THE RELATIONSHIP BETWEEN PECTINASE AND CELLULASE AND POST-HARVEST NEEDLE ABSCISSION IN BALSAM FIR (*Abies balsamea* (L.))

by

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ABSTRACT

Post-harvest needle abscission is a major challenge for the Christmas tree industry in Atlantic Canada. To further elucidate the physiological basis of needle loss in Balsam fir, experiments were conducted to investigate the possible link between certain hydrolytic enzymes and post-harvest needle abscission in balsam fir. This study focussed on the role of enzymes, pectinase and cellulase, in post-harvest needle abscission. Following harvest, cumulative cellulase activity increased over time prior to needle loss on both high and low needle retention duration (NRD) clones. Total cellulase activity of low NRD clone was much higher than that of high NRD clone. Pectinase does not affect needle abscission in this study. If cellulase activity is linked to post-harvest needle abscission, then inhibiting cellulase enzyme would reduce needle loss. Pre-treating branches with 2.5g 1-methylcyclopropene (1-MCP) enhanced needle retention.

LIST OF ABBREVIATIONS USED

ABA	Abscisic acid
ACC	1-aminocyclopropane-l-carboxylic acid
AdoMet	S-adenosyl-L- methionine
ANOVA	Analysis of variance
ASA	Acetyl salicylic acid
AVG	Aminorthoxyvinylglycine
AWU	Average water use
AZ	Abscission zone
CMC	Carboxymethyl-cellulose
DNS	3,5-dinitrosalicylic acid
EDTA	Ethylene diamine tetra acetic acid
NAR	Needle abscission resistance
NCTA	National Christmas Tree Association
NRD	Needle retention duration
PBS	Phosphate buffer solution
RO water	Reverse osmosis water
SAS	Statistical analysis system
TRIS	Tris (hydroxymethyl)aminomethane
1-MCP	1-methylcyclopropene
2, 4-D	Dichlorophenoxyacetic acid

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CHAPTER 1 INTRODUCTION

Christmas trees are an important part of Christmas since 1776 (Chastager and Benson, 2000). About 33 to 36 million Christmas trees are produced in North America (Chastager and Benson, 2000). The most common species of Christmas trees in the US are Douglas-fir, white pine, Fraser, noble, Virginia, and balsam fir (Chastager and Benson, 2000). It is approximately 2381 Christmas trees farms and 22 hectares of land per farm are used to grow Christmas trees in Canada (Statistics Canada, 2016).

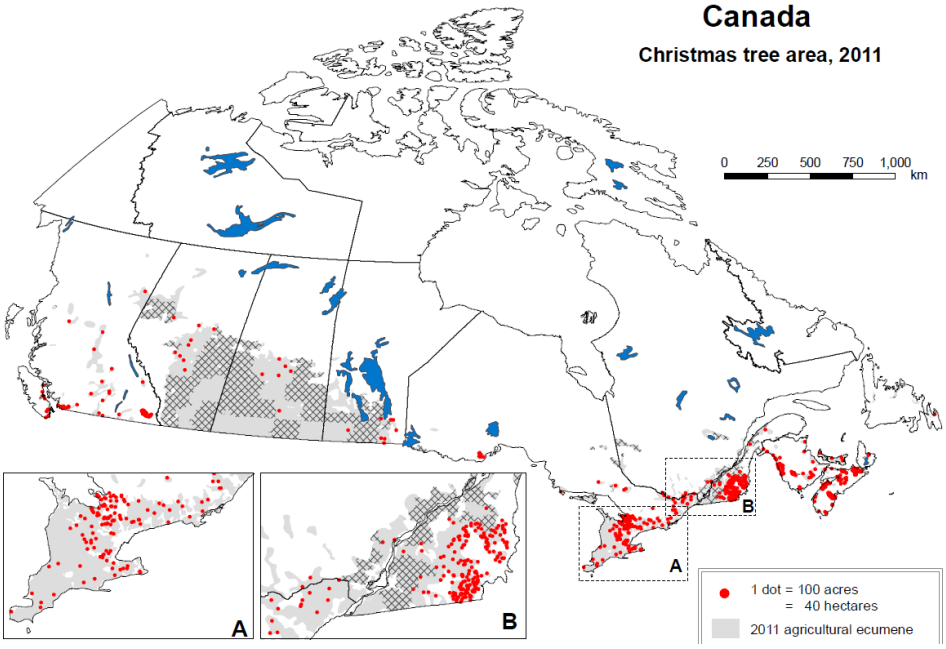

Figure 1. 1 Map of Christmas tree area in Canada and United States (Statisstics Canada, 2016)

It is estimated that 1,719,735 fresh-cut Christmas trees were exported from Canada to the rest of the world in 2015, including 1,634,249 trees which were exported to the United

States, valued at $ 41.4 million (Statistics Canada, 2016). It was nearly, 2 million produced in Nova Scotia and it created nearly, $30 million. Nearly, 365,095 trees were exported from Nova Scotia and it created around $8.6 million (Statistics Canada, 2016). Balsam fir (*Abies balsamea* (L.)) is a popular native Christmas tree species of Nova Scotia. Consumers choose Balsam fir as a Christmas tree because of its attractive needle color and pleasant fragrance. Fresh-cut Christmas trees were exported to the rest of the world including the US, Aruba, Jamaica and Russian Federation and so on. Due to long-distance transport, farmers usually harvest Christmas trees as early as October. However, early harvested trees resulted in poor needle retention, which may be due to a lack of cold acclimation (MacDonald and Lada, 2012). Physical (harvest handling) and environmental factors (drought, temperature, plant hormone changes) also impact post-harvest needle abscission independently or in combination (Lada and Adams, 2009; Thiagarajan et al., 2013; MacDonald et al., 2011). Post-harvest needle abscission is identified as a big challenge by the Christmas tree industry.

In nature, organ abscission is an important developmental process, which maintains homeostasis on plant vegetative parts (Addicott, 1982). Based on previous research, there are several factors that may induce needle abscission, such as environmental, nutritional and hormone factors (MacDonald et al., 2011). The hormone, ethylene has been demonstrated to induce post-harvest needle abscission in balsam fir. MacDonald et al. (2011) found that ethylene induces post-harvest needle abscission in Balsam fir. They also found that ethylene increases cellulase activity during the abscission process. Hydrolytic enzymes were also found to relate to abscission in other species, such as bean leaf abscission and tomato fruit abscission. Durbin et al. (1981) reported that the form of

cellulase with an isoelectric point of 9.5 was involved in bean leaf abscission. Also, other hydrolytic enzymes, such as pectinase also implicated to be involved in inducing citrus' leaf and fruit abscission. Riov (1974) reported that polygalacturonase was involved in the citrus leaf abscission process. Hydrolytic enzymes play a negative role in organ abscission. Plant cell wall can provide support for tissue and the main compounds of plant cell wall are pectin and cellulose. Pectinase and cellulase can degrade pectin and cellulose to decrease cell wall strength, which can make plants organs, such as needles, easier to separate from plants. Based on the previous research, several hydrolytic enzymes are involved in abscission in other species but the nature of hydrolytic enzymes and their dynamics of change in post-harvest needle loss in balsam fir is not clear. It is also not known whether the changes in the hydrolytic enzyme levels would explain the needle retention resistance among high and low needle abscision resistant clones. Identifying the dynamics of change would be a useful tool to manipulate the synthesis of hydrolytic enzymes prior to initiation of the abscission process through inhibiting the synthesis of these enzymes thus, we could promote postharvest needle retention.

Currently, there are limited or no detailed studies to understand the dynamics of changes in hydrolytic enzymes in relation to post-harvest needle abscission, and whether the changes in the levels would explain the needle retention in clones with contrasting needle abscission resistance (NAR). This research is designed to determine the link between the synthesis of hydrolytic enzymes and post-harvest needle retention, and explain the needle abscission resistance.

This leads to several hypotheses:

1) Cellulase activity negatively affects post-harvest needle retention.

2) The intensity of needle abscission is proportional to the cumulative activity of hydrolytic enzymes (cellulase and/or pectinase).
3) The activity of hydrolytic enzymes will be lower in high NAR clones compared to low NAR clones.
4) Application of hydrolytic enzyme inhibitors will promote needle retention.

Based on these hypotheses, the specific objectives were to:

1) determine and quantify hydrolytic enzyme activity (cellulase and pectinase) in balsam fir needles, post-harvest;
2) establish the relationship between hydrolytic enzyme dynamics and clonal variation in post-harvest needle abscission;
3) establish the linkage between hydrolytic enzymes (cellulase and pectinase) and post-harvest needle abscission in balsam fir; and to
4) determine the effectiveness of using cellulase inhibitors to reduce post-harvest needle abscission.

CHAPTER 2 LITERATURE REVIEW

2.1 The Balsam Fir

Balsam fir (*Abies balsamea* (L.)) was first described in 1768, which grows naturally from central Canada to West Virginia and Viginia (NCTA, 2017). Balsam fir has thin and ash-grey and numerous blisters in the bark that contain a sticky and liquid resin. Its name was given from resin, which can produce sweet smell fragrance.

Balsam fir is a medium-sized tree with a relatively dense, dark-green and pyramidal crown. On the lower branches, needles are arranged in two ranked along sides of the branch and not crowded. The needles on the older branches are shorter and curved upward to cover the twigs (NCTA, 2017).

Figure 2. 1 An example of the balsam fir tree.
(Adapted from: http://www.realchristmastrees.org/dnn/Education/Tree-Varieties/Balsam-Fir)

Balsam fir is usually found from sea level to 1,524 meters in elevation. Cooler climates with abundant soil moisture and humid atmosphere are good for Balsam fir (NCTA, 2017). Growth is best in well-drained, sandy loam soil. Male and female flowers occur on the same tree and flowers are receptive in the late May to early June (NCTA, 2017). After ripening in September to November, cones drop and leave an erect central core on the branches (NCTA, 2017).

2.2 Abscission

2.2.1 Organ abscission

The separation of cells, tissues or organs from the plant body is called "abscission" (Addicott, 1982). During the plant growth period, leaf abscission plays an important role in keeping balance on the plant vegetative parts. It is a process to remove old and injured leaves to supply more nutrients for new leaves. When soil nutrients are limited, shed leaves contribute to the mineral nutrients in the soil (Addicott, 1982). Thus, abscission is a beneficial ecological process for plant growth and defend infections by natural enemies and pathogens (Addicott, 1982). However, the post-harvest needle abscission is caused by many different factors, such as environmental, nutritional, hormonal and biochemical factors. There are two phases during the abscission period, which are the lag phase and the separation phase. The first phase is the lag phase, which is the induction of separation and the first signs of effect. In this stage, the strength of the plant cell wall has no perceptible decline (Sexton and Roberts, 1982). During the separation phase, the strength of the separation layer reduces rapidly. Leaf abscission is completed in 10 to 48 hours, once initiated (Sexton and Roberts, 1982). According to previous research, there was no needle

loss during the first four days after harvest and the needle abscission occurs on day 6 and then peaked on day 24. On day 24, about 60% needles abscised and all needles dropped on day 28 (MacDonald and Lada, 2014). However, the abscission length was also impacted by harvest season, genotype or experimental treatments, such as exposure to ethylene (MacDonald and Lada, 2014). For example, there are over 220 genotypes of Balsam fir in the Debert Tree Orchard (Thiagarajan et al., 2013). Some trees have better needle retaining ability, whereas some have lower needle retention. For example, some harvested trees can keep their needles for approximately 60 days, while some can retain their needle for only ten days (Lada and Veitch, 2009). Abscission causes several cell structure changes and the separation area is commonly called the abscission zone (AZ).

2.2.2 Abscission zone

The region that is located at the base of the leaf, flower branch or other plant part is called the abscission zone (AZ). The abscission zone consists of a separation layer and a protective layer. Cells of the abscission zone are smaller and denser than adjacent cells. Before abscission, cells in the abscission zone cannot become larger and vacuolated like other cells. During the abscission process, cells in the abscission zone become larger and begin to divide to form the functional separation layer. Due to cell divisions, the middle lamella of the cell wall becomes weakened and is dissolved, which causes organs to shed (Sexton and Roberts, 1982).

2.2.3 The structure of cell wall

Cell walls are an important structure of plant cells, which can provide protection and support for plants. The plant cell wall is made up of three layers: the middle lamella, the primary wall and the secondary wall. The first layer is the middle lamella, which is formed

during cell division. It consists of pectin and protein. The primary and secondary walls are formed after the middle lamella. The primary wall layer is composed of a rigid skeleton of cellulose microfibrils and the secondary wall is made of cellulose, hemicellulose and lignin (Lodish et al., 2000). The secondary cell wall is thick and provides compression strength. Hydrolytic enzymes of the cell wall are involved in plant abscission by degrading the cell wall to break down cell wall strength (Greenberg, 1975). Cellulase and pectinase were reported to play a negative role in plant abscission in other plant species. However, there is little or no information on the role of hydrolytic enzymes on post-harvest needle abscission in balsam fir.

2.2.4 Ultrastructural changes at the abscission zone

With the development of the electron microscope, the study of ultrastructure changes in the organelles of cells has been undertaken for different plant processes. During abscission, several ultrastructural changes happen in the abscission zone (Addicott, 1982). When the nucleolus becomes conspicuous in the abscission zone, the ultrastructural changes begin. Smooth and rough endoplasmic reticula become conspicuous and increase rapidly following RNA increase in the abscission zone. Dictyosomes and vesicles also become numerous, particularly in the leaf abscission zone. Vesicles near the plasmalemma fuse with them. Intact vesicles are often found in the periplasmic region and rarely in the primary cell wall and the middle lamella. The numbers of mitochondria and chloroplasts change little during abscission. However, the amount of starch increases rapidly when abscission begins and largely disappears during the separation period. Some of the starch can provide energy and some are involved in forming protective layers (Addicott, 1982).

Among the three layers in the cell wall, the middle lamella is the first part to be affected during abscission. When abscission approaches, cells in the middle lamella swell and this leads to separation by the weakened middle lamella. The cells in the separated surfaces are rounded and completely free (Addicott, 1982). There are few changes in the primary wall. In previous research, some cells in the primary wall became thick during abscission. Those thick cells have a similar function to transfer cells (Addicott, 1982). There is usually little or no breakdown of the primary wall during separation.

2.3 Factors that influence abscission in balsam fir

There are several factors that may influence abscission in a plant, including genetic variation; and environmental conditions such as temperature, drought and soil nutrition and post-harvest factors like handling and dehydration.

2.3.1 Genetic variation

Conifer species have different needle abscission characteristics depending on genetic variations. Various Christmas tree species, such as Douglas-fir (*Pseudotsuga menziesii*), Fraser (A. *fraseri*), noble (A. *procera*), and balsam firs, Scot's, Virginia (P. *virginiana*), and white pines (P. *monticola*) have different needle retention characteristics. Noble and Fraser fir have a better post-harvest needle and moisture retention than Scot’s pine and Douglas-fir, which result in increasing production of Noble and Fraser fir in North America (Chastagner and Benson, 2000). Nordmann fir, an important Christmas tree species in Europe, was reported that it had superior post-harvest needle qualities than Noble fir (Chastagner and Riley, 2003). MacDonald and Lada (2008) reported that some genotypes

of balsam fir completed needle abscission within 6 days while some would complete it in 60 days. Similarly, the average needle abscission resistance (NAR) was found between 12 days to 60 days (MacDonald et al., 2014). Based on the difference in needle abscission resistance, genotypes of balsam fir were classified as low NAR clone if they completed abscission within 20 days, moderate NAR if they completed abscission between 20 to 40 days, and high NAR if they completed abscission over 60 days (Lada and MacDonald, 2015).

In Europe, Christmas trees are displayed indoor just for several days, while they are displayed for 4-6 weeks in North America (Chastagner and Riley, 2003). Long-time display increases the potential for needle abscission of trees. Superior genotypes can provide more opportunity for farms of Christmas trees to expand the export, and increase consumers' satisfaction.

2.3.2 Harvest date

Plants have different tolerance to chilling (0 to 15 °C) and freezing (below 0 °C) conditions. Cold stress can influence plant growth in several ways: metabolic dysfunction, plasma membrane disintegration, growth inhibition, solute leakage and cell dehydration (Quinn 1985; Beck et al., 2004). When temperature drops below 0 °C, ice formation would initiate in the intercellular space (Thomashow, 1999). Plant cellular membranes are fluid structures and cold temperature can reduce their fluidity, which would cause rigidity, decreasing water potential in plants. When water became more viscous, it would cause a physiological drought in the cells and the apoplast water would freeze, which would cause dehydration of the cells and solute leakage from cells.

Cold acclimation is a process triggered by low temperature to help plants to adapt to cold climate. The cold tolerant plants have a higher accumulation of sugar in their tissue. Sugars such as trehalose and polyols such as sorbitol are considered as antifreeze compounds. They can decrease freeze point and bind to the ice crystals and inhibit recrystallization in the plant cells. Sugars can also balance water potential and keep water in cells to adjust the osmotic potential of cells. Conifers growing in the colder climate have high needle retention compared to trees growing in the warmer climate.

