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ORIGINALARBEITEN ORIGINALS

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Tracheid length prediction in *Pinus palustris* by means of near infrared spectroscopy: the influence of age

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Abstract The prediction of tracheid length using near infrared (NIR) wavelengths can provide either useful or misleading calibrations depending on the context. This can happen since tracheid length is not directly related to the absorbance at any wavelength but is instead the result of a secondary correlation with some unknown chemical constituent. In this work, the effect of tree age and height on NIR predictability was investigated since tracheid length and chemistry may vary as a function of location within the tree. It was found that tracheid length predictability did not change with height but decreased with age. As a result, predicting tracheid length regardless of age was good (R^2 = 0.72) while predictability holding age and height constant was mostly low to moderate with the exception of rings 1 and 4 which was quite strong.

Vorhersage der Tracheidenlänge in *Pinus palustris* mittels NIR-Spektroskopie: Einfluss des Alters

Zusammenfassung Das Abschätzen der Tracheidenlänge mittels NIR-Spektroskopie kann je nach Sachlage zu brauchbaren oder auch irreführenden Kalibrierungen führen. Das kommt daher, dass die Tracheidenlänge nicht unmittelbar mit der Extinktion bei irgendeiner Wellenlänge in Beziehung steht, sondern über eine indirekte Korrelation mit unbekannten Inhaltsstoffen. In dieser Arbeit wird der Einfluss des Baumalters und der Höhe auf die Verlässlichkeit der NIR-Messung untersucht, da sowohl die Tracheidenlänge als auch die chemischen Bestandteile je nach Position im Baum variieren können. Es zeigte sich, dass die Vorhersagbarkeit der Tracheidenlänge sich nicht mit der Baumhöhe ändert, jedoch mit dem Alter abnimmt. Insgesamt war die Vorhersagbarkeit der Tracheidenlänge unabhängig vom Alter recht gut (R^2=0,72); bei konstantem Baumalter und -höhe war sie jedoch mäßig bis niedrig außer für die Jahrringe 1 und 4, wo die Korrelation sehr streng war.

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1 Introduction

Rapid measurement of tracheid length is desirable by tree improvement programs who wish to assess tracheid length variation from increment cores. It is well established that tracheid length, in most cases, is more heritable than density, growth rate, or disease resistance (Jackson and Greene 1958; Zobel et al. 1960; Dadswell et al. 1961; Nicholls et al. 1964; Smith 1967; Matziris and Zobel 1973; Loo et al. 1984; Cown et al. 1992; Whiteman et al. 1996; Hannrup and Ekberg 1998; Miranda and Pereira 2002; Pot et al. 2002).

Near infrared spectroscopy (NIR) to measure fiber morphology is emerging as an alternative to sometimes more precise, but time-consuming, measurement techniques for tree improvement groups. For example, NIR and FT-Raman Spectroscopy appears to detect tracheid length by accounting for more than 70% of the variation when tested across rings for Norway spruce (Hauksson et al. 2001; Ona et al. 2003). However, before scanning, the wood was milled to a particle size for both studies. Since tracheid length could be estimated from small particles, it becomes apparent that NIR is dependent on some secondary relationship since tracheids would have been cut to smaller sizes during grinding. To date, no attempt has been made to understand the basis for how a spectroscopic technique is related to tracheid length.

Nexus 670 FTIR spectrometer (Thermo Nicolet Instruments, Madison, WI, USA) at wavelengths between 1,000 nm and 2,500 nm at 1 nm intervals using reflectance spectroscopy. Forty scans were collected and averaged into a single spectrum curve. The size of the scan was 5 mm in diameter. The scan was executed on the latewood portion next to the maceration site. These scan sites were located on strips, that were sawed from just below the maceration site, to facilitate counting, scanning at the right age, and to create a smooth surface. To reduce computation time using Statistical Analysis Software (SAS), the data set was averaged and categorized into 10 nm intervals (Osborne and Fearn 1986; SAS 2001).

