 0.001$) over the rest was Clone 506. All four clones contained the same 20 FA. The USFA present in the highest percentage were as follows: in all genotypes 18:3n3 (α – linolenic acid), 18:2cis (linoleic acid), and 18:1cis (oleic acid) were the predominant USFA and the 16:0 was the main saturated FA. The mean % FA are presented in Table 19 showing the FA composition of the four clones on September 18th and the results of ANOVA showing significant differences between clones at the beginning of the experiment. By that time there had been only 4 nights between 0

and 5°C and three below 0°C. Several significant differences were noted in the means of some FA in the superior NRD clone (506) setting it apart from some of the other clones. Clone 506 had a mean % of 16:0 higher than Clones 9 and 566. It also had higher mean 7 – 16:1 and lower 18:2cis. The mean of the most plentiful FA, 18:3n3, was higher than the mean of both 9 and 566. However, the mean of 5,9,12 – 18:3 was significantly lower in Clone 506 than in 9 or 566. Clone 506 was lower in mean 20:0 than Clones 9 and 566, lower than all others in mean 20:2. Mean 20:3 was lower in Clone 506 than in Clones 37 or 566, and mean 22:0 was lower in Clone 506 than 37.

Δ5-polymethylene-interrupted FA (Δ5-UPIFA) were found in the needles. Δ5-UPIFA include 5,9-18:2 (taxoleic), 5,9,12-18:3 (pinolenic), 5,9,12,15-18:4 (coniferonic), 5,11,14,17-20:4 (juniperonic), and 5,11,14-20:3 (sciadonic). These together made up approximately 20% of the total FA in the needle. There was no overall difference in total Δ5-UPIFA between clones, however significant differences were present in the means of individual ones (Table 19). There is no 16:3 in any clones, a characteristic of plants using the prokaryotic pathway for lipid synthesis. There was 17:0 in minute amounts, which has been found in small quantities in conifer seed oils and identified as 14-methylhexadecanoic (anteiso-17:0) acid.

Table 19. Mean per cent FA present in balsam fir needles from four different genotypes of balsam fir collected initially on September 18th to show the FA profile at the beginning of the experiment. Horizontal letter groupings on the chart show significant differences between genotypes. Clone **506** – high NRD.

FA	Clones				P-*value*
	37	9	566	**506**	
14:0	1.6 $_{a}$	1.0 $_{b}$	1.4 $_{ab}$	**1.5** $_{ab}$	*
16:0	11.8 $_{a}$	9.6 $_{b}$	9.0 $_{b}$	**10.9** $_{a}$	*
7-16:1	0.8 $_{b}$	0.7 $_{b}$	0.7 $_{b}$	**1.1** $_{a}$	**
14methyl-16:0	5.2 $_{b}$	5.5 $_{ab}$	6.0 $_{a}$	**4.8** $_{b}$	**
17:0	0.3	0.2	0.3	**0.3**	
18:0	2.8	2.7	2.9	**2.5**	
18:1cis (9-18:1)	12.0	13.5	12.3	**11.1**	
5,9-18:2	2.9	3.1	3.0	**3.2**	
18:2cis (9,12-18:2)	14.5 $_{b}$	16.2 $_{a}$	17.0 $_{a}$	**14.5** $_{b}$	**
5,9,12-18:3	4.9 $_{b}$	6.3 $_{a}$	6.9 $_{a}$	**5.1** $_{b}$	**
20:00	0.5 $_{bc}$	0.8± $_{a\ b}$	1.0 $_{a}$	**0.4** $_{c}$	**
18:3n3 (9,12,15-18:3)	27.1 $_{ab}$	25.3 $_{b}$	24.3 $_{b}$	**30.4** $_{a}$	**
5,9,12,15-18:4	4.0 $_{b}$	5.1 $_{a}$	4.7 $_{ab}$	**4.4** $_{ab}$	**
20:2	0.5 $_{a}$	0.5 $_{a}$	0.4 $_{a}$	**0.3** $_{b}$	**
5,11,14-20:3	4.5 $_{a}$	4.2 $_{ab}$	4.5 $_{a}$	**3.7** $_{b}$	*
22:0	1.5	1.4	1.4	**1.3**	
20:3n3 (11,14,17-20:3)	0.5 $_{a}$	0.2 $_{b}$	0.4 $_{ab}$	**0.4** $_{ab}$	**
5,11,14,17-20:4	2.5$_{a}$	1.9 $_{b}$	1.9 $_{b}$	**2.0** $_{ab}$	*
23:0	0.9	0.8	0.9	**0.8**	
24:0	1.2	1.0	0.9	**1.2**	

* indicates that differences were significant at 5% level

** indicates that differences were significant at 1% level

8.4.2 Genotype and Sampling Date

Genotype had a significant main effect on several mean FA (14:0; 5,9,12,15-18:4; 20:2; 5,11,14-20:3; 22:0; 5,11,14,17-20:4; Table 20). Of particular note was 5, 11, 14-20:3, which was significantly lower in the high needle retaining genotype (506) than any other genotype. Sampling date also had a significant effect on several FA (20:2; 5, 11, 14-20:3; 22:0; Table 21). Both 20:2 and 22:0 increased in per cent throughout the experiment, while 5, 11, 14-20:3 decreased.

Table 20. List of FA in balsam fir with significant differences due to genotype.

FA	9			37			506			566		
14:0	0.982	±	0.038	1.482	±	0.087	1.380	±	0.066	1.262	±	0.058
5,9,12,15-18:4	5.146	±	0.070	4.574	±	0.117	4.629	±	0.090	4.947	±	0.087
20:2	0.535	±	0.014	0.597	±	0.021	0.463	±	0.020	0.478	±	0.018
5,11,14-20:3	4.044	±	0.069	4.334	±	0.079	3.550	±	0.108	4.139	±	0.121
22:0	1.772	±	0.069	1.970	±	0.074	1.661	±	0.055	1.631	±	0.041
5,11,14,17-20:4	1.898	±	0.033	2.203	±	0.083	1.814	±	0.078	1.753	±	0.042

Table 21. List of FA in balsam fir with significant differences due to date.

FA	Day 1	Day 40	Day 68	Day 106	Day 144
20:2	0.420± 0.019	0.560± 0.019	0.544± 0.020	0.539± 0.020	0.532± 0.026
5,11,14-20:3	4.249± 0.111	4.321± 0.120	3.815± 0.104	3.773± 0.136	3.908± 0.107
22:0	1.388± 0.028	1.596± 0.050	1.903± 0.057	1.914± 0.047	2.005± 0.072

8.4.3 Interactions

There were several significant interactive effects to note (Fig. 33, 34), especially between the highest needle retaining clone (506) and one of the lowest needle retaining clones (566). FA 16:0 was lowest in Clone 566 and relatively high in Clone 506, but between days 106 and 144 decreased by 24% in Clone 506 and increased by 20% in Clone 566. Conversely, FA 18:1 increased by 35% in Clone 506 and decreased by 10% in Clone 566 in the same time span. FA 18:2 increased in all clones in the first 68 days, then decreased in all clones with the exception of Clone 506. Other key interactions include a decrease in 5, 9, 12-18:3 in Clone 566 while all other clones increased and an increase in 18:3n3 in Clone 566 while all other clones decreased.

8.4.4 Correlations

Each month, when the opposite branches were kept in the laboratory to measure needle retention, several FA present when sampling took placer were significantly correlated with needle retention (Fig. 35). FA 16:0, 7-16:1, 18:3n3, 20:3n3, and 24:0 were positively correlated, meaning that higher concentrations were associated with better needle retention. FA 14-methyl-16:0, 18:1 cis, 5,9,12-18:3, 20:0, 5,9,12,15-18:4, and 5,11,14-20:3 were negatively correlated, meaning that higher concentrations were associated with lower

needle retention. Despite being statistically significant, all correlations were relatively weak.

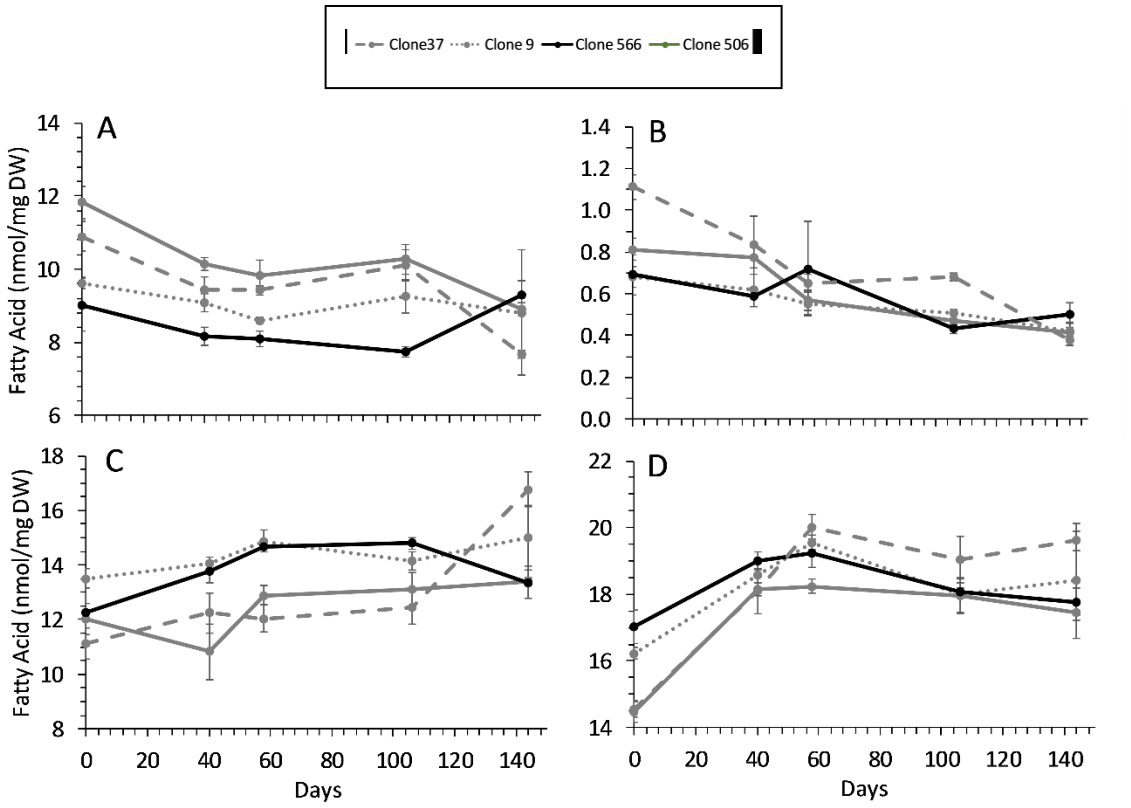

Figure 33. Interactive effects between genotype and collection date for several FA concentrations of balsam fir. A) 16:0; B) 7-16:1; C) 18:1 cis; D) 18:2 cis. Data points correspond to day 1 (Sept. 18), day 40 (Oct. 28), day 68 (Nov. 25), day 106 (Dec. 30), and day 144 (Feb. 4).

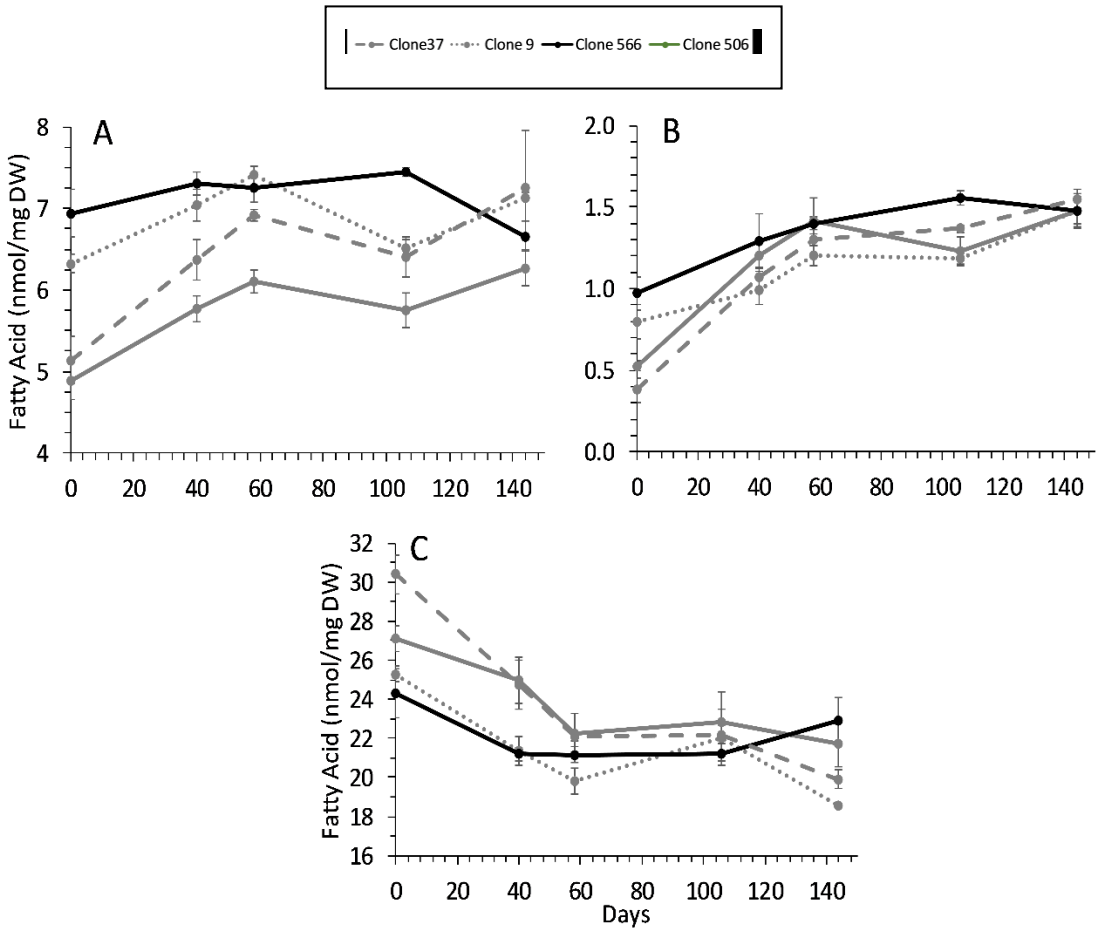

Figure 34. Interactive effects between genotype and collection date for several FA concentrations of balsam fir. A) 5,9,12-18:3; B) 20:0; C) 18:3n3. Data points correspond to day 1 (Sept. 18), day 40 (Oct. 28), day 68 (Nov. 25), day 106 (Dec. 30), and day 144 (Feb. 4).