Harvesting time is another important factor for needle retention. Christmas trees harvested in late autumn or winter shows higher needle retention than those harvested in early autumn due to a lack of natural cold acclimation. MacDonald and Lada (2008) reported that balsam fir harvested in January had an improved needle retention compared to those harvested in October. Branch needle retention decreased when branches was exposed to 20ºC, while needle retention increased when branches were exposed to 5ºC (MacDonald and Lada, 2012).

2.3.3 Post-harvest factors

There are also several post-harvest factors that may influence needle retention of balsam fir. Based on the previous research, this problem was caused by different factors, such as post-harvest handling (Lada and Adams, 2009), dehydration (Thiagarajan et al., 2013), ethylene (MacDonald et al., 2011). Rough handling, such as shaking and baling trees, increased needle loss compared to those trees with less handling (Lada and Adams, 2009).

Drought stress has been found to trigger needle abscission by affecting stomata (Chave, 1991). It is known that plants shed leaves to keep water balance under dehydrating conditions as an adaptation (Addicott, 1982). Dehydration can cause several changes to

hormones, such as ethylene and auxin (Addicott, 1982). Ethylene is a plant hormone, which can accelerate abscission in plants and it can reduce the strength of the cell wall at the abscission zone to trigger needle abscission (Addicott, 1982). Abscisic acid (ABA) is another hormone, which accelerates abscission in plants by increasing cellulase activity in the abscission zone (Cracker and Abeles, 1969). MacDonald et al (2014) also reported that the amount of ABA increased 38-fold during the post-harvest prior to needle abscission. MacDonald et al. (2011) also found that ethylene can increase cellulase activity during the peak needle abscission. Cellulase plays a negative role on bean leaf abscission (Durbin et al., 1981). Bean leaf abscission increased when the activity of cellulase was high (Durbin et al., 1981). Pectinase is also involved in abscission process. Polygalacturonase, for instance, impacts leaf and fruit abscission (Valdovinos and Muir, 1965; Greenberg et al., 1975). However, there is limited research on the dynamics of change of cellulase and pectinase on post-harvest needle abscission in balsam fir. Also, there is no information on the expression of cellulase and other hydrolytic enzymes that would explain the needle abscission resistance among different clones.

2.4 Plant cell wall and hydrolytic enzymes

2.4.1 Plant cell wall

Plant cell wall is a complex and rigid structure, which consists of many polysaccharides such as pectin, cellulose and hemicellulose (Aro et al., 2005). Pectin contains a backbone of α (1→4) linked to D- galacturonic acid (Aro et al., 2005). It consists of two regions: smooth and hairy regions (Figure 2.2). The homogalacturonan parts of the polymer are called “smooth region”, that the D- galacturonic acid residues can be methylated or

acetylated. Whereas there are two main structures in the rhamnose-rich zone ("hairy region"): Rhamnogalacturonan-I, and substituted galacturonans (Aro et al., 2005; Ridley et al.,2001).

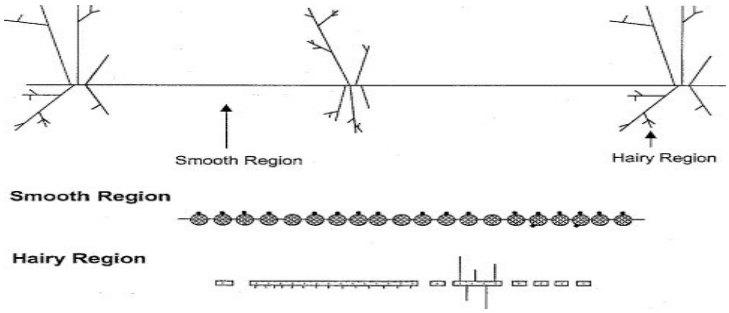

Figure 2. 2 The smooth and hairy regions of pectin (Pérez et al., 2000).

Cellulose is the most abundant polysaccharide in nature and it provides rigidity for plant cell wall. It consists of β (1→4) linked D-glucose units that form a linear chain of about 8,000 to 12,000 glucose unit (Figure 2.3). In crystalline cellulose, these chains are linked by hydrogen bonds to form an insoluble structure (Aro et al., 2005).

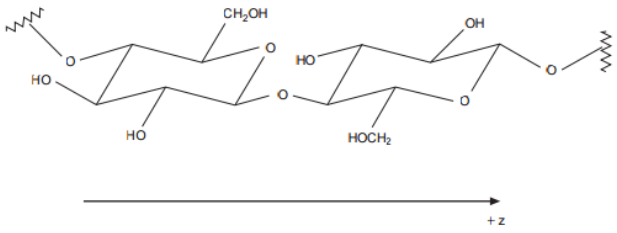

Figure 2. 3 Repeating unit of cellulose (O'sullivan, 1997).

Hemicellulose is the other main biopolymer in the plant cell wall, which is a copolymer composed of various sugar unit (Aro et al., 2005). The structures of hemicellulose in various plants are different that xylan hemicellulose found in hardwood and cereals and

(galacto) glucomannan found in hardwood and softwood. The main sugar chain of hemicellulose is linked to different groups, such as 4-O-methylglucuronic acid, arabinose, galactose, and acetyl to form various structure (Aro et al., 2005).

2.4.2 Ethylene

Ethylene, a simple organic molecule, is a plant hormone that regulates many diverse metabolic and developmental processes including senescence in plants (Abeles et al. 1992). Ethylene in higher plants is synthesized by this pathway: L-methionine → S-adenosyl-L-methionine (AdoMet) → 1-aminocyclopropane-l-carboxylic acid (ACC) → ethylene (Adam and Yang, 1979). The two main enzymes are required in this pathway: ACC synthase, converting AdoMet into ACC, and ACC oxidase, converting ACC to ethylene (Mathooko, 1996).

Ethylene is considered as a key regulatory molecule in fruit ripening (Alscher and Cumming, 1990). Fading of flowers and abscission of petals are also regulated by ethylene (Bleecker and Kende 2000). Ethylene also plays an important role in leaf abscission. MacDonald et al. (2011) showed that continuous exposure of branches to exogenous ethylene induced abscission and greatly reduced needle retention in balsam fir. Leaf abscission is also associated with hydrolytic enzymes, which can break down cell wall strength. Ethylene is also related to hydrolytic enzymes, such as cellulase and polygalacturonase. Taylor et al., (1993) showed that polygalacturonase was associated with ethylene-promoted leaf abscission, and the activity of polygalacturonase increased during abscission period. A similar result was also found in balsam fir that cellulase activity largely increased when branches were exposed to ethylene (MacDonald et al, 2011).

2.4.3 Hydrolytic enzymes

Pectin is an important component of a cell wall, which is abundant in the primary cell wall and middle lamella of plants. Pectinase can induce abscission by degrading pectin reducing cell wall strength. Pectin methylesterase is a kind of pectinase, which has been reported in association with plant abscission. Valdovinos and Muir (1965) noticed that activity of pectin methylesterase was high during the abscission period. Polygalacturonase is another major pectinase, which is involved in the ripening process. Based on the previous research, Riov (1974) reported that polygalacturonase was involved in citrus leaf abscission process. Greenberg et al. (1975) found that polygalacturonase played a negative role in citrus fruit abscission and its behavior was similar to cellulase. The activity of polygalacturonase increased after fruit abscission occurred and showed a positive correlation with citrus fruit abscission.

Cellulose (β-D-glucose) is a polymer of glucose, which is located in the middle layer of the secondary wall (Xue et al. 1999). Cellulase can cleave the β-1, 4-D-glycosidic bond to degrade cellulose and decrease the break strength of the cell wall to allow abscission (MacDonald et al., 2011). MacDonald et al. (2011) indicated that ethylene increased cellulase activity in needles of Balsam fir branches. When branches were exposed to endogenous ethylene, cellulase activity increased 8-fold compared to the control, while it increased by 12-fold when exposed to exogenous ethylene. Similar results have been reported in other species, such as cotton and bean (Durbin et al. 1981; Mishra et al. 2008). Durbin et al. (1981) reported that the form of cellulase with an isoelectric point of 4.5 (4.5 cellulase) was not involved in abscission, while the other forms with isoelectric point 9.5

(9.5 cellulase) were. MacDonald et al. (2011) have identified that two different cellulases, 75 and 125 kDa, were involved in Balsam fir abscission.

2.5 Inhibitors of hydrolytic enzymes

Enzymes are extraordinarily efficient, selective biological catalysts. Enzymes play an important role in enhancing the rates of reactions. Enzyme inhibitors are low molecular weight chemical compounds, which can stop or reduce a particular enzyme activity (Berg et al., 2002). There are two types of enzyme inhibitors, reversible and irreversible. Reversible enzyme inhibitors can attach to non-covalent bonds of enzymes molecules and temporarily block enzyme function, while irreversible inhibitors can bind to covalent bonds of enzyme molecules and permanently block enzyme function (Berg et al., 2002).

2.5.1 Natural enzyme inhibitors

In nature, many plants and animals can produce natural inhibitors, which are small organic molecules (Sharma, 2012). Plants develop natural inhibitors against pathogens, which can degrade cell walls to enter plant cells (Ximenes et al., 2009). The leaves from muscadine grape, persimmon, blueberry and raspberry contain water-soluble inhibitors, which can inhibit the activity of pectinase and cellulase (Bell et al., 1962; Mandel and Reese, 1963). Bell et al. (1962) reported that fourteen species inhibited both pectinase and cellulase activity (Table 1) and more plant sources inhibited pectinase than cellulase.

Table 2.1 Pectinase and cellulase inhibited by plant-leaf extracts (Bell et al., 1962).

Scientific name	Common name	Degree of enzyme inhibition	
		Pectinase	Cellulase
Acer rubrum L.	Red maple	Weak	Weak
Antirrhinum majus L.	Snapdragon	weak	-
Capsicum frutescens L.	Bell pepper	-	-
Carya illinoensis Koch.	Stuart pecan	Weak	-
Chaenomeles japonica Lindl	Flowering quince	Weak	-
Cornus florida L.	Flowering dogwood	strong	Strong
Dianthus caryophyllus L	Carnation	-	-
Diospyros virginiana L.	Persimmon	Strong	Strong
Euphorbia pulcherrima Willd.	Poinsettia	Weak	
Fragaria chiloensis Duchesne.	Albritton strawberry	Moderate	Weak
Ipomoea batatas Lam	Sweet potato	-	-
Iris hybrid	German iris	-	-
Lespedeza cuneata Don.	Sericea	Strong	Strong
Ligustrum lucidum Ait.	Privet	Weak	-
Parthenocissus quinquefolia	Virginia creeper	Moderate	-
Pelargonium hortorum.	Geranium	Weak	Weak
Persea americana Mill.	Avocado	Weak	-

Scientific name	Common name	Degree of enzyme inhibition	
		Pectinase	Cellulase
Phaseolus vulgaris L.	Snap bean	Weak	-
Prunus domestic L.	Plum	weak	-
Prunus persica Batsch	Hale Harrison peach	-	-
Punica granatum L.	Pomegranate	Weak	Weak
Pyrus communis L.	Pear	-	-
Rosa odorata Sweet.	Tea rose	Moderate	Weak
Rubus hybrid	Carolina blackberry	Moderate	Weak
Rubus strigosus Michx.	Latham raspberry	Moderate	Weak
Thea sinensis L.	Tea	Weak	Weak
Vaccinium ashei	Rabbiteye blueberry	Strong	Strong
Vitis labrusca L.	Concord grape	Moderate	Weak
Vitis rotundifolia Michx	Muscadine grape	Strong	Strong

2.5.2 Chemical enzyme inhibitors

Chemical compounds are one type of enzyme inhibitor. Enzymes can be inhibited by heavy metals, such as mercury, silver, chromium and copper (Mandel and Reese, 1963). Heavy metals are nonspecific salt formations and they can bind tightly with active sites of enzymes to reduce enzyme activity. They are nonspecific inhibitors and they can widely affect a variety of enzymes (Sharma, 2012). Large organic molecules, such as acids or basic

dyes, can also inhibit enzymes by affecting pH during reactions. Enzymes can also be inhibited by phenolic compounds, such as chlorophenol and tannin.

According to the previous research, cellulase and pectinase activity were inhibited with increasing concentration of ethylene diamine tetra acetic acid (EDTA) (Vatanparast et al., 2014). Dichlorophenoxyacetic acid (2, 4-D) also reduces polygalacturonase and cellulase activity of citrus fruit. 2, 4-D delayed the fruit abscission of citrus plants by inhibiting polygalacturonase and cellulase activity in the cell wall. It played a similar effect on hydrolytic enzymes (Greenberg et al., 1975). $CaCl_2$ and $MgCl_2$ were also reported to reduce pectinase activity of tomato (Vatanparast et al., 2014). Cellulase activity has been reported to be inhibited by $NaSO_4$ from 10^{-2} to 10^{-3} M (Mandel et al., 1963).

2.5.3 Ethylene inhibitors

The activity of hydrolytic enzymes is also associated with ethylene. Several ethylene inhibitors, such as aminorthoxyvinylglycine (AVG), 1-methylcyclopropene (1-MCP) and salicylic acid, have also been shown to reduce ethylene synthesis and hydrolytic enzymes' activity.

In the ethylene synthesis, S-adenosyl-L-methionine (AdoMet) is an intermediate, which is converted into 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase. AVG can inhibit ACC synthase to reduce ethylene production (MacDonald et al., 2010). Unlike the AVG, 1-methylcyclopropene (1- MCP) is an ethylene receptor blocker. The function of 1-MCP is competitively binding to the ethylene receptors to reduce ethylene production (MacDonald et al., 2010). Exposure to exogenous ethylene significantly reduced needle retention in balsam fir, while when ethylene production was inhibited by AVG and 1- MCP, needle retention increased 73% and 147%, respectively (MacDonald et al., 2010). Salicylic

acid, a phenolic compound, was also shown to inhibit ethylene and cellulase activity (Leslie and Romani, 1986; Ferrarese et al., 1996). Salicylic acid and its derivative acetyl salicylic acid (ASA) can block the conversion of 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene (Leslie and Romani, 1986). They have been reported to inhibit ethylene production in pear fruit (Leslie and Romani, 1986). The effect of salicylic acid on cell wall degrading enzymes, cellulase, polygalacturonase and xylanase have been investigated during ripening of banana fruits (Srivastava and Dwivedi, 2000). The inhibition of cellulase and polygalacturonase was stronger than xylanase. Ferrarese et al., (1996) also reported that salicylic acid reduced leaf abscission in both peach and pepper plants by decreasing the cellulase activity.

2.6 Summary

Needle abscission is a major challenge for the Christmas tree industry. Abscission is regulated by cell wall hydrolytic enzymes. The hydrolytic enzymes such as polygalacturonase and cellulase have been reported to induce organ abscission in many other plant species. These cell wall degrading enzymes have also shown the strong relationship with ethylene, which is related to plant organ abscission. However, the dynamics of hydrolytic enzymes in post-harvest needle abscission, the clonal differences between high and low NAR clones are unknown. It is also unknown, whether inhibiting the hydrolase enzyme activity would promote needle retention.

2.7 References

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CHAPTER 3 GENERAL METHODOLOGY

3.1 Sampling site

Balsam fir branches were used for this study. These branches were collected from a balsam fir tree germplasm site at the Tree Breeding Centre, Department of Natural Resources, Debert, NS (45° 25' N, 63° 28' W). The orchard is approximately 4 ha and approximately about 200 genotypes were assembled on this site. All genotypes had been classified as low (0-20 days), moderate (21-40 days) and high (41-60 days) needle retention duration (NRD) clones based on previous studies (MacDonald and Lada,2008; Lada and Veitch, 2010). All clones were grafted and transplanted at the same time, thus the entire orchard is 21 years old.

3.2 Sample collection and transportation

The low NRD clone (clone #1) and the high NRD clone (clone #330) were used in Experiment 1 (Chapter 6). All collected branches were of second-year growth and cut on the same day. All branches were cut from 1.5 m above the ground on the same side of the trees. The same low NRD clone #1 was used in Experiment 2 (Chapter 7). A branch cutting from 2-year growth, was randomly collected from the 4 trees. Samples were immediately immersed in a bucket filled with reverse osmosis (RO) water and transported to the laboratory.

3.3 Sample preparation

For every experiment, green freshly cut branches were initially weighed and then placed in a 150mL amber bottle with approximately 100 mL RO water. All bottles were sealed with cotton gauze to prevent evaporation (Figure 4.1). Finally, the entire apparatus (branch, bottle and RO water) were weighed. The light intensity ranged from 15 to 25 $\mu mol*m^{-2}*s^{-1}$ and temperature ranged from 21.5 ºC to 22.5 ºC in the lab. Due to non-homogeneous light and temperature conditions in the lab, branches in the same table was randomly moved every two days.

Figure 3. 1 Cut branches are placed in an amber bottle with water.

3.4 Measured Response Variables

3.4.1 Average water use (AWU)

In every experiment, the apparatus (branch, bottle and RO water) was re-weighed on each sampling day as the final mass (g). All needles lost from natural abscission and due to finger-run test were weighed as needle loss (g). The difference between the current day's weight of the apparatus and the weight of the apparatus from the previous sampling (Initial

mass) day was calculated as average water usage. Average water use (g/d) was calculated as (MacDonald et al. 2010):

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

3.4.2 Cumulative water use

The cumulative water use (g) was calculated as the water loss through the duration of the whole experiment by adding each measured AWU value (MacDonald et al. 2010).

