Scientific Research Publishing

American Journal of Plant Sciences, 2023, 14, 1009-1028
https://www.scirp.org/journal/ajps
ISSN Online: 2158-2750
ISSN Print: 2158-2742

Qualitative Assessment of Postharvest Stomata and Chloroplast Degradation in Contrasting Abscission Resistant Balsam Fir (*Abies balsamea* (L.) Mill.)

Gaye MacDonald[1], Rajasekaran R. Lada[1], Claude D. Caldwell[1], Chibuike C. Udenigwe[2], Mason T. Maconald[1*]

[1]Faculty of Agriculture, Dalhousie University, Bible Hill, Canada
[2]School of Nutritional Sciences, Faculty of Health Sciences, University of Ottawa, Ottawa, Canada
Email: *mason.macdonald@dal.ca

How to cite this paper: MacDonald, G., Lada, R.R., Caldwell, C.D., Udenigwe, C.C. and Maconald, M.T. (2023) Qualitative Assessment of Postharvest Stomata and Chloroplast Degradation in Contrasting Abscission Resistant Balsam Fir (*Abies balsamea* (L.) Mill.). *American Journal of Plant Sciences*, **14**, 1009-1028.
https://doi.org/10.4236/ajps.2023.149069

Received: July 15, 2023
Accepted: September 23, 2023
Published: September 26, 2023

Abstract

Balsam fir is an important Christmas tree species, especially in eastern Canada. The natural Christmas tree industry faces a challenge in postharvest needle abscission. Though there have been many studies describing the physiological triggers and consequences in postharvest balsam fir, there have been no studies describing morphological or ultrastructural changes. Therefore, the objective of this study was to examine changes in stomata and chloroplast of postharvest needles. Branches were collected from low and high needle abscission resistance balsam fir genotypes, placed in water, and displayed in typical household conditions for 11 weeks. Needle abscission, chlorophyll fluorescence, and water uptake were monitored throughout. Needles stomata and chloroplasts were examined under a scanning and transmission electron microscope, respectively, each week. All branches had increased abscission, decreased chlorophyll fluorescence, and decreased water uptake over time. Needle surfaces accumulated fungal hyphae, especially in stomata. Chloroplasts demonstrated some dysfunction within two weeks, with notable decreases in chloroplast starch and increases in plastoglobulins. Within several weeks thylakoid membranes had been dismantled as chloroplasts transformed into gerontoplasts. All biophysical and structural changes were more pronounced in low needle abscission resistant genotypes. This research identifies a potential role for needle fungi in postharvest needle abscission and confirms the postharvest senescence of chloroplasts. Though it was previously speculated that chloroplasts must senesce postharvest, this study identifies how quickly this process occurs and that it occurs at different rates in contrasting genotypes.

Keywords

Abies balsamea, Balsam Fir, Christmas Tree, Fluorescence, Needle Abscission Resistance, Scanning Electron, Transmission Electron

1. Introduction

Christmas trees are a culturally and economically important specialty crop. Germans began using conifers in Christmas celebrations in the 16th century, which has since evolved to require millions of trees each year [1]. The Christmas tree industry in Canada alone was worth $163.3 million in 2021 with balsam fir (*Abies balsamea* (L.) Mill.) as one of the most popular species [2]. Larger Christmas tree producing areas, such as the United States and Europe, have over $1 billion in annual sales [3]. However, post-harvest needle abscission remains a major challenge for the industry with estimated economic losses exceeding $600 million [4].

Postharvest needle abscission is a complex physiological phenomenon that is triggered by root detachment but modified by several factors. Cold acclimation [5], soil nutrition [6], volatile terpenes [7], and a plethora of hormones [8] all affect postharvest abscission. In any case, ethylene biosynthesis is increased while auxins decrease [8] [9], which activates several hydrolytic enzymes that weaken abscission zone cell walls [10]. Once weakened, needles will often fall off by themselves or with minor mechanical stimulation (e.g. brushing against or shaking a branch). There are also significant genotypic differences in needle abscission, which led to the identification of balsam fir genotypes with low and high needle abscission resistance (NAR) [11].

Reduced water uptake is one of the earliest indicators that postharvest needle abscission will begin. Water uptake is generally high (~0.20 $mL \cdot g^{-1} \cdot d^{-1}$) in freshly harvested balsam branches [8] [12]. However, stomatal conductance and water uptake decrease by 80% and 75%, respectively, within the first 2 weeks postharvest and coincide with needle abscission commencement [12] [13]. Similarly, postharvest decreases in water potential have been well-documented in many other conifers [14] [15]. Decreases in water uptake have often been attributed to blockages in the xylem, due to air embolism or bacterial contamination [12] [16] [17]. Attempts to mitigate xylem blockage by preventing bacteria accumulation have had some success in delaying abscission [12]. It has also been noted that changes in water uptake could be attributed to ABA-induced stomatal closure as trees attempt to mitigate postharvest water deficit [4] [18]. Less attention has been placed on physical changes in needle stomata, but it is possible that stomata could also be blocked due to fungal contamination. Aerial plant organs, such as conifer needles, often provide a suitable habitat for epiphytes, saprophytes, or pathogens [19]. Some fungal secretions or high concentrations of bacteria effectively restrict the stomatal aperture [20] [21] [22], which could also contribute to

reduced water uptake in balsam fir.

Membranes are one of the primary sensors for abiotic stresses, such as water deficit [23]. Reactive oxygen species (ROS) production is dramatically increased during stress due to increased photorespiration or excessive reduction in the electron transport chain [24] [25]. These ROS often work as signals of stress but also cause damage to cellular membranes, such as in chloroplasts, resulting in cellular dysfunction [26] [27]. Early indicators of postharvest stress in balsam fir include decreases in chlorophyll index [8], decreases in chlorophyll fluorescence [5] [28], and increased membrane injury [8] [29]. Changes to chlorophyll index and fluorescence are likely caused by damage to chloroplasts.

Qualitative changes in stomata and chloroplasts are not well established in balsam fir postharvest. Transmission electron microscopy (TEM) allows detailed studies of ultrastructural changes within a cell, including nuclear alteration, cytoplasmic reorganization, and loss of membrane integrity. Of particular interest is the structural change in chloroplasts [30]. Meanwhile, scanning electron microscopy can be used to observe surface changes in the cell, such as membrane blebbing or stomatal aperture [31]. Both TEM and SEM were used to observed cellular changes postharvest in balsam fir. It was hypothesized that stomata and chloroplast display increasing dysfunction postharvest. It was also hypothesized that that chloroplast changes will occur more rapidly in a low NAR genotype than a high NAR genotype. Therefore, the objective of this study is to compare changes in stomata and chloroplasts in postharvest balsam fir needles from contrasting NAR genotypes.

