CHARACTERIZATION AND PHYSIOLOGICAL SIGNIFICANCE OF VOLATILE TERPENE COMPOUNDS (VTCs) IN POSTHARVEST NEEDLE ABSCISSION OF BALSAM FIR (*ABIES BALSAMEA* (L.) MILL.)

by

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for the degree of Master of Science

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The undersigned hereby certify that they have read and recommend to the Faculty of Graduate Studies for acceptance a thesis entitled "CHARACTERIZATION AND PHYSIOLOGICAL SIGNIFICANCE OF VOLATILE TERPENE COMPOUNDS (VTCs) IN POSTHARVEST NEEDLE ABSCISSION OF BALSAM FIR (*ABIES BALSAMEA* (L.) MILL.)" by Ernest Asante Korankye in partial fulfillment of the requirements for the degree of Master of Science.

Dated: March 12, 2013

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Signature of Author

Dedication

This thesis is dedicated first of all to my lovely wife, Mrs. Anita Asante Korankye for her immense support, encouragement and love. To my parents, Mr. and Mrs. Korankye Sarfo for their prayers and all who have supported me throughout this journey.

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Abstract

In the quest to understand the physiological basis of postharvest needle loss in balsam fir, we hypothesized that, volatile terpene compounds (VTCs) have a role in needle abscission. This study focused on understanding the role of VTC's in postharvest needle abscission. We demonstrated that balsam fir contains twelve VTCs with varying concentrations depending on whether it is a seedling or a clonal tree branch. Total VTC concentration consistently increased prior to needle loss. Five specific VTCs (β-Pinene, β-Terpinene, Fenchyl acetate, Camphene and 3-Carene) have been identified as possible key signal molecules in needle abscission. VTCs were synthesized independently of ethylene, thus VTCs can be a possible signal molecule to needle abscission. Exposure of branches to ethylene showed an increase in both ethylene and VTC however, total VTC concentration was below the threshold required to cause needle abscission.

List of Abbreviations Used

ACC	1-aminocyclopropane-l-carboxylic acid
ANOVA	Analysis of variance
AVG	Aminoethoxyvinylglycine
AWU	Average water use
AZ	Abscission zone
BWA	Balsam woolly adelgid
CO_2	Carbon dioxide
CRC	Christmas tree Research Centre
CTCNS	Christmas tree Council of Nova Scotia
DCM	Dichloromethane
DPP	Dimethylallyl pyrophosphate
FID	Flame ionization detector
FPP	Farnesyl pyrophosphate
GC	Gas chromatography
GGPP	Geranylgeranyl pyrophosphate
GLM	General linear model
GAP	Glyceraldehyde 3-phosphate
GPP	Geranyl pyrophosphate
IAA	Indole-3-acetic acid (auxin)
IPP	Isopentenyl pyrophosphate
JA	Jasmonic acid
MeJA	Methyl jasmonate
MEP	2-C-methyl-D-erythritol 4-phosphate
MS	Mass spectrophotometer
MVA	Mevalonic acid
NAR	Needle abscission resistance
NRD	Needle retention duration
NSERC	Natural Sciences and Engineering Research Council
NV	Needle volatiles
O_2	Oxygen
PA	Peak area
PGR's	Plant growth regulators
PIN	Production of proteinase inhibitor
PR	Pathogenesis-related
SAM	S-adenosyl methionine
SAS	Statistical analysis system

SPME	Solid-phase microextraction	VTCs	Volatile terpene compounds
TPP	Thiamine pyrophosphate	XPP	Xylem pressure potential
USDA	United States Department of Agriculture		

Acknowledgements

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CHAPTER 1: INTRODUCTION

Balsam fir (*Abies balsamea* L. (Mill.)) has been a major contributor to specialty horticulture, the Christmas tree and greenery industries since, 1851 (Albers and Davis, 1997). It is native to eastern North America, extending from Atlantic and central Canada to northern Virginia and West Virginia (Figure 1). It is one of the most popular Christmas trees and preferred over Douglas fir (*Pseudotsuga menziesii*, Mirb.), white spruce (*Picea glauca*, Schwer.) and eastern red cedar (*Juniperus virginiana*, L.) due to its shape, blue-green color and a distinctive pleasant fragrance (CTCNS, 2011).

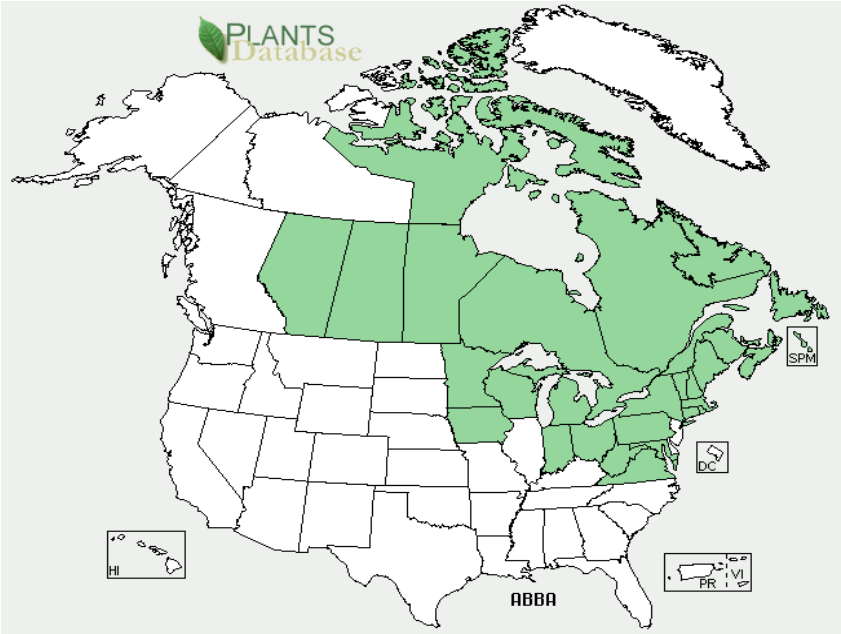


Figure 1: Map of Canada and United States. Naturally occurring balsam fir can be found in the green shaded region, largely in eastern and central Canada (adopted from United States Department of Agriculture, NRCS (2012)).

It is estimated that approximately 6 million trees are produced annually in Canada (Statistics Canada, 2010), and over 20 million in the United States (USDA, NRCS (2012)). Out of that, Nova Scotia alone produces 1.8 million trees annually on 23,450 acres of land. The trees are valued at approximately $30 million of which 95% is

exported to the United States, Antigua, Aruba, Bahamas, Barbados, Bermuda, Cayman Islands, Cuba, France, Jamaica, Netherlands Antilles, Panama, Thailand, United Arab Emirates and Venezuela (CTCNS, 2011 and Statistics Canada, 2012). Overall, the world market value is estimated to be $1.85 billion with Canada contributing $50 to $100 million, annually (Statistics Canada, 2012). It is noted that demand for fresh cut Christmas trees in Canada has declined tremendously in recent years. Since 2001, farm cash receipts for fresh cut Christmas trees in Canada alone have declined by more than 62.5%. The current value of farm cash receipts for Christmas trees in Canada is $51.3 million, which is a 9% decrease from $56.6 million in 2010. On the other hand, the importation of artificial Christmas trees currently stands at $47 million, a 43% increase since 2001. Out of that, 98% comes from China and the rest from Thailand, United States, Mexico and Vietnam (Statistics Canada, 2012).

Postharvest needle abscission has been an enormous problem in the Christmas tree industry in recent years despite the much celebrated culture of Christmas trees during Christmas around the world as well as its multi-million dollar industry. The problem of postharvest needle abscission has been noted as a significant source of consumer dissatisfaction of natural trees (Chastagner and Riley, 2003), and may have contributed to a reduction in their demand and increase in demand for artificial trees since 2001 (Statistics Canada, 2012).

Postharvest needle abscission has been attributed to several environmental, biological, genetic and post-harvest factors such as rigorous harvesting techniques, rough handling and long transportation of the trees before they get to consumers (Sexton, 1982). It is also speculated that a lack of enough cold acclimation of trees due to warmer fall temperatures

along with early harvesting of trees to satisfy high demand of consumers have the potential to induce needle abscission (Mitcham-Butler et al. 1987a; Rajasekaran and Thiagarajan, 2006; MacDonald and Rajasekaran, 2008). Another area which has been explored relates to post-harvest plant defense mechanisms to help overcome mechanical injury caused by harvesting and insects. Linked to plant growth regulators (PGR's), these trees respond to injuries by producing phytohormones such as ethylene and/or jasmonic acid (Schmeltz et al. 2003), which can speed up the process of needle abscission (MacDonald et al. 2010).

In drawing the big picture on needle abscission, nothing is known about the role of organic volatile terpene compounds (VTCs) in postharvest needle abscission. It is well established that balsam fir is one of the most preferred conifer species due to its distinctive aroma, which is caused by the release of VTCs from the foliage and other tissues (Schmeltz et al. 2003). Under stresses such as insect attack and mechanical injuries, balsam firs are known to produce excess VTCs for their defense (Carlow et al. 2006). Although the emission of plant volatiles is speculated to be dependent on both ethylene and jasmonic acid (Schmeltz et al. 2003), little is known about the role of VTCs in postharvest needle abscission. A study by Horiuchi et al. (2001) demonstrated that the exogenous application of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid to detached lima bean leaves enhanced jasmonic acid (JA)-induced VTCs emission, suggesting a possible relationship between ethylene and VTCs.

Thus, the need to establish a relationship between VTCs and postharvest needle abscission and its inter-relationship between ethylene, the signal molecule that induces postharvest needle abscission in balsam fir and VTCs.

CHAPTER 2: LITERATURE REVIEW

2.1 Abscission in plants

2. 1.1 Process of abscission

Abscission is described as a natural separation of cells, tissues, or organs (e.g. leaves, flowers, fruits, seeds) from the parent plant (Addicott, 1982). In plants, abscission often occurs as a beneficial process, which provides a mechanism for the removal of senescing or otherwise damaged organs to help promote growth (Bleecker and Patterson, 1997), especially under stress. Under water stress, plants go through the process of abscission to shed their leaves in order to maintain balanced gas exchange and transpiration (Taylor and Whitelaw, 2001). Invasive stresses from wounds or pathogen attacks and diseases have also been found to result in abscission of infected plant parts to avoid the spread of infection (Taylor and Whitelaw 2001), although in some cases, intact and healthy organs after harvest may shed as a response to the process of plant defense mechanism (Bleecker and Patterson, 1997).

Abscission is a coordinated sequence of multiple changes in cell structure, metabolism and gene expression (Brown and Addicott, 1950) leading to wall digestion in well-defined cell layers known as an abscission zone (AZ). Similar to broad-leafed plant species, the AZ in conifer species often evolves during the development of associated organ system and is generally characterized as a band of small, square shaped, densely cytoplasmic cells ranging from a few to many cell layers thick (Figure 2a) (Sexton and Roberts, 1982; Taylor and Whitelaw, 2001). The separation of organs from the parent plant is often preceded by the enlargement of cells in the AZ. The middle lamellae

between the cells then dissolves, resulting in the formation of a fracture plane across the stem (Figure 2b) (Addicott, 1982; Bleecker and Patterson, 1997). In combination with mechanical forces such as wind, abrasion or internally generated force by the cells, effective abscission is achieved. Abscission is then followed by the continuous enlargement of the cells on the proximal face of the fracture plane and, ultimately, the differentiation of these cells into suberized scar tissue (Addicott, 1982). Unlike broad-leafed trees, conifers do not shed all of their needles annually, but retain needles for two to three years before shedding them making them one of the best plant species in retaining leaves (Sexton and Roberts, 1982).

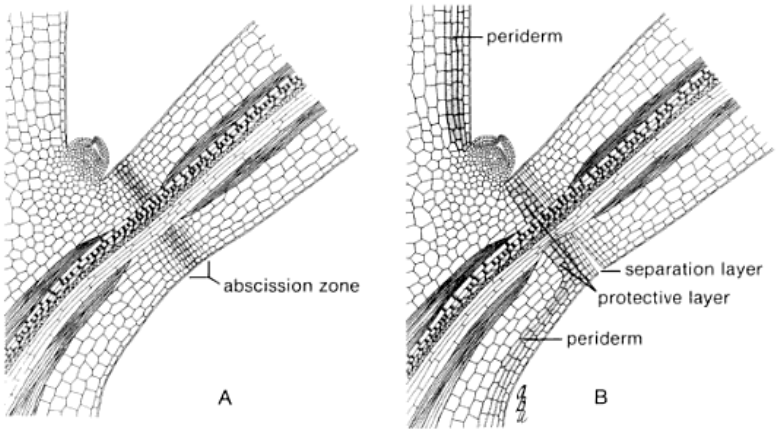


Figure 2: Diagrams of the tissues in a typical leaf abscission. a) Shortly before separation, with smaller and less differentiated cells in the AZ. b) At time of abscission with compressed vascular tissues by developed protective layer. Adopted from Addicott (1982).

2.2 Biotic and abiotic factors causing abscission in plants

2.2.1 Genotypic variation influence on abscission

The number of cells that make up an abscission zone has been surprisingly found to vary from species to species (Addicott, 1982). Research shows that the separation process in

the pedicel abscission zone of tomato flowers takes place between only two discrete, closely packed layers of cells (Tabuchi and Arai, 2000). In comparison, the abscission zone at the base of the leaflet of *Sambucus nigra* is composed of up to 50 cell layers, which during the abscission process separate over many cell layers (Tabuchi and Arai, 2000). The number of cells that make up an abscission zone is known to be fixed for particular organs in a species, but varies among species (Addicott, 1982).

Conifer species are known to distinctively differ in needle abscission characteristics depending on the age of tree, season and post-harvest environmental exposure. When five different Christmas tree species; *Pinus sylvestris* L., *Picea abies* L., *Tsuga heterophylla*, *Chamaecyparis lawsoniana* and *Thuja plicata* were studied for their needle qualities, it was discovered that needles of younger trees were high in moisture and nitrogen but low in fiber. Therefore, younger trees lost needles more easily when compared to mature trees, and this varied among all the species (Hatcher, 1990). Similar work done by Chastagner and Riley (2003) also reported that Nordmann fir (*Abies nordamnniana*) trees have high quality foliage and better needle retention characteristics than Noble fir (*Abies procera*) trees. In this same work, it was proposed that needle retention is a highly hereditary characteristic and through breeding strategies, genotypes with superior needle retention traits can be identified. However, the knowledge on the genetic makeup of ecotypes in governing the abscission processes in Christmas trees especially, balsam firs, is limited. Studies investigating genotypic influences on the abscission processes would provide opportunity to identify superior genotypes with better postharvest qualities as well as ability to withstand water, temperature and invasive stresses.

2.2.2 Stress influences on needle abscission

Drought and other stresses such as high temperatures, cold and salt cause a decline in plant water status, and promote organ abscission (Taylor and Whitelaw, 2001). As water deficit develops, there is a rapid decline in leaf expansion resulting in stomatal closure (Chaves, 1991) with consequences on transpiration, photosynthesis, growth and developmental processes that promote abscission (Jones et al. 1980; Thimann et al. 1982). A study by MacDonald et al. (2011) showed that there is a consistent positive relationship between water use and needle abscission in balsam fir. Branches with low needle retention (as a result of long-term ethylene exposure) used 80% more water than non-ethylene treated branches. This confirmed reports by several studies that, exposure to ethylene slows down the closure of stomata (Tanaka et al. 2005) thereby, increasing the rate of transpiration (Azuma et al. 2003).

During invasive stresses, which involve penetration of the plant tissue either through wounding or pathogen attack, plants respond by inducing a defense response through a substantial alteration in gene expression. This reinforces the cell wall by deposition of callose, lignin and hydroxyproline rich in glycoproteins, synthesis of antimicrobial compounds such as phytoalexins (terpenoids, glycosteroids and alkaloids) and production of proteinase inhibitor (PIN) and pathogenesis-related (PR) proteins such as chitinases and endo-β1-1,3-glucanases (Ryan, 1987). If the defense response is unsuccessful and pathogen invasion occurs, then the plants shed their infected organ, in order to prevent the spread of infection throughout the plant and its neighbors (Taylor and Whitelaw, 2001). Such a defense is speculated to have a possible relationship with organic volatile compounds. Studies by Carlow et al. (2006) showed that Fraser fir trees infected by

balsam woolly adelgid tend to produce 50% more volatile compounds than non-infected trees, eventually causing drop of most of the needles.

2.2.3 Postharvest factors on needle abscission

Dehydration has long been known to trigger abscission, which may subsequently affect postharvest needle retention (Addicott, 1982). A study of conifers such as Atlantic white cedar for their postharvest qualities has found that needles dry rapidly after harvesting resulting in rapid loss of needles mass (Hinseley and Snelling, 1991). Moisture loss from trees harvested during warm periods is known to rapidly lead to heavy needle losses (Hinesley and Chastagner, 2004). For that reason, baling of trees with moisture proof materials during storage and transportation is suggested to decrease the moisture loss and hence improve the needle retention qualities (CTCNS, 2011). Although detailed information on postharvest storage and transport conditions is not available for balsam fir, controlled atmospheric storage has been shown to effectively reduce the rates of oxidation, transpiration, respiration, and ethylene production in order to delay abscission (Gorny, 1997). Much of the work investigating how these conditions induce organ shedding has focused on the associated hormonal changes in an attempt to establish a causal link.

2.3 Role of plant growth regulators on abscission of plants

2.3.1 Ethylene

Ethylene, a simple unsaturated hydrocarbon, regulates many diverse metabolic and developmental processes in plants (Abeles et al. 1992). It is derived from the amino acid, methionine, which is converted to S-adenosyl-methionine (SAM) by SAM synthase.

SAM serves as an intermediate in a variety of synthetic pathways, including polyamines. SAM can be converted into 1-aminocyclopropane-l-carboxylic acid (ACC) through ACC synthase, and it is the first committed step in the production of ethylene (Adams and Yang, 1979). The final step converts ACC to ethylene with the enzyme ACC oxidase in the presence of oxygen (Yang and Hoffman, 1984).

Plant growth and development is known to be profoundly affected by ethylene, although it is usually associated with fruit ripening (Alscher and Cumming, 1990). However, there are several senescence processes that are regulated by ethylene; such as fading of flowers and abscission of petals (Bleecker and Kende 2000). From the limited work done on the role of ethylene in postharvest needle abscission in conifers, it is clear that ethylene induces needle abscission in much the same manner as it induces fruit, petal, and leaf abscission in other species (MacDonald et al. 2009, 2010, 2011). Several approaches have been used to confirm the role of ethylene in abscission such as, the use of ethylene synthesis inhibitor, aminoethoxyvinylglycine (AVG) and the ethylene receptor blocker, 1-methylcyclopropene (1- MCP) to inhibit the conversion of S-adenosyl-L-methionine (SAM) to 1-aminocyclopropane-1-carboxylic acid (ACC) to delay senescence and reduce abscission (Beyer, 1976; Rath et al. 2006; MacDonald et al. 2010).

Ethylene has not only been found to be an important regulator of abscission but also it is known to work concurrently with auxin (IAA). IAA is known to retard abscission, whilst ethylene is a potent accelerator of the abscission process (Addicott, 1982). The general rule depicts that, provided the flux of IAA to the abscission zone region is maintained, cell separation is inhibited and abscission does not occur (Sexton et al. 1985).

2.3.2 Jasmonic acid

Jasmonic acid (JA) is another PGR that is believed to be derived from fatty acids. It has wide ranging natural effects on higher plants especially in plant defense against insects, pathogen and wounds (Meyer et al. 1984). In the target cell, systemin, an 18-amino acid polypeptide hormone, produced during the initial stages of tissue wounding, binds to a site on the plasma membrane to initiate biosynthesis of JA (Creelman and Mullet 1997). JA is also known to regulate a variety of plant development processes, including root growth, tendril coiling, and abscission (Andresen et al. 1992). In conifers such as Norway spruce [*Picea abies* L. (Karst)], using methyl jasmonate (MeJA) to induce defensive responses cause a 2-fold increase in monoterpene and sesquiterpene accumulation in needles without changes in terpene composition (Martin et al. 2003). JA has also been shown to stimulate production of ethylene and volatile organic compounds (Schmeltz et al. 2003). In this same study, results strongly support the role of JA in the regulation of volatile emissions but also suggest that ethylene production regulates the magnitude of volatile emissions during postharvest plant defense. Despite the body of work on JA and its relation to abscission in many plants such as oats and barley (Weidhase et al. 1987), there is little information on its role in postharvest needle abscission.

2.4 Secondary metabolites: the role of volatile organic compounds (terpenes) in organ abscission

2.4.1 Secondary metabolites

Unlike plant primary metabolites such as chlorophyll, amino acids, nucleotides, simple carbohydrates and membrane lipids, secondary metabolites are made up of diverse array

of organic compounds that differ in distribution and appear to have no direct function in plant growth and development (Taiz and Zeiger, 1998). For many years, the adaptive significance of most plant secondary metabolites was a mystery, until more recently, when it was suggested that they help to protect plants against herbivores, infections by microbial pathogens and mechanical damage such as wounding (Taiz and Zeiger, 1998; Vereen et al. 2000). Secondary metabolites are made up of three distinct groups: Phenolics (aromatic substances), nitrogen-containing compounds (alkonoids), and terpenes (Buchanan et al. 2000).

2.4.2 Terpene biosynthesis and distribution

Terpenes are biosynthesized from primary metabolites through two different pathways: mevalonic acid (MVA) and 2-C-methyl-D-erythritol 4-phosphate (MEP) pathways (Buchanan et al. 2000). In the well-studied mevalonic acid pathway, three molecules of acetyl CoA are joined together stepwise to form mevalonic acid (Figure 3a) (Taiz and Zeiger, 1998). The key six-carbon intermediate is then phosphorylated, decarboxylated, and dehydrated to yield isopentenyl pyrophosphate (IPP) (Lichtenthaler et al. 1997). The second pathway is the pyruvate/ glyceraldehyde 3-phosphate pathway, which uses the plastid-localized route to produce IPP. Pyruvate reacts with thiamine pyrophosphate (TPP) to yield a two carbon fragment, hydroxyethyl-TPP, which condenses with glyceraldehyde 3-phosphate (GAP). TPP is then released to form a five-carbon intermediate, 1-deoxy-D-xylulose 5-phosphate, which is rearranged and reduced to form 2-C-methyl-D-erythritol 4-phosphate and subsequently transformed to yield IPP (Figure 3b) (Buchanan et al. 2000). Inhibition of IPP by either mevastatin (inhibitor of IPP from

MVA pathway) or fosmidomycin (inhibitor of IPP from MEP pathway) is known to obstruct volatile terpene synthesis (Hampel et al. 2005).

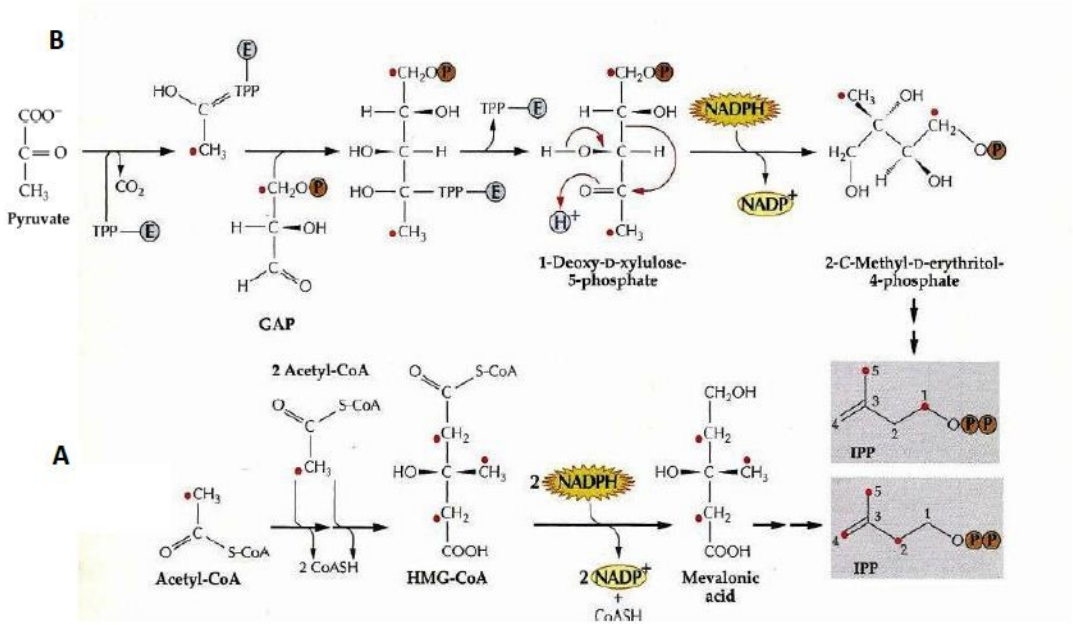


Figure 3: (a) Mevalonic acid (MVA) and (b) 2-C-methyl-D-erythritol 4-phosphate (MEP) pathways for the formation of IPP. Adopted from Buchanan et al. (2000).

