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FINAL PROGRESS REPORT

"ANALYSIS OF CHEMICAL COMPONENTS FROM PLANT TISSUE SAMPLES"

Contract No. NAS 9-11339

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Facility & Laboratory Support Branch

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INDEX

ITEM		PAGE
I.	Introduction	2
II.	Methods	
	(a) Preparation of Methyl Esters	2
	(b) Preparation of Trimethylsilyl Ethers and Acetates	2
III.	Instrumentation	
	(a) Gas Chromatography	3
	(b) Mass Spectrometry	3
	(c) Computer	3
IV.	Literature Cited	4
V.	Financial	5
VI.	Results	
	(a) Tabulation of Data (Example)	6-10
	(b) Publications and Manuscripts Produced which Summarize the Findings of the Program	Appendix

(This Index is for Volume IV only.)

I. Introduction: This progress report includes information as to the type and concentration of sterols, free fatty acids and total fatty acids in samples received during the duration of the contract. All samples were analyzed by gas chromatography and then by gas chromatography-mass spectrometry combination. In each case the mass spectral data was accumulated as a computer data printout (relative intensity vs m/e) and as a plot. Typical gas chromatogtams are included for reference purposes.

II. (a) Preparation of methyl esters: All fatty acid samples were converted to the corresponding fatty acid methyl esters by the use of a methanol-BF_3 reagent (1).

(b) Preparation of trimethyl silyl ethers and acetates: All sterols reported in this document were run as the free sterols. However, in two cases, in order to achieve better gas chromatographic separation, the extracted sterols were converted to the corresponding trimethyl silyl ethers. Preparation of the ethers was accomplished by treatment of the sterol (0.1 - 1 mg) in dry pyridine (10 - 20 µl) with hexamethyldisilazane (10 - 20 µl) and trimethylchlorosilane (2 - 5 µl) at room temperature. The reagents were removed in a stream on nitrogen and the residue was extracted with hexane or chloroform. The resulting solution was used directly for chromatography (2).

Acetylation of the sterols was accomplished by treating 1-10 mg portions of the sterol fraction with (1 ml) acetic anhydride and (5 ml) pyridine followed by heating at 40°C for 10 min. The resulting solution was directly injected in the gas chromatograph.

III. Instrumentation:

(a) Gas Chromatography: Stainless steel columns packed with 12% ethylene glycol succinate were used, which were 9' x 1/8". Chromosorb W was the solid support. Sterols were separated on 1% OV-17 glass column 6' x 2mm using chromosorb W as the solid support. Separations were accomplished using Hewlett-Packard 7620 gas chromatographs with flame detectors.

(b) Mass Spectrometry: Mass spectrometry was carried out using duPont 21-491 double focusing mass spectrometer with Hewlett-Packard 7620 and 5750 gas chromatograph inlet.

(c) Computer: All mass spectrometric data was reduced using a PDP-1050. Fortran language was used. Print-out was accomplished using a LP-12 high speed line printer coupled to a Houston complot plotter.

IV. Literature: Literature outlining the procedures employed in this program are cited in the following section.

LITERATURE CITED

(1) Morrison, N. R. and L. M. Smith. J. Lipid Res. 5: 600-08 (1964).

(2) Brooks, C. J. W., E. C. Horning, and J. S. Young. Lipids, 3 (5) 391-402 (1968).

(3) Benveniste, P., L. Hirth, and G. Ourisson. Phytochem. 5, 31-44 (1966).

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V. Financial: No items of equipment costing more than $100.00 was purchased with funds from Contract No. NAS 9-11339.

VI. Results:

(a) Tabulation of data is illustrated in the following section, Tables I-IV.

(b) Publications and manuscripts produced which summarize the findings of the program are attached as an Appendix.

TABLE I. FREE FATTY ACID COMPOSITION OF TISSUE CULTURE EXTRACTS AS METHYL ESTERS

SAMPLE NUMBER	FATTY ACID (%)														
	C_{14}	$C_{14:1}$	C_{15}	$C_{15:1}$	C_{16}	C_{17}	$C_{17:1}$	C_{18}	$C_{18:1}$	C_{19}	$C_{18:2}$	$C_{18:3}$	C_{20}	C_{21}	C_{22}
1	tr	tr	0.5	0.9	22:2	2.2	7.2	3.6	5.1	tr	34.1	26.1	3.1	tr	-
2	tr	tr	0.4	0.5	20.2	2.0	1.5	5.3	5.4	0.9	30.5	31.1	2.0	tr	-
3	tr	tr	0.5	0.5	24.2	1.0	1.7	5.6	7.5	tr	34.5	22.9	0.9	tr	0.7
4	tr	tr	0.5	0.5	24.8	1.8	2.2	5.3	6.1	tr	31.7	25.8	0.7	0.3	0.3
5	tr	tr	0.2	0.3	22.0	1.8	1.0	5.1	5.5	tr	34.1	29.7	0.3	-	-
6	tr	tr	0.5	1.0	20.4	1.4	tr	3.1	3.4	tr	32.0	37.2	1.0	-	-
7	tr	tr	0.5	0.8	19.7	2.3	2.0	5.7	5.7	tr	31.6	28.6	2.3	-	0.8
8	tr	tr	0.6	0.7	20.4	1.5	0.6	4.4	3.4	tr	30.1	37.3	1.0	tr	-
9	tr	tr	0.5	0.9	21.4	1.8	1.7	2.1	5.5	tr	29.5	35.7	0.8	-	-

