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Project Title: Use of pyroligneous acid to improve postharvest needle retention in balsam fir Christmas trees.

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1. INTRODUCTION

Christmas trees are a culturally and economically important specialty crop. Conifers were used in Christmas celebrations beginning 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 [2]. Larger Christmas tree producing areas, such as the United States and Europe, have over $1 billion in annual sales [3]. However, postharvest needle retention is a major challenge for the industry with estimated economic losses exceeding $600 million [4].

Postharvest needle retention is a complex physiological process that presents a major challenge for Christmas tree producers. Factors such as cold acclimation [5], light [6], nutrition [7], hormonal differences [8], and genotype [9] all influence postharvest needle retention. However, the driving factor for postharvest needle abscission appears to be dehydration and/or water uptake deficit through embolism despite the provision of water [10–12]. Freshly cut branches uptake water at 0.20 mL g^{-1} d^{-1}. However, water uptake, stomatal conductance, and water potential all decrease within the first 2 weeks postharvest, which directly coincides with commencement of leaf abscission [12,13].

The original premise behind postharvest dehydration was that if water could be conserved by blocking stomata, then dehydration would decrease, and needle retention would increase. However, anti-transpirants have generally been only effective at reducing transpiration, with no effect on water status and needle retention [14,15]. Another strategy was delivering water to trees via an intravenous type device, which was also ineffective [16]. Several species face postharvest challenges in water uptake due to bacterial accumulation in the water and xylem of the cut end [12,17]. Xylem blockages (embolism) can be mitigated to some extent by antibacterial leachates from the plant into the water, practices such as changing water daily, or addition of low concentration silver nitrate to standing water [12,18]. However, such strategies are inconvenient and not guaranteed to work. Further, recent investigations with electron microscopy have shown that fungi accumulate on stomata shortly after harvest and causes stomatal dysfunction (Fig. 1) [19]. There may be a benefit to limiting fungi accumulation to promote water uptake and needle retention.

Pyroligneous acid (PA) is a by-product of pyrolysis where plant biomass undergoes thermal degradation in the absence or near absence of oxygen. The condensed organic vapors form an aqueous liquid fraction, PA, that has several desirable properties [20]. PA has been an effective organic biostimulant, fertilizer, insecticide, fungicide, bactericide, and antioxidant [21–23]. As such, PA has effectively increased growth, maintained chlorophyll membranes, reduced the impact of stress, and inhibited fungal and bacterial growth in agricultural species. Furthermore, the use of PA in agriculture is desirable because it can be produced sustainably and has virtually no environmental footprint [24,25]. The combination of PA's beneficial properties has high potential to improve postharvest characteristics of balsam fir, specifically by inhibiting growth of contaminants as a bactericide/fungicide and membrane protection as an antioxidant. It is hypothesized that PA could inhibit bacteria blockage in balsam fir xylem, inhibit fungal contamination of stomata, and protect chloroplast membranes. All 3 hypothesized mechanisms could lead to improved postharvest needle retention. The specific objectives of this research are to determine (1) the effectiveness of xylem-fed PA on balsam fir needle retention, (2) the effectiveness of foliar applied PA on balsam fir needle retention, and (3) any effect on phytochemical composition.

2. METHODS

2.1. Experiment 1: Preliminary Work to Determine Application Method and Concentration Range

2.1.1. Sample Collection

Branches were collected from the Christmas tree clonal orchard in Plumdale research facility at Dalhousie University on December 19, 2022. Branches were cut to include 2 full years of growth and were collected from the same genotype. Samples were taken to the lab, provided a fresh cut, and then placed in glass vases to simulate household conditions.

2.1.2. PA as a Water Additive

This experiment followed a completely randomized design where the factor of interest was PA concentration. Treatment levels consisted of 0% (water only control), 0.25%, 0.5%, 1%, and 2% PA solutions. Each branch was provided 1L of its respective treatment solution. The base of the branch was wrapped in aluminum foil at the mouth of the vase, to reduce evaporation and provide stability to the branch. Since PA is a naturally derived product containing multiple organic compounds. specific water quality parameters pH, salinity, total dissolved solids (TDS), and electrical conductivity (EC) of solutions were measured at the beginning of the experiment. This experiment was replicated 5 times.

2.1.3. PA as a Foliar Spray

This experiment followed a completely randomized design where the factor of interest was PA concentration. Treatment levels consisted of a no spray control, 0% (water spray), 0.5% PA, 1% PA, 2% PA, and 4% PA. Each branch was provided 1L of distilled water and the base of the branch was wrapped in aluminum foil at the mouth of the vase, to reduce evaporation and provide stability to the branch. Branches were sprayed with their respective treatment as the beginning of the experiment and then once per week throughout the remainder of the experiment. As above, spray solutions were evaluated for pH, salinity, TDS, and EC. This experiment was replicated 5 times.

2.1.4. Needle Abscission

Since these experiments were preliminary in nature, the major response variable was only needle abscission. Needle abscission was determined by performing a finger run test [10,16]. The first finger run test for both experiments was conducted on January 4, 2023. The water additive experiment was terminated at this point. A second finger run test was conducted January 23, 2023, on the remaining foliar spray experiment, after which the foliar spray experiment was also terminated. In each case, abscised needles and branches were collected, dried in an oven at 80°C for 24h, and then weighed. Needle abscission was then expressed a percentage of total dry mass of a branch.

2.1.5. Statistical Analysis

Data from each experiment were submitted to an analysis of variance to determine significant differences. The null hypothesis was that there was no difference between treatments and the alternative was that there was at least one difference. Specific differences were then determined through Fisher's multiple means comparison at 5% significance. All statistical assumptions were confirmed through the analysis.

2.2. Experiment 2: Effect of Foliar PA on Postharvest Needle Abscission

2.2.1. Experimental Design

The experiment was conducted in the Plant Ecophysiology lab in Cox institute, Dalhousie University Agriculture Campus in Bible Hill. The experiment was arranged in a completely randomized block design with five treatments. The treatments were no spray, water spray (0% PA), 1% PA, 2% PA and 4% PA based on preliminary work in Experiment 1. Genotype was used as the blocking factor, with branches collected from 5 different genotypes and evenly distributed among treatments.

A total of 25 balsam fir tree cuttings were collected from the Plumdale Orchard located on College Road in Bible Hill, Nova Scotia. The cuttings were taken from the second-year growth of balsam fir trees from a height of 1.5 m above the ground. Samples were immediately placed in distilled water, immersing the cut ends in the water, and transported to the lab. Response variables were needle abscission and water uptake.