IPP and its isomer, dimethylallyl pyrophosphate (DPP), are the activated five-carbon building blocks of terpene biosynthesis that join together to form larger molecules (Figure 4). First, IPP and DPP react to give geranyl pyrophosphate (GPP), the ten-carbon precursor of all the monoterpenes. GPP can then link to another molecule of IPP to give 15-carbon compound farnesyl pyrophosphate (FPP), the precursor of all the sesquiterpenes. Addition of yet another molecule of IPP gives the 20-carbon compound geranylgeranyl pyrophosphate (GGPP), the precursor of the diterpenes. Finally, FPP and GGPP can dimerize to give the triterpenes (C_{30}) and the tetraterpenes (C_{40}), respectively (Figure 4) (Buchanan et al. 2000).

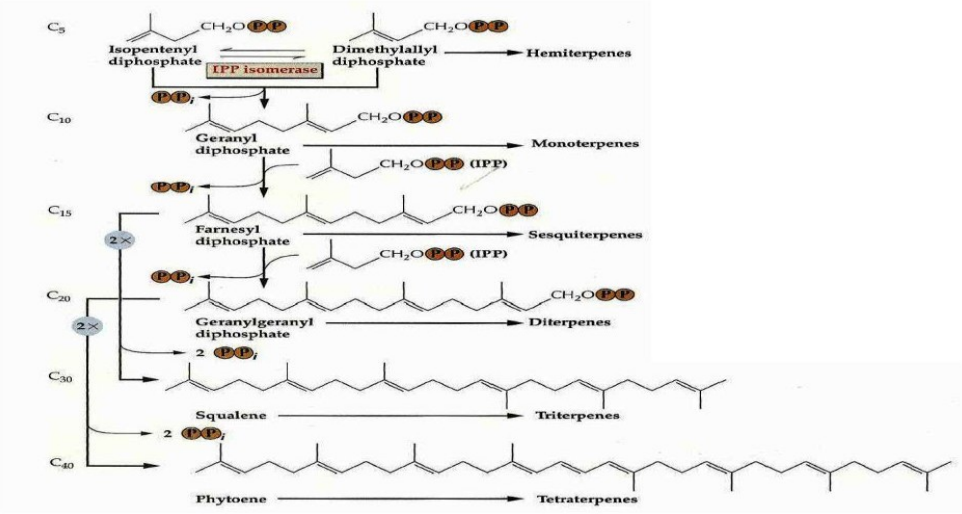


Figure 4: The major subclasses of terpenes. Adopted from Buchanan et al. (2000).

Terpenes constitute the largest class of secondary products commonly produced by conifers through the union of 5-carbon elements that have the branched carbon of isopentane (Figure 5a). Since terpenes can decompose at higher temperatures to give isoprene (Figure 5b), their basic structural elements are sometimes called isoprene units.

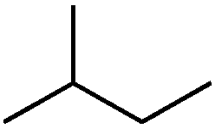
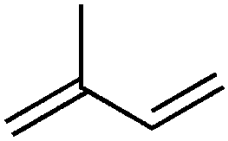
a. Isopentane  b. Isoprene 

Figure 5: Chemical structure of a. Isopentane and b. Isoprene. Adopted from Buchanan et al. (2000).

Terpenes are classified by the number of five carbon units they contain: Ten-carbon terpenes (two C_5 units) are called, monoterpenes. They are best known as components of volatile essence of flowers and essential oils of herbs and spices, which make up as much as 5% of plant dry weight. Common examples of monoterpenes are pinene (α and β-pinene), nerol, citral, camphor, menthol, limonene and myrcene. Fifteen-carbon terpenes

(three C_5 units) are called sesquiterpenes. Like monoterpenes, many sesquiterpenes are found in essential oils and have also been found as phytoalexins, antibiotic compounds produced by plants in response to microbial challenge, and anti-feedants to discourage herbivore. Common examples are nerolidol and farnesol. Twenty-carbon terpenes (four C_5 units) are diterpenes, with common examples as phytol, a hydrophobic side chain of chlorophyll, gibberellin hormone, and resin acids of conifer and legume species. Larger terpenes include triterpenes (30 carbons), tetraterpenes (40 carbons), and polyterpenoids ($[C_5]n$) carbons, where $n > 10$) (Buchanan et al. 2000).

2.4.3 Physiological roles of volatile terpene compounds in plants

Volatile terpene compounds (VTCs) are toxins and feeding deterrents to a large number of plant-feeding insects and mammals thus, they play important defensive roles in the plant kingdom (Gershenzon and Croteau, 1991). Many monoterpenes and their derivatives are important agents of insect toxicity, for example, pyrethroids, which are monoterpene esters produced in the leaves and flowers of *Chrysanthemum* species show very striking insecticidal activity, therefore, are often used in commercial insecticides (Buchanan et al. 2000). In conifers such as pine and fir, it is commonly reported that VTCs such as α, β-pinene, limonene and mycrene accumulate in resin ducts found in needles, twigs, and trunk, posing as toxic compounds to serious pests of conifer such as bark beetle and balsam woolly adelgid (Taiz and Zeiger, 1998; Arthur and Hain, 1987; Carlow et al., 2006).

Many plants such as peppermint, lemon, basil, sage, and balsam fir contain essential oils (mixtures of volatile monoterpenes and sesquiterpenes), which give them a characteristic odor of their foliage. These odors have well-known insect repellent properties towards

herbivores (Taiz and Zeiger, 1998). However, they are also isolated and used in flavor and perfume industries. Turlings et al. (1995) reported that VTCs are not only for defense but also provide a way for plants to elicit defensive help from other organisms such as predatory and parasitic insects.

2.4.4 The proposed link between VTCs, ethylene, jasmonic acid and needle abscission

Ethylene and JA have long been considered as endogenous regulators of plant organ abscission, as exogenous applications of ethylene and JA promote foliage abscission (Creelman and Mullet 1995; MacDonald et al. 2010). In work by Schmelz et al. (2003), exogenous application of JA promoted ethylene and VTCs emission in numerous excised-leaf bioassays. Signaling interactions between JA and ethylene have also been demonstrated to result in either synergistic (Penninckx et al. 1998) or antagonistic interactions (Winz and Baldwin 2001) in the expression of plant defense responses to pathogens, insects and mechanical wounds. However, in the case of VTCs emission in plants, the role of ethylene remains unclear. Using detached lima bean leaves (*Phaseolus lunatus* L), Horiuchi et al. (2001) demonstrated that exogenous applications of the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid, enhance JA-induced VTC emission. However, in tobacco (*Nicotiana attenuata*), Kahl et al. (2000) did not detect any significant interactions between exogenous methyl jasmonate and ethylene upon induced emission of sesquiterpenes. This suggests a promising trend that jasmonic acid induces ethylene and VTCs during stress such as mechanical wounding and it is possible that needle abscission may be triggered by VTCs through ethylene or JA dependent or independent pathways. Since most of these works were conducted under pre-harvest

conditions, there is no clear understanding of the inter-relationship between these phytohormones, VTC and needle abscission postharvest.

2.5 VTC identification and quantification

2.5.1 Methylene chlorine extraction

Methylene chloride, also known as dichloromethane (DCM), is a colorless volatile liquid with a low boiling point of 40°C. Its volatility and ability to dissolve a wide range of organic compounds makes it a useful solvent for many chemical processes such as paint and varnish remover formulations, aerosol applications and as a general cleaning solvent. However, over the years it has been used by scientists in the extraction of organic VTCs from plant tissues (Carlow et al. 2006; Thomson et al. 2006). Although it is time consuming (24 h), it is known to be very effective (analyte concentration of 0.03 µg/L) because of the ability of methylene chloride to dissolve pure single lipid classes by overcoming the strong forces of association between tissue lipids and other cellular constituents, such as proteins and polysaccharides. This is mainly achieved by mechanical agitation (freezing, heating and overnight shaking) of a mixture of the solvent and plant tissue (Thomson et al. 2006).

2.5.2 Solid phase microextraction

Solid-phase microextraction (SPME) is a relatively new extraction method, developed by Pawliszyn and co-workers in 1989 and made commercially available in 1993. SPME is a non-solvent extraction method that employs a fused silica fiber coated with a thin film of extraction phase which can be either liquid (polymer) or solid (sorbent), to extract

volatile analytes from a sample matrix by adsorption or absorption of the compounds (Wells, 2003). The fiber is housed within a syringe needle that protects the fiber and allows for easy penetration of sample and gas chromatography (GC) vial septa. Most published SPME work has been performed with manual devices (Vereen et al. 2000; Beck et al. 2008), although automated systems are also available. There are two approaches to SPME sampling of volatile organics: direct and headspace. In direct sampling the fiber is placed directly into the sample matrix, and in headspace sampling the fiber is placed in the headspace of the sample. SPME has several advantages in the analysis of VTCs; no additional instruments or hardware required and fibers can be reused from several to thousand times, depending on extraction and desorption conditions. Despite the ability of methylene chloride to extract all available VTCs, it is only used in destructive experiments. It is therefore advantageous to use SPME for head space analysis in a nondestructive study where VTC will be monitored over a post-harvest period.

2.5.3 Gas Chromatography

Gas chromatography (GC) is a quantitative analytical tool used in the determination of sample components that can vaporize without decomposition (Carlow et al. 2006). In its operational process, the sample is first injected into the injection port of the temperature programmed GC. The sample then vaporizes onto the head of the chromatographic column. Molecules within the sample are then transported through the column by the flow of an inert (e.g. nitrogen, helium, argon or carbon dioxide) gaseous mobile phase. When they come in contact with the microscopic layer of liquid or polymer on an inert solid support (solid phase) inside the column, molecules are separated into their

individual component and recorded by a detector (Vereen et al. 2000). There are several detectors that are used in GC but the common one is a flame ionization detector (FID). In some cases, the GC is connected to a mass spectrophotometer (MS), which acts as a detector popularly known as GC-MS. Since each type of molecule has a different rate of progression, they elute at different times known as retention time. Chromatographic data is generated as graphs (chromatogram) of detector response against the retention time (Vereen et al. 2000).

Several studies have adopted these methods for the extraction and analysis of VTCs. The most common and useful ones for this study are shown in Table 1.

Table 1: References to show methods of extraction and analysis of organic volatile compounds

Reference	**Extraction method**	**Analysis tool**	**Column/Carrier gas**	**Programmed temperature**
Vereen et al. (2000)	Methylene chloride	GC-MS	DB-1701 (0.25mm i.d.×60m) and DB-5 (0.25mm i.d.×30m) – with helium gas	50°C for 3 min, 10°C/min ramp to 250°C and held for 1min (24 min)
Carlow et al. (2006)	Methylene chloride	GC-FID and GC-MS	DB-1701 (0.25mm i.d.×60m) and DB VRX (0.45mm×75m) – with helium gas	90°C for 15 min, 10°C/min ramp to 200°C and held for 5 min (31 min)
Beck et al. (2008)	SPME	GC-MS	DB-wax (0.32mm i.d×60m) and DB-1 (0.32mm i.d×60m) -with helium gas	40°C for 0.0 min, 4°C/min ramp to 200°C, and held for 40 min (90 min)

CHAPTER 3: OBJECTIVES

The overall goal of this study was to characterize balsam fir volatile terpene compounds (VTCs) and determine their role in postharvest needle abscission and to understand the relationship between needle VTCs, ethylene and needle abscission. Several hypotheses were drawn based strongly on the current literature;

1. Balsam fir seedlings and branches of different clones contain distinct VTCs.
2. Total VTC and individual VTC evolution increases with increasing postharvest duration in balsam fir branches
3. Inhibiting ethylene will reduce or inhibit VTCs evolution thereby extend postharvest needle retention in balsam fir.

Based on these hypotheses, specific objectives were developed to accomplish the goal.

1. To identify and quantify VTCs in balsam fir seedlings and branches of two genotypes that differ in their needle retention duration (NRD) and establish differences in individual VTCs.
2. To establish possible linkages between individual VTCs, total VTC and postharvest balsam fir needle abscission.
3. To determine the effect of ethylene on VTCs and the impact of that on postharvest needle abscission of balsam fir. This was achieved through;
    a. Inhibition of ethylene synthesis by exogenous application of ethylene synthesis inhibitor AVG.
    b. Stimulation of ethylene action by the exogenous application of ethylene gas.

CHAPTER 4: GENERAL METHODOLOGY

4.1 Materials and Sample sites

Three year-old seedlings of 0.25m in average height obtained from T&D Nursery in New Ross, NS were selected for the study. These seedlings were maintained in a greenhouse at the Christmas tree Research Centre (CRC). Branch samples were collected from a balsam fir tree stand at the Tree Breeding Centre, Department of Natural Resources, Debert, NS (45° 25' N, 63° 28' W). These were 16 year-old genotypes that had been classified as being low (0-20 days), moderate (21-40 days), or high (41-60 days) needle retention duration (NRD) clones based on needle retention screening studies done under dehydration by MacDonald and Rajasekaran (2008), and Rajasekaran and Veitch (2010). The orchard is approximately 4 ha and consists of 21 rows, each with 75 trees and a total of approximately 200 classified clones. Each row is spaced 3 m apart and trees are spaced 2 m apart within a row.

4.2 Sample collection, transportation and preparation

A two-year branch with terminal growth from high NRD (clone # 9) and low NRD (clone # 608) trees were used for this study. Samples were collected from the south eastern side of the tree from a height of 1.5 m as done by MacDonald and Rajasekaran (2008); MacDonald et al, (2009); (2010); (2011). Samples were immediately placed in zip lock bags and closed tightly to prevent mechanically-induced volatile compounds and to prevent volatile interaction among branches (Figure 6). To prevent immediate water stress, the branch stalks were exposed at the bottom of the zip lock bag and immersed in a

container filled with distilled water to transport to the laboratory within an hour (Figure 7). For each experiment, freshly cut seedlings or branches were used.

For every experiment, seedlings and or branches were initially weighed, and then placed in a 150 mL conical flask filled with 100 mL of water, which was sufficient for the entire experiment. The neck of each jar was then sealed with cotton gauze to minimize water evaporation and provide added stability to the seedlings and branches. Finally, the entire apparatus was weighed and placed in a volatile incubation chamber, which was tightly sealed. The samples were then kept in the continuous airflow system throughout the entire experiment except 30 minutes every other day when the samples were removed from the system for VTC and ethylene extractions, and data collection such as needle drop and water use.

Figure 6: Cut branch sealed in a zip lock bag

Figure 7: Transportation of branches in water filled buckets

4.3 Design of volatile incubation chamber

After several trials, it was determined that all the experiments required a method to incubate samples to aid in trapping volatile terpene compounds. An incubation chamber

system was designed large enough to accommodate balsam fir branches and seedlings, allowing light penetration, provide complete air circulation, and monitor conditions such as temperature and humidity (Figure 8a). Unless otherwise specified, all studies were conducted under supplemented lightning of 100µmol·m^{-2}·s^{-1} light intensity from overhead fluorescent bulbs. The continuous air flow chamber system was designed and built on the light rack using clear glass jars and pvc gas pipes, maintaining an average of 30% relative humidity and 22°C in each jar from a temperature regulated and compressed air at a rate of 50 mL·min^{-1} (Figure 8b). The air flow rate was constantly maintained by building a back pressure system using water (Vines and Oberbacher, 1963). This was to avoid CO_2 build up and or O_2 depletion, which could inhibit ethylene production thus affecting the abscission process (Abeles et al. 1992).

Figure 8: a. Continuous air flow system with regulated light intensity, humidity and airflow (left). b. Air compressor attached to the system to supply regulated air continuously to the system (right).

Using 4L glass jars (Figure 9a), an air tight plastic lid on each jar was drilled to fix inlet and outlet air tubes for inflow and outflow of air through the jars (Figure 9b). The plastic

lids were again drilled to insert a rubber septum to allow the injection/extraction of volatiles using a syringe and SPME sampler respectively, and/or injection of ethylene gas (Figure 9b). To filter the in-flowing air, a carbon filter (Sigma-Aldrich Co. LLC) was fixed on the main inlet tube to trap any VTCs as well as any foreign gases in the air (Figure 10). The outlet tubes were also joined to a main outlet tube that was channeled into a 10 L bucket containing activated charcoal to purify the out flowing air of any VTC (Figure 11). In each chamber, an LCD humidity thermometer (TechMart Electronics, Canada) was attached to monitor the humidity as well as the temperature in the chambers over the entire experimental period.

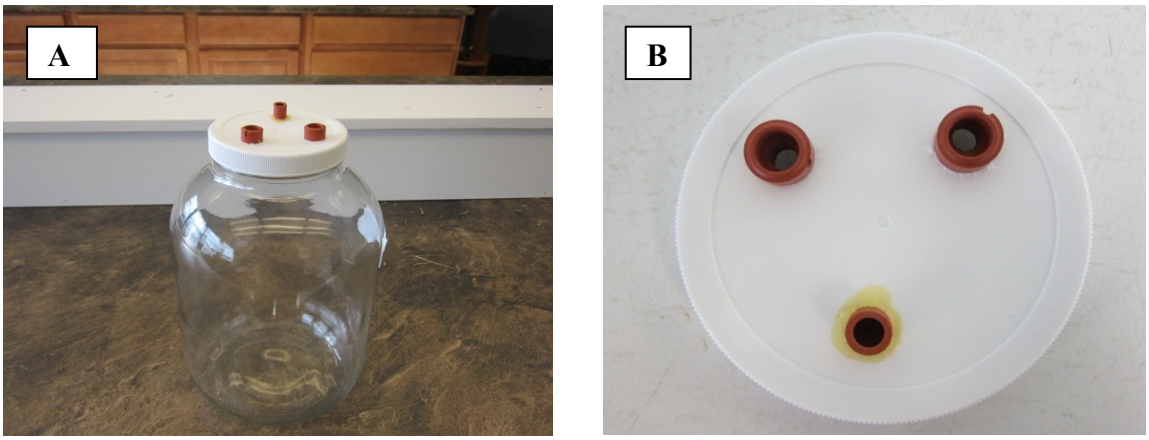


Figure 9: a. 4L jar used for building the continuous air flow system (left). b. Air tight plastic lid with septa (right).

Figure 11: Carbon filter attached to the system to filter inflow-air of any contamination

Figure 10: Bucket of activated charcoal to filter out-flow air of any contamination

4.4 Sample extractions

4.4.1 Solid phase microextraction (SPME)

Extraction method that was used in this study is a non-solution extraction procedure called SPME. This was conducted using a portable SPME sampler (Sigma-Aldrich, Canada) with a liquid phase (polymer) coated StableFlex fiber. The extraction process always started by closing both the inlet and outlet tubes using the rubber stop cocks. After that, the SPME sampler was inserted into the chamber through the rubber septum on the lid using its sharp protective needle that contains the extraction fiber. The fiber was then released out of the protective needle by pushing down the plunger on the handle. This exposed the fiber to the head space, for the adsorption of emitted volatile compounds in the chamber. For each extraction, the fiber was exposed to the headspace for an equilibration time of 30 minutes from there it was sent to the GC for analysis.

4.4.2 Ethylene extraction

The 4 L jars with the branches in them were first taken off the continuous airflow system and tightly sealed for an equilibration period of 30 minutes at room temperature using a lid that had rubber septa which did not release ethylene. This was done to allow ethylene to accumulate in the jars to a detectable concentration. A 1-ml air sample was drawn from the jar with a gas-tight syringe (Sigma-Aldrich Co. LLC, Canada) and injected onto a FOCUS 3420 gas chromatograph (Thermo Scientific, Canada) equipped with flame ionization detector as described in Section 4.5

4.5 Instrumentation and materials

A FOCUS 3420 gas chromatograph (Thermo Scientific, Canada) at the Christmas tree Research Centre (CRC), Truro, Nova Scotia, Canada was used for both VTCs and ethylene measurements. For VTCs measurements, it was equipped with a flame ionization detector (FID) and a Stabilwax capillary column (60 m × 0.25 mm ID × 0.25 µm, RESTEK, Bellefonte, PA). A splitless method was employed with helium as the carrier gas at the rate of 1.5 ml min^{-1}. GC temperature program for VTCs analysis included an injector temperature of 200 °C and an initial oven temperature of 40 °C held for 0 min, a ramp at 4 °C min^{-1} to 140 °C and held for 1 min, a second ramp of 3 °C/ min to 200 °C and held at for additional 4 min, a total run time of 50 minutes (Beck et al. 2008). A Varian 4000 gas chromatograph-mass spectrometer at the Agriculture and Agri-Food Canada Research Centre, Kentville, NS, was used for validation of all the VTCs by reanalyzing branches and seedlings as well as standards.

For ethylene analysis, the GC was equipped with an Agilent J&W GC 30m×0.32mm, GS-Gaspro column (Chromatographic Specialties Inc, Canada) packed with fused silica. Helium was used as a carrier gas at 1.5 $mlmin^{-1}$, with injector, column and detector temperatures at 200, 65 and 250 °C respectively. A total run time of 5 minutes was used (Modified: Klintborg et al. 2001).

4.6 Response variables

4.6.1 Volatile terpene evolution

In experiments to establish evolution of volatile compounds and their respective concentrations in postharvest balsam fir branches and seedlings, sample extraction was done once using the SPME procedure described in section 4.5. In experiments where volatile terpene evolution was monitored over the duration of the experiment, extraction using SMPE was conducted every three days. Using the GC, chromatographic data of peak area (PA) against retention time (minutes) for each of the samples was defined. Confirmation of terpene compounds were provided by comparison of retention times and peak areas with that of known standards. The respective relative abundances for each sample were normalized against β-Pinene standard (Sigma-Aldrich Co. LLC, Canada). Detailed standardization of β-Pinene is found in Appendix II.

4.6.2 Percentage needle loss

Every other day, needle loss was accounted for by weighing the dropped needles. A finger run test was first conducted and the dropped needles weighed (MacDonald et al. 2010). This was monitored throughout the experimental duration to estimate the percentage needle loss for each sample.

4.6.3 Needle retention duration (NRD)

The primary measurement of abscission was NRD, which was defined as the number of days required for complete needle abscission (MacDonald et al. 2009; MacDonald et al. 2010).

4.6.4 Average water use (AWU)

Average daily water use ($g \cdot d^{-1}$) was calculated as the sum of the change in mass of the apparatus (excluding mass loss due to abscission) per unit fresh weight of needles over the duration of the experiment or until a branch had lost all its needles (MacDonald et al. 2010):

$$AWU = \frac{(Initial\ Mass - Final\ Mass) - Needle\ Mass}{Time}$$

Where *Needle Mass* is weight of needle dropped after each finger run test.

4.6.5 Ethylene evolution

Ethylene evolution was determined in the respective experiments every week. Evolution rates from branches were calculated by the following equation:

$$Ethylene\ evolution = \frac{Ethylene\ concentration\ (Initial - Final) \times 4L}{0.5h \times Mass}$$

Where ethylene evolution is in $\mu L \cdot g^{-1} \cdot h^{-1}$, concentration is in $\mu L \cdot L^{-1}$, and mass is the fresh weight (g) of a branch. Detailed standardization of ethylene is found in Appendix III.

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CHAPTER 5: IDENTIFICATION AND CHARACTERIZATION OF VOLATILE TERPENE COMPOUNDS (VTCs) IN BALSAM FIR SEEDLINGS AND MATURE TREES

5.1 Abstract

Postharvest needle abscission is a major challenge for Atlantic Canada's Christmas tree and greenery industry. It was hypothesized that the nature and concentration of volatile terpene compounds (VTCs) differ among balsam fir seedlings and mature branches. The objective was to identify and quantify the VTCs in balsam fir seedlings and branches of two contrasting, high and low needle abscission-resistant (NAR) genotypes and to identify differences in individual VTCs and how they relate to needle abscission resistance (NAR). An experiment was designed using two balsam fir genotypes (low and high NAR with branches of two-year old growth) and three-year old seedlings with five replicates. Harvested branches and seedlings were sealed in a 4L air tight glass jar to equilibrate for 30 minutes. Concentrations of VTCs were determined by a head space solid phase microextraction (SPME) procedure, followed by analysis using gas chromatography. The results showed twelve distinct VTCs in balsam fir seedlings and mature trees; out of which, ten were monoterpenes and two were terpenoids. Individual VTC concentrations varied among samples used, ranging from 0.02 nMg^{-1} (β-terpinene) to 0.39 nMg^{-1} (3-Carene). High NAR genotype contained the highest concentration of 3-Carene, suggesting a potential role in NAR. Total concentration of monoterpenes in seedlings was 53.3% higher than monoterpene concentration in mature tree.

[1]This chapter won the second best graduate student poster competition at the 2012, American Society of Horticultural Science (ASHS) Annual Conference. Abstract "The Nature and Role of Volatile Terpene Compounds in Postharvest Needle Abscission" is published in ASHS Newsletter, August 2012, Vol. 28, No. 8.