Cumulative water use (g) = $AWU_1 + AWU_2 + AWU_3 + \ldots + AWU_n$

Where, AWU was calculated on the n^{th} sampling date.

3.4.3 Needle loss percentage

On each sampling date, abscised needles were collected and put into the oven for 24 hours to get the dry weight:

$$Needle\ loss\ (\%) = \frac{dry\ weight\ of\ needle\ loss\ (g)}{Dry\ weight\ of\ total\ needle\ loss\ (g)} * 100$$

3.4.4 Cumulative needle loss percentage

The cumulative needle loss percentage (%) was calculated as the needle loss (%) through the duration of the whole experiment by adding each measured needle loss (%) value.

Cumulative needle loss (%) = Needle $loss_1$ + Needle $loss_2$ + …+ Needle $loss_n$

Where, Needle loss (%) was calculated on the n^{th} sampling date.

3.4.5 Optimization of pectinase and cellulase digestion

Spotting gel plates infused with cellulose and pectin were used to determine optimum pH for enzyme assay with cellulase from *Aspergillus niger* (EC 3.2.1.4) and pectinase from *Aspergillus niger* (EC 3.2.1.15) (Sigma-Aldrich, Oakvile, ON, Canada). For cellulase assay, each plate contained 0.7% Gelzan (PhytoTechnology Laboratories, Lenexa, KS, USA) and 0.05% carboxymethyl-cellulose (CMC) (Sigma-Aldrich, Oakvile, ON, Canada) were made to the volume of 20 mL in a buffer. For pectinase assay, each plate contained 0.7% Gelzan (PhytoTechnology Laboratories, Lenexa, KS, USA) and 0.2% pectin (Sigma-Aldrich, Oakvile, ON, Canada) were made to the volume of 20 mL in a buffer. There are three different buffers used to determine the optimum pH for cellulase and pectinase: 0.1M sodium acetate (pH = 5.5), 0.1M phosphate buffer solution (PBS) (pH = 6.5), and 0.1M Tris(hydroxymethyl)aminomethane (TRIS) (pH = 7.4). Plates were spotted with 0.01, 0.02, 0.04, 0.08, 0.1, 0.2, 0.4, 0.8, and 1 unit of cellulase and pectinase activity, respectively. Plates were incubated for 20 hours at 37 ºC. After incubation, plates were stained with 0.1% Congo Red (Sigma-Aldrich, Oakille, ON, Canada) stain for 20 minutes and then destained with 1M NaCl for 1 hour.

3.4.6 Enzyme extraction

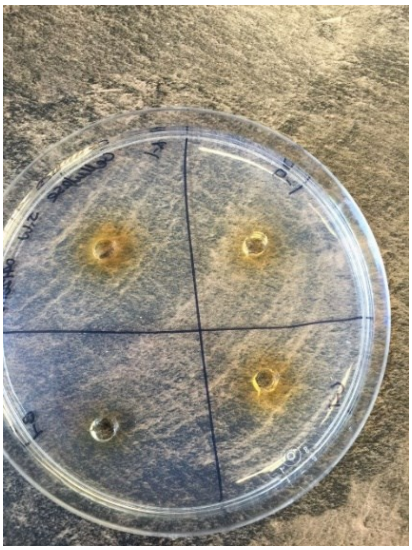
Needles were frozen dried for 20 hours then 0.5g needles were ground with liquid nitrogen using a mortar and pestle (Figure 3.2). After grinding, 0.2g ground needles were mixed with 1.5 mL PBS buffer (pH = 6.5). The homogenate was centrifuged at 7500 rpm for 15 minutes at 4 ºC, then the supernatant was collected. This extraction was repeated 3 times. The extraction solutions were frozen dried for 48 hours and then re-suspended in 1.5 mL PBS buffer.

Figure 3. 2 Needles were ground with liquid nitrogen.

3.4.7 Analysis of enzyme activity

A mixture of 0.07% Gelzan and 0.05% CMC or 0.2% pectin was made in 20 mL PBS buffer. There were 4 wells (0.16 cm^2 per well) in each plate and these wells were filled with 30 μL extracted enzyme solutions. After 20 hours incubation and staining procedure, the diameter of digested area of each sample was measured by digital caliper (0.01 mm) for both vertical and horizontal directions. Standard curves were made by using 0.01, 0.02, 0.04, 0.08, 0.1, 0.2, 0.4, 0.8, and 1 unit of purchased cellulase and pectinase activity, respectively.

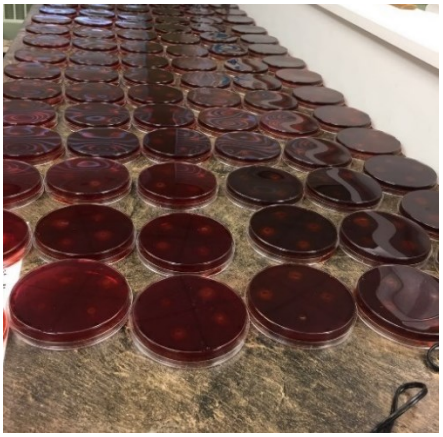
a



b

Figure 3. 3 a. 4 wells in each plate for enzyme activity analysis (left) b. 20 hours incubation at 37 ºC.

a



b

Figure 3. 4 a. Plates stained with 0.1% congo red then destained with 1 M NaCl. b. The diameter of digested area was measured by digital caliper.

3.5 References

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CHAPTER 4 HYDROLYTIC ENZYME EXTRACTION AND QUANTIFICATION PROTOCOL DEVELOPMENT

4.1 Introduction

Pectinase and cellulase are a group of enzymes involved in the breakdown of pectin or cellulose in the cell wall of plants. In plant organ abscission, they play a role in degrading cell walls to reduce strength of cell wall to induce abscission. Assays for determining enzyme activity have been classified differently over years of enzyme research. 3,5-dinitrosalicylic acid (DNS) assay was used to measure pectinase (Bernfeld, 1955) and cellulase activity (Miller, 1959; Wood and Bhat, 1988). The DNS reagent is used as a colorimetric method to determine the reducing sugars. In the enzyme quantification assay, DNS was added to the sample as a stop buffer and samples need to be kept at 45°C for 60 minutes to promote full color development. The absorbance of sample will be read at 540 nm (Zhang et al., 2009; Vatanparast et al., 2014). The advantage of this method is that it is simple, and the reagent is not very expensive. However, the disadvantage of this method is that DNS reagent requires appropriate temperature control for color development and stability (Miller, 1959). Enzyme activity can be also measured by using a soluble substrate such as soluble pectin and carboxymethyl cellulose (CMC) (Mandels et al., 1976). Dye can be added to solid agar plates to determine enzyme activity. MacDonald et al. (2011) used Congo red (Sigma-Aldrich, Oakille, ON, Canada) to measure cellulase activity of balsam fir's needle in agar plates. The cellulase activity can be also measured by using cellulase enzyme kit, which is an easy and quick method to detect cellulase activity by using long wavelength fluorescent substrate. When cellulase degrades cellulose, the β-1, 4-D-glycosidic bond of cellulose is broken and the fluorescent compound comes out. Then the

activity of cellulase can be measured by a microtiter plate fluorescent reader at Ex/Em = 530/595 nm.

4.2 Materials and methods

4.2.1 Optimization of pectinase and cellulase digestion

Pectinase and cellulase standardization was measured by using the method described in section 3.4.5.

4.2.2 Enzyme extraction

Method 1: The enzyme extraction was modified from a method described by MacDonald et al. (2011). 10g fresh needles were homogenized in 30 mL RO water by using a mortar and pestle. The homogenate was layered onto a 10% glycerol solution for 3 hours. Then the cell wall pellet was suspended in RO water and washed 3 times with repeated centrifugation. The pellet was suspended in 10 mL of 0.2 M $CaCI_2$ and then the supernatant was collected by repeated centrifugation. After that, collected supernatant was dried down and then re-suspended in 1 mL PBS buffer (pH = 6.5) when doing analysis.

Method 2: 1.0g fresh needles were ground with 1.5 mL PBS buffer (pH = 6.5) by using homogenizer for 30 seconds (Figure 4.1). The homogenate was centrifuged at 7500 rpm for 15 minutes at 4 ºC, then the supernatant was collected. This extraction was repeated 3 times.

a

b

Figure 4. 1a. Fresh needles (1g) were ground by using homogenizer. b. Ice was used for reducing temperature during grinding process.

Method 3: 0.5g fresh needles were ground with liquid nitrogen using a mortar and pestle. After grinding, 0.3g ground needles were mixed with 1.0 mL PBS buffer (pH = 6.5). The homogenate was centrifuged at 7500 rpm for 15 minutes at 4 ºC, then the supernatant was collected. This extraction was repeated four times.

Method 4: Needles were freeze dried for 20 hours then 0.5g needles were ground with liquid nitrogen using a mortar and pestle. After grinding, 0.2g ground needles were mixed with 1.5 mL PBS buffer (pH = 6.5). The homogenate was centrifuged at 7500 rpm for 15 minutes at 4 ºC, then the supernatant was collected. This extraction was repeated 3 times. The extraction solutions were frozen dried for 48 hours and then re-suspended in 1.5, 1.0, 0.5, 0.2, 0.15 mL PBS buffer, respectively.

4.2.3 Enzyme quantification

Enzyme activity was measured by using the method described in section 4.4.6.

4.3 Results and discussion

Calibration curves were constructed by using enzyme activity of purchased products (Figure 4.2 and Figure 4.3). Interpretation curves were created to calculate of pectinase and cellulase activity of samples (Figure 4.4 and Figure 4.5)

4.3.1 Standard curves for pectinase and cellulase activity

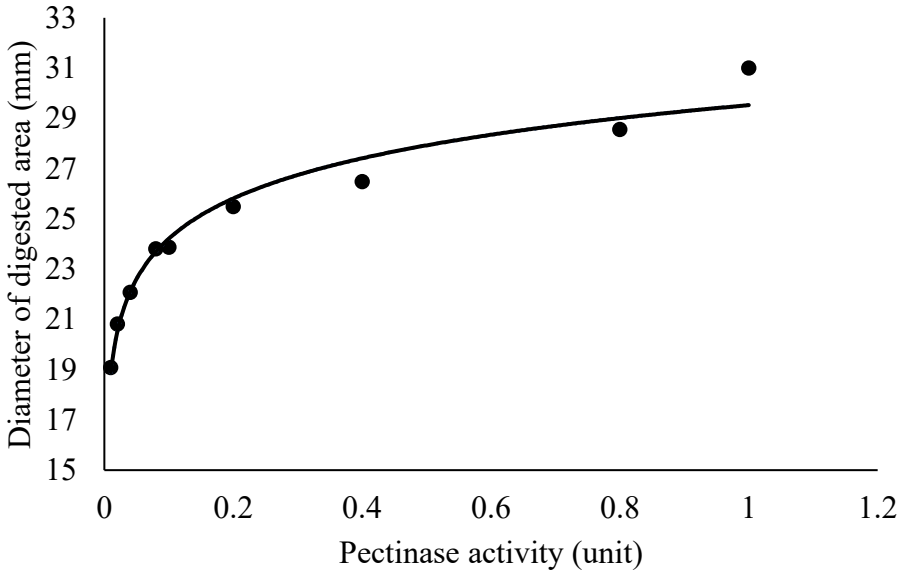

Figure 4. 2 Calibration curve of pectinase activity.

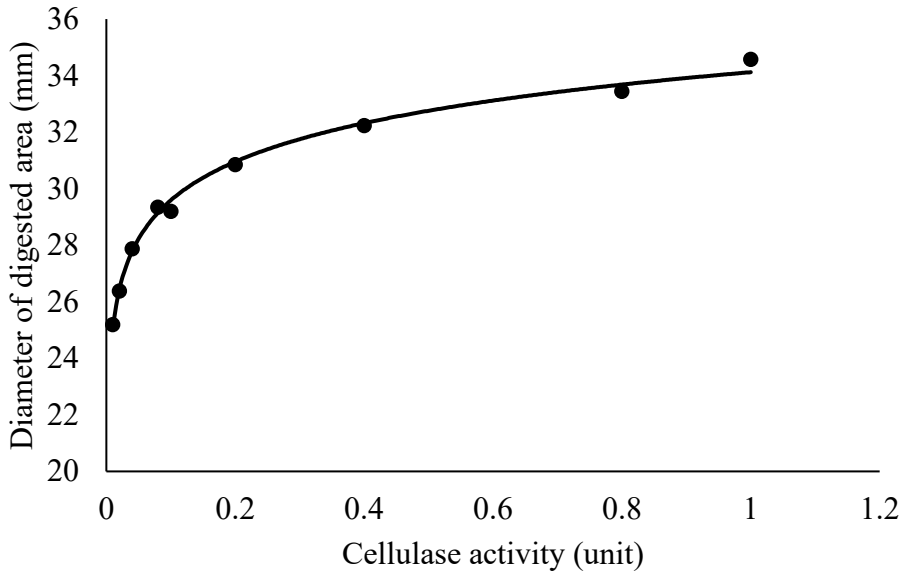

Figure 4. 3 Calibration curve of cellulase activity

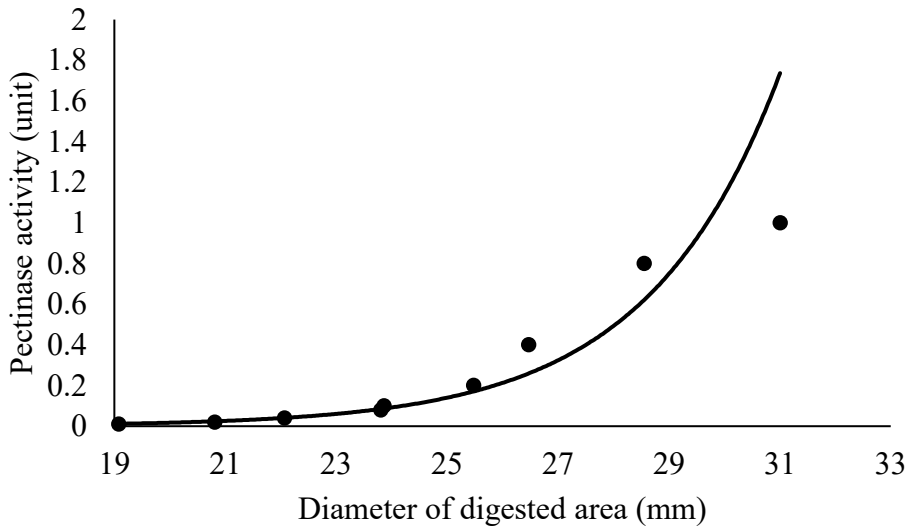

Figure 4. 4 Interpretation curve used to measure pectinase activity

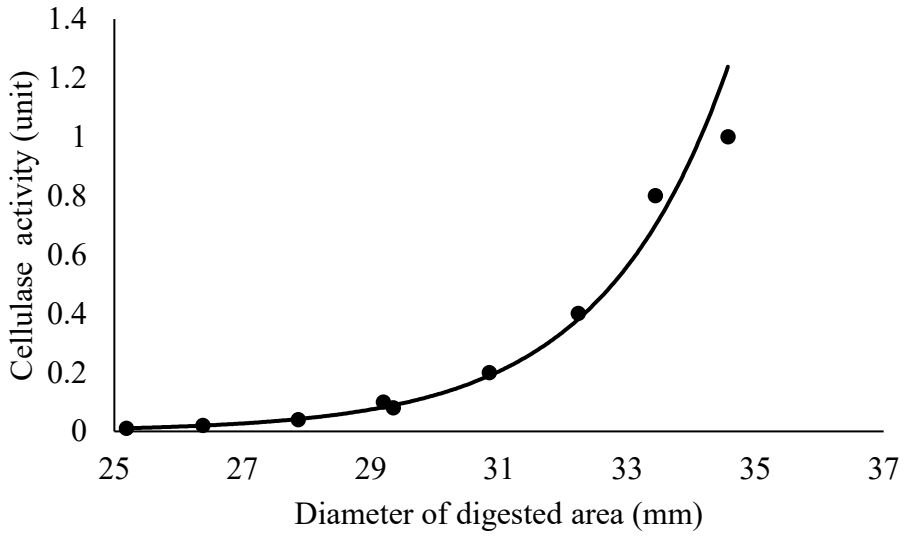

Figure 4. 5 Interpretation curve used to measure cellulase activity

Calculation of pectinase and cellulase activity from calibration curve:

Pectinase activity: $\mathrm{X} = e^{\frac{diameter\ of\ digested\ area - 29.524}{2.3079}}$

Cellulase activity: $\mathrm{X} = e^{\frac{diameter\ of\ digested\ area - 34.121}{1.9679}}$

4.3.2 Enzyme extraction

Method 1: Cellulase and pectinase activity was hard to be detected by using this method. The possible cause may be the amount of needle was too small or the enzyme could not be extracted completely.