tr - TRACE

TABLE II. TOTAL FATTY ACID COMPOSITION OF TISSUE CULTURES AS METHYL ESTERS

SAMPLE NUMBER	FATTY ACID (%)													
	C_{14}*	C_{15}	$C_{15:1}$	C_{16}	$C_{16:1}$	C_{17}	$C_{17:1}$	C_{18}	$C_{18:1}$	C_{19}	$C_{18:2}$	$C_{18:3}$	C_{20}	C_{22}
10	0.4	0.7	2.0	21.4	7.8	1.5	0.9	2.6	2.8	4.7	28.3	20.0	4.0	3.1
11	0.3	0.5	0.8	17.7	0.8	1.1	1.4	0.6	3.3	1.8	30.4	29.6	4.5	6.1
12	0.4	0.4	0.3	19.5	2.2	3.5	0.5	0.7	3.0	2.2	40.4	21.8	2.8	2.6
13	1.7	0.7	1.3	21.5	3.8	2.0	1.5	3.8	4.9	2.6	25.7	26.5	5.4	3.7
14	1.6	0.4	1.0	19.3	2.0	1.4	1.1	3.9	3.9	2.5	30.2	24.6	4.6	0.7
15	0.9	0.6	1.2	16.8	1.2	1.4	1.9	1.8	4.1	1.5	27.0	31.4	0.5	8.3
16	0.6	0.5	0.8	20.2	0.6	0.8	1.0	0.9	3.6	1.6	36.2	29.8	0.2	2.6
17	0.4	0.4	0.9	15.4	0.5	1.5	1.1	--	2.7	2.7	31.0	36.2	--	4.6
18	0.5	0.5	0.7	19.6	0.6	1.2	1.9	1.5	4.2	0.8	28.5	35.6	0.3	--

* Structure confirmed by MS but concentration too low for computer printout of data and plot.

TABLE III. RELATIVE PERCENT (%) DISTRIBUTION OF THE TOTAL STEROLS FROM TISSUE CULTURES

	SAMPLE NUMBER								
	19	20	21	22	23	24	25	26	27
UNKNOWN 1	0.9	0.8	tr	0.1	tr	tr	tr	---	---
" 2	1.4	0.6	tr	0.3	0.3	0.2	0.1	0.3	tr
" 3 **	2.6	1.1	1.8	2.3	1.7	3.0	1.7	2.7	1.6
CAMPESTEROL	9.3	22.7	6.4	19.2	8.1	21.6	5.8	11.8	7.2
STIGMASTEROL	11.3	27.5	8.7	13.9	15.1	18.1	13.7	10.6	8.8
β-SITOSTEROL	71.0	44.5	60.9	63.2	42.7	55.5	47.1	73.5	81.0
UNKNOWN 4	tr	tr	---	tr	tr	tr	---	---	tr
" 5	0.7	2.0	7.4	1.3	9.3	0.5	12.4	0.3	0.8
" 6	1.9	2.6	17.1	1.0	22.2	0.3	19.0	0.3	2.5
" 7	0.7	0.1	tr	0.3	tr	0.9	tr	---	tr
" 8	tr	2.4	tr	tr	0.7	---	tr	---	---
CAMPESTEROL/ STIGMASTEROL (C/S)	1.21	1.21	1.35	0.72	1.82	0.84	2.36	0.89	1.22

** - MIXTURE WITH MOLECULAR WEIGHTS OF 320 and 322 WITH RETENTION TIMES SIMILAR TO CHOLESTEROL

tr - TRACE

TABLE IV TOTAL FATTY ACID, FREE FATTY ACID AND STEROL COMPOSITION OF SAMPLES (µg/g fresh weight)

SAMPLE	CONCENTRATION
FREE FATTY ACIDS:	
1	64.6
2	*
3	*
4	48.3
5	40.8
6	64.2
7	103.0
8	48.8
9	54.9
TOTAL FATTY ACIDS:	
10	87.7
11	88.3
12	78.8
13	89.5
14	45.2
15	82.6
16	116.5
17	82.3
18	119.5

STEROLS:

19	165.8
20	220.1
21	4
22	[illegible]
23	97.1
24	150.4
25	120.8
26	1568.6
27	823.7

* A portion samples lost and therefore impossible to determine accurate ies.

