

Article

Wood Quality and Growth Characterization across Intra- and Inter-Specific Hybrid Aspen Clones

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Abstract: Trembling aspen (*Populus tremuloides* Michx.) is one of the most abundant poplar species in North America; it is native, displays substantial breadth in distribution inhabiting several geographical and climatic ecoregions, is notable for its rapid growth, and is ecologically and economically important. As the demand for raw material continues to increase rapidly, there is a pressing need to improve both tree quality and growth rates via breeding efforts. Hybridization is considered one of the most promising options to simultaneously accelerate these tree characteristics, as it takes advantage of heterosis. Two aspen species showing particular promise for hybridization with trembling aspen are European aspen (*P. tremula*) and Chinese aspen (*P. davidiana*) because their native climates are similar to that of *P. tremuloides* and are also very easy to hybridize. In 2003, aspen clones were planted in Athabasca, Alberta from the following species crosses: open pollinated (OP) *P. tremuloides* (NN), OP *P. davidiana* (CC), *P. tremula* × *P. tremula* (EE), *P. tremula* × *P. tremuloides* (EN), and *P. tremuloides* × *P. davidiana* (CN). In November 2010, growth measurements and core samples were taken from seven-year field grown clones. Comparisons of the mean growth and cell wall traits were made between crosses using generalized linear model least squares means tests for stem volume, fiber length, fiber width, coarseness, wood density, microfibril angle, total cell wall carbohydrate and lignin content, and lignin composition. The results clearly indicated that the inter-specific

crosses offer a means to breed for more desirable wood characteristics than the intra-specific *Populus* spp. crosses.

Keywords: *Populus*; *P. tremuloides*; *P. tremula*; *P. davidiana*; hybridization; wood quality

1. Introduction

1.1. *Populus tremuloides*

Populus comprised of both poplars and aspens, is Canada's most widespread and fastest growing tree genus [1]. It constitutes over 50% of all Canadian-grown hardwoods and approximately 11% of all timber resources in Canada [2]. It can be found in North America, Europe, and Northeast Asia. Specifically, trembling aspen (*Populus tremuloides* Michx.) is Canada's most abundant aspen species; it is native nationwide and is considered a commercially important hardwood species in western Canada [3–5]. Its importance is due to a rapid growth rate resulting in shorter rotation times compared to other hardwood species grown in similar environments [6]. The primary uses for trembling aspen include oriented strand board (OSB) manufacture and bleached Kraft hardwood pulp production [5,7]. There is also potential for aspen to be used as a source for energy production through its conversion into biofuels such as ethanol. However, challenges remain which currently limit the effective conversion of lignocellulosic material into liquid bioenergy, which need to be overcome before aspen can be employed in such applications [8]. As the demand for raw materials increase, there is an added incentive to improve tree quality and growth rates through breeding, particularly in fast-growing species suitable for Northern climates.

1.2. *Aspen Breeding*

One putative mechanism of enhancing aspen wood quality and productivity is breeding focused on improvements in desirable wood attributes and growth rates. Early work on aspen improvement successfully demonstrated increased growth rates, and to a lesser extent improved wood fiber quality traits [4,9,10]. Naturally, there is substantial genetic diversity available in native aspen that can be exploited via selective breeding, inter-specific hybridization, and cloning; and, a combination of the three would be ideal for substantial realized gains [11]. In particular, hybridization is considered the best approach to improve productivity and wood quality [9,12,13].

Programs attempting to utilize aspen hybridization strategies have been successful in the United States, Germany, and China [9,12–14], as well, testing in Finland showed faster juvenile growth in hybrid aspen trees compared to the parental material [15,16]. More specifically, productivity in the United States was substantially improved and as such decreased rotation age from 40 to 20 years [12]. There is also potential for improved wood quality in combination with superior growth using hybrid aspen breeding strategies [17]. Similar breeding programs also appear to be possible in Canada, if well designed programs are developed [12,18].

Two promising species for hybridization with trembling aspen are European aspen (*P. tremula* L.) and Chinese aspen (*P. davidiana*) because their native climates are similar to that of Canadian grown

P. tremuloides. Previously in the United States, *P. tremuloides* and *P. tremula* crosses demonstrated improved growth, form, and wood quality; while, *P. tremuloides* and *P. davidiana* crosses had superior growth characteristics on relatively poor and dry sites [12]. Using a similar strategy, it was postulated that similar results could be achieved in Canada, if the appropriate inter-specific crosses of parent trees from northern European countries such as Finland and from north-eastern China were employed [19].

1.3. Heterosis

A theory for the improved growth observed in inter-specific hybrid crosses of aspen is heterosis. Numerous studies have shown both significant and stable heterosis in F1 hybrids of *Populus* spp. However, the genetic cause is not yet fully understood [10,12,20–22]. There are two main hypotheses commonly used to explain the genetic basis of heterosis: dominance and overdominance effects [23]. A review of the mechanisms involved with the heterosis phenomenon proposes there is additional, epigenetic machinery regulating multigenic traits causing increased efficiencies and greater growth in hybrid plants both through increased energy input, by photosynthetic efficiency, and less energy expenditure for basic metabolism [24].

Hybrid aspens demonstrating heterosis were analyzed using a quantitative approach to determine the genetic cause [25]. The results suggest heterosis between *P. tremuloides* and *P. tremula* may be caused by overdominance. In contrast, improved growth in hybrids compared to their parents could be accounted for by a later bud set in hybrid aspen [26]. Similar findings by Yu *et al.* [15] also concluded that the perceived hybrid vigor was a function of the observed extended growth period [15]. As such, the authors suggested the improved vigor observed was not necessarily true heterosis commonly assumed to occur in aspen. Nonetheless, there was a desirable increase in growth in the hybrids.

1.4. Study Objectives

The aim of this study was to (1) characterize the wood quality and tree growth of *P. tremuloides*, *P. tremula*, and *P. davidiana* clones grown in Alberta, Canada, and (2) investigate the effects of *P. tremula* and *P. davidiana* hybridization with *P. tremuloides* on wood quality and tree growth.

Productivity was quantified by an estimation of volume calculated from tree height and breast height diameter. Wood quality attributes were quantified through examination of increment cores taken at breast height to specifically quantify fiber length, fiber width, coarseness, wood density, microfibril angle, total cell wall carbohydrate and lignin content, and lignin composition.

The characterized tree and wood properties offer insight into whether or not inter-specific crossing of these aspen species can be achieved in western Canada and, more importantly, if this breeding strategy can be used to improve potential future generations of aspen on the northern landscape.