Method 2: Cellulase activity was detected by using this method, while pectinase activity was still not detected. It is possible that pectinase activity may have been diluted by the buffer added (Table 4.1).

Table 4. 1 The cellulase and pectinase activity of samples.

Clone	Extraction 1 (+ 1.5ml buffer)		Extraction 2 (+ 1.5ml buffer)		Extraction 3 (+ 1.5ml buffer)	
	Pectinase activity (unit/g)	Cellulase activity (unit/g)	Pectinase activity (unit/g)	Cellulase activity (unit/g)	Pectinase activity (unit/g)	Cellulase activity (unit/g)
High	NA	0.0030	NA	0.0018	NA	0.0014
Low	NA	0.0018	NA	0.0018	NA	0.0007

Table 4. 2 The cellulase and pectinase activity of samples.

Day	Clone	Extraction 1 (+ 1.0ml buffer)		Extraction 2 (+ 1.0ml buffer)		Extraction 3 (+ 1.0ml buffer)		Extraction 4 (+ 1.0ml buffer)	
		Pectinase activity (unit/g)	Cellulase activity (unit/g)	Pectinase activity (unit/g)	Cellulase activity (unit/g)	Pectinase activity (unit/g)	Cellulase activity (unit/g)	Pectinase activity (unit/g)	Cellulase activity (unit/g)
0	High	0.1233	0.0051	0.0336	0.0024	0.0271	0.0002	NA	NA
18	Low	0.1531	0.0067	0.0002	0.0011	NA	0.0007	NA	0.00003
33	High	0.1530	0.0015	0.0518	0.0024	NA	0.0003	NA	0.00004
36	Low	0.0518	0.0040	NA	0.0019	NA	0.0005	NA	0.00005
54	Low	0.0336	0.0086	0.0092	0.0024	NA	0.0005	NA	0.00005
63	High	0.0799	0.0019	0.0141	0.0011	NA	0.0005	NA	NA

Method 3: Cellulase and pectinase both were detected by using this method, while both cellulase and pectinase activity was low in extracted solution 4.2.

Method 4: Cellulase and pectinase activity were both detected by using this method. Enzyme activity was the highest when sample was re-suspended with 1.5 mL buffer (Table 4.3). The viscosity of solution was high when sample was re-suspended with 0.15 and 0.2 mL buffer. It was difficult to take samples from tubes and add samples to plates for analysis.

Table 4. 3 The cellulase and pectinase activity of samples with different re-suspended buffer.

Enzyme activity	Re-suspended buffer (mL)				
	1.5	1.0	0.5	0.2	0.15
Pectinase (unit/g)	0.7130	0.1290	0.2951	0.0951	0.0241
Cellulase (unit/g)	0.0416	0.0036	0.0231	0.0029	0.0025

Four complementary methods were used to extract pectinase and cellulase to detect enzyme activity. Reducing sugar method detected both pectinase and cellulase activity from needles of balsam fir during post-harvest needle abscission. Needles were frozen dried before grinding to remove moisture from needle and improve extraction efficiency. When doing the extraction, samples need to repeat centrifugation (3 times) to extract enzyme from needles completely.

4.4 Conclusion

Reducing sugar method with soluble substrate can be used to detect pectinase and cellulase activity. Removing moisture from needles and grinding needles with liquid nitrogen would improve extraction efficiency. This was the method adopted in all the experiments in this thesis.

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CHAPTER 5 NATURE, DYNAMICS OF CHANGE OF CELLULASE AND PECTINASE AND THEIR RELATIONSHIP WITH POST-HARVEST NEEDLE ABSCISSION IN BALSAM FIR (*Abies balsamea, L*)

5.1 Abstract

Hydrolytic enzymes have been shown to relate to organ abscission in plants. However, it is not clear about the nature of hydrolytic enzymes and their dynamics of change in balsam fir post-harvest needle abscission. It is also not known whether the changes in the levels or the dynamics of change would explain the post-harvest needle retention resistance in contrasting balsam fir clones. An experiment was conducted to evaluate the dynamics of change in hydrolytic enzymes (cellulase and pectinase) during post-harvest period and uncover their relationship to the post-harvest needle abscission resistance (NAR). A total of 264 branches were collected from both low and high genotypes with 4 replicates. The treatments included genotypes and days. Water was supplied to branches during a 72-day period. Water usage and needle loss were recorded every 2 days. Water usage decreased ($P<0.05$) in post-harvest. The cumulative needle loss percentage was significantly affected by the interaction between clone and day ($P<0.05$). In this experiment, the low NAR clone began to lose needle faster and reached 50% of needle loss than that of the high NAR clone. Cellulase (EC 3.2.1.4) and pectinase (EC 3.2.1.15) activities in the needle were evaluated every 3 days. Cellulase activity of low NAR clone was significantly higher ($P<0.05$) than that of the high NAR clone throughout the whole experimental period. The pectinase activity was not affected by neither the genotypes nor days in post-harvest. These results indicate that higher cellulase activity could be the reason for higher needle abscission in

balsam fir, post-harvest. Cellulase activity was also found to be significantly higher in the low NAR clone than the high NAR clone, which could be a possible reason why the low NAR clones loose needles early.

5.2 Introduction

Balsam fir (*Abies balsamea* (L.)) is a native Christmas tree species in Nova Scotia. Earlier harvesting met the high demands of Christmas tree, but also accelerated post-harvest needle abscission (MacDonald and Lada, 2008). Genotypes, post-harvest handling, dehydration, and ethylene also affect needle retention (Lada and Adams, 2009; MacDonald et al.; 2011 Thiagarajan et al., 2013).

Studied by MacDonald et al. (2011) have shown that needle retention declined when ethylene concentration increased. Ethylene plays a key role in the plant growth, development and senescence. It triggered the post-harvest needle abscission in balsam fir. It is believed ethylene increased the activity of hydrolytic enzymes, such as cellulase and pectinase, and reduced cell wall strength (Sexton and Roberts, 1982). Pectinase (EC 3.2.1.15) can degrade pectin and increase soluble pectin fraction in the middle lamella of the cell wall. Cellulase (EC 3.2.1.4) is required for abscission due to degrading cellulose which can provide rigidity for plant cell wall. The relationship between hydrolytic enzymes and plant abscission has been shown in many species, including citrus, cotton and bean (Riov 1974, Durbin et al. 1981; Mishra et al. 2008) however, little or no information is available on the nature or relationship of hydrolytic enzyme activity in post-harvest balsam fir.

Cellulase, is ethylene- sensitive, and increase in ethylene levels has been reported to increase cellulase activity. When branches were exposed to endogenous and exogenous ethylene, cellulase activity increased 8-fold and 12-fold, respectively, compared to the control (MacDonald et al., 2011).

Several studies have established the relationship between hydrolytic enzymes in plant abscission, but it is not clear the nature of cellulase and pectinase and their dynamics of changes post-harvest in balsam fir. It is also not known whether the activity of hydrolytic enzymes would explain the needle retention resistance in different genotypes.

This study investigates the possible link between hydrolytic enzymes and needle loss in post-harvest balsam fir. Several objectives must be met to understand the role of hydrolytic enzymes in needle abscission. Firstly, determine and quantify hydrolytic enzyme activity in the post-harvest needle of two clones. The changes of enzyme activity during the abscission period can provide evidence for explaining the needle retention resistance. The second one is modeling relationships of water usage, needle loss and enzyme activity. Low and high needle retention duration (NRD) clones were used in this study.

5.3 Materials and methods

5.3.1 Sample collection and preparation

Two hundred and sixty-four branches of two-year-old growth from eight trees (33 branches per tree) were collected from Orchard at Debert, NS (45° 25' N, 63° 28' W) on September 10, 2015. Two genotypes (4 trees per genotype) that were low NRD (clone #1) and high NRD (clone #330) were selected for this experiment. Branches were immediately placed

in a container with reverse osmosis (RO) water and transported to the laboratory. Eight branches (1 branch per tree) were cut and immediately placed in separate plastic bags to determine the initial enzyme activity.

All freshly cut branches were initially weighed and then placed in a 150 mL amber bottle with approximately 100 mL RO water. All bottles were sealed with cotton gauze to prevent evaporation. Initial measurements were taken for the total weight of the entire apparatus.

5.3.2 Experimental design

The experiment followed a completely randomized block design. The experiment consisted of two treatments (high NRD clone and low NRD clone) and each treatment had four replicates. Several response variables, such as water usage and needle loss, were measured every two days from separate 24 branches for the entire duration of the experiment. Enzyme activity was analyzed every three days on a separate set of 240 branches. There were eight branches that were randomly selected on each sampling day and all needles were collected and immediately frozen in liquid nitrogen, then stored at -80 ºC for enzyme activity analysis. After sampling, these branches were discarded from this experiment.

5.3.3 Average water use (AWU) and cumulative water use

Measurements of water consumption were taken every three days using methods described in Section 3.4.1, average water usage and cumulative water usage were measured and calculated using formulas from Section 3.4.1 and 3.4.2.

5.3.4 Needle loss percentage and cumulative needle loss percentage

Measurements of needle loss were taken every three days using finger run test, needle loss percentage and cumulative needle loss percentage were measured and calculated using formulas from Section 3.4.3 and 3.4.4.

5.3.5 Enzyme extraction and analysis of enzyme activity

Enzyme extraction was taken by using the method described in section 3.4.5. Pectinase and cellulase activity was measured by using the method described in section 3.4.6.

5.3.6 Statistical analysis

Data were analyzed using analysis of variance (ANOVA) with repeated measures in statistical analysis system (SAS) 9.4 (SAS Institute, NC, USA). Data were transformed to a normal distribution. If main effects or interaction effects were found to be significant ($P \leq 0.05$), then Tukey-Kramer test was used to differentiate the means at $\alpha = 0.05$ (Gbur et al., 2012).

The relationship between cumulative needle loss and cumulative enzyme activity was analyzed by using linear regression analysis in Minitab 17 (Minitab 17, Minitab Inc., PA, USA).

5.4 Results

The average water usage was not affected by genotypes (Table 5.1), while it was significantly affected by day (Table 5.1). Dynamics of average water use (Figure 5.1) showed a decline trend with post-harvest day increase. There was a sharp decline from Day 2 to Day 6. After Day 6, the average water usage decreased gradually until the end of the experiment.

Table 5. 1 The main and interaction effects of clones (high NRD and low NRD) and day in post-harvest on water usage, cumulative water usage.

Effect	**P-value**	
	Water usage	**Cumulative water usage**
Block	0.5352	0.5989
Clone	0.1081	0.1527
Day	<.0001	<.0001
Clone*Day	0.7903	0.0001

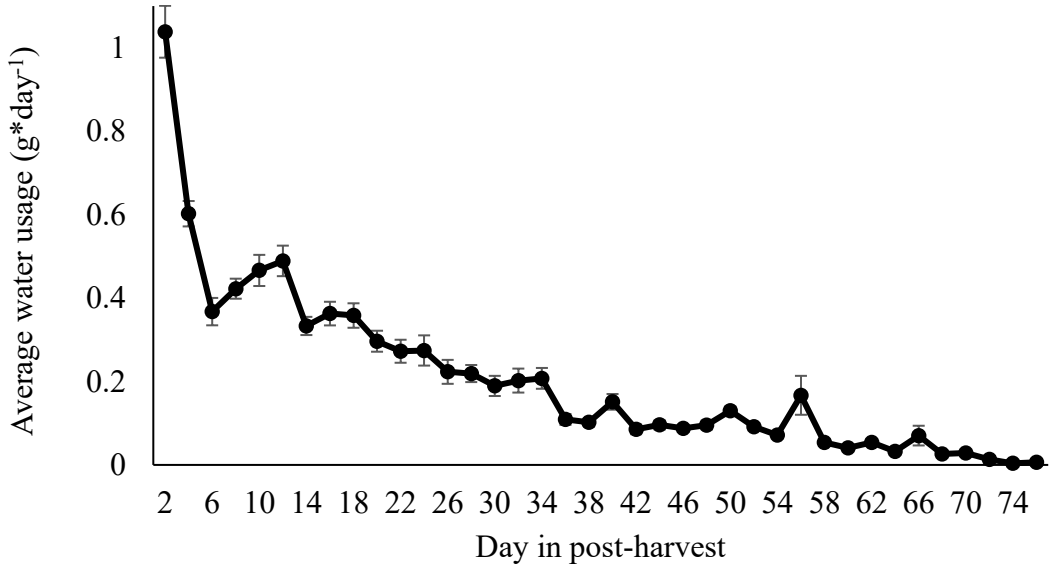

Figure 5. 1 Dynamics of change in average water usage of branches over a 76-day experimental period in balsam fir.

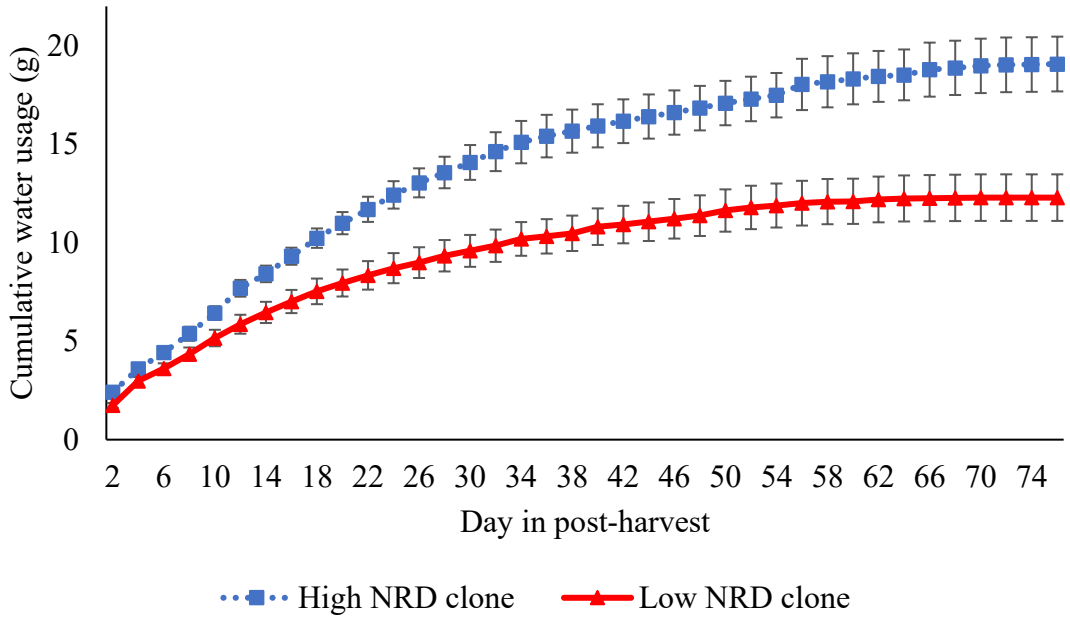

Figure 5. 2 Dynamics of cumulative water use of branches over a 76-day experimental period in balsam fir.

The cumulative water usage was significantly (p=0.0001) affected by the interaction between genotypes and time (Table 5.1). High NRD clone consumed more water than low NRD clone (Figure 5.2) for the entire duration of the experiment. Both genotypes used most water from Day 4 to Day 36, while cumulative water use continued to increase but at a slower pace after 36 days.

Table 5. 2 The main and interaction effects of clones (high NRD and low NRD) and day in post-harvest on needle loss%, and cumulative needle loss%.

Effect	**P-value**	
	Needle loss%	**Cumulative needle loss%**
Block	0.0484	0.652
Clone	0.5207	0.080
Day	<.0001	<0.0001
Clone*Day	<.0001	0.002

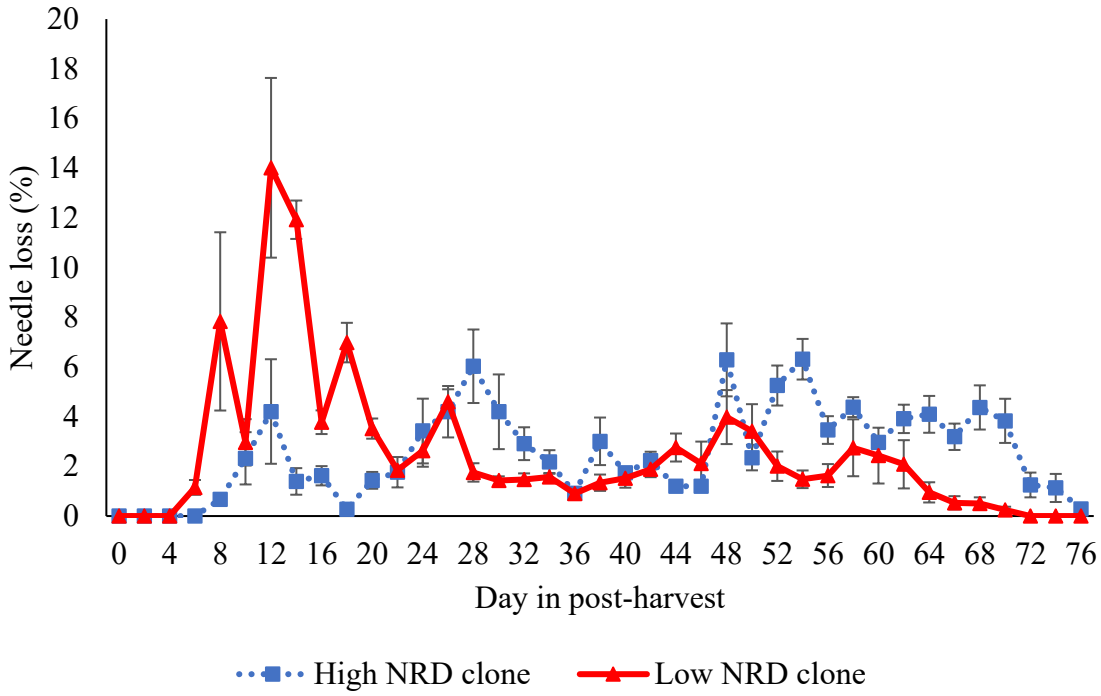

Figure 5. 3 Dynamics of needle loss (%) of branches over a 76-day experimental period in balsam fir.