As a first step, least square regression was used to determine how much variation in tracheid length was accounted for by age. Scatter plots were reviewed to ensure that a linear trend was appropriate. Then, multiple linear regression was used to determine if peaks and valleys, from the first derivative, were important for determining tracheid length. For all NIR analysis, after preliminary tests, the first derivative yielded superior equations over the original and second derivative data. Therefore, absorbance across all wavelengths was transformed into the first derivative. PROC PRINCOMP was used to run principal components regression (SAS 2001). A p-value less than 0.05 was used as the determining criteria for a principal component being a significant predictor. After determining which principal components (PCs) were useful in predicting tracheid length, the analysis was ran for (a) the whole tree including calibration ($n = 300$) and validation ($n = 153$) models, and (b) each age and height group. For calibration modeling, two thirds of the data was randomly chosen leaving one third of the data for validation. For each age and height group, the same PCs were used for prediction as those chosen for the overall calibration and validation model.

3 Results and discussion

3.1 Whole tree modeling

Figure 1 shows the plot of predicted versus measured tracheid length for the calibration and validation samples. A one-to-one diagonal line was drawn to indicate any offset or bias. There exists a slightly higher frequency of data points above the diagonal line of approximately a 0.2 mm offset. The calibration and validation data appeared to follow the same slope, which was not deemed to be significantly different at the $\alpha = 0.05$ level ($p > 0.05$). All tracheid lengths greater than 4.2 mm were below the diagonal line for the validation data indicating an underestimation by the model in this region (Fig. 1). The underestimation was either due to an over fit of the calibration data or perhaps the data was slightly non linear in behavior above 4.2 mm. Using 6 PCs, the calibration model gave a good fit with an R^2 value of 0.72 while the validation test set gave a slightly lower R^2 value of 0.65.

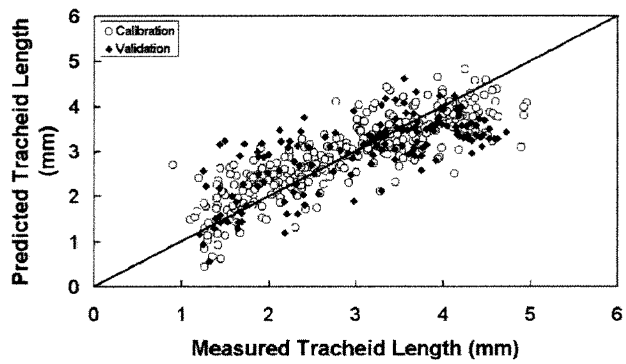

Fig. 1 Predicted versus measured tracheid length for calibration (*Open circle*) and validation samples (*filled diamond*)
Abb. 1 Berechnete und gemessene Tracheidenlänge bei der Kalibrierung (*Open circle*) und Messung (*filled diamond*)

Table 1 lists the coefficients for PC variables used for whole tree regression analysis. These coefficient estimates represent the calibration model used to predict tracheid length in Fig. 1. When the calibration model was used to predict the validation data, similar coefficients emerged supporting a sound calibration model. Due to a low correlation with tracheid length ($p > 0.05$), PC 1 was not included in the model. While PC 1 represented the largest axis of variation, for a linear combination of all wavelengths, it didn't prove significant in predictive equations. Inclusion of PC 1 into the backward stepwise selection procedure did not significantly change the coefficients of the remaining PCs and further justified leaving it out.

By taking the 1st derivative and then calculating PCs, the statistical noise attributable to high multicollinearity between wavelengths was reduced. The extreme peaks and valleys in Fig. 2 identified the wavelengths most associated with tracheid length. Multiple linear regression, on data not transformed into principal components, revealed all the major peaks and valleys to be significant in predicting tracheid length, with the exception of wavelength 2,045 nm. The following wavelengths 1,895, 1,415, 1,965, 2,245, 2,295, 2,315, and

Table 1 Coefficient and error estimates for the principal component (PC) regression model
Tabelle 1 Koeffizienten und Fehlerschätzung der Hauptkomponenten (PC) des Regressionsmodells