2.2.2. Needle Abscission

A finger run test was performed for the needle loss data collection. The test was performed by gently brushing the branches using both index finger and thumb fingers. Percent needle loss was determined gravimetrically as performed by Lada et al. [12]. The abscised needles were collected every alternate day separately for every treatment and every replication, dried and weighed. Percentage needle loss was calculated as the cumulative sum of needle loss divided by total mass of needles upon complete abscission. Data was expressed at the length of time required to reach critical needle abscission values (1, 5, 10, 50 and 100%).

2.2.3. Water Uptake

Water uptake was determined gravimetrically as suggested by Lada et al. [12]. To determine water uptake the branches were gently removed from the amber flasks and the remaining contents (water and flask together) were weighed every alternate day. Since bottles were sealed, any loss in mass indicated water uptake by the branches. The water uptake was reported in mL g^{-1} d^{-1}.

2.2.4. Statistical Analysis

The data collected from the experiment was analyzed statistically using the Minitab software. Analysis of variance (ANOVA), general linear model was used to determine if the foliar application of PA helps to improve needle retention in balsam fir. The mean separation was determined using Fisher's method with a 95% confidence interval at $p \leq 0.05$ after a significant ANOVA result was obtained.

2.3. Experiment 3: Effect of Foliar PA on Stress-related Biochemical Parameters in Balsam Fir

2.3.1. Sample collection

The branches of balsam fir were collected from two different tree (genotype) in the clonal orchard at the Plumdale research facility, Dalhousie faculty of Agriculture, Truro, NS. Each branch was collected from the most recent 2-year growth. All the samples were screened for the absence of diseases and nutrient deficiencies, and they were placed in a container with cut ends submerged in distilled water. Needle samples from the fresh branches were collected and frozen in liquid nitrogen for the biochemical analysis.

2.3.2. Laboratory setup

The samples collected in the field were given a fresh slant cut and placed in a 250 mL flask filled with distilled water. The lab was maintained at room temperature (22°-24°C) with fluorescent light. The neck of the flask was sealed with aluminium foil to reduce evaporation and to keep the branches stable. The samples were arranged in two rows of each genotype for all the treatments.

2.3.3. Preparation of PA for spray

The experiment setup consists of five treatments with each of five replications. The four treatments were –Water, 1%, 2%, and 4% of PA. The solution of PA concentrations 1%, 2%, and 4% were made by dissolving PA in distilled water and making up to one litre of solution for each concentration. The pH, solubility, total

dissolved solids (TDS), and electrical conductivity (EC) of PA were measured before spraying. The spraying was at one week interval.

2.3.4. Experimental design and needle collection

The experimental treatments were arranged in a completely randomized block design where the explanatory variable was the concentration of PA and time. Different response variables such as chlorophyll a and b, carotenoids, phenolics, flavonoids, proline, ROS and membrane injury require independent samples to determine the initial data for these variables before spraying. The intact needles were forcibly removed the branches. After that the needles were collected in two-week interval throughout the eight-week period of the experiment. The collected needles were frozen in liquid nitrogen and stored in - 80 ℃ freezer until the biochemical analysis.

2.3.5. Chlorophyll a and b, carotenoids

Chlorophyll a, b and carotenoids were measured in reference to Lichtenthaler [25]. A 0.2 g of ground samples were transferred into a sterile 50 mL falcon tube and 10 mL of 80% acetone was added. The mixture was vortexed for 1 min and centrifuged at 12000 g for 15 min. 1 mL of supernatant was transferred into a cuvette. The absorbance was measured at 646.8 and 663.2 nm with a UV-Vis spectrophotometer against 80% acetone as blank. The concentration of chlorophyll a, b, and carotenoids was calculated as µg g^{-1} FW using the formulae:

$$\text{Chlorophyll a } \left(\frac{\mu g}{mL}\right) = 12.25 \times A663.2 - 2.79 \times A646.8$$

$$\text{Chlorophyll b } \left(\frac{\mu g}{mL}\right) = 21.50 \times A646.8 - 5.1 \times A663.2$$

$$\text{Carotenoids } \left(\frac{\mu g}{mL}\right) = \frac{1000 * A470 - 1.8 \times chla - 85.02 \times chlb}{198}$$

2.3.6. Phenolic and flavonoid content

The total phenolic content was determined by the Folin-Ciocalteu assay described by Ainsworth & Gillespie [27] with a slight modification. A 0.2g of sample was homogenized in a 2 mL of ice-cold methanol and incubated in the dark at room temperature for 48 hr. The mixture was centrifuged at 13000 g for 5 min and 100 µL of supernatant was transferred into a new microfuge tube 200 µl of 10% Folin-Ciocalteau reagent was added and vortexed for 5 min. 800 µl of 700 nM sodium carbonate (Na_2CO_3) was added, vortexed for a min, and incubated at room temperature (25°C) for 2 hr. The absorbance of the resultant mixture was measured at 765 nm. Total phenolic content was estimated using gallic acid equivalents standard curve and was expressed as mg gallic acid equivalents per g of the sample.

Total flavonoids was determined as described by Chang et al. [28] with some modifications. A 0.2 g of ground samples were homogenized with 2.5 mL of 95% methanol. The mixture was centrifuged at 13000 g for 10 min and 500 µL of the supernatant was transferred into a new tube. To each tube, 1.5 mL of 95% methanol, 0.1mL of 10% $AlCl_3$, 0.1mL of 1 M potassium acetate, and 2.8 mL of distilled water was added. The mixture was vortexed and incubated for 30 min at room temperature and absorbance was measured at 415 nm against a blank. Flavonoids content was estimated using a quercetin standard curve and expressed in µg of quercetin per g of sample.

2.3.7. Proline content

A 0.5 g of ground samples were mixed with 1 mL of 70% ethanol. Then the mixture was centrifuged at 12000 g for 15 min at 4°C and 500 µl of supernatant was added to 1 mL of the reaction mixture (ninhydrin 1% (w/v) dissolved in 60% acetic acid (v/v) and 20% ethanol (v/v)). The tubes were sealed and vortexed for 30 s. The mixture was incubated in a water bath at 95°C for 30 mins. After cooling at room temperature, the absorbance was measured at 520 nm using a spectrophotometer against a blank containing ethanol and reaction mixture. Proline content was estimated using the L-Proline standard curve. The absorbance was plotted against L-Proline concentration to obtain the standard curve. Total proline content can be calculated using the formula:

$$\text{Proline}\left(\frac{\mu\text{mol}}{\text{gFW}}\right) = \frac{\text{Abs}_{ext} - \text{blank}}{\text{Slope}} \times \text{Vol}_{ext}/\text{Vol}_{aliquot} \times \left(\frac{1}{\text{FW}}\right)$$

where Abs_{ext} is the absorbance of plant extract, blank is the absorbance of clear extraction solution, the slope is determined by linear regression of a calibration curve, $Vol_{extract}$ is the total extract volume, $Vol_{aliqout}$ is the extract volume used for the assay, FW is the weight of the plant material.

