CLONAL VARIATION OF TANNIN AND PHENOLIC CONCENTRATIONS OF CONES FROM A SELECTED POPULATION OF LOBLOLLY PINE

T. N. Trembath 1/ and G. R. Askew 2/

Abstract.--Cone samples were collected from 19 clones in a chemically treated loblolly pine seed orchard every 2 months between March 1985 and May 1986. Tannin and phenolic concentrations of the cones was quantified by chemical analysis to examine the clonal and seasonal variation and to determine if variability in tannin and phenolic concentration was related to insect susceptibility. Both tannin concentration and phenolic concentration varied throughout the cones lifecycle. Tannin concentration consistently peaked for all 14 clones at age 10 mo. Total phenolics were not found to be as consistent for all clones as were the tannins. Both tannin concentration and phenolic concentration had significant variation associated with cone age. No single clone had a consistently higher or lower overall mean tannin or mean phenolic concentration than any other clone and no relationship was found between mean tannin concentration or mean phenolic concentration of cones and seed bug and coneworm infestation levels.

Coneworm and seed bug damage is a major problem in loblolly pine seed orchards. Cone and seed damage estimates as high as 90% have been found in some untreated southern pine seed orchards (Ebel et al. 1981), with Dioryctria amatella, D. disclusa, D. merkeli, Leptoglossus corculus and Tetyra bipunctata being the primary damaging insects. Coneworms, Dioryctria spp., destroy the entire cone by tunnelling and feeding within the cones. Seed bugs, Leptoglossus corculus and Tetyra bipunctata destroy individual seeds by puncturing the conescales with needle-like mouthparts and extracting nutrients from the seeds.

Application of insecticides is the current control practice in seed orchards, but because of the high monetary cost and potential adverse environmental effects of pesticides, there has been increased interest in exploiting genotypic variation in coneworm and seed bug susceptibility to reduce losses from these pests. Askew et al. (1985) noted significant genetic variation in coneworm and seed bug attacks among loblolly pine clones.

Many factors, including the levels of defensive plant chemicals, may determine the reduced susceptibility of some lobolly pine trees to pest attack. Schultz stated (in Maugh 1982) that chemicals produced by plants may be more important than any other single factor in controlling insects in nature and phenolics and tannins have been shown to be related to the level of herbivore damage in some plant species (Feeny 1976).

1/ Graduate Research Assistant, Clemson University, Clemson, South Carolina.

2/ Associate Professor of Forestry, Belle W. Baruch Forest Science Institute of Clemson University, P. 0. Box 596, Georgetown, South Carolina 29442.

Many studies of phenolics of coniferous tree species have been completed, but nearly all the work has been with wood, bark, or foliage (Hillis 1962, Forrest 1975, Hergert 1960). Little information has been reported about phenolic compounds in conifer cones. Hillis (1962) stated that concentrations of various polyphenols in different tissues of the same tree can vary considerably and that once formed, translocation of these substances is improbable. Information about the occurrence and variation of phenolic compounds in cones is needed in order to learn more about the relationship of phenolic compounds with cone and seed insects. In this paper we will discuss the variations of tannin and phenolic concentration of cones among clones in a loblolly pine seed orchard and measurements of tannin and phenolic concentrations as the cones age. We will also examine the variability in tannin and phenolic concentration of cones as it is related to susceptibility to insect attack.

STUDY AREA

Loblolly pine cone samples were collected from 19 clones at a coastal seed orchard located near Georgetown, South Carolina. The orchard is 10.52 ha in size, with a 4.57 m by 9.14 m original tree spacing. An average spacing of 9.14 m was achieved through orchard roguing, with some spacing being as wide as 18.29 m by 9.14 m. The trees were 22 years old at time of initial sampling and had an average height of 19.81 m. The orchard was chemically treated from the ground with a mist blower on a monthly basis from April through August, using Guthion 2S for seed bug control and Pydrin for coneworm control.

Cone Collection

NW Two ramets from each of the 19 clones in the orchard were selected for sampling throughout the study. Ramets were selected such that several trees could be sampled without moving the equipment. Due to orchard roguing and unavailability of cones from some trees, the number of ramets per clone and the number of clones varied throughout the study. Whenever possible, a new ramet was selected for those that could no longer be sampled.

We attempted to collect 4 cones per ramet every 2 months, beginning May 1985 when the cones were 2 months old and ending in March 1986, but the actual number of cones collected in a sampling month varied depending on cone availability. Cones of two different ages were collected during the sample months of May 1985 and July 1985. In May 1985 2 month old and 14 month old cones were collected, and in July 1985 4 month old and 16 month old cones were collected. By simultaneously sampling both young and old cones, all age groups were collected in one year rather than across an 18 month period. The 6 month old cones were not collected due to the unavailability of collection equipment in September 1985. Cones were randomly selected from each tree without regard to position. After collection, the cones were transported to the lab in a portable cooler and then stored in a walk-in cooler until they were processed.

