ween the 3rd and 9th and between the 9th and 14th months of planting for the two parameters. A negative correlation was obtained between the 3rd and 14th month of planting. The negative correlation might be due to an increased improvement in performance of source materials that had an unfavourable beginning. A weakening in correlation between early and later nursery vigour measurements have also been reported by SENANAYAKE *et al.* (1975), though negative correlations were not obtained.

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Chemogenetic study of phenolic compounds extracted from loblolly pine (Pinus taeda L.) needles[1])

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Summary

The objectives of this study were to examine the variation of phenolic constituents of pine needles among selfed progenies of loblolly pine and to determine the inheritance of phenolic constituents in loblolly pine needles. Most of the compounds found in the grafts were found in the selfed progenies. Four needle constituents showed segregation patterns in the selfed families. The observed segregation ratios of compound 53 in the selfed progenies fit a single gene hypothesis best. The inheritance patterns of the other three compounds are not clear from this study. Compound 53 was identified as dihydrokaempferol 7-0-glucoside by UV, NMR and mass spectra. Because dihydrokaempferol 7-0-glucoside might be a precusor of taxifolin 7-0-glucoside whose aglycone has been shown to inhibit the growth of a variety of pathogens, either compound could be correlated with disease resistance.

Key words: flavanoids, dihydrokaempferol, phenolics, grafts, selfs, thin-layer chromatography.

Zusammenfassung

Der Zweck dieser Untersuchung war, die Variation von phenolischen Bestandteilen in Kiefernnadeln von Selbstungen von *Pinus taeda* zu beschreiben und die Vererbung dieser phenolischen Stoffe zu bestimmen.

Die meisten in den Pfropfreisern vorhandenen Stoffe waren auch in den Selbstungen vorhanden. Vier der Inhaltsstoffe zeigten in den Selbstungen Aufspaltungen. Die beobachteten Spaltungsverhältnisse von Substanz 53 in den Selbstungen deuten am besten auf die Ein-Gen-Hypothese hin. Die Vererbungsmodi der anderen drei Stoffe waren nicht eindeutig.

Substanz 53 wurde mit Hilfe von UV, NMR und Massenspektroskopie als Dihydrokaempferol 7-0-Glucosid identifiziert. Weil Dihydrokaempferol 7—0-Glucosid eine Vorstufe von Taxifolin 7-0-Glucosid sein könnte, deren Aglycone das Wachstum verschiedener Pathogene hemmt, könnte diese Substanz mit der Krankheitsresistenz zusammenhängen.

Introduction

In recent years, many biochemicals, such as phenols, terpenoids (VON RUDLOFF 1975; SQUILLACE 1976), enzymes (HARE and SWITZER 1969; YANG *et al.* 1977), and DNA (MIKSCHE and HOTTA 1977) have been used in genetics studies. Because of their chemical complexicity, variable occurrence among species and ready detection in plant extracts as complex patterns of spots on two-dimensional chromatograms, phenolic compounds have often been used for the verification of species (HANOVER and WILKINSON 1970), races (FROST *et al.* 1977), cultivars (GRANT 1973), and hybrids (HOFF 1968), for introgression studies (DUNCAN 1975) and for studies of genetic differences in disease resistance (TJIA and HOUSTON 1975; NOMURA and KISHIDA 1978).

If a specific biochemical trait can be detected at an early age and is correlated with disease resistance, it can be of considerable value as a mean of indirect selection for disease resistance. Phenolic compounds may be particularly useful in this respect (KAUFMAN *et al.* 1974).

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To effectively use a phenolic compound as a genetic marker for a physiological character, it is necessary to know its inheritance pattern. In view of the need for early testing procedures in loblolly pine, we investigated the inheritance of foliage phenolic compounds with the hope that we could lay the groundwork for later studies of correlations between phenolic compounds and traits of importance in breeding programs. This study was carried out to examine the variation of phenolic constituents of pine needles among the selfed progenies of loblolly pine and to determine the inheritance patterns of phenolic compounds in loblolly pine needles.

Materials and Methods

The selfed progenies and the grafts used in this experiment were from a heritability study initiated in the early 1960's. In this study six forest stands were sampled from Mississippi, Louisiana and Texas to determine the inheritance of quantitative characters in natural populations of loblolly pine. Ten trees from each stand were randomly chosen and mated in a modified half-diallel design as described by GRIFFING (1956). In addition a number of parents were self-pollinated in a separate study. The selfed progenies and a number of grafts of the parents, were established at the Southern Institute of Forest Genetics, Gulfport, Mississippi from 1970 to 1972. Three grafts of each parent and approximately 12 individuals from each self-pollinated family were sampled. Ten families were included in this study. One-year-old needles were collected on August 1 and August 2, 1977. The needles were placed in sealed plastic bags and stored in a freezer.