2.3.8. ROS determination (H_2O_2 concentration)

ROS was estimated using the H_2O_2 concentration by following the procedure described by Patterson et al. [29]. 0.1 g of ground samples were mixed with 1 mL of 0.1%(w/v) TC. The mixture was vortexed and centrifuged at 16000 rpm for 10 min. After that 0.2 mL of supernatant was added to 200 µL of 100mM potassium phosphate buffer (pH 7.0) and 800µL of 1 M KI. The mixture was incubated in the dark for 1 hr. The absorbance was measured at 390 nm against the blank (0.1 TCA in the absence of needle extract). The H_2O_2 concentration was calculated in according to the standard curve (H_2O_2 dissolved in 0.1% TCA) and expressed in µM/g of fresh weight.

2.3.9. Membrane integrity

Membrane integrity was performed in reference to the study by MacDonald & Lada [13]. The percentage of electrolyte that leaks into the solution was used by the membrane injury index (MII) to measure membrane integrity. Approximately 30 mL of distilled water was added to test tubes, which were then let to warm up to room temperature (25 °C). Using a CDM 2e Conductivity Meter, the electrical conductivity of the distilled water (ECw) alone was measured Then, 0.4 g of needles was taken from each branch and immersed entirely in a centrifuge tube. After being sealed, the tubes were kept at room temperature for 24 hr. To estimate how much of the electrolytes are leached into the solution, the initial conductivity (EC0) was measured. After 4 hr at 90 °C in a forced-air oven to kill tissues, sealed tubes was then cooled to room temperature. To estimate the maximum leakage, final conductivity measurements (ECf) were made after the system had reached equilibrium at 25 °C. The following formula was used to determine MII:

$$\text{MII} = \text{EC0} - \text{ECw}\ /\text{ECf} - \text{ECw} \times 100$$

2.3.10. Statistical analysis

Data were submitted to general linear model using Minitab (ver. 19.0, Minitab Inc., State College, Pa.). Foliar application and time were entered as explanatory variables while genotype was entered as a blocking variable. A 2-way interaction between spraying and time was also included in the model. Means were compared using Fisher's exact test when significant differences were found from the ANOVA at $p \leq 0.05$. Statistical assumptions of normality, homogeneity, and independence were confirmed.

3. RESULTS AND DISCUSSION

3.1. Experiment 1

3.1.1. PA as a Water Additive

There was a significant (P <0.001) difference in all water quality response variables due to PA concentration (Table 1). The significant differences in water quality parameters were expected since they were based on carefully made concentrations. This is also reflected in the low standard error in pH, salinity, TDS, and EC. It is noteworthy that there is a clear trend that pH greatly decreases with even a small percentage of PA, while salinity, TDS, and ES all increase.

Visual differences in abscission due to foliar spray are shown in Figure 1. There was a significant (P < 0.001) difference in needle abscission due to PA concentration (Table 1), but not the trend we were expecting. The 0.25% PA solution had no impact on abscission compared to the control, but every other concentration significantly increased abscission. It is possible that PA altered water chemistry too much at concentration above 0.25%. The pH was very low compared to distilled water, while salinity, TDS, and EC had all significantly increased.

Table 1. Water quality parameters and abscission due to PA added to stand water. Different letters indicate significant differences between treatments at 5% significance.

Concentration (%)	pH	Salinity (ppm)	TDS (ppm)	EC (µS)	Abscission (%)
0	6.15 ± 0.09 A	441.0 ± 0.6 A	634.7 ± 1.2 A	908.3 ± 1.3 A	1.0 ± 0.5 A
0.25	3.54 ± 0.06 B	490.0 ± 1.2 B	703.4 ± 1.2 B	1008.3 ± 1.76 B	1.3 ± 0.5 A
0.50	3.29 ± 0.02 C	505.7 ± 0.3 C	724.7 ± 0.9 C	1035.3 ± 0.3 C	44.7 ± 5.7 B
1.00	3.23 ± 0.03 C	550.7 ± 0.9 D	784.7 ± 0.7 D	1122.0 ± 1.0 D	67.9 ± 9.5 C
2.00	3.06 ± 0.01 D	577.0 ± 0.6 E	821.0 ± 1.0 E	1174.7 ± 2.3 E	75.2 ± 9.9 C
p-value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