Extractions

v- Phenolic compound extraction was conducted on a cross-section sample of individual cones or on the entire cone if they were small enough to be easily handled. Samples were pooled by ramets, chopped, frozen in liquid nitrogen

and then ground by hand using a mortar and pestle. The mature 18 month old cones that were collected in September 1985 were too woody to be ground by the liquid nitrogen method, so they were pooled together by ramets, chopped, and then ground in a Wiley Mill to pass through a 1 mm mesh screen.

Extraction of the tannin and phenolic compounds was accomplished by placing 1.25 grams of the ground tissue (or less if the cones were small) from each pooled ramet sample into 50 ml of 80% methanol maintained at 80°C for ten minutes (Buchsbaum et al. 1984). Two sequential extractions were completed if there was sufficient tissue. After extraction, all samples were filtered by gravity through No. 1 Whatman filter paper and the sequential extractions were pooled.

Extraction of all cone samples for a collection date was completed within 4 to 6 weeks after cone collection. Two 8 ml vials of the pooled extractions were stored in a refrigerator until they could be analyzed. All analyses were completed within 4 to 6 weeks after extraction.

Tannin Analysis

Tannin content of the extract was determined as follows: 1) 100 ml of fresh steer blood was collected and immediately lysed by the addition of 50 ml of cold distilled water. The mixture was returned on ice to the laboratory, and then centrifuged to produce a solution that was free of cell membranes. A 5% blood solution for use in the analysis was then prepared in distilled water from this stock. 2) Tannic acid standards of 0, 500, 750, 1000, 1250, 1500, 1750, and 2000 ppm were prepared in distilled water and used for the Hemoglobin test. Three ml of tannin extracts or standard solutions were added to 3 ml of 5% blood in a centrifuge tube, vortexed until the solution was homogenous (about 10 seconds), and then centrifuged at 9000 rpm at 21 C for 30 minutes. Absorbances of the solutions were determined at 578 nm on a SPECTRONIC 88 spectrophotometer calibrated to an absorbance of 2.0 with a blood blank. Tannic acid equivalents (TAE) were determined for each sample from a plotted curve of the tannic acid standards. TAE was defined as the ratio of the sample concentration predicted from the standard curve to the actual dry weight concentration of the extracted material (Bate-Smith 1973).

Phenolic Analysis

Total phenolic content of the extracts was determined by using the Folin-Denis procedure as described by Swain and Hillis (1959). Aliquots of the extracts were diluted with distilled water to 1%. Tannic acid standards of 0, 20, 40, 60, 80, and 100 ppm were used. Equal volumes (usually 3 ml) of tannin extracts and Folin-Denis reagent were combined in a centrifuge tube. After 3 minutes, an equal volume of 2N sodium bicarbonate solution was added as a fixative. Tubes were vortexed to homogenize the liquids after a 1 hour incubation at room temperature. Sample absorbances were determined at 725 nm on a SPECTRONIC 88 spectrophotometer calibrated to an absorbance of 2.0 with a 100 ppm standard.

Seed Analysis

Ten mature cones free of coneworm damage were collected from each of the clones in October 1985. Cones were dried at the Southeastern Forest

Experiment Station Forestry Sciences Lab in Athens, Georgia. In January of 1986 the seeds were extracted from the dried cones and x-ray analysis was used to determine the degree of seed bug damage for each clone. The seed bug damage was categorized as percent full, percent empty, percent abort (second year abortion of ovules early in the second growing season), percent seed bug (damage to the seeds late in the second growing season), percent seedworm (Laspeyresia spp.), and percent fungi.

Cone Analysis

All coneworm infested cones were separated from the 1985 bulk collection of each ramet and the species of coneworm was identified. The total number of cones harvested for each ramet was not determined, so the proportion of infested cones for each clone was calculated based on a bushel estimate from the previous three years.

Data Analysis

Analysis of variance was used to evaluate the effects of age on the average tannin and phenolic concentration of all clones and to examine the interactions of age by clone and age by ramet within clone, with Type I error set at 0.05. Correlation analysis was used to examine the relationship of tannin and phenolic concentrations at each age with the seed but damage levels for each clone. Only those clones that had tannin and phenolic data for all ages, excluding 6 and 18 months, were analyzed.