Variable	Coefficient and intercept	Standard error	T-value	Pr > |t|
Intercept	2.969	0.031	94.31	<0.0001
PC 2	0.080	0.005	17.55	<0.0001
PC 3	0.138	0.009	16.08	<0.0001
PC 5	0.144	0.015	9.48	<0.0001
PC 6	0.123	0.022	5.64	<0.0001
PC 7	0.134	0.026	5.19	<0.0001
PC 9	−0.150	0.032	−4.64	<0.0001

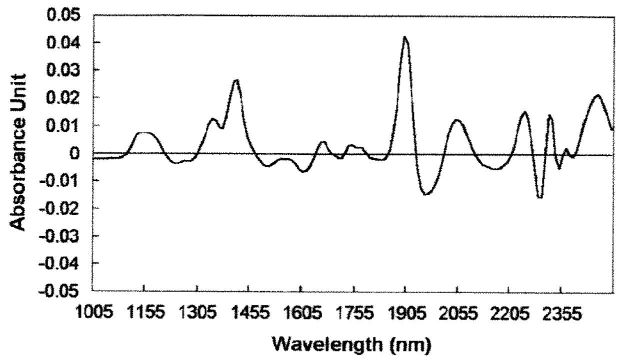

Fig. 2 Wavelength versus 1st derivative absorbance of calibration samples ($n = 300$)
Abb. 2 Beziehung zwischen Wellenlänge und 1. Ableitung der Extinktion für Kalibrierproben ($n = 300$)

2,455 nm gave a combined R^2 of 0.65 for predicting tracheid length. While regression with PC variables exhibited a more accurate model, multiple linear regression facilitated the understanding of which wavelengths were fundamentally important in predicting tracheid length. Had only one or 2 PC's been needed to predict tracheid length, one could have alternatively used the coefficients of the PC linear combination.

The first two PC's, in Table 1, were plotted against one another to distinguish if clusters occurred as a function of age (Fig. 3). PC1 was not used for prediction since it did not show a significant p-value. The presence of clusters for juvenile and mature wood was an indication that physiological age, hereafter referred to as ring number or age, may have some influence on the ability to predict tracheid length from NIR spectra. Rings 16 and 30, throughout the tree, were categorized as mature wood, while juvenile wood was categorized as rings 1, 4, and 8. This grouping scheme was chosen since 2 populations seemed to best provide superior discrimination. As Fig. 3 shows, PC 2 did most of the separation of mature and juvenile wood. Figure 3 was important because it showed that juvenile and mature wood may be separated when the data lay outside the overlap zone. Since tracheid length increased sharply from juvenile to mature wood one could categorize tracheid length as short in the juvenile wood zone and long in the mature wood zone.

The variability of the juvenile wood data in Fig. 3 was greater than the mature wood population which was tightly bunched. Sixty-nine percent of the juvenile wood data fell outside the mature wood cluster zone while 31% overlapped. The additional variability of the juvenile wood population implied that many juvenile samples could be separated with certainty. Given that tracheid length increased with age and that PC 2 depended on age, it was evident that the model in Fig. 1 and Table 1 was confounded by age. What was also interesting was that when age was accounted for by using PC 2, five more PC's were significant in predicting tracheid length (Table 1) and represents alternative variation in the spectra that predicts tracheid length, independent of age.

3.2 Age and height modeling

It was shown in Fig. 3 that radial position within the tree was influential on NIR spectra which influenced tracheid length prediction (Table 1). It was considered possible that height may play a critical a role. As a result, the apparent strong correlation in Fig. 1 may be an artifact of changing chemistry and fiber morphology with age. It is known that lignin decreases, while cellulose and tracheid length increases from pith to bark (Koch 1972; Megraw 1985). So first, the effect of height on regression coefficients was examined. If height were to play a part in predicting tracheid length, then the regression coefficients may change significantly with