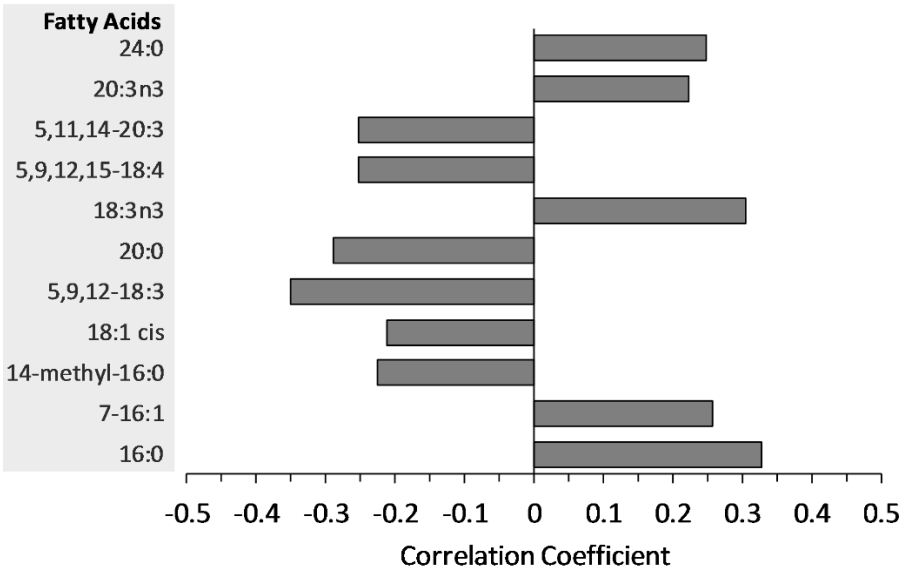

Figure 35. Significant correlation coefficients of FA compared to needle retention in balsam fir.

8.5 Discussion

8.5.1 Fatty Acid Profiles

As discussed in Chapter seven, Clone 506 was found to be the superior clone for needle retention postharvest. All four Clones, 9, 37, 566 and 506 contained the same 20 FA as past studies for balsam fir needles at the Christmas Tree Research Center, Truro, NS (MacDonald et al., 2015, 2017). This profile is unique to balsam fir, as FA can be used as taxonomic markers for species. In 2017, a study compared the FA in four conifer species, larch, Scot's and Korean Pine, and white spruce needles. The FA spectrum for these species consisted of 24 - 25 acids between C14 – C22 as compared to 20 in the balsam fir. The major characteristics that varied in the balsam fir in this current study from the 1997 study by Wolff et al., was in the quantities of SFA. SFA were lower in balsam fir, 23 – 25% as opposed to 30 – 38 % in the other conifers. There was considerably more 18:1 in the balsam fir, a fairly stable FA, 14.5 – 17% in balsam fir as compared to the other conifer species at 3.2 -7.4%.

It is difficult to compare profiles, as they are the result of adaptation to the environment (Wolff et al., 1997). However, any difference in the high NRD clone from the three other

clones in September was of interest to us and the adaptation of this clone could possibly be linked to its superior postharvest quality. There is more 16:0 and 7-16:1 in clone 506 than in the clones with poor NRD. More of the FA 16:0 and 16:1 could be important for oxidative stability of the membranes. Manufacturers of oils will often try to enhance palmitic acid to increase shelf life (Liu et al., 2016). In September, there may be no reason to increase the fluidity of the membranes as the temperature is often quite warm in NS (between 10 – 20 °), so more stable lipids could be more desirable. There was more mean 18:3n3 in the high NRD clone, than in two other low NRD clones, 9 and 566. However, the opposite situation exists with 5, 9, 12–18:3, so these two could balance each other. Other differences exist with FA > 20. FA above 20 are not common in membrane lipids, but do exist in small quantities. These longer chained FA, particularly the unsaturated ones could be the reason why the High NRD clone, 506, is better postharvest. Unsaturated very long chained FA are extremely unstable and prone to oxidation (Yeats and Rose, 2013).
Δ5-UPIFA, previously thought of as rare, are common in the oil and needles of conifers (Makarenko et al., 2014). They were all present in balsam fir needles with predominant Δ5-UPIFA being pinolenic, coniferonic and sciadonic. Together they composed approximately 20% of the FA in the balsam fir needles as in previous studies (MacDonald et al., 2015, 2017). In a chemotaxonomic study done by Wolff et al. in 1997, FA were analyzed in seed oils. Pinoleic acid was found in quantities three times as high as in the balsam fir needles in this present study. Not much is known about the function of these Δ5-UPIFA. It is known that they are a common component of triacylglycerol. This compound is commonly found in seed oils, but not in needles.

8.5.2 Overall Genotype Changes

Although there are several significant differences in genotype during this experiment, the objective of this research was to identify clonal differences that might set a high NRD clone apart from low NRD clones. In that respect, 3, 11, 14 -20:3 is significantly lower in the high NRD clone. This FA does have a significant but weak negative correlation with needle retention meaning that it is generally lower when needle retention is greater. The reason is not clear, but this long chain USFA could add instability to the membranes and thus be linked with poorer needle retention when present in larger quantities.

8.5.3 Changes in Fatty Acid and Sampling Date

FA 20:2n6 content increased significantly between September and October. This USFA is called eicosadienoic acid, an omega – 6 FA. This FA, 20:2n6, is not commonly associated with plants but has been identified in pine nuts (Vanhanen et al., 2017). Usually, if a FA accumulates, then it is being produced at a faster rate than it is metabolized. Eicosdienoic acid is the result of elongation of linoleic acid, 18:2n6, and is metabolized in animals into dihomogamma-linolenic acid, 20:3n6 (Metamatrix Clinical Laboratory, n.d.). The pathway in balsam fir is not clear as there is no 20:3n6 in plants. There is a significant decrease in 5, 11, 14 - 20-3, a Δ5-UPIFA with cold acclimation as well as an increase in 22:0,

8.5.4 Interactive Effects Due to Cold Acclimation

As for significant interactive effects, FA 16:0 was the lowest in Clone 566 and relatively high in Clone 506, but between days 106 and 144 decreased by 24% in Clone 506 and increased by 20% in Clone 566. Palmitic acid is a stable FA, not as susceptible to oxidation (Zheng et al., 2011; Liu et al., 2016). In the high NRD clone, palmitic acid stayed higher than in one of the poorest clones, possibly giving it greater stability which could have affected in turn its postharvest quality. The 24% decrease in 16:0 between the last two sampling dates is interesting as the needle retention postharvest for needles collected on Day 144 was significantly lower for Clone 506, so this could be related. The fact that FA 18:1 increased by 35% in Clone 506 and decreased by 10% in Clone 566, would relate to a faster conversion by the low NRD clone, possibly as an attempt to maintain homeostasis, and could be linked to an "often found" increase in needle retention postharvest (Lada and MacDonald, 2015). FA 18:2 increased in all clones in the first 68 days, then decreased in all clones with the exception of Clone 506. There has been an increase in 18:2 in other studies on cold acclimation, however there are so many factors involved that it is hard to compare. The balsam fir has been successfully adapting to the cold for many years, and could react totally different than an angiosperm (Strimback et al., 2015). As said earlier, when a FA increases at a rate faster than it can be desaturated and elongated into another FA, the quantity will be higher. It seems after 68 days, in all clones except the better clone, conversion must be occurring. Another interactions includes a decrease in 5, 9, 12-18:3 in

Clone 566 while all other clones increased, and an increase in 18:3n3 in Clone 566 while all other clones decreased. This seems to indicate a different metabolic pathway in one of the poorer clones, Clone 566, and a potential role of 5, 9, 12-18:3 during cold acclimation in balsam fir. Metabolic pathways are necessary to maintain homeostasis in a cell and can be dependent on substrates available (Zheng et al., 2011). Most other plants have been reported to increase in 18:3n3 as seen in Clone 566 (Hamada et al., 1998; Kodama et al., 1994). The changes mediating desaturation are not totally understood. We know that it involves the activation of desaturation enzymes, FA desaturase (FAD), but we are not sure how it is activated (Falcone et al., 2004). A study to identify the genes in balsam fir identified the gene for FAD in balsam fir needles (Personal Communication, Sherin Jose, Dalhousie Agricultural Campus, Truro, NS). To confuse things more, the precise roles of FA in chloroplast lipids are not fully understood. Clearly in MacDonald et al., 2015 and 2017, there was a redistribution of membrane lipids involving a change in the ratio of MGDG and DGDG. More research is required to see where the FA fit into this picture.

The most surprising change in FA during cold acclimation was the fact that there was no increase in linolenic acid in most clones. The unsaturated species that significantly increased were linoleic acid, pinoleic acid and eicosodienoic acid. Unsaturation of membranes usually occurs in plants to increase membrane stability in stressful situations such as low temperatures. Since for other plants, there is a reported increase in linolenic acid, it would make sense that there was a different metabolic pathway occurring in the balsam fir during cold acclimation – one that results in an increase in pinolenic acid as opposed to linolenic.

8.6 Conclusions

Clone 506 had significantly better needle retention than all other genotypes in each of the sampling periods. All genotypes commenced needle abscission significantly earlier when collected in February than when collected in September through December, possibly related to a January thaw. The USFA present in the largest proportion was 18:3n3, and the most abundant USFA was 16:0. Δ5-UPIFA composed > 20% of FA in all clones, 5,9-18:2 (taxoleic), 5,9,12-18:3 (pinolenic), 5,9,12,15-18:4 (coniferonic), 5,11,14,17-20:4

(juniperonic), and 5,11,14-20:3. Genotype had a significant main effect on 14:0; 5,9,12,15-18:4; 20:2; 5,11,14-20:3; 22:0; 5,11,14,17-20:4). FA 5, 11, 14-20:3, was significantly lower in the high needle retaining genotype (506) than any other genotype. Date also had a significant effect on 20:2; 5, 11, 14-20:3 and 22:0. Both 20:2 and 22:0 increased in concentration through the experiment, while 5, 11, 14-20:3) decreased. FA 16:0 was lowest in Clone 566 and relatively high in Clone 506, but between days 106 and 144 decreased by 24% in Clone 506 and increased by 20% in Clone 566. Conversely, FA 18:1 increased by 35% in Clone 506 and decreased by 10% in Clone 566 in the same time span. FA 18:2 increased in all clones in the first 68 days, then decreased in all clones with the exception of Clone 506). Other key interactions include a decrease in 5, 9, 12-18:3 in Clone 566 while all other clones increased and an increase in 18:3n3 in Clone 566 while all other clones decreased. This suggests the possibility of different metabolic pathways.

CHAPTER 9 - SCANNING ELECTRON MICROSCOPE (SEM) AND TRANSMISSION ELECTRON MICROSCOPY (TEM) STUDY OF LOW AND HIGH NRD GENOTYPES OF BALSAM FIR NEEDLES POSTHARVEST

9.1 INTRODUCTION

Postharvest lipid and FA changes have been reported in Chapters 5 and 6 of this thesis. Significant postharvest changes have occurred in chloroplast lipids, MGDG and DGDG, in all three clones tested (Chapter 5, 6). There has been a significant reduction in the percentage of MGDG 36:6 and 36:7, a decrease in DGDG 36:6, and a significant decrease in the MGDG/DGDG ratio (MacDonald et al, 2015). MGDG and DGDG are important in the dynamic organization of the thylakoid membranes in the chloroplasts. It is thought that maintaining a constant MGDG to DGDG ratio is crucial to the stability of the chloroplasts (Dörmann and Benning, 2002). Therefore, observed changes in MGDG and DGDG in Chapters 5 and 6 point towards chloroplast breakdown. Reduced chlorophyll fluorescence has also has been confirmed postharvest in many balsam fir studies also suggesting chloroplast breakdown (Lada et al., 2015). In addition to chloroplast degradation, there is evidence of cell membrane loss of integrity leading to ion leakage. This has been related to postharvest abscission in balsam fir (Lada and MacDonald, 2015).