The needle loss was significantly affected by the interaction between genotypes and time (Table 5.2). The needle loss versus time was graphed for both clones to show their patterns of needle loss (Figure 5.3). Low NRD clone started to abscise needle sooner and lost most needles from day 12 to day 26 following the harvest, while the most needle loss of high NRD clone happened after day 48 following the harvest.

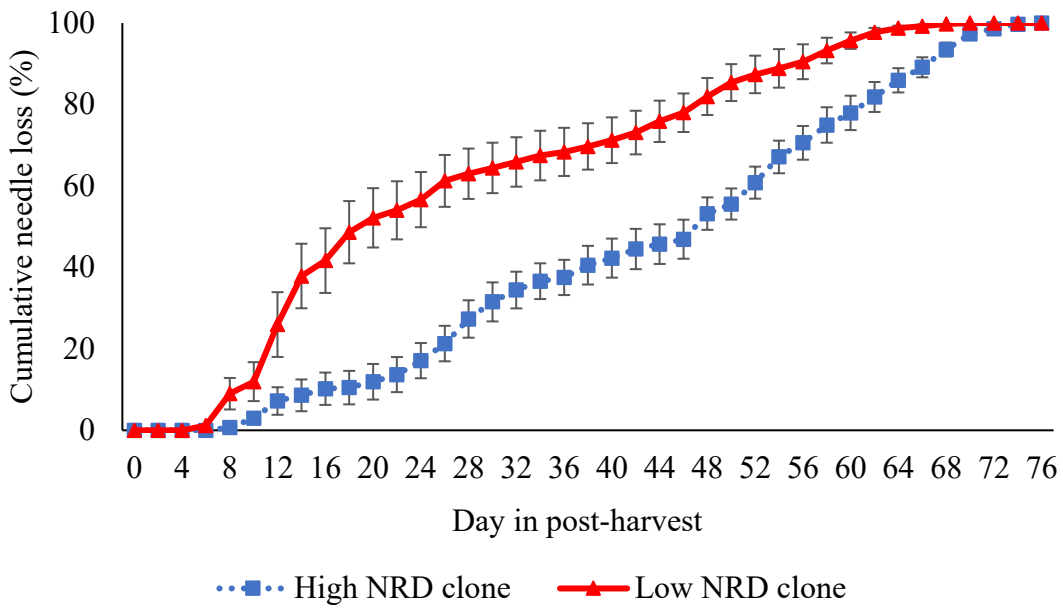

Figure 5. 4 Kinetics of cumulative needle loss (%) of branches in two balsam fir genotypes.

The needle loss was significantly affected by different genotypes during post-harvest period. Both clones had generally a similar pattern for cumulative needle loss (Figure 5.4). The needle loss pattern consisted of three phases: little needle loss at first, gradual increasing in needle loss at second, and then leveling off at the end. Low NRD clone began to lose needle sooner and reached 50% of needle loss at day 20 while high NRD clone got 50% of needle loss at day 48 after harvesting.

Table 5. 3 The main and interaction effects of clones (high NRD and low NRD) and day in post-harvest on enzyme activity.

Effect	P-value	
	Pectinase activity	**Cellulase activity**
Block	0.765	0.190
Clone	0.700	0.001
Day	0.253	0.057
Clone*Day	0.766	0.386

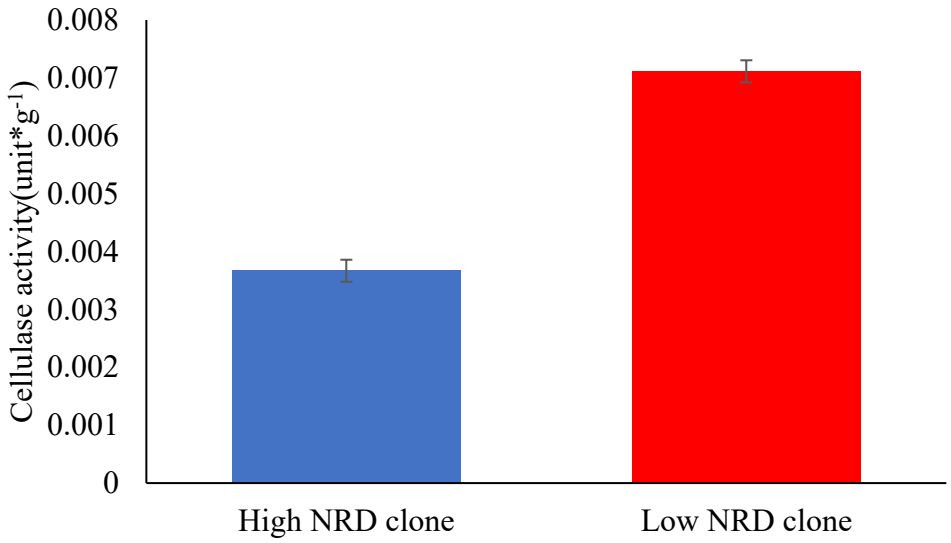

Figure 5. 5 The effect of genotypes on cellulase activity of needles in balsam fir.

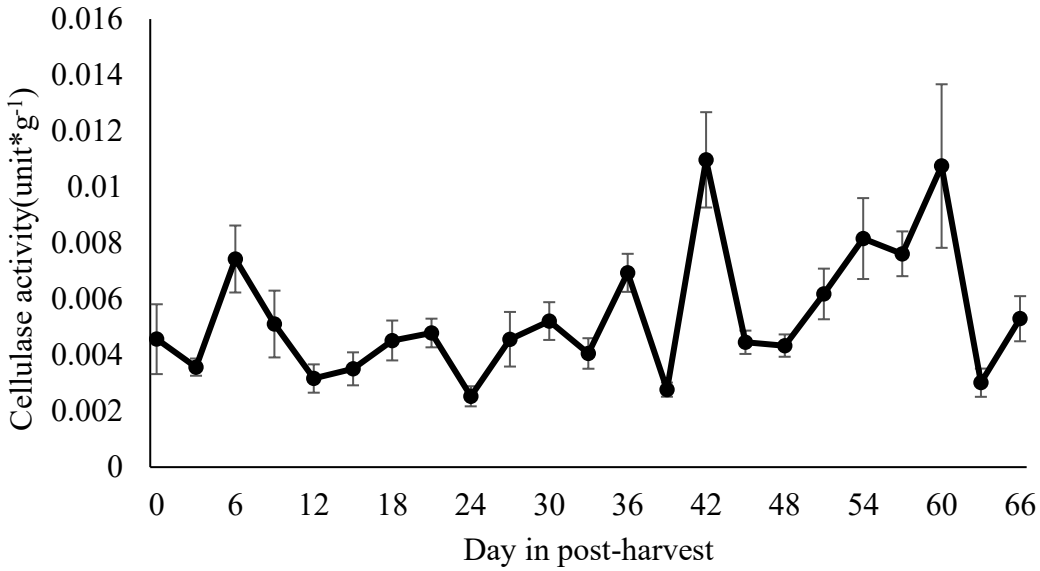

Figure 5. 6 The effect of days on cellulase activity of needles during post-harvest period in balsam fir

The pectinase activity was not significantly affected either time (Table 5.3) or genotype ($P > 0.05$). The cellulase activity, however was significantly affected by both genotype ($P < 0.05$) and time ($P = 0.05$). The cellulase activity of low NRD clone was significantly higher than that of high NRD clone (Figure 5.5), where low NRD clone had 47% more cellulase activity than high NRD clone. However, the interaction between clone and duration was not significant for cellulase.

Table 5. 4 The main and interaction effects of clones (high NRD and low NRD) and day in post-harvest on cumulative cellulase activity.

Effect	P-value
	Cumulative cellulase activity
Block	0.7211
Clone	0.0023
Day	<.0001
Clone*Day	<.0001

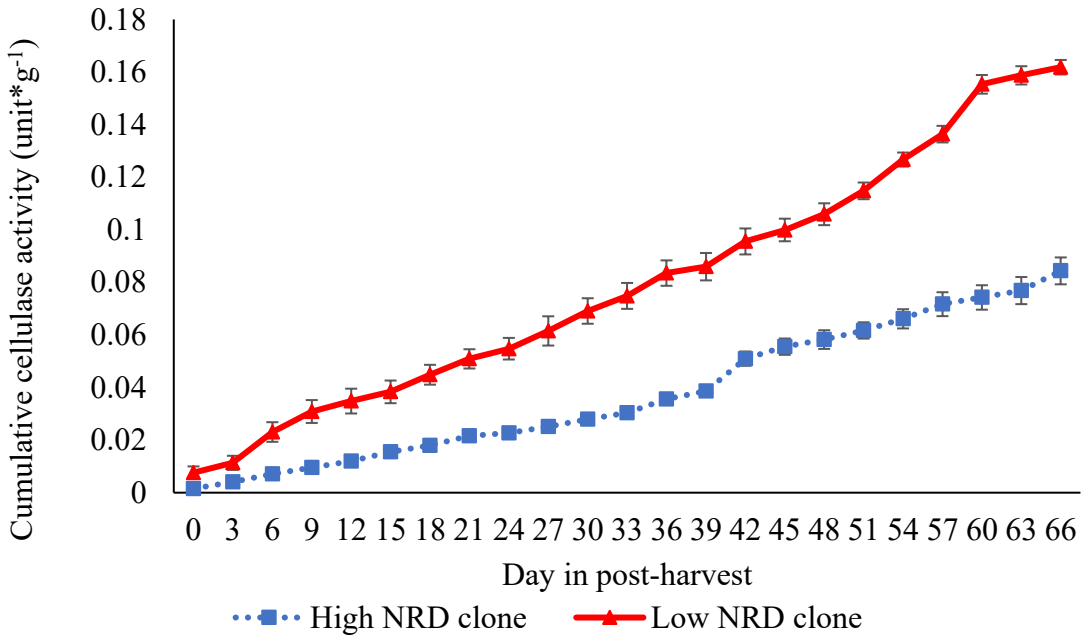

Figure 5. 7 Dynamics of genotype and time on cumulative cellulase activity of needles in balsam fir.

The cumulative cellulase activity was significantly affected by the interaction between genotypes and day ($P < 0.05$). Low NRD clone had higher cumulative cellulase activity than high NRD clone in the entire duration of the experiment increasing at a linear pace throughout the experimental time frame. From day 30 to day 66 after harvest, the cumulative cellulase activity of low NRD clone was significantly higher ($P < 0.05$) than that of high NRD clone.

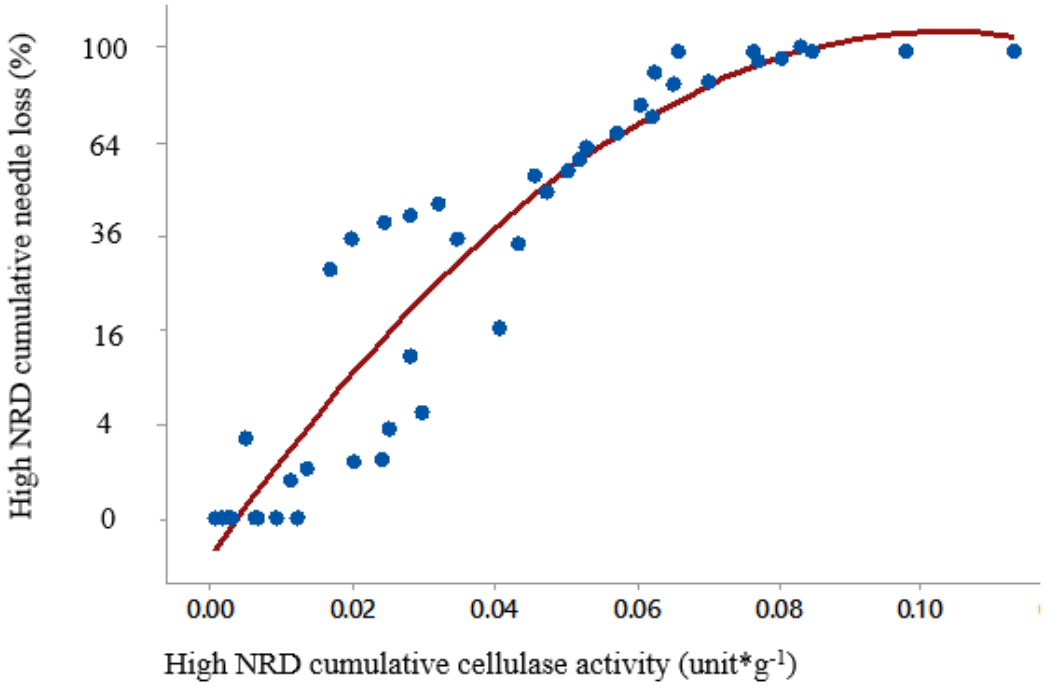

Figure 5. 8 A quadratic relationship between cumulative needle loss (%) and cumulative cellulase activity (unit*g^{-1}) for high NRD clone ($P < 0.05$).

The relationship between cumulative needle loss and cumulative cellulase activity in high NRD clone was significant, which showed a positive relationship with a high R^2 (R^2 = 0.885) suggesting that higher the cumulative cellulase activity higher the needle loss would be. This relationship also suggests that the accumulation was linear initially but at a higher cumulative activity of cellulase, the needle loss is not proportional indicating at higher concentration of cellulase the cellular response can be desensitised.

High NRD cumulative needle loss (%) $^{\wedge 0.5}$ = 0.7736 + 215.6 High NRD cumulative cellulase activity (unit/g) - 1047 High NAR cumulative cellulase activity$^{\wedge 2}$ (unit/g)

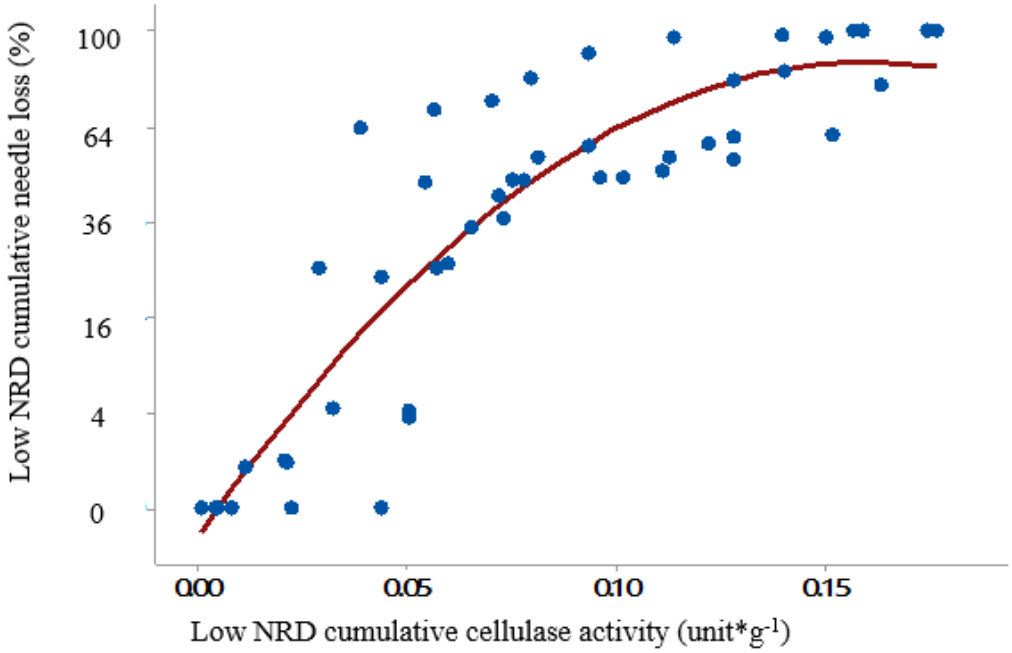

Figure 5. 9 A quadratic linear regression between cumulative needle loss (%) and cumulative cellulase activity (unit*g^{-1}) for low NRD clone ($P < 0.05$).

The relationship between cumulative needle loss and cumulative cellulase activity in low NRD clone was also significant, which also showed a positive relationship with a high R^2 (R^2 = 0.791) indicating again as the cellulase activity increased, post-harvest needle loss can increase progressively.