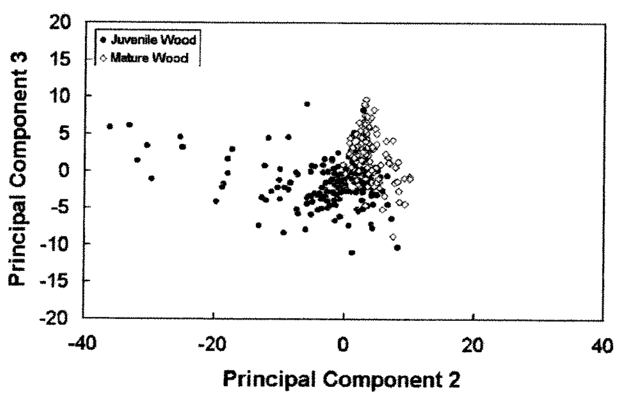

Fig. 3 Classification of juvenile (*diamond*) and mature wood (*filled circle*) using principal component 2 vs. 3 in a scatter plot
Abb. 3 Klassifizieren von juvenilem (*diamond*) und reifem Holz (*filled circle*) anhand der Verteilung der Hauptkomponenten 2 und 3

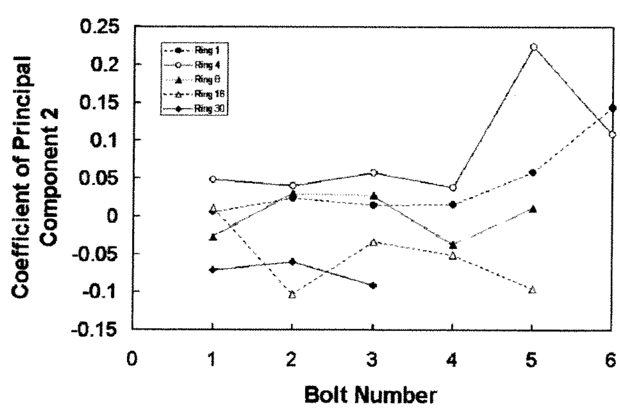

Fig. 4 Regression coefficients for principal component 2 across all heights for different age groups
Abb. 4 Regressionskoeffizienten der Hauptkomponente 2 über alle Baumhöhen für unterschiedliche Altersklassen

height. Figure 4 demonstrates the coefficient response with height, for different age groups. As revealed, the regression coefficients remained fairly stable regardless of height (bolt number). While the coefficients of rings 1, 4, and 8 appeared to increase between bolt 4 though 6, it was not statistically significant as determined by overlapping 95% confidence intervals. Nevertheless, perhaps the change in coefficients was attributable to the crown effect. It was interesting to note that juvenile rings 1, 4, and 8 had higher regression coefficients than mature wood rings 16 and 30. For most bolt and age comparisons, these regression coefficients were significantly different. As a result, perhaps different models would enhance ones ability to predict tracheid length in juvenile and mature wood populations.

To determine if physiological age (ring number) had an effect, a least squares regression was run for the calibration data. Ring number accounted for 66% of the variation in tracheid length. When height was used to predict tracheid length, an R^2 of 0.015 emerged supporting that age from pith was much more influential on tracheid length than height.

To demonstrate the effect of ring number and height on NIR models of tracheid length, Fig. 5 was constructed and shows the R^2 response at each position. No trend in R^2 was apparent with tree height while R^2 values generally decreased from pith to bark. Close to the pith, the NIR spectra showed good predictive ability for tracheid length while by ring 8, models were at best moderate and quite often poor. Nevertheless, even for older tree rings, the PCs consistently had p-values less than 0.05, meaning that some fundamental chemical constituent, independent of age, must be related to tracheid length. As discussed earlier, PC's greater than 2 somehow explain the overall variation independent of age and deserves further attention in future studies. The higher R^2 values at rings 1 and 4 were unexplainable but were perhaps attributable to the physiological response of the tree. At that time, the cambial initials were growing at a faster rate, resulting in different fiber morphology from a chemical point of view (Megraw 1985). The higher R^2 value for rings 1 and 4 were opposite of what was expected. Since resin levels were very high during the early years of growth, as discerned visually, increased noise and decreased R^2 values were expected. Despite the already moderately high correlations at rings 1 and 4, perhaps removal of extractives for all ring numbers would yield better models and merits further research.