2. Materials and Methods

2.1. Experimental Design and Set Up

This experiment was designed to investigate 2 factors: NAR genotype and time postharvest. The experiment used a low and high NAR genotype as described in [11]. Branches were monitored for 11 weeks postharvest, and each week was used as a separate time point for analysis. The experiment was replicated 10 times. Due to the destructive nature of some measurements 120 branches from each genotype were collected. Measurements and electron microscope observations were made on 20 branches each week (10 from each genotype). This process was repeated each subsequent week on 20 different branches.

All branches were collected from a clonal balsam fir orchard at the Tree Breeding Center, Department of Natural Resources, Debert, Nova Scotia, Canada (45˚25'N, 63˚28'W) in December. Each branch was cut from 2-year growth at 1.5 m aboveground. Collected branches were placed in a container with distilled water immediately after harvest to equilibrate water status. All branches were then transported to the lab.

All branches were provided a fresh cut 2.5 cm above the previous cut to ensure good water uptake. The fresh cut was made with the original cut end submerged in distilled water to reduce risk of introducing air into the xylem and

disrupting water flow. Branches were weighed, placed in an amber bottle filled with 100 mL of distilled water, then the neck of each bottle was sealed with cotton wool to reduce direct water evaporation and provide added stability to a branch. All branches were randomly placed in a growth room at 21˚C constant temperature, 32% relative humidity, and 99 μmol·m^{-2}·s^{-1} light intensity.

2.2. Quantitative Response Variables

Needle abscission was measured gravimetrically using dry mass [8] [29]. Each day the branches used to determine needles loss were subjected to a "finger run" test to dislodge loose needles, which was conducted by gently passing a branch between the index and middle finger 3 times. Fallen needles were collected and placed in an oven for 24 h at 90˚C. This process was repeated until the end of the 11th week when the experiment was terminated. Any needles still attached to a branch after the 11th week were removed from the branch, dried, then weighed so that percentage needle loss was expressed as a percentage of total needle dry mass. The length of time to abscise 1% of needles was defined as needle abscission commencement (NAC) [5] and was also reported in our experiment.

Water uptake was estimated gravimetrically. The mass of each bottle with water was weighed at the beginning of the experiment. Branches were removed from their bottles each week and the bottles were weighed again. Any difference in mass was estimated as water uptake. The rate of water uptake was calculated using Equation (1) and expressed in mL·g^{-1}·d^{-1}. In Equation (1), M_n is the mass of the flask on any given week, M_{n+1} is the mass of the flask on the subsequent week, and M_b is fresh weight of the branch [29]. The difference in mass is also divided by a factor of 7 to convert the rate to daily uptake as opposed to weekly uptake.

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

Dark-adapted chlorophyll fluorescence is used to assess maximum potential quantum and stress status of photosystem II [32]. Chlorophyll fluorescence in balsam fir needles was determined using an OS1p fluorometer (Opti-Sciences, Tyngsboro, MA) as described in [5]. Branches were placed in darkness for 15 min before fluorescence measurements, where a low intensity light pulse measured minimal fluorescence (F_0) and a saturating light pulse measured maximal fluorescence (F_m). Variable fluorescence (F_v) was calculated as the difference between F_0 and F_m. Dark adapted fluorescence was calculated as F_v/F_m.

2.3. SEM

Fully expanded needles from the previous year of growth were sampled immediately after harvest, 2-weeks postharvest, 4-weeks postharvest, and during abscission at 10-weeks postharvest. Needles were fixed by placing them in 2 mL vials of 2.5% glutaraldehyde then stored between 0 - 4˚C for at least 2 h. Needles from each genotype were placed in separate wells in a plastic 12-well plate tray at

room temperature and then washed with tap water three times to remove the fixative with a plastic pipette. The needles were then fixed a second time with 2% osmium tetraoxide for 1 h at room temperature, after which needles were washed three more times with tap water. Needles were gradually dehydrated by coating and removing increasing concentrations of ethanol (25%, 50%, 70%, 80%, 90%, 95%, 100%). The highest concentration was used three times. The needles were then critical point dried using Leica EM CPD300 (Leica Microsystems Inc., Concord, ON). The dried needles were mounted on to SEM specimen stub with needle's abaxial face facing up using conductive carbon cement. Three to four needles were mounted on each stub and coated with 12 nm of gold palladium alloy using a Lucia EM ACE200 (Leica Microsystems Inc., Concord, ON). Samples were viewed using a LEO 1455VP SEM (Carl Zeiss Canada Ltd., Toronto, ON).

2.4. TEM

Fully expanded needles from the previous year of growth were taken initially at the orchard and then each week from the lab for TEM. Needles were sampled at the same time of day (16:00 - 18:00). Needles were cut transversely and longitudinally and then fixed in 2.5% glutaraldehyde. Needles were fixed a second time in 1% osmium tetroxide and then dehydrated with acetone. Finally, needles were embedded in an epoxy resin for thin sectioning as described by [33]. Thin sections were observed in a JEM-1230 TEM (JEOL Canada Inc., Saint-Hubert, QC) equipped with an ORCA-HR high-resolution (2000 by 2000 pixels) digital camera (Hamamatsu Corp., Bridgewater, NJ).

2.5. Statistical Analysis

This experiment was analyzed as a 2 × 12 factorial with 2 genotypes and 12 sample times. Statistical assumptions of normality and homogeneity were confirmed for quantitative analysis using Minitab 19 software (Minitab, LLC., Pennsylvania State College, PA). Independence was assured using different branches each week. Data were submitted to an analysis of variance using the general linear model feature in Minitab 19. Tukey's multiple mean comparison was used to separate means at 5% significance. SEM and TEM photographs were described qualitatively.