Prior to extraction the needles were cut into approximately 1-cm lengths. Three grams of needles were placed in the container of a Waring Blendor and 50 ml of boiling water were added into the container and allowed to stand for two minutes. The mixture was then blended for two minutes. The resulting pulp was centrifuged and the supernatant was transferred to a separatory funnel. The aqueous solution was extracted with 25 ml of ethyl ether and then with 10 ml of n-butanol. Five ml of water were added to the n-butanol fraction and the azeotropic mixture was taken to dryness at temperatures between 30° C and 35°C with a flash evaporator. The residue was dissolved in 0.2 ml of 95 % ethanol. The ethanol solution was stored in a freezer ready for chromatography.

Five μl of the phenolic extract in ethanol were chromatographed on cellulose TLC plate (ANALTECH, Newark, Del.). The plate was developed with n-butanol: acetic acid: water (65:10:25) followed by sodium formate: formic acid: water (10:1:200, w/v/v) in the second dimension. Compounds were located by viewing the chromatogram under UV light (360 nm) before and after exposure to ammonia vapor.

For isolation of compound 53, six kg of air-dried needles were ground in a Waring Blendor. The neeedle powder was extracted with acetone. After acetone was removed from the acetone solution, the residue was dissolved in water and extracted with hexanes. The aqueous solution was treated with lead acetate. After lead phenolates, which precipitated out, were removed, the aqueous solution was further treated with phosphoric acid to remove the excess lead acetate. After lead phosphate was removed by filtering, the filtrate was concentrated in vacuo. The concentrated aqueous solution was chromatographed on a 4.5 × 32 cm polyamide column (polyamide-CC6, 0.07 mm; Brinkmann; Westbury, New York), eluted with water and aqueous methanol solution, followed by chromatography on a 2.4 × 30 cm Sephadex G-25 column (Pharmacia Fine Chemicals, Piscataway, New Jersey), eluted with 10 % methanol. The fractions, which contained compound 53 were taken to dryness and the residue was dissolved in 95 % ethanol. Compound 53 in ethanol was further purified on silica gel G preparative TLC plate (ANALTECH) with chloroform:methanol: water (14:6:1), followed with ethyl acetate:methanol:water (130:17:13).

NMR spectrum and UV spectra of compound 53 were taken. Compound 53 was hydrolyzed in a 100°C water bath for 45 minutes. The hydrolyzate was extracted with ethyl acetate. The sugar in the aqueous fraction was identified

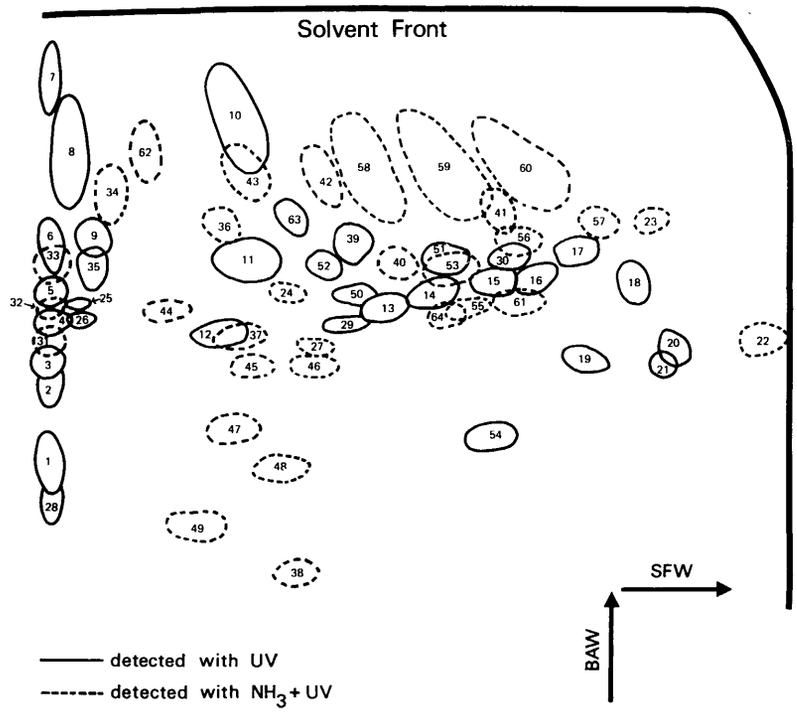

Figure 1. — Two-dimensional composite chromatogram of loblolly pine needle constituents.

Table 1. — Chromatographic properties of foliage constituents of *Pinus taeda*.