APPENDIX

The following articles and manuscripts resulted from NASA Contract NAS 9-11339 and summarize the findings.

(1) Apollo 12 Lunar Material: Effects on Lipid Levels of Tobacco Tissue Cultures. John D. Weete, Charles H. Walkinshaw, and John L. Laseter. Science, 175, 623 (1972). (A72-27626)

(2) Fatty Acid Ethyl Esters of Rhizopus arrhizus. John L. Laseter and John D. Weete. Science, 172, 864 (1971). (A71-29352)

(3) Gas Chromatographic Determination of Trimethylsilyl Derivatives of Free Amino Acids from a Botanical Source. J. L. Laseter, J. D. Weete, A. Albert, and C. H. Walkinshaw. Anal. Letters, 4(10), 671 (1971). (Not Attached)

(4) Response of Tobacco Tissue Cultures Growing in Contact with Lunar Fines. John D. Weete, Charles H. Walkinshaw, and John D. Laseter. Space Life Sciences, 3 (4), 38 (1972). (Not Attached)

(5) Volatile Terpenoids from Aeciospores of Cronartium Fusiforme. J. L. Laseter, J. E. Weete, and C. H. Walkinshaw. Phytochemistry (in press) (1973). (Attached)

(6) Fatty Acids of Slash Pine Tissues. J. L. Laseter, G. C. Lawler, C. H. Walkinshaw, and J. D. Weete. Phytochemistry (in press) (1973). (Attached)

(7) Gas Chromatography-Mass Spectrometry Study of Sterols from Slash Pine Tissues. J. L. Laseter, R. Evans, C. H. Walkinshaw, and J. D. Weete. Phytochemistry (in press) (1973). (Attached)

PREPRINT

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VOLATILE TERPENOIDS FROM AECIOSPORES OF CRONARTIUM FUSIFORME

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ABSTRACT

Aeciospores of *Cronartium fusiforme* isolated from slash pine (*Pinus elliottii*) trees were analyzed for volatile terpenoids by gas chromatography and gas chromatography-mass spectrometry. α-Pinene, β-pinene, Δ^3-carene, myrcene, linonene, β-phellandrene, and δ-terpinene were the major monoterpenoid hydrocarbons present with only traces of camphene. A number of monoterpenoid alcohols were also present of which terpinen-4-ol predominated. Among the various acyclic sesquiterpenes present, β-farnesene and β-citronellol were identified. Several compounds with aromatic rings were also observed.

INTRODUCTION

When the steam volatile constituents of *Ceratocystis*, *Penicillium*, and *Fusarium* species grown on synthetic media were investigated by Sprecher[1], no detectable quantities of monoterpenes were present. He further demonstrated that the lack of monoterpenes was not a result of their preferential degradation in the culture media by the fungi investigated. More recently, however, simple terpenoids have been reported as constituents of the volatile fraction of two wood-rotting species of *Ceratocystis* and *Trametes* grown in culture, but no cyclic monoterpenes were detected among the volatile products reported.[2,3] Little work has been reported which deals with volatile hydrocarbons from other fungal organisms and no attention has been given to those fungi which are pathogenic on members of the terpenoid producing Pinaceae family.

In recent years, considerable information has been published by Smith[4], Roberts[5], and others[6-8] on the volatile terpenoids of various *Pinus* species. Predominate monoterpenoids indigenous to the Pinaceae family include acyclics such as myrcene and ocimene; monocyclics like limonene, β-phellandrene, δ-terpinene, and bicyclics such as α-pinene, camphene, β-pinene, Δ^3-carene. Many of the above carbon skeletons also occur in hydrated forms such as borneol and terpinen-4-ol. In addition, a large number of both acyclic and cyclic sesquiterpenes have also been described for the pinyons.

At this point it is interesting to note that volatile terpenoid constituents of oleoresin are among the factors thought to be related to susceptibility of Pinus species to attack by wood-inhabiting fungi.[9,10] There is also some evidence which suggest that the terpenoid composition can vary in pine trunk xylem as a result of wounding.[11] For these reasons it seemed timely to determine if a pine rust disease causing organism such as C. fusiforme elaborates volatile terpenoids and if so, whether or not the terpenoids are similar to those reported for the gymnosperm host.