3. Results

3.1. Needle Abscission

The low NAR genotype had significantly higher needle abscission throughout the experiment than the high NAR genotype (**Figure 1**). Postharvest needle abscission began gradually in both genotypes during the first week, though the low NAR genotype lost 3.2 times more needles than the high NAR genotype. The low NAR genotype had lost over 1% of its total needles by week 2, which was 4.7 times more needles than the high NAR genotype. By comparison, the high NAR

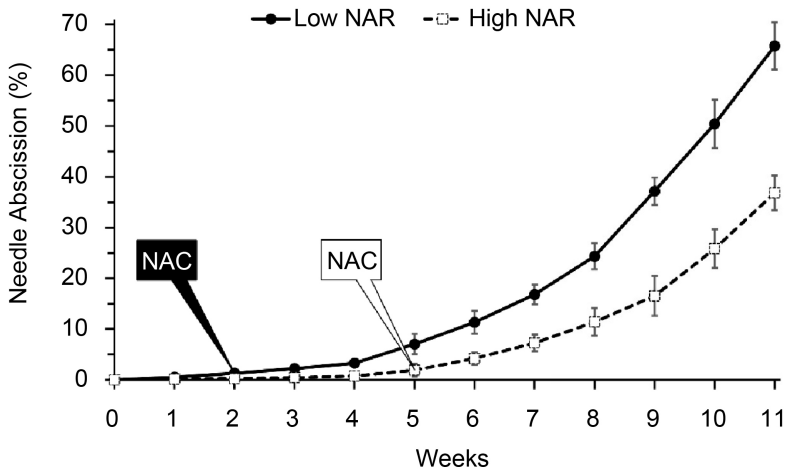


Figure 1. Progression of postharvest needle abscission in balsam fir genotypes with high and low needle abscission resistance. Data points represent the mean and error bars represent the standard error, all calculated from 10 replicates each week. NAC refers to needle abscission commencement, the time at which a branch lost 1% of its needles. NAR refers to needle abscission resistance of genotypes.

genotype didn't lose 1% of its total needles until week 5. Needle abscission dynamics for both genotypes followed a similar pattern, but the high NAR genotype was roughly 2 - 3 weeks slower than the low NAR genotype throughout the experiment.

3.2. Chlorophyll Fluorescence

The low NAR genotype had significantly lower chlorophyll fluorescence throughout the experiment than the high NAR genotype (**Figure 2**). The chlorophyll fluorescence of the high NAR genotype was 18% higher than the low NAR genotype immediately after harvest. Fluorescence increased in both genotypes during the first 3 weeks, then fluorescence decreased faster in the low NAR genotype. By week 7, chlorophyll fluorescence was 37% higher in the high NAR genotype than the low NAR genotype. Fluorescence started to significantly decrease in the high NAR genotype by week 9 but remained higher in high NAR genotype versus low NAR genotype. The high NAR genotype had 92% higher fluorescence than the low NAR genotype in the final week.

3.3. Water Uptake

There was no significant difference in water uptake between genotypes in the first week (**Figure 3**). However, water uptake decreased by 79% in the low NAR genotype during the second week compared to only a 9% decrease in the high NAR genotype. The high NAR genotype maintained significantly higher water uptake than the low NAR genotype until week 5. There were no differences in water uptake between genotypes from week 5 until completion of the experiment.

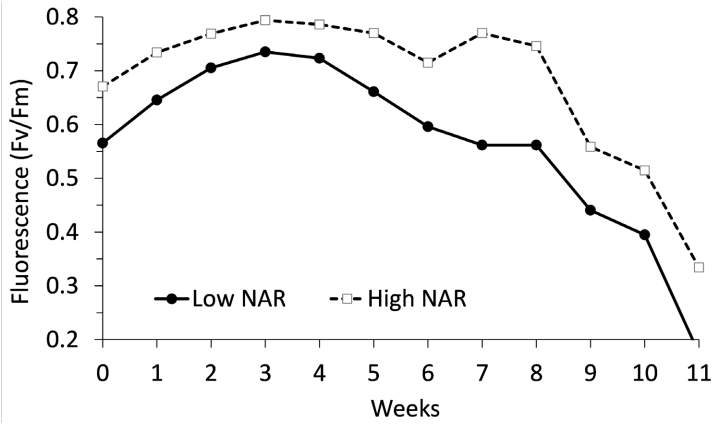


Figure 2. Postharvest changes in chlorophyll fluorescence (determined as the ratio of variable fluorescence by maximum fluorescence) of balsam fir genotypes with high and low needle abscission resistance. Data points represent the mean and error bars represent the standard error, all calculated from 10 replicates each week. NAR refers to needle abscission resistance of genotypes.

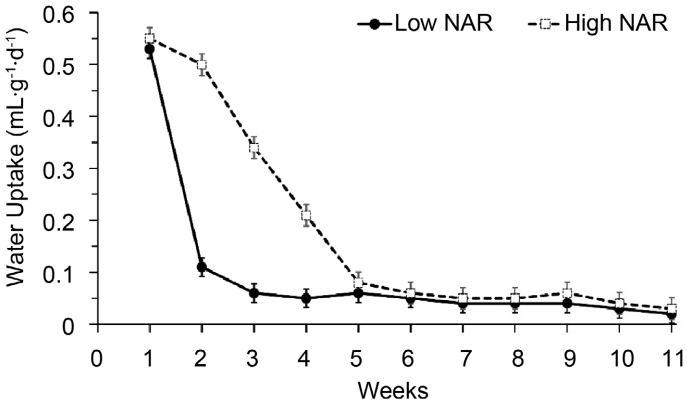


Figure 3. Postharvest decrease in water uptake in balsam fir genotypes with high and low needle abscission resistance. Data points represent the mean and error bars represent the standard error, all calculated from 10 replicates each week. NAR refers to needle abscission resistance of genotypes.

3.4. SEM

Although there was relatively little fungus on balsam fir needles during harvest, there was noticeable more fungal contamination on low NAR genotypes versus high NAR genotypes during harvest (**Figure 4**). There was considerably more evidence of fungus in both genotypes by 2-weeks postharvest, though fungus was much more visible in low NAR genotypes (**Figure 5**). Fungus could be observed around stomata of low NAR genotypes at 151x magnification, which was further confirmed at 1010x magnification (**Figure 5**). Some stomata were also observed to have an unidentified waxy build up (**Figure 6**).