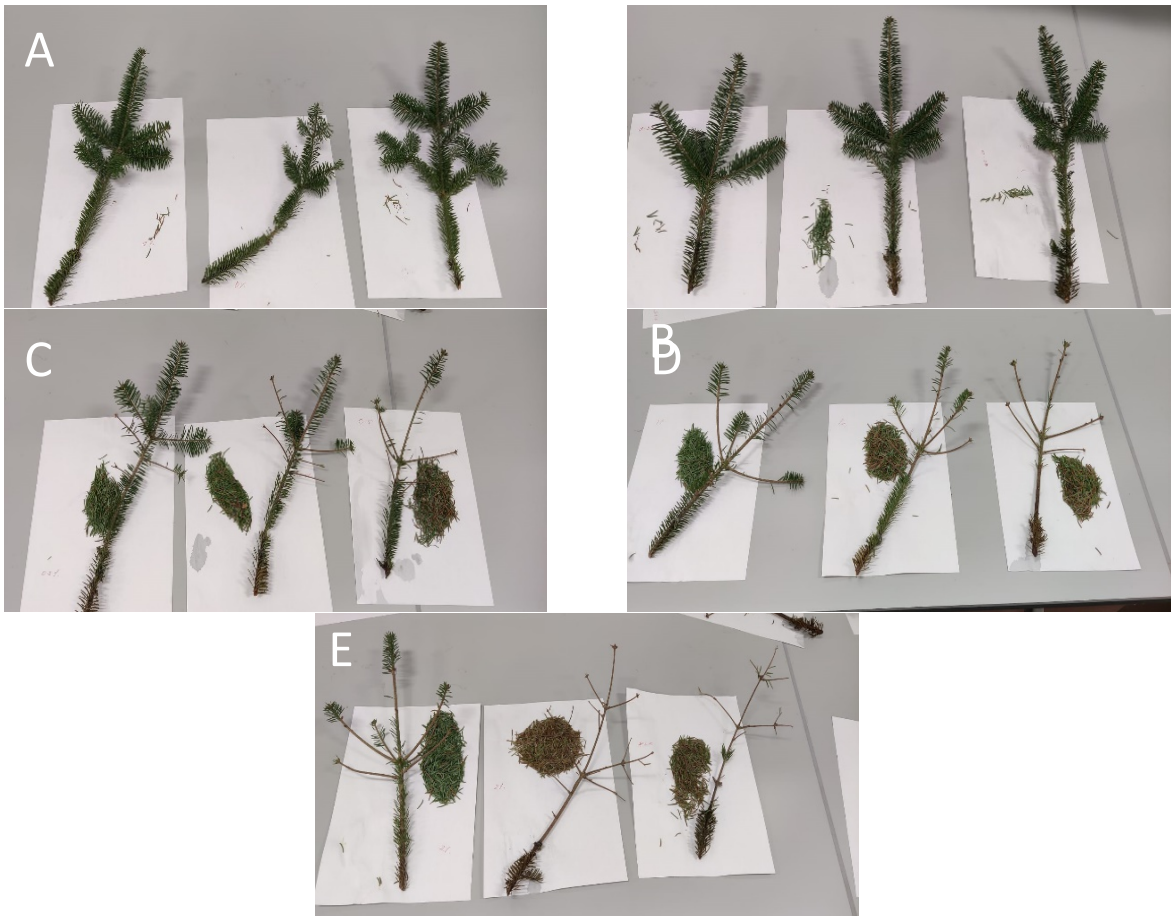

Figure 1. Visual inspection of branches after finger run test when displayed in A) 0%, B) 0.25%, C) 0.5%, D) 1%, or E) 2% pyroligneous acid.

3.1.2. PA as a Foliar Spray

There was a significant (P <0.001) difference in all water quality response variables due to PA concentration (Table 2). These changes were consistent with the other preliminary experiment using PA as a water additive.

Table 2. Water quality parameters of foliar PA spray. Different letters indicate significant differences between treatments at 5% significance.

Concentration (%)	pH	Salinity (ppm)	TDS (ppm)	EC (µS)
0	6.15 ± 0.09 A	441.0 ± 0.6 A	634.7 ± 1.2 A	908.3 ± 1.3 A
0.5	3.39 ± 0.03 B	522.3 ± 0.7 B	745.0 ± 1.0 B	1068.3 ± 1.3 B
1	3.30 ± 0.02 B	561.5 ± 1.5 C	800.7 ± 0.9 C	1143.3 ± 3.5 C
2	3.10 ± 0.05 C	614.7 ± 0.7 D	871.3 ± 4.2 D	1244.3 ± 4.2 D
4	3.04 ± 0.05 C	680.0 ± 14.2 E	958.7 ± 17.6 E	1370.3 ± 26.3 E
p-value	< 0.001	< 0.001	< 0.001	< 0.001

There was also a significant different (P = 0.035) in needle abscission due to foliar spray treatments (Fig. 2). The no spray control lost significantly more needles than any other treatment, while 1% and 4% PA sprays lost significantly less needles. The relative differences are impressive. The 1% PA treatment lost 78% fewer needles than the no spray control and 69% fewer needles than the water only spray. The 4% PA treatment lost 66% fewer needles than the no spray control and 55% fewer needles than the water only spray. However, it is also important to realize that the raw numbers are all very small percentages of total needles. Ultimately, all branches retained needles reasonably well throughout the approximately 5 weeks of this experiment. But the 1% and 4% PA foliar treatments performed significantly better statistically, though perhaps not practically.

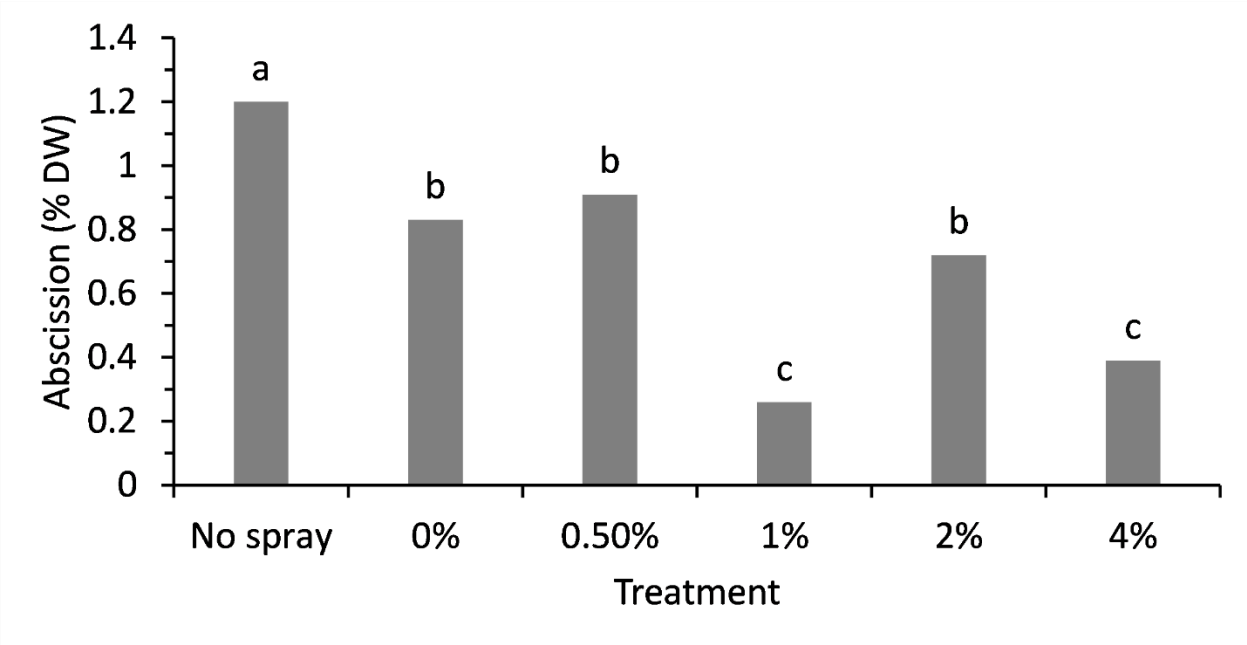

Figure 2. Needle abscission percentage in balsam fir seedlings after foliar spray with various concentrations of pyroligneous acid. Each treatment mean was calculated from 5 replicates and those bars with different letter groupings are significantly different (P < 0.05).

3.2. Experiment 2

It was determined that 1% needle loss occurred between 6 to 8 days (Table 3). The 5% needle loss occurred between 16 to 18 days and 10% needle loss occurred between 20 to 24 days. So, after approximately 3 weeks there is no marketable produce as producers prefer less than 10% needle loss. The P-values for the percent needle loss for the critical values was also more than 0.05, which means that PA application had no significant (P > 0.05) effect on the percent needle loss of the balsam fir trees.

The P-Value for the average water uptake was 0.405. There was an increasing trend in the water uptake from 0 - 4% (Figure 3). Although there is a trend the average water use has been noted to be insignificant over the 56 days since the P value was greater than 0.05. From the Figure 2 we can see that there was significant overlapping between the error bars indicating that the variability within the data points is high reinforcing that the results are not statistically significant.