5.2 Introduction

Balsam fir (*Abies balsamea* L (Mill)), like other confers, is characterized by a distinctive fragrance caused by the release of volatile terpene compounds (VTCs) from the foliage and other tissues (Schmeltz et al. 2003). They are mostly lipids that are synthesized from acetyl CoA or from the basic intermediates of glycolysis (Taiz and Zeiger, 1998). They constitute the largest class of secondary metabolites produced by conifers and are known to play a major physiological role in plant natural defense (Carlow et al, 2006; Lagalante et al, 2007). Although most commonly associated with resistance to pest infestation, VTCs are speculated to defend plants against mechanical injuries and abscission (Winz and Baldwin 2001; Horiuchi et al. 2001). This is achieved by protecting the photosynthetic apparatus of the plant against oxidative stress (Loreto and Velikova, 2001) and also stabilizing the membrane lipid bilayer against external destruction (Alberts, 2002).

Postharvest needle abscission is a major problem in balsam fir and other Christmas tree species. Nevertheless, balsam fir is one of the most planted and prized conifer trees in eastern and central Canada, and the northeastern United States, with more than 180 genotypes (MacDonald and Rajasekaran 2008, and Veitch and Rajasekaran 2010). These genotypes are mostly asexually propagated through grafting and for most parts are variants selected from natural or cultivated populations screened for their shape, color and needle retention. In addition to morphological differences created by selective propagation in balsam fir, the technique may have introduced variations in the composition and concentrations of foliar terpenes. Campeol et al. (2003) showed that the chemical composition of volatile fractions in three cultivars of *Olea europaea* L. differ

from each other at harvest and in stems and leaves, suggesting a possible variation in terpene profile for different plant genotypes. Also confirming this speculation is a study by Lagalante et al, (2007). After analyzing thirteen cultivars of *Tsuga canadensis* L. (Carriere), it was speculated that cultivars in eastern North America have adapted their terpenoid chemistry for protection against endemic defoliators while some cultivars of the same specie had a terpenoid profile that resembles that of the resistant eastern North American species serving as candidates for biological screening for resistance. Whether this is the case in balsam fir remains to be seen.

Most studies have also shown that conifers synthesize high concentrations of monoterpenes, sesquiterpenes and terpenoids in defending the plants against insect predators (Bowman et al. 1997; Vereen et al. 2000; Carlow et al. 2006) but none has been able to bridge the gap between the concentrations of individual terpene compounds in balsam fir, the differences in total terpene concentration among genotypes and seedlings, and ultimately, the role of these terpenes in post-harvest needle abscission. If volatile terpenes can be demonstrated to be involved in post-harvest needle abscission in balsam fir, needle abscission mitigating technologies by using terpene synthesis inhibitors such as mevastatin and or fosmidomycin (Hampel et al. 2005) can be developed for practical use in Christmas tree and greenery industries.

It was hypothesized that volatile terpene compounds (VTCs) are available in balsam fir seedlings and matured branches but in varying concentrations. However, several objectives must be met to understand the role of volatile terpene in balsam fir needle abscission. First, it will be important to establish the volatile terpene profile in balsam fir.

5.3 Objective

To identify and quantify VTCs in balsam fir seedlings and branches of two genotypes that differ in their needle retention duration (NRD) and establish differences in individual VTCs.

5.4 Materials and Methods

Ten branches of two-year old growth from two contrasting balsam fir genotypes that are low (clone #680) and high (clone #9) NAR and five three-year old seedlings were collected from a population of randomly selected trees and seedlings on February 7, 2012. There were five replications for each of the genotypes, where a single branch represented a replicate. Harvested branches and seedlings brought to the lab as described in Section 4.2 were sealed separately in a 4L airtight glass jar and equilibrated for thirty minutes, (based on standardization experiment) (Figure 12a). Following equilibration, head-space extraction of volatile terpenes was done using the SPME procedures (Section 4.4.1) followed by analysis using gas chromatograph as described in Section 4.5 and shown in Figure 12b.

Figure 12a: Volatile terpene extraction setup with SPME

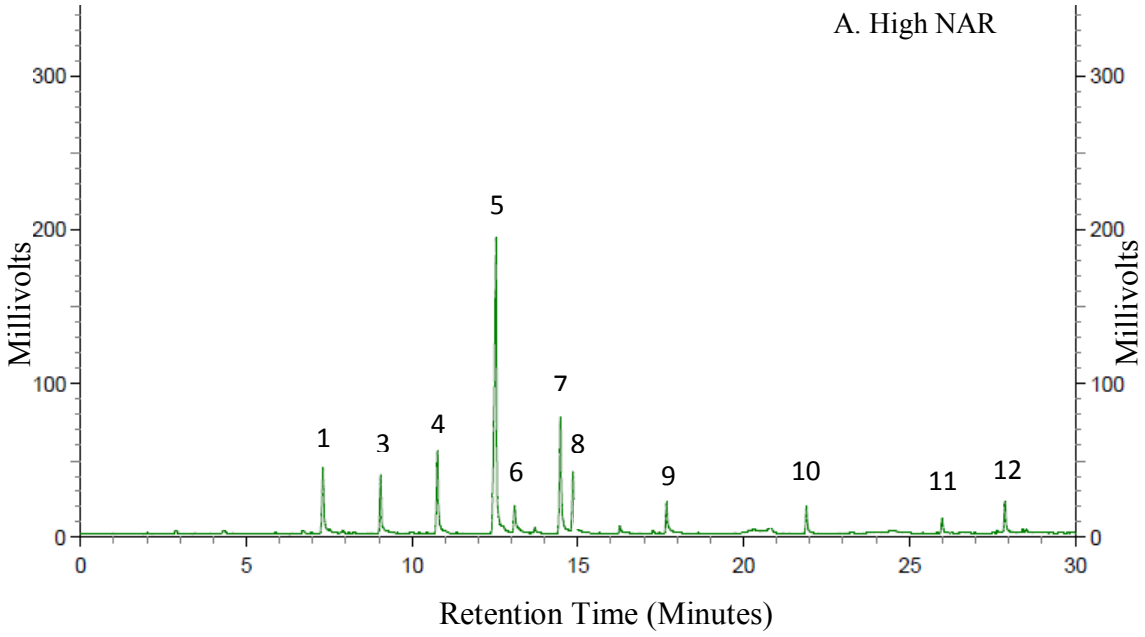
Figure 12b: Analysis of terpene using GC-FID

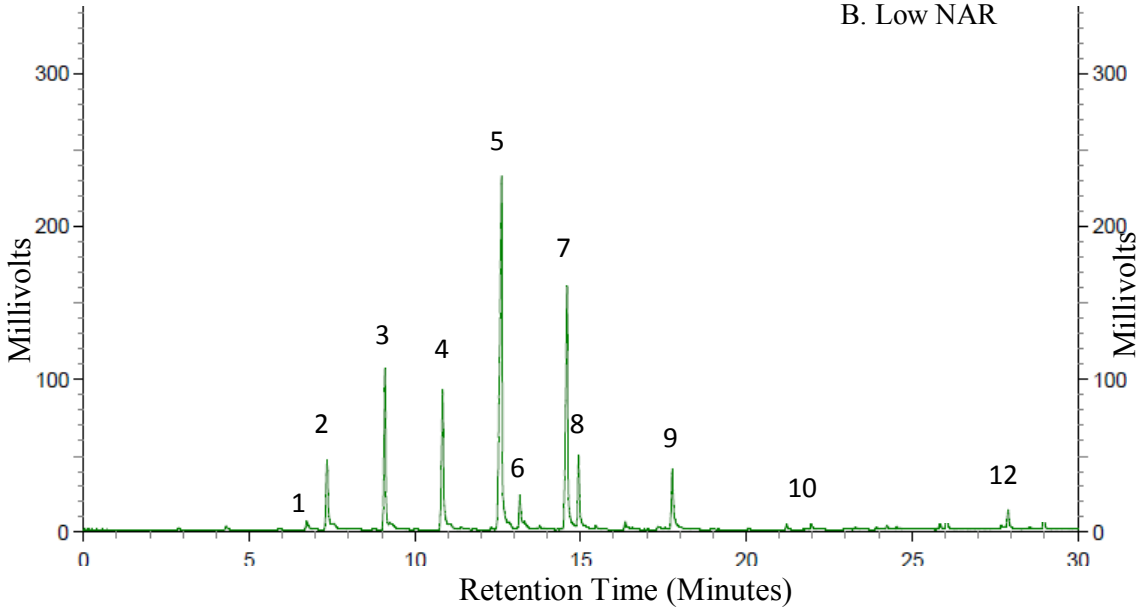
The response variables in this experiment were concentration of individual VTCs, total monoterpenes and terpenoid concentrations in the two contrasting genotypes and the seedling as described in Section 4.6.1.

Normality and variance of the responses were checked using Minitab 15 (Minitab 15, Minitab Inc., PA, USA). Volatile terpene concentrations were submitted to an analysis of variance (ANOVA), general linear model (GLM) using statistical analysis system (SAS) 9.1 (SAS Institute, NC, USA). Significant differences were then analyzed using Tukey's multiple mean comparison at 5% significance level.

5.5 Results

Comparisons of terpene profiles (Figure 13) showed that a total of 12 VTCs were consistently present in high and low NAR genotypes as well as the seedlings used for this study. The results also showed that the high NAR genotype had eleven out of the twelve VTCs present with only 3-Thujene being absent (Figure 13a). With the exception of Fenchyl acetate, low NAR genotype and seedling also contained eleven VTCs (Figure 13b and c). Out of the twelve identified VTCs, ten (peaks 1 to 10) of them were monoterpenes whereas the remaining two (peaks 11 and 12) were terpenoids (Figures 13a, b and c).


A. High NAR
Millivolts
300
200
100
0
5
10
15
20
25
30
1
3
4
5
6
7
8
9
10
11
12
Retention Time (Minutes)


B. Low NAR
Millivolts
300
200
100
0
5
10
15
20
25
30
1
2
3
4
5
6
7
8
9
10
12
Retention Time (Minutes)

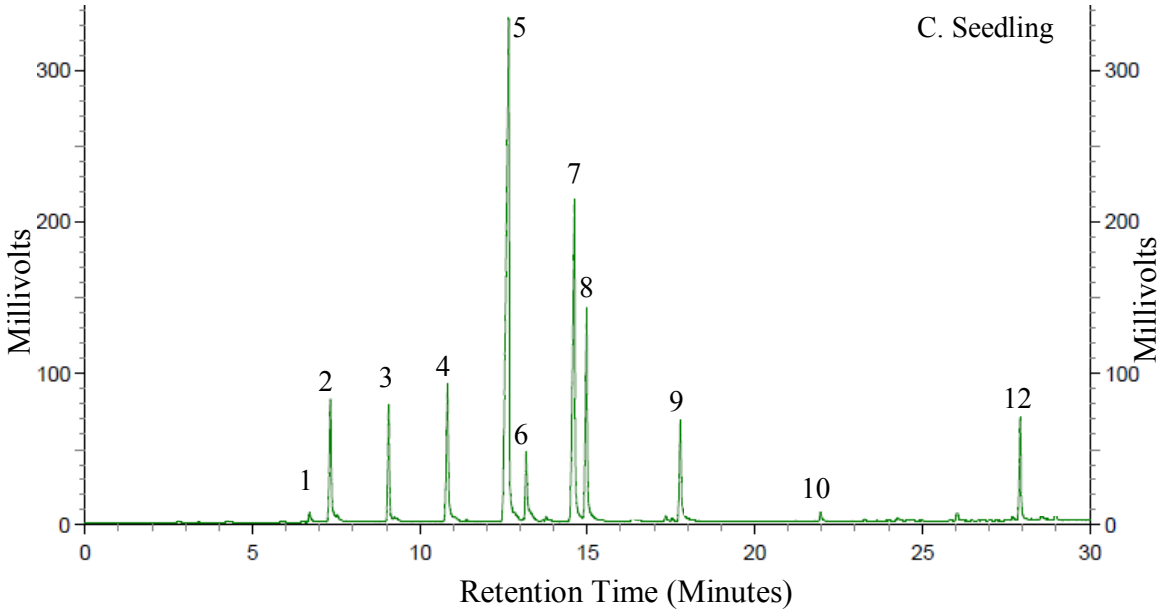


Figure 13 a, b, c: Volatile terpene compound profiles for high and low needle abscission resistant balsam fir genotypes and seedlings using SPME with headspace sampling after 30 mins equilibration. Compounds determined were: (1) α-Pinene; (2) 3-Thujene; (3) Camphene; (4) β-Pinene; (5) 3-Carene; (6) β–Terpine; (7) D-Limonene; (8) β –Phellandrene; (9) ɣ -Terpinene; (10) Terpinolene; (11) Fenchyl acetate; (12) Bornyl acetate.

Analysis of variance (ANOVA) showed that seedlings were significantly high in total monoterpene concentration with 1.94 nano molar per gram (nMg^{-1}) compared to 0.93 and 0.77 nMg^{-1} in the high and low NAR genotypes, respectively. On the other hand, the high NAR genotype was high in total terpenoid concentration (0.09 nMg^{-1}), however it was not significantly different from that of the low NAR genotype (0.04 nMg^{-1}) and the seedling (0.08 nMg^{-1}) (p=0.077) (Table 2).

Among the identified individual VTCs, 3-Carene, which is a monoterpene, was the highest of all VTCs in both the high and low NAR genotypes with 0.39 and 0.20 nMg^{-1}, respectively. However, it was ranked fourth highest concentrated VTC in the seedlings with 0.24 nMg^{-1}. ANOVA showed a significant difference between the 3-Carene in the high NAR genotype compared to the low NAR genotype and the seedlings as shown in

Table 2. In the seedlings, ɣ -Terpinene was the highest concentrated VTC with 0.37 nMg^{-1} and was significantly higher than the concentrations obtained from both the low (0.07 nMg^{-1}) and high (0.05 nMg^{-1}) NAR genotypes.

There were few other individual VTCs that were significantly different when a comparison was made between the high and low genotypes and the seedlings. The results showed that concentration of β-Pinene was significantly higher (0.34 nMg^{-1}) in seedlings than in the high NAR (0.08 nMg^{-1}) and the low NAR (0.09 nMg^{-1}) genotypes. In addition, β-Terpinene and D-Limonene were also significantly higher in the seedlings than in the high and low NAR genotypes with p-values of 0.026 and 0.014 respectively (Table 2). Individual VTCs such as α-Pinene, 3-Thujene, Camphene, β–Phellandrene, Fenchyl acetate and Bornyl acetate although present, were not significantly different among all the samples analyzed.

Table 2: A comparison of volatile emission profiles of balsam fir seedling and branches of matured tree

Compound information[b]				Compound presence			Compound Concentration (nM/g)			*P-value*
No.	Library/ID	Source [c]	RI [d]	High NAR	Low NAR	Seedling	High NAR	Low NAR	Seedling	
1	α-Pinene	Ald	1020	+	+	+	0.09 a	0.06 a	0.23 a	0.065
2	3-Thujene	Ald	1034	*	+	+	NA	0.04 a	0.10 a	0.335
3	Camphene	Ald	1079	+	+	+	0.05 a	0.09 a	0.07 a	0.070
4	β-Pinene	Ald	1123	+	+	+	0.08 b	0.09 b	0.34 a	0.047
5	3-Carene	Ald	1165	+	+	+	0.39 a	0.20 b	0.24 b	0.031
6	β-Terpinene	Ald	1176	+	+	+	0.02 b	0.03 b	0.07 a	0.026
7	D-Limonene	Ald	1217	+	+	+	0.05 b	0.03 b	0.10 a	0.014
8	β-Phellandrene	Ald	1227	+	+	+	0.12 a	0.14 a	0.33 a	0.397
9	ɣ-Terpinene	Ald	1265	+	+	+	0.07 b	0.05 b	0.37 a	0.045
10	Terpinolene	Ald	1302	+	+	+	0.05 a	0.04 a	0.08 a	0.116
11	Fenchyl acetate	Ald	1492	+	*	*	0.03	NA	NA	
12	Bornyl acetate	Ald	1605	+	+	+	0.06 a	0.04 a	0.08 a	0.121
All monoterpenes							0.93 b	0.77 b	1.94 a	<0.001
All terpenoids							0.09 a	0.04 a	0.08 a	0.077

[a]Volatile amounts are normalized to the internal standard, β-pinene and are average of 5 replications, [b]Compound identification by retention index on Stabil-Wax Column, retention times, mass fragment libraries and comparison to authentic samples. [c] Source of compound for authentication: Ald = Sigma Aldrich or Fluka. [d] Calculated retention index relative to n-alkanes. +,* = VTCs presence or absence in samples, respectively. *P-values* were derived by ANOVA at α = 0.05. Highlighted *p-values* are significant and means that do not share the same letters in a row are significantly.different.

5.6 Discussion

The first ten VTCs (α-Pinene up to Terpinolene) (Figures 13a, b and c) are examples of monoterpenes, with a chemical formula $C_{10}H_{16}$. The last two VTCs, Fenchyl acetate and Bornyl acetate are oxygenated derivatives of monoterpenes known as terpenoids. Thus, confirms the dominance of monoterpenes in conifer species as reported by Taiz and Zeiger (1998) and Buchanan et al. (2000). A new discovery was made in this study with the identification of 3-Thujene in balsam fir samples. 3-Thujene is a monoterpene found in essential oils of a variety of plants (Maarse and Kepner, 1970; Bowman et al, 1997; Carlow et al, 2006) contributing to the pungency of their flavor. Although it has been identified in a variety of plants such as coriander (*Coriandrum sativum*) and eucalyptus as well as some conifers such as *Thuja occidentalis* (Civjan, 2012), it has never been identified or reported in balsam fir. A study by Carlow et al. 2006 reported nineteen VTCs in fraser fir when methylene chloride extraction was used. However, Vereen et al. 2000 had earlier on reported twelve VTCs as in fraser fir when SPME was used as done in this study. This suggests the advantage methylene chloride extraction has over SPME to extract a lot more VTCs but it can only be used in a destructive experiment. Since our study was a nondestructive one, SPME was the best method for VTC extracting.

Interestingly, not all the individual VTCs were identified in each sample used as shown in Figs. 8a, b and c as well as Table 2. Although 3-Thujene was present in the low NAR genotype and the seedlings at lower concentrations, it was not identified in the high NAR genotype (Table 2). On the other hand, Fenchyl acetate, which was present in very low concentration in the high NAR genotype, could not be detected in the low NAR genotype and in the seedlings. The absence or the presence of these VTC could suggest a possible

lead to a significant physiological role of these individual VTC such as 3-Thujene and Fenchyl acetate in needle abscission resistance.

A study by Carlow et al. (2006) concluded that, 3-Carene is the most abundant VTC in seedlings, saplings and mature trees that were subjected to mechanical stress through insect infestation. This was similar to the ones found in this study, where seedlings and branches were mechanically injured through cutting and hence one would expect higher concentration levels of 'protecting' compounds in the sample. Examination of Table 2 showed the highest concentration of 3-Carene in the high NAR genotype compared to low NAR genotype signifying the potential role for 3-Carene in needle retention. On the other hand, α-Pinene, β-Pinene, β-Terpinene, D-Limonene, β–Phellandrene and ɣ-Terpinene had higher concentrations in the seedling but significantly lower concentrations in the high and low NAR genotypes. This can be explained by the age difference of the samples used. Coley, (1983); Puttick, (1986); Hatcher, (1990) and Carlow et al. (2006) all reported of high VTC concentrations in younger conifer tress than mature trees. Such a consistent trend may also suggest a possible linkage of these specific VTCs to needle abscission resistance characteristics of the samples.

Balsam fir seedlings have not received much attention in relation to needle abscission; however, Thiagarajan et al. 2012 reported that after harvest, seedlings go through senescence by discoloration rather than abscission. Hatcher (1990) also discovered that younger trees tend to lose their needles faster compared to mature trees as a result of low levels of nitrogen and fiber in young trees as well as less toughness of their needles. Although this work does not focus on needle abscission, we have demonstrated that seedlings have higher concentrations of monoterpenes (two fold) than that of the low and

high NAR genotypes (Table 2). This supports a report by Carlow et al, (2006), that seedlings contain higher levels of VTCs, serving as secondary metabolites for development and defense. We can therefore speculate that high levels of monoterpenes in seedlings might also explain the faster loss of needles in young trees compared to mature trees.

The study also showed that high NAR trees had higher terpenoids than low NAR and seedling. It is speculated that this could also be a contributing factor to their differences in needle abscission resistance but there is no current literature to support this hypothesis.

5.7 Conclusion

Prior to this study, VTC profile for balsam fir and their linkage to the regulation of needle abscission resistance in other plant species had never been established. This study uncovers that there are twelve distinct VTCs present in the balsam fir used in this study, with ten of them being monoterpenes and two terpenoids. In addition, 3-Thujene, a monoterpene, which had never been identified or reported in balsam fir, was discovered in this study.

Clearly, two distinct trends of individual VTC concentrations evolved, some with higher concentrations in the seedling but lower in mature trees (α-Pinene, β-Pinene, β-Terpinene, D-Limonene, β-Phellandrene and ɣ-Terpinene), but others with higher concentrations in mature trees but lower in seedlings (3-Carene). Both trends can, therefore, be speculated to support a possible link to post-harvest needle abscission

resistance in balsam fir. Among all VTCs, 3-Carene is significantly higher in the high NAR genotype suggesting a potential role in needle retention.

Nonetheless, the mechanism through which VTCs either induce or inhibit needle abscission in balsam fir remains unclear. A further study on dynamics of the individual VTCs over the entire postharvest shelf life of balsam fir may establish any linkage between VTCs and post-harvest needle abscission resistance.

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CHAPTER 6: DYNAMICS OF VOLATILE TERPENE EVOLUTION AND LINK WITH POSTHARVEST NEEDLE ABSCISSION

6.1 Abstract

Postharvest needle loss is a major problem for balsam fir Christmas tree species. Our recent study has identified several key volatile terpene compounds (VTCs) in high and low needle abscission resistant (NAR) genotypes and seedlings and suggested a possible link with postharvest needle abscission. However, the postharvest dynamics of VTC evolution and their physiological role as a potential trigger of postharvest needle abscission still remain unknown. The objective of this study was to establish possible linkages between individual VTCs, total VTCs and postharvest balsam fir needle abscission. A randomized repeated measures experiment was designed with two contrasting genotypes with ten replicates each. Branches were kept in the VTC incubation chamber on a continuous airflow system throughout the experiment. Key response variables measured in this study included needle loss, needle retention duration, and concentration of individual and total VTCs. β-Pinene, β–Terpinene, Fenchyl acetate, Camphene and 3-Carene all were released in significant quantities prior to initiation of needle abscission suggesting they could play a role in abscission. Total VTCs also followed similar trends thereby, suggesting that VTCs may be involved in possible signaling for triggering postharvest needle abscission.

6.2 Introduction

Postharvest needle abscission is a major challenge in the Christmas tree industry. Balsam fir trees popularly used as Christmas trees, like many other conifers, are known by their unique aroma as a result of their ability to release volatile terpene compounds (VTCs) (Schmeltz et al. 2003). VTCs in conifers include monoterpenes, sesquiterpenes and terpenoids (Rudloff, 1996), and are stored in the gladular trichomes of the resin ducts after synthesis with concentrations ranging between 1 and 3% of dry mass (Penuelas et al. 1995). Although nothing substantive is known on the physiological role of VTCs in balsam fir, some studies (Shiojiri et al. 2006; Carlow et al, 2006; Lagalante et al, 2007) have shown that most plants, especially conifers, respond to stress such as pathogenic and insect attacks as well as mechanical injury by emitting high levels of VTCs. In our previous study (Chapter 5), twelve VTCs were discovered and characterized in the balsam fir, with monoterpenes as the dominant VTCs.

It was established in our previous study that the concentration of 3-Carene is higher in mature high NAR trees compared to mature low NAR trees. This supports work done by Carlow et al. (2006) on VTCs in relation to insect attack, which reported higher levels of VTCs in mature trees than in seedlings. Our study also showed higher concentrations of α-Pinene, D-Limonene, ɣ-Terpinene, Terpinolene and Bornyl acetate in high NAR trees in significant quantities, compared to the low NAR clone. In contrast, Camphene, β-Pinene, β-Terpinene and β-Phellandrene were higher in the low NAR than the high NAR clone. These trends do not clearly suggest a possible role of individual VTCs in postharvest needle abscission. 3-Thujene, a monoterpene found in essential oils of a variety of plants contributing to their pungency (Maarse and Kepner, 1970; Bowman et

al, 1997) has been discovered in balsam fir. Though nothing is known about its physiological role in postharvest it was speculated to have a possible role in needle abscission.

Although it is known that there is a difference in evolution of specific VTCs between high and low NAR genotypes, we do not know if they are linked to needle abscission characteristics. In order to ascribe the physiological role for any VTC as a trigger, VTC should accumulate or be released prior to needle loss, or the dynamics of VTC evolution should follow needle loss pattern. However, such information is not available. There is, therefore, the need to study the postharvest dynamics of VTCs evolution simultaneously with needle loss. This unprecedented study will lead us to understand the role of specific VTCs in balsam fir needle loss. In addition, this knowledge can move us closer to curbing the problem of postharvest needle loss in balsam fir and perhaps other conifer species.