The mechanisms of chloroplast and cell membrane breakdown are not established in balsam fir postharvest. The TEM allows detailed studies of ultrastructural changes within the cell, nuclear alterations, cytoplasmic reorganization, and loss of membrane integrity. In particular, the structural changes in chloroplasts can be observed clearly. The SEM is useful to see surface changes in the cell such as membrane blebbing and blistering (Burattini and Falcieri, 2004). These two microscopic analyses have been chosen as a method of determining cellular changes postharvest in balsam fir. One hypothesis of this study is examination of needles postharvest using SEM and TEM will reveal changes that occur on the surface of the needles such as membrane blabbing, or changes to the stomata, as well as ultrastructural chloroplast or cell membrane deterioration postharvest. It was also hypothesized that that chloroplast changes will occur more rapidly in a low NRD clone

than a high NRD clone. Therefore, the objective of this study was to study the needle surface to observe the condition of the stomata, check for invasion by microorganisms, and membrane blabbing. A further objective was to observe the cell's internal ultrastructure to observe chloroplast and any signs of other cellular damage postharvest. An overall goal was to possibly relate these changes observed using the TEM and SEM to lipid and FA changes postharvest.

9.2 Materials and Methods

9.2.1 Sample Collection

A total of 20 branches, 10 from Clone 506, a high NRD clone, and 566, a low NRD clone were collected from a 16-year-old balsam fir orchard at the Tree Breeding Center, Department of Natural Resources, Debert, Nova Scotia, Canada (lat. 45°25′ N, long. 63°28′ W) in December, 2017. Each branch was cut from 2-year growth at 1.5 m aboveground. These were to determine the abscission curve for each clone to verify that Clone 506 had better needle retention than Clone 566. Extra branches were collected for sampling and sacrifice for fluorescence. The branches were immediately placed in a container with distilled water to equilibrate water status and transported to the laboratory.

9.2.2 Experimental Setup and Design

Once in the laboratory, all branches were given a fresh cut 2.5 cm above the previous cut while stem ends were put in water to reduce risk of cavitation, weighed, and then placed in a 100-mL amber bottle filled with 100 mL of distilled water. The neck of each flask was plugged with cotton wool to reduce direct water evaporation and provide added stability to a branch. For needle loss and water uptake, the branch and entire apparatus was weighed, which allowed for quantification of water uptake throughout the experiment. All branches were randomly placed on a light rack and changed weekly. Branches were provided water throughout the experiment and the growth room was maintained at conditions similar to typical display conditions for balsam fir (i.e. 21 - 22°C constant temperature, 32% relative humidity, and a light intensity of 99 µmol m^{-2} s^{-1}).

This experiment for needle loss was set up as a 2 x 11 factorial experiment where the factors of interest were time and clone. The time began at harvest and the clones were chosen due to results in Chapter 7. In that Chapter, Clone 506 was the superior NRD clone every month it was sampled between September and December, and Clone 566 was consistently the lowest NRD clone, however equal to others statistically. Therefore, in addition to the results of Chapter 7, trees were personally inspected for quality.

9.2.3 Per Cent Needle Loss (PNL)

Needle loss was calculated as a percentage of the fresh and dry weight of abscised needles to the fresh the lowest clone weight of initial branch weight (MacDonald and Lada, 2012). Each day the branches used to determine needle loss were subjected to a "finger run" test, and this dislodged loose needles from the branch (MacDonald, 2010). To perform the "finger run" test, each branch was gently passed three times through the index and middle fingers, and the fallen needles were quantified gravimetrically and recorded to arrive at a fresh needle mass lost (g).

9.2.4 Water Uptake

Water uptake was estimated for each clone by weighing each bottle at the beginning of the experiment after being filled with water and then, weekly, each branch was removed from its flask and the flask was weighed again. The difference in mass was estimated as water uptake and the rate of water uptake was calculated as water uptake (mL g^{-1} d^{-1}):

$$Water\ Uptake = \frac{M_n - M_{n+1}}{7 \cdot M_b}$$

M_n is the mass of the flask on any given week while M_{n+1} is the mass of the flask on the subsequent week. M_b is fresh weight of the branch. The critical value for water uptake is 0.05 mL g^{-1} d^{-1}, below which is the point at which a branch will commit to abscission (Lada et al. 2016).

9.2.5 Chlorophyll Fluorescence

Maximum photochemical efficiency or optimum quantum yield of photosystem II (Fv/Fm), was fluorometrically determined using a MINI-PAM Photosynthesis Yield Analyzer (Heinz Walz, Effeltrich, Germany). Plants were dark-adapted for 15 min before Fv/Fm measurements with illumination by application of a saturation flash. Three random replicates for each experimental unit were done, at the time of sampling

9.2.6 Scanning Electron Microscope (SEM)

Samples were taken onsite, and postharvest, just before abscission and abscised, to prepare for SEM observation. All needles taken onsite were still attached to the branch. At peak abscission, some needles that were still attached to the branch were sampled as well as needles that had abscised. Some needles were cut transversely, while some were cut longitudinally on the coronal plane. They were fixed by placing them in 2 mL vials containing 2.5% glutaraldehyde and stored between 0-4 °C for at least 2 hours. Cutting was necessary for absorption of this fixative, as well as an attempt to see the surface with less noise. Samples were prepared for the SEM partially at the Dalhousie Agricultural Campus, and partially at the Life Science Building at Dalhousie, Halifax. Needles from each genotype were placed in separate wells in a plastic 12 well plate tray at room temperature. Needles were washed with tap water three times to remove the fixative (water in, water out using a plastic pipette). The needles then were covered with the second fixative, 2% osmium tetraoxide for one hour at room temperature. A second washing was performed three times to remove the fixative. The needles then were gradually dehydrated by covering them (and removing) with increasing concentrations of ethanol (25, 50, 70, 80, 90, 95, 100 %). The highest concentration was used three times. The needles were then critical point dried using Lucia EM CPD300. The dried needles were mounted on to SEM specimen stub with needle's back side facing up using Neubauer conductive carbon cement. Three to four needles were mounted on each stub. They were coated with gold/palladium alloy for a thickness of 12 nm using Lucia EM ACE200 to ensure electrical conductivity (Personal Communication, 2017, Ping Li, Director, Scientific Imaging Suite, Department of Biology, Dalhousie University). Samples were viewed on a Zeiss 1455 VP Scanning Electron

Microscope. (LEO 1455 VP). Approximately 50 microphotographs were taken during observation.

9.2.7 Transmission Electron Microscope (TEM)

Samples of balsam fir needles from Clones 506 and 566 were taken from the initial site and from the laboratory weekly postharvest for observation using the TEM. Needles were cut transversely and longitudinally, fixed in 2.5% glutaraldehyde for transport to Dalhousie Medical School. There, they were post fixed in 1% osmium tetroxide, dehydrated with acetone, and embedded in epoxy resin for thin sectioning (Faulkner and Garduno, 2002). Thin sections were observed in a JEOL JEM-1230 transmission electron microscope equipped with a Hamamatsu ORCA-HR high-resolution (2,000 by 2,000 pixels) digital camera, and images were saved as TIFF files. Approximately 200 microphotographs were taken.

9.3 RESULTS

9.3.1 Needle Loss

Per cent cumulative needle loss confirms that Clone 506 is a better clone in regards to needle retention than Clone 566 ($p < 0.001$; Figure 36). In Clone 506, needle loss continued to increase slowly, remaining under 2% for the first four weeks. Between week four and weeks five needle loss increased and then paused until week nine when it started to increase exponentially. In Clone 9 the first four weeks were similar as in Clone 506, but after the first four weeks, needle loss increased exponentially without delay. Needle loss in Clone 9 increased quickly after the commencement of abscission over the 11 weeks of study (Fig. 36).

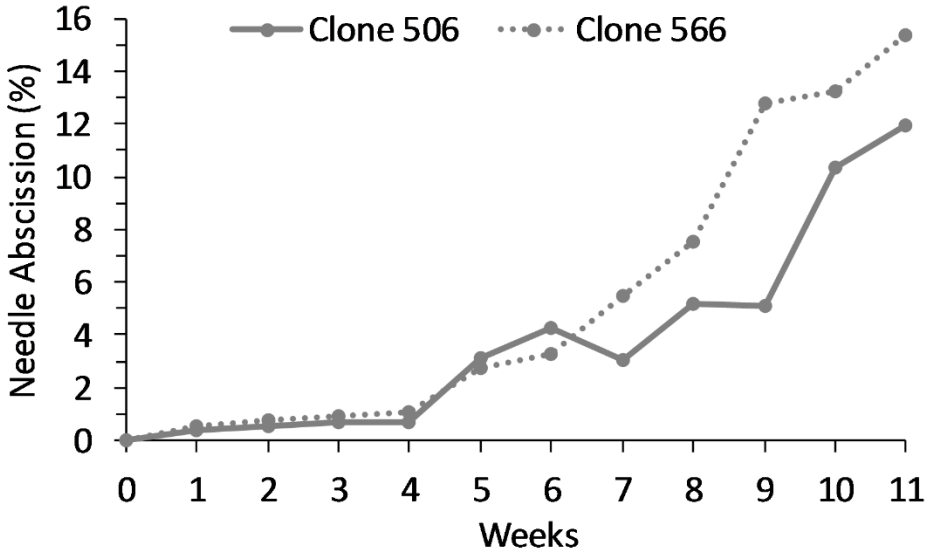

Figure 36. Progression of abscission in two different balsam fir clones. Data points were averaged from 5 replicates.

9.3.2 Chlorophyll Fluorescence

Chlorophyll fluorescence improved by one week postharvest after being acclimated to room temperature and given light. Branches were collected in December in Nova Scotia when the days are short and it is very cold. Clone 566 deteriorated by week five and clone 506 by week 9 postharvest (Fig. 37).

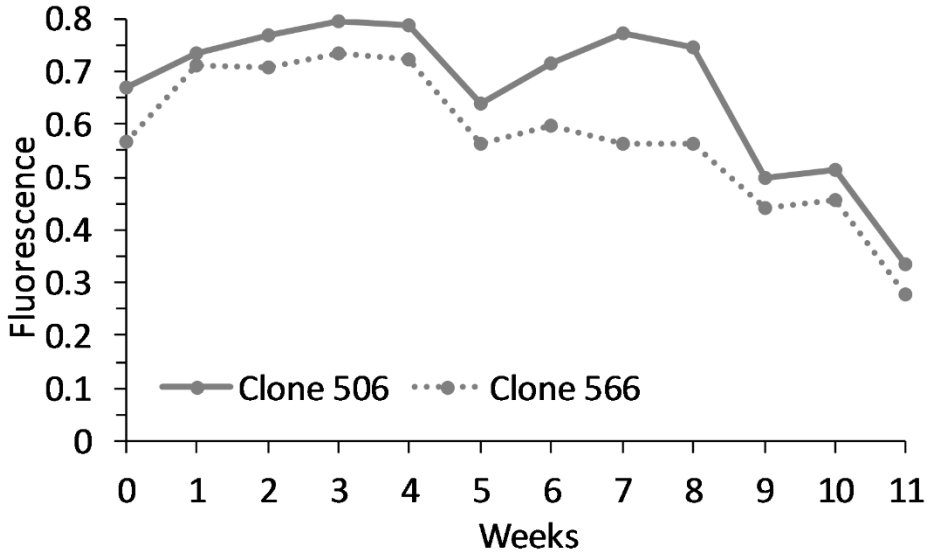

Figure 37. Fluorescence of two balsam fir clones.

9.3.3 Scanning Electron Microscopy

Initially, in Clone 506, there was little evidence of invasion by mold. One hypha adhered to the needle surface when scanning the surface of the needles in the initial samples (Fig. 38 A). In Clone 566, there was greater evidence of invasion by mold (Fig. 38 B). Hyphae were very easy to find with extensive branching. Stomata were closed all but one in Clone 506 and one in 566 (Fig. 38 C, D). Stomata were clearly seen on the underneath side of the needle and raised off the surface of the needle (Fig. 39 A, C).

By pre-abscission, there was much more evidence of the presence of mold hyphae (Fig. 39B, D). This was visible in both clones, but more so in Clone 566. Also wax or a similar substance was observed built up on the surface of the stoma possibly in an attempt to prevent total moisture loss (Fig. 40).

At post-abscission, it seemed that hyphae branching was more extensive in Clone 506 (Fig. 41 A), however, still not as extensive as observed in Clone 566 (Fig. 41 B). It appeared that hyphae were trying to grow into the needle through the stomata in both clones (Fig. 41A, C). Abscised needles seemed to have their stomata clogged with wax in both clones (Fig. 41 C, D). This could have been an attempt by the needle to protect the clones from complete dehydration, By post-abscission, bacteria, mold spores, pollen, and a great abundance of wax was observed in both clones (Fig. 41 C, D). The abscission zone was observed from a needle that had not yet abscised. This revealed what appeared to be many bacteria cells at the tip of the needle where it was attached to the branch (Fig. 42 A, B).