2. Experimental Section

2.1. Sample Procurement

In 2003, poplar clones were planted in two adjacent plots, consisting of the main growth trial plot and a secondary plot for wood evaluation, which were located within one kilometer of each other near the Alberta-Pacific pulp mill in Athabasca, Alberta (54°53' N, 112°51' W, 575 m). The trees represent clones from one of the following seven species crosses: two open pollinated (OP) *P. tremuloides* families, two OP *P. davidiana* families, a single *P. tremula* × *P. tremula* family, a single *P. tremula* × *P. tremuloides* family, and a single *P. tremuloides* × *P. davidiana* family. All four OP crosses are assumed to be intra-specific crosses due to the mother parent's proximity to its own species (Table 1).

Table 1. Species crosses, their associated abbreviations, and geographical origin of parents.

Genetics classification	Species	Abbreviation	Mother Origin	Father Origin
Intra-specific (OP)	<i>P. tremuloides</i> × <i>P. tremuloides</i>	NN	55°25' N, 113°21' W	-
Intra-specific (FS)	<i>P. tremula</i> × <i>P. tremula</i>	EE	60°32' N, 24°29' E	60°37' N, 24°31' E
Intra-specific (OP)	<i>P. davidiana</i> × <i>P. davidiana</i>	CC	47° N, 128° E	-
Inter-specific (FS)	<i>P. tremula</i> × <i>P. tremuloides</i>	EN	60°32' N, 24°15' E	54°50' N, 119°32' W
Inter-specific (FS)	<i>P. tremuloides</i> × <i>P. davidiana</i>	CN	55°7' N, 111°28' W	47° N, 128° E

OP = Open Pollinated, FS = Full Sibling.

In November 2010, growth measurements were taken from the trial plot, while the secondary plot was used to collect increment wood cores. Twenty to 30 trees from each cross type were measured for height and diameter at breast height (DBH). At the same time, 20 to 30 trees from each cross type were cored (5 mm diameter increment core) twice from bark to bark on the north side of the tree, with the exception of the *P. tremula* × *P. tremula* cross, which only had 12 trees available for coring.

2.2. Growth Traits and Core Sampling

Tree height was measured using an extendable meter stick. Trunk diameter was measured at breast height (1.3 m) using electronic calipers. Stem volume was calculated for all crosses using equation (1) for aspen according to British Columbia Forest Inventory Division [27]:

$$\log V = -4.538904 + 1.834410 \log DBH + 1.208970 \log H \quad (1)$$

where, V is the volume (m^3), DBH is the diameter at breast height (cm), and H is the tree height (m) [27]. One increment core taken from the wood quality plot trees was used for density, microfibril angle, and fiber measurements, while the second core was employed for chemical analysis.

2.3. Wood Density

The average wood density was measured from the southern section of one core from pith to bark, along the radial face. The cores were precision sawn to 1.68 mm thick sections using a twin blade pneumatic circular saw. Cut sections were then extracted in a Soxhlet apparatus with hot acetone for 24 h and allowed to acclimate to 7% moisture. The average density was determined for the entire length of the section, using an X-ray densitometer scanned at a 0.254 nm resolution (Quintek Measurement Systems Inc., Knoxville, USA).

2.4. Microfibril Angle

Microfibril angle (MFA) was measured on the radial face of the growth ring closest to the bark by X-ray diffraction. The 002 diffraction spectra were obtained for T-value distribution using a Bruker D8 discover X-ray diffraction unit equipped with a general area array detector (GADDS). Measurements were performed using wide-angle diffraction in the transmission mode with $\text{CuK}\alpha_1$ radiation ($\lambda = 1.54 \text{ \AA}$). The X-ray source was fit with a 0.5 mm collimator and the scattered photon were collected by the GADDS detector. Both the X-ray source and detector were set to $\theta = 0^\circ$. The average of the T-values from each of the two 002 diffraction arc peaks was used to calculate the MFA.

2.5. Fiber Length, Width, and Coarseness

Samples, representing wood from pith to bark, were macerated in Franklin's solution (1:1 glacial acetic acid and 30% hydrogen peroxide) at 70 °C for 48 h. The fibers were then washed with deionized water to remove residual Franklin's solution and suspended in deionized water and gently stirred in a Waring blender until fully separated. The fibers in suspension were then collected by vacuum on filter paper and dried in an oven at 50 °C for 24 h. Approximately 0.350 mg of dried fiber was re-suspended in deionized water and gently stirred in a Waring blender and their attributes were determined on a Fiber Quality Analyzer (OpTest Equipment Inc., Hawkesbury Ontario, Canada).

2.6. Cell Wall Chemistry

Wood from pith to bark was ground in a Wiley mill until it passed through a 40 mesh screen and Soxhlet extracted with hot acetone for 24 h and then oven dried at 105 °C for 24 h. Three mL of 72% (w/w) H_2SO_4 was pipetted into a test tube containing approximately 200 mg of dried material and was mixed for 30 sec every 10 min. After two h, the contents of the test tube were transferred to a serum bottle using 112 mL nanopure water. The serum bottles were then sealed and autoclaved at 121 °C for 60 min. After autoclaving, the contents of the bottles were allowed to cool, then vacuum filtered through a pre-weighed medium coarseness crucible (Pyrex, USA) and 15 mL filtrate collected for further analysis. The retentate was rinsed with 60 mL of deionized water to remove any residual sugars and acid. The crucibles containing retentate were oven dried at 105 °C for 24 h and then re-weighed to obtain the insoluble lignin content of the wood gravimetrically. A sample of filtrate was analyzed for acid-soluble lignin at 205 nm. The total lignin (soluble and insoluble) is expressed as a proportion of the initial extractive free wood.

Approximately 0.9 mg of the solubilized filtrate and 0.1 mg of fucose (5mg/mL) internal standard was mixed and filtered through a 0.45 µm nylon filter into a glass vial. The total carbohydrate content (arabinose, rhamnose, galactose, glucose, mannose, and xylose) was determined using an anion exchange high-performance liquid chromatograph (Dx-600; Dionex, Sunnyvale, CA, USA) equipped with an ion exchange PA1 (Dionex) column, a pulsed amperometric detector with a gold electrode, and a SpectraAS3500 auto injector (Spectra-Physics, USA). The concentrations of arabinose, rhamnose, galactose, glucose, mannose, and xylose were calculated in proportion to the initial extractive free wood and combined as total carbohydrate concentration.