Table 3. Number of days to reach critical needle loss values for each PA treatment. Means and SE were calculated from 5 replicates.

Treatment	1% NL	5% NL	10% NL	50% NL	100% NL
Control	6.8±2.73	16±3.85	20±3.74	34±4.69	41.2±5.39
0%	8.8±1.36	18.8±1.5	24±2.68	36.4±5.04	43.6±5.08
1%	6.4±2.15	16.8±4.18	24±5.87	33.6±2.93	40.8±3.07
2%	8.8±3.2	17.2±4.59	21.2±5.16	31.6±3.92	44±5.25
4%	7.6±2.64	17.6±1.94	20.8±1.62	34.4±4.75	44.8±6.09
P- Value	0.773	0.9	0.665	0.843	0.91

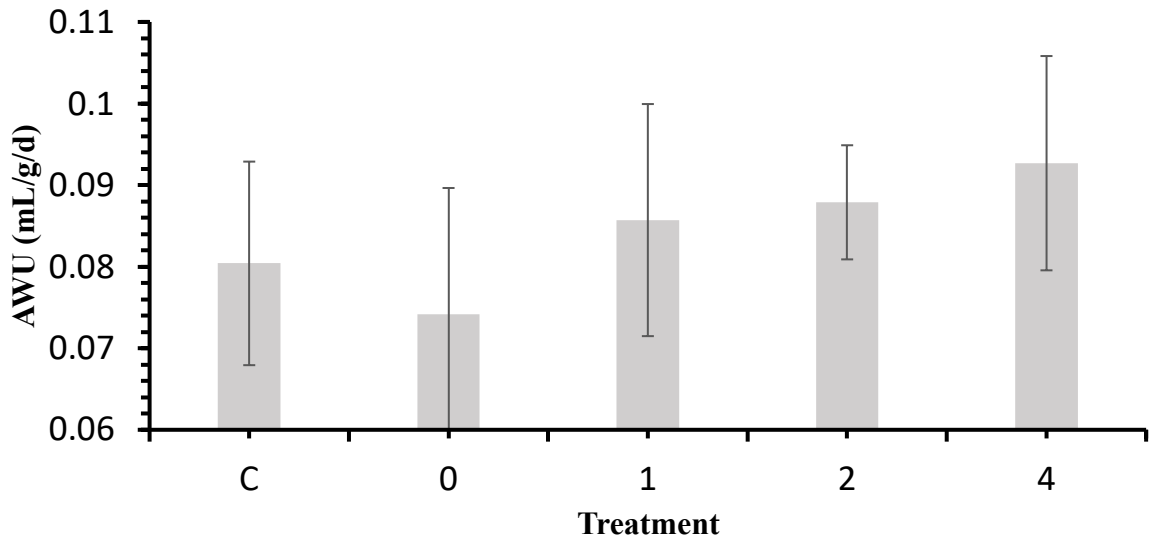

Figure 3. Average water use of balsam fir branches exposed to foliar treatment of PA. Bars were calculated from 5 replicates.

Present methods of crop cultivation utilize natural substances to enhance overall plant well-being and improve the quality of produce, while maintaining environmental and agroecological integrity. As a result, the functional properties of several natural substances, including PA, have piqued the interest of producers and scientists. The effect of bamboo pyroligneous acid (BPA) on the intrinsic antioxidant capacity and defense responses of harvested apple fruit is investigated in a paper by Liu et al. [30]. The study discovered that BPA treatment improved the intrinsic antioxidant capacity of the fruit and induced defense responses, which could potentially alleviate quality deterioration and control diseases caused by fungal pathogens post-harvest. Tomatoes pre-treated with pyroligneous acid also showed lesser weight loss post-harvest in tomatoes [31]. However, the benefits of pyroligneous acid application may vary across different plant species. In contrary to the observed positive outcomes in apples and tomatoes, the experiment performed with balsam fir did not yield statistically significant results.

Despite the lack of statistically significant effects on the percent needle loss and water uptake post-harvest, PA seemed to have slightly increased the average water uptake and this is beneficial as increased water uptake leads to decrease in needle retention [32]. Even though the water uptake was not statistically significant, in Figure 2 we can see an upwards trend which does show that it might have increased the water uptake to a certain extent.

There are several improvements to the experimental design that could be of help if repeated in the future. One such improvement is the application of a surfactant along with the spray solution. The cuticle in conifers is a continuous extracellular membrane composed of biopolymer cutin and wax-like lipids, embedded within the cutin matrix or covering its outer surface. In addition to preventing excessive transpiration and loss of nutrients, the cuticle protects the inner tissue from penetration of substances and pathogens [33]. Since they have thick cuticles, a surfactant should be used to help PA retain on the needles for longer, which would give it the time to penetrate into the needles. Using a surfactant might produce better results. We could design the experiment with more replicates. More replicates could be used to give a better idea as we may lessen the influence of random variation and achieve a more accurate assessment of the treatment effects by increasing the number of replicates in the experiment. This may help establish whether or not the treatment is effective.

Even though there were no statistically significant results, the average water uptake (Fig. 3) showed an increasing trend as the PA concentration increased. Henceforth we could design the experiment with different concentrations of PA. The PA concentration range could be altered and the experiment could be repeated again since there was an increasing pattern in water uptake we could try using higher concentrations of PA > 4%.

The results of the preliminary and the main experiment were not the same and we expected this result since balsam fir trees are known to lose needles faster in the summer months than in the winter months since they have cold acclimation during the winter months [34]. The experiment could be conducted in October to both simulate the harvest period for long distance transport and to avoid cold acclimation. There also could be some sources of error such as too much mechanical pressure applied during finger run test on some branches than the others. Also, some needles falling into the amber flasks and increasing the mass of water leading to incorrect results in the water uptake. As much as the blocking factor is important, genotype might have also been a reason for the insignificant results.

3.3. Experiment 3

The study investigated the impact of different concentrations of PA on various biochemical components in balsam fir needles over an eight-week experiment. The main effect graph (Fig. 4) shows that the chlorophyll a content increased significantly ($P < 0.001$) when the PA concentration increased. There is also a significant ($P < 0.05$) increase in chlorophyll a content as the time increases. The chlorophyll b content had similar results as chlorophyll a. Chlorophyll b content in balsam fir needles increased significantly ($P < 0.001$) as the PA concentration increased. In terms of time, the chlorophyll b content decreased from the second week to the fourth week and stayed consistent through the sixth week. After the sixth week of the experiment, the chlorophyll b content increased and reached its peak in the eighth week (Fig. 5).