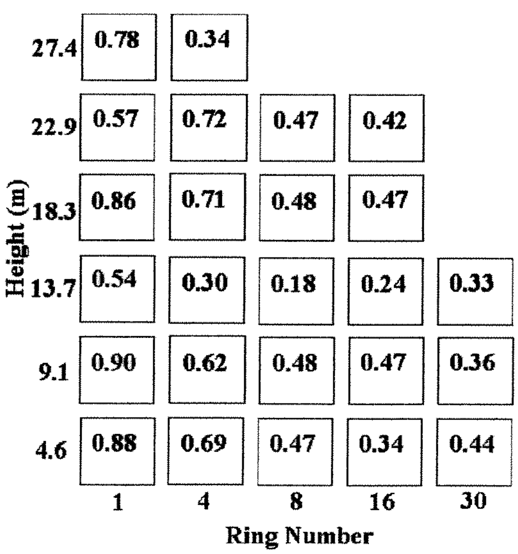

Fig. 5 Diagram showing the R^2 value (in *boxes*) of predicting tracheid length from NIR spectra at each ring and height combination ($n = 20$ per box)
Abb. 5 Diagramm der Bestimmtheitsmaße (in den Kästen) zum Vorhersagen der Tracheidenlänge aus NIR-Spektren für alle Jahrring- und Höhen-Kombinationen

Taking the first derivative appeared to partially remedy some of the strong multicollinearity between wavelengths associated with both extractives and tracheid length. This was judged by the decrease in variance inflation factor (VIF), which fell between 10 and 30 for many of the chosen wavelengths. Ten is a common threshold used in multiple regression modeling to evaluate if excessive multicollinearity between variables exist (Neter et al. 1996). While high multicollinearity still existed between wavelengths, the VIF was considerably lower with 1st derivative processing. High multicollinearity was undesirable since it escalated the variance of the regression coefficient estimates making interpretation difficult to impossible.

The lower R^2 values, at ages greater than 8 rings from the pith, suggest that NIR is not adequate for modeling tracheid length, in older portions of the tree. However, improvement of tracheid length in older portions of the tree(s), has limited utility since older trees are harvested for lumber while thinnings go to pulp mills. In genetics studies, replicates are usually no greater than 10–20 trees per family, making the precision of the model highly essential. The high correlations, exhibited in the first years of growth of the cambium, may be high enough to detect differences in means between families with sufficient replication. Given that trees are thinned as early as 8 years into a rotation, measurement and improvement of tracheid length with NIR spectroscopy may be useful.

The significant relationship exhibited in Fig. 1 was mostly attributable to an underlying interrelationship between age, wood chemistry, and tracheid length. Specifically, three of the six wavelengths significant in predicting tracheid length were associated with lignin and cellulose and occurred at 1,415, 1,965, and 2,315 nm.

4 Conclusions

The ability to estimate tracheid length from NIR spectra was primarily due to the change in NIR signal with age. When relationships were investigated holding ring position and height constant, the relationship was mostly weak for ages 8 to 30 and moderate to strong for ages 1–4. When all PC's were used to predict tracheid length, it was shown that NIR could successfully model tracheid length using 6 principal components and yielding an R^2 of 0.72.

The modeling of tracheid length, for tree improvement applications needs more justification, particularly for mature wood zones. Without the variation in xylem age, NIR does not generally have an acceptable precision to estimate tracheid length. The exception to the rule was the rings adjacent to the pith which had a mean $R^2 = 0.75$. As a result, NIR spectroscopy may be suitable for tree improvement in this juvenile zone. The predictability of tracheid length (R^2) decreased rapidly from ring 1 to 8 and then continued to decrease at a gradual rate. Thus, by ring 30, PC models accounted for approximately 40% of the variation in tracheid length.

Finally, when PC 2 and 3 were plotted it was found that juvenile and mature wood could be separated with some overlap. This demonstrated that linkages exist between NIR absorbance and tracheid length when samples from all parts of the trees were analyzed.

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It is clear that tracheid length detection with NIR must have some level of indirect relationship to the NIR signal, particularly since the absorbance capacity at a given wavelength depends on the stretching or bending nature of bonds between molecules and not a macro feature like tracheid length. One possible connection between tracheid length and NIR spectra is the change in tracheid length and chemistry with age. Often, as the cambium matures, the concentration of cellulose and fiber length increases while the amount of lignin decreases (Bendtsen and Senft 1986; Shupe et al. 1997). Such covariance between traits with age may result in a secondary correlation between the NIR signal and tracheid length.