2.7. Syringyl-Guaiacyl Ratio

Ten mg of oven dried extract-free wood was used to determine the lignin monomer composition. One mL of a reaction mixture (8.75 mL dioxane, 250 µL BF₃, and 1 mL ethaninol) was added to a 6 mL reaction vial containing the dried material and purged with N₂ gas before the lid was tightly sealed. Vials were placed in a heating block at 100 °C for 4 h with periodic (hourly) agitation. The vials were transferred to a −20 °C fridge for 5 min to halt the reaction. Then, 200 µL of internal standard (5 mg tetracosane/1 mL methylene chloride) and 300 µL 0.4 M NaHCO₃ were added to the vial to bring the pH between 3 and 4. Next, 2 mL of nanopure water and 1 mL methylene chloride were added to the vial, which was then recapped, vortexed, and allowed to separate into two phases. One mL of the lower phase was drawn by pipette, filtered through anhydrous Na₂SO₄, and finally transferred directly into a 2 mL polypropylene safe-lock microfuge tube. The sample was evaporated to dryness in a Speedvac set to 45 °C and then resuspended in 700 µL of methylene chloride. Twenty µL of resuspended sample was derivatized by combining it with 20 µL of pyridine and 100 µL of N,O-bis(trimethylsilyl) acetamide in a glass insert within an amber-glass vial. The vial was sealed and inverted to mix, and allowed to incubate for at least 2 h at 25 °C prior to analysis. Finally, 1 µL of solution was analyzed by a gas chromatograph (HP 5890 Series II, Agilent Tech., Ontario, Canada) on an HP 6890 series II column equipped with an auto injector and flame ionization detector (FID) (Agilent Tech., Ontario, Canada) as per Robinson & Mansfield [28].

2.8. Data Analysis

The phenotypic traits assessed in this study compared species cross types for statistical differences among their mean values. In order to simplify the comparisons, the observations from clones with the same intra-specific cross, but different parents, were grouped together reducing the number of comparisons. A generalized linear model (GLM) was utilized using PROC GLM in SAS (SAS Institute V 9.2). The following linear model (2) was used for analysis of each phenotypic trait:

$$Y_{ij} = \mu + T_j + E_{ij} \quad (2)$$

where, Y_{ij} is the individual phenotypic observation of the i th clone of the j th species cross, μ is the overall mean, T_j is the class variable for species cross, and E_{ij} is the residual error of the observation.

Each trait was tested to meet the modeling assumptions. The dependent variable was transformed and/or outliers were removed in order to meet the model assumptions when necessary. The pre-planned comparisons between species cross types were performed using the least squares means

procedure. The comparisons were performed using an alpha level set to 0.0036 to account for testing the effect of cross type on multiple traits simultaneously. Finally, the SAS CORR procedure was used to determine the correlations between wood quality phenotypic traits for all species cross types.

3. Results and Discussion

3.1. Models

Table 2 summarizes the results for the PROC GLM class variable models, Kolmogorov-Smirnov tests for normality, and Bartlett's tests for homoscedasticity. A PROC GLM model ANOVA F-test for significance was performed for each regression. Each model's p -value was smaller than alpha; therefore, all comparisons were considered significant. Next, each PROC GLM model's residuals were tested for normality using the Kolmogorov-Smirnov test. The p -value for each model was again greater than alpha, thus, each model's residuals were normally distributed. Finally, each PROC GLM model's species groups were tested for homoscedasticity using the Bartlett's test. The p -value for all but one model was greater than alpha and had homogenous variance. The exception was the model for volume, where the Bartlett test p -value was 0.005. However, given that this value is extremely close to alpha, the model can and was still be used for the least squares (LS) means comparisons.

Table 2. PROC generalized linear model (GLM) ANOVA of traits using the class variable species (Ho: regression is not significant, Ha: regression is significant, Alpha = 0.05), Kolmogorov-Smirnov test for normality (Ho: data is normally distributed, Ha: data is not normally distributed, Alpha = 0.01), and Bartlett's test for homogeneity (Ho: variances are homogeneous, Ha: variances are not homogeneous, Alpha = 0.01).

Trait	Proc GLM			Kolmogorov-Smirnov	Bartlett
	R-squared	RMSE	F -value (P -value)	D-statistic (P -value)	Chi-Square (P -value)
MFA	0.12	1.07	5.18 (0.001)	0.07897 (0.058)	3.2338 (0.520)
D	0.10	29.25	4.86 (0.001)	0.056371 (>0.150)	8.8251 (0.066)
FL	0.41	0.05	22.06 (<0.001)	0.055896 (>0.150)	2.1548 (0.707)
FW	0.13	1.60	4.66 (0.002)	0.058263 (>0.150)	8.3866 (0.078)
CS	0.11	0.23	3.63 (0.008)	0.054734 (>0.150)	3.1562 (0.532)
S:G	0.52	0.25	47.71 (<0.001)	0.058548 (0.132)	6.7663 (0.149)
VOL	0.47	0.000746	34.28 (<0.001)	0.051991 (>0.150)	14.84 (0.005)

Table 2. Cont.

Trait	Proc GLM			Kolmogorov-Smirnov	Bartlett
	R-squared	RMSE	F-value (P-value)	D-statistic (P-value)	Chi-Square (-value)
TC	0.10	4.53	4.42 (0.002)	0.055846 (>0.150)	9.9356 (0.042)
TL	0.13	1.14	6.27 (<0.001)	0.041626 (>0.150)	3.9646 (0.411)
SOL	0.36	0.51	21.37 (<0.001)	0.0556349 (>0.150)	12.9529 (0.012)
INSOL	0.32	1.1459	18.04 (<0.001)	0.047491 (>0.150)	3.6516 (0.455)

MFA = Microfibril Angle, D = Wood Density, FL = Fiber Length, FW = Fiber Width, CS = Coarseness, S:G = Syringyl-Guaiacyl Ratio, VOL = Volume, TC = Total Carbohydrate Concentration, TL = Total Lignin Concentration, SOL = Soluble Lignin Concentration, INSOL = Insoluble Lignin Concentration, R-squared = Explained Variance, RMSE = Root Mean Squared Error.

3.2. Least Squares Means Comparisons

Least squares means comparisons were performed between pre-determined pairings of the five species cross types to ensure the overall protection level of the test (Table 3). The three intraspecific crosses were compared to one another, and the two interspecific crosses were compared to their corresponding intraspecific crosses for a total of seven species comparisons. The LS-means comparison t-tests and corresponding *p*-values are found in Table 4.