The effect of time and PA concentration was not significant ($P > 0.05$) on the carotenoid content of balsam fir needles. The interaction between time and treatment was also not significant ($P > 0.05$) in the carotenoid content of balsam fir needles. The main effects of increase in PA concentration have no significant ($P > 0.05$) effect on phenolic content in balsam fir needles, but there was a significant ($P < 0.001$) effect due to time (Fig 6). Phenolic increased significantly by week 4, but then decreased by week 6.

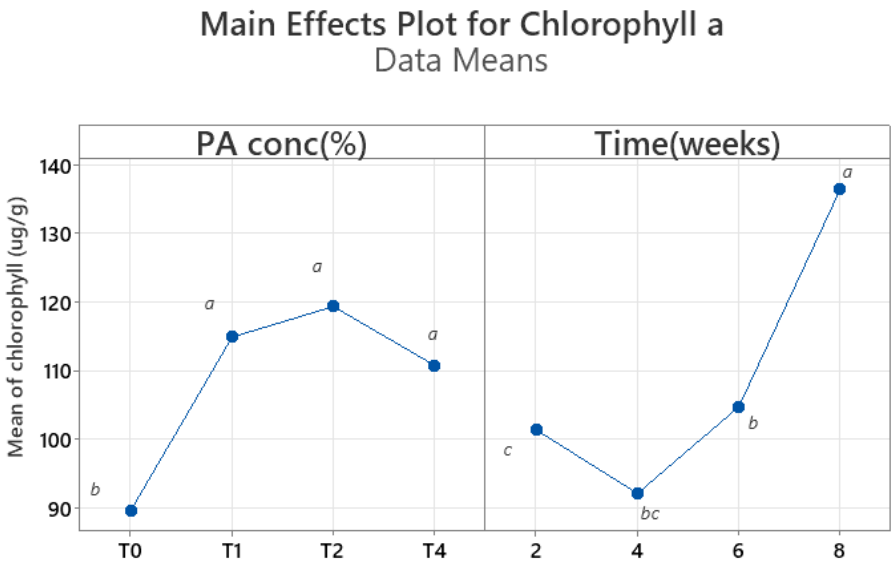

Figure 4. Main effects plot for time and PA concentration on Chlorophyll a content in balsam fir needles. The different letters indicate the significant (P < 0.05) difference according to the Fisher's significant difference (LSD) post hoc test. The means that share the same letters are not significantly different.

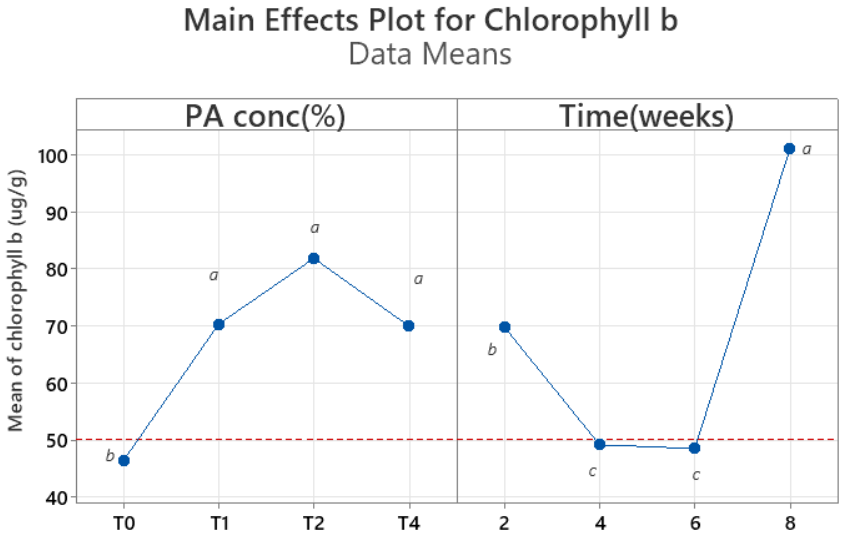

Figure 5. Main effects plot for time and PA concentration on Chlorophyll b content in balsam fir needles. The different letters indicate the significant (P < 0.05) difference according to the Fisher's significant difference (LSD) post hoc test. The means that share the same letters are not significantly different.

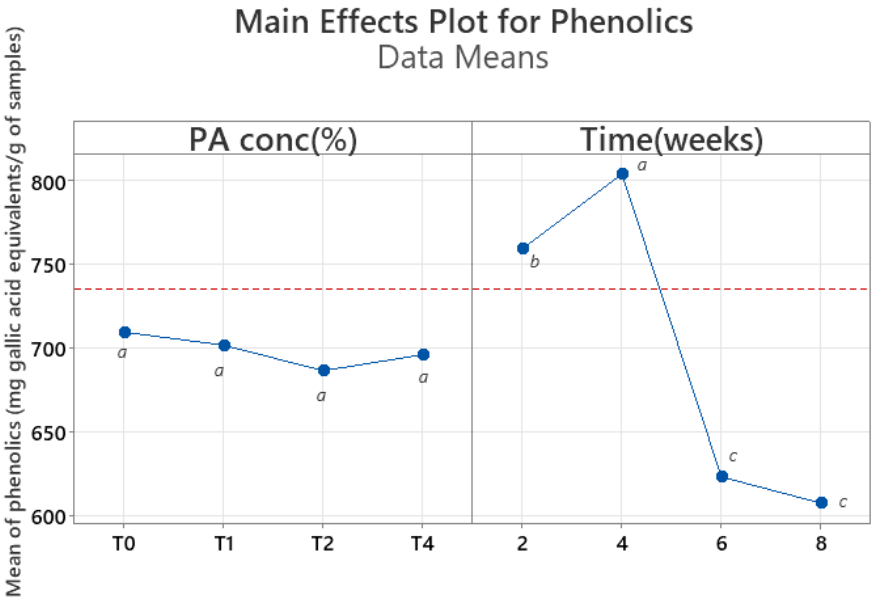

Figure 6. Main effects plot for time and PA concentration on phenolics content in balsam fir needles. The different letters indicate the significant (P < 0.05) difference according to the Fisher's significant difference (LSD) post hoc test. The means that share the same letters are not significantly different.

The flavonoid content increased significantly ($P < 0.05$) with the increase in PA concentration especially when the PA is applied at 1% and 2% concentration (Fig. 7). Flavonoid content decreased significantly ($P < 0.05$) from the second week to the fourth week and increased from the fourth to the eighth week. PA concentration and time each had a significant ($P < 0.001$) effect on proline content (Fig. 8). Proline content increased significantly ($P < 0.001$) over time with increasing PA concentration.