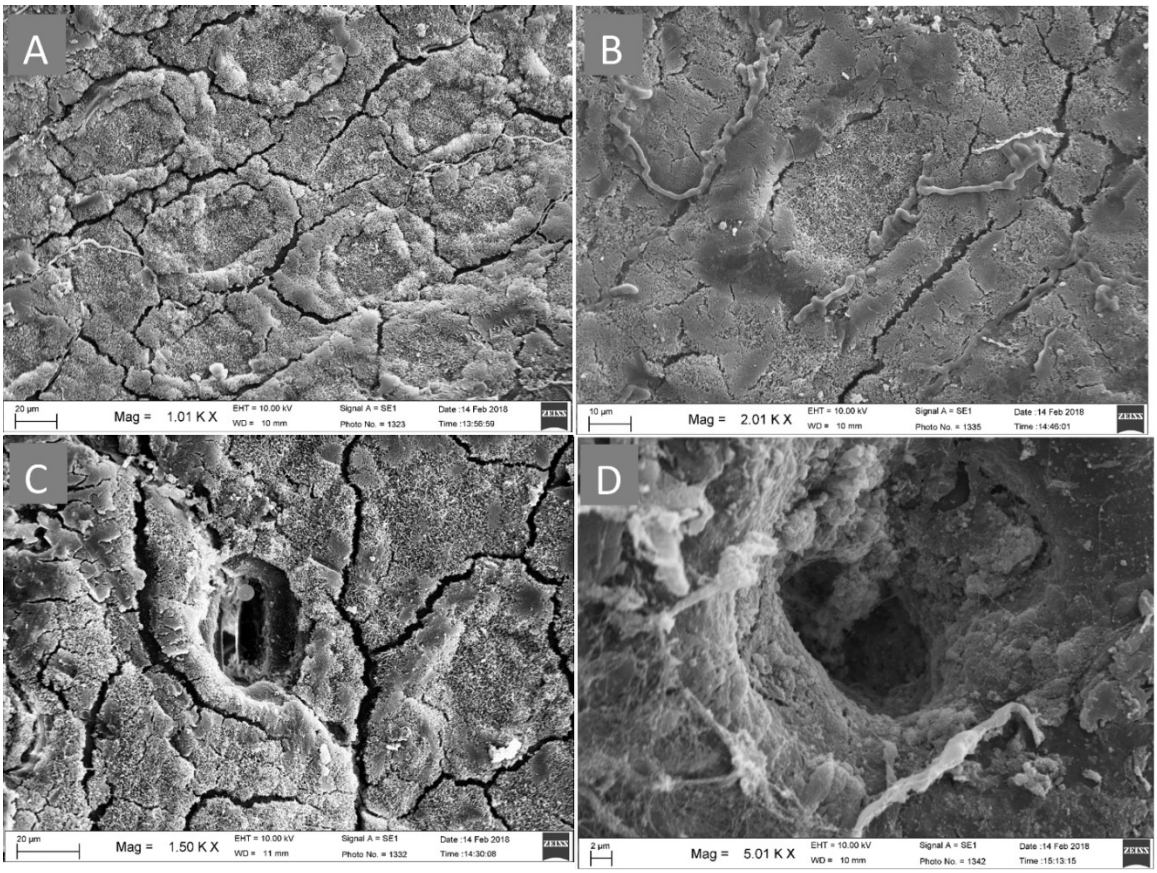

Figure 38. Initial SEM images of balsam fir needles. A) Clone 506, closed stomata; B) Clone 566, closed stomata; C) Clone 506, open stomata; D) Clone 566, open stomata

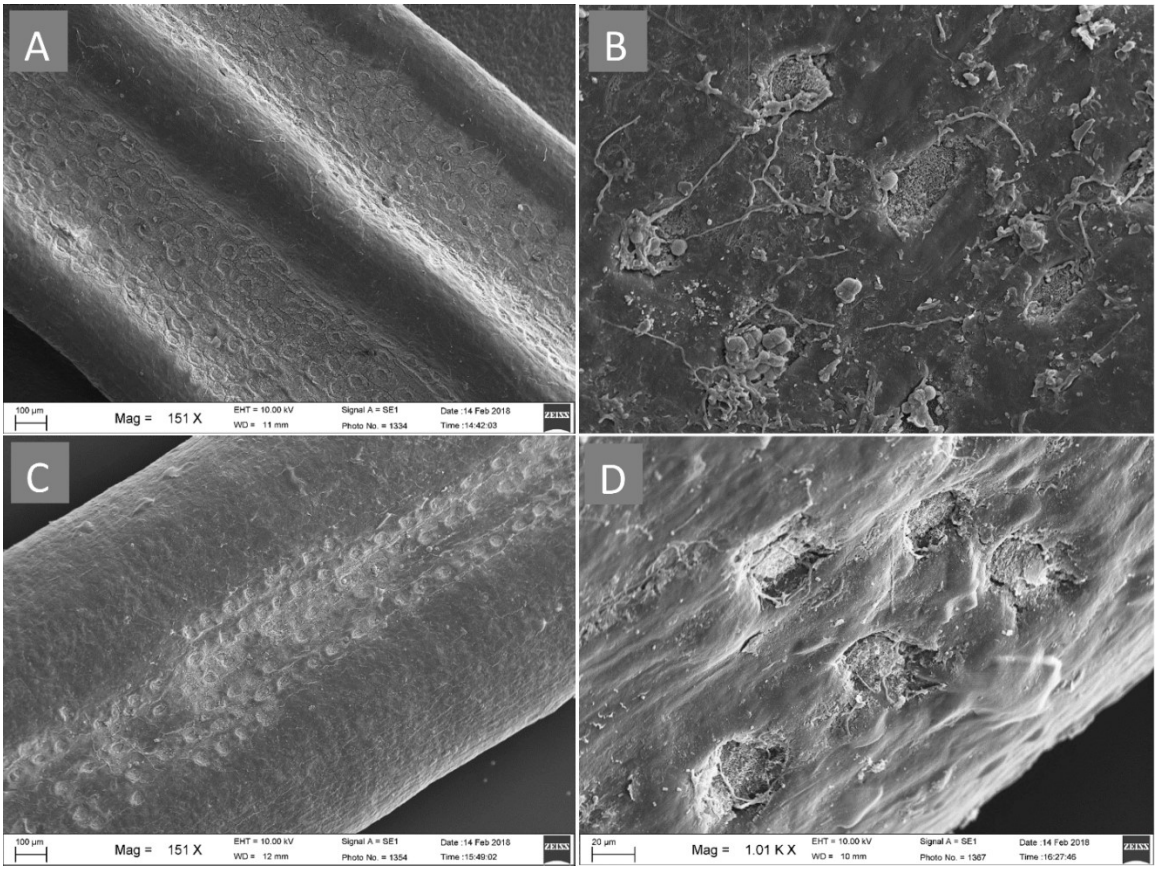

Figure 39. SEM image of stomata on balsam fir needles just prior to abscission. A) & C) Clone 506; B) & D) Clone 566

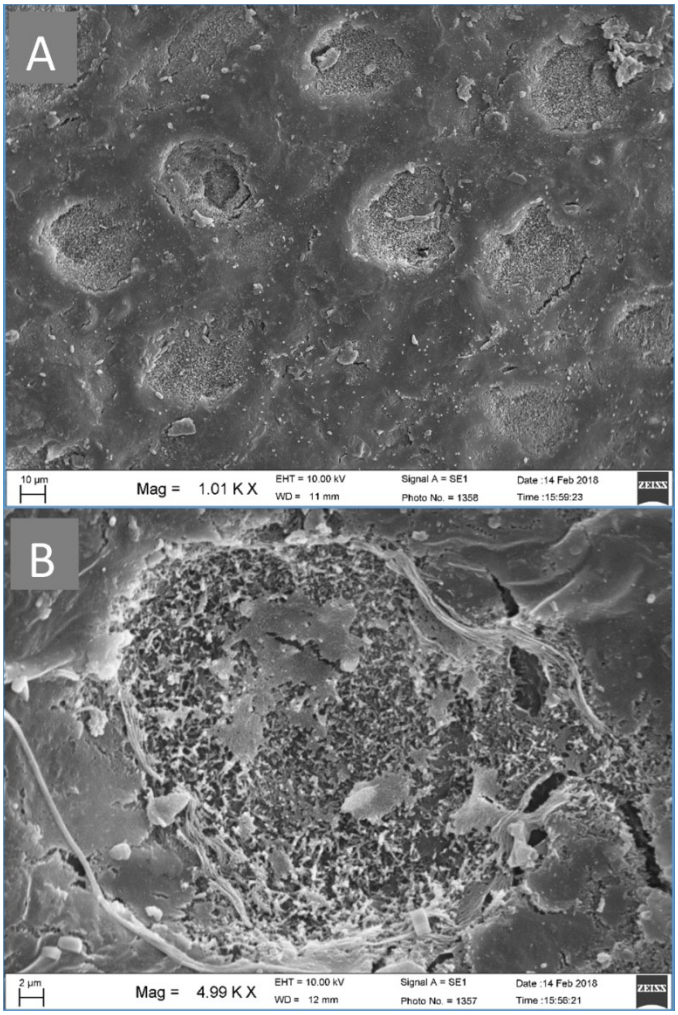

Figure 40. A) Stomata of balsam fir stomata just prior to abscission showing waxy build up; B) magnified image of wax covered stomata

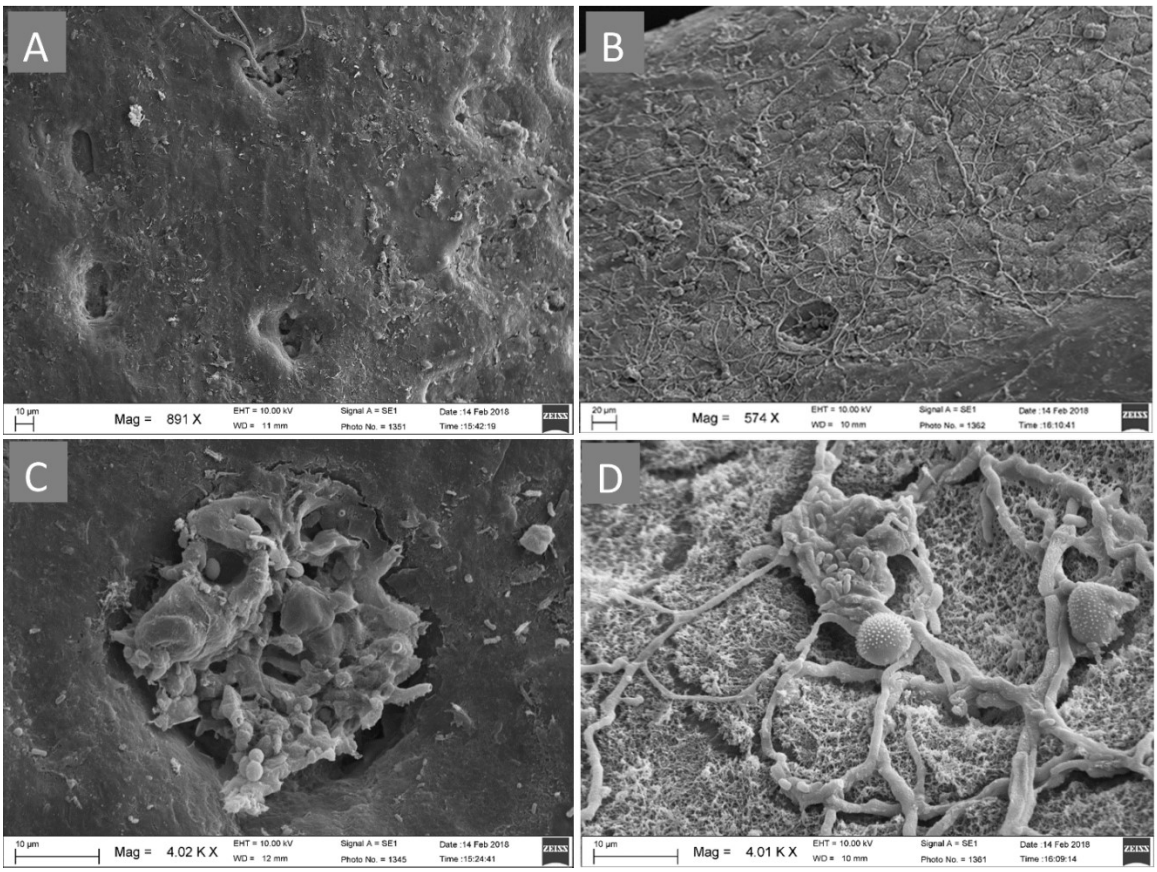

Figure 41. A) Stomata of abscised needles from Clone 506; B) stomata of abscised needles from clone 566; C) magnified image of Clone 506 clogged stomata; D) magnified image of Clone 566 clogged stomata.