3.2.1. Volume

The mean individual tree volume of the species crosses ranged from 1.14×10^{-3} to 3.11×10^{-3} m³ (Figure 1). The EN cross had the largest average volume, while the NN cross had the lowest average volume. The LS-means tests (Table 4) indicated that volume was the trait with the greatest number of differences. The NN cross displayed significantly less growth compared to the CC and EE crosses, accruing 55.0% and 43.8% less volume, respectively, and the volume of the CC and EE crosses were not significantly different. The EN cross had 172.8% and 53.1% larger volume than the NN and EE crosses, respectively. The CN cross was 51.8% larger than the NN cross, but the CN cross was significantly smaller than the CC cross (31.6% less growth).

Table 3. Least squares means and standard error (in brackets) for each trait by species.

Cross Type ¹	D ²	MFA	FL	FW	CS	S:G	VOL	TC	TL	SOL	INSOL
CC	407.10 (±3.78)	22.5 (±0.1)	0.531 (±0.007)	21.7 (±0.2)	0.072 (±0.002)	1.85 (±0.03)	0.00253 (±0.00011)	64.12 (±0.61)	22.01 (±0.15)	- -	- -
CN	399.10 (±5.43)	23.1 (±0.2)	0.496 (±0.012)	21.4 (±0.4)	0.063 (±0.003)	1.74 (±0.05)	0.00173 (±0.00015)	64.50 (±0.91)	22.93 (±0.23)	2.51 (±0.07)	20.37 (±0.23)
EE	436.22 (±8.44)	23.7 (±0.4)	0.511 (±0.027)	19.4 (±0.8)	0.068 (±0.008)	2.56 (±0.07)	0.00203 (±0.00014)	66.63 (±1.31)	21.36 (±0.33)	- -	- -
EN	413.33 (±5.34)	22.2 (±0.2)	0.537 (±0.011)	22.2 (±0.3)	0.075 (±0.003)	2.22 (±0.05)	0.00311 (±0.00014)	67.69 (±0.83)	21.59 (±0.21)	3.42 (±0.10)	18.12 (±0.19)
NN	398.72 (±4.14)	22.8 (±0.2)	0.428 (±0.010)	20.7 (±0.3)	0.065 (±0.003)	2.29 (±0.04)	0.00114 (±0.00012)	63.82 (±0.66)	21.87 (±0.17)	2.79 (±0.09)	19.04 (±0.19)

¹ CC = *P. davidiana* × *P. davidiana*, CN = *P. tremuloides* × *P. davidiana*, EE = *P. tremula* × *P. tremula*, EN = *P. tremula* × *P. tremuloides*, NN = *P. tremuloides* × *P. tremuloides*; ² D = Wood Density (kg/m³), MFA = Microfibril Angle (degrees), FL = Fiber Length (mm), FW = Fiber Width (μm), CS = Coarseness (mg/m), S:G = Syringyl/Guaiacyl (ratio), VOL = Volume (m³), TC = Total Carbohydrate Concentration (%), TL = Total Lignin Concentration (%), SOL = Soluble Lignin Concentration (%), INSOL = Insoluble Lignin Concentration (%).

Table 4. Least squares means comparison *t*-values and *p*-values (in brackets). Ho: LS-means are equivalent, Ha: LS-means are not equivalent, Alpha = 0.0036. Values in bold are statistically significant.

Cross Type ¹	D ²	MFA	FL	FW	CS	S:G	VOL	TC	TL	SOL	INSOL
NN vs CC	-1.497 (0.136)	1.225 (0.222)	-8.275 (<0.001)	-2.545 (0.012)	-2.048 (0.043)	9.152 (<0.001)	-8.345 (<0.001)	-1.919 (0.057)	-0.651 (0.516)	- -	- -
NN vs EE	-3.988 (<0.001)	-2.471 (0.015)	-2.904 (0.004)	1.539 (0.126)	-0.085 (0.932)	-3.302 (0.001)	-4.740 (<0.001)	-0.336 (0.737)	1.379 (0.170)	- -	- -
CC vs EE	-3.148 (0.002)	-3.282 (0.001)	0.759 (0.449)	2.738 (0.007)	0.840 (0.403)	-8.898 (<0.001)	2.707 (0.008)	-1.742 (0.083)	1.806 (0.073)	- -	- -
EN vs NN	2.163 (0.032)	-2.010 (0.046)	8.229 (<0.001)	3.317 (0.001)	2.328 (0.022)	-1.10323 (0.271)	10.766 (<0.001)	3.659 (<0.001)	-1.032 (0.304)	4.922 (<0.001)	-3.245 (<0.001)

Table 4. Cont.

Cross Type ¹	D ²	MFA	FL	FW	CS	S:G	VOL	TC	TL	SOL	INSOL
EN vs EE	-2.291 (0.023)	-3.711 (<0.001)	1.323 (0.188)	3.223 (0.002)	1.105 (0.272)	-3.853 (0.002)	5.449 (<0.001)	0.686 (0.493)	0.599 (0.550)	- -	- -
CN vs NN	0.056 (0.955)	1.300 (0.196)	4.553 (<0.001)	1.402 (0.163)	-1.036 (0.302)	-9.373 (<0.001)	3.023 (0.003)	0.606 (0.546)	3.782 (<0.001)	-1.679 (0.095)	4.460 (<0.001)
CN vs CC	-1.210 (0.228)	2.547 (0.012)	-2.326 (0.022)	-0.713 (0.477)	-2.976 (0.004)	-1.925 (0.056)	-4.212 (<0.001)	0.347 (0.729)	3.357 (0.001)	- -	- -

¹ NN = *P. tremuloides* × *P. tremuloides*, CC = *P. davidiana* × *P. davidiana*, EE = *P. tremula* × *P. tremula*, EN = *P. tremula* × *P. tremuloides*, CN = *P. tremuloides* × *P. davidiana*; ² D = Wood Density, MFA = Microfibril Angle, FL = Fiber Length, FW = Fiber Width, CS = Coarseness, S:G = Syringyl–Guaiacyl Ratio, VOL = Volume, TC = Total Carbohydrate Concentration, TL = Total Lignin Concentration, SOL = Soluble Lignin Concentration, INSOL = Insoluble Lignin Concentration.

Figure 1. Boxplots of growth and wood quality traits by species cross type. CC = *P. davidiana* × *P. davidiana*, CN = *P. tremuloides* × *P. davidiana*, NN = *P. tremuloides* × *P. tremuloides*, EN = *P. tremula* × *P. tremuloides*, and EE = *P. tremula* × *P. tremula*. From left to right, top to bottom: volume, density, microfibril angle, fiber length, fiber width, coarseness, SG, carbohydrate, and lignin. Whiskers indicate the maximum and minimum values, the top of the blue box indicates the 75th percentile, the line between the boxes represents the median, and the bottom of the green box indicates the 25th percentile.