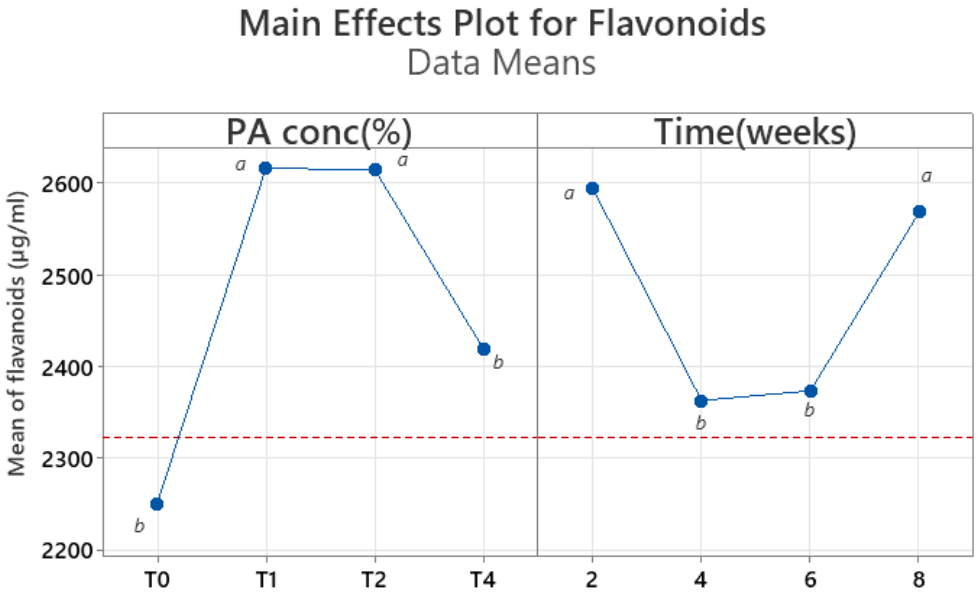

Figure 7. Main effects plot for time and PA concentration on flavonoid content in balsam fir needles. The different letters indicate the significant (P < 0.05) difference according to the Fisher's significant difference (LSD) post hoc test. The means that share the same letters are not significantly different.

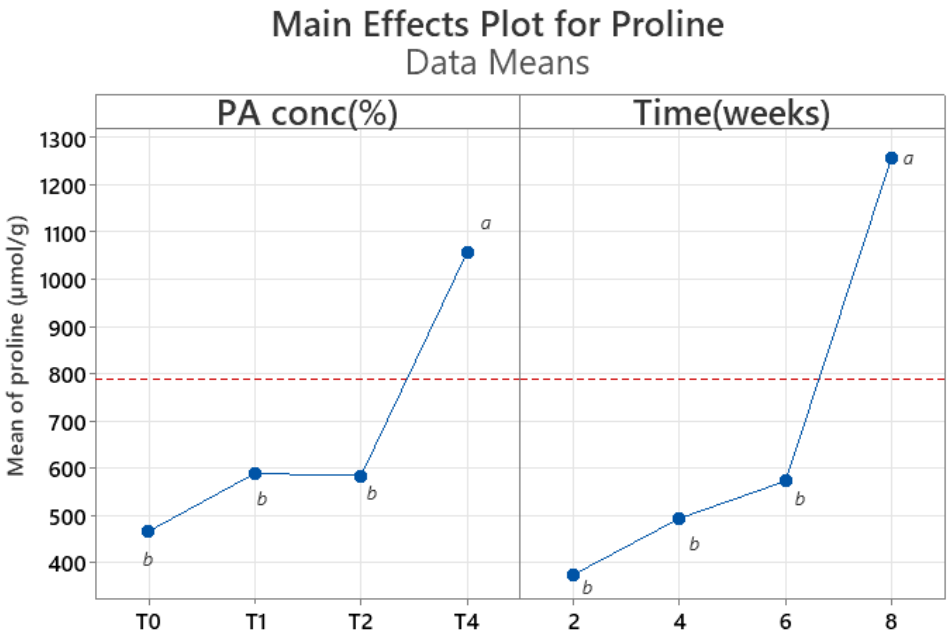

Figure 8. Main effects plot for time and PA concentration on proline content in balsam fir needles. The different letters indicate the significant (P < 0.05) difference according to the Fisher's significant difference (LSD) post hoc test. The means that share the same letters are not significantly different.

Regarding ROS concentration, a consistent increase was observed from the fourth to the eighth week with increasing PA concentration (Fig. 9). There was also an increase in ROS concentration when 1% or 2% PA was applied. However, foliar PA significantly ($P < 0.001$) decreased the membrane injury in balsam fir needles, though membrane injury increased over time postharvest (Fig. 10).

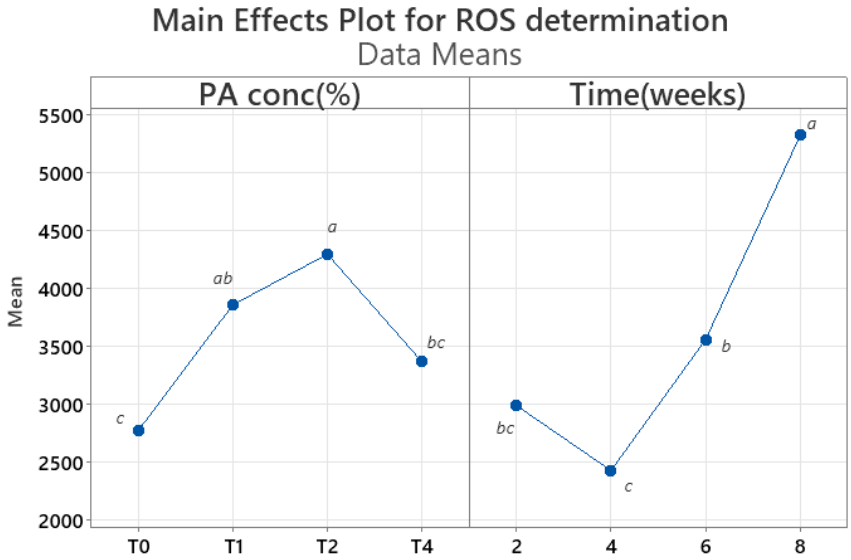

Figure 9. Main effects plot for time and PA concentration on ROS concentration in balsam fir needles. The different letters indicate the significant (P < 0.05) difference according to the Fisher's significant difference (LSD) post hoc test. The means that share the same letters are not significantly different.