Compound number	Rf (BAW)	Rf (SFW)	Color in UV[a]	Color in UV + NH_3[a]
1.	.37	.01	BB	B
2.	.47	.01	RY	RY
3.	.51	.01	RY	RY
4.	.57	.01	BB	GY
5.	.61	.01	RY	RY
6.	.67	.01	B	GY
7.	.91	.00	R	R
8.	.79	.03	P	G
9.	.68	.07	GY	GY
10.	.85	.26	B	B
11.	.66	.27	P	B
12.	.55	.23	OY	OY
13.	.59	.45	BB	B
14.	.61	.52	RY	RY
15.	.63	.60	GB	GB
16.	.64	.66	B	-
17.	.68	.72	RY	RY
18.	.63	.79	V	V
19.	.52	.73	V	-
20.	.54	.85	V	V
21.	.51	.83	B	-
22.	.56	.96	-	B
23.	.71	.81	-	B
24.	.61	.32	-	GY
25.	.60	.04	P	RY
26.	.57	.05	RY	RO
27.	.54	.36	-	Y
28.	.31	.01	RY	-
29.	.57	.40	GB	GB
30.	.66	.64	G	-
31.	.54	.01	-	GY
32.	.59	.01	-	GY
33.	.66	.01	-	GY
34.	.75	.09	-	RY
35.	.64	.07	GY	OY
36.	.71	.24	-	GY
37.	.55	.26	-	R
38.	.22	.34	-	P
39.	.67	.41	P	GY
40.	.65	.48	-	GB
41.	.73	.61	-	GY
42.	.78	.37	-	GB
43.	.78	.27	-	GY
44.	.59	.16	-	P
45.	.51	.27	-	P
46.	.51	.36	-	P
47.	.42	.25	-	P
48.	.37	.32	-	P
49.	.29	.20	-	P
50.	.61	.41	P	GY
51.	.66	.54	BG	BG
52.	.65	.38	GB	-
53.	.65	.54	-	GY
54.	.41	.60	B	-
55.	.59	.57	-	B
56.	.69	.64	-	B
57.	.71	.74	-	BB
58.	.78	.43	-	P
59.	.78	.54	-	P
60.	.78	.65	-	P
61.	.60	.64	-	B
62.	.80	.14	-	GY
63.	.72	.33	B	B
64.	.58	.54	-	GY

[a] B = blue, G = green, P = purple, R= red, V = violet, Y= yellow, BB = bright blue, BG = blue green, GB = green blue, GY = green yellow, OY = orange yellow, RO = red orange, and RY = red yellow.

by cellulose TLC, eluted with ethyl acetate:pyridine:water (12:5:4). The ethyl acetate fraction, which contained the aglycone, was taken to dryness. A high resolution mass spectrum of the aglycone was taken.

Results and Discussion

Phenotypes of the grafts and the self-pollinated families

The two-dimensional composite chromatogram of the chemical constituents in loblolly pine needles is shown in *Figure 1*. The R_f values and fluorescent colors of needle constituents are shown in *Table 1*.

The phenotypes of the selfs generally reflect the phenotypes of the grafts. Four phenolic compounds occurred in the needles of only part of the self-pollinated families. The frequency of occurence of these compounds was calculated and the results are shown in *Table 2*. To calculate the frequency of occurrence, the parents whose grafts do not contain a particular compound are placed in another group. The frequency of a compound occurring in each corresponding group of progenies are then calculated from the number of trees which contained the particular compound divided by the total number of trees examined. In a few cases the grafts of a parent did not show a uniform phenotype. These parents and their progenies are excluded from this calculation. The probability of finding a particular compound in the selfed progenies of a parent whose grafts contain the compound is significantly higher than that in the selfed progenies of a parent in which the compound is not detected in the grafts. Apparently, the frequency of occurence of these compounds is genetically controlled.

Inheritance patterns:

Phenolic compounds in pine foliage have a high degree of heritability. Most of the phenolics found in the grafts were found in the selfed progenies. THIELGES (1972) suggested that some phenolic compounds in pine needles followed monogenic inheritance pattern. Nevertheless, the inheritance patterns of phenolic compounds in pine needles are not very clear.

The segregation ratios of compound 41, 42, 43 and 53 are shown in *Table 3*. Chi-square values are calculated using the expected 3 to 1 ratio for one heterozygous gene pair. Among the segregation ratios that were significantly different from 3 to 1 ratio at the 5% level, five had only one phenotype, e. g., compound 41 was not detected in any needle samples from family A3PT34. Such families most likely are homozygous. Nevertheless, it is difficult to rationalize the discrepancy of the remaining nine segregation ratios.

The observed segregation ratios of compound 53 agree with a monogenic inheritance pattern, but the observed segregation ratios of compounds 41, 42 and 43 do not fit a single gene hypothesis well. Compounds 41, 42 and 43 exhibit a discontinuously segregation distribution. The concentrations of these compounds do not appear to follow the

Table 2. — Relation between the occurrence of four needle constituents in parental grafts and their frequency in the corresponding self-pollinated families.