It was hypothesized that headspace concentrations of individual VTCs, in addition to total VTC concentration, increase with increasing postharvest needle loss in balsam fir.

6.3 Objective

The objective of this study was to establish possible linkages between individual VTCs, total VTC and postharvest balsam fir needle abscission.

6.4 Materials and Methods

A completely randomized design was used with twenty (two-year old growth) lateral branches from contrasting balsam fir genotypes of low (clone #608) and high (clone #9) NAR. Branches from the Tree Breeding Centre, Department of Natural Resources, Debert, NS (45º 25' N, 63º 28' W) were collected from a population of randomly selected 20 years old trees on April 3, 2012 for this experiment. Each genotype had ten replications, where a single weighed branch represented a replicate, with average weight of 9.01g. Harvested branches were brought to the lab as described in Section 4.2, and sealed separately in the volatile incubation chamber (Section 4.3) throughout the experiment.

The response variables in this experiment were VTC, NRD, percentage needle loss and AWU as described in Section 4.6. Head space VTC concentrations were also measured every other day from the first day the experiment was set up till the end of the experiment by first equilibrating the VTCs in the chambers for thirty minutes and extracted using the SPME procedures (Section 4.5) followed by analysis using gas chromatograph as described in Section 4.4. Needle loss and AWU were measured every other day from the first day the experiment was set up till the end of the experiment when branches lost all needles (Section 4.6).

Repeated measures were conducted where postharvest needle loss, NRD and VTC concentrations were submitted to analysis of variance (ANOVA) using PROC Mixed of SAS 9.1 (SAS Institute, NC, USA). Significant differences were then analyzed with Tukey's multiple means comparison at 5% significance level. Regression analysis was

done using Minitab 15 (Minitab 15, Minitab Inc., PA, USA) to analyze percentage needle loss using transformed VTC data.

6.5 Results

Comparison of postharvest needle loss and total VTC concentration showed intriguing trends. The low NAR clone for the first 30 days suffered a low needle loss with no significant difference in needle loss and values ranging between 0.1 and 2.1% (Figures 14a and 15). During that same period, the total VTC concentrations although fluctuated between 12.8 and 69.5mMg^{-1}, these were not significantly different. After 30 days postharvest, there was a slight increase in needle loss from 2.2 to 17.3% until day 51 as shown in Figure 14b. As days progressed, there was a significant increase in needle loss, which was significantly higher ($p<0.001$) than the needle loss during the first 30 days (Table 3). On day 86, 100% of the needles on the branches were lost (Figure 14c). Total VTC concentration peaked to all-time high with concentrations reaching 200.3mMg^{-1} on day 42, which was sustained at 111.0mMg^{-1} until day 45 thereafter followed an increase in needle abscission (Figure 15). However, regression analysis showed no relationship between postharvest total VTC concentrations and needle loss in the low NAR genotype.

Table 3: P values; Low NAR branches subjected to analysis of variance at $\alpha = 0.05$

Source of	P value (Day 1-29)		P value (Day 30-86)	
Variance	Needle Loss	VTC	Needle Loss	VTC
Day	0.402	0.108	<0.001	0.594

The table gives *p values* of needle loss for low NAR branches for days 1-29 and 30-86

a b c

Figure 14: Progressive needle loss (calculated using fresh weight) in a low needle retainer balsam fir genotype after daily finger run test: a) 0 days (0% needle loss); b) 51 days (17.3% needle loss); c) 86 days (100% needle loss).

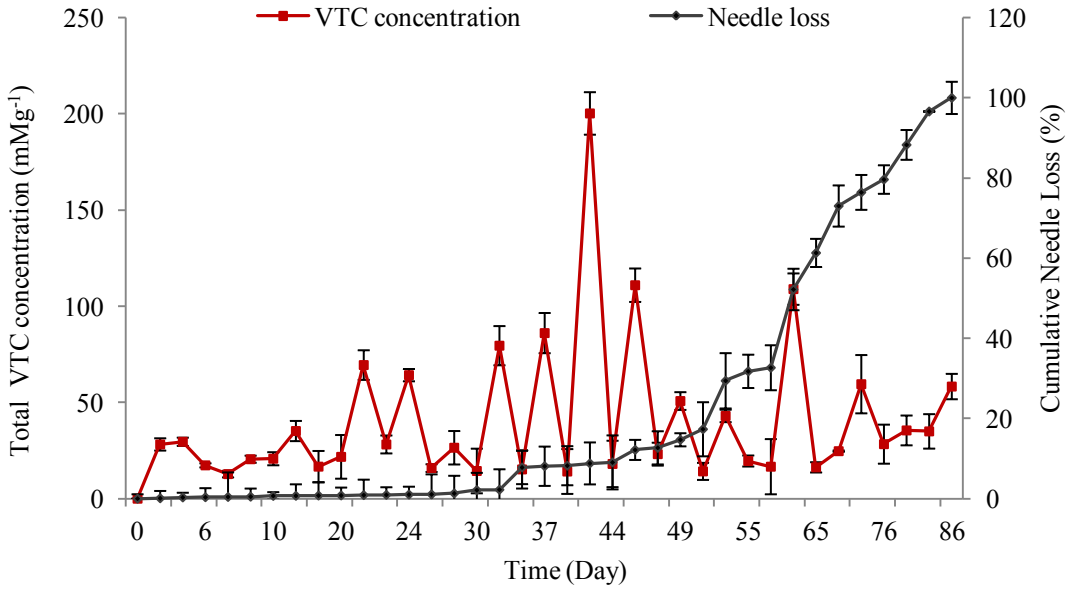
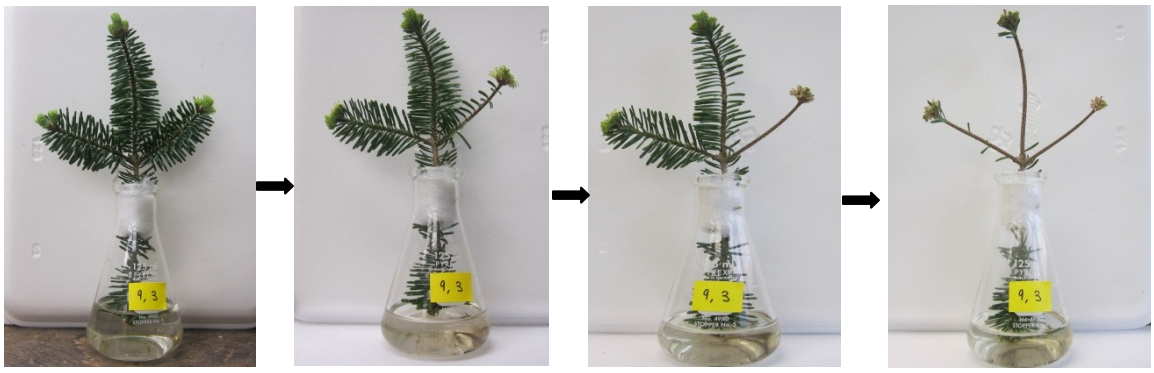


Figure 15: The progression of needle loss (as a percentage of needle fresh weight) and total VTC evolution in low needle retainer (Low NAR) balsam fir genotype with a peak VTC evolution of 42 days and NRD of 86 days (n=10). The trends observed comparing needle loss to total VTC evolution in this genotype were consistent with other genotypes.

The high NAR clone followed a similar trend with low needle loss between 0.2 and 4.0% in the first 52 days (Figures 16 a and b), which corresponded with lower total VTC evolution ranging between 8.3 and 29.1mMg^{-1} within the same period (Figure 17). The ANOVA showed no significant change in needle loss and total VTC concentrations

within the first 52 days. However, after 52 days, needle loss started to increase gradually reaching significantly higher levels ($p<0.001$) (Table 4) until 100% needles on the branches were lost (Figure 16c). Within that same period, VTC peaked to significantly high ($p < 0.001$) concentrations of 86.2, 50.6 and 45.0 mMg^{-1} on days 73, 84 and 101, respectively (Table 4).



a **b** **c** **d**

Figure 16: Progressive needle loss (calculated using fresh weight) in a high NAR clone (# 9) after daily finger run test: a) 0 days (0% needle loss); b) 52 days (4.4% needle loss); c) 73 days (39.3% needle loss); d) 132 days (100%).

Table 4: P values; High NAR branches subjected to analysis of variance at $\alpha = 0.05$

Source of	P value (Day 1-50)		P value (Day 51-132)	
Variance	Needle Loss	VTC	Needle Loss	VTC
Day	0.093	0.091	<0.001	<0.001

The table gives *p values* of needle loss for high NAR branches for days 1-29 and 30-86 days

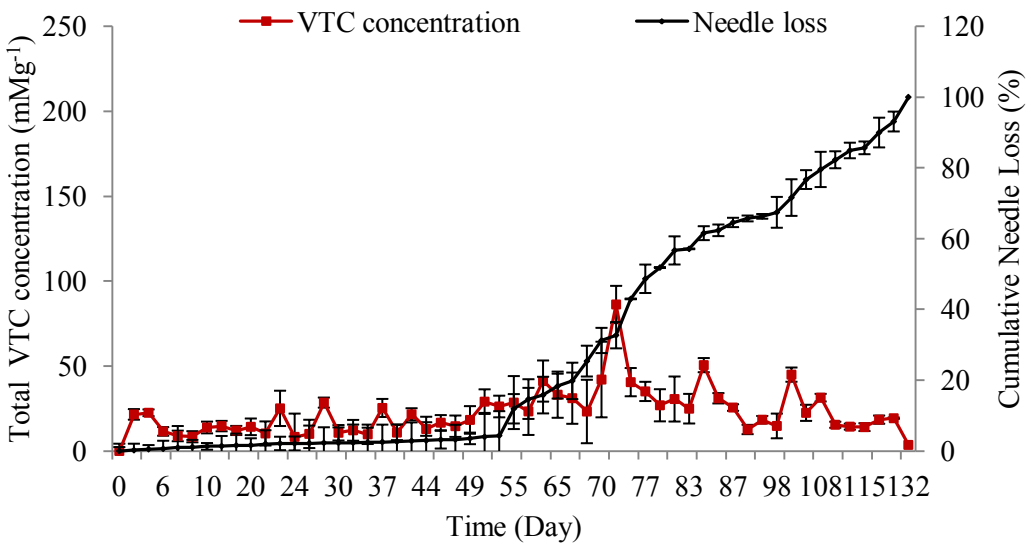


Figure 17: The progression of needle loss (as a percentage of needle fresh weight) and total VTC evolution in high needle retainer (High NAR) balsam fir genotype with a peak VTC evolution on day 73 and NRD of 132 days (n=10). The trends observed comparing needle loss to total VTC evolution in this genotype were consistent with other genotypes.

Our regression analysis suggested both positive and negative relationships between total VTC concentration and needle loss over postharvest duration of the branches (Figures 18 and 19). From day 0 to day 73 (0 to 33% needle loss), there was a highly significant ($p<0.001$) (Table 5), strong, and positive relationship between total VTC concentration and needle loss ($r^2 = 0.679$) (Figure 18). However, between day 74 and 132 (33 to 100% needle loss) a significant ($p=0.004$) but negative relationship between total VTC concentration and needle loss was identified ($r^2=0.478$) (Figure 19).

Table 5: P values; Low and High NAR branches subjected to regression analysis at $\alpha = 0.05$

Source of Variance	Regression P value Needle Loss and VTC
Low NAR	0.093
High NAR	<0.001

The table gives *p values* for regression analysis between needle loss and VTC for both low and high NAR branches.

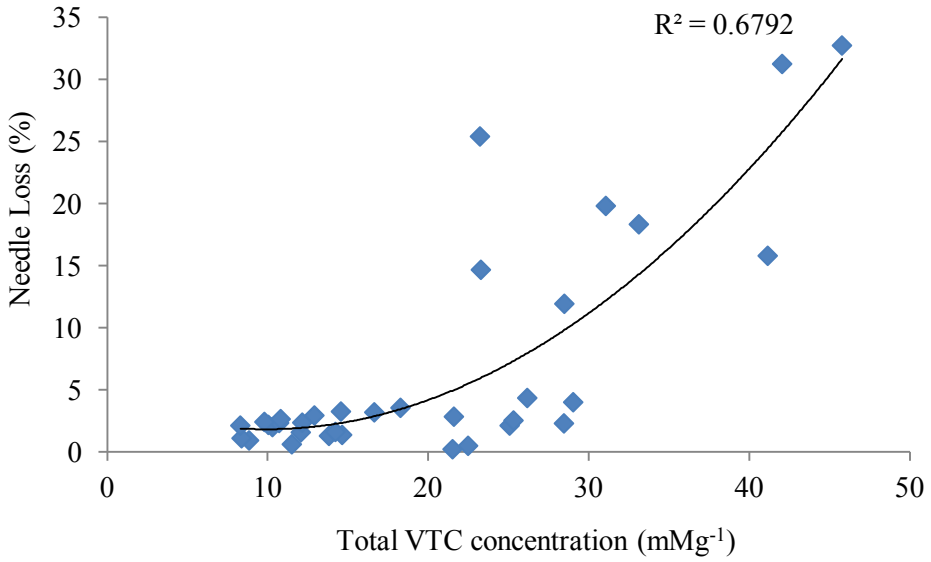


Figure 18: Significant ($p<0.001$) quadratic relationship between total VTC concentrations and percentage needle loss in high NAR balsam fir branches from day 0 to day 73 (0 to 33% needle loss) (n = 10). The relationship is described by Needle Loss - 0.023*VTC concentration

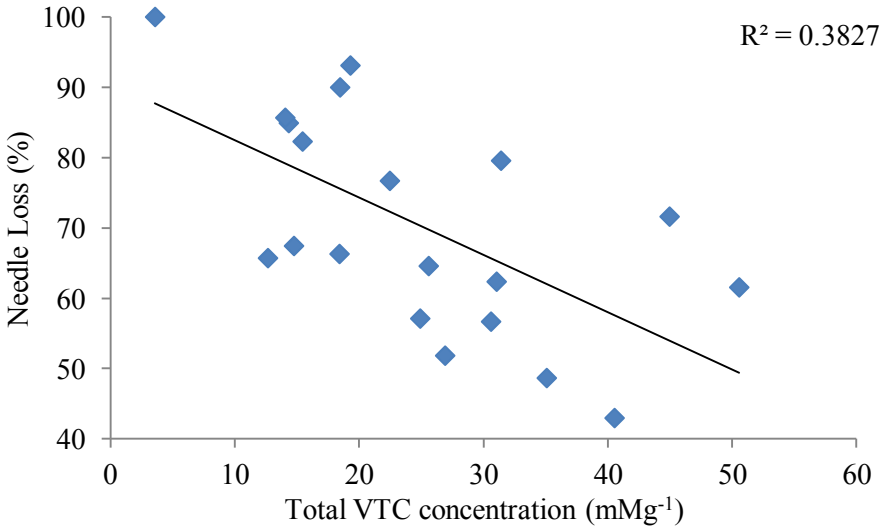


Figure 19: Significant ($p=0.004$) linear relationship between total VTC concentrations and percentage needle loss in high NAR balsam fir branches from day 74 to day 132 (33 to 100% needle loss) (n = 10). The relationship is described by Needle Loss + 0.816*VTC concentration

Comparison of daily average needle loss, AWU and VTC concentration identifies the trends of water use by postharvest balsam fir branches. The graph (Figure 20) shows an initial high volume (10.27 g) of water consumed by the branches in the first 8 days. It dropped to about 1.33g on day 9 and fluctuated between 1.30 and 3.71 g in the first 51 days. During the same period, the daily average VTC concentration evolved was significantly higher ($p<0.001$) than the last 78 days of postharvest needle loss. Just before needle loss started (day 62) AWU increased again to 4.13 g but dropped consistently till all needles were lost (Figure 20).

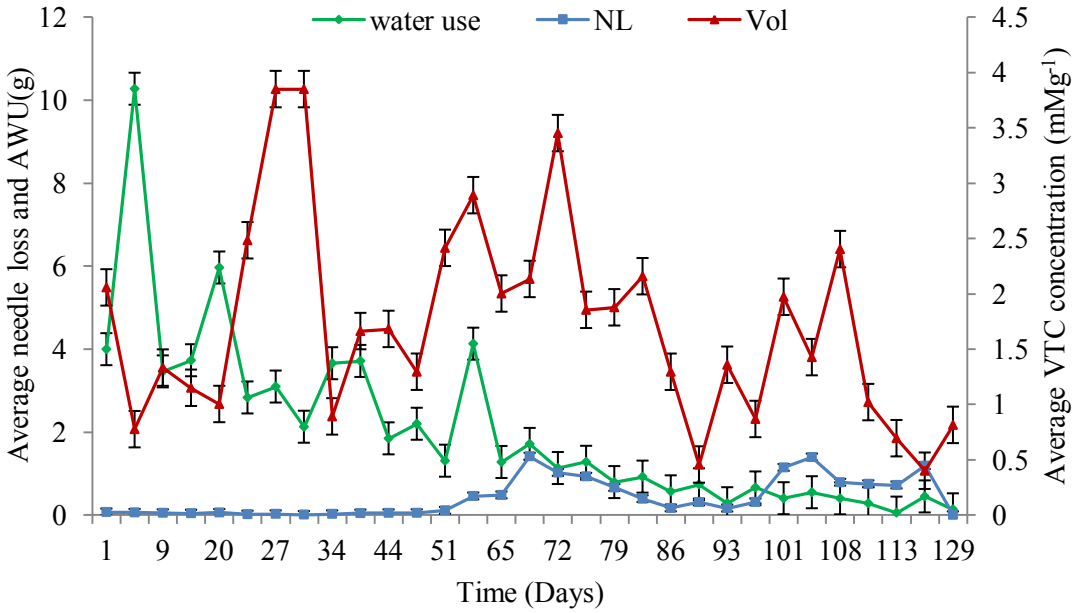


Figure 20: Progression of needle loss (average needle fresh weight), average VTC evolution and average water use in high NAR balsam fir genotype (n=10).

All individual VTCs were analyzed and the data showed similar trends in some individual VTCs as with total VTCs. Data from Low NAR genotype showed a significant peak in the concentration of β-Pinene ($p=0.026$) (Table 6) on day 41 (29.99 mMg^{-1}) and decreased to as low as 2.5 mMg^{-1} on day 65 until 100% needle loss (Figure 21). Although

a significant increase in β-Pinene on day 41 preceded needle abscission, the regression analysis showed no relationship between needle loss and evolved β-Pinene, postharvest. In the same genotype, β-Terpinene peaked on days 23 (2.10 mMg^{-1}), 34 (2.91 mMg^{-1}) and 62 (3.14 mMg^{-1}), which were significantly (p=0.011) (Table 6) higher than concentrations on the first thirteen days (Figure 22). No relationship however was identified between needle loss and postharvest β-Terpinene evolution when a regression analysis was conducted. Fenchyl acetate also peaked at the later days (day 83) at 95.5% needle loss and was significantly (p=0.05) (Table 6) higher than concentrations for the first 82 days (Figure 23) although regression analysis shows no relationship with needle loss. Comparatively, concentration of β-Pinene was highest among the three significant VTCs in the low NAR genotype.

Table 6: VTC of low NAR branches subjected to ANOVA and regression again needle loss at α = 0.05

	Volatile Terpene Compounds	P – Value Analysis of variance	P – Value Regression
1	α-Pinene	0.299	-
2	3-Thujene	0.102	-
3	Camphene	0.154	-
4	β-Pinene	0.026	0.876
5	3-Carene	0.097	-
6	β-Terpinene	0.011	0.415
7	D-Limonene	0.105	-
8	β –Phellandrene	0.189	-
9	ɣ -Terpinene	0.910	-
10	Terpinolene	0.920	-
11	Fenchyl acetate	0.050	0.697
12	Bornyl acetate	0.950	-

The table gives *p values* for ANOVA and regression analysis on individual VTCs of low NAR branches.

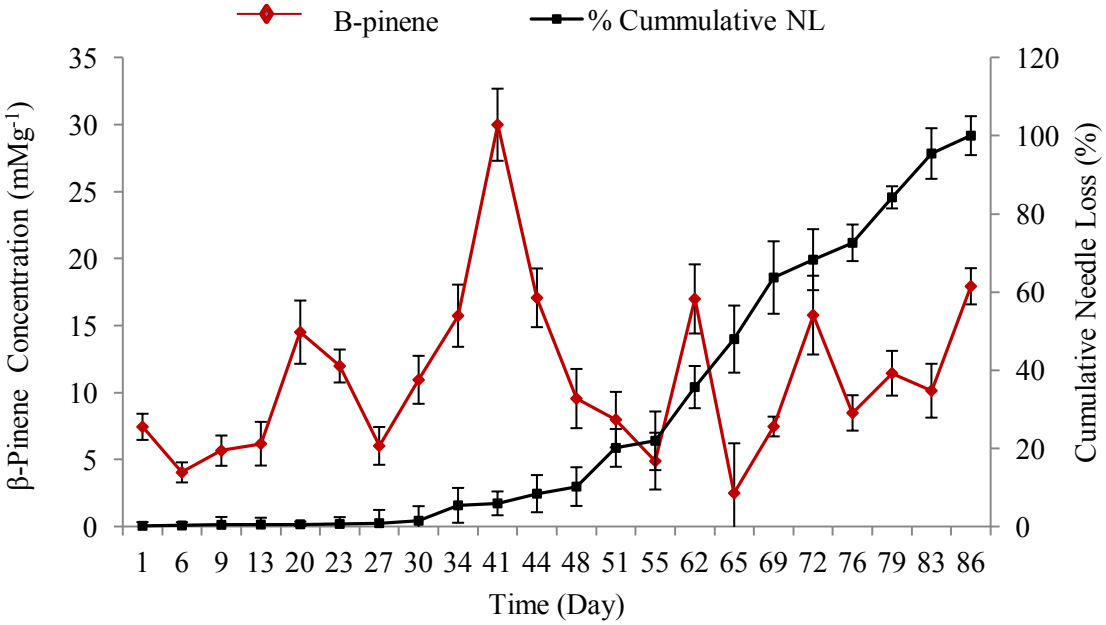


Figure 21: The progression of needle loss (as a percentage of needle fresh weight) and daily average of β-Pinene evolution in low needle retainer (Low NAR) balsam fir genotype with a peak β-Pinene evolution on day 41 and NRD of 86 days (n=10).

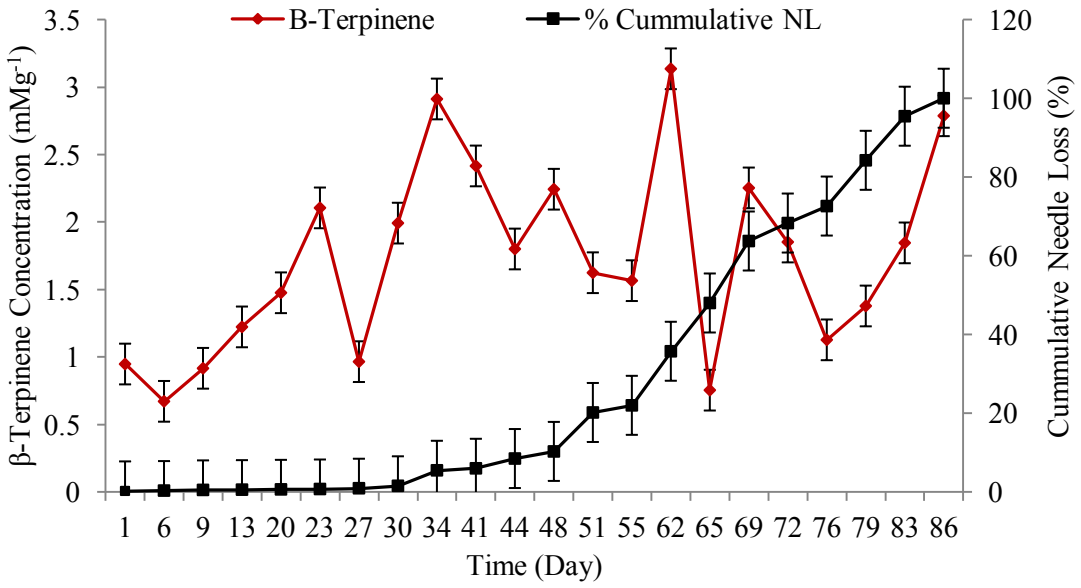


Figure 22: The progression of needle loss (as a percentage of needle fresh weight) and daily average of β-Terpinene evolution in low needle retainer (Low NAR) balsam fir genotype with a peak β-Terpinene evolution on day 62 and NRD of 86 days (n=10).