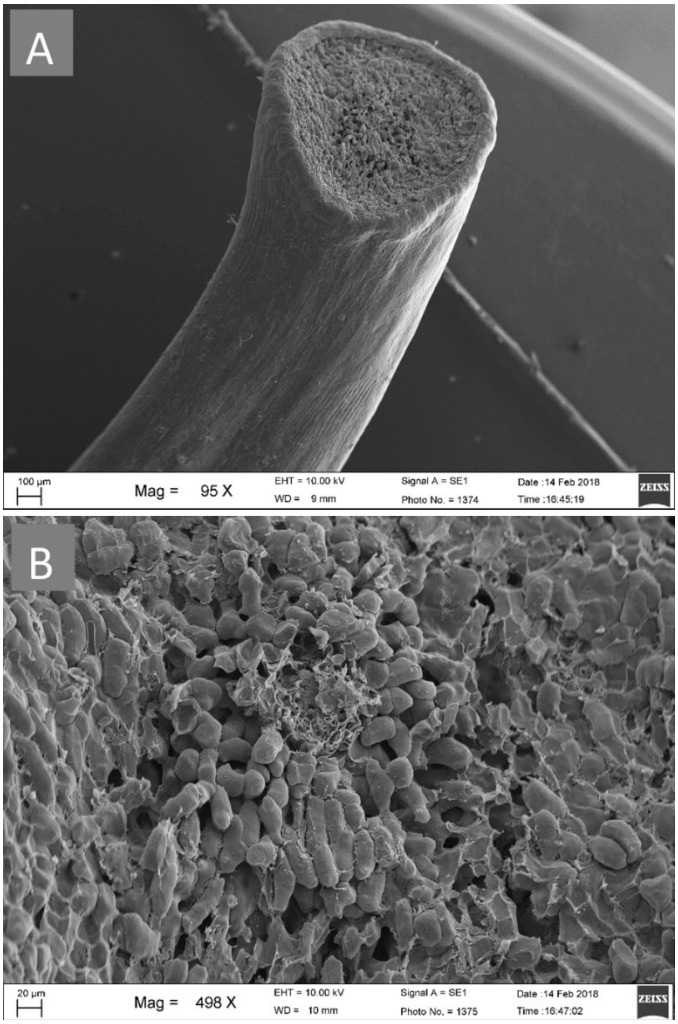

Figure 42. A) Abscission zone of balsam fir needle; B) magnification of balsam fir abscission zone and accumulation of bacteria.

9.3.4 Transmission Electron Microscopy

The first specimens viewed were samples collected and preserved onsite. Initial measurements for each clone are in Table 22. In sample one, Clone 506, many chloroplasts were viewed (Fig. 43 A, C), with a mean length of 3667 nm. Plastoglobuli (Fig. 43 A) were observed in the chloroplast, and were approximately 150 x 100 nm. Thylakoids were generally oriented parallel to the long axis of the chloroplast. In Clone 566, the chloroplasts were more difficult to observe, but the mean size of the chloroplasts was 3725 nm (Fig. 43 B; Table 22). Plastoglobuli were less plentiful, and the size was smaller than in Clone 506, 100 x 75 nm. In both Clone 506 and Clone 566, there was some residual starch present but

it was not plentiful indicating chloroplast inactivity (Fig. 43). Very little starch was in the chloroplast. Clone 566 had large starch deposits in the cells, possibly amyloplasts (Fig. 43 D). Other parts observed in the cells of both clones were cell wall, cell membrane, vesicles, nucleus, golgi body, and what was thought to be mitochondria (Fig. 43).

One week postharvest, having one week of normal lighting, chloroplasts in both clones appeared to be actively producing starch (Fig. 44 A, B). There seemed to be a clear separation of the stroma lamellae in the chloroplasts with many starch granules in addition to larger starch deposits primarily in between the stroma lamellae. Thylakoids appear to be swollen (Fig. 44 A). Plastoglobuli still appear to be present but are difficult to measure or quantify due to numerous starch grains blocking the view. In both Clones 506 and 566, chloroplasts are smaller, approximately 2000 nm in length.

In samples prepared from subsequent weeks postharvest, observations of the starch grains were fewer and fewer. By week two postharvest it was more difficult to find chloroplasts or starch in Clone 566, but a few were visible (Fig. 45). In week three postharvest, the chloroplasts in Clone 506 looked like they were overfilled with starch grains and chloroplast breakdown was evident (Fig. 46 A), but again very difficult to find in Clone 566 and it look like it had ruptured (Fig.46 B). By week four postharvest in Clone 506, chloroplasts were difficult to find and looked very much like they did in the beginning showing plastoglobuli and little starch indicating inactivity. In Clone 566 by week four, many chloroplasts are visibly damaged. Throughout the next six weeks, it became increasing difficult to find active chloroplasts. There were some in Clone 506, but virtually none in Clone 566 (Fig. 47).

Table 22. Quantification of chloroplast size and plastoglobuli number and size using TEM for Clones 506 and Clone 566 harvested onsite at Debert, NS orchard in December, 2019.

Clone	Chloroplast Mean Size (nm)	Plastoglobuli Mean Size (nm)	Number of Plastoglobuli
506	3667 nm	150 x 100	50 -100
566	3725 nm	100 x 75	15 - 30

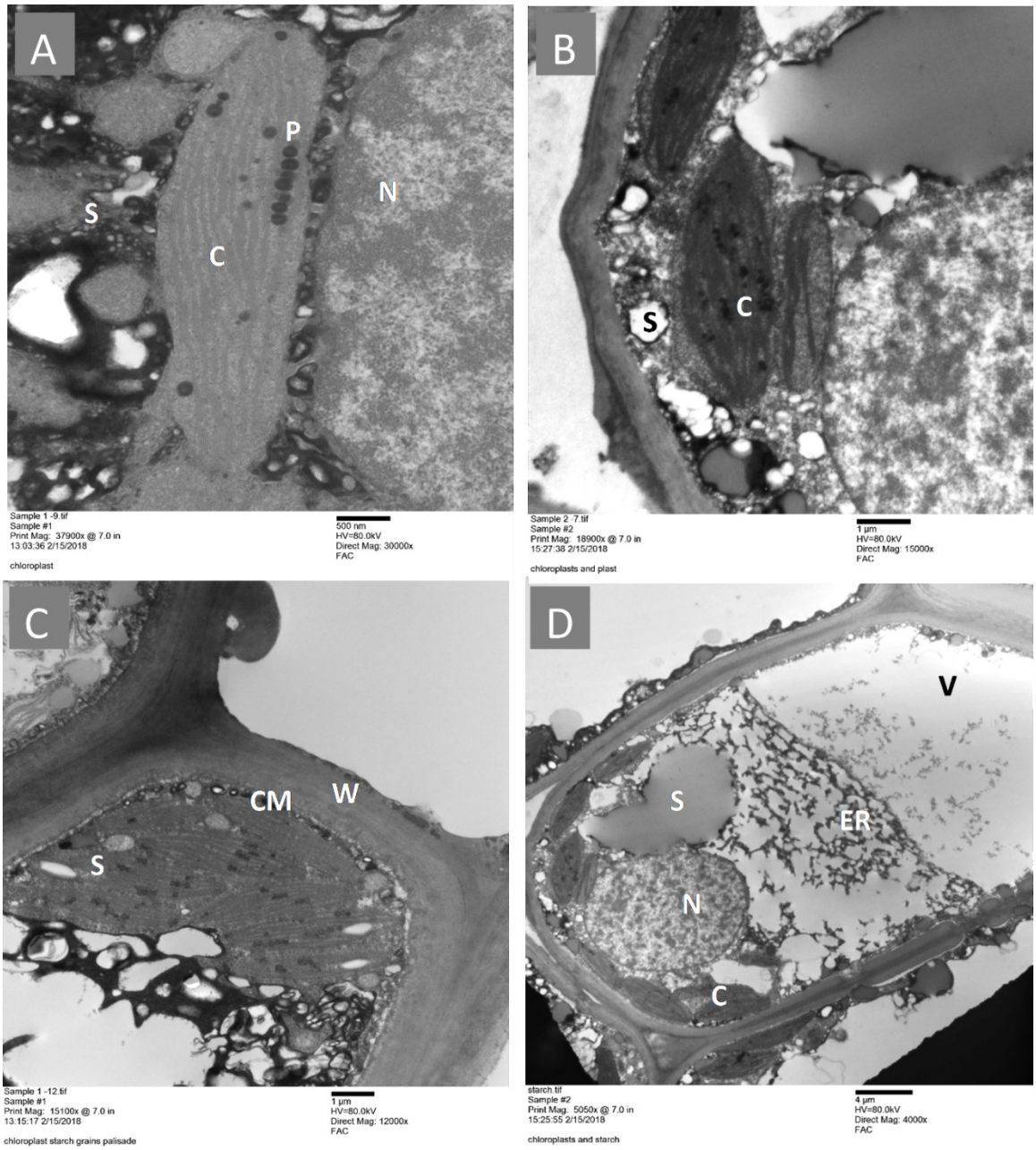

Figure 43. Initial TEM of balsam fir needles on-site of A) & C) Clone 506; B) & D) Clone 566. C = chloroplast, S = starch granule, N = nucleus, W = cell wall, CM = cell membrane, ER = endoplasmic reticulum, V = vacuole.

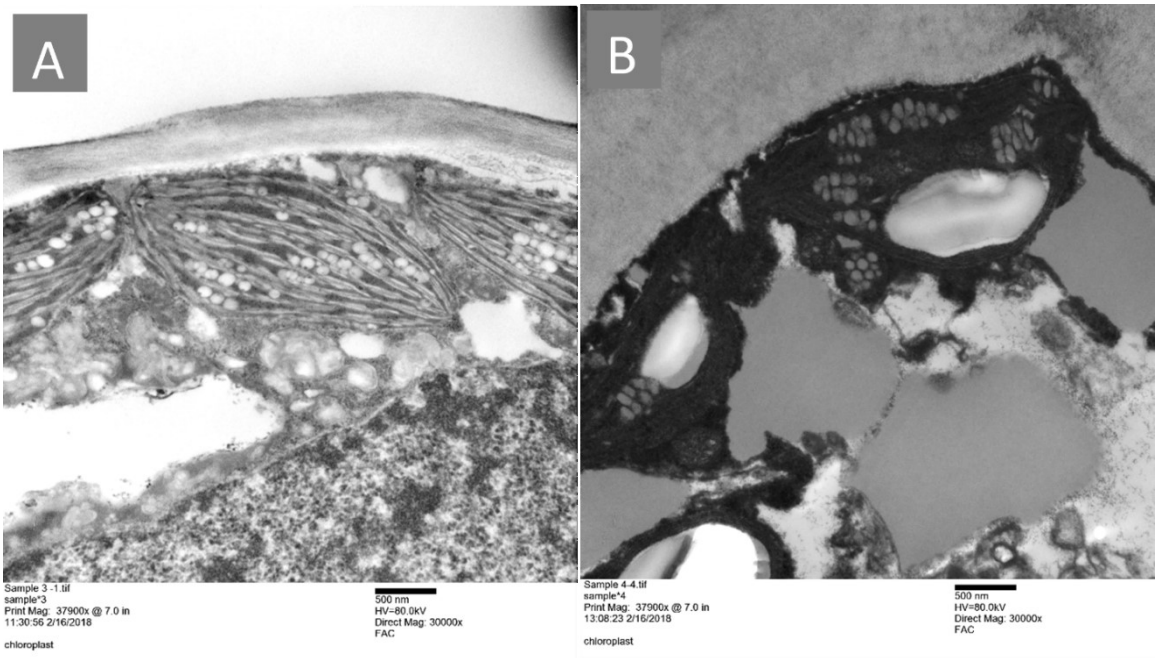

Figure 44. TEM of balsam fir needles 1-week postharvest. A) Clone 506. B) Clone 566.

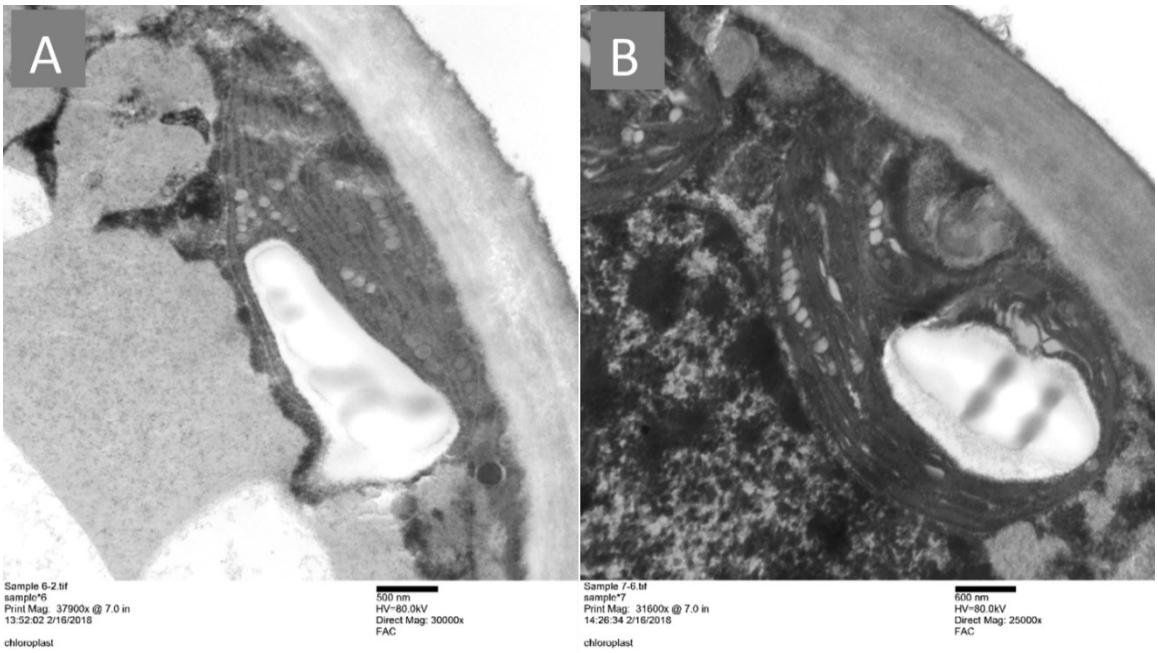

Figure 45. TEM of balsam fir needles 2-weeks postharvest. A) Clone 506. B) Clone 566.