Both genotypes had easily observable fungus by 10-weeks postharvest (**Figure 7**). The high NAR genotype had several stomata clogged with fungus (**Figure**

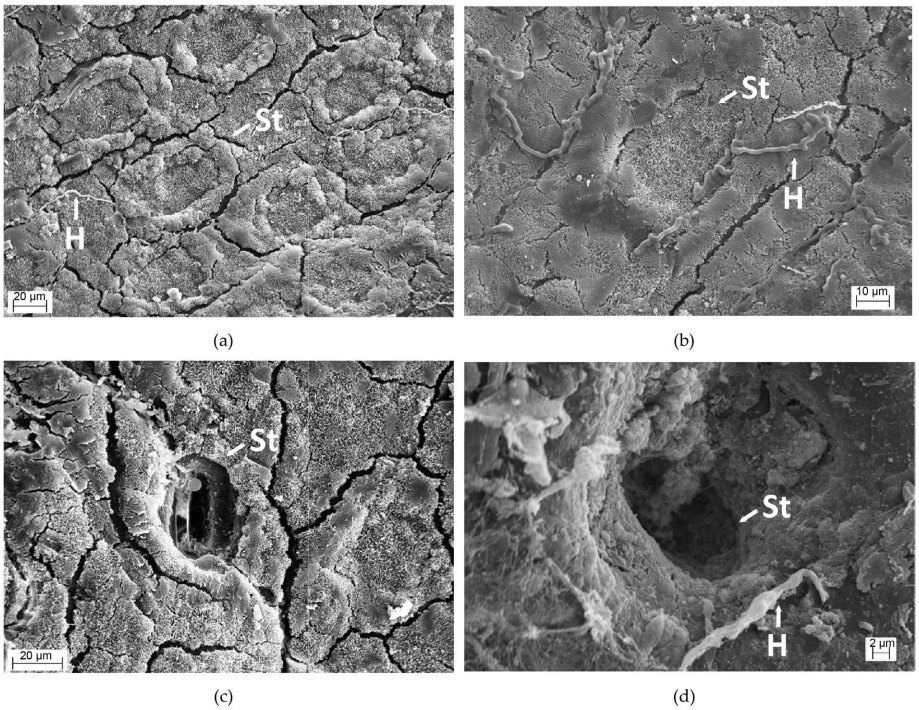


Figure 4. Balsam fir needles immediately after harvest: (a) high NAR genotype with closed stomata (1010× magnification, scale bar = 20 µm); (b) low NAR genotype with closed stomata (2010× magnification, scale bar = 10 µm); (c) high NAR genotype with open stomata (1500× magnification, scale bar = 20 µm); (d) low NAR genotype with open stomata (5010× magnification, scale bar = 2 µm). St = stomata and H = fungal hyphae.

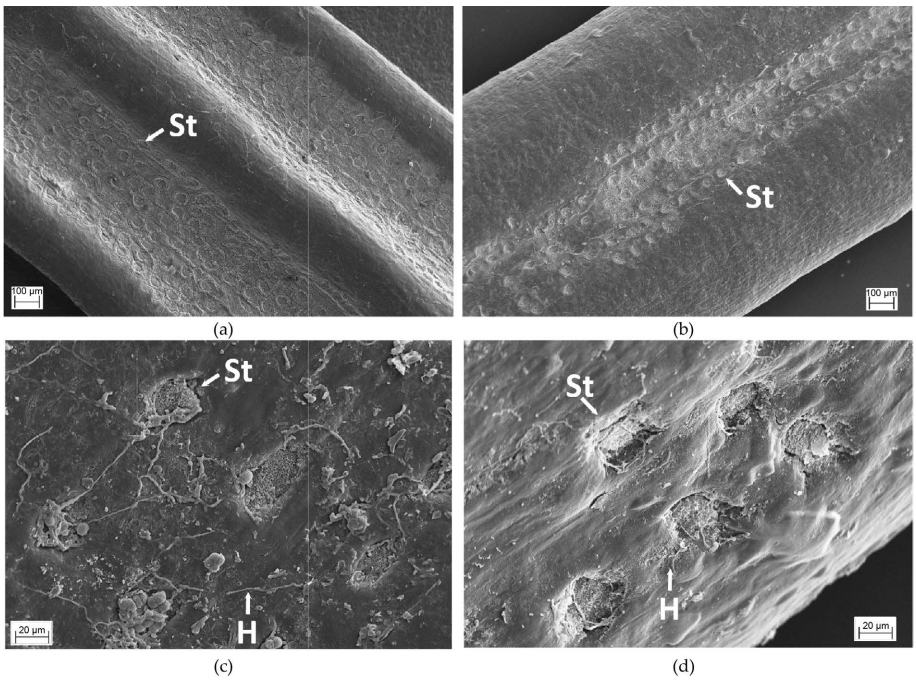
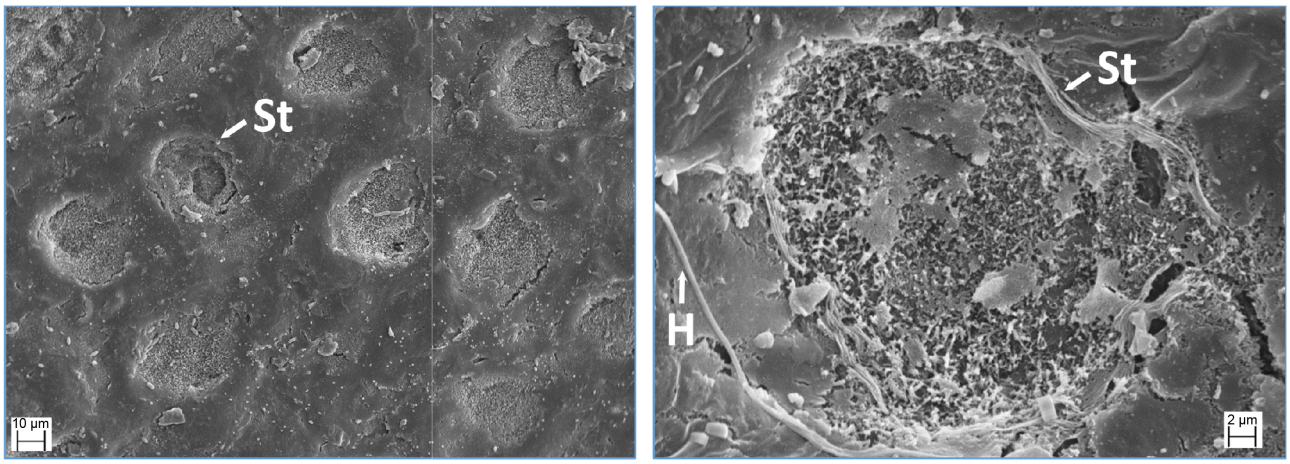
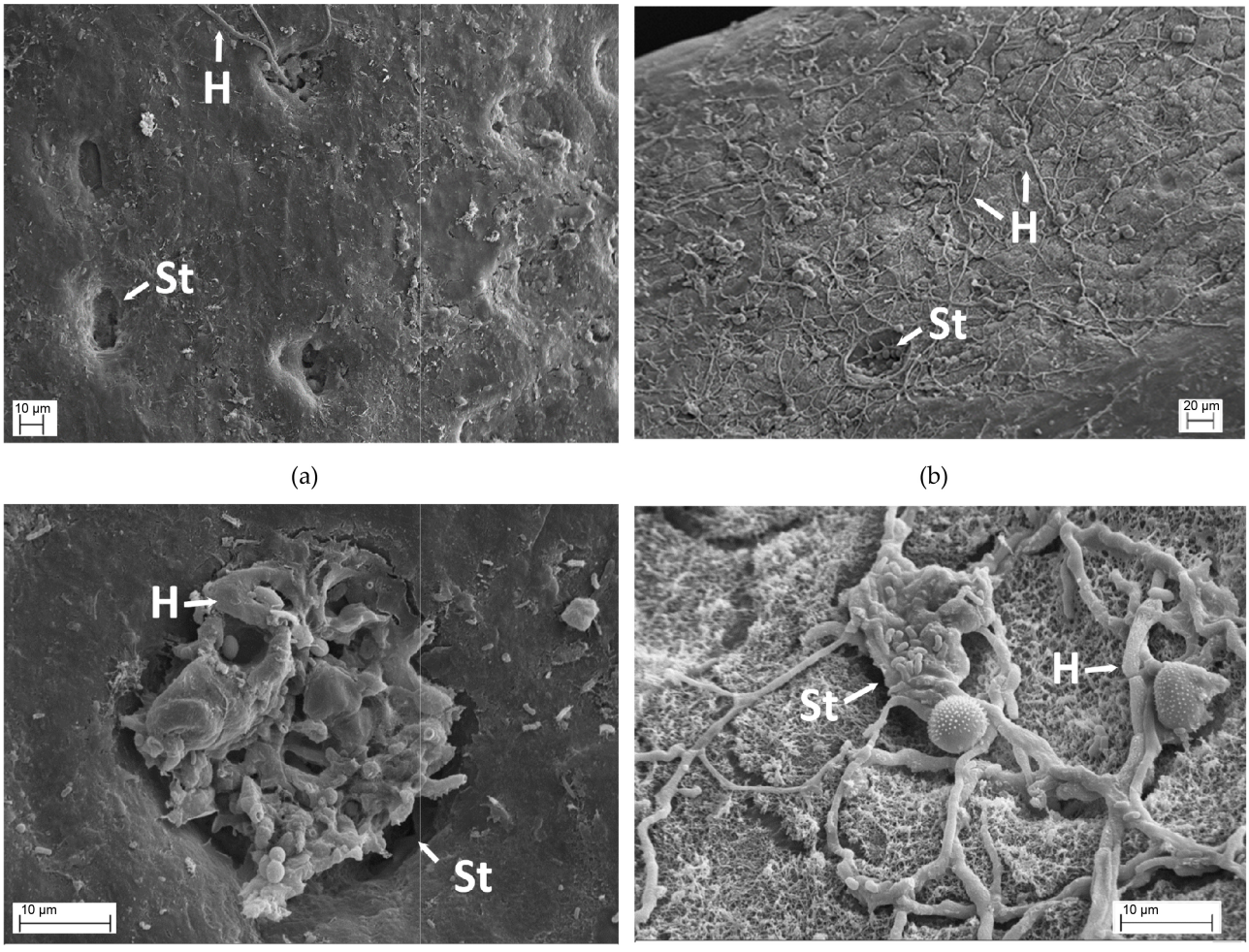