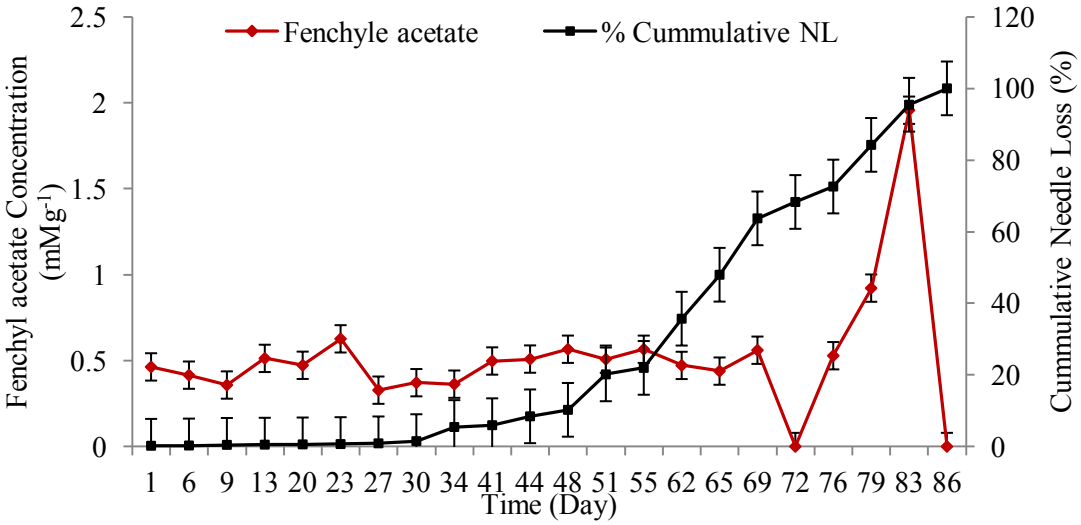


Figure 23: The progression of needle loss (as a percentage of needle fresh weight) and daily average of Fenchyl acetate evolution in low needle retainer (Low NAR) balsam fir genotype with a peak Fenchyl acetate evolution on day 83 and NRD of 86 days (n=10).

In the High NAR genotype, β-Pinene, β-Terpinene, Fenchyl acetate and other VTCs such as Camphene, and 3-Carene also showed significant peaks during the postharvest needle loss (Table 7).

Table 7: Individual VTC of high NAR branches subjected to ANOVA and regression at α = 0.05

	Volatile Terpene Compounds	*P – Value* Analysis of variance	*P – Value* Regression
1	α-Pinene	0.501	-
2	3-Thujene	0.424	-
3	Camphene	0.002	<0.001
4	β-Pinene	<0.001	0.014
5	3-Carene	<0.001	0.002
6	β-Terpinene	<0.001	0.001
7	D-Limonene	0.096	-
8	β –Phellandrene	0.534	-
9	ɣ -Terpinene	0.820	-
10	Terpinolene	0.660	-
11	Fenchyl acetate	<0.001	0.043
12	Bornyl acetate	0.950	-

The table gives *p values* for ANOVA and regression analysis on individual VTCs of high NAR branches

Camphene started off with lower concentrations, as low as 0.56 mMg^{-1} on day 20 for the first 48 days postharvest and peaked to significant (p=0.002) (Table 7) concentration (4.23 mMg^{-1}) on day 72 at 30.1% needle loss (Figure 24). High and significant concentrations were maintained for 11 days after which Camphene concentrations started dropping to as low as 0.56 mMg^{-1} on day 91 at 50% needle loss. Lower concentrations were maintained till day 101 right before needle abscission. Concentrations then dropped till 100% needle loss (Figure 24). Regression analysis showed a significant (p<0.001) relationship between needle loss and Camphene evolved postharvest (Table 7).

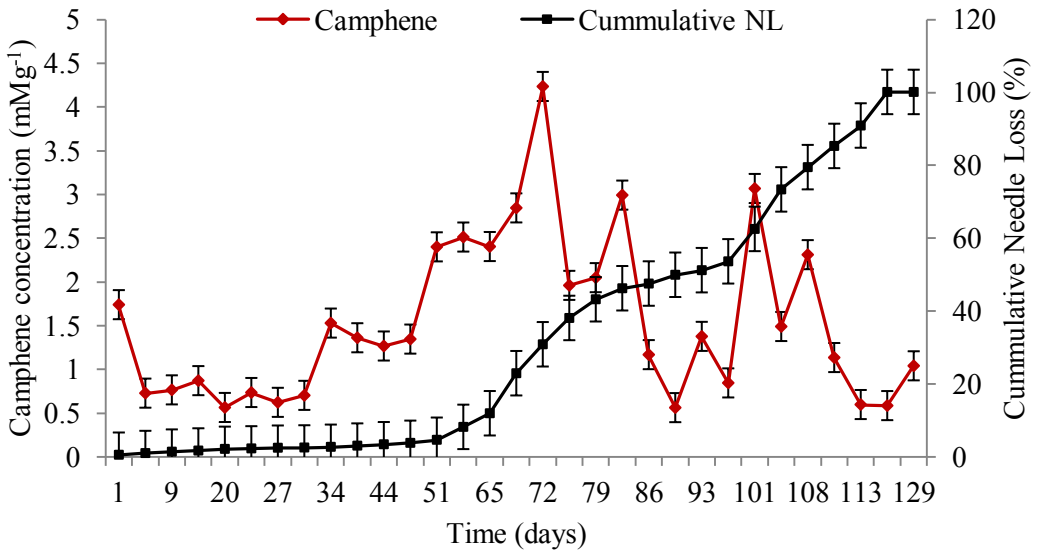


Figure 24: The progression of needle loss (as a percentage of need fresh weight) and daily average of Camphene evolution in high needle retainer (High NAR) balsam fir genotype with a peak Camphene evolution on day 72 and NRD of 132 days (n=10).

On day 1, concentration of β-Pinene was comparatively higher than that of Camphene, however concentration dropped to as low as 0.8mMg^{-1} on day 6 and lower concentrations were maintained for the first 48 days postharvest (Figure 20). β-Pinene concentration peaked to a significant (p<0.001) (Table 7) concentration of 6.15mMg^{-1} on day 62 at 8.2% needle loss, right before significant initiation of needle loss (Figure 25).

Significantly high concentrations were maintained for 21 days after which β-Pinene concentrations started dropping to as low as 0.79mMg^{-1} on day 129 at 100% needle loss (Figure 25). Regression analysis showed a significant (p=0.014) positive relationship between needle loss and concentration of β-Pinene evolved in the first 55 days. After that, there was a negative linear relationship in which β-Pinene concentration decreased with increased needle loss.

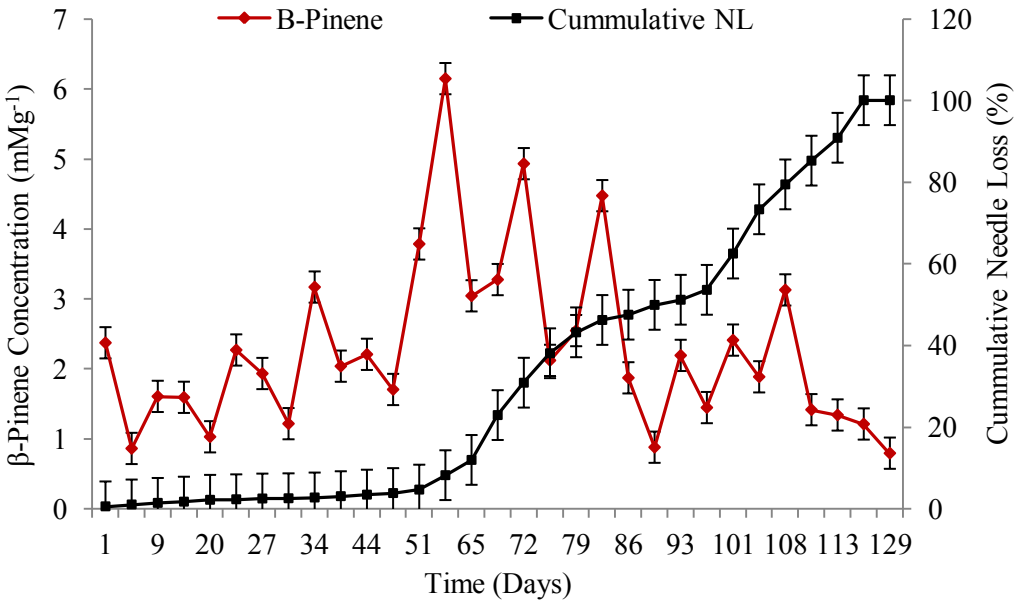


Figure 25: The progression of needle loss (as a percentage of branch fresh mass) and daily average of β-Pinene evolution in high needle retainer (High NAR) balsam fir genotype with a peak β-Pinene evolution on day 62 and NRD of 132 days (n=10).

The trend of 3-Carene (Figure 26) concentration during postharvest needle loss was very similar to that of Camphene (Figure 24). Initial concentrations for the first 51 days dropped to as low as 1.31mMg^{-1} on day 20. Right before the rise in needle loss (30.84 %), 3-Carene peaked to a significant (p<0.001) (Table 7) concentration of 15.40 mMg^{-1} on day 72 and started decreasing after that to lower concentrations ranging between 9.42 and 1.06 mMg^{-1} before 100% needle loss (Figure 26). Regression run also showed a

significant (p=0.002) positive relationship between needle loss and concentration of 3-Carene within the first 72 days and negative relationship from day 76 to last day postharvest (Table 7).

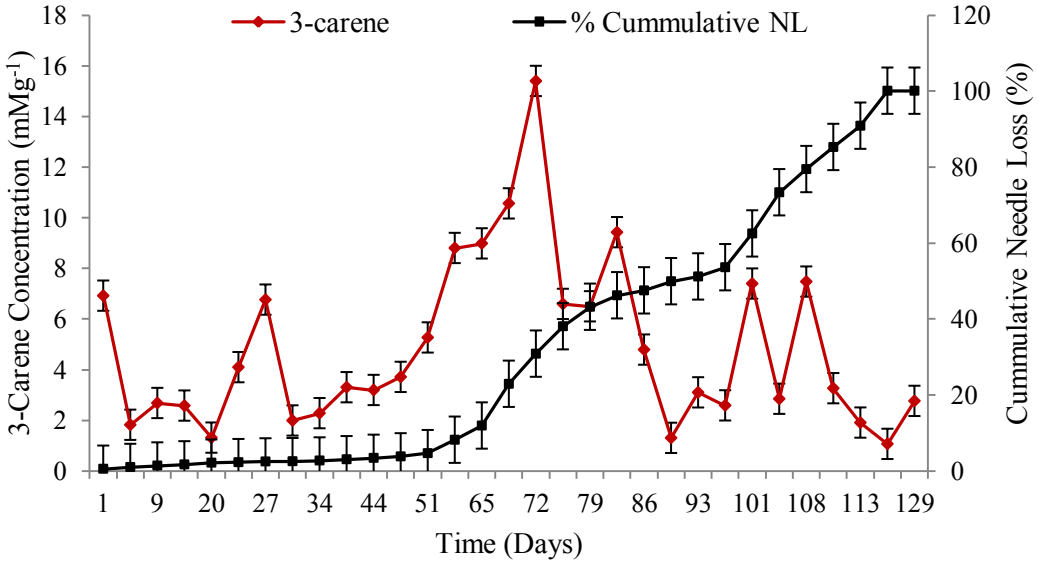


Figure 26: The progression of needle loss (as a percentage of branch fresh mass) and daily average of 3-Carene evolution in high needle retainer (High NAR) balsam fir genotype with a peak 3-Carene evolution on day 72 and NRD of 132 days (n=10).

Just as Camphene, β-Terpinene in the first 65 days fluctuated at very low concentrations between 0.40 and 2.19 mMg^{-1}. On day 72, the concentration of β-Terpinene peaked to a significant ($p<0.001$) (Table 7) high of 4.49 mMg^{-1}. Right after that, the concentration started decreasing while the needle loss increased, following similar trends of Camphene and 3-Carene (Figure 27). Regression analysis indicated a strong significant (p=0.001) positive relationship between needle loss and β-Terpinene in the first 72 days postharvest at 30.8% needle loss (Table 7). However, in the last 57 days where needle loss was high there was a negative relationship between the needle loss and β-Terpinene (Figure 27).

This follows the same trends as the other individual VTC's in both the high and low NAR genotypes.

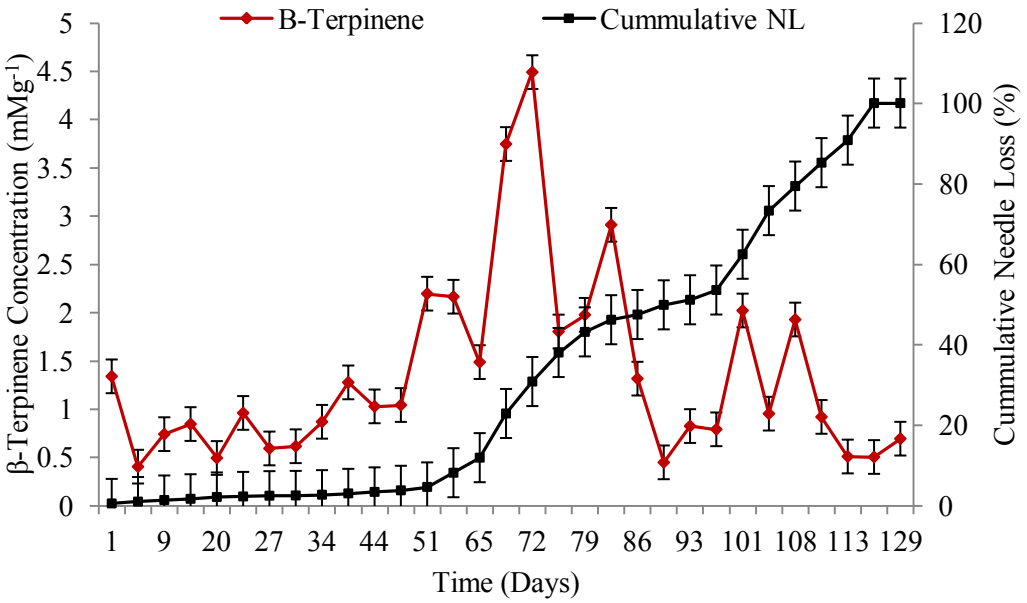


Figure 27: The progression of needle loss (as a percentage of branch fresh mass) and daily average of β-Terpinene evolution in high needle retainer (High NAR) balsam fir genotype with a peak β-Terpinene evolution on day 72 and NRD of 132 days (n=10).

The evolution trend of Fenchyl acetate gives a clearer understanding how the majority of the individual VTCs under this study responded to needle loss in the postharvest balsam fir branches. The branches in the first 69 days produced very low concentrations of Fenchyl acetate between 0.31 and 0.52mMg^{-1}. Within the same period the branches loss 0.54 to 11.91% needles (Figure 28). Prior to the initiation of an increasing percent needle loss (22.90%), there was a significant ($p<0.001$) (Table 7) peak in the concentration of Fenchyl acetate (6.55 mMg^{-1}) on day 72. Right after that, concentration of Fenchyl acetate dropped while needle loss increased until all needles on the branches were lost (Figure 28). Regression analysis showed a significant ($p=0.043$) but weak positive relationship between needle loss and Fenchyl acetate (Table 7).

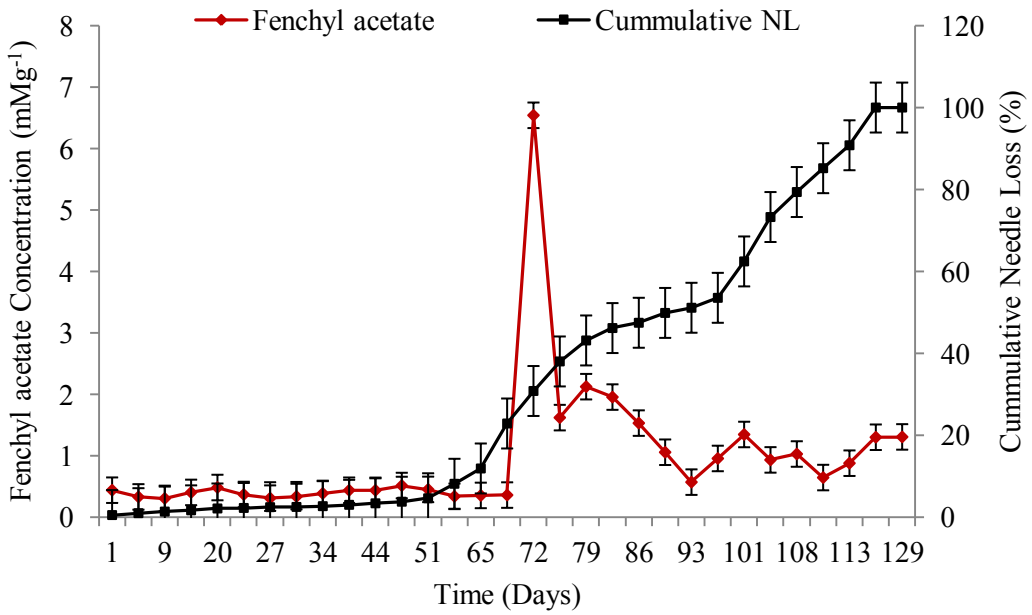


Figure 28: The progression of needle loss (as a percentage of branch fresh mass) and daily average of Fenchyl acetate evolution in high needle retainer (High NAR) balsam fir genotype with a peak Fenchyl acetate evolution on day 72 and NRD of 132 days (n=10).

6.6 Discussion

Both genotypes used for this study did not depict their expected needle retaining abilities. A study by Veitch and Rajasekaran (2010) identified the low NRD clone (# 608) and the high NRD clone (# 9) under dehydrated conditions (not fed with water throughout the experiment) to lose 100% needles on 18 and 41 days, respectively. However, in this study where branches were hydrated, low NRD clone lasted for 86 days while the high NRD clone lasted for 132 days, about 68 and 91 days higher compared to what was reported by Veitch and Rajasekaran (2010). Needle retention trends were however similar between both studies. One of the obvious reasons contributing to the high NRD in this study was the hydration of the branches. Studies have shown that in the presence of water, temperature (20-26°C) and humidity, plants are able to go through metabolisms thereby slowing down the abscission process (Buchanan et al. 2000; Taylor and Whitelaw 2001).

This was proven when branches during the initial days of postharvest consumed higher volumes of water, and these volumes dropped with time when branches were losing a large number of needles. Again, harvesting of branches right after the winter season meant they were acclimatized to the cold. It was shown that balsam firs tend to retain their needles longer after exposure and acclimatization to lower temperatures (MacDonald and Lada, 2008; Thiagarajan et al. 2012).

There was a distinct pattern in total VTC evolution, which was consistent with needle abscission pattern. During the early stages of postharvest, VTC concentration was generally low. However, a few days before initiation of needle abscission the total VTC concentration peaked, which then dropped after a few days but continued until all needles were lost on the branches. These trends were consistent in both the high and low needle retainers. Since VTCs spike prior to needle abscission, this may indicate their actions as a potential signal to induce needle abscission.

A detailed analysis of the individual VTCs showed a similar trend to total VTC during postharvest needle abscission. Among all VTCs, five individual VTCs (β-Pinene, β-Terpinene, Fenchyl acetate, Camphene and 3-Carene) showed significant increases in both clones, 26 days prior to needle abscission. Out of these five individual VTCs, only three (β-Pinene, β-Terpinene and Fenchyl acetate) were identified in the low NRD clone to be significantly higher in concentration prior to needle abscission but then they showed no relationship with postharvest needle abscission. This is not unusual since it is well documented in other signal molecules such as ethylene that it is not always necessary for signal molecules to remain high in concentration unless it is required for a direct involvement in abscission process (MacDonald et al. 2010). On the other hand, all five

VTCs (β-Pinene, β-Terpinene, Fenchyl acetate, Camphene and 3-Carene) were consistently and significantly higher in concentration in the high NAR genotype prior to needle abscission. Just as in the low NAR clone, β-Pinene, β-Terpinene, Fenchyl acetate, and 3-Carene except Camphene all showed a significant relationship with postharvest needle abscission. However, the concentration of β-Pinene, β-Terpinene and Fenchyl acetate in the high NAR were two to three fold higher than the low NAR clone. These individual VTCs were also identified to be higher in concentration in our previous study, confirming our speculation made in the previous study that VTCs such as β-Pinene, β-Terpinene, and 3-Carene may have a role to play in postharvest needle abscission.

Although 3-Thujene was identified as a new VTC in balsam fir in the previous study, in this study over the entire postharvest period it was not identified. This tells us that 3-Thujene might be an early (less than 24 hours) signal molecule synthesized when balsam fir trees are injured such as in our case where branches were cut from the main trees. 3-Thujene is known for its high level of pungency (Maarse and Kepner, 1970; Bowman et al. 1997) therefore, it can be speculated to serve a common role just like other VTCs such as 3-Carene and β-Pinene, and sesquiterpenes that provide balsam firs defense against insect feeding (Vereen et al. 2000; Carlow et al. 2006).

Since no work has been done on how these individual VTCs can affect needle loss, the mechanisms by which specific individual VTCs regulate needle abscission or whether they play a role is unclear. Studies have shown that monoterpenes, which in previous studies are known to be dominant in balsam fir serve as a molecule to protect the photosynthetic apparatus against oxidative damage (Loreto and Velikova, 2001; Lorento et al. 2004). Since oxidative stress is known to cause cell degradation and eventually

abscission (Valko et al. 2005), we can speculate that the consistent increase in individual VTC concentrations prior to needle abscission is a signal to abscission. Therefore, the plant at the point of cellular degradation during postharvest needle loss induces the synthesis of VTCs or stimulates the release of existing VTCs.

6.7 Conclusion

The previous study identified twelve distinct VTCs in postharvest balsam fir. Out of that, this study can conclude that five individual VTCs (β-Pinene, β-Terpinene, Fenchyl acetate, Camphene and 3-Carene) may have a role in triggering needle abscission postharvest. A look at total VTCs showed similar trends as individual VTCs, indicating a possibility that VTCs may trigger or signal needle abscission postharvest.

While this study clarified that 3-Thujene did not play a role in postharvest balsam fir needle abscission it may possibly act as an early signal in balsam fir in response to wounds or provide defense against insect attacks.

This groundbreaking finding does not end here; it is now necessary to confirm the specific role of these identified VTCs in postharvest balsam fir and to establish if VTC evolution is dependent or independent of ethylene synthesis. A future study to monitor the evolution of VTCs when ethylene synthesis is inhibited or induced may help establish relationships between VTC, ethylene and postharvest needle abscission.

6.8 Reference

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CHAPTER 7: THE LINK BETWEEN VOLATILE TERPENE COMPOUNDS, ETHYLENE AND POSTHARVEST NEEDLE ABSCISSION

7.1 Abstract

Despite limited work in the area of postharvest needle abscission, we now know that release of volatile terpene compounds (VTCs) into the headspace peak prior to needle abscission, ethylene triggers needle abscission and long-term exposure to ethylene induces postharvest needle abscission. It is not known however, whether or not ethylene works independently or whether needle abscission is triggered by ethylene-induced VTCs. It was therefore hypothesized that inhibiting ethylene will inhibit or reduce VTC synthesis thereby extending postharvest needle retention. Two experiments were conducted; the first used aminoethoxyvinylglycine (AVG) (0, 0.1, 1, 10, 100 ppm) with 3 replicates at each level. The second experiment used ethylene (0 or 1000 ppm) with 3 replications at each level. The measured response factors included needle retention duration (NRD), percent needle loss, ethylene and VTC evolution. The study confirmed the ethylene inhibitory ability of AVG at 10 ppm. At this concentration, a significant (34.7%) needle loss was observed on day 28 however NRD was extended by 26 days compared to the control. AVG at 10 ppm inhibited ethylene evolution but there was no significant change in VTCs seen at any AVG concentration. Despite undetectable levels of ethylene, needle abscission occurred accompanied by three distinct peaks of VTCs thus, suggesting that ethylene and VTC evolution are independently regulated and postharvest needle abscission can be triggered by VTCs, independent of ethylene evolution. It was also confirmed that β-Pinene, β-Terpinene, Camphene and 3-Carene may be the possible signal molecules to trigger and sustain needle abscission since their evolution prior to needle abscission was consistent with our previous study. We also demonstrated that exposure to exogenous ethylene induces VTCs but was ineffective at that concentration in inducing needle abscission.

7.2 Introduction

Postharvest needle loss in balsam fir has been a major bottleneck for the Christmas tree and greenery industry for decades. Little was understood on the physiological functions of the tree and how it affects postharvest needle abscission until recently. Our previous studies identified twelve VTCs in balsam fir (Chapter 5). Out of which, β-Pinene, β-Terpinene, Fenchyl acetate, Camphene and 3-Carene, all showed a consistent increase in evolution prior to needle abscission (Chapter 6). Their evolution appears to be similar to ethylene during postharvest needle abscission as reported by MacDonald et al. (2010). Therefore, it has been hypothesized that VTCs may trigger and/or regulate needle abscission. However, it has not been confirmed whether postharvest needle abscission is a consequence of VTC emissions or ethylene evolution alone, or the actions of VTCs and ethylene are dependent on each other.