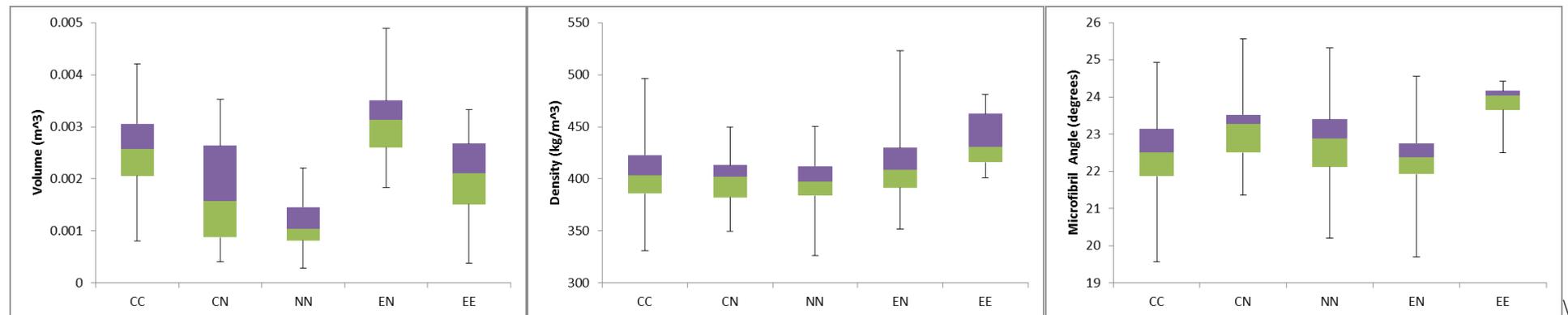
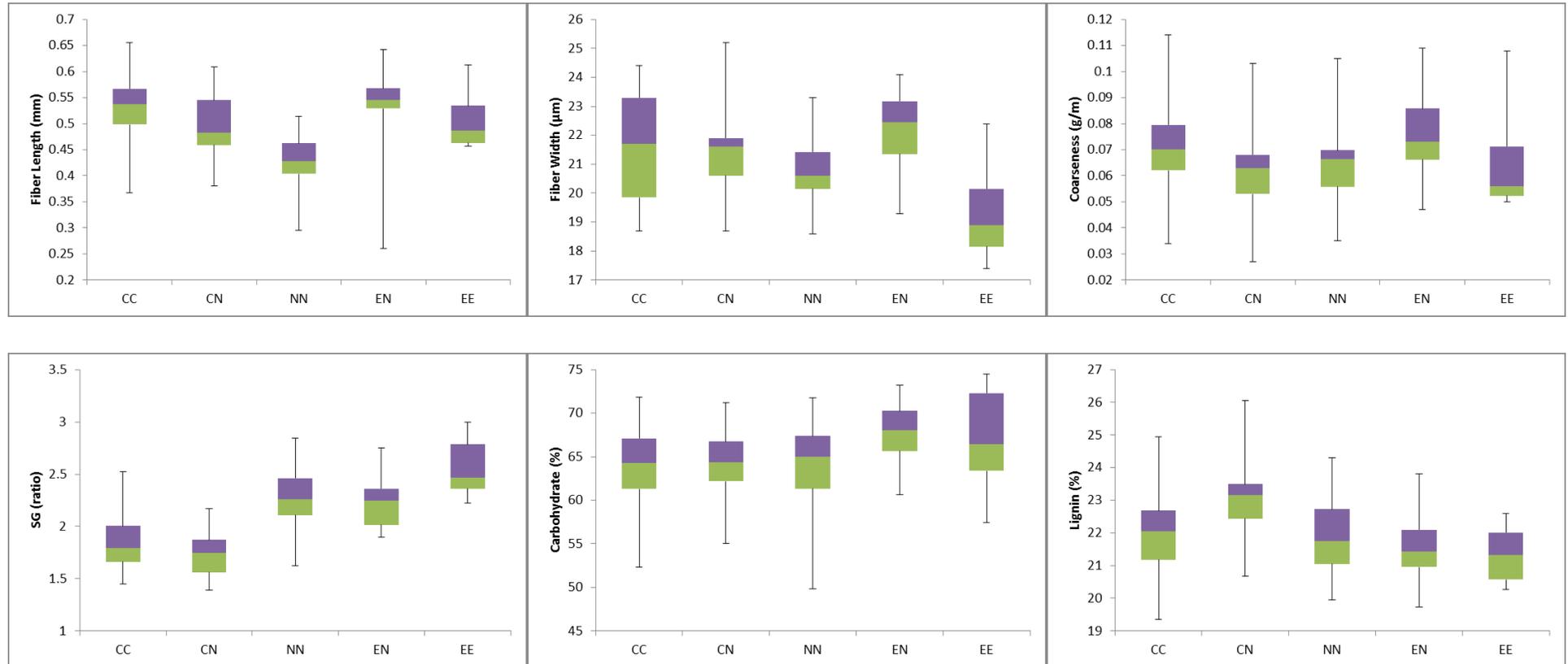


Figure 1. Cont.



When comparing the intra-specific crosses, the average growth of the NN cross was markedly less than the growth of either the EE or CC crosses, while the EE and CC crosses displayed similar growth rates. Li *et al.* [12] previously estimated that the average rotation age of *P. tremuloides* was 40 years and the expected rotation age for hybrid crosses were based on the *P. tremuloides* rotation. For example, the *P. tremuloides* × *P. tremula* inter-specific cross growth doubled that of the pure *P. tremuloides* cross. Therefore, it was predicted that the hybrid's minimum expected rotation was 20 years. In Canada, native stands of *P. tremuloides* typically have a rotation of 60–80 years, which, in this case, can be assigned to the NN cross. Similar rotation ages have also been recommended for hybrid aspen in Northern Europe [16]. Using the aforementioned method of projecting rotation age, the relatively superior growth of the CC and EE crosses over the NN cross could potentially reach merchantable volumes within 27 and 34 years, respectively.

The CN hybrid volume was intermediate, and significantly different from the observed volumes of the NN and CC crosses. In contrast, the volume of the EN cross was greater than the NN and EE crosses. The improved growth observed in the EN and CN crosses is in agreement with other studies investigating heterosis using hybridization of *P. tremuloides* with *P. tremula* or *P. davidiana* [12,13,15]. The superior growth of the EN cross suggests its progeny could reach harvestable size within as few as 22 years. These findings are consistent with Li *et al.* [12] who concluded that stand rotation could be reduced by half in the Lake States region of the United States (US) (*ca.* 20 years). However, the relative growth improvements observed in the EN hybrid over the NN cross exceeds the relative improvements observed by Li *et al.* [12], who examined *P. tremuloides* × *P. tremula* hybrids over intraspecific *P. tremuloides* clones. These findings imply the effects of heterosis may be more pronounced in Canada than the US, or the original parents have a higher breeding value than those used in the US study. Although the CN hybrid was not superior to the CC cross in volume, its improved growth over the NN cross could reduce rotation times from 60 to 40 years, which is a marked improvement over native aspen stands. In addition, the CN cross may have inherent drought tolerance traits derived from the *P. davidiana* parent, something not expected for the EN cross, which could improve its relative performance on drier sites [12].