Figure 5. Stomata of balsam fir needles 2-weeks postharvest: (a) high NAR genotype (151× magnification, scale bar = 100 µm); (b) low NAR genotype (151× magnification, scale bar = 100 µm); (c) high NAR genotype (1010× magnification, scale bar = 20 µm); (d) low NAR genotype (1010× magnification, scale bar = 20 µm). St = stomata and H = fungal hyphae.



(a) (b)

Figure 6. Stomata of balsam fir needles with waxy build at 4-weeks postharvest: (a) 1010× magnification, scale bar = 10 µm; (b) 4990× magnification, scale bar = 2 µm. St = stomata and H = fungal hyphae.



(a) (b)

(c) (d)

Figure 7. Stomata of balsam fir abscised needles: (a) high NAR genotype (891× magnification, scale bar = 10 µm); (b) low NAR genotype (574× magnification, scale bar = 20 µm); (c) high NAR genotype (4020× magnification, scale bar = 10 µm); (d) low NAR genotype (4010× magnification, scale bar = 10 µm). St = stomata and H = fungal hyphae.

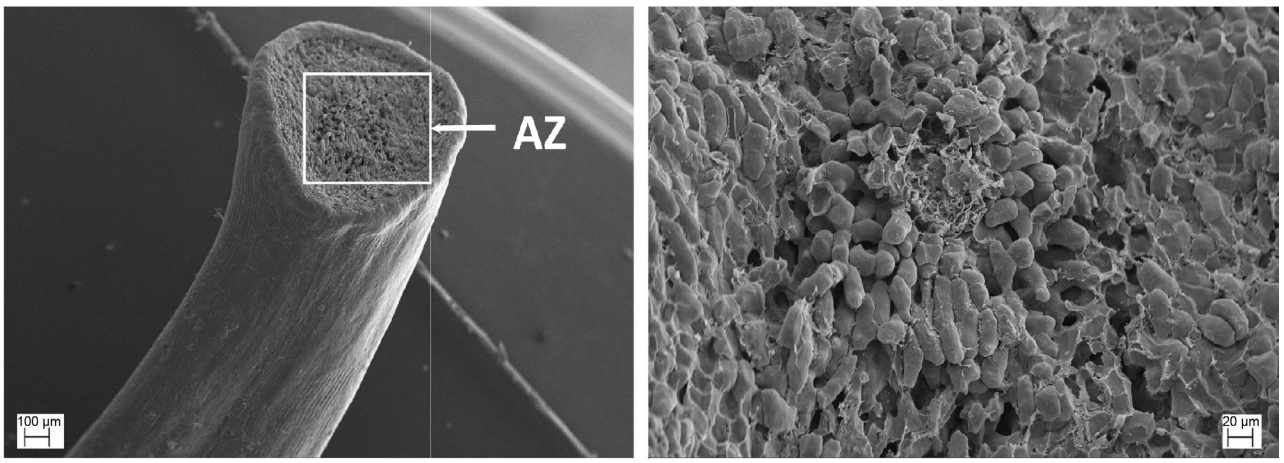
7(a) and Figure 7(c)). However, virtually all stomata of low NAR genotypes were clogged and hyphae branching was more extensive than high NAR genotypes (Figure 7(b) and Figure 7(d)), It appeared that hyphae were trying to grow into the needle through the stomata in both clones. There were also bacteria, mold spores, pollen, and wax observed in both genotypes by 10-weeks postharvest (Figure 7). An examination of the abscission zone of a needle that had not yet been abscised by 10-weeks postharvest revealed the presence of many bacteria at the abscission zone (Figure 8).