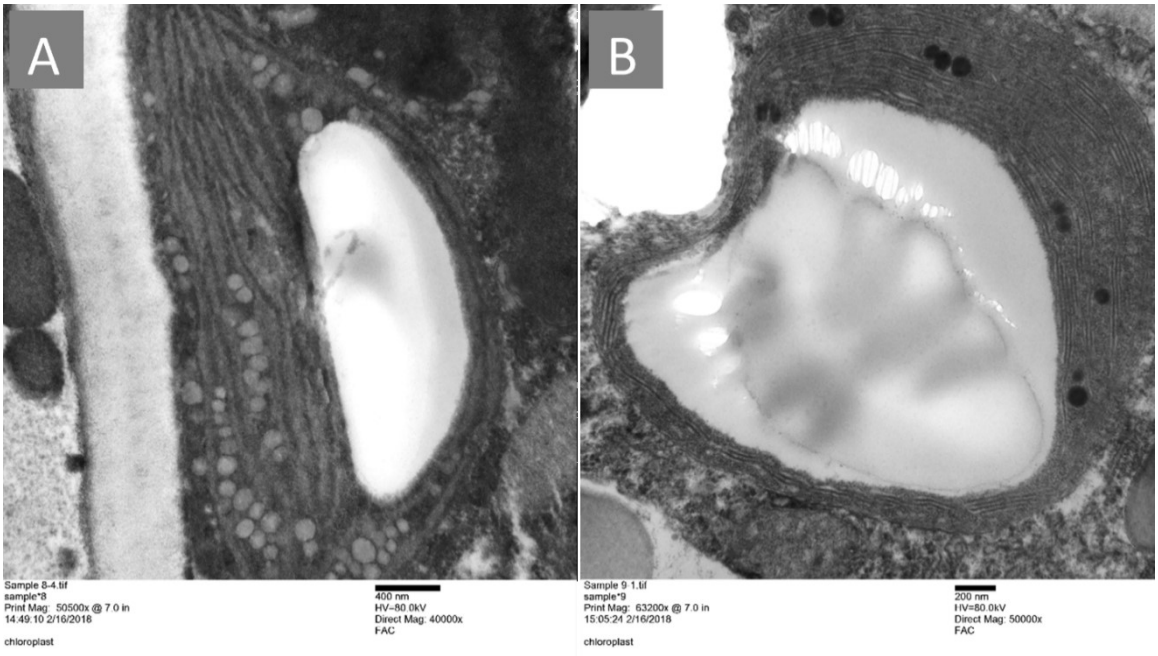

Figure 46. TEM of balsam fir needles 3-weeks postharvest. A) Clone 506. B) Clone 566.

Figure 47. TEM of balsam fir needles from Clone 506 6 weeks postharvest.

The last samples were particularly clear. Needles from Clone 506 showed small chloroplasts (approximately 2000 nm in length), many somewhat more globular (Fig. 48 A). Starch grains are slightly visible but thylakoids are not clear. Clone 566, showed a complete cellular and chloroplast disorganization (Fig. 48 B).

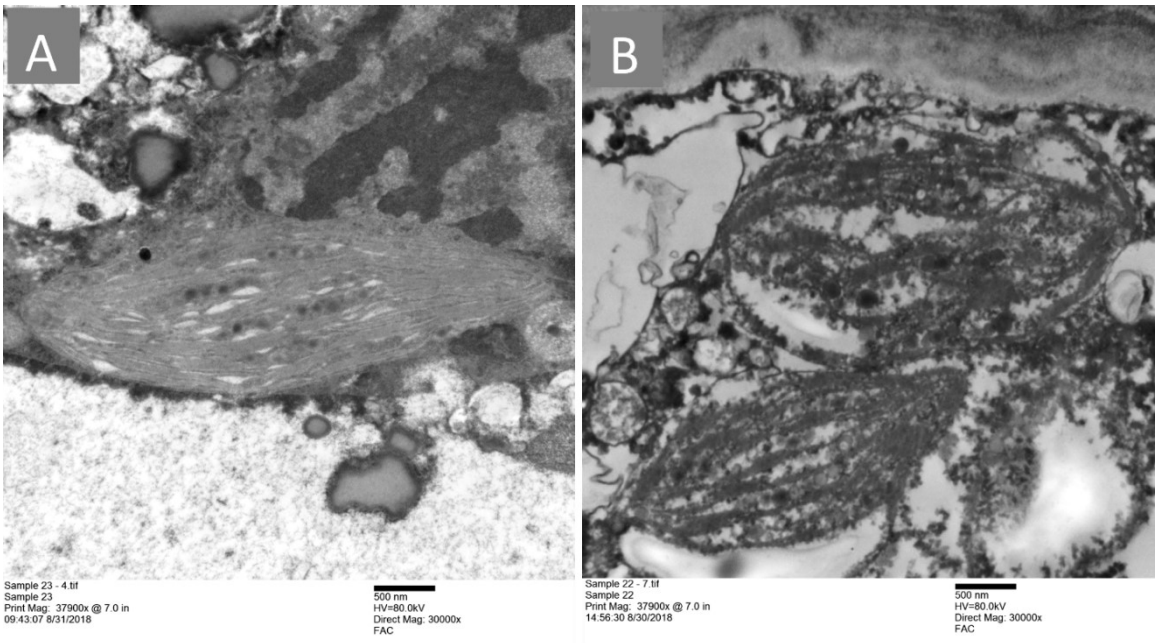

Figure 48. TEM of balsam fir needles final weeks postharvest. A) Clone 506. B) Clone 566.

9.4 Discussion

9.4.1 Scanning Electron Microscopy

The closed stoma in the initial samples are not unexpected in either clone as these balsam fir trees have undergone cold acclimation being harvested in December in Nova Scotia. Cold – induced closures occur in many plants (Honor et al., 1995). More interesting is the adhering of fungal hyphae on the exterior of the needle surface, more prevalent in Clone 566, the clone with lower NRD postharvest. It is difficult to tell whether these fungi are harmful or helpful, but microorganisms are involved in the ecology of balsam fir. If these are harmful fungi, it would help explain why this clone is a poor NRD clone. Many years ago in the balsam fir industry, there was concern of pathogenic fungi as a cause of economic loss (Bakusis and Hansen, 1965). More recently studies have been done, using

the balsam fir in New Brunswick, Canada, on fungal endophytes. Endophytic fungi have a mutual relationship with the plant and contribute to the defense of the host plant (Pirtiiä and Frank, 2011). Endophytic bacteria have the ability to enter the cells and colonize the interior of the plant without causing disease symptoms (Johnson and Witney, 1989). In this study there was no way to determine the species of fungus, but due to the increase in this fungal hyphae postharvest, another possibility that exists is that it is a saprophytic fungus that was observed 11 weeks postharvest, along with bacteria cells (Bakuzis and Hansen, 1965). Saprophytic fungi, *Coniophora puteana* and *Coriolus versicolor,* which commonly colonize beech and pine were studied using the EM. It was found that after colonizing a tree, these fungi produce what is called extracellular mucilaginous material (ECM) that aids in decay (Ali et al., 1999). The microphotographs of ECM shown in the paper by Ali, bear a resemblance to what was termed "waxy build up" described earlier in the SEM results section (9.3.3.3). It seems most likely that the fungus observed in the balsam fir SEM samples postharvest is saprophytic, as opposed to pathogenic or endophytic, due to the accumulation observed 11 weeks postharvest and the fact that is was more abundant on the surface of the poorer clone. This could be related to postharvest needle abscission. In addition, there was no observation of fungal hyphae occupying intracellular spaces in the TEM results. However, more research and diligent classification would be required to determine the fungal type. No membrane blabbing or blistering was observed using the SEM in the samples from pre- or post-abscission, possibly due to the hard cuticle on the balsam fir needle.

9.4.2 Transmission Electron Microscopy

This study is the first of its kind, observing balsam fir needles postharvest using electron microscopy. This is primarily a qualitative study, but changes are obvious in the chloroplast postharvest in both clones of balsam fir, and this study was necessary to confirm changes the postulation formed when looking at lipids and FA reported in past postharvest studies. The chloroplast has been found to be the main target of abiotic stress, inhibition of photosynthesis, as well as, degradation of photosynthetic apparatus (Rivero et al., 2007). However, there can be various reactions to stress. One possibility in root detached balsam fir is that stress will induce senescence, which in turn leads to abscission. Another

possibility is ethylene induced chloroplast breakdown, similar to what happens when fruit ripens (Shimokawa et al., 1978). Either would result in cell breakdown and death, as great importance is attached to the role of functioning chloroplasts in preventing plant cell death (Thomas et al., 2003).

Plants have not been studied in reference to cell death and breakdown as much as animal cells have (Reap et al., 2008) It has been recognized in balsam fir postharvest studies, that sometimes senescence is evident with yellowing or browning of needles, but other times abscission occurs without noticeable changes in the color of the needles (Lada and MacDonald, 2015). There has been some discussion as to the meaning of senescence and cell death – senescence seems to be the preferred term when there are visible changes, but there is a chance of reversal, whereas programmed cell death (PCD) is used when the damage is not reversible. PCD means there is a programmed breakdown within the cell that leads ultimately to death (van Doorn, and Woltering, 2004). PCD in plant cells can be attributed to apoptosis, autophagy, or necrosis (Lockshin and Zakeri, 2004). Apoptosis is quite fast and is characterized by cell shrinkage, and formation of apoptotic bodies. Necrosis happens as the result of overwhelming stress and results in swelling and eventually total cellular disorganization, whereas autophagy type ll death is much slower. Autophagy is a normal part of cell recycling, but this can turn into cell death (van Doorne and Woltering, 2004). It is quite clear in this study that a cell breakdown is occurring but quite slowly, relating this to abscission is somewhat more difficult. There is thought to be involvement with elevation of peroxidases. Peroxidases can regulate ROS production in a cell and chloroplast, and perhaps make it a target for extermination. Interestingly, peroxidase and ROS production has been related to, cell wall metabolism, creating loosening of the cell wall and abscission, in some plants (Cosio and Dunnand, 2009). This could be the link.

9.4.3 Initial Samples Taken Onsite

Neither sample, 506 (high NRD clone) or 566 (lower NRD clone) were showing active starch production. When microphotographs were compared to others taken in previous research, the chloroplasts look very similar (Alberts et al., 2002). The structure of the

chloroplast was made up of two main membranes. The other components visible were stromal lamellae, thylakoid grana, stroma, and plastglobuli. The difference between Clone 506 and Clone 566 was in the size of the chloroplast and the number of plastoglobuli. Clone 506 had larger chloroplasts and more plastoglobuli. The sampling time was December in Nova Scotia. This would account for the photoinhibition observed in the chloroplast. There was significantly higher fluorescence in Clone 506 in the initial samples, and it is known that cold tolerance and chilling requirement for each cultivar can vary for cold acclimation to occur (Ignacio Calzadilla et al., 2019). In a study by Li et al., (2018), on cold tolerant and cold sensitive cultivars of sugar cane, there was an accumulation of starch in the less tolerant cultivar. The obvious collection of starch in Clone 566, could mean it is not coping as well with the cold. Clone 566 had significantly lower fluorescence as well. However, this extra starch could be helping Clone 566 postharvest, as needle retention improves after cold acclimation in poor NRD clones (MacDonald and Lada, 2008). Organization of microtubules was not clear in our samples, however reorganization of microtubules during cold can result in better fluorescence as was noted in Clone 506. This has been noted in cold tolerant clones of sugarcane (Li et al., 2018).

Plastoglobuli are lipoproteins present in the plastids. These are rich in lipids, but not polar lipids, instead prenylquinones, and TAG. Plastoglobuli are typically anywhere from 30 – 500 nm (Lichtenthaler, 2013). The plastoglobuli observed under the TEM are well within the normal size range. It has been found that plastoglobuli can reach sizes up to four μm under stressful conditions and this can indicate beginning of cell death. There is a strong relationship between enlargement of plastoglobuli and dismantling of the thylakoid (Lichtenthaler, 2013; van Wijk and Kessler, 2017). Our samples seemed quite normal, as mentioned earlier.