RESULTS AND DISCUSSION

This paper reports the occurrence of terpenoids present in the volatile fraction from aeciospores of the gall rust fungus C. fusiforme. Due to small sample sizes, the common steam-distillation techniques were not employed. Volatiles were driven off by gentle heating in a helium gas stream and trapped at acetone-dry ice temperatures. The same procedure was used with mature slash pine tissues and the results compared favorably with those previously reported. The isolation method was a modification of that used to monitor the volatile components of human breath and urine.[12]

Table 1 lists the volatiles identified from a 0.2 gm aeciospore sample with the relative concentration of each computed from gas chromatographic peak areas. As can be seen,

not only are a number of common monoterpenes present but oxygenated monoterpenes and sesquiterpenes are also found in the volatile fraction. Mass spectral data of the monoterpene hydrocarbons compared favorably with those previously published.[13,14] Even though differences between the spectra of the monoterpenes are not great, the contrast is sufficient to allow one to easily differentiate them from each other. As an added precaution, however, the volatiles from young slash pine needles and stems were also extracted and analyzed. The electron impact data coupled with information from authentic standards provided confirmation for most of the terpenoid structures reported. α-Pinene is the major monoterpene component and is over 2.5 times the concentration of β-pinene. Roberts[5] observed that the α-pinene percentage was greater than that of β-pinene in all xylem oleoresin originating from 19-year-old Pinus elliottii trees. In no instance did he observe, on a relative basis, myrcene values above 18 percent of the total monoterpene hydrocarbon content of the various tissues studied. Myrcene, however, is a major component of the xylem monoterpenes of P. monophylla and is frequently present in low quantities in other pine species.[6,8] On the other hand, the fact that the bicyclics such as Δ^3-carene and camphene as well as the monocylic compound limonene are present in small amounts relative to the other monoterpenes seems to be in good

agreement with previous reports for P. elliottii.[5] Taken as a class, however, the monoterpenes appear to be lower in concentration than the higher-boiling volatiles when compared to slash pine needles, stem cortex and stem xylem tissues extracted and analyzed by the same technique.

Molecular ions of m/e 156 were common in scans made of compounds with relative retention volumes higher than δ-terpinene and were taken as indicative of monoterpene alcohols. Terpinen-4-ol was positively identified and the resulting spectra agreed with that previously published.[15] A compound with a base peak at m/e 69 and major fragments at m/e 41 and 55 with a molecular ion at m/e 156 was also observed and identified as β-citronellol.[15] It appeared that several other acyclic alcohols were also present, but in lower concentrations. Mass spectra of these alcohol components were typical of acyclic compounds by demonstrating high intensity fragmentation at low mass. Additional evidence was provided by meta-stable ions at m/e 89.2, 78.1, and 37.2 which are characteristic of acyclic monoterpene alcohols. Citronellol has been reported by Collins and Halim[2] as the major volatile monoterpene component in Ceratocystis variospora and Trametes odorata. β-Farnesene appears to be present in relatively high concentrations in the aeciospores of this study and produced a molecular ion at m/e 204 and fragments characteristic of a 3,7,11-methyl branched structure. This acyclic

sesquiterpene has been previously reported as a minor component of the higher-boiling terpenoids of P. monophylla.[5]

A compound having a relatively high retention volume and a molecular ion at m/e 108 was identified as o-cresol. Other major confirming fragments were present at m/e 79 and 77 which correspond to the benzenium and $C_6H_5^+$ ions respectively. Fragments at m/e 90, corresponding to the loss of water, were very evident and typical of the ortho isomer.[17] Scans of other less well resolved chromatographic peaks with strong molecular ions at m/e 108 were also observed and associated with intense peaks at m/e 78 corresponding to $C_6H_6^+$ fragments. Such fragments are known to be present with aromatic ethers like anisole.[18] In no instance were fragments characteristic of methyl esters (m/e 74, 87, and 143) or ethyl esters (m/e 88 or 101) observed in any spectra. Likewise, if there were any normal alkanes present they were below the limits of detectability.

METHODS AND MATERIALS

Aeciospores of C. fusiforme were collected in the field from infected slash pine (Pinus elliottii) trees averaging 15 years of age. The aeciospores, collected in 1972, were maintained at 5°C until analyzed. Pine tissues used in this study were collected locally from 5-6-year old trees.