So far there is little or no information on the relationship between VTCs and ethylene, the role of VTCs in postharvest abscission or the effect of VTC evolution on synthesis of plant metabolites. A study by Horiuchi et al. (2001) successfully demonstrated that VTCs can be produced through jasmonic acid when detached lima bean leaves (*Phaseolus lunatus*) are subjected to the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid. However, in some plants such as tobacco, exogenous application of ethylene and jasmonic acid does not induce VTC production (Kahl et al. 2000). Apart from these studies, the majority of previous studies have focused on VTCs as defense compounds against pathogens and herbivores (Vereen et al. 2000; Carlow et al. 2006). Earlier, VTCs were found to act as signals between different parts of the same plant, between plants, and between plants and animals as well as micro-organisms (Penuelas and Llusia, 2001).

The role of phytohormones such as abscisic acid, jasmonic acid, auxin and even ethylene in postharvest balsam fir needle abscission is not completely understood, although MacDonald et al. (2010; 2011) established that, prior to abscission, ethylene evolution increases, exogenous ethylene accelerates abscission, and blocking ethylene synthesis or ethylene action with aminoethoxyvinylglycine (AVG) and 1-methylcyclopropane (1-MCP) delays postharvest needle abscission in balsam fir. They proposed that ethylene acts as a potential signal molecule to induce postharvest needle abscission in balsam fir. As of now, there are no known studies that satisfactorily implicate ethylene as the only signal molecule to induce postharvest needle abscission in balsam fir.

With limited background work on the effects of ethylene on the evolution of VTCs and how it affects abscission, it is therefore difficult to predict whether postharvest needle abscission in balsam fir is a consequence of a complex relationship between VTC and ethylene. Thus, it is prudent to conduct a study that will investigate the link between ethylene, VTC evolution and postharvest needle abscission. It was hypothesized that inhibiting ethylene will reduce or inhibit evolution of VTCs.

7.3 Objective

The objective of this study was to determine the effect of ethylene on VTCs and the impact on postharvest needle abscission of balsam fir.

This was achieved through;

a. Inhibition of ethylene synthesis by exogenous application of the ethylene synthesis inhibitor AVG.

b. Stimulation of ethylene action by the exogenous application of ethylene gas.

7.4 Materials and Methods

A random sample of twenty one (two-year old growth) lateral branches from a low needle abscission resistance (NAR) genotype (clone #608) were harvested on August 21, 2012 from a population of 20 year-old trees at the Tree Breeding Centre, Department of Natural Resources, Debert, NS (45º 25' N, 63º 28' W). Branches were individually sealed in zip lock bags in the field and transported to the lab as described in Section 4.2. Two experiments were conducted to achieve the objectives of this study. The first experiment was set up in the volatile incubation chamber as described in Section 4.3 with a completely randomized design. Branches were treated with AVG (Sigma-Aldrich, Oakville, ON, Canada) at concentrations (0, 0.1, 1, 10, 100 ppm). Each treatment was replicated three times with each branch as a replicate. The second experiment was also a completely randomized design set up in the volatile incubation chamber. However, branches were exposed to ethylene (Air Liquid, Truro, NS, Canada) at 0 and 1000 ppm concentrations. Each treatment was replicated three times with each branch serving as replicate.

AVG was applied through xylem feeding by dissolving a known concentration into the water used. In this case, each branch except the control was provided AVG for the entire duration (84 days) of the experiment (MacDonald et al. 2010). Controls were fed 150 ml reverse oxidation water throughout the experiment.

In the experiment where samples were exposed to 1000 ppm exogenous ethylene, the chambers were injected with 4 ml ethylene and sealed for 12 hours daily, to maintain a concentration of 1000 ppm (MacDonald et al. 2010). In order to prevent excess CO2 and humidity build up, chambers were placed back on the continuous airflow system after the 12 hours of ethylene exposure. This continued for 14 days since it was previously identified as an effective way to accelerate needle abscission in postharvest balsam fir (MacDonald et al. 2009).

Response variables were NRD, percent needle loss, ethylene and VTCs evolution as describes in chapter 4. Repeated measures analysis was conducted using general linear model (GLM) with Minitab 16 (Minitab Inc., State College, PA) to determine the relationship between volatile evolution, ethylene evolution and needle loss. All statistical assumptions, such as normal distribution, and independence were confirmed.

7.5. Results

7.5.1 Effect of ethylene inhibition on VTC evolution in balsam fir

The data showed that AVG inhibited endogenous ethylene evolution, depending on the concentration (Figure 29). Branches subjected to 0 ppm AVG had an average peak ethylene evolution of 6.5 $\mu L \cdot g^{-1} \cdot h^{-1}$ (Figure 29), which was significantly ($p=0.035$) higher than 1 and 10 ppm AVG treated branches (Table 8). However, ethylene evolution in branches with 10 ppm AVG application was below detectable limits and therefore, considered as the most effective concentration for ethylene inhibition (Figure 29). AVG concentration of 1 ppm also inhibited ethylene evolution with an average ethylene

evolution of 2.12 µL·g-^{1}·h^{-1} but was not significantly different from 100 ppm (4.03 µL·g^{-1}·h^{-1}) (Figure 29). Average VTC concentration did not differ significantly (p=0.24) among various AVG treatments. However, average VTC evolution was higher (2.98 mMg^{-1}) in 1 ppm AVG treatment and lower (1.74 mMg^{-1}) in the control (Figure 29).

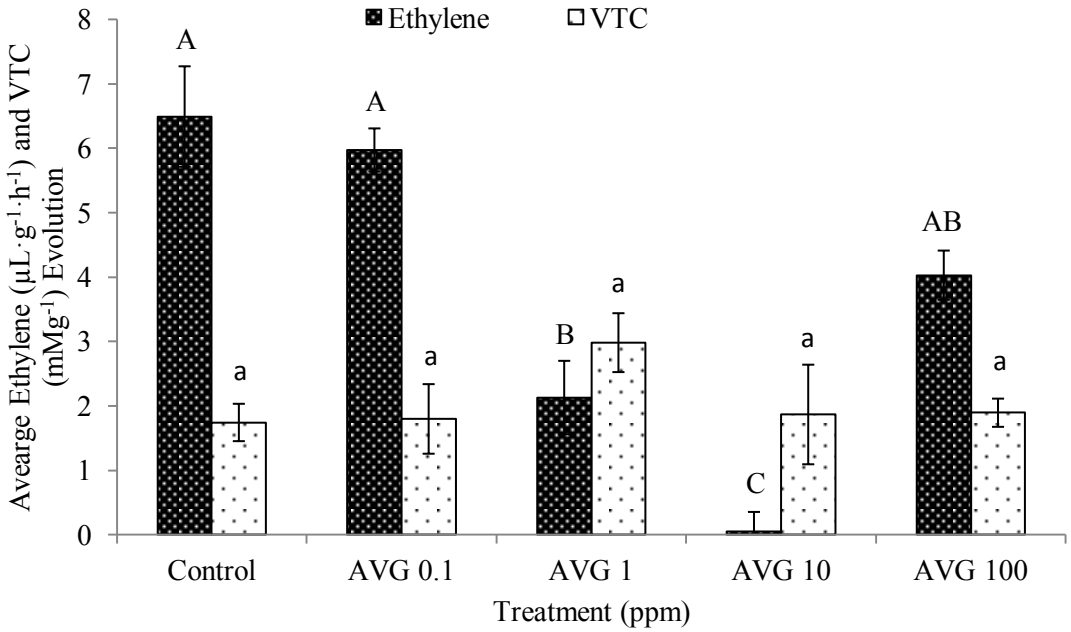

Figure 29: Average ethylene and VTC evolution of postharvest balsam fir branches with no AVG (Control), 0.1, 1, 10 and 100 ppm AVG treatments. Significant differences were determined using Tukey's multiple mean comparison of 3 balsam fir branches at α = 0.05. Overall average of measurements was used. In a particular color shade, bar charts that do not share the same letters are significantly different.

Table 8: P values; AVG treated branches subjected to analysis of variance at α = 0.05

Source of	P value (Day 1-35)			P value (Day 36-84)		
Variance	Needle Loss	Ethylene	VTC	Needle Loss	Ethylene	VTC
Treatment	0.08	0.035	0.240	0.343	0.333	0.330
Day	< 0.001	0.052	0.002	0.615	0.715	0.086

The table gives *p values* of needle loss, ethylene and VTC under AVG and ethylene treated branches for days 1-35 and 36-80. Two time ranges were used since AVG 100 ppm treated branches loss 100% needles on day 35 and ethylene 1000 ppm on day 84.

In the absence of exogenous ethylene, 1 and 10 ppm AVG concentrations were effective in delaying postharvest needle abscission. AVG concentration of 10 ppm was the most effective, increasing NRD significantly ($p<0.001$) by 26 days compared to branches subjected to 0 ppm AVG (Figure 30). Branches treated with 0.1 and 100 ppm AVG performed poorly with AVG 0.1 and 100 ppm retaining needles for only 56 and 35 days, respectively (Figures 30 and 31).

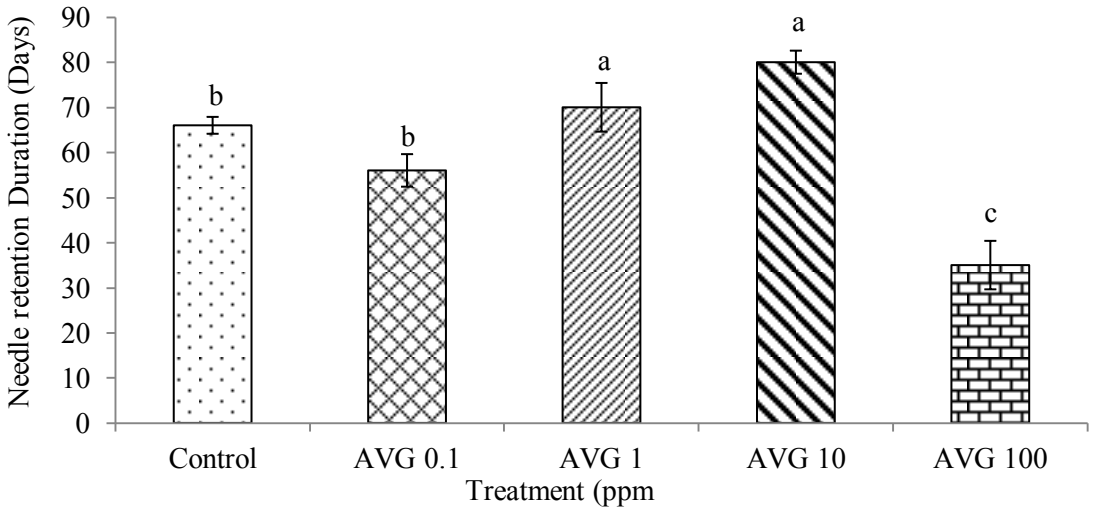

Figure 30: Needle retention duration of root detached balsam fir branches with no AVG (Control), 0.1, 1, 10 and 100 ppm AVG treatments. Significant differences were detected using Tukey's multiple mean comparison of 3 balsam fir branches at $\alpha = 0.05$. Bar charts that do not share the same letters are significantly different.

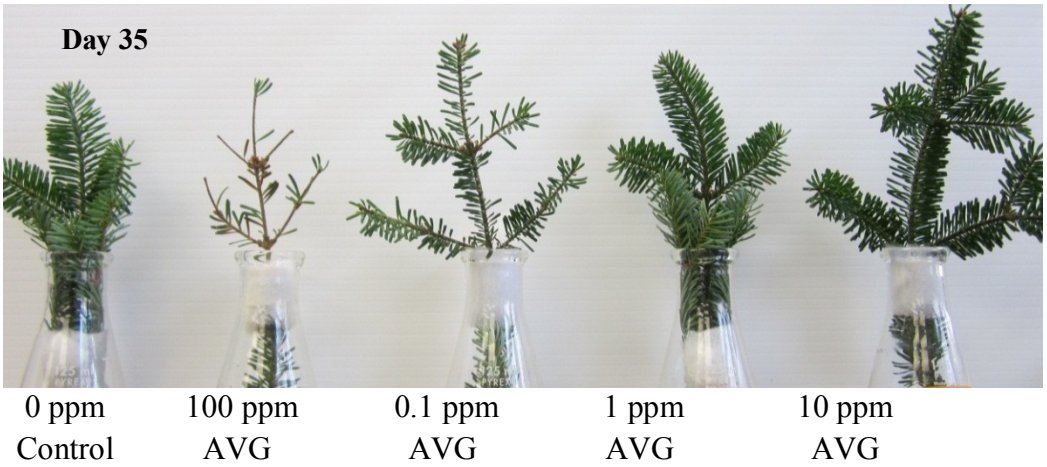

Figure 31: Balsam fir branches treated with AVG on day 35 in the absence of exogenous ethylene.

Analysis was also conducted on the effect of AVG treatment on total VTC evolution, ethylene evolution and needle loss. Although within the first 35 days, AVG application at any concentrations did not have a significant effect on total VTC whereas the control showed a significant ($p=0.002$) increase (3.1 mMg^{-1}) in VTCs on day 28 (Figure 32). Ethylene evolution in the control was also significantly ($p=0.05$) higher in the first few days but declined drastically and remained at a low level throughout the rest of the period. While this was so, there was a significant increase in VTC evolution, which corresponded to a significant ($p<0.001$) increase in postharvest needle loss of 43.6% on day 28 (Figure 32).

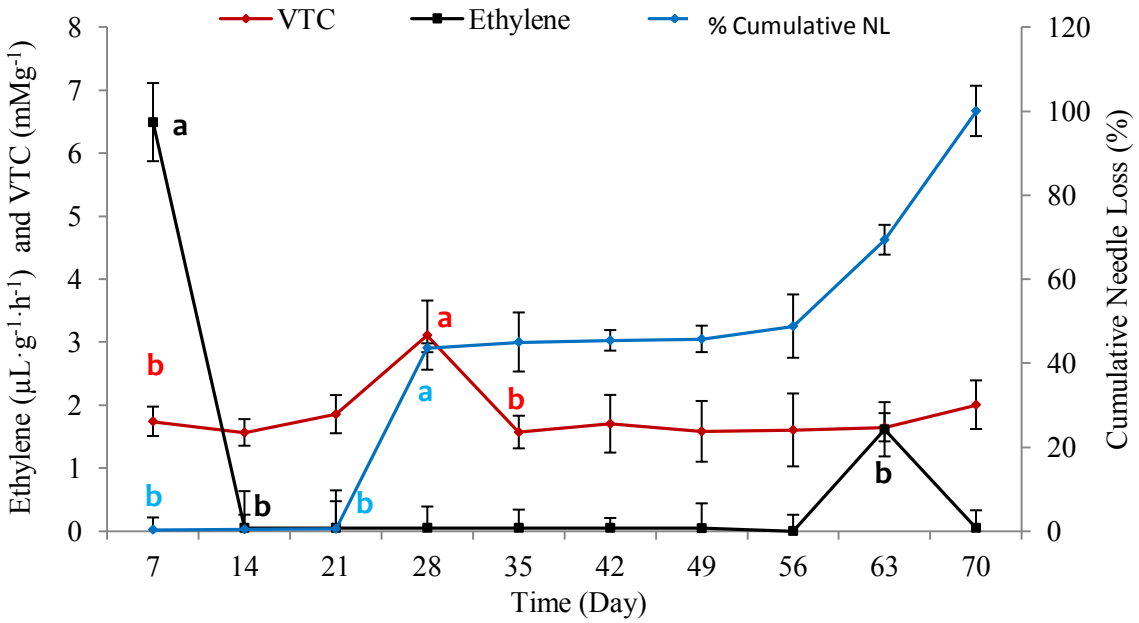

Figure 32: The progression of needle loss (as a percentage of branch fresh mass), total VTC and ethylene evolution of 0 ppm AVG (control) treated balsam fir branches. Peak VTC and ethylene evolution on days 28 and NRD of 66 days (n=3). Graph points that do not share the same letters in a particular color are significantly different.

While 10 ppm AVG treatment inhibited ethylene evolution, it promoted VTC evolution significantly ($p<0.001$) to as high as 4.99 mMg^{-1} on day 28 (23.4% higher than control)

(Figure 33). This again corresponded to a significant ($p<0.001$) increase in needle loss of 34.8% on the same day. There was also high VTC peaks on day 56 (4.2 mMg^{-1}) and 70 (3.2 mMg^{-1}) that corresponded with a high cumulative needle loss (84 and 91%, respectively) (Figure 33). Since ethylene evolution in this treatment was inhibited, there were no significant differences in ethylene evolutions over the entire postharvest period. The increases in ethylene evolution on day 42 (0.6 $\mu L \cdot g^{-1} \cdot h^{-1}$) and day 63 (0.8 $\mu L \cdot g^{-1} \cdot h^{-1}$) were not statistically significant (Figure 33).

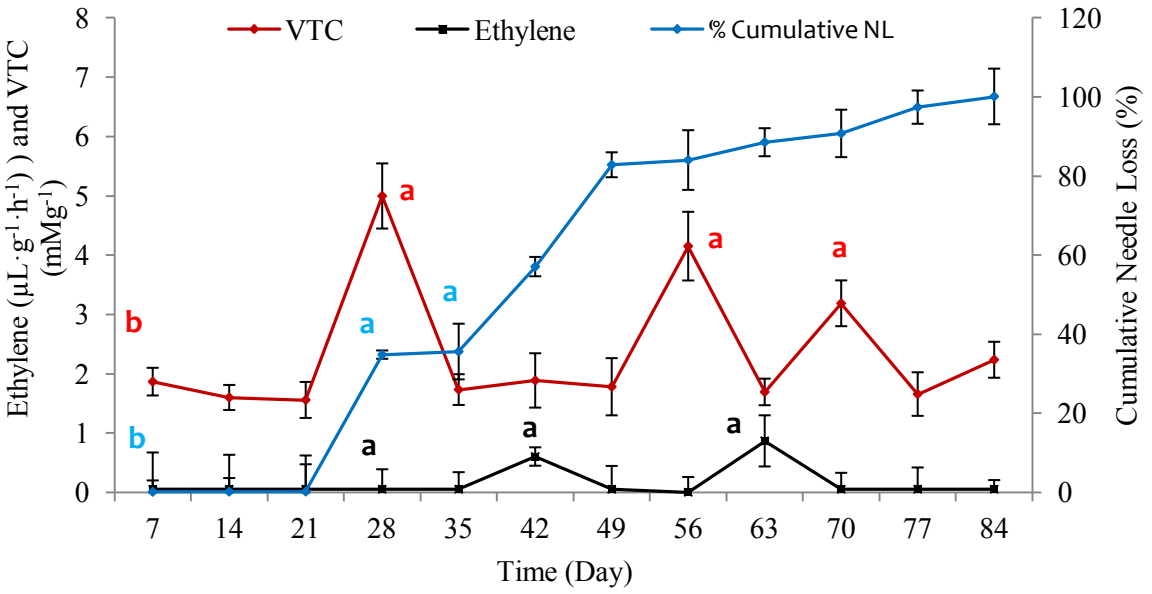

Figure 33: The progression of needle loss (as a percentage of branch fresh mass), total VTC and ethylene evolution of 10 ppm AVG treated balsam fir branches. Peak VTC evolution on days 28, 56 and 77, and NRD of 84 days (n=3). Graph points that do not share the same letters in a particular color are significantly different

Detailed analysis was also conducted on specific VTCs (β-Pinene, β-Terpinene, Camphene and 3-Carene) that were identified to have a possible role in postharvest needle abscission. The results showed similar trends to the previous study where VTCs increased prior to needle abscission. Although Camphene concentrations for all

treatments were between 1.5 and 2.2 mMg^{-1} in the first 17 days, it increased in the control and 0.1 ppm AVG treatments on day 21 to 5.8 mMg^{-1} and 6.8 mMg^{-1}, respectively, prior to needle abscission on day 28 (Figure 34). The increase in Camphene concentration however was not significantly (p=0.258) different from other days (Table 9).

Table 9: P values; AVG treated branches subjected to analysis of variance at α = 0.05

Source of Variance	P Value			
	Camphene	β-Pinene	β-Terpinene	3-Carene
Treatment	0.172	0.326	0.089	0.157
Day	0.258	0.020	<0.001	<0.001

The table gives *p values* of Camphene, β-Pinene, β-Terpinene and 3-Carene under Control, 0.1, 1, 10 and 100 ppm AVG treated branches for the entire postharvest duration.

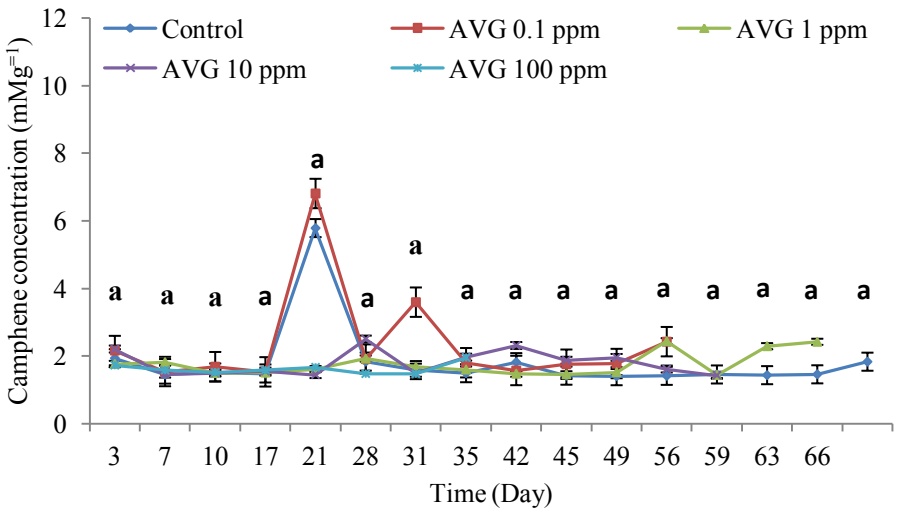

Figure 34: The progression of Camphene (mMg^{-1}) in 0, 0.1, 1, 10 and 100 ppm AVG treated root detached balsam fir branches. Peak Camphene evolution on day 21 (n=3). Graph points that do not share the same letters are significantly different

Similar trends to that of Camphene were observed for β-Pinene however the evolution of β-Pinene showed a significant increase (p=0.02) (Table 9) on day 28 for 0.1, 1 and 10

ppm AVG treatments with 3.9, 4.8 and 4.9 mMg^{-1} evolved, respectively (Figure 35). Evolution of β-Pinene on day 28 corresponded with a significant increase in needle loss in those AVG treatments with 32, 23 and 30% needle loss, respectively as presented earlier. β-Pinene concentrations in AVG 10 ppm treatment declined after that and remained similar until day 49 when β-Pinene concentrations increased. The increase in β-Pinene concentration on day 49 was not significant statistically.

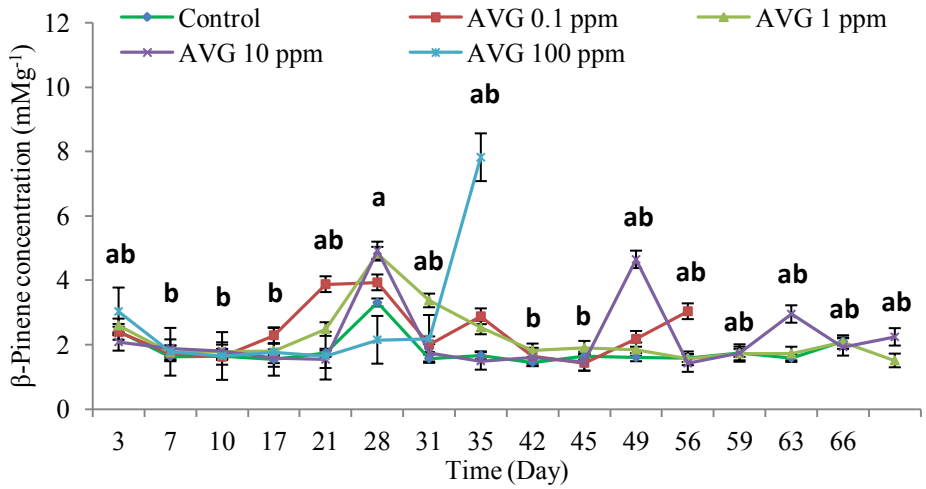

Figure 35: The progression of β-Pinene (mMg^{-1}) in response to 0 ppm, 0.1, 1, 10 and 100 ppm AVG treated root detached balsam fir branches. Peak β-Pinene evolution on day 28 (n=3). Graph points that do not share the same letters are significantly different

3-Carene concentrations also increased significantly ($p<0.001$) on day 28 like Camphene and β-Pinene (Table 9). AVG 10 ppm treated branches synthesized higher concentration of 10.4 mMg^{-1} compared to AVG 100 ppm (2.8 mMg^{-1}), Control (4.9 mMg^{-1}), 0.1 ppm (6.9 mMg^{-1}), and 1 ppm (7.1 mMg^{-1}), respectively, (Figure 36). They all corresponded to a high increase (23 and 45 %) in needle loss.