	Frequency of occurrence in the S. progenies			
	Compound 41	Compound 42	Compound 43	Compound 53
P	0.85	0.82	0.75	0.93
A	0.28	0.39	0.16	0.44

P = Compounds were present in grafts
A = Compounds were absent grafts

Table 3. — Segregation ratios and their Chi-square (X^2) values of four phenolic compounds in the pine needles of the selfed progenies.

Family	Compound 41		Compound 42		Compound 43		Compound 53	
	P:A[a]	X^2	P:A	X^2	P:A	X^2	P:A	X^2
A3PT31	4:1	0.07	4:1	0.07	2:3	1.67	4:1	0.07
A3PT34	0:9	23.15*	0:9	23.15*	0:9	23.15*	4:5	3.00
A3PT35	9:3	0.11	8:4	0.11	6:6	2.78	12:0	2.78
A3PT37	1:11	25.00*	3:9	13.44*	1:11	25.00*	10:2	0.11
A3PT38	5:7	5.44*	3:9	13.44*	0:12	32.11*	12:0	2.78
A3PT40	14:0	3.43	12:2	0.38	14:0	3.43	14:0	3.43
A6PT25	9:1	0.53	6:4	0.53	0:10	26.13*	9:1	0.53
A6PT26	2:8	13.33*	2:8	13.33*	1:9	19.20*	5:5	2.13
A6PT28B	7:4	0.27	8:3	0.03	1:10	22.09*	11:0	2.45
A6PT30	10:2	0.11	12:0	2.78	9:3	0.11	12:0	2.78

* Significantly different from the 3:1 ratio at the 5 percent level.
[a] P:A The segregation ratio in terms of presence or absence of a particular compound.

Correction: In line 8. 13.33* should read 11.67* and in line 9 2.45 should read 3.54.

normal distribution as expected of multigenic inheritance, however, it is not possible to tell the inheritance patterns of compounds 41, 42 and 43 from this study. Although the segregation ratios of compound 53 in families A3PT35, A3PT38, A3PT40 and A6PT30 do not significantly differ from the 3 to 1 ratio, it is very likely that many of these families are homozygous dominant.

Compound 53:

Compound 53 was not normally observed on TLC chromatograms under UV light. However, when a sufficient amount of compound 53 was accumulated during purification, it showed purple color. Compound 53 was soluble in water and methanol.

The UV spectrum had the following features:

λ_{max}^{MeOH} (ε): 215 (24260), 227 sh, 285 (14000), 331 sh nm

λ_{max}^{MeOH} + NaOMe: 243 sh, 286, 350 sh nm

λ_{max}^{MeOH} + $AlCl_3$: 220, 268, 310, 353 nm

λ_{max}^{MeOH} + $AlCl_3$ + HCl: 220, 270 sh, 305, 353 nm

λ_{max}^{MeOH} + NaOAc: 285, 326 nm

λ_{max}^{MeOH} + NaOAc + H_3BO_3: 285, 360 nm

The NMR spectrum of compound 53 in D_2O showed a 5-proton multiplet at δ6.00 (H-6) and δ6.2 (H-8) one-proton singlets at δ6.00 (H-6) and δ6.2 (H-8) and two two-proton doublets at δ6.92 (H-3' and H-5') and δ7.36 (H-2' and H-6') with a coupling constant of 8 cps.

The sugar moiety of compound 53 was identified as D-glucose by TLC.

The molecular formula of the aglycone of compound 53 was determined by high resolution mass measurement (calculated for $C_{15}H_{12}O_6$: 288.0634; found: 288.0638). Major ionic fragments were noted at m/e 288 (30 %), m/e 259 (31 %), m/e 165 (15 %), m/e 153 (100 %), m/e 136 (32 %).

Compound 53 was identified as dihydrokaempferol 7-0-glucoside. This was the first time dihydrokaempferol 7-0-glucoside was observed in a forest tree. The closely related compound taxifolin is well known to inhibit the growth of a variety of pathogens (Nomura and Kishida 1978; Ramaswamy et al. 1972). It differs from dihydrokaempferol by one hydroxyl group. Because dihydrokaempferol 7-0-glucoside is found in loblolly pine needles, there is a chance to find taxifolin 7-0-glucoside as well. On the other hand, trees that do not synthesize dihydrokaempferol 7-0-glucoside in needles are less likely to synthesize taxifolin 7-0-glucoside. Thus, should taxifolin 7-0-glucoside be found to correlate with other physiological characters, such as resistance to a certain disease, taxifolin 7-0-glucoside or dihydrokaempferol 7-0-glucoside could be used as a genetic marker for early selection of trees that are resistant to this particular disease.

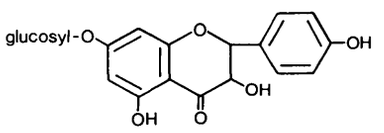

Figure 2. — Chemical structure of Compound 53.

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