Volatiles were collected by passing a stream of ultra-pure helium across approximately 0.2-0.3 gm of sample in a 6 mm x 10 cm pyrex tube at a rate of 10 ml/min. After purging for 5 min. at room temperature the glass tube containing the spores was heated to approximately 40°C by means of a heat gun for 20 min. The efluent gas containing the volatile components passed directly through a cold trap emmersed in an acetone-dry ice bath. The trapping apparatus was 2 ft. in length and constructed of stainless steel. The inlet segment was 0.125 in. o.d., 0.10 in. i.d. tubing silver soldered to an outlet segment of 0.0625 in. o.d., 0.04 in. i.d. stainless steel tubing. The trapping apparatus is a modification of that employed by Teranishi, et al. for evaluation of volatiles from breath and urine.[12] The trap was vented to the atmosphere during sample collection. Injection onto the gas chromatograph column was accomplished by means of a stainless steel capillary 6-way gas sampling value (Varian Aerograph, Walnut Creek, Calif.) as the acetone-dry ice was removed and the trap heated by a heat gun. The sampling value and all transfer lines were maintained at a constant 85°C with the injection port of the gas chromatograph held at 100°C.

Chromatographic separation was achieved by means of a Hewlett-Packard Model 5750 gas chromatograph equipped with a 50 ft. x 0.01 in. stainless steel capillary column coated with

IGEPAL CO-880 and programmed from room temperature. Approximately 90% of the chromatographic efluent was allowed to enter a duPont 21-491 double focusing mass spectrometer by means of jet-type separator. The separator and transfer lines were held at 125°C. The ion source was 100°C with a filament current of 40uA. All spectra were obtained at 70eV with scan speeds of 2 sec./decade.

CONCLUSIONS

This preliminary study points out the fact that aeciospores of C. fusiforme isolated from its natural host contain many of the common volatile terpenoid components present in the genus Pinus. However, the amounts of monoterpenes in the rust spores appear to be lower than one might expect in terms of the higher-boiling terpenoids when compared to the distribution of terpenoids common for pine species. Even though fungi are known to synthesize oxygenated monoterpenes, the evidence available suggest that they lack the ability to either form and/or accumulate the cyclic monoterpenes.[2,3] This may be due to their inability to form the postulated 1-p-methene-8-carbonium ion intermediate leading to compounds such as α- and β-pinene, Δ^3-carene and limonene.[7] It has been suggested[18] that myrcene formation does not proceed through the carbonium ion but rather is formed directly from the common precursor, neryl-pyrophosphate. If this latter pathway is active in wood-inhabiting fungi then

it could explain the relatively high myrcene values observed in the aeciospores.

The mono- and bicyclic terpenoids as well as the acyclic types may, however, simply be accumulated from the pine host. If such is the case then the fungus does so on a selective basis exhibiting a marked tendency to accumulate the higher-boiling terpenoids. This fact is supported by a recent observation on the total fatty acids of both the rust spores and slash pine tissues.[19] Total fatty acids present in the aeciospores and slash pine stem tissues were extracted and analyzed by gas chromatographic and mass spectrometric methods previously described and the results indicate that fatty acids from these two sources are qualitatively and quantitatively distinct.[20] For example, if lipids are simply accumulated then one would expect to see fatty acids occurring in rust tissues such as the 3-methyl branched hexadecanoic acid, which is common in the pine host tissues. However, no methyl branched fatty acids were observed in the aeciospores used in this study.

TABLE 1. Relative Percentage of Volatile Components Identified by Gas Chromatography-Mass Spectrometry from *Cronartium fusiforme* Aeciospores

Identified Compound	Relative Percent*
α-Pinene	8.9
Camphene	>0.1
β-Pinene	3.3
Δ^3-Carene	1.4
Myrcene	5.5
Limonene	3.0
β-Phellandrene	4.0
Terpinen-4-ol	6.8
β-Farnesene	4.2
β-Citronellol	2.3
o-Cresol	2.7

*Computed from gas chromatographic peak areas of total volatiles.

ACKNOWLEDGMENT

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PHYTOCHEMISTRY

FATTY ACIDS OF SLASH PINE TISSUES

by

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ABSTRACT

The total fatty constituents of slash pine (Pinus elliottii) tissue cultures, seeds, and seedlings were examined by gas chromatographic and mass spectrometric methods. Qualitatively, the fatty acid composition of these tissues was very similar and typical of those previously reported for other pine species. Although quantitative differences were noted, the tissue cultures more closely resembled the seedling tissues. In addition to the fatty acids common to botanical materials, Δ^5-C_{18} and -C_{20} non-methylene-interrupted polyunsaturated acids were present in low relative abundances. The branched-chain C_{17} acid reported for several other Pinus species was confirmed as the anteiso isomer.

INTRODUCTION

It has been established by gas chromatographic means using relative retention values that the most common fatty acids present in mature pine tree xylem are 16:0, 16Δ^1, 18:0, 18Δ^1, 18Δ^2, 18Δ^3, 20:0, and 22:0.[1-3] In addition to the more common saturated acids, a number of reports indicate the presence of a branched-chain C_{17} acid. Hemingway and Hillis[3,4] found that a branched acid was present in equal or greater amounts than palmioleic in Pinus radiata wood. More recently, Jamieson[5] observed that eleven of the conifer species studied contained considerable proportions of a saturated C^{17} branched-chain acid in leaf lipids which was assumed to be the anteiso isomer based on gas chromatographic behavior. McDonald and Porter[6] also reported a branched C_{17} in the bark lipids of P. radiata.