3.5. TEM

Branches were preserved on site immediately during harvest and examined for chloroplasts and plastoglobuli (Figure 9). Chloroplasts were not significantly different in size between the two genotypes (Table 1). There were differences in both the size and number of plastoglobuli present. Plastoglobuli in high NAR genotypes were approximately 2x larger and occurred 3x more frequently than in low NAR genotypes. There was also some residual starch present in both genotypes, but it was not plentiful in either.

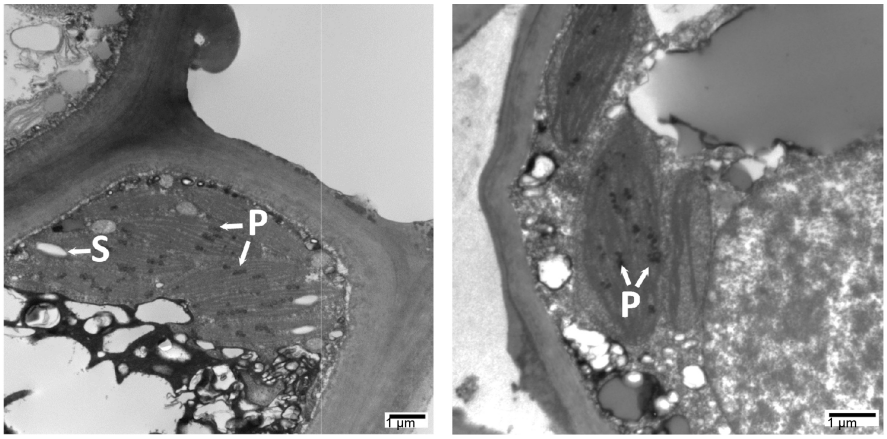
Chloroplasts in each genotype were actively producing starch grains 1-week postharvest (Figure 10). There appeared to be a clear separation of the stroma lamellae in the chloroplasts with larger starch deposits observed between the stroma lamellae. Thylakoids also appear to be swollen (Figure 10). Plastoglobuli were still present but were difficult to measure or quantify due to the increased number of starch grains. Chloroplasts were 46% smaller in both genotypes 1-week postharvest, but there were no differences in chloroplast size between genotypes.

The number of starch grains present in chloroplasts decreased in subsequent weeks postharvest. By 2 weeks postharvest it was more difficult to find chloroplasts or starch in the low NAR genotype (Figure 11). By 3 weeks postharvest, the



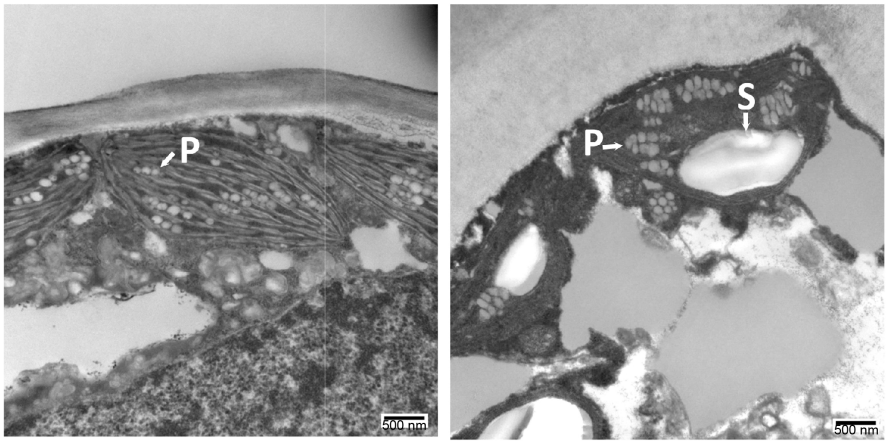
(a) (b)

Figure 8. Abscission zone of balsam fir needle: (a) 95× magnification, scale bar = 100 µm; (b) 498× magnification, scale bar = 20 µm. AZ = abscission zone and the white rectangle indicates the magnified area shown in Figure 8(b).



(a) (b)

Figure 9. Initial TEM of balsam fir needles postharvest: (a) high NAR genotype (12,000× magnification, scale bar = 1 µm); (b) low NAR genotype (15,000× magnification, scale var = 1 µm). S = starch and P = plastoglobuli.



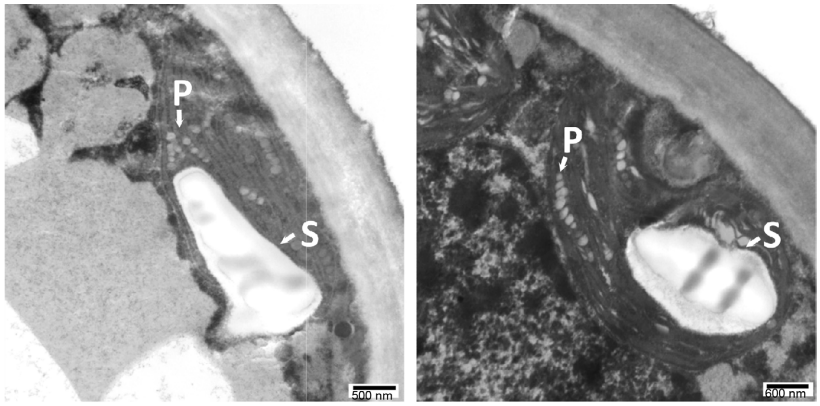
(a) (b)

Figure 10. TEM of balsam fir needles 1-week postharvest: (a) high NAR genotype (30,000× magnification, scale bar = 500 nm); (b) low NAR genotype (30,000× magnification, scale bar = 500 nm). S = starch and P = plastoglobuli.

Table 1. Quantification of chloroplast size and plastoglobuli number and size using TEM for high and low NAR genotypes harvested in Debert, NS.