The biomass of each species cross could be estimated by multiplying their average wood density by their average volume to determine the expected mass derived from a stem. The average biomass estimates for each cross follows similar trends to the volume, but not with density. This trend is likely because the differences in average stem volume between crosses were more pronounced than the differences in wood density. Therefore, species cross types with superior volume growth would also be expected to have greater potential for rapid production of biomass for bioenergy applications, such as ethanol production.

3.2.2. Wood Density

Across all cross types, the mean wood densities ranged from 399 to 436 kg/m³ (Figure 1). The EE cross had the highest average wood density and was greater than aspen estimates reported in the literature for *P. tremula* [29,30], while the NN cross had the lowest average wood density and was in agreement with the upper range reported for mature aspen trees originating from Alberta, Canada [29,31,32]. The LS-means tests (Table 4) indicated the EE cross was significantly different

from NN and CC crosses. The higher density of the EE cross over the NN cross was in agreement with previous reports in the literature [29,30,33]. The average wood density of the EE cross exhibited a 37.5 kg/m^3 increase over the NN cross, which reflects an approximate 10% increase in wood density.

Yanchuk and Micko [34] studied the variation in radial wood density in aspen selected for their extremes (high and low density values) [34]. Density was averaged in five-year increments from pith to bark, and the authors found that density showed large variability both among and between trees of some clones. There were also varied density profiles from pith to bark among clones; some displaying high densities near the pith, which decreased, and then stabilized; while others steadily increased from pith to bark. Also, decreases in wood density generally occurred between years 6–10. In comparison, wood density in both *P. tremula* and *P. tremula* × *P. tremuloides* from Finland increased radially outward from the pith [31]. The specimen densities investigated in this study were measured as averages of seven years of growth, and therefore, if they follow similar patterns of growth trends for native North American or European aspen, their current overall wood density may not be an accurate predictor of their mature average wood density; it may be an overestimation, or they could further increase as they mature if they follow the European trend.

3.2.3. Microfibril Angle

The species cross type means for MFAs ranged from 22.2° to 23.7° (Figure 1). The EE cross had the largest average MFA, while the EN cross had the smallest average MFA. The average MFA for each cross was in agreement with several studies evaluating juvenile trembling aspen MFA, which has been shown to range from 18° to 22° [35,36]. The LS-means test (Table 4) shows statistically significant differences between the EE cross and both the CC and EN crosses, with the EE cross being significantly greater than the CC or EN crosses by 5.3% and 6.8%, respectively. All other species cross comparisons evaluated were similar.

Microfibril angle is generally greatest near the pith and decreases radially toward the bark [37]. The MFA of poplar clones was shown to consistently decrease with age, from 28° in the first four years of growth to 7.8° at year 11 [38]. Similarly, in two 31-year-old natural aspen trees, the mean MFAs were estimated at 12.5° and 14.7° [39].

3.2.4. Fiber Length, Width, and Coarseness

The mean fiber lengths ranged from 0.428 to 0.537 mm (Figure 1) for all cross types. The EN cross had the longest average fiber length, while the NN cross had the shortest average fiber length. All the crosses displayed average fiber lengths that were consistent with the lower end of ranges reported for *P. tremuloides* [34,40]. Previously, it was shown that trembling aspen fiber length followed a common trend: fiber length near the pith was short and slowly increased toward the bark [41]. Therefore, it is likely that the average fiber length of the aspen clones investigated will increase as they mature, and become more consistent with values reported in other studies. However, their current values do permit a ranking among crosses, to project superiority.

The LS-means tests (Table 4) showed that the NN cross was significantly different from several other species cross types. The NN cross was 20.3%, 19.4% and 13.7% shorter than the EN, CC and CN crosses, respectively, while it was similar to the EE cross. The remaining species cross

comparisons were not significantly different from one another. Fiber length was generally longer in crosses that contained *P. davidiana* compared to the intraspecific *P. tremuloides* cross. The CN cross was slightly shorter, but statistically equal to the CC cross; while both the CN and CC crosses were significantly longer than the NN cross, which were 0.103 mm and 0.068 mm longer, respectively. Clones with *P. davidiana* for one or both parents, may therefore, be better suited for hardwood pulp production, as this cell trait has been shown to contribute to superior mechanical properties of paper [42,43]. Increased fiber length, along with decreased coarseness, was found to promote fiber flexibility and collapse which further aids in inter-fiber bonding [44]. Therefore, the relatively longer fibers inherent in the CC and CN crosses offers a means to breed for traits that will generate increased inter-fiber bonding and improve paper performance; increasing tensile and tear strength [45].

The mean fiber width of the species crosses ranged from 19.4 to 22.2 μm (Figure 1). All crosses average fiber widths were also consistent with the range previously reported for aspen [40]. The EN cross had the largest average fiber width, while the EE cross had the smallest average fiber width. The LS-means tests (Table 4) revealed the EN cross had significantly wider fibers compared to the NN and EE crosses, 7.2% and 14.4% respectively. There were no other significant differences between the species cross type comparisons. The lower density observed in the EN cross when compared to the EE cross indicates the wider fibers observed in EN crosses may be due to a difference in morphological traits such as increased fiber lumen area as opposed to a thicker cell wall. Therefore, it may be of interest to investigate differences in cell wall thickness and density concurrently.

The mean fiber coarseness (a measure of cell weight per unit length, which acts as a surrogate for cell wall thickness) of the species crosses ranged from 0.063 to 0.075 mg/m (Figure 1), and the EN cross had the highest average fiber coarseness values, while the CN cross had the smallest values. The average coarseness estimates of all species crosses were below reported values for *P. tremuloides* in Canada and the US [43,46,47]. The LS-means test (Table 4) indicated that the CN cross was significantly different from the CC cross; the average coarseness of the CN cross was 12.5% lower than the CC cross. However, the *p*-value was 0.004 and very close to alpha, and it would be too presumptuous to expect differences in their pulp properties based on differences in coarseness.