Also using chromatographic retention data, a number of reports suggests the presence of C_{18} and C_{20} acids with non-methylene-interrupted polyunsaturation containing Δ^5 bonds for Pinus species. A 18:3 $\Delta^{5,9,12}$ was reported in the wood of P. radiata[3] and a 20:3 $\Delta^{5,11,14}$ in the bark of the same species.[6] The leaf lipids of several Pinus species were all found to contain 18:2 $\Delta^{5,9}$, 18:3 $\Delta^{5,9,12}$ and 18:4 $\Delta^{5,9,12,15}$ which represents the common C_{18} series of fatty acids with an additional Δ^5 olefinic bond.[5] In addition, several polyunsaturated C_{20} acids

containing the Δ^5 double bond have also been observed in pine leaves. Lehtenen and co-workers[7,8] reported the presence of small amounts of 18:2 $\Delta^{5,9}$ and 18:3 $\Delta^{5,9,12}$ fatty acids in tall oil and pine seed extracts as well as 20:1 Δ^{11} and 20:3 $\Delta^{5,11,14}$ from the pine extracts.[9,10] All double bonds had the cis configuration. Fatty acids with 16 to 20 carbon atoms containing the cis-Δ^5 olefinic bond have been also identified in other seed oils,[11-14] a slime mold,[15] and bacteria.[16]

In our continuing studies to characterize the slash pine tissue cultures grown in our laboratory, we have compared the fatty acid composition of tissue culture seed, needle, and stem tissues of P. elliottii with particular interest in the branched-chain and non-methylene-interrupted polyunsaturated acids.

RESULTS AND DISCUSSION

The tissue cultures employed in this study were originally initiated from an embryo explant of slash pine and have been maintained in germ-free cultures by periodic transfer for two years on modified Brown and Lawrence media. The callus tissue culture is composed of parenchyma cells with infrequent expressions of differentiation by the presence of xylem elements. The cultures are characterized by a light brown and less often a green pigmentation in healthy actively growing tissues and by a very dark

brown and highly osmophilic pigmentation reminiscent of injured pine cells in the senescencing cultures.

When compared to the seed, needle, and stem tissues, the total lipid composition of the 6-week old tissue culture materials was low at 4.01 percent of the dry tissue weight and more closely resembled lipid levels found in the stem tissues (Table I). This value is approximately twofold higher than other tissue cultures such as tobacco and soybean which have been examined in our laboratory.[17] As one would expect, the seed tissues contained the highest total lipid composition at 65.02 percent.

The total fatty acid constituents from each of the above tissue types obtained by alkaline hydrolysis of the total lipids were analyzed by gas chromatography and mass spectrometry (table II). The principal fatty acids from the tissues of this study were typical of those reported for higher plants[18] and consistent with previous reports of members of the Pinaceae family. Qualitatively, the fatty acid composition of the tissue culture materials was very similar to that of the seed, needle, and stem tissues. Each tissue type had both saturated and unsaturated acids ranging in carbon chain length from C_{12} to C_{22} with the even numbered carbon chains predominant (Table II). Because the fatty acid composition varies considerably from tissue to tissue within the same plant[19] and this composition

is also influenced by environmental conditions, the tissues of this study would not be expected to be quantitatively comparable. However, the principal fatty acids of the pine tissue cultures were C_{16}, $C_{18:1\Delta^{9}}$, and $C_{18:2\Delta^{9,12}}$ which quantitatively more closely resembled the stem and needle tissue acids. The predominant seed fatty acids were the unsaturated C_{18} isomers containing double bonds in the Δ^{9}, $\Delta^{6,9}$, and $\Delta^{5,9,12}$ positions with the linoleic acid representing almost half of the fatty acid components. The minor acids, particularly the unsaturated components of each tissue studied appeared to be characteristic of conifer species in general and more specifically the C_{18} and C_{20} polyunsaturated acids whose double bonds are separated by two methylene units and contain an olefinic bond at the Δ^{5} position.[5-7] An unsaturated acid containing the Δ^{5} double bond was a major component in only the seed tissues; $C_{18:3\Delta^{5,9,12}}$ at 16.9 percent of the total fatty acid fraction.