RESULTS AND DISCUSSION

Seasonal Variation of Tannin and Phenolic Concentration

Tannin concentrations and phenolic concentrations (Table I) varied throughout the life cycle of loblolly cones. Tannin concentration consistently peaked for all 14 clones in January samples (Age 10 mo.), while July samples (Age 16 mo.) showed the lowest tannin concentration in all but one clone. Total phenolics were not as consistent for all clones as were the tannins. Nine of the 14 clones peaked in mean phenolic concentration in January samples (Age 10 mo.), two clones peaked in November samples (Age 8 mo.), 2 clones peaked in July samples (Age 16 mo.), and 1 clone peaked in March samples (Age 12 mo.). Age of cones at time of lowest mean phenolic concentration was more variable among all clones than peak concentration. Six clones had the lowest mean phenolic concentration in May samples (Age 4 mo.), 2 clones in July samples (Age 16 mo.), and 1 clone in May samples (Age 2 mo.).

Analysis of variance revealed an age effect for both tannin concentration and phenolic concentration and a clonal effect for phenolic concentration alone (Table II). These results were as expected due to the obvious peak in both tannin and phenolic concentrations at age 10 mo. The clonal effect for phenolics was not of interest by itself, but will be discussed later in connection with the cone and seed insect data.

The large reduction in phenolic concentration from January samples (Age 10 mo.). to May samples (Age 14 mo.) may be attributed to the depletion of starch caused by the vegetative flush of growth in the spring. Phenolic concentration rapidly increased from May samples (Age 14 mo.) to July samples

(Age 16 mo.). This increase may be explained by the results of a study by Chung and Barnes (1980) who found that the time of rapid increase in the fraction of photosynthate in loblolly pine allocated to protection constituents (phenolics) late in the growing season seemed to be correlated with the termination of axis and needle elongation.

Table I.--Mean tannin and phenolic concentrations of cones for each age.

Age (Mo.)	Mean Tannin concentration (ppm TAE)	Std Error	Mean Phenolic concentration (ppm TAE)	Std Error
2	1432	23.16	48	2.54
4	1258	14.03	48	3.70
8	1259	13.23	52	2.72
10*	1832	36.99	67	2.25
12	1450	16.81	59	2.27
14	1417	15.37	43	2.34
16	970	36.65	53	3.53

*Indicates peak age for tannin and phenolic concentrations

Table II.--Analysis of variance for tannin and phenolic concentrations.

		Tannin		Phenolic	
Source	df	MS	F	MS	F
Clone	13	32217.62	1.46	665.04	3.07*
Ramet (Clone)	14	22022.11	1.44	216.49	0.93
Age	6	1919211.80	140.91*	1776.82	12.66*
Clone x Age	78	13620.56	0.89	140.40	0.60
Error	84	15298.39		232.30	

*Calculated F exceeds tabulated F with a Type I error rate of 0.05

Correlation of Mean Tannin and Mean Phenolic Concentration of Cones To Coneworm and Seedbug Infestation Levels

No relationship was found between mean tannin concentration or mean phenolic concentration (Table III) of cone tissue and percent of dead cones attributed to attack by coneworms. Specifically, per bushel average of percent small dead cones attributed to attack by Diorctria merkeli in late (May samples) was compared to the tannin and phenolic concentrations of cones at ages 14 mo. and 16 mo. for these clones. No relationship of low percent

dead cones to high mean tannin concentration or mean phenolic concentration was apparent. The same was true for high percent dead cones and low mean tannin concentration or mean phenolic concentration.

A per bushel average was also determined for the percentage of large dead cones attributed to attack by Dioryctria amatella in mid to late summer and compared with mean tannin concentration and mean phenolic concentration at age 16 mo. (July samples). Again, no relationship was found between high or low percent dead cones and low or high mean tannin concentration or mean phenolic concentration. For example, Clone 11 (ramet 1) had the same per bushel average (7%) of small dead cones (May attack) as large dead cones (July attack), but the tannin concentration of 1359.3 ppm at age 14 mo (May sample) was much higher than the tannin concentration of 948.7 ppm at age 16 mo (July sample). Ramet 2 of the same clone had a 79% per bushel average of small dead cones (May attack) and a 51% per bushel average of large dead cones (July attack). However, the tannin concentration of 1384.9 ppm at age 14 mo (May sample) was much higher than the tannin concentration of 954.7 ppm at age 16 mo (July samples).

Table III.--Per bushel average of percent small dead cones attributed to attack by Dioryctria merkeli in late May to early June (Age 14 mo.) and percent large dead cones attributed to attack by Dioryctria amatella in mid to late summer (Age 16 mo.) with corresponding mean tannin and phenolic concentrations.