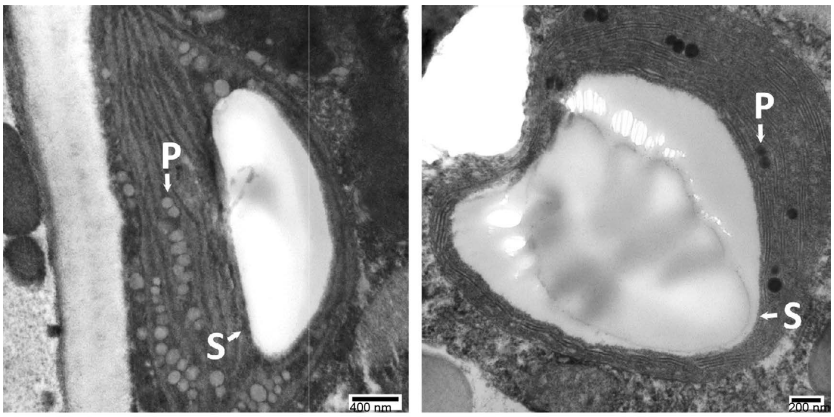
Clone	Chloroplast Thickness (nm)	Plastoglobuli Size (nm^2)	Number of Plastoglobuli
High NAR	3667 ± 51	15,050 ± 274	75 ± 16
Low NAR	3725 ± 47	7489 ± 197	25 ± 10

chloroplasts were over-filled with starch grains and chloroplast breakdown was evident (**Figure 12**). By 4 weeks postharvest chloroplasts in the high NAR genotype looked very much like they did at harvest with obvious plastoglobuli and little starch, which indicated inactivity. However, after 4 weeks postharvest the chloroplasts of low NAR genotypes displayed appreciable damage. Throughout



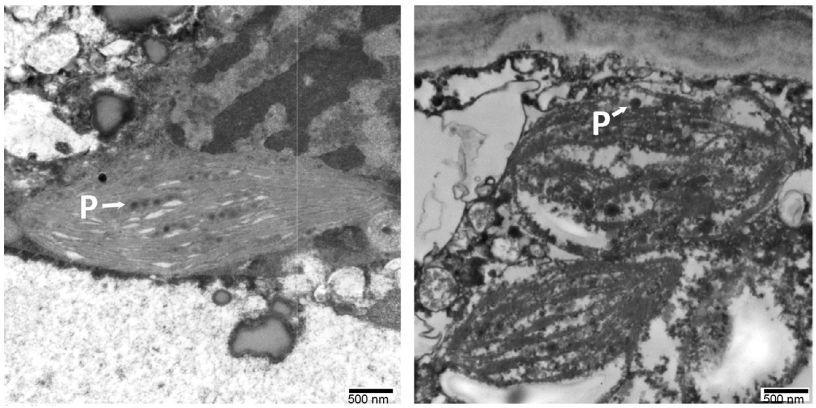
(a) (b)

Figure 11. TEM of balsam fir needles 2-weeks postharvest: (a) high NAR genotype (30,000× magnification, scale bar = 500 nm); (b) low NAR genotype (25,000× magnification, scale bar = 600 nm). S = starch and P = plastoglobuli.



(a) (b)

Figure 12. TEM of balsam fir needles 3-weeks postharvest: (a) high NAR genotype (50,500× magnification, scale bar = 400 nm); (b) low NAR genotype (63,200× magnification, scale bar = 200 nm). S = starch and P = plastoglobuli.



(a) (b)

Figure 13. TEM of balsam fir needles 9-weeks postharvest: (a) high NAR genotype (37,900× magnification, scale bar = 500 nm); (b) low NAR genotype (37,900× magnification, scale bar = 500 nm). P = plastoglobuli.

the next six weeks, it became increasingly difficult to find active chloroplasts in either genotype.

The final TEM images were taken 9-weeks postharvest and were particularly clear (**Figure 13**). Needles from the high NAR genotype had chloroplasts approximately 50% the size of chloroplasts at harvest. The chloroplasts in high NAR genotypes were also more globular and some starch grains remained visible (**Figure 13(a)**). However, the low NAR genotype displayed complete cellular and chloroplast disorganization 9 weeks postharvest (**Figure 13(b)**).

4. Discussion

4.1. Postharvest Quality of Genotypes

Low NAR genotypes abscised needle faster, had lower chlorophyll fluorescence, and decrease water uptake earlier than high NAR genotypes. The general trend in postharvest quality between contrasting genotypes is consistent with previous work [11] [29] [34], though our study had a smaller difference in needle retention between low and high NAR trees. The difference in NAC in [34] was approximately 5 weeks, while our current study noted only a 3-week difference in NAC. There is significant variation in needle retention based on time of year, with cold acclimation often reducing the gap in postharvest needle abscission between genotypes [11]. Thus, it is possible that the time of harvest might help explain this discrepancy. Regardless, the purpose of studying postharvest characteristics was to confirm the difference between contrasting differences, which was successful in this study. From a practical perspective, this reaffirms that there is significant variance in needle retention characteristics of different balsam fir trees. Identification of trees with superior needle retention remains an important task.

4.2. Stomatal Changes

It is well established that stomatal conductance rapidly decreases in balsam fir postharvest, which coincides with abscission commencement [12] [18]. There seems to be a degree of dehydration stress that can be observed through increases in abscisic acid and ethylene postharvest [8] [9], but the contributing factors are not completely understood. One hypothesis was that bacterial accumulation in Christmas tree stand water was impeding uptake through the xylem, which was mitigated in part by antibacterial agents [12]. It can be suggested from our current study that stomatal dysfunction may also play a role in dehydration stress and needle abscission.

There were more fungal hyphae present on low NAR genotypes compared to high NAR genotypes. The exact nature of these fungi remains unknown, but vulnerability to harmful fungi could help explain why low NAR genotypes abscise needles so much earlier. Economic losses due to pathogenic fungi were once a concern in the balsam fir industry [35], though more recent research has focused on fungal endophytes and balsam fir in New Brunswick, Canada. En-

dophytic fungi can have a mutual relationship with the plant, contributing to the defense of the host plant while not causing disease symptoms or additional distress [36] [37]. It is also possible for endophytic bacteria to modify certain phytohormones in host plants, such as abscisic acid [38], which have been associated with postharvest abscission in balsam fir [8].