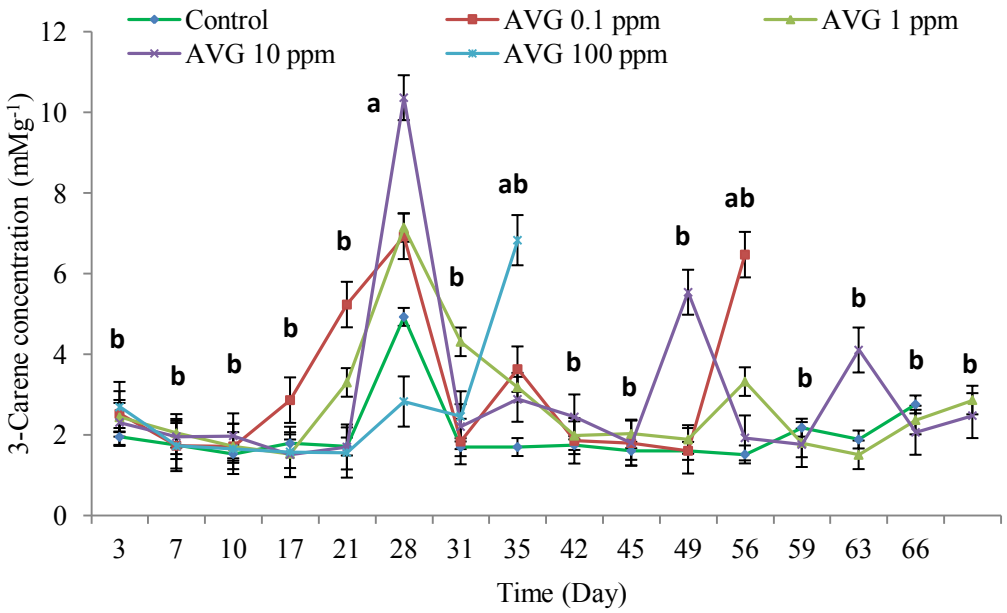

Figure 36: The progression of 3-Carene (mMg^{-1}) in 0 ppm, 0.1, 1, 10 and 100 ppm AVG treated root detached balsam fir branches. Peak 3-Carene evolution on day 28 (n=3). Graph points that do not share the same letters are significantly different

Trends of β-Terpinene among the AVG treatments were of no difference compared to other VTCs. In week 4 (day 28) there was a convergence with a significant ($p<0.001$) increase in β-Terpinene concentrations in all AVG treatments except the 100 ppm treatment (Table 9). B-Terpinene concentration ranged between 3.5 mMg^{-1} in control treatment to 7.1 mMg^{-1} in 10 ppm AVG treatment (Figure 37). Increases in β-Terpinene concentrations also corresponded to increases in needle loss between 23 and 45 % for the AVG treatments.

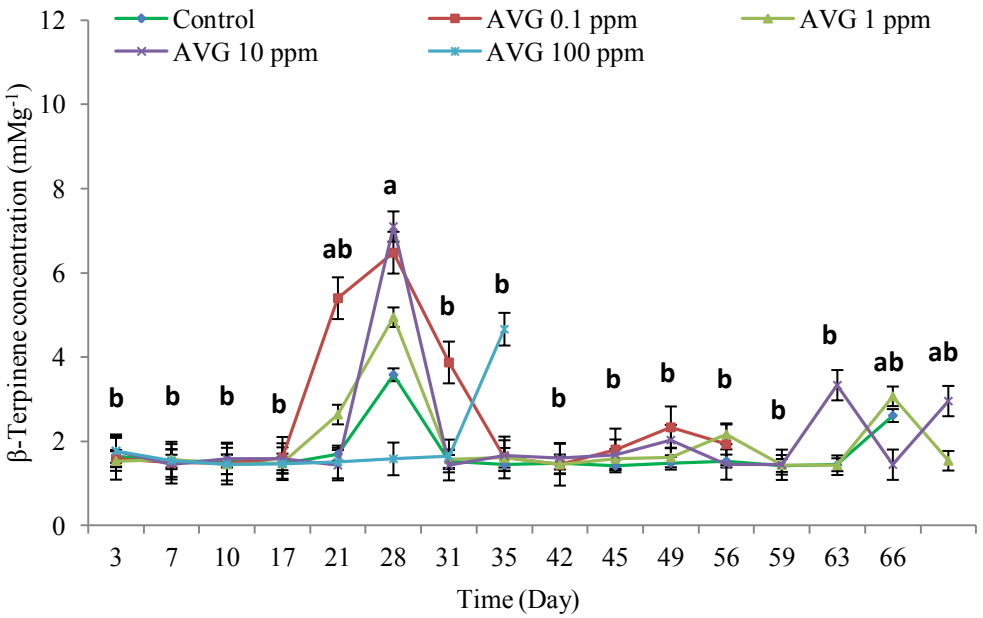

Figure 37: The progression of β-Terpinene (mMg^{-1}) in 0 ppm, 0.1, 1, 10 and 100 ppm AVG treated root detached balsam fir branches. Peak β-Terpinene evolution on day 28 (n=3). Graph points that do not share the same letters are significantly different

7.5.2 Effect of ethylene exposure on VTC evolution in balsam fir

In this experiment, branches were subjected to 0 and 1000 ppm exogenous ethylene. There was no significant (p=0.384) effect of ethylene exposure on needle loss (Table 10), however, 1000 ppm treated branches lost their needles 4 days earlier than the control (Figures 38 and 39). In branches exposed to 1000 ppm exogenous ethylene there was a significant (p=0.027), two fold increase in VTCs (3.72 mMg^{-1}) compared to the control, which had 1.84 mMg^{-1} (Figure 38). Ethylene evolution in the 1000 ppm treatment was about 20 fold significantly (p=0.008) higher (25.24 $\mu L \cdot g^{-1} \cdot h^{-1}$) than the control (1.29 $\mu L \cdot g^{-1} \cdot h^{-1}$) (Figure 38).

Table 10: P values; Ethylene treated branches subjected to analysis of variance at $\alpha = 0.05$

Source of	P value (Day 1-35)			P value (Day 36-84)		
Variance	Needle Loss	Ethylene	VTC	Needle Loss	Ethylene	VTC
Treatment	0.384	0.008	0.027	0.062	0.445	0.448
Day	0.004	0.048	0.347	0.567	0.163	0.335

The table gives *p values* of needle loss, ethylene and VTC under ethylene treated branches for days 1-35 and 36-84.

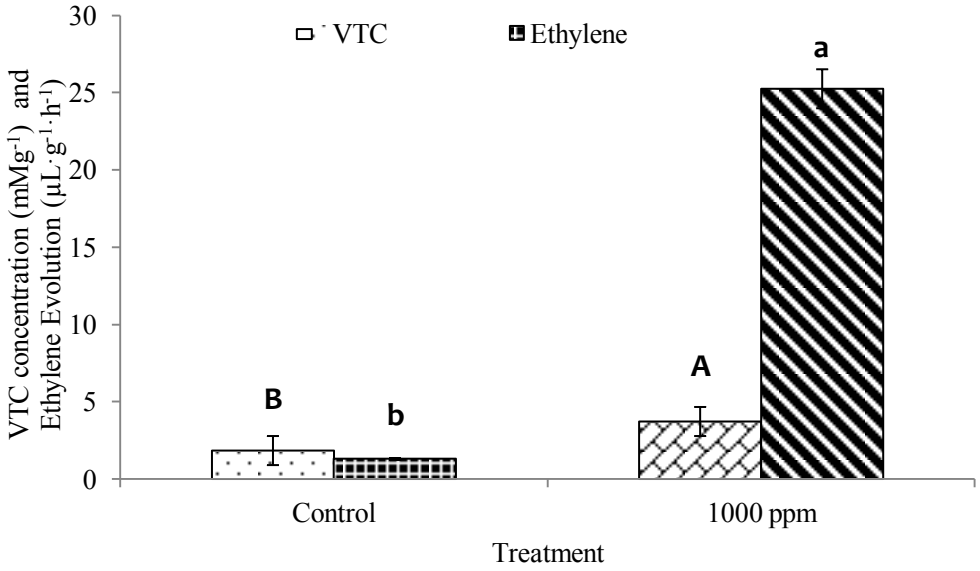

Figure 38: Average VTC and ethylene evolution of root detached balsam fir branches with no exogenous ethylene (Control) and 1000 ppm exogenous ethylene treatments. Significant differences were determined using Tukey's multiple mean comparison of 3 balsam fir branches at $\alpha = 0.05$. Average of overall measurements. In a particular color shade, bar charts that do not share the same letters are significantly different.

The trends also show that, in the control there was little activity over the first 21 days. However needle abscission increased significantly (p=0.004) (Table 10) on day 28 to 16.5% but was subsequently delayed until day 63 (Figure 39). Ethylene evolution increased to a significantly (p=0.048) high rate (7.43 $\mu L \cdot g^{-1} \cdot h^{-1}$) on day 35 prior to the needle drop on day 63 (Figure 39). Exogenous ethylene treatment at 1000 ppm started with high levels of ethylene evolution but dropped to a non-detectable concentration on

day 21. Ethylene evolutions rose to rates as high as 64.90, 57.10 and 62.08 $\mu L \cdot g^{-1} \cdot h^{-1}$ on days 28, 35 and 42 respectively prior to significant ($p<0.001$) needle loss of 19.8% on day 70 (Figure 40).

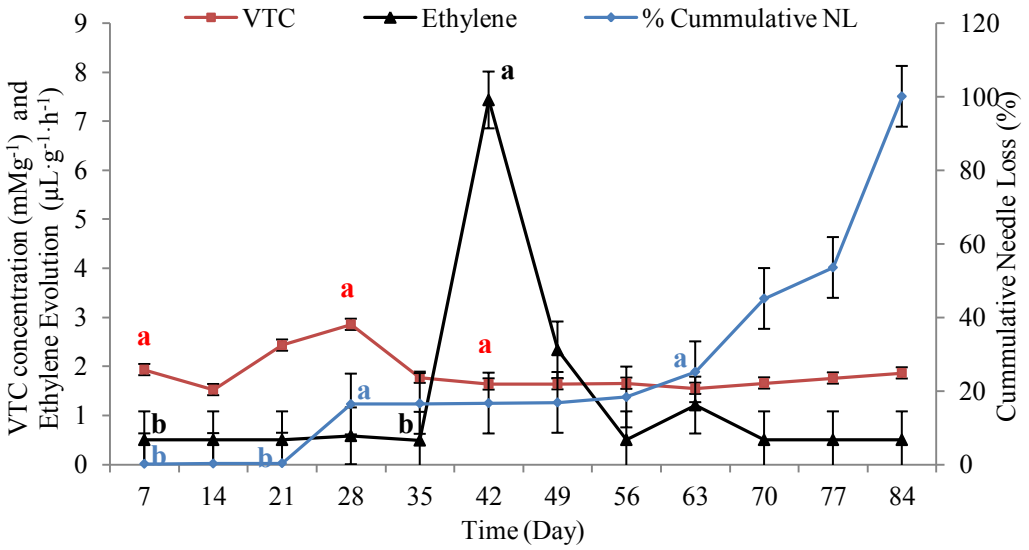

Figure 39: The progression of needle loss (as a percentage of branch fresh mass), total VTC and ethylene evolution of 0 ppm (control) ethylene treated balsam fir branches. Peak ethylene evolution on day 42 and NRD of 84 days (n=3). Graph points that do not share the same letters in a particular color are significantly different.

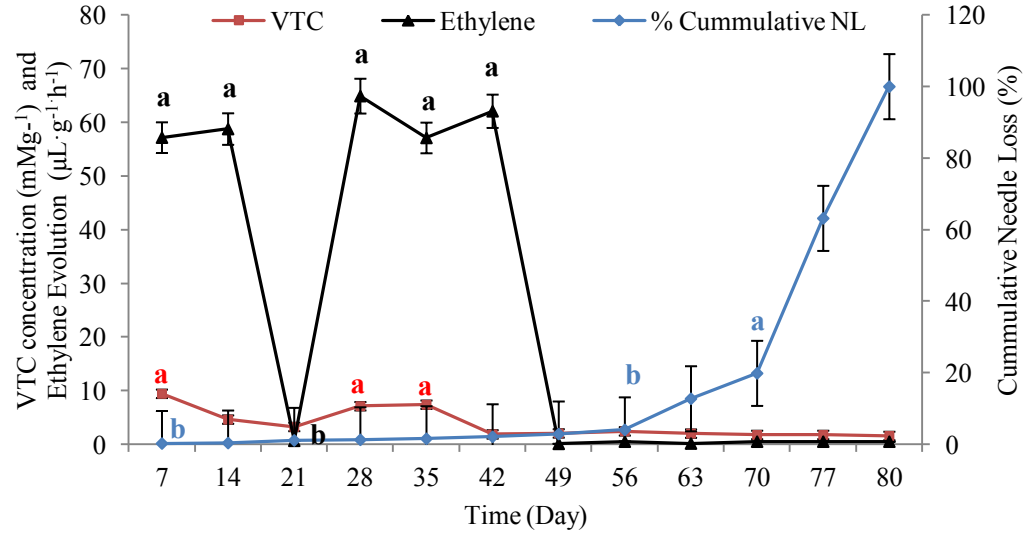

Figure 40: The progression of needle loss (as a percentage of branch fresh mass), total VTC and ethylene evolution of 1000 ppm (control) ethylene treated balsam fir branches. Peak ethylene evolution on days 7, 14, 28, 35 and 42, and NRD of 80 days (n=3). Graph points that do not share the same letters in a particular color are significantly different.

Individual VTCs were also analyzed for both the control and 1000 ppm exogenous ethylene treated branches. Analysis of variance showed that, evolution of all individual VTCs in the 1000 ppm exogenous ethylene treatment was significantly higher than the control (Table 11). With exception of Camphene and Fenchyl acetate, the remaining VTCs (β-Pinene, β–Terpinene, and 3-Carene) in the control experiment peaked at significantly higher concentrations (Table 11) ranging between 2.83 and 4.93 mMg^{-1} on days 17, 21 and 28, respectively (Figure 41). This increase in VTCs was prior to the significant increase in needle loss observed on the same day.

Table 11: P values; Ethylene treated branches subjected to analysis of variance at α = 0.05

Source of Variance	P Value				
	Camphene	β-Pinene	β-Terpinene	3-Carene	Fenchyl acetate
Treatment	0.024	0.043	0.010	<0.001	0.044
Day	0.172	0.026	0.009	<0.001	0.243

The table gives *p values* of Camphene, β-Pinene, β-Terpinene, 3-Carene and Fenchyl acetate under Control and 1000 ppm exogenous ethylene treated branches for 14 days.

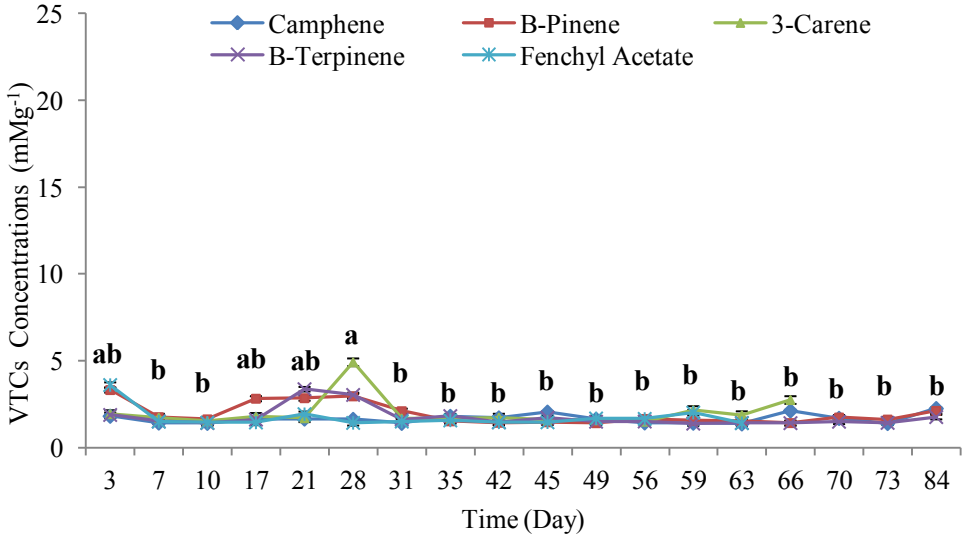

Figure 41: The progression of individual VTCs in 0 ppm (control) ethylene treated root detached balsam fir branches. Peak VTC evolution on days 17, 21 and 28 (n=3). Graph points that do not share the same letters are significantly different

With 1000 ppm exogenous ethylene treatment, there was initially a significant increase in evolution for all the individual VTCs in the first 7 days after commencement of the experiment (Figure 42). This increase ranged between 9.2 and 19.79 mMg^{-1}. There were increases in VTCs between day 21 and 35, however they were not significantly higher than the last 40 days of the experiment.

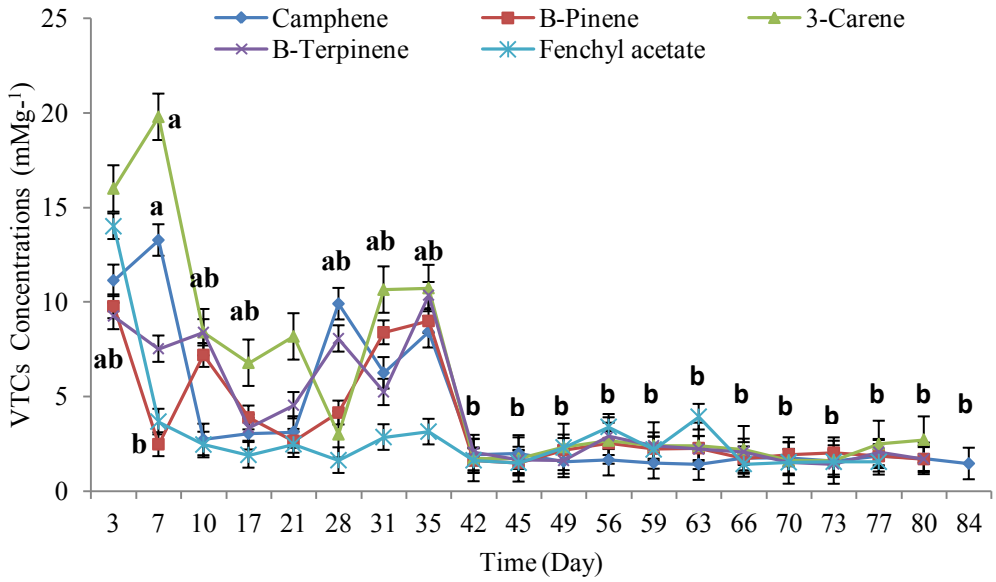

Figure 42: The progression of individual VTCs in 1000 ppm ethylene treated root detached balsam fir branches. Peak VTC evolution on days 3, 7, and 10 (n=3). Graph points that do not share the same letters are significantly different

7.6 Discussion

AVG, in many studies, has been reported to inhibit the conversion of S-adenosyl-methionine (SAM) to 1-aminocyclopropane-l-carboxylic acid (ACC) in plant species, including conifers (Boiler et al. 1979; Yu et al. 1979; MacDonald et al. 2010). As with other studies, when branches were supplied with the ethylene synthesis inhibitor AVG, ethylene synthesis was reduced and at 10 ppm it was completely inhibited below the

detectable limit of 0.05 ppm. It was observed that increasing concentration of AVG inhibited ethylene synthesis until 10 ppm; at 100 ppm AVG, ethylene was detected. Similar observation has been reported by MacDonald et al. (2010) and may be due to high concentrations of AVG. Ethylene has been identified as a signal molecule in triggering needle abscission (MacDonald et al. 2010). Thus, inhibition of ethylene synthesis using AVG increased NRD by 33%. Nevertheless, in this study, needle abscission eventually occurred in 10 ppm AVG-treated branches in the absence of ethylene evolution. Thus, it is possible that postharvest needle abscission can occur in the absence of endogenous ethylene evolution.

This study also sheds more light on the relationship between ethylene, VTCs and needle abscission. In our previous study, we proposed that VTC may be a possible signal molecule, apart from ethylene, triggering needle abscission since there was a consistent increase in β-Pinene, β-Terpinene, Camphene, 3-Carene and Fenchyl acetate prior to needle abscission (Chapter 6). However, it was not understood whether VTCs trigger ethylene or ethylene triggers VTCs and cause needle abscission. These results show that, despite inhibition of ethylene synthesis, VTC concentrations were not significantly affected. Kahl et al. (2000) did not detect any significant effect of exogenous ethylene or ethephon (an ethylene releaser) upon induction of VTCs in tobacco (*N. attenuate*). This then suggests that for VTC synthesis to occur, ethylene is not required. Alternatively, VTCs are synthesized independently of ethylene synthesis.

This study also revealed that, prior to needle abscission there was a consistent increase in total VTC and individual VTCs such as β-Pinene, β-Terpinene, Camphene and 3-Carene confirming results from our previous study (Chapter 6). With the exception of Camphene,

average concentrations of β-Pinene, β-Terpinene, Camphene and 3-Carene in ethylene inhibited treatments increased between 2 to 5 folds higher than the control suggesting that these specific VTCs may trigger needle abscission and needle abscission can occur independent of ethylene synthesis. A study done at CRC in 2010 showed that when balsam fir branches were xylem fed with β-Pinene, it rapidly promoted needle abscission. This can be explained by the possible needle abscission triggering role for VTCs (Jacob et al, 2011). Although, the signaling interaction between JA, ethylene and abscisic acid have been demonstrated to result in either synergistic (Xu et al. 1994; Penninckx et al. 1998) or antagonistic (Winz and Baldwin 2001) interactions in the expression of plant defense causing needle abscission, the case of VTCs emission in plants and the role in needle abscission remains unclear.

One would expect a quick response of needle abscission in ethylene treated branches since ethylene is known to trigger abscission (MacDonald et al. 2010). On the contrary, the ethylene- treated branches retained their needles for 84 days just as the control. Similar reports on delayed needle abscission were made by Macdonald et al. (2011) when branches were exposed to ethylene for a short term of 24 hrs. It was also noted that total VTC was twofold higher in branches exposed to ethylene. However needle abscission was still delayed. This may be explained by a lower concentration of VTC in branches exposed to ethylene compared to the VTC threshold that resulted in needle abscission when ethylene was inhibited. Retention of needles in these branches under ethylene treatment may also be due to high humidity (85%) build up in the ethylene treatment chambers as similar trends were again reported by MacDonald and Rajasekaran (2012) when balsam fir branches were subjected to 90% humidity at 19.7 °C. However in their

work, branches were not subjected to exogenous ethylene as in this case. In this study, humidity was built up since the chambers were tightly closed after exposing the branches to ethylene gas in order to avoid escape of the gas and also to allow branches to take up the gas. Plants respond to high humidity by reducing transpiration and thus dehydration thereby decelerating the abscission processes (Buchanan et al. 2000). Humidity also delays the evolution of ethylene and maintaining higher xylem pressure potential (XPP) can therefore delay postharvest needle abscission (MacDonald and Rajasekaran, 2012).

7.7 Conclusion

It can be concluded that both ethylene and VTC synthesis are independently regulated. It is also confirmed that specific VTCs such as β-Pinene, β-Terpinene, Camphene and 3-Carene are released prior to needle abscission and this does not require the presence of ethylene. Exposure to exogenous ethylene however induced VTCs but was ineffective at that concentration in inducing needle abscission.

7.8 Reference

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CHAPTER 8: GENERAL DISCUSSION, SUMMARY AND CONCLUSIONS

8.1 General Discussion

In solving the important issue of postharvest needle abscission in balsam fir, it was realized that little is known about the role of volatile terpene compounds (VTCs) and postharvest needle abscission. However, it is known that balsam fir Christmas trees have a unique aroma whereas, Fraser firs, a similar ecotype known to keep its needles for longer have less aroma or fragrance. Thus, it was hypothesized that balsam fir's VTC may have a role in postharvest needle abscission. We have demonstrated that balsam fir contains twelve distinct VTCs with concentrations varying between 0.02 nMg^{-1} and 0.39 nMg^{-1} among individual VTCs and clones (Chapter 5). With 95.3% of the VTCs being monoterpenes, it confirms high levels of monoterpenes in balsam fir reported by Taiz and Zeiger, (1998). Monoterpenes make up as much as 5% of plant dry weight and are produced by an enzyme called monoterpene synthase (Buchanan et al. 2000). An important feature of this enzyme is its ability to produce more than one monoterpene. Majority of these monoterpenes are commonly known for their dominant defensive role in insect and pathogenic attacks (Pare and Tumlinson, 1999; Carlow et al. 2006), attractants for pollinators (Buchanan et al. 2000) and intermediates for synthesis of alkaloids (Didna et al. 2007). The easy synthesis or reproducibility and an array of uses of monoterpenes may contribute to high levels of monoterpenes in balsam fir. Seedlings also showed high concentration of total VTCs compared to the low and high needle abscission resistant genotypes used. Studies by Coley, (1983), Puttick, (1986), Hatcher, (1990) and Carlow et al. (2006) all have reported high VTCs concentration in younger conifer tress than mature trees. This increase can be explained by strong defense

mechanisms in younger trees since they are more susceptible to insect, pathogen and other herbivore attacks. 3-Carene, one of the twelve individual VTCs identified in this study is documented as showing higher concentration in most conifer species (Carlow et al. 2006). This is explained by its known key role in the defense of conifers against insects such as balsam woolly adelgid (BWA), an exotic insect commonly found in Northern America. This trend is consistent with our study. However, VTCs were significantly higher in the high NAR clone than low NAR clone and seedling.