When the nature of a relationship is not understood, there is increased chance for misleading calibrations (Osborne and Fearn 1986). As a result, one needs to understand how a correlation exists and when it can be used for maximum advantage. Koubaa et al. (1998) demonstrated that age from the pith accounted for more than 80% of the variation in fiber length for hybrid poplar (*Populus* × *euramericana*). Many other studies of various softwood species demonstrate similar trends showing a distinct increase in tracheid length at a decreasing rate from pith to bark and from breast height to crown (Megraw 1985). It is possible that since tracheid length increases with age and various chemistry components change with age, than an indirect relationship may exist.

The chemical morphology in the primary wall is also quite different than the secondary wall. Given that primary wall development affects cell length, there may be some relationship between the NIR signal and the primary cell wall. For instance, as the primary cell wall develops in length, it needs to be plastic in nature to allow stretching thus requiring a more amorphous polymer network than the secondary layer (Roelofsen 1969; Cosgrove 1999). Arabinose and galactose are two sugar units common in the primary cell wall of southern pines while mostly absent in the secondary cell wall (Koch 1972).

The objective of this work was to explore the potential of using NIR absorbance data to predict tracheid length from (a) all samples collected from experimental trees and (b) data categorized by age and height. Two thirds of the data, (n = 300) were used to develop calibration equations while the remaining data (n = 153) were used to validate the model. Alternatively, when age and height were held constant, 20 data points were available to develop principal component regressions (PCR) for each age-height group for a total of 25 replicate equations. Trends in principal component coefficients, and R^2 values, for age and height were explored. This objective had utility since it determined the role that age and/or height played in the secondary correlation between tracheid length and NIR absorbance data. Such information is needed to help conclude how NIR can predict tracheid length and when calibration equations should or should not be used.

2 Materials and methods

2.1 Sample preparation

Ten longleaf pine (*Pinus palustris*) trees were harvested from a plantation on the Harrison Experimental Forest, which is maintained and owned by the USDA Forest Service near Saucier, Mississippi (USA), at approximately 30.6 north and 89.1 west coordinates. These 41-year-old trees were bucked into five to seven bolts, depending on tree height, to yield a disk every 4.57 m in height. The trees were handpicked to yield extreme and typical diameter and height values for the stand. A double blade was used to rip bark to pith to bark strips approximately 20×20 mm in tangential and longitudinal dimensions. These strips were then scanned by NIR and will be further discussed in the NIR spectroscopy section.

For tracheid separation, a small portion of the latewood, from the remaining part of the disk, was chiseled into random matchstick-sized dimensions and macerated in 1:1 mixture of glacial acetic acid and hydrogen peroxide and heated for 24 h at 60°C or until bleached white. This was performed on samples at rings 1, 4, 8, 16, and 30 from the pith for both the north and south face of the tree. The fibers were then rinsed with two to three cycles of water; i.e., the glacial acetic acid and hydrogen peroxide were completely replaced by water. The samples were stored in small vials and then refrigerated at 4.4°C until further processing.

2.2 Tracheid length measurement

Macerated samples were processed through a Fiber Quality Analyzer, in which each tracheid was suctioned through a flow cell and automated image analysis was used to determine tracheid length. Control samples were periodically run to ensure no drift in tracheid length measurement. A minimum of 1,000 tracheids per sample were measured except for a handful of vials that were broken during transportation but still yielded 300 plus tracheids. The mean number of tracheids measured was 4,700 tracheids per sample. A total of 475 samples were run. Length weighted averages were calculated using Eq. 1.

$$L_{avg} = \frac{\sum \left(l_i^2 n_i\right)}{\sum \left(l_i n_i\right)} \quad (1)$$

where l_i represents the tracheid length of the ith bin and n_i represents the total bin tracheid count. The length weighted measurements were used to adjust for the bias caused by the small distribution of cut fibers inherent when using a 12 mm increment borer during sampling.

2.3 NIR spectroscopy and analysis

Before scanning, samples were air dried to a common moisture content. NIR absorbance was obtained using a