3.2.5. Syringyl-Guaiacyl Ratio

The mean S:G ranged from 1.74 to 2.56 (Figure 1) for all cross types. The S:G estimates for the NN and EN crosses were slightly lower than previously reported *P. tremuloides* estimates; while they were similar to a more expansive group of British Columbian *P. tremuloides* from several sites [47,48]. The S:G of the EE cross was comparable to *P. tremuloides* clones from both previous studies, but the CC and CN crosses were below the ranges in the studies. The discrepancies in S:G ratio in this study and those from the literature might be explained by regional differences between trees from British Columbia and those from Alberta, or possibly due to inherent differences in lignin composition between *P. tremuloides* and *P. davidiana*.

The EE cross had the highest molar ratio of syringyl monomers in the lignin polymer, while the CN cross had the lowest ratio. The LS-means tests (Table 4) showed several statistically distinct differences among the crosses. The S:G for the NN cross was 23.8% and 31.6% greater than those of the CC and CN crosses, respectively. The EE cross was 11.8% and 15.3% greater than those of the NN

and EN crosses, respectively. The greatest comparative difference in S:G was between the EE and CC crosses; where the EE cross was 38.3% greater than the CC cross. The comparisons between the NN and EN crosses, as well as between the CN and CC crosses were not significantly different.

Species crosses with *P. davidiana* generally showed lower S:G. To increase the clarity in the magnitude of differences in the S:G between species crosses, the S:G ratio can be expressed in percent syringyl lignin monomers. When compared to the NN cross, the percent syringyl lignin monomer content for the CN and CC crosses were 6.1% and 4.7% lower, respectively. In contrast, the EE cross had 7.0% and 2.3% greater syringyl lignin monomers composition than the CC and NN crosses, respectively.

The differences in lignin composition would have notable implications on the chemical pulping efficiency of the clones as syringyl monomers have a higher propensity to form β -O-4 linkages among subunits, and ensuing lignin with a higher proportion of S units has been shown to have a lower overall molecular weight which will affect pulping efficiency [48,49]. Moreover, β -O-4 linkages are among the most labile bond to cleave during chemical pulping operations [48,49]. Lignin composition may also be a factor in the efficiency of wood conversion to ethanol. Specifically, variation in lignin monomer composition in transgenic hybrid poplar was associated with a 10% increase in cellulose recovery in organosolv pre-treatment, and it was more easily hydrolyzed by enzymes with less inhibition to fermentation [50]. Therefore, crosses in this study with higher S:G would have higher potential yield if used as a source for such biofuel production.

3.2.6. Total Carbohydrate Concentration

The species crosses mean carbohydrate concentrations ranged from 63.8% to 67.7% (Figure 1). The total carbohydrate content of each cross was less than the general average for *P. tremuloides*, and was less than ranges reported for trembling aspen clones in British Columbia [47,48,51]. The EN cross had the highest average carbohydrate content and the NN cross had the lowest average carbohydrate concentration. The LS-means test (Table 4) suggested that the EN cross was significantly different from the NN cross. The EN cross was 3.9% greater than the NN cross. All other species cross comparisons evaluated were similar.

The differing degrees of accessibility of monomers which comprise the total carbohydrate content in the cell wall will have an effect on the availability for moisture sorption and pulping, and affects carbohydrate's ability to be hydrolyzed [52,53]. The carbohydrate composition will play a role in yield for pulp manufacture and ethanol biofuel applications; but, in general, greater total cell wall concentration results in higher yields. Overall, the clones in this study would have lower yields in pulp manufacture or biofuel applications compared to native *P. tremuloides* according to the concentrations measured. In a cross comparison, the carbohydrate concentration of the EN cross was significantly higher than the NN cross. Using the EN hybrid instead of the NN cross as a fiber source for Kraft pulp processing would improve the relative yield. The EE cross had higher carbohydrate concentrations than the NN cross, but they were statistically equal, which is likely due to the EE cross's large standard error. In addition, the CN and CC crosses had slightly higher carbohydrate contents than the NN cross, but were again statistically equal and would likely have similar pulp yields.

3.2.7. Total Lignin Concentration

The mean total cell wall lignin contents for all cross types ranged from 21.4% to 22.9% (Figure 1). The cell wall lignin contents for all crosses were similar to those previously reported for *P. tremuloides* [47,48,51]. The CN cross had the highest average lignin concentration and the EE cross had the lowest average lignin concentration. The LS-means tests (Table 4) revealed the CN cross was significantly higher than the NN and CC crosses. The CN cross had 1.06% and 0.92% greater average lignin content, by weight, than the NN and CC crosses, respectively. All other species cross comparisons were similar.

The soluble and insoluble lignin concentrations were compared between the inter-specific crosses and the NN cross. The soluble and insoluble lignin contents of the EN cross were 0.64% greater and 1.08% less (by total wood mass) than the NN cross, respectively. In contrast, the insoluble lignin concentration of the CN cross was 1.33% greater than the NN cross, and its soluble lignin content was not significantly different. The increased total lignin content of the CN cross is largely a result of the observed increased insoluble lignin. Whereas, the soluble lignin concentration of the EN cross was greater than the NN cross. Further, the EN cross's insoluble lignin concentration was lower than the NN cross.

Lignin content will have adverse effects on cellulose yield, as lignin removal during the pulping process is accompanied with some cellulose degradation [54]. Also, in bioconversion processes to ethanol, lignin both significantly obstructs enzymatic accessibility and binds with cellulolytic enzymes which negatively impact sugar release [50]. Recently, it was shown that transgenic poplar trees with lowered lignin content showed as much as 15% improvement in their conversion efficiency of cellulose to the corresponding monomers. While the differences in lignin concentration of species crosses in this study were small, any difference will have an impact on processing operations of large scale pulp mills. Therefore, the lignin content of the EN cross, while equal to the NN cross, is more desirable than the NN cross, as it has the potential to improve pulping efficiency due to its lower insoluble lignin contents [48]. With respect to their total lignin concentrations, all the intra-specific crosses, as well as the EN hybrid, would be better suited for bleached Kraft hardwood pulp production or as a biofuel source than the CN hybrid.

3.3. Correlations

Table 5 summarizes correlation coefficients and their level of significance between the traits studied.