Low NRD cumulative needle loss (%)$^{\wedge 0.5}$ = 0.5867 + 124.7 Low NRD cumulative cellulase activity(unit/g) - 391.3 Low NRD cumulative cellulase activity$^{\wedge 2}$ (unit/g).

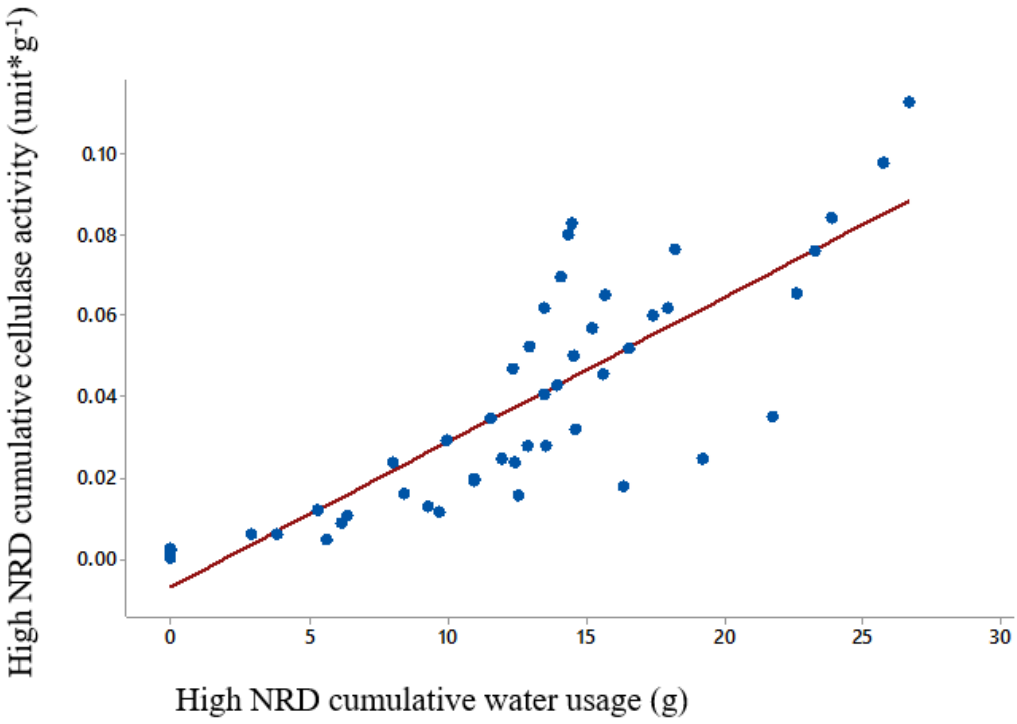

Figure 5. 10 A linear regression between cumulative cellulase activity (unit*g^{-1}) and cumulative water usage (g) on high NRD clone ($P < 0.05$).

The relationship between cumulative cellulase activity and cumulative water usage in high NRD clone was significant, which was also showing a positive relationship with a high R^2

(R^2 = 0.681). The relationship suggests that the cumulative water usage increased, the higher cumulative cellulase activity would be.

High NRD cumulative cellulase activity (unit*g^{-1}) = - 0.007120 + 0.003584 high NRD cumulative water usage (g).

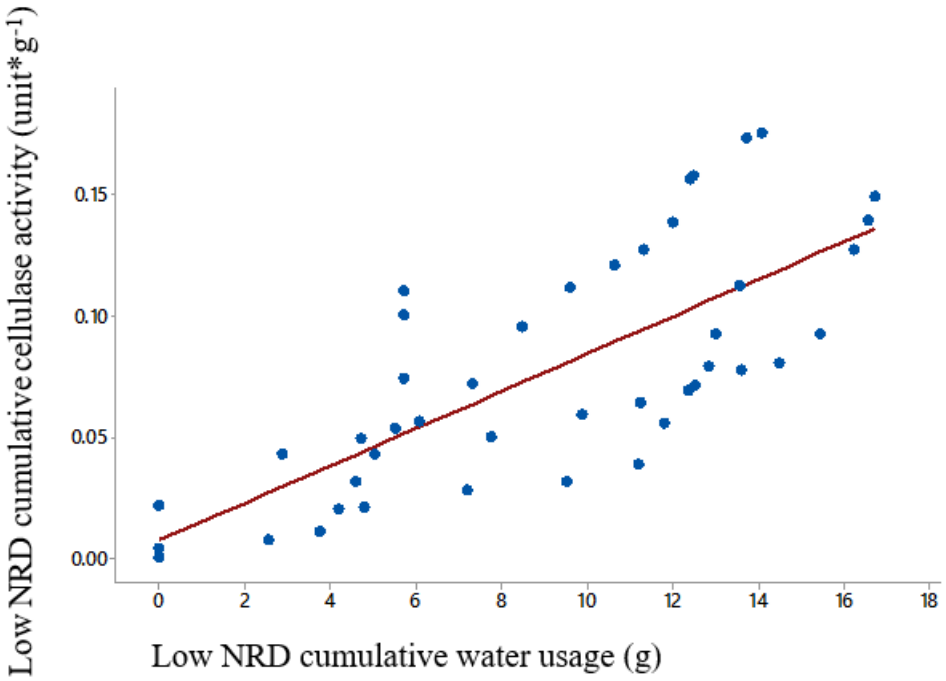

Figure 5. 11 A linear regression between cumulative cellulase activity (unit*g^{-1}) and cumulative water usage (g) on low NRD clone ($P < 0.05$).

The relationship between cumulative cellulase activity and cumulative water usage in low NRD clone was significant, which was also showing a positive relationship with a high R^2 (R^2 = 0.569) indicating again as the water consumption increased, cellulase activity can increase linearly.

Low NRD cumulative cellulase activity (unit*g^{-1}) = 0.00733 + 0.007713 low NRD cumulative water usage (g).

The regression analysis of cumulative water usage and cumulative cellulase activity suggests that the cumulative water usage of low NRD clone was less than high NRD clone's and the increase rate of cumulative cellulase activity was also higher than that of high NRD clone. It may suggest that higher cumulative water usage is related to low cumulative cellulase activity.

5.5 Discussion

Dynamics of average water use was significantly related to time with a decline in average water usage. Harvested branches consumed more water during the initial days and then decreased with time when needle abscission begins. This could possibly be due to stomata closure of needles during the post-harvest period or decreasing of surface area of evaporation due to needle loss. After harvesting, branches lost the positive water pressure, which was provided by the root system. This could have caused the stomata to close to reduce water loss by transpiration to prevent dehydration. The stomatal closure was related to the plant hormone, abscisic acid (ABA) and one of the important roles is to regulate stomatal aperture (MacRobbie, 1995). Due to the fact that the average water usage for both clones were higher initially and then declined later in this experiment, it is also possible that there may be xylem blockage after harvest or some microbial growth at the cut end of branches, which can cause less water uptake (Van Doorn and Cruz, 2000; Van Ieperen et al., 2002).

While there were no significant differences in average water usage between high and low NRD genotypes, the cumulative water usage of high NRD clone was higher than low NRD clone. As post-harvest dehydration leads to needle abscission in balsam fir, this suggests a possible link between needle retention and cumulative water usage that low cumulative water usage can be associated with poor needle retention.

While the average water usage was not affected by genotypes, the results of needle loss and cumulative needle loss were both significantly affected the interaction between the genotypes and time. The low NRD clone lost approximately 60% needles during the first 26 days and it was 3-fold than higher that of high NRD clone (20%). This result depicted their expected needle retaining ability, that low NRD clone abscised needles sooner and reached 50% needle loss quicker than high NRD clone.

The results of hydrolytic enzymes' activity indicated that pectinase was not linked to needle abscission of balsam fir in this study. The role of pectinase in abscission was not clear yet. Pectinase was reported to have a correlation with citrus fruit abscission (Greenburg et.al, 1975) and citrus leaf explants (Riov, 1974). While Berger and Reis (1979) reported that no increase in pectinase activity in bean prior to leaf abscission and an increase after flower abscission in tomato (Tucker et al., 1984). The result of this experiment suggests that pectinase is not a key factor in post-harvest needle abscission in balsam fir. However, cellulase activity in needles of low NRD clone was significantly higher than that of high NRD clone. The initial cellulase activity in needle of low NRD clone was approximately 4-fold higher than that in needle of high NRD clone. This suggests that low cellulase activity is associated with better needle retention. Interestingly, cellulase activity in needles of low NRD branches induced abscission increased by approximately 0.015 unit/g (300%)

compared to initial, while it increased by approximately 0.007 unit/g (450%) in needles of high NRD branches to induce abscission. It suggests that low NRD clone need to accumulate a higher amount of cellulase activity to induce needle loss and it need less time to accumulate same amount of cellulase activity compared to high NRD clone. It also suggests that high NRD clone is more sensitive to cellulase that high NRD clone abscised more needles than low NRD clone at the same level of cellulase activity. The cumulative water usage was found to have a relationship with cumulative cellulase activity for both clones that the cumulative water usage of low NRD clone was lower than high NRD clone while the cumulative cellulase activity of low NRD clone was higher than high NRD clone. The cumulative cellulase activity increased to 0.152 unit/g in low NRD clone during experimental period, while the cumulative cellulase activity increased to 0.082 unit/g in high NRD clone. The increase rate of cumulative cellulase activity of low NRD clone was higher than that of high NRD clone. It suggests that higher cumulative water usage is related to lower increase rate of cellulase activity. The cumulative cellulase activity was also found to have a strong relationship with the cumulative needle loss for both genotypes in this study. The total amount of cellulase activity in low NRD clone was approximately 2-fold higher compared to high NRD clone. This could possibly explain the link between cumulative needle loss, cumulative water usage and cumulative cellulase activity. Low cumulative water usage may be associated with high needle loss and high cellulase activity. The important role of cellulase in organ abscission is to degrade cellulose and reduce the strength of cell wall to allow abscission (Sexton et al, 1985). It has been reported that cellulase involved organ abscission in many species, such as bean leaf abscission (Durbin

et al., 1981), red raspberry fruit abscission (Sexton et al, 1997) and tomato fruit abscission (Iwai et al, 2013), and now including balsam fir.

5.6 Conclusion

Reducing sugar method was able to detect pectinase and cellulase activity from needle of balsam fir. The results of this study suggest that pectinase is not a signal for post-harvest needle abscission in balsam fir. The cellulase activity of balsam fir may be the trigger for needle loss. Low NRD clone had more cellulase activity to trigger needle loss than the high NRD clone. High cellulase activity was related to low water consumption and high needle loss in this study. Cellulase inhibitor will be applied to confirm the effects of needle abscission in balsam fir. It is expected that when cellulase activity is inhibited post-harvest needle abscission could be delayed. Investigating the effect of inhibitors on cellulase activity may help to further understand the effect of cellulase and post-harvest needle abscission.

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CHAPTER 6 THE EFFECT OF CELLULASE INHIBITORS ON POST-HARVEST NEEDLE ABSCISSION IN BALSAM FIR

6.1 Abstract

Post-harvest needle abscission is a major problem for balsam fir Christmas trees. In our recent study, it was found that the activity of cell wall degrading enzyme, cellulase is associated with the post-harvest needle abscission in balsam fir. It was hypothesized that reducing cellulase activity will delay needle abscission. An experiment was conducted to evaluate the effects of various cellulase inhibitors on needle abscission during post-harvest period. A total of 198 branches were collected from a low NRD genotype with 3 replicates. The treatments included two inhibitors and days in post-harvest. Water was supplied to branches during a 36-day period. Water usage and needle loss were recorded every 3 days. Water usage decreased ($P < 0.05$) in post-harvest. The cumulative needle loss percentage was significantly affected by the interaction between treatment and time ($P < 0.05$). Needle retention significantly increased when branches treated with the ethylene receptor blocker, 1-methylcyclopropene (1- MCP). Needle loss however increased when branches are treated with salicylic acid compared to branches provided with water. Cellulase (EC 3.2.1.4) activity in the needle was evaluated in this study. Branches treated with 2.5g 1-MCP significantly reduced cellulase activity and delayed post-harvest needle loss compared to control.

6.2 Introduction

Post-harvest needle abscission is a major challenge in the Christmas tree industry and it is complex phenomenon triggered by various internal and external factors including genetic variations (MacDonald et al., 2014), harvest date (MacDonald and Lada, 2008), post-harvest handling (Lada and Adams, 2009) and dehydration (Thiagarajan et al., 2013). In root-detached branches, the water pressure provided by the root system is disappeared. Limited water uptake can increase ethylene concentration to trigger needle abscission (Addicott, 1982). Cellulase is an ethylene- sensitivity enzyme and it is believed that ethylene can increase cellulase activity to accelerate abscission in balsam fir (MacDonald et al., 2011). Cellulase was involved in organ abscission in many species, including bean leaf abscission (Durbin et al., 1981), red raspberry fruit abscission (Sexton et al., 1997), and tomato fruit abscission (Iwai et al., 2013).

The experiment in Chapter 5 revealed that pectinase (E.C. 3.2.1.15) is not related to the post-harvest needle abscission, while cellulase was directly involved in promoting needle abscission. It was found that low NRD clone had higher cellulase activity than high NRD clone (Chapter 5). Cellulase activity in low NRD clone increased earlier, which could be a possible reason why low NRD clone had a poor needle retention (Chapter 5). In addition, cellulase activity in both clones increased prior to the peak of needle loss, which could be a possible triggering signal for post-harvest needle abscission.

If cellulase is a possible signal or a trigger for post-harvest needle abscission in balsam fir, then inhibiting cellulase activity should delay or defer abscission. As cellulase is linked to ethylene synthesis and ethylene is implicated in post-harvest needle abscission (MacDonald et al., 2011), inhibiting ethylene using ethylene synthesis inhibitors or

blocking ethylene receptors can be used to reduce cellulase activity. The ethylene inhibitors: 1-methylcyclopropene (1-MCP) and salicylic acid, both have been shown to reduce ethylene synthesis and cellulase activity. 1-methylcyclopropene (1-MCP) has been shown to reduce ethylene production by competitively binding to the ethylene receptors (MacDonald et al., 2010). MacDonald et al. (2010) reported that ethylene production was reduced when branches treated with 1-MCP and balsam fir needle retention increased 147%.

Salicylic acid is a phenolic compound, which can inhibit ethylene production by blocking the conversion of 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene (Leslie and Romani, 1986). Salicylic acid has also been shown to decrease hydrolytic enzymes in many species, such as pear (Leslie and Romani, 1986), banana (Srivastava and Dwivedi, 2000), peach and pepper (Ferrarese et al., 1996).

The objective of this experiment was to investigate the effect of various concentrations of 1-MCP and salicylic acid on cellulase activity and needle abscission in balsam fir.

6.3 Materials and method

6.3.1 Sample collection and preparation

A low NRD (clone #1) was selected in this experiment. One hundred and ninety-eight branches of two-year-old growth from four trees were collected from Orchard at Debert, NS (45° 25' N, 63° 28' W) on November 8, 2016. After cutting, branches were immediately placed in a container with reverse osmosis (RO) water to transport to the laboratory.

All freshly cut branches were initially weight and then placed in a 150 mL amber bottle with approximately 100 mL RO water. All bottles were sealed with cotton gauze to prevent evaporation. Initial measurements were taken for the total weight of the entire apparatus as described in Chapter 3.3.

6.3.2 Experimental design

The experiment followed a completely randomized design. The experiment consisted of two enzyme inhibitors (1-MCP and salicylic acid) with 3 concentration levels and each treatment had three replicates. Several response variables, including water usage and needle loss, were measured every three days from 9 separate branches for the entire duration of the experiment. Cellulase activity was analyzed on day 0, 6, 12, 18, 21, 24, 27, 30, 33, 36 post-harvest on a separate set of 180 branches. Nine branches were randomly selected on each sampling day and all needles were collected and immediately frozen in liquid nitrogen, then stored at -80 ºC for cellulase activity analysis. After sampling, these branches were discarded from this experiment.

6.3.3 Treatment preparation

1-MCP and salicylic acid (Sigma-Aldrich, Oakvile, ON, Canada) were used in this experiment. The 1-MCP treatment was applied by dissolving a known mass of EthylBloc ^{TM} (Florlife Inc., Walterboro, SC, USA) in 500 mL RO water. 1-MCP is a gas and it is produced when EthylBloc ^{TM} is dissolved in water. In this experiment, 0, 2.5 and 7.5g mass of EthylBloc ^{TM} were used. Each EthylBloc ^{TM} solution was placed in a 4L glass jar with 3 branches for a 24-hour exposure period. After 24 hours, solutions were discarded from this experiment.

There were three concentration levels of salicylic acid in this experiment: 0, 0.5 and 1.0 mM. Salicylic acid was dissolved in the water supplied to branches and branches were provided a salicylic acid solution for the entire duration of the experiment.

A  B 

Figure 6. 1 A: 4L jar used for pre-exposing 1-MCP. B: 3 branches were pre-treated 1-MCP for 24 hours.

6.3.4 Average water use (AWU) and cumulative water use

Measurements of water consumption were taken every three days using methods described in Section 3.4.1, average water usage and cumulative water usage were measured and calculated using formulas from Section 3.4.1 and 3.4.2.