The mass spectrometric techniques employed in this study will not provide information on the position or the configuration of the double bonds. However, with knowledge derived from relative retention times and supported by electron impact data one can position the points of unsaturation and their stereochemistry with reasonable accuracy. Recent reports have suggested that systematic relationships exists between the gas chromatographic retention times, the chain length, and double bond position

in the non-methylene-interrupted polyunsaturated methyl esters.[20] Similar relationships have been shown for the methylene-interrupted polyunsaturated esters.[21] Appropriate polyolefin standards were therefore employed using both packed and high resolution capillary columns to support the identifications made in this study.

There were several compounds with retention times in the range similar C_{20} to C_{22} acids, but mass spectra of these compounds revealed that they were neither fatty acids esters nor the common esters of resin acids. Molecular weights ranged from 342 to 370 with M-15 fragments which are characteristic of the loss of a methyl group common to several spectra.

The C_{17} branched-chain fatty acid characteristic of pine was not detected in the tissue culture and seed tissues which suggests that the branched acid may not be formed and/or accumulated in these tissues. This compound was present in both the stem (< 0.1%) and needle (0.2%) tissues. The branched C_{17} acid has been reported for several conifer species and this communication confirms the anteiso isomer by mass spectrometry. The M-29 fragment representing the loss of an ethyl group upon electron impact is characteristic of the typical 3-methyl branched acid and was observed in our analyses. A molecular ion at m/e 284 indicated no unsaturation. Although a branched-chain C_{15} acid has been reported for leaf lipids of several conifer species,[5] no other acids of this type were detected in the tissues of this study.

A major portion of the total fatty acid components isolated from each of the tissues examined were unsaturated and when compared, the tissue cultures more closely resembled the stem and needle tissues (Table III). Using the relative concentrations of Δ^5 double bond containing acids as a marker, the tissue culture materials again closely resembled these tissues.

We can conclude from these results that the slash pine tissue cultures employed in this study are composed of parenchyma cells which are typical with respect to the fatty acid composition of those found in healthy intact pine plants and that culturing in the manner described above does not result in serious alterations in these constituents. In a similar study, Weete[22] found quantitative variations when comparing the fatty acid composition of habituated tobacco tissue cultures and seedlings but qualitatively the tissues were very similar. A significant application of the pine tissues cultures grown in germ-free culture may be their use in elucidating the mechanisms involved in the formation of the Δ^5 double bond and particularly, the study of single pathogen-host interactions.

EXPERIMENTAL

Tissue preparation: Slash pine tissue cultures were maintained on modified Brown and Lawrence media in a Percival

incubator at 22°C under constant illumination as previously described.[23] Seeds (slash pine) were obtained from the field and the wings were removed prior to extraction. Stem and needle tissues were obtained from 8-10 month old slash pine seedlings grown under greenhouse conditions.

Lipid extraction: All tissues except seeds employed in this study were first homogenized in a Waring Blender in absolute methanol. The tissue was then collected by centrifugation and extracted for 1-2 hours with chloroform:methanol (1:1) on a magnetic stirrer. The tissue was collected and further extracted with chloroform as before. Pine seeds were crushed and then extracted in a microsoxlet apparatus with the same series of solvents described above. The combined extracts of each tissue were reduced in volume under nitrogen and partitioned between n-hexane and a saturated NaCl solution. The n-hexane phase was washed with water and taken to dryness under nitrogen and used as the total lipid extract. The total fatty acid components were obtained by alkaline hydrolysis of the total lipids by the methods described by Wilde and Stewart.[24] Fatty acid methyl ester derivatives for GLC-MS analysis were prepared using BF_3-MeOH.[25]

Separation and Identification: Chromatographic separation was achieved by use of Hewlett-Packard Model 5750 gas chromatograph equipped with either a 50 ft. x 0.01 in. stainless steel capillary

column coated with IGEPAL CO-880 or a 9 ft. x 0.125 in. stainless steel column packed with 12% ethylene glycol succinate on a 60/80 mesh GAS-CHROM-P support. The capillary column was programmed from 80°C to 170°C at 4°C/min. while the packed column was operated isothermally at 195°C. The injector port was maintained at 225°C and the detector temperature at 250°C. Approximately 90% of the chromatographic effluent was allowed to simultaneously enter duPont 21-491 double focusing mass spectrometer by means of a jet-type separator. The separator and transfer lines were held at 250°C. The ion source was 200°C with a filament current of 40 μA. All spectra were obtained at 70 eV with scan speeds of 2 sec./decade. Chromatographic standards were obtained from Applied Science Laboratories, College Park, Pennsylvania (U.S.A.).

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TABLE I. Total extractable lipids and fatty acids from slash pine tissue cultures, seed, and seedling needle and stem tissues.