It has clearly been identified that VTCs that are synthesized in balsam fir varies in nature and quantity in various samples tested. The important question however, summons the physiological significance or role of these VTCs in postharvest needle abscission in balsam fir. It was therefore hypothesized that concentrations of individual VTCs in addition to total VTC increases will promote postharvest needle loss in balsam fir. This study has established that VTC evolution increases between 0 to 7 days prior to postharvest needle abscission (Chapter 6). Consistency of total VTC as well as individual VTCs such as β-Pinene, β–Terpinene, Fenchyl acetate, Camphene and 3-Carene with needle loss indicates the possible role of VTCs in promoting needle abscission. This can be explained by two main mechanisms; VTCs as causal or defense molecules to needle abscission. As a causal molecule, there is no information to explore the possibilities of VTCs to promote needle abscission. However, previous work done in our lab shows that β-Pinene, one of the identified common monoterpenes can possibly promote rapid needle loss when branches are exposed to β-Pinene through xylem feeding (Jacob et al. 2011). On the other hand, it is well established that the majority of cell death that results in abscission is caused by oxidative stress (Valko et al. 2005). One of the secondary

metabolites that have been identified in protecting the photosynthetic apparatus against oxidative stress is VTC (Loreto and Velikova, 2001). There is also an indication in other studies, which proves that in the presence of VTCs, membrane lipid bilayer is stabilized (Logan and Monson, 1999) as a mechanism to protect cells from external destruction (Alberts, 2002). Thus, as a possible defense molecule, one would expect plants to switch on this defense mechanism when cells begin to degenerate prior to needle abscission resulting in the synthesis of high levels of VTCs to retard needle abscission process. However, cell degradation and needle abscission eventually took place in these branches despite the high levels of VTCs as shown in all chapters. This makes the second argument a non-plausible one. Instead we see in all our studies, evolution of VTCs prior to needle abscission, befitting the criteria for a molecule to be considered as a trigger or signal to any developmental phase such as abscission. If it is true VTCs have a causal effect on postharvest needle abscission then it could be linked to ethylene, a well-known molecule that has been identified in several works to cause postharvest needle abscission in minute concentrations (Macdonald et al. 2010; Macdonald et al. 2011).

A diverse array of plant responses to mechanical injuries has been demonstrated to be linked to signal molecules such as VTCs (Schmelz et al. 2003), ethylene and jasmonic acids (JA) (Creelman et al. 1992). The signal interaction between ethylene and JA has been established to result in either antagonistic (Zhu-Salzman et al. 1998; Winz and Baldwin 2001) or synergistic interaction (Xu et al. 1994; O'Donnell et al. 1996; Penninckx et al. 1998) in the expression of plant response to mechanical damage. In the case of VTC evolution, a study by Kahl et al. (2000) in tobacco (*N.attenuata*) showed no interaction between JA, ethylene or ethephon when VTC emission was induced. Most of

these studies were geared towards pathogenic and insecticidal effects on plants leaving the interactive role of ethylene on the evolution of VTC and its effect on abscission still unclear. Our work (Chapter 7), however, clearly showed that when ethylene is successfully inhibited by 10 ppm AVG treatment, VTCs (β-Pinene, β–Terpinene, Camphene and 3-Carene) still evolved at significantly high concentrations prior to needle abscission. This suggests that VTCs evolution and its role in needle abscission is independent of ethylene and needle abscission can occur independent of ethylene. In the same work, we expected that if ethylene is inhibited then needle abscission would stop; however, needle abscission continued eventually with high VTC evolution. This also suggests that even in the absence of ethylene, needle abscission can occur and VTCs such as β-Pinene, β–Terpinene, Camphene and 3-Carene may be the potential candidate signal molecules triggering postharvest needle abscission. This is also in line with previous reports by Jacob et al. (2011) which showed β-Pinene to promote postharvest needle abscission.

It is well established that, there are independent pathways for the synthesis of ethylene (Adams and Yang, 1979; Abeles et al. 1992) and VTCs (Buchanan et al. 2000) but there might be a common cascade of molecule(s) triggered by either of the two to result in the initiation of needle abscission. Hence, in the absence of one of these two molecules the plant switches on the synthesis of the other to promote needle abscission. The plant goes through these abscission processes at all cost in response to environmental stresses such as change in temperature, light intensity and water availability as a defense mechanism to be able to allocate limited resources efficiently and effectively.

It is also reported that VTCs in most conifer species are released from resin ducts (Shao et al. 2001and Loreto and Schnitzler 2010). Resin ducts are usually <2 μm in diameter and lie on the surface of the needles (Holopainen, 2011). Since these ducts are exposed on the surface of the needles, any form of contact to the needles that disturbs the ducts may cause the release of VTCs. Again any contact that results in the separation of needles from branches exposes the abscission zone and may also expose resin ducts, resulting in the leaking of VTCs. This raises the question as to whether the finger-run tests performed promote VTCs as such a mechanical stress may have erupted resin ducts causing VTC evolution to be mechanically induced rather than a physiological significance to postharvest needle abscission. However, in our studies, finger-run tests were done after extraction of VTC and thus, it is unlikely that VTC evolution was induced mechanically. Therefore, it cannot be assumed that the relationship between VTC evolution and postharvest needle abscission is coincidental.

Given the limited information on the issue of VTCs relation to needle abscission, it is intricate to hypothesize a strong relation of VTCs with needle abscission. However we can draw inferences from other signal molecules that trigger needle abscission such as ethylene and abscisic acid. It is possible that when trees are detached from their roots causing mechanical wounding, defensive signals are sent throughout the tree for the protection of limited resources. One way the plant manifests such a defense is by shedding of organs such as leaves through abscission. To stimulate abscission processes, VTCs may then be synthesized by the plant to promote the production of cell wall hydrolytic enzymes such as cellulase that may weaken the cell wall to cause needle

abscission. However, VTC synthesis may play a direct role in weakening the cell walls and the mechanical forces required to cause abscission.

8.2 General Conclusion

This study has identified twelve distinct VTCs (α-Pinene, 3-Thujene, Camphene, β-Pinene, 3-Carene, β-Terpinene, D-Limonene, β-Phellandrene, ɣ -Terpinene, Terpinolene, Fenchyl acetate and Bornyl acetate) present in balsam fir. The first ten are monoterpenes and the last two are terpenoids. Clearly, VTC evolution increases significantly prior to postharvest needle loss and out of the twelve VTCs identifies, five (β-Pinene, β–Terpinene, Fenchyl acetate, Camphene and 3-Carene) may have a role in triggering needle abscission after harvest. Both VTC and ethylene synthesis are independently regulated. It is also confirmed that specific VTCs such as β-Pinene, β–Terpinene, Camphene and 3-Carene are released in significant quantities prior to needle abscission and this does not require the presence of ethylene. Exposure to exogenous ethylene however induced VTC production but was ineffective at that concentration in inducing needle abscission.

8.3 Recommendations for future research

As an extension to this study, further studies are recommended to consider the inhibition of VTCs to be able to demonstrate effect on needle abscission. Such a study will further identify the possible role of VTCs as a signal to trigger needle abscission.

Also future studies may test the effect of exogenous application of β-Pinene, β-Terpinene, Fenchyl acetate, Camphene and 3-Carene on needle abscission. This would also clarify our understanding of VTCs in promoting needle abscission.

Since studies have suggested the possibility of JA-induced VTC production or evolution, further study may look at the role of JA in VTC evolution and postharvest needle abscission.

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Appendix I: Fiber selection and determination of incubation time

In selecting the best SPME fiber for this study, a StableflexTM fiber assortment kit (Supelco, USA) was acquired. It contained four fibers of different film thickness and fiber coating; 85µm Carboxen/polydimethylsiloxane (CAR/PDMS), 65µm Polydimethylsiloxane/Divinylbenzene (PDMS/DVB), 85 µm Polyacrylate (PA) and 50/30 µm (DVB/CAR/PDMS). Each fiber was exposed to the headspace of the volatile incubation chamber that contained balsam fir branch for an incubation period of 15 min, 30 min, 45 min and 1 hr. Comparing the chromatograms generated (Figures 43, 44, 45 and 46), 85 µm (CAR/PDMS) fiber was selected since it showed clearer and well separated peaks. 30 minutes of incubation showed all available VTCs in the highest concentrations, thus, was selected for VTC extraction.

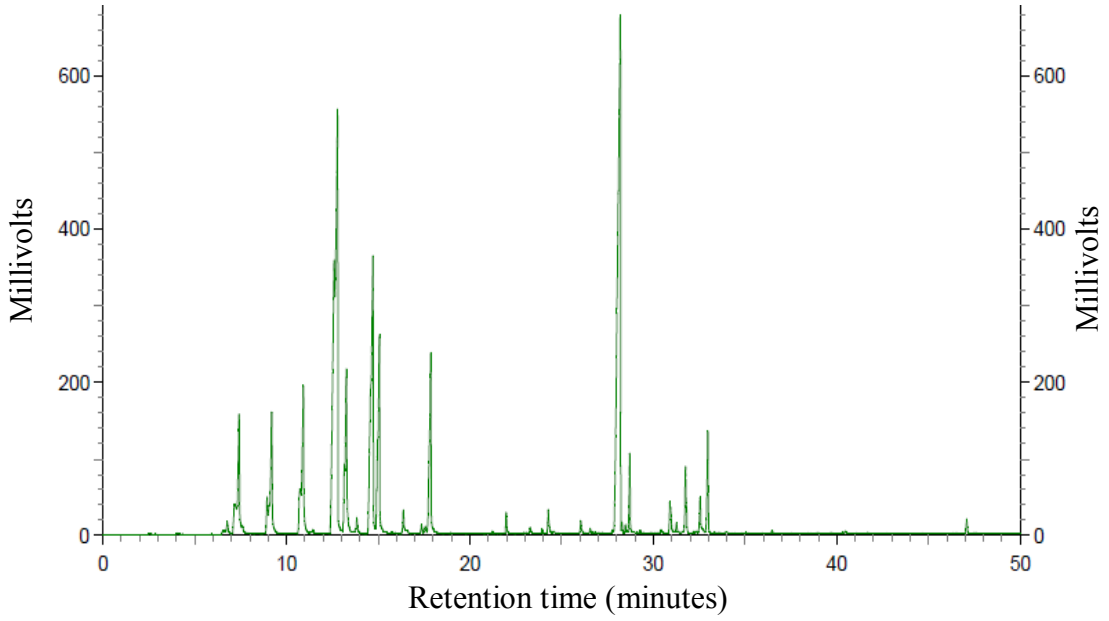

Figure 43: Chromatogram for 85 µm CAR/PDMS fiber after 30 minutes of incubation

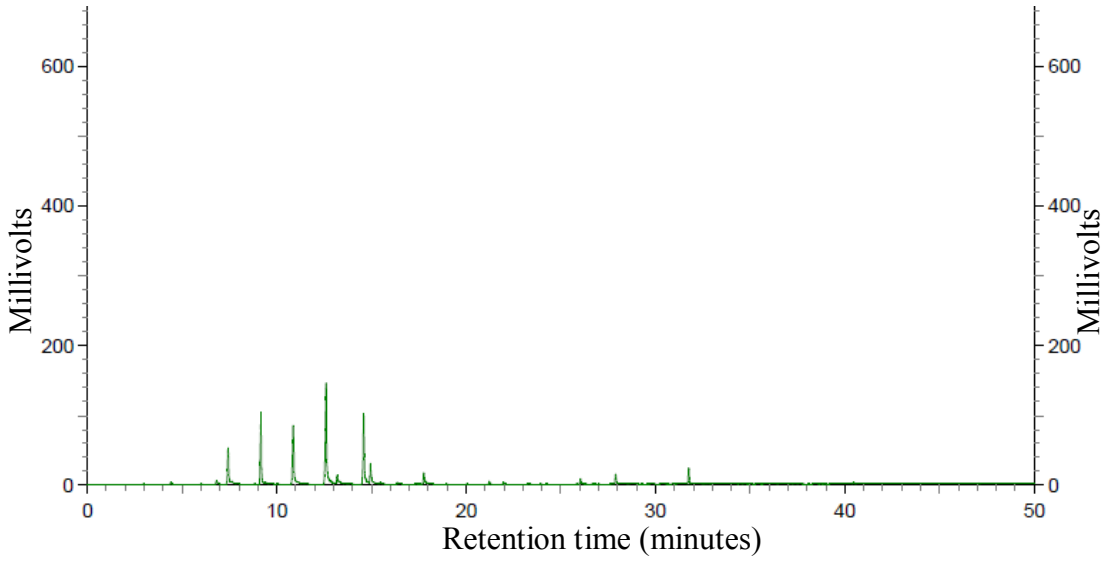

Figure 44: Chromatogram for 65µm PDMS/DVB fiber after 30 minutes of incubation

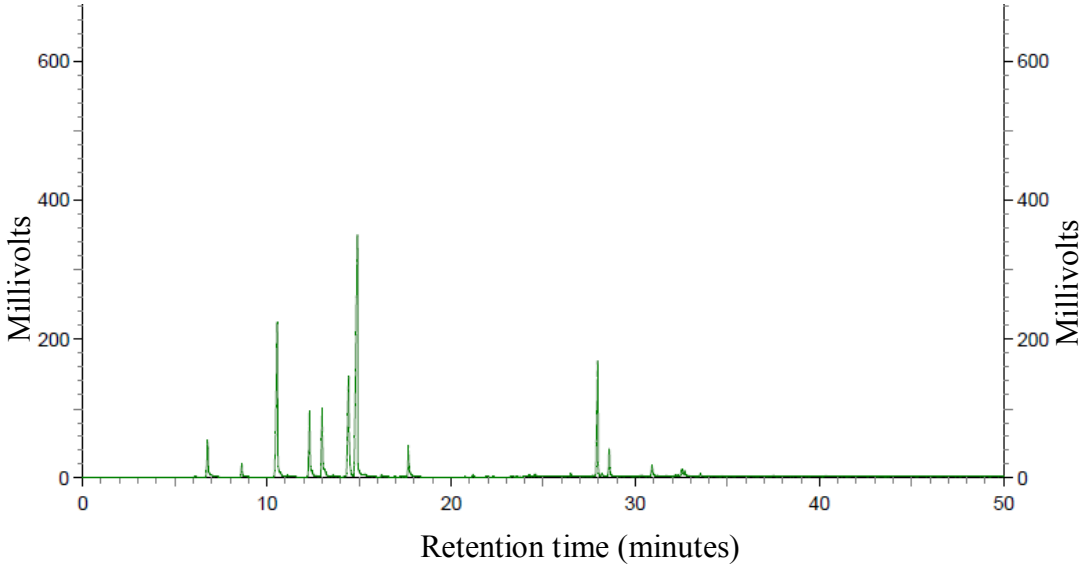

Figure 45: Chromatogram for 50/30 µm DVB/CAR/PDMS fiber after 30 minutes of incubation

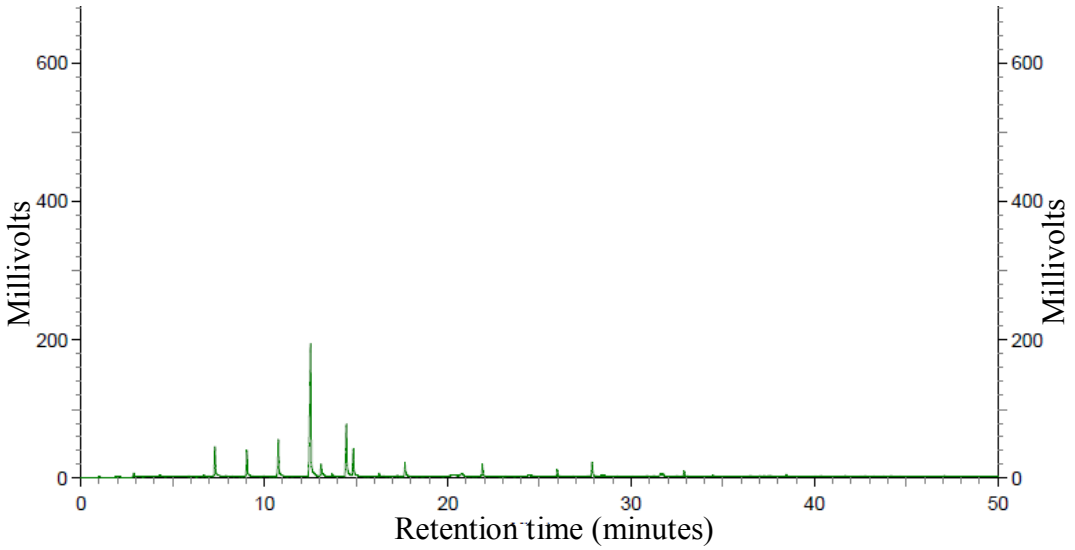

Figure 46: Chromatogram for 85 µm PA fiber after 30 minutes of incubation

Appendix II: Standardization of Gas Chromatograph method for VTC analysis

The GC was standardized in each experiment by injecting 10µl of internal standard (β-pinene) onto a filter paper placed in the incubation chamber and left sealed for 30 min with the SPME holder on the jar for headspace analysis. Since the volume of each incubation chamber was known, the concentration of injected terpene standard could be calculated and plotted against the concentration determined by the GC analysis. For each experiment, standards were made and tested each day of VTC analysis to account for the degradation of the SPME fiber and the daily variations in the GC. All standards were purchased from Sigma-Aldrich Co. LLC, Canada.

Chromatograms generated throughout the experiments showed a nice and distinct peak. Figure 47 shows one of the chromatograms with a 95.9% peak area at 6min. 6sec. and a baseline free of any noise. This shows the efficiency of the method developed for the terpene analysis.

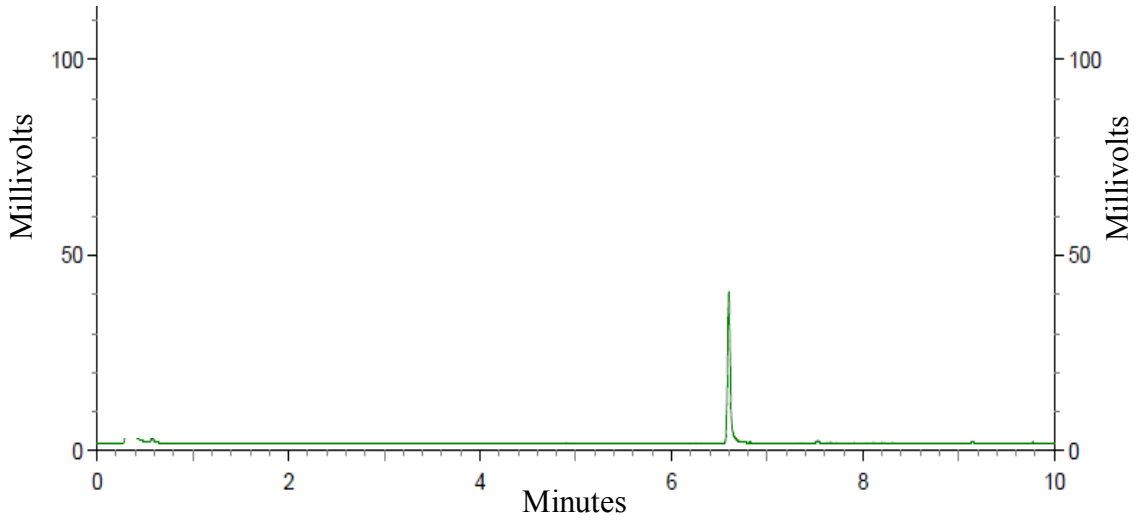

Figure 47: Chromatogram for 10mM β-pinene by SPME at 30 min equilibration time

A linear relationship was found between 0mM and 10mM β-pinene (Figure 48). However, the detected concentrations slightly diverged from the injected concentrations. For example, a concentration of 4mM was measured a 3mM by the GC. The largest discrepancy was found at the highest injected concentration of 10mM which was measured at only 8mM. The slope of the linear regression was determined to be 1.215 with $R^2 = 0.991$. In this case a perfect linear relationship would have a slope of exactly 1. The GC can be concluded to have performed excellent therefore should be accurate at detecting VTCs released from balsam fir. Determination of all VTC concentrations throughout this study was done in equivalence to β-pinene standard concentrations detected.

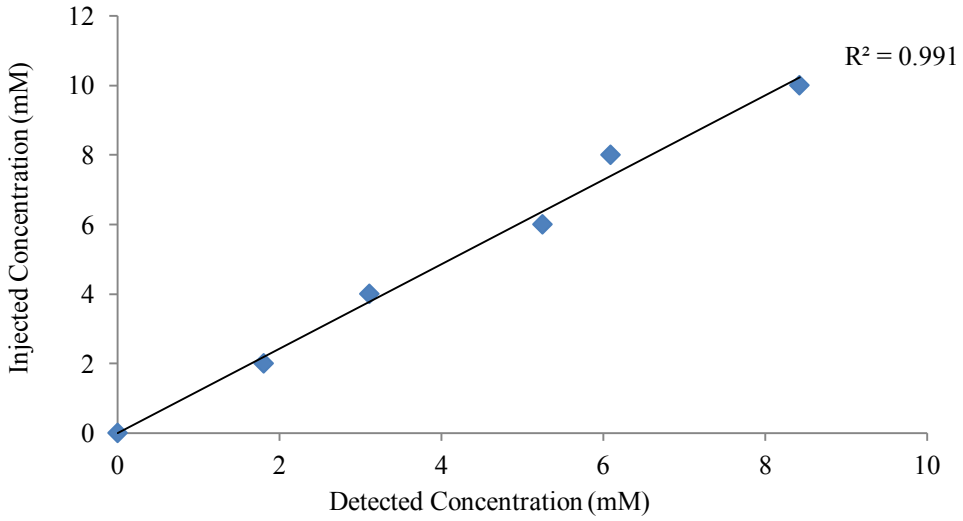

Figure 48: Detection limits of VTCs using β-pinene standard at different concentrations.

Appendix III: Standardization of Gas Chromatograph method for ethylene analysis

Ethylene was standardized by injecting known volumes of ethylene gas into the incubation chamber. Since the volume of each incubation chamber was known, the concentration of injected ethylene could be calculated and plotted against the concentration determined by the GC (Figure 49).

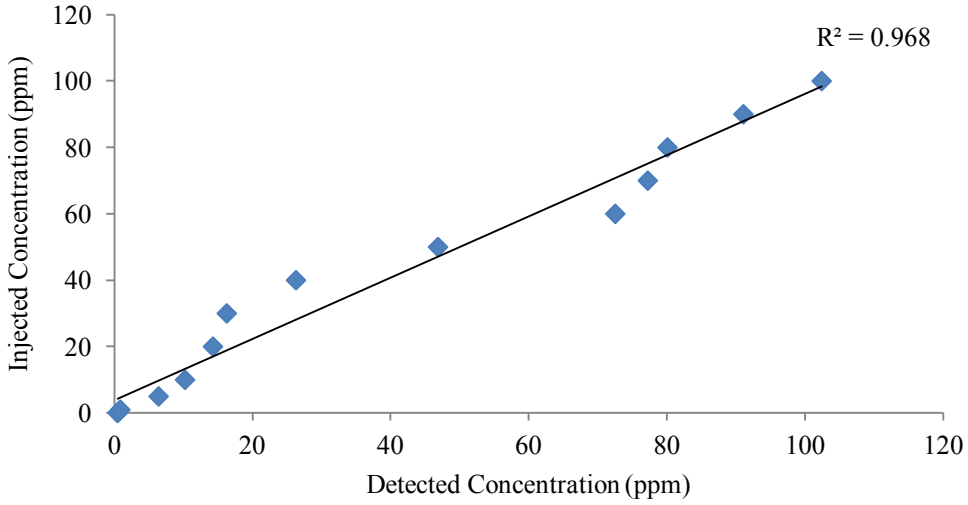

Figure 49: Detection limits of ethylene using ethylene standard at different concentrations.

A linear relationship was found between 0 ppm and 100 ppm ethylene. However, the detected concentrations begin to diverge from the injected concentrations after 10 ppm. For example, a concentration of 100 ppm was measured at 102 ppm by the GC. The largest discrepancy was found as the concentration of injected ethylene was increased. The GC performed excellent between 0 ppm and 100 ppm. The slope of the linear regression was determined to be 0.02 with R2 = 0.968. In this case a perfect relationship would have a slope of exactly 1. The GC should be accurate at detecting ethylene released from balsam fir provided concentrations remain below 100 ppm.