6.3.5 Needle loss percentage and cumulative needle loss percentage

Measurements of needle loss were taken every three days using finger run test; needle loss percentage and cumulative needle loss percentage were measured and calculated using formula from Section 3.4.3 and 3.4.4.

6.3.6 Enzyme extraction and analysis of enzyme activity

Enzyme extraction was performed using the method described in section 3.4.5. Cellulase activity was measured by using the method described in section 3.4.6.

6.3.7 Statistical analysis

Data were analyzed using analysis of variance (ANOVA) with repeated measure in statistical analysis system (SAS) 9.4 (SAS Institute, NC, USA). Data were transformed to a normal distribution. If main effects or interaction effects were found to be significant ($P \leq 0.05$), then Tukey-Kramer test was used to differentiate the means at $\alpha = 0.05$ (Gbur et al., 2012).

The relationship between cumulative needle loss and cumulative enzyme activity was analyzed using linear regression analysis in Minitab 17 (Minitab 17, Minitab Inc., PA, USA).

6.4 Results

The average water usage was significantly affected by the interaction between treatment and time (Table 6.1). The average water usage of all treatments, took a sharp decline initially until day 9 or 15, then showed an increase with a small peak on day 24 or 27 followed by a decline until the end of the experiment. 7.5 g MCP treated branches had the lowest AWU compared to the control. The trends in all treatments were similar with a dramatic decline initially until day 9 and remaining low until the end of the experiment. The treatment with 2.5g MCP treatment retained high AWU until day 12 compared to other treatments.

Table 6. 1 The main and interaction effects of treatments and day in post-harvest on average water usage and cumulative water usage.

Effect	P-value	
	Average water usage	**Cumulative water usage**
Treatment	0.351	0.243
Day	<.0001	<0.0001
Treatment*Day	0.001	0.147

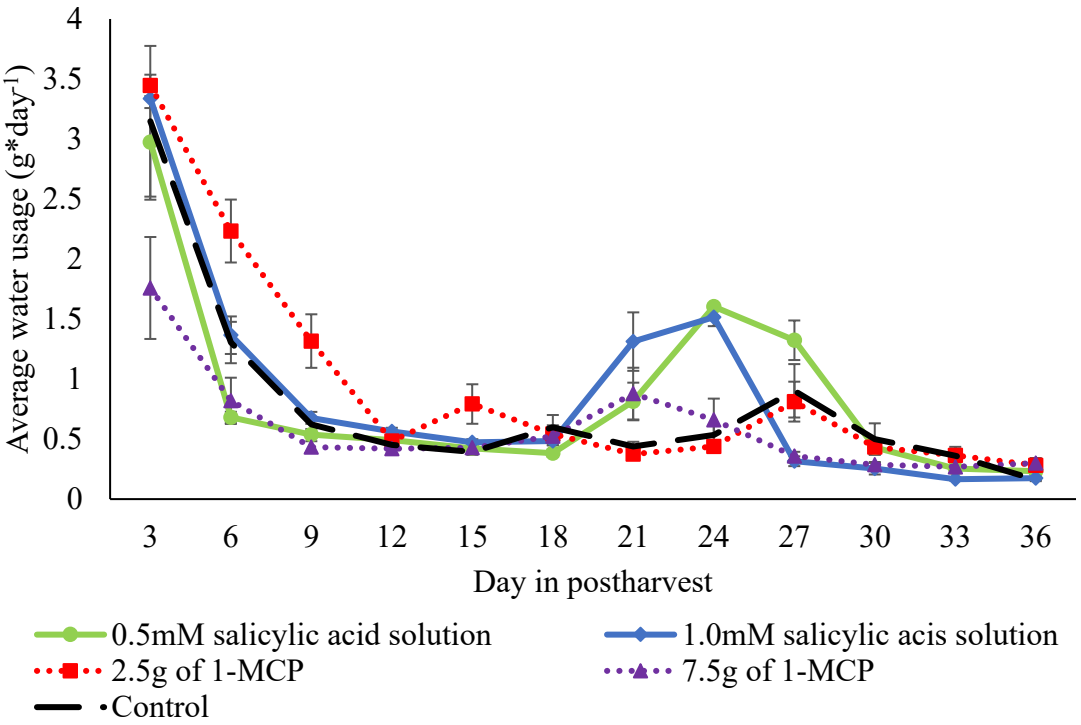

Figure 6. 2 Dynamics of average water usage of branches over a 36-day experimental period in balsam fir.

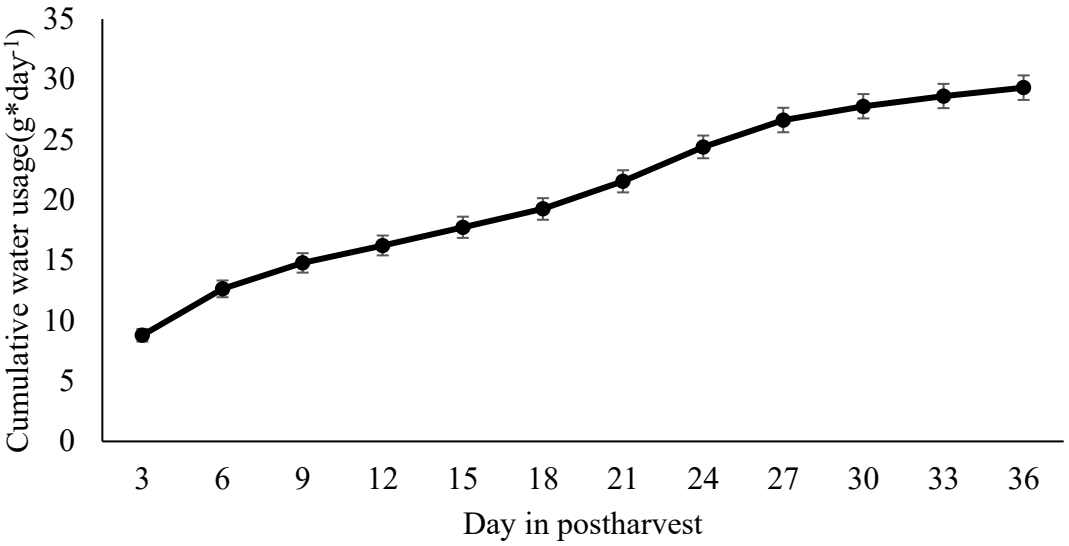

Figure 6. 3 Dynamics of cumulative water use of branches over a 36-day experimental period in balsam fir.

The interaction between treatment and days for cumulative water use was not significant statistically. However, the cumulative water usage was significantly affected by time (Table 6.1). The cumulative water usage showed an increase within the first 24 days following harvest, with a slower increase until the end of the experiment (Figure 6.3). This suggests that water uptake in all treatments combined progressed by rather slowly.

Table 6. 2 The main and interaction effects of treatments and day in post-harvest on needle loss% and cumulative needle loss%.

Effect	P-value	
	Needle loss%	**Cumulative needle loss%**
Treatment	0.564	0.019
Day	<.0001	<.0001
Treatment*Day	0.0002	0.022

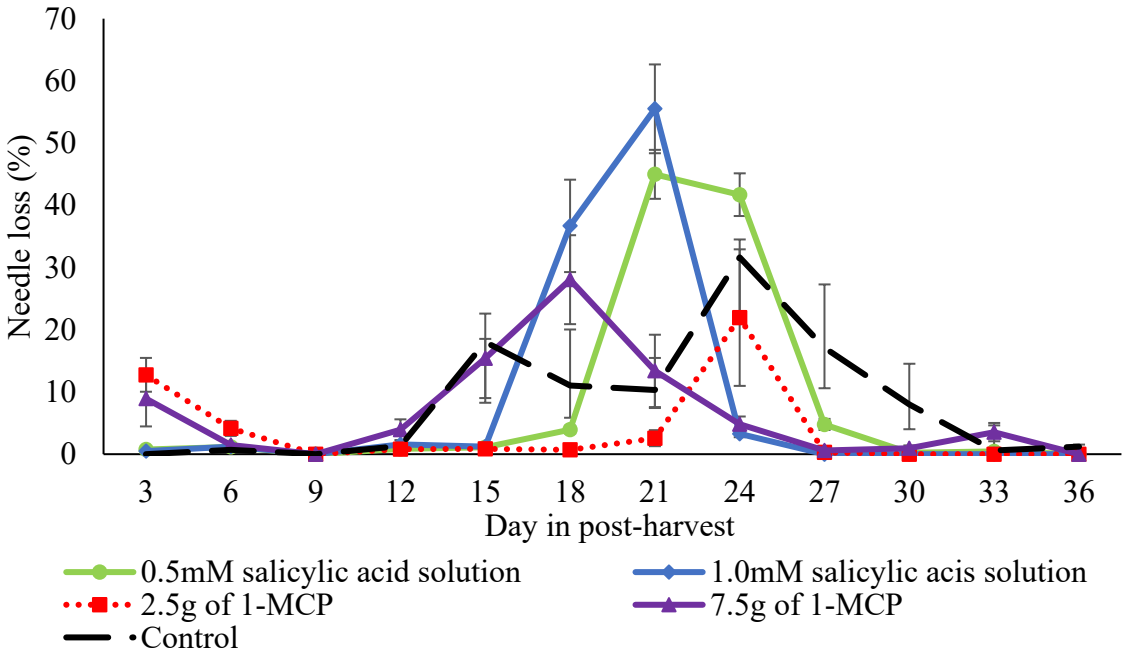

Figure 6. 4 Dynamics of needle loss of branches over a 36-day experimental period in balsam fir.

The interaction between treatment and time for needle loss was significant (P-Value < 0.05).

The pattern of needle loss differed among the treatments. The control branches lost most

needles at days 15 and 24, while other branches got most needle loss after 18 days. Branches treated with salicylic acid at both concentrations lost their needles faster than the branches treated with 2.5g of 1-MCP. Needle loss commenced on day 12 in control branches compared to day 21 in 2.5g 1-MCP treated branches. Overall, 2.5g 1-MCP treatment was most effective in delaying and reducing needle loss compared to other treatments. There was nearly 10-day advantage due to 1-MCP treatment compared to the control.

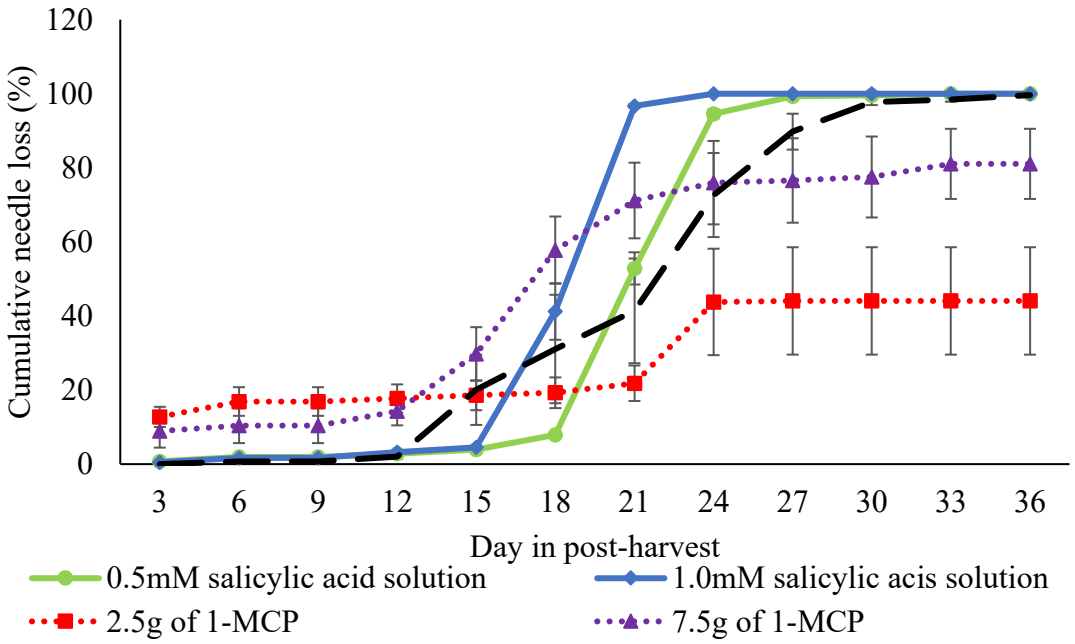

Figure 6. 5 The interaction effect of treatments and time on cumulative needle loss of branches over a 36-day experimental period in balsam fir.

The cumulative needle loss was significantly affected by the interaction between treatments and time (Table 6.2). The cumulative needle loss of all treatments followed a similar

pattern (Figure 6.5). All patterns of treatments showed a slower increase followed a sharp increase and then leveling off at the end.

Branches treated with salicylic acid solution got the 100% needle loss sooner than control, while branches treated with 2.5g 1-MCP abscised lost only 20% needles when all needles were lost under salicylic acid. Overall, there was a significant reduction in needle loss in branches treated with 2.5g 1-MCP. There was no further increase in needle drop beyond 40% when the control branches lost all its needles on day 30.

Table 6. 3 The main and interaction effects of treatments and day in post-harvest on cellulase activity and cumulative cellulase activity.

Effect	**P-value**	
	Cellulase activity	**Cumulative cellulase activity**
Treatment	0.098	0.135
Day	0.002	<0.0001
Treatment*Day	0.837	0.988

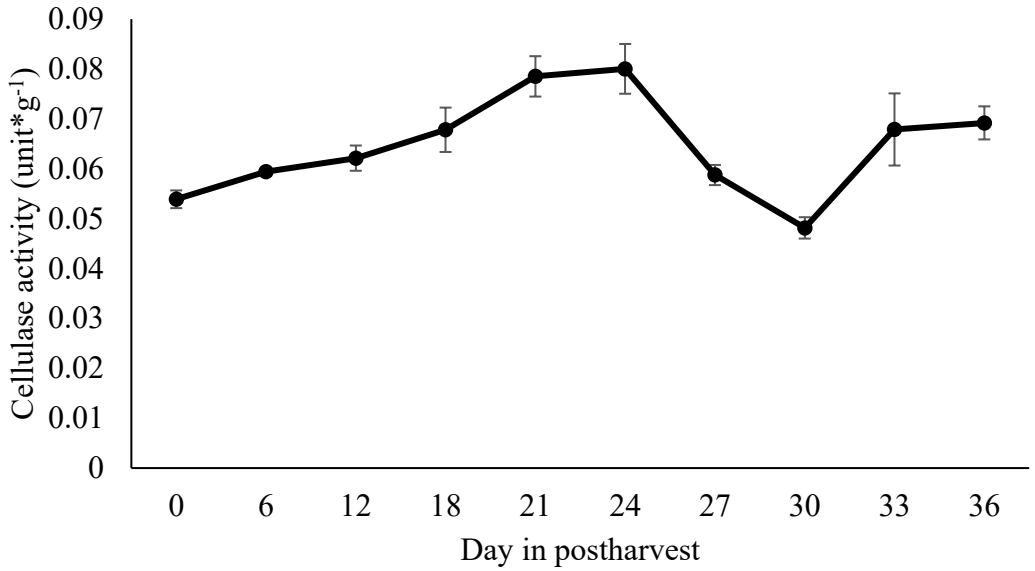

Figure 6. 6 The effect of days on cellulase activity of needle loss during post-harvest period in balsam fir ($P < 0.05$).

Figure 6. 7 The effect of days on cumulative cellulase activity of needle loss during post-harvest period in balsam fir ($P < 0.05$).

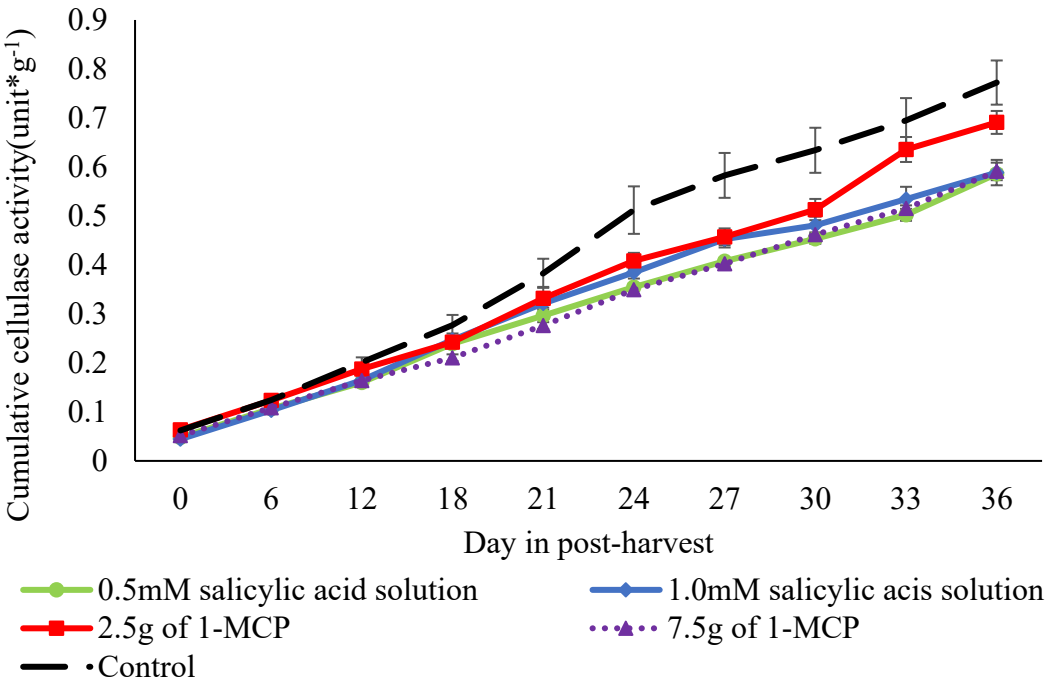

Figure 6. 8 Dynamics of cumulative cellulase activity of branches over a 36-day experimental period in balsam fir ($P > 0.05$).