The growth characteristics (height and DBH) were the only two traits that displayed high correlations. Unfortunately, the growth traits could not be correlated to the wood quality traits because measurements were performed on different sets of trees. The trial plot (measured for growth traits) was established to permit a long-term study of tree performance, and therefore these specific trees were not available for wood coring. The fiber characteristics had the highest correlations among of the wood properties. Each fiber characteristic was positively correlated with one another, which is in agreement with several previous studies [55–57]. Syringyl-guaiacyl ratio correlations with cell wall composition was favorable; it was inversely correlated with lignin concentration and directly correlated with

carbohydrate concentration, and these relationships are in agreement with recent studies [58–60]. Correlations of S:G with soluble and insoluble lignin were also investigated. Examining the lignin traits as their individual components had stronger correlations compared to the total lignin concentration. The direct correlation of soluble lignin and inverse correlation of insoluble lignin with S:G ratio indicates S:G ratio could be a desirable trait to breed for; the clones in this study with higher S:G ratio would likely have improved pulping efficiency, less lignin, more easily removable lignin, and a greater yield of cellulosic fiber.

Table 5. Pearson’s correlation coefficient between traits and *p*-value (in brackets). Ho: regression is not significant, Ha: regression is significant, Alpha = 0.05. Values in bold are statistically significant.

	D	FL	FW	CS	S:G	TL	TC	SOL	INSOL	DBH
MFA	0.09 (−0.26)	−0.02 (0.83)	−0.27 (0.04)	−0.06 (0.55)	−0.1 (0.2)	−0.02 (0.77)	−0.02 (0.81)	-	-	-
D		0.2 (0.02)	−0.25 (0.04)	0.19 (0.03)	0.11 (0.14)	−0.11 (0.14)	0.18 (0.02)	-	-	-
FL			0.4 (<0.001)	0.45 (<0.001)	−0.11 (0.2)	−0.2 (0.03)	0.12 (0.18)	-	-	-
FW				0.4 (<0.001)	0.02 (0.83)	−0.19 (0.03)	0.09 (0.34)	-	-	-
CS					0.06 (0.48)	−0.15 (0.09)	0.05 (0.59)	-	-	-
S:G						−0.39 (<0.001)	0.3 (<0.001)	0.43 (<0.001)	−0.58 (<0.001)	-
TL							−0.19 (0.01)	-	-	-
HT										0.88 (<0.001)

MFA = Microfibril Angle, D = Wood Density, FL = Fiber Length, FW = Fiber Width, CS = Coarseness, S:G = Syringyl-Guaiacyl Ratio, VOL = Volume, TC = Total Carbohydrate Concentration, TL = Total Lignin Concentration, SOL = Soluble Lignin Concentration, INSOL = Insoluble Lignin Concentration, HT = Height, DBH = Breast Height Diameter.

3.4. Heterosis

The interspecific species cross types in this study were bred from different parent crosses than those of the intraspecific crosses. As such, the interspecific crosses do not reveal true heterosis when compared to their relative intraspecific crosses. However, the comparisons are indicative of heterosis that could be prevalent in offspring of interspecific crosses. The EN cross showed signs of heterosis, as the growth was superior to both the EE and NN crosses. In contrast, the CN cross growth was not suggestive of heterosis as the growth was intermediate between the respective intraspecific crosses. While heterosis is primarily used to qualify growth improvements, the wood quality characteristics were also investigated to determine if the hybrid crosses’ phenotypes were beyond that of the intraspecific crosses. There were signs of the hybrids surpassing the intraspecific crosses when

investigating some of the wood quality traits. However, even though the wood quality values of the interspecific crosses were beyond both intraspecific crosses, in most cases the interspecific crosses' trait was only significantly different from one of the two intraspecific crosses to which it was compared, according to the LS means tests (Table 4). The trend of dominance in the CN cross was prevalent for lignin concentration and S:G. Furthermore, the apparent dominance for these traits was unfavorable for wood used in pulp manufacture and ethanol biofuel applications. In contrast, the EN cross showed the trend of dominance in MFA, fiber length and width, and carbohydrates; and, the dominance for the wood quality traits was favorable. Overall, it appears any improved growth in the interspecific hybrid crosses does not negatively affect the wood quality characteristics in the clones.

4. Conclusions

There is an escalating need to improve tree fiber quality and growth rates as the demand for raw material increases globally. The research performed in this study demonstrates the potential of using hybridization strategies to significantly increase the growth rates of aspen in Alberta, Canada. It also provides insight beyond growth characteristics of hybrid aspen crosses through the extensive characterization of their wood quality traits.

The most notable difference between the species crosses was in volume growth and large improvements could be realized using hybrid aspen over native aspen trees. The NN cross had lower volume growth than the CC and EE crosses with 55.0% and 43.8% less volume, respectively. The EN and CN crosses had 172.8% and 51.8% greater volume than the NN cross, respectively and could potentially decrease rotations from 60 years to 22 and 40 years, respectively. The species crosses with improved growth exhibited similar or improved wood density to the NN cross. Thus, the improved volume growth is a substantial improvement in total lignocellulosic biomass produced by the clones. Furthermore, improved growth in the interspecific hybrid crosses does not appear to negatively affect the wood quality characteristics in the clones.

There were significant correlations between S:G ratio, both soluble and insoluble lignin concentration, and total carbohydrate concentration; clones with higher S:G ratio would likely have improved pulping efficiency, with less lignin, more easily removable lignin, and more cellulosic fiber at a given time in the Kraft pulp process.

The EN cross had longer, wider fibers, higher carbohydrate concentration, a more favorable distribution of soluble and insoluble lignin compared to the NN cross, and it could potentially create better quality pulp with higher yield. The EN cross had slightly lower MFA and greater fiber width, but lower S:G compared to the EE cross. The processing efficiency of the EN cross would likely be lower compared to the EE cross. The CN cross had greater fiber length and lignin concentration in the form of insoluble lignin, and lower S:G compared to the NN cross which indicates the CN cross would have higher Kraft pulp processing demands and be less desirable as a biofuel source due to the higher lignin concentration and decreased S:G. However, the CN cross's longer fibers may create paper with superior strength properties compared to using the NN cross fibers.

The results clearly indicate that the hybrid species crosses produce a more desirable wood source than the pure *P. tremuloides* cross. Specifically, *P. tremula* × *P. tremuloides* displayed superior growth rates, while either equaling or surpassing the *P. tremuloides* × *P. tremuloides* wood quality attributes.

In contrast, the *P. tremuloides* × *P. davidiana* cross, which also displayed superior growth compared to the *P. tremuloides* × *P. tremuloides* cross, appeared to have less desirable lignin content and composition that may affect processing energy requirements and yield in bleached Kraft hardwood pulp production or potential biofuel applications. Therefore, the *P. tremula* × *P. tremuloides* cross appears to be the most desirable candidate cross that should be considered for use to improve potential future generations of aspen on the Canadian landscape.

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Conflicts of Interest

The authors declare no conflict of interest.

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