9.4.4 Week One Postharvest

Swelling of the thylakoids as seen in both clones can be a marker of stress, and evidence of the beginning of necrosis. This marker can show damaging effects in the cell long before visible symptoms occur (Alves et al., 2016). Stress in root detached balsam fir can be caused by physical injury and water deficit (Lada et al., 2016). However, one week is quite

early after detaching from the branch. There is an indication however, that water status does goes down within the first two weeks (Lada et al., 2016). Another stress to be considered is that we are taking plants that have gone through cold acclimation and adjusted to low light, and bringing them inside to room temperature and also providing light. Even normal lighting of approximately 100 µmol m^{-2} s^{-1} as given in this experiment can cause light stress. The chloroplasts are actively producing starch grains and the starch has aggregated into large starch masses in the chloroplast. Some chloroplasts appear to have a normal starch buildup, however with swollen thylakoids (Okansen, 2010).

The size change in chloroplasts by approximately half could be the result of two factors. First of all, when the balsam fir needles are in the field in December in Nova Scotia, when the light is low, these chloroplasts may have an increased size in an attempt to get light (Wang and Blumwold, 2014). They are also reported to be in a cluster on one end of the cell. When provided with light perhaps the chloroplast shrink. Also, it has been found that one of the ethylene induced changes in chloroplasts is a rapid reduction in size of the chloroplasts (Shimokawa et al., 1978). Also typical of ethylene, chloroplast degradation is the separation of the thylakoid membrane layers as seen in the balsam fir starting at week one. This occurs due to the degradation of the granal and lamellar membranes (Shimokawa et al., 1978). There have been cases of gradual decrease of chloroplast size leading up to senescence, however, a fast decrease in one week is more specific to the action of ethylene (Shimokawa et al., 1978).

9.4.5 Weeks Two to Nine Postharvest

Definite signs of stress are apparent in micrographs taken during this period. Actually chloroplasts in 566 are very difficult to find. This could be that they are starting to go through autophagy, a process to get rid of damaged organelles (Nakamura and Izumi, 2018). As mentioned in an earlier chapter (6) discussion, in stressful times such as water deficit, the chloroplasts of a cell can become damaged and it is thought that ROS are formed (Bailey-Serres and Mittler, 2006). It is also thought that these damaged cells result in the formation of an enzyme, ubiquitin ligase, that acts as a signal degrade ROS producing chloroplasts (Woodson et al., 2015; Lavelle, 2019). If it is only a small amount of damage

chloroplasts can be repaired, or once they are beyond repair there is a recycling process, as 70% of the chloroplasts are composed of valuable proteins, which could be used in other cells (Wang and Blumwald, 2014). Another possibility is that often the osmophilicity of tissues changes when they are damaged or senescing. This means the ability of osmium tetroxide (OsO_4) to achieve the proper staining intensity in TEM images can be compromised. Although the same protocol was used for all slide preparation, there can be batch to batch differences and these last samples were processed separately.

Stress has been found to alter the ultrastructure and contribute to the degradation of chloroplasts. The degree of stress seems important in regards to ultrastructural changes in the cell (Reap et al., 2008). In *Thellungiells salsuginea* exposed to varying degrees of salt stress, similar changes were seen in chloroplast structure. There was a swelling of the thylakoids and starch production. Results also showed a change in photosynthetic efficiency (Goussi et al., 2018). Zechmann (2019) studied effects of three different stresses, senescence, high light and, Botrytis infection, on the ultrastructure of chloroplasts. They all showed similar results which were reduced chloroplast size and reduced production of starch. These are all similar to the microphotograph results of the balsam fir postharvest.

9.4.6 Week Ten and Eleven Postharvest

Chloroplast breakdown is occurring. All chloroplasts in both clones are showing disorganization of the chloroplast. There was no enlargement of plastoglobuli or globules or cell enlargement as seen in apotosis. Membrane blebbing was evident at this stage which was anticipated due to increased membrane injury that has been seen around the time of peak abscission. There is also a greatly reduced chlorophyll fluorescence at this point indicating no photosynthesis was occurring. This all could be related to reduced capacitance that occurred in past Chapter 6 and other similar measurements that indicated water deficit in balsam fir postharvest (Lada and MacDonald, 2015). It seems possible that water deficti could be the stressor. Chloroplasts are one of the first parts of a plant cell to react to stress. Therefore, chloroplasts start to be broken down. Cell death occurs very slowly in plant cells (Reap et al., 2008). It appears that Clone 566 is farther along in chloroplast disintegration than Clone 506. Since we are seeing a reduction in fluorescence

earlier in Clone 566, it is possible that the cell is farther along in its program that occurs before death, possibly due to a decrease in water status. It is difficult to say which type of cell death is occurring, but the result is necrosis of the cell. It is possible that when chloroplast manifest ROS, that autophagy occurs (Nakarmurand and Izumi, 2018). This could be related to peroxidase production, which could result in the weakening of the cell wall. It seems the timing is right, but much more research is necessary in the area of plant cell death.

9.5 Conclusions

Stomatal pores were almost all closed in Clones 506 and 566 in initial and subsequent weeks. Fungi were more evident on Clone 566 than Clone 506 in SEM studies and very common by week 11, along with bacteria. It was thought most likely this was related to death of the cell. No membrane blabbing or blistering was noted in SEM studies. TEM showed evidence of stress in balsam fir cells postharvest, characterized by smaller chloroplasts and less starch production over time. In addition, the thylakoids were swollen, a microscopic sign of stress, and the lamellae were separated. Many characteristics observed were typical of those seen in other plants during ethylene production. Clone 566 was found to decline faster than Clone 506, although this was quite subjective. There was a possibility of autophagy occurring due to the difficulty of finding chloroplasts especially, in the lower NRD clone. It also appeared that necrosis of cells had occurred by week 11, but it was more evident in Clone 566.

CHAPTER 10 – GENERAL DISCUSSION AND CONCLUSIONS

10.1 DISCUSSION

Postharvest abscission is a major limitation in the balsam fir Christmas tree industry in Atlantic Canada. This has been attributed to several factors, such as the environment, genetic variations, endogenous factors, rough postharvest handling, and nutrition (Mitcham-Butler et al., 1987; Hatcher, 1990; Thiagarajan and Lada, 2010; MacDonald et al., 2014; Georgeson, 2013). This thesis has revealed changes that occur postharvest in balsam fir needles in polar lipids and fatty acids that fill in gaps in knowledge that were missing before this study began. These gaps were shown as question marks in the introduction (Figure 2).

Plant cell membranes contain diverse lipid molecular species and these all have a fatty acid component. There were more than 230 polar lipid species and approximately 20 FA identified in each study performed during the duration of this research. This included three new lipid species that have not been reported in plant studies prior to this, DGDG 36:7, DGDG 38:7, and MGDG 36:7, which have been confirmed to be real (Personal Communication: Dr. Ruth Welti, Kansas Lipidomic Research Center, Kansas State University, Kansas). Changes in polar lipids and FA help explain changes that have been observed in past research with balsam fir needles postharvest, such as increased membrane injury, and decreased chlorophyll fluorescence (Lada and MacDonald, 2015). Many of these lipid changes, as you will see, are typical of water deficit stress experienced in other plants, but some major differences have been identified due to the presence of FA only found in conifers and a few other plants, Δ5-UPIFA. In addition, more lipid species have been identified in this thesis than have been found in conifers before.

Water deficit and dehydration have been identified as an immediate concern in balsam fir postharvest, and the major problem leading to abscission. This has been confirmed in many prior balsam fir studies by testing per cent moisture, xylem pressure potential and relative water content. Water deficit stress can lead to biophysical changes such as decreased stomatal conductance, decreased fluorescence, and increased membrane injury. New knowledge has been added through this thesis about the changes in polar lipids and fatty acids (Figure 49) that can further our knowledge and help explain these biophysical

changes. On the other hand, changes found in polar lipids and balsam fir postharvest can also be related to confirmed PGR changes that have been noted postharvest, such as elevated ABA, ethylene, terpenes, and decline of IAA.

It has been generally accepted, but never tested in balsam fir, that stresses trigger the generation of free radicals, and ROS in turn trigger lipid peroxidation (Hodges et al., 2004). This thesis confirms, in all clones tested, that there is a decline in acyl lipids postharvest. Loss of acyl lipids has been known to occur during water-deficit stress, but it is impossible to say that such changes are reflective of progressive dehydration or water deficit. This could be linked to ABA, phospholipase D production, and lipolysis (Kar, 2011). Hanrouni et al. (2001) studied water-deficit stress in safflower aerial parts and found that severe stress caused a sharp decrease in polar lipids. Studies prior to that showed comparable results (Svenningson and Liljenberg, 1986; Hubac et al., 1989). More recently, Martins Junior et al. (2008) found that there was a gradual loss of polar lipids during water-deficit stress. In this thesis, water deficit was confirmed by a sharp drop in capacitance after week one postharvest and subsequent decreases.

Cellular membranes are the first part of the cell to perceive a change, therefore, the main target of water deficit damage. However, in plants, the chloroplast membranes are usually the first membranes to deteriorate during stress. Lipid composition is the main determinant of the ability of plants to deal with water deficit stress. This thesis is the first study on balsam fir polar lipids postharvest, and the results show significant MGDG breakdown postharvest increasing the MGDG: DGDG ratio in three clones of balsam fir. This suggests lipolysis, which could be induced by ABA, or ABA *via* ethylene, production (Lada and MacDonald, 2015). The poorer NRD clone shows a decline by week four postharvest, earlier than the high NRD clone. These results in balsam fir postharvest are typical of water deficit stress in other plants. Drought provokes a decrease in polar lipid content of leaves, the GL being more affected than the phospholipids (Campos and Thu, 1995). The most susceptible lipid class is MGDG. Moreover, this thesis has revealed the lipid species that are most affected during postharvest in balsam fir, MGDG 36:5, 36:6 and 36:7. I would recommend taking 20 low NRD and 20 high NRD clones, and doing further testing on

MGDG, DGDG, and the MGDG: DGDG ratio to search for an eventual correlation between the ability of balsam fir postharvest to resist water stress and some characteristics of their membrane lipids. The decrease in GL content could be due to an increase in galactolipase activities and/or to a decrease in galactolipid biosynthesis (Campos and Thu, 1995).

Typically, MGDG contains large amounts of linolenic acid (α-18:3n3). As previously stated, the results in this thesis identify MGDG 36:6 and 36:7 as the main lipid species reduced postharvest, so reduction in linolenic acid makes sense. The FA tails would be 18:3-18:3, and 18:4-18:3, respectively. The content of linolenic acid in leaf cells from other plants in past studies dramatically decreased under water deficit, due to an inhibition of the biosynthesis of linoleic acid (C18:2) and C18:3 (Pham Thi et al., 1987), and possibly due to lipid degradation (Monteiro de Paula et al., 1993). It was thought that these were possible related to the drought activation of MGDG-hydrolases (El-Hafid et al., 1989; Sahsah et al., 1998), and to the fact that polyunsaturated fatty acids are preferential substrates for peroxidative breakdown (Ferrari-Iliou et al., 1993). However, in balsam fir, it has been found that there are Δ5-UPIFA, and one of these is pinolenic acid, 5,9,12 – 18:3 confuses the issue. There has been very little study on pinolenic acid, so its role during postharvest lipid degradation is not known. There have also been genotype differences in other compounds that are reduced postharvest. Some show a reduction in 18:2 *cis* and some 5,9,12 15-18:4. To determine if Δ5-UPIFA are present in the chloroplasts, it would be useful to isolate intact chloroplasts from balsam fir needles and analyze the lipids and fatty acids present.

The premier environmental factor to play a role in postharvest needle abscission is cold acclimation. In this thesis, it is evident that there is a co-occurring change in DGDG and MGDG, possibly related to the known increase in ABA during cold acclimation. DGDG significantly increased by 13% and MGDG decreased by 35%. There were significant changes in four lipid species causing the change in the ratio: DGDG 36:6, MGDG 36:5; MGDG 36:6, and MGDG 36:7, the first two significantly increasing and the latter two decreasing. This was similar in all clones. In addition, there was an decrease in the GL: PL

ratio. However, the objective of this thesis was to identify differences in low and high NRD. The main difference was found in the FA unsaturation that occurred during cold acclimation. It has been found that the Clone 566, the clone that consistently had the lowest NAR , increases its percentage of α-linolenic acid during cold acclimation, where as the other three clones increase their percentage of a Δ5-UPIFA, pinolenic acid (5, 9, 12 – 18:3), primarily found in conifers. An increase in α-linolenic acid is known to increase membrane fluidity which perhaps explains why sometimes poor clones perform better after cold acclimation (Thiagarajan, 2012). There is not much known about the role of pinolenic acid in trees, however, pinolenic acid in pine seeds has been found to have huge health benefits in humans by activating certain enzymes (Xi et al., 2016). If pinolenic acid is used to add to membrane stability to chloroplasts in better needle retaining clones, possibly these clones are getting added protection from this accumulation of pinolenic acid. Pinolenic acid does not degrade postharvest, so could be more stable than α-linolenic acid.

There is a fine line between membrane fluidity and photosynthetic efficiency in leaves. The degradative nature of PUFA make them undesirable as part of the thylakoids. It may be that better clones have achieved photosynthetic efficiency with less PUFA. Another important finding in this thesis is that the best clone has more of the most plentiful SFA, 16:0. There seems to be an apparent opposition between a good fluidity and high thylakoid content, and membrane tolerance to water deficit stress. The fact that chloroplast membranes of resistant genotypes contain less polyunsaturated MGDG molecules and this could be related to the well-known lower yield of drought-resistant plants compared to sensitive ones when they are placed in irrigated conditions (Parsons, 1982).