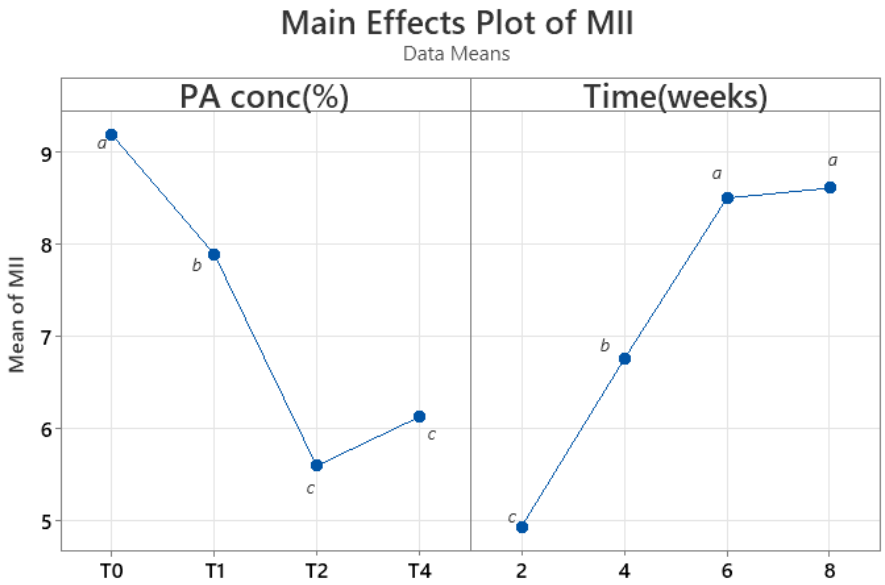

Figure 10. Main effects plot for time and PA concentration on membrane injury index in balsam fir needles. The different letters indicate the significant (P < 0.05) difference according to the Fisher's significant difference (LSD) post hoc test. The means that share the same letters are not significantly different.

Studies have demonstrated the biophysical, hormonal change and physiology of needle abscission in balsam fir [13,32] but there are no studies conducted to understand the change in the phytochemicals such as phenolics, flavonoids, proline, and ROS in balsam fir needles. Usually in conifers including balsam fir, the chlorophyll contents and the fluorescence decrease gradually after harvesting [8]. However, the results of the present study showed that 2% PA application increases chlorophyll a and b contents in balsam fir needles after harvesting. A study by Benzon and Lee [35] in Indian mustard showed that PA has a positive effect in increasing the chlorophyll content. This suggests that PA as a foliar application can increase the chlorophyll contents and thereby increase the photosynthetic efficiency of balsam fir needles. This will help keep the needles fresh and intact for a longer time.

This study found that 4% PA increased the proline content in the eighth week. Proline, an amino acid found in plants, serves as a crucial asset when plants face stressful conditions. It performs multiple essential functions during stress by acting as an osmolyte, a metal binder, an antioxidant, and a signalling molecule [36]. PA has a positive effect in accumulating proline as a response to stress to counteract production and accumulation of H_2O_2 [37].

Pyroligneous acid at 2% foliar application was found to be effective in increasing the flavonoids content in the balsam fir needle after harvesting. Flavonoids play significant role as antioxidants in stressed plants by reducing the generation of ROS and exhibit ROS scavenging properties [38]. The result showed that in the eighth week the flavonoid content was increased significantly by foliar application of 2% PA. This is result is backed by a study in tomato, where foliar application of 2% PA significantly increased the flavonoids contents under aluminium stress conditions [37].

Reactive oxygen species like H_2O_2 is the reason for cell damage and oxidative stress in plant cells [39]. In this experiment the 2% PA increased the concentration of H_2O_2 in the eighth week. PA is acidic in nature and is rich in acetic acid that might induce moderate stress to improve the production of antioxidants by inducing the Nrf2 pathway in the cells which in turn will help in maintaining the redox balance [40]. The previous results also show an increase in flavonoids content when PA was applied at 2% in the eighth

week which suggests that the production of H_2O_2 induced the production of flavonoids and thereby maintained redox balance in the balsam fir needles.

Membrane injury index increases as balsam fir branches are exposed to stress and dehydration (Lada & MacDonald, 2015). The ROS produced during stress can induce lipid peroxidation and thereby, cause membrane damage and increased ethylene production [41]. The production of antioxidative enzymes such as super oxide dismutase will produce antioxidants to counteract the ROS produced and reduce membrane damage. Application of PA at 4% concentration on balsam fir needles reduced membrane damage significantly in the eighth week. A study in canola seed by Yang et al. [42] showed that PA has a positive effect in increasing the SOD activity and reducing oxidative stress. Therefore, the antioxidant property of PA reduced oxidative stress in needles and also reduced membrane damage. These results suggest that PA can be a good substance for maintaining the phytochemical composition of balsam fir after harvest.

4. CONCLUSION AND RECOMMENDATIONS

It was concluded after preliminary tests that PA was not effective at delaying abscission in balsam fir when fed via the xylem. If anything, abscission was increased through such an application. It was speculated that significant changes to water chemistry, specifically a decrease in pH, caused damage to balsam fir. If water pH was adjusted to neutral after PA was added, then perhaps PA could have a positive effect. Though any benefit is purely speculative; xylem-fed PA was not explored further in this study.

It was concluded after preliminary tests that PA had potential to delay abscission in balsam fir when applied as a foliar spray. PA concentrations of 1% and 4% significantly delayed abscission. However, our own results were contradicted in a second experiment where there was no effect on abscission. The mixed results on needle retention warrant further investigation. If work continues PA in balsam fir, it is recommended that additional surfactant additives be tested to allow PA the best opportunity to be absorbed.

PA significantly modified some other characteristics of balsam fir. Chlorophyll and flavonoid concentration in balsam fir increased after PA was applied, which may have been linked to an observed decrease in membrane injury. Although this did not consistently translate to improved needle retention, the above changes should be beneficial to balsam fir and help to justify further studies on the effectiveness of PA.

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EXECUTIVE SUMMARY OF RESEARCH REPORT (FOR PUBLIC RELEASE)

Three experiments were conducted to determine the effect of pyroligneous acid (PA) on balsam fir Christmas trees. Experiment 1 compared PA applied via the xylem as a Christmas tree stand additive to a foliar spray. PA was ineffective when fed via the xylem, but 1% and 4% foliar sprays significantly delayed abscission. Experiment 2 was conducted to confirm the effect of foliar sprayed PA on needle retention but did not have the same success as Experiment 1. There was no significant effect on needle retention in Experiment 2. Finally, Experiment 3 examined changes to certain phytochemicals and other characteristics after a PA spray. Though PA did not improve needle retention, there were significant increases in chlorophyll a, chlorophyll b, and flavonoids. These changes were associated with a decrease in membrane injury.

Overall, PA was beneficial to balsam fir through increases in beneficially phytochemicals and membrane stability. However, such increases did not consistently translate to improved needle retention. It is recommended that PA be explored further as a foliar spray using different surfactant agents that may help PA permeate into balsam fir needles.

LIST OF PUBLICATIONS

Nothing has been published to date, but one manuscript is being prepared:

MacDonald MT, Abbey L, Kasu R, Senthilkumar N. 2024. Effect of foliar pyroligneous acid applications on postharvest needle abscission and phytochemical composition of balsam fir. Plants. Expected submission date September 2024.

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