It is also possible that we observed saprophytic fungi 11 weeks post-harvest. Saprophytic fungi, such as *Coniophora puteana* and *Coriolus versicolor*, are common in beech and pine [39]. Saprophytic fungi produce extracellular mucilaginous material (ECM) after colonizing on a tree, which accelerates senescence and decay [39]. The micro-photographs of ECM bear a resemblance to the "waxy build up" described in **Figure 6** and **Figure 7**. It would be useful to identify the type of fungi present in future studies. Further, if fungi are contributing to postharvest abscission, then antifungal agents might delay abscission.

4.3. Chloroplast Degradation

This study was the first to observe balsam fir needle chloroplasts postharvest using transmission electron microscopy. The chloroplast is a main target of abiotic stress, which often inhibits photosynthesis and degrades the photosynthetic apparatus [40]. Endogenous abscisic acid quickly increases in balsam fir postharvest, presumably to close stomata and conserve water [8]. Ethylene biosynthesis inevitably increases afterwards and promotes the synthesis of hydrolytic enzymes [9] [10]. Though not confirmed in balsam fir, it is likely that any increase in abiotic stress and ethylene evolution causes an imbalance in the redox state of plant cells as observed in other species [41]. A shift in the production of ROS without the complementary ability to sequester ROS will promote senescence [41] [42], which is clearly demonstrated through a postharvest increase in abscission and a decrease in chlorophyll fluorescence in balsam fir. There are also several changes in balsam fir chloroplasts indicative of postharvest senescence.

There were changes in chloroplast starch postharvest. Starch is synthesized during the day and is indicative of active photosynthesis [43]. Starch may then be mobilized at night to help provide carbon for continued growth when not photosynthesizing [43]. However, starch is an emerging key molecule in abiotic stress response. Abiotic stress often limits photosynthesis and starch may be mobilized to provide energy, sugars, or other metabolites to help mitigate stress [44]. Little starch was present in balsam fir immediately after harvest suggesting limited photosynthesis, which was not surprising considering the cooler temperatures in December [45] [46] [47] [48]. Starch accumulated in balsam fir chloroplasts during the first week postharvest since plants were placed in warmer, lighted conditions. However, the low NAR and high NAR genotypes stopped producing starch in the second and fourth weeks postharvest, respectively. This lack of starch suggests that photosynthesis was inhibited and starch would likely have been mobilized from the chloroplast [44].

There are several morphological changes to the chloroplast that are indicative

of senescence. The three most relevant ultrastructural changes to describe the senescence-induced change of chloroplast into gerontoplasts are: 1) thylakoid membrane degradation, 2) increased plastoglobules number and size, and 3) disruption of the plastid envelope [41] [49]. All the above changes were observed in balsam fir chloroplasts postharvest but were more pronounced in the low NAR genotype. Further, chloroplasts decreased in size postharvest and began to transition from ellipsoid to circular morphology. Ethylene synthesis is increased in balsam fir postharvest [9] [10] and is also known to rapidly reduce the size of the chloroplasts [50] [51]. Further, ethylene causes the separation of thylakoid membrane layers due to degradation of granal and lamellar membranes [51]. Such a separation is evident in balsam fir as early as one week postharvest. There have been cases of gradual decreases in chloroplast size leading up to senescence, however, a fast decrease in one week is more specific to the action of ethylene [51].

As mentioned earlier, abiotic stresses (e.g. dehydration) can induce ROS [41] [42] and chloroplasts are often one of the first organelles to be damaged during times of stress [52]. Degradation of chloroplasts accounts for postharvest decreases in chlorophyll fluorescence and helps explain potential differences in NAR genotypes observed in this study. Changes in lipids and fatty acids were linked to postharvest abscission in balsam [29] [34]. It was speculated that changes in the ratio of unsaturated and saturated fatty acids might explain genotypic differences in abscission [53]. Such changes in fatty acids would also help protect chloroplasts [53], potentially explaining why chloroplasts were protected to some degree in high NAR genotypes

Chloroplasts became especially difficult to detect several weeks postharvest in the low NAR genotype. This could indicate the start of autophagy to remove damaged organelles [54]. Damaged cells may also induce production of ubiquitin ligase, an enzyme that signals for the degradation of ROS producing chloroplasts [55] [56]. Another possibility for difficulty in detecting chloroplasts is that often the osmophilicity of tissues changes when they are damaged or senescing, which means that osmium tetroxide may have been less effective when preparing samples for TEM. However, it seems less likely that deficiencies in osmium tetroxide staining would be a problem since the same protocol was used for all slide preparation and weekly samples of each genotype were prepared at the same time.

5. Conclusion

Balsam fir demonstrated increased abscission, decreased chlorophyll fluorescence, and decreased water uptake postharvest, all of which were more pronounced in low NAR genotypes. Both genotypes accumulated fungi on their needles and in their stomata, though more hyphae were found on low NAR genotypes. Finally, each genotype showed typical signs of chloroplast dysfunction postharvest, as indicated by the decrease in chloroplast starch, an increase in

plastog-lobulins, and the dismantling of thylakoid membranes. It was quite clear that chloroplasts were undergoing a conversion to gerontoplasts postharvest.

This research contributes to our understanding of cellular postharvest changes in balsam fir needle abscission. Ultrastructural changes in balsam fir chloroplasts postharvest have never been observed before. Documenting postharvest chloroplast senescence helps confirm the effect of abiotic stress in balsam fir and underscores the difference in how NAR genotypes can tolerate that stress. Chloroplast senescence also emphasizes a gap in our knowledge regarding the role of ROS in postharvest balsam fir. It would be advantageous to study a potential ROS imbalance that occurs postharvest and identify redox system differences in contrasting genotypes.

The accumulation of fungi on balsam fir stomata, especially with greater abundance in low NAR genotypes, indicates a potential role in postharvest abscission. There remain questions about the impact of fungi on postharvest trees. Does the fungi impact water up-take? Are the fungi causing biotic stress that has not be investigated postharvest? Or are fungi taking advantage of a situation where needles are already undergoing senescence and may have weakened defenses? There may also be a practical benefit to testing the effect of fungicides on postharvest Christmas trees and whether that has any effect on maintaining water uptake and/or delaying postharvest abscission.

Acknowledgements

The authors thank NSERC, (CRDPJ-364061-07/09) and ACOA (195310) to for funding this research. The authors gratefully acknowledge the Nova Scotia Department of Natural resources for access to the clonal orchard. The authors also acknowledge Mary Ann Trevors of the Faculty of Medicine at Dalhousie University for her technical expertise.

Conflicts of Interest

The authors declare no conflicts of interest.

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