Other notable changes that occurred in both high and low clones postharvest was the decrease in 7-16:1, and the increase in PA by peak abscission. MUFA 7-16:1 is associated with TAG. We also saw plastoglobuli, lipoproteins in TEM studies. This stored lipid may be used up during stress. As for PA, it of great metabolic importance. Signal transduction is at the heart of stress response. PA has been found to stimulate ABA production, which in turn results in stomatal closure and also inhibits them from opening (Bastian et al., 2006). However, according to Ruth Welti et al, 2002, there is often a 20 times increase in times

of stress, but PA is very quick to form and not long lasting so can be difficult to capture in analysis.

Another biophysical change realized in this research was stomatal closure. Using the SEM, it was clear that almost all stoma were closed. This is typical of water deficit stress, in an effort to conserve water. In balsam fir this is commonly found postharvest, stomatal conductance decreasing by 50% within four days of harvest and 80% by the end of the first week (Lada and MacDonald, 2015). This SEM study showed a clogging of the stomatal pores with what appeared to be wax by the time of abscission, possibly in an effort to conserve water. It appeared that there was more buildup in the low NRD clone that might mean they were suffering more from the water-deficit stress, but this was a very subjective study with only observations made at the first and last of the experiment. It would be more conclusive to look at temporal samples postharvest at more frequent intervals. There also seemed to be more contamination by fungi and bacteria in the low NRD clone, again very subjective.

TEM studies showed thylakoid swelling one week postharvest, an indication of stress. There was also a reduced size by almost 50% of the chloroplast. The changes seen in this study are typical of changes caused by ethylene production. In senescence the opposite is usually seen – plastoglobuli greatly enlarged as well as cell enlargement. Differences between clones were, i) noticeable starch stores, week one in the low NRD clone, ii) by week two, there were fewer chloroplasts in the low NRD clone, possibly due to ROS marking them for recycle in the cell, iii) more disorganization of the chloroplast at peak abscission. This study was very subjective, but it did seem like the high NRD clone was better able to cope postharvest.

In summary, most specifically, there is a huge decline in MGDG, the monolayer lipid, and a significant decrease in the MGDG/DGDG ratio postharvest in balsam fir (Fig. 49). This thesis suggests that there is a strong negative relationship between fluorescence and needle abscission postharvest, and both MGDG and DGDG, the main chloroplast lipids, have declined postharvest. TEM, in turn, confirmed chloroplast breakdown. Timing of the

breakdown seems to be one difference between the low and high NRD clone, with the low clone showing earlier MGDG degradation and more progressive decline when observed under the TEM. Linolenic acid, a major component of MGDG, is reduced in all postharvest studies in this thesis. However, in cold acclimation the high NRD clone assimilates pinolenic acid, not linolenic, suggesting a genotypic preference. More study is needed in the area of lipids and fatty acids as related to postharvest needle abscission in balsam fir in the future. It is also worth mentioning, in regards to future studies, that lipid and fatty acid analysis is very labor intensive and difficult to make sure all the lipids are extracted. Another method that is not invasive could be used, Raman Bifocal Spectroscopy. This helps to quickly give you a chemical image with out all the intensive labor. There are, however, some limitations as it is difficult to determine lipid species. If you want to analyze only for lipid classes and FA this could save a lot of time (Personal Communication: Dr. Ruth Welti, Kansas Lipidomic Research Center, Kansas State University, Kansas).

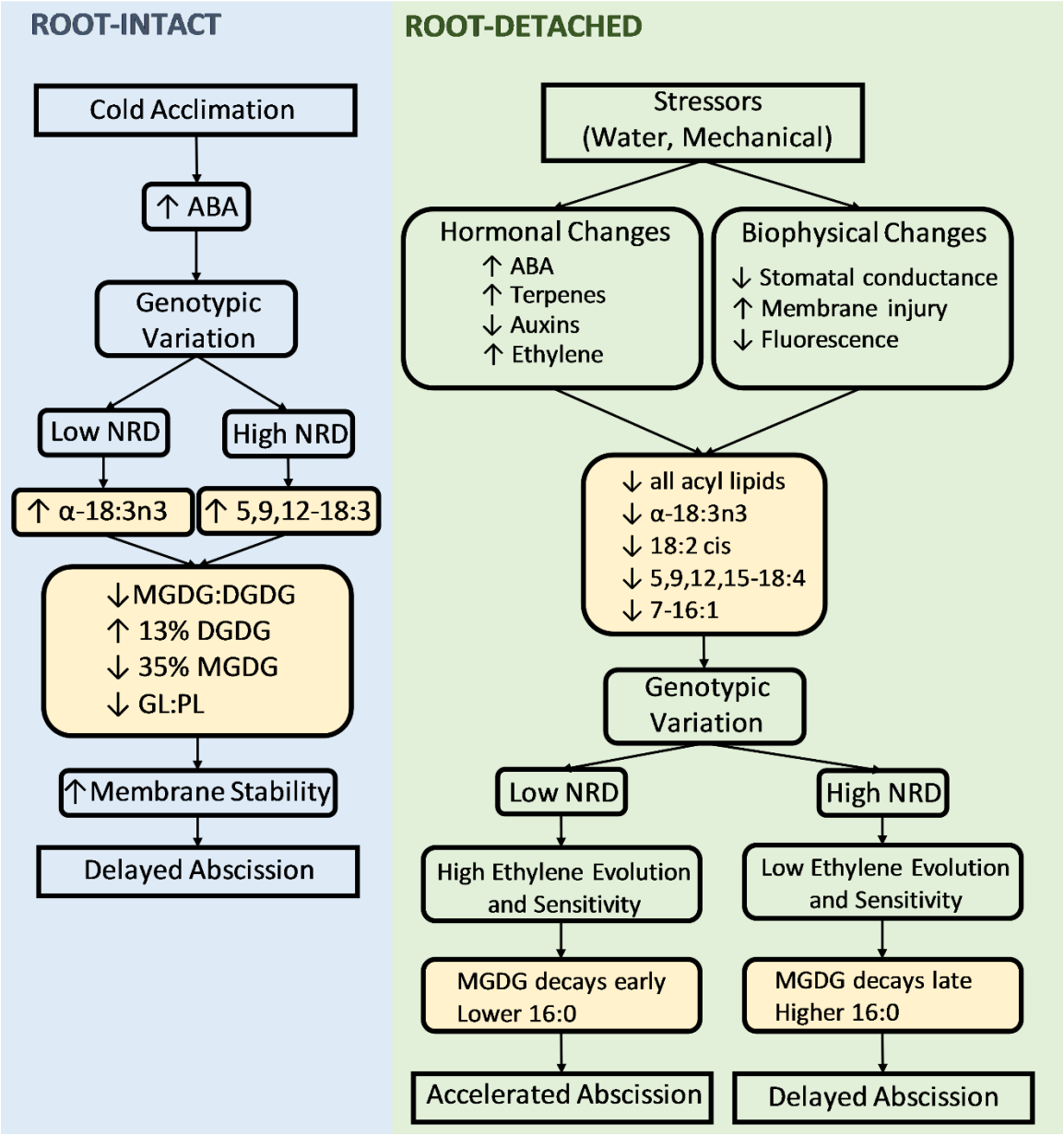

Figure 49. Proposed schematic of role of lipids in abscission. 5,9,12-18:3 and 5,9,12,15 -18-4 are both Δ5-UPIFA.

10.2 CONCLUSIONS

(i) To characterize the nature of polar lipids and FA (FA) in balsam fir to determine links between polar lipid and FA changes in balsam fir postharvest and needle loss.

There were 240 polar lipids identified by mass spectrometry and 20 FA identified by gas chromatography. In the needles of balsam fir postharvest in Clone 706, there was a decrease in total polar lipids, total FA, and total PUFA postharvest. MGDG and DGDG both showed a significant drop in overall percentage, and the percentage of PC increased. There was a significant decrease particularly in 18:3n3. Loss of 18:3n3 correlated strongly with postharvest needle abscission, as did 18:2 *cis*. USFA: SFA ratios also changed significantly from 4:1 to 1:1 from initial sampling to peak abscission significantly lowering the UI suggesting more SFA and fewer unsaturated. There was a high negative correlation between the ratio of USFA: SFA and postharvest needle abscission further suggesting a decline in USFA postharvest in balsam fir.

(ii) To determine the differences in polar lipid and FA in contrasting genotypes of high (Clone 506) and low (Clone 9) needle retention duration (NRD)

There was a decrease in polar lipids in balsam fir over time in a high and low NRD clones postharvest suggesting a degradation of cellular lipids in both. There was a major decline in MGDG pointing towards chloroplast breakdown and lowering the MGDG to DGDG ratio. Changes were clearly seen in a few lipid species, a decline in MGDG 36:6, MGDG 36:7, DGDG 36;6 and an increase in MGDG 36:5. PA also increased significantly by week ten in both clones. There was a decrease over time postharvest in mean percentage of USFA 5,9,12 15-18:4, 18:3n3, and 7-16:1. Other FA, SFA 22:0 and 20:0, and PUFA 5,9 18:2, and cis18:2, showed a significant increase in both clones. 7-16:1 in

both clones dropped approximately 40% and in the first week postharvest. There were some differences between the high and low NRD clones that could be important. In polar lipids, there was a decline in MGDG and DGDG species by week four in the low NRD clone, but these occurred later in the high NRD clone. This suggests a breakdown of the chloroplast membranes sooner postharvest in the lower NRD clone than in the higher NRD clone.

(iii) To determine changes in polar lipids and FA profiles in needles sampled monthly in four different clones, 2 high (Clones 506 and 37) and 2 low (Clones 9 and 566) NRD, onsite from September through to February in Nova Scotia, to determine differences in lipid metabolism.

There was a redistribution of polar lipids during cold acclimation in all genotypes of balsam fir tested. The polar lipid MGDG decreased during the fall and early winter with a subsequent increase in DGDG and a significant decrease in the MGDG: DGDG ratio.

(iv) To identify genotypic differences in polar lipids and FA, if any, onsite and at each sampling period during the process of cold acclimation.

There was significantly less DGDG in clone 506 than in Clone 9. PC and PI were significantly less in clone 506 than in Clones 9 and 37. LPG was significantly higher in 506 and 566. There is no one lipid class unique to just Clone 506. PC 36:3, PC 37:3, and 38:5 are significantly less in Clone 506 than all other clones assessed in this work. PG 35:3 and PG 35:2 are less in Clones 506 and 37 than 9 and 566. LPG 16:1 is eight times as much in Clone 506 than some of the poorer clones, although only slightly more than Clone 566. This data could potentially be linked to greater needle retention postharvest in high NAR clones.

FA 16:0 was lowest in Clone 566 clone, a lower NRD clone, and relatively high in Clone 506, a high NRD clone, but between days 106 and 144 decreased by 24% in Clone 506 and increased by 20% in Clone 566. Conversely, FA 18:1 increased by 35% in Clone 506 and decreased by 10% in Clone 566 in the same time span. FA 18:2 increased in all clones in the first 68 days, then decreased in all clones with the exception of Clone 506.There is a significant increase in FA unsaturation in some FA. The mean percentage 5, 9, 12 – 18:3 (pinolenic acid) increased in Clones 506, 9, and 37 and there was a significant decrease in α – linolenic acid (18:3n3). This occurred in reverse in Clone 566, a significant increase in α – linolenic acid with a decrease in pinolenic acid. This suggest a different mode of metabolism in one of the poorer Clones. FA, 5, 11, 14-20:3, was significantly lower in the high needle retaining genotype (506) than any other genotype.

(v) To examine the ultrastructure of membranes in a low and high NRD clone of balsam fir postharvest using scanning and transmission electron microscopy, SEM and TEM, respectively.

TEM showed evidence of stress in balsam fir postharvest, characterized by smaller chloroplasts, swollen thylakoids, and less starch production over time. Clone 566 was found to show signs of breakdown sooner than Clone 506, with intact chloroplasts becoming very difficult to find. There was a possibility of autophagy due to the difficulty of finding chloroplasts especially, in the lower NRD clone. It also appeared that necrosis of cells had occurred by week 11 in both clones, but the lower NRD clone showed greater disorganization of the chloroplast. The stomata were closed, typical of a plant with water deficit stress. However, it was week 11 so more research is needed to find out when the stoma close. Fungi was adhered particularly to the low NRD clone. By week 11 postharvest there was hyphae on both clones in addition to bacteria, possibly related to decomposition. Membrane blebbing was noted in week 11 using the

TEM, but no evidence was seen in the SEM observations, possibly due to the hard cuticle.

Research should continue in this area of lipids and fatty acids to confirm their role in abscission postharvest in balsam fir. There have been many postulations made in this thesis. Testing more genotypes for lipid and fatty acid changes, in combination with TEM and SEM work could substantiate the evidence found in this thesis. Electron microscopes are powerful tools, particularly the combination of SEM and TEM. The amalgamation of quantitative and qualitative studies is of great value for scientific research.

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