There was no significant interaction between treatments and days for cellulase activity for the entire duration of the experiment. Also, there was no significant differences between treatments in cellulase activity of needles (Figure 6.6). However, the main effects showed a significant increase in cellulase activity starting day 6 progressing gradually until day 24, declining thereafter until day 30 and increasing until the end of the experiment.

The interaction between treatments and days was not significant for the cumulative cellulase activity as well. The cumulative cellulase activity increased significantly and linearly with days in post-harvest (Figure 6.7). Although, there was no statistically significant increase with various treatment over days, the cumulative cellulase activity for all treatments showed an increasing trend during the post-harvest period (Figure 6.8).

Table 6. 4 The main and interaction effects of treatments (control and 2.5g of 1-MCP) and day in post-harvest on cumulative needle loss% and cumulative cellulase activity.

Effect	P-value	
	Cumulative needle loss%	**Cumulative cellulase activity**
Treatment	0.6965	0.022
Day	<0.0001	<0.0001
Treatment*Day	0.0002	0.941

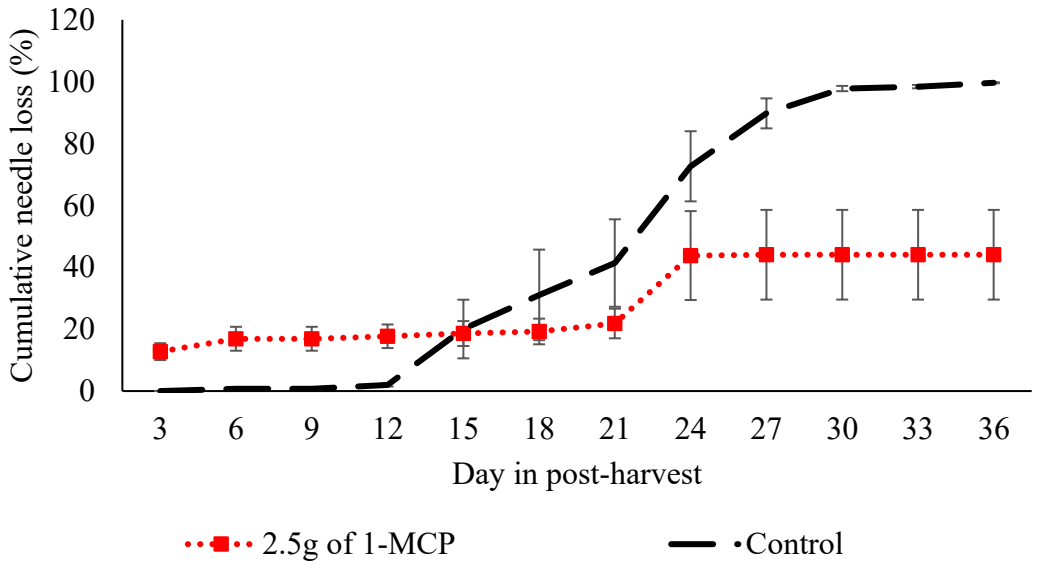

Figure 6. 9 The interaction effect of treatment and time on cumulative needle loss (%) of branches in balsam fir.

Since there was a distinct difference in the cellulase concentration between control and 2.5g 1-MCP treatment, a contrast analysis was done. The contrast analysis showed that

cumulative needle loss was significantly affected by the interaction between treatment (control and 2.5g 1-MCP) and day in post-harvest (Table 6.4). The needle loss percentage versus time was graphed for both treatments (control and 2.5g of 1-MCP) to show their patterns of needle abscission (Figure 6.9). Branches treated with 2.5g of 1-MCP started to abscise needles at day 3 after harvest following a leveling off until day 21. Branches treated with 2.5g of 1-MCP got a sharp increase from day 24 to day 27 after harvest then leveling off until the conclusion of the experiment. However, branches in control group started to abscise needles at day 12 and almost lost 100% of needles on day 30.

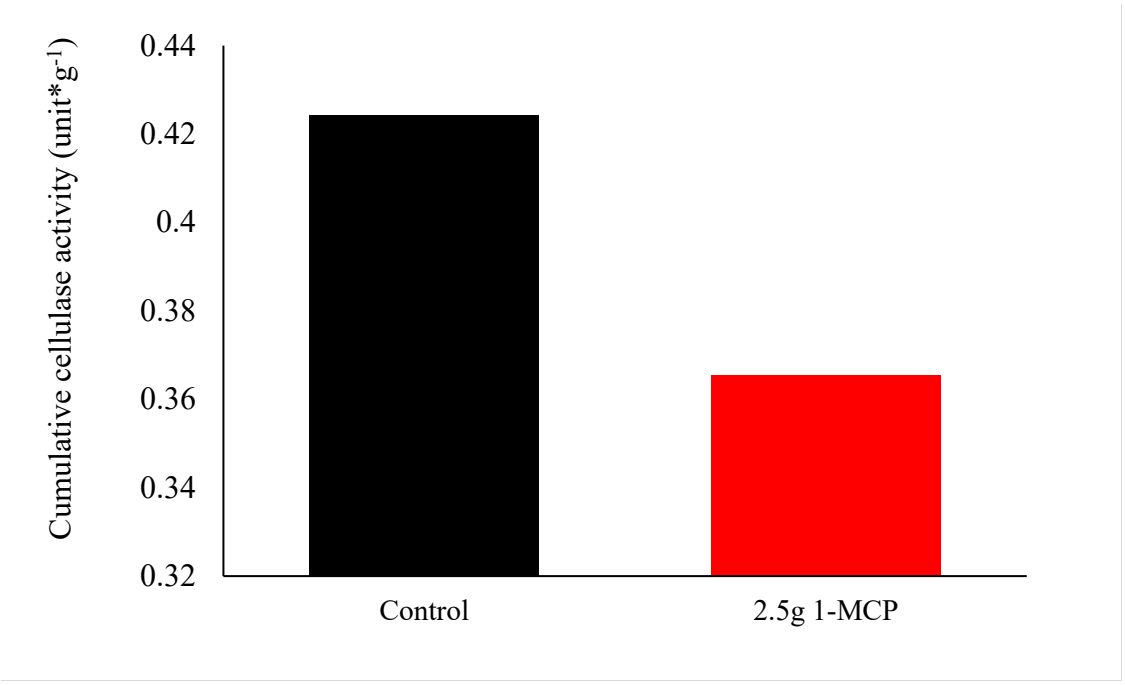

Figure 6. 10 The effect of treatment on cumulative cellulase activity in balsam fir.

The contrast analysis showed that the cumulative cellulase activity of control group was significantly higher than that of branches treated with 2.5g of 1-MCP (Figure 6.10). The

branches treated with 2.5g 1-MCP had a 15% reduction in cellulase content compared to the control.

6.5 Discussion

When comparing needle abscission trends for each level of the treatments, branches treated with 1-MCP were found to lose needles at a slower rate than branches treated with water. Branches treated with 1-MCP -were also found to have lower cumulative needle loss at the end of the experiment and branches treated with 2.5g of 1-MCP had lower cumulative needle loss than branches treated with 7.5g of 1-MCP. The contrast analysis between control and 2.5g 1-MCP showed that cellulase in needle of control treatment was significantly higher than that of branches treated with 2.5g 1-MCP confirming that reducing cellulase activity will reduce post-harvest needle loss. Branches treated with 1-MCP had approximately 10% needle loss on day 3 after the branch was cut. It was possibly caused by an external factor during preparation. Branches were pre-exposed to with 1-MCP for 24 hours in 4L jars in this experiment. Branches may get some physical force when they were placed in or taken out jars, which might have caused some needle loss in the early stage after harvest.

Cellulase has been shown to be regulated by ethylene. Ethylene plays a role on the synthesis of cellulase and control the secretion or release of cellulase to the cell wall (Abeles and Leather, 1971). The ethylene receptor block, 1-MCP has been reported to compete with ethylene for binding to ethylene receptor to reduce ethylene production (Sisler and Wood,

1988). In this study, 1-MCP reduced cumulative cellulase activity and thus, delayed needle abscission.

While 1-MCP reduced needle loss, salicylic acid (SA) did not. On the contrary, SA hastened needle loss compared to the control. Salicylic acid was reported to have a positive effect on improving plant growth and productivity (Arberg, 1981) and reduced cellulase activity in many species including pear (Leslie and Romani, 1986), banana (Srivastava and Dwivedi, 2000), peach and pepper (Ferrarese et al., 1996). Ferrarese et al. (1996) reported that 1 mM exogenous salicylic acid decreased the cellulase activity and reduced leaf abscission in both peach and pepper plants. Although salicylic acid was reported to inhibit ethylene biosynthesis and reduce cellulase activity in many studies, the recommended concentration of salicylic acid and their inhibitory effects were variable among species. Roustan et al. (1989) found the maximum inhibitory concentration of salicylic acid in carrot cell is 100 μM, while Nissen (1994) found that 1 mM salicylic acid inhibited ethylene production. However, Srivastava and Dwivedi (2000) observed an inhibition of ethylene at a higher salicylic acid concentration (> 10^{-4} M). Fariduddin et al. (2003) reported that low concentration (10^{-5} M) of exogenous salicylic acid increased day mass production in *Brassica juncea*, and enhanced plant growth. While high concentration (10^{-3} M) showed a negative effect on plant growth, which causes a permanent change at the level of membrane organization of the cells. Hinesley and Blankenship (1991) have also reported that aspirin (acetyl salicylic acid) increased needle loss post-harvest compared to distilled water in Fraser fir. In this experiment, salicylic acid also found to reduced cellulase activity in this study while it did not delay needle abscission in balsam fir. It is possible that the concentration of salicylic acid used in this study may be too high thus negatively

affecting needle retention. Salicylic acid may have negatively affected post-harvest needle retention possibly through a non-enzymatic process.

The results of this experiment showed a possible link between needle loss, cellulase activity and inhibitors. While 1-MCP reduced cellulase activity and needle loss in balsam fir.

6.6 Conclusion

In this study, pre-treated branches with an inhibitor, 2.5g of 1-MCP, delayed needle abscission by significantly decreasing cellulase activity. However, salicylic acid promoted needle loss faster, despite lower cellulase activity suggesting a direct effect of salicylic acid in promoting needle abscission. The results of this experiment suggest that reducing cellulase activity may delay needle abscission. It is necessary to confirm the specific role of salicylic acid in post-harvest balsam fir and to establish the link between salicylic acid, cellulase activity and needle loss. A future study to investigate the different concentration of salicylic acid may help to further understand the relationship between cellulase activity on post-harvest needle abscission.

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CHAPTER 7 CONCLUSION

7.1 General discussion

Until recently, post-harvest needle abscission in balsam fir still is a major threat to the Christmas tree industry. It was understood that post-harvest needle abscission can be triggered by ethylene and ethylene increases needle cellulase activity promoting abscission (MacDonald et al., 2011). However, little was known about the nature of hydrolytic enzymes, dynamics of accumulation and the relationship between the hydrolytic enzymes and genotypes sensitivity to post-harvest needle abscission in balsam fir. It was known that cellulase and pectinase have an effect on organ abscission in bean (Durbin et al., 1981), red raspberry (Sexton et al, 1997), tomato (Iwai et al, 2013) and citrus (Riov, 1974). Thus, it was hypothesized that hydrolytic enzymes in needle may have a role in post-harvest needle abscission of balsam fir.

Before harvest, abscission is a beneficial ecological process to keep balance on the plant vegetative parts and defend infections by natural enemies and pathogens under natural condition. After harvest, abscission can be triggered by rough handling (Lada and Adams, 2009), dehydration (Thiagarajan et al., 2013), ethylene (MacDonald et al., 2011) or by volatile terpene compounds (Korankye, 2013). After cutting, branches placed in water had a better needle retention compared to branches without water. Water deficit is an important issue in post-harvest needle abscission and branches supplied with water can reduce the risk of dehydration. Freshly cut branches consumed more water at the beginning, approximately 1g* day^{-1} and rapidly decreased after harvest. As shown in Chapter 5, daily water usage experienced a decline over time in post-harvest. Cumulative water consumption of high NRD clone was higher than low NRD clone, which suggests that high

water consumption is related to better needle retention. Lower water uptake could lead to dehydration, possibly triggering cellulase accumulation and promoting needle loss. Dehydration can increase ethylene concentration (Addicott, 1982) and cellulase was reported to be regulated by ethylene (Abeles and Leather, 197). However, pectinase in needles did not affect needle loss in this experiment (Chapter 5). The role of pectinase in plant abscission is varied. Greenburg et.al. (1975) reported that pectinase had a correlation with fruit abscission in citrus. Berger and Reid (1979) found that polygalacturonase was not related to leaf abscission in bean. In this study, no correlation between pectinase activity and balsam fir post-harvest needle abscission was observed. While cellulase had a significant effect on needle abscission in both clones, and a positive relationship was found between cumulative cellulase activity and needle loss. High cumulative cellulase activity suggests a high needle loss. However, high NRD clone was detected to have lower cumulative cellulase activity over a 76-day experimental period, which was approximately 2-fold less than low NRD clone. Also, accumulation of cellulase was slower in high NRD clone than the low NRD clone. There is a possible link between water usage, cellulase activity and needle abscission. The high NRD clone is more sensitive to cellulase and the mechanism is through delayed accumulation and the low concentration of cellulase in high NRD clone.

The experiment involving cellulase inhibitor, 1-MCP and salicylic acid attempted to delay needle abscission by reducing cellulase activity (Chapter 6). Branches treated with 2.5g of 1-MCP delayed the needle abscission and decreased needle loss. Untreated branches (control) started needle abscission at day 12 and following a sharp increase in needle loss until day 30 after cutting, while branches treated with 2.5g of 1-MCP only abscised

approximately 44% needles at the end of this experiment. The cumulative cellulase activity of untreated branches was 1.2-fold higher than that of branches treated with 2.5g of 1-MCP. 1- MCP is an ethylene receptor blocker which was reported to prevent ethylene action and enhance needle retention in balsam fir (MacDonald, 2010). In this study, cellulase activity reduced when ethylene production decreasing. However, branches treated with salicylic acid promoted needle loss in this study. The recommended concentration of salicylic acid for different species is highly variable and ≥ 0.5 mM salicylic acid showed a negative effect on needle abscission in this study. The benefit of applying inhibitors can be seen in branches treated with 1-MCP and 2.5g 1-MCP had most effective on improving needle retention.

Overall, water uptake of branches would decrease after harvest which would increase ethylene synthesis. Although ethylene was not measured in this study, high concentration of ethylene also is known to increase cellulase activity which would have triggered needle loss. Based on the results, the cumulative water usage of low NRD clone is less than that of high NRD clone. It maybe a reason why low NRD clone accumulated cellulase sooner and more than the high NRD clone. 1-MCP is an ethylene receptor blocker, which would have reduced cellulase activity by decreasing ethylene action. High salicylic acid (≥ 0.5 mM) also reduced cellulase activity but negatively impacted needle retention post-harvest. It suggests that salicylic acid affect post-harvest needle abscission by non-enzymatic process.

7.2 General conclusion

This study has investigated the relationship between cellulase activity and needle loss. Clearly, increasing cellulase increased postharvest needle loss, and it may have a significant role in triggering and promoting post-harvest needle abscission. High NRD clone is more sensitive to cellulase post-harvest but it is slow in accumulation of cellulase. There is also some evidence of a link between water consumption, cellulase activity and needle loss. Low water consumption increased cellulase activity, promoting needle loss. It is also confirmed that 2.5g of 1-MCP delays needle abscission, lowering cellulase activity while ≥ 0.5 mM salicylic acid would promote needle loss in balsam fir.

7.3 Future study

More future research is recommended to understand their effects of other hydrolytic enzymes on postharvest needle abscission. More knowledge is needed on cell wall change of abscission zone, which may explain why high NRD clone is more sensitive to cellulase. Results from this study suggest that salicylic acid had a negative effect on needle loss. This brings several questions about the concentration of salicylic acid - What is the recommended concentration of salicylic acid for balsam fir? Can low level of salicylic acid reduce cellulase activity? Does low concentration of salicylic acid delay needle abscission? In this study, the inhibitor was applied only on a low NRD clone. Future studies are needed to better understand the effect of various cellulase inhibitors on needle abscission on a large number of low NAR clones.

7.4 References

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