Clone	Small Dead Cones	Large Dead Cones	Tannin 14	Tannin 16	Phenol 14	Phenol 16
	%	%	ppm	ppm	ppm	ppm
1	2.7	5.7	1400	1231	34	50
2	11.0	23.0	1533	924	48	65
5	5.0	4.0	1425	826	45	57
6	5.0	6.5	1455	1073	42	65
7	13.0	14.0	1501	1056	48	63
9	2.0	1.3	1444	846	56	57
10	3.5	5.0	1419	762	44	42
11	42.0	29.0	1372	970	41	54
Ramet 1	7.0	7.0	1359	985	38	64
Ramet 2	79.0	51.0	1385	955	44	43
14	2.5	6.0	1268	1111	39	41
15	6.0	21.0	1378	870	47	52

A significant correlation was found between mean phenolic concentration at age 16 mo. (July samples) and percentage of aborted, full, and empty seeds ($r = -.54$, $.76$, and $-.74$, respectively). Reasons for this correlation are unapparent at this time. A correlation earlier in the growing season would be expected because the percent abort figure represented the percentage of aborted ovules early in the second growing season. Also, no correlation was

found between percent seed bug infestation and mean phenolic concentration at age 16 mo. (July samples), which would be late in the second growing season.

There are several possible explanations for not finding a relationship of mean tannin and mean phenolic concentration to seed bug and coneworm infestation levels. Most importantly, the orchard was sprayed from the ground using a mist blower from April through August with Guthion 2S for seed bug control and Pydrin for coneworm control. Expected effectiveness of this treatment is 80 to 90 percent. Because some trees in the orchard may not have been as effectively sprayed as others due to differing total heights and crown depths, any differences in infestation levels among clones found in this study may not be the same if the entire orchard was untreated or sprayed at a consistent level. However, even if the orchard was sprayed at a consistent level, differences in infestation levels would still be expected. Another explanation may be that quality of tannins and phenolics, rather than quantity, is responsible for susceptibility or resistance to insect attacks. In trees with low tannin levels, phenolic levels, and low infestation levels, a specific phenolic compound may be present which is responsible for the tree's decreased susceptibility. The opposite may be true for trees with high tannin and phenolic concentrations with high infestation levels. Some trees that had a high infestation level also had high concentrations of mean tannins and mean phenolics. If these trees had lower mean tannin and mean phenolic concentrations the infestation level may have been even greater. One last possibility may be that once a tree is attacked it will increase production of phenolic compounds as a response to the attack. Schultz and Baldwin (1982) reported that mechanically damaged poplar (Populus x euromericana) ramets and sugar maple (Acer saccharum Marsh) seedlings showed increased concentration and rates of synthesis of phenolic compounds.

Drought or other growth stress can influence phenolic production (Forrest 1975). Because of the high energy cost associated with carbohydrates being used for phenolic production rather than for growth, a stressed tree would probably produce less phenolic compounds than would a healthy, vigorous tree. Available carbohydrates would be allocated to growth and foliage production rather than secondary plant substances (Hillis 1968). For this reason, we feel that cones would be more susceptible to insect attack during stress years. However, a more vigorous tree would generally produce more cones than would a stressed tree, thus there would be more available infestation sites on a more vigorous tree.

SUMMARY AND CONCLUSIONS

Tannin and phenolic concentrations varied throughout the lifecycle of loblolly cones. Tannin concentration was highest for all clones at age 10 mo. and lowest for all but 1 clone at age 16 mo.

The pattern of phenolic concentration was less consistent for all clones than was the tannin concentration. Most clones peaked in phenolic concentration at age 10 mo.; however, the age of cones at time of lowest phenolic concentration varied among clones. Depletion of starch caused by the vegetative flush of growth in the spring may be responsible for the decrease in phenolic concentration in May.

Overall analysis of variance of mean tannin cor centration and mean phenolic concentration of cones revealed age to be a significant factor. Variation of tannin and phenolic concentration of cones among ramets within clones and interactions of clone by age were not significant, but a significant level of clonal variation was found for mean phenolic concentration of cones.

No relationship was found between mean tannin concentration or mean phenolic concentration of cones and seed bug and coneworm infestation levels. Possible reasons for the lack of correlation may be non-uniform chemical treatment of the orchard studied or that quality of tannins and phenolics, rather than quantity, was responsible for susceptibility or resistance to insect attacks.

Further research on tannin and phenolic concentration of loblolly pine cones should include an examination of the relationships of crown position of cones and their tannin and phenolic concentrations. Cones from orchards not treated with insecticides need to be examined and the phenolic concentration of seeds, cone axes, and conescales should be studied individually to better understand the relationship of tannin and phenolic concentration to insect resistance or susceptibility. We feel that the most valuable information to be gained from future research will be the delineation of the specific phenolic compounds that are present in the cones throughout their lifecycle and the correlation between these specific phenolics and insect resistance or susceptibility.

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