SAMPLE	AGE (Mo.)	DRY WEIGHT EXTRACTED (gms.)	TOTAL EXTRACTABLE LIPIDS (% of Dry Weight)
TISSUE CULTURE	1.5	6.40	4.01
SEEDS	-	1.59	65.02
NEEDLES	8-10	1.28	7.43
STEMS	8-10	0.63	5.03

TABLE II. Fatty acid composition of the total lipids from slash pine tissues.

TISSUE	12:0*	13:0	14:0	14:1	15:0	16:0	16:1 (Δ^{7})	16:2 ($\Delta^{7,11}$)	16:3 ($\Delta^{7,11,14}$)	17:0	17:0 anteiso	18:0	18:1 (Δ^{9})
TISSUE CULTURE	tr	-	4.3	-	0.2	22.1	0.8	0.4	tr	-	-	3.9	26.1
SEED	tr	-	0.1	tr	tr	6.2	0.1	tr	tr	tr	-	2.3	21.6
LEAF	0.4	tr	1.4	-	0.1	17.7	1.1	0.8	0.2	0.3	0.2	2.6	14.1
STEM	0.8	tr	1.0	tr	0.5	17.2	2.2	0.8	2.6	tr	tr	2.9	17.9

*Expressed as relative percent (%) of the total fatty acids from gas chromatographic data using both packed and capillary colums.

tr = Less than 0.1 percent.

18:2 ($\Delta^{5,9}$)	18:2 ($\Delta^{9,12}$)	18:3 ($\Delta^{5,9,12}$)	18:3 ($\Delta^{9,12,15}$)	20:0	18:4 ($\Delta^{5,9,12,15}$)	20:1	20:2 ($\Delta^{11,14}$)	20:3 ($\Delta^{5,11,14}$)	20:4 ($\Delta^{5,11,14,17}$)	22:0
0.2	30.4	1.6	6.3	tr	0.2	tr	1.8	1.4	tr	tr
2.9	47.9	16.9	1.1	0.5	0.1	tr	0.5	tr	tr	tr
tr	24.8	tr	30.6	0.1	0.2	tr	1.4	1.9	0.2	1.5
tr	30.3	tr	12.9	7.6	-	tr	tr	0.9	tr	2.2

TABLE III. Unsaturated fatty acid relationships between slash pine tissue culture, seed, and seedling needle and stem tissues.

SOURCE	UNSATURATED ACIDS AS PERCENT OF TOTAL FATTY ACIDS	Δ^5 ACIDS AS PERCENT OF TOTAL FATTY ACIDS	Δ^5 ACIDS AS PERCENT OF TOTAL UNSATURATED FATTY ACIDS
TISSUE CULTURE	73.1	7.5	10.2
SEED	91.1	19.9	21.8
NEEDLE	76.2	8.2	10.7
STEM	67.6	5.9	8.7

PREPRINT (7)

PHYTOCHEMISTRY

GAS CHROMATOGRAPHY-MASS SPECTROMETRY STUDY OF STEROLS FROM SLASH PINE TISSUES

by

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STEROL	RELATIVE PERCENT*		
	Tissue Culture	Seeds	Seedlings
I. CHOLESTEROL	0.34	5.25	2.90
II. DESMOSTEROL	0.15	0.66	1.74
III. LOPHENOL	-	2.64	0.67
IV. CAMPESTEROL	5.56	7.90	6.95
V. STIGMASTEROL	6.69	1.31	8.70
VI. UNKNOWN	-	0.52	6.90
VII. 24-METHYLENE LOPHENOL	tr	31.85	16.13
VIII. β-SITOSTEROL	80.78	38.07	47.83
IX. CYCLOEUCALENOL	2.01	6.58	4.63
X. CYCLOARTENOL	4.47	3.96	2.83
XI. 24-METHYLENE CYCLOARTANOL	tr	1.26	0.72
XII. 24-ETHYLIDENE LOPHENOL (CITROSTADIENOL)	-	-	tr

*Expressed as relative per cent (%) of the sterols from gas chromatographic data.

tr = less than 0.1%.

TABLE I. Relative sterol concentrations of the freely extractable lipids of slash pine tissue cultures, seeds, and seedlings.

ABSTRACT

A comparative study of the sterol components of slash pine (Pinus elloittii) callus tissue cultures, seeds, and seedlings was carried out using gas chromatographic-mass spectrometric techniques. Cholesterol, desmosterol, campesterol, stigmasterol, β-sitosterol, and cycloeucalenol were identified from each of the tissues studied while lophenol and 24-methylene lophenol were identified in only the seed and seedling tissues. 24-Ethylidene lophenol was detected in trace concentrations in only the seedlings. β-Sitosterol was the predominant sterol component in each of these tissues i.e. 80.8, 38.1, and 47.8 percent of the tissue culture, seed, and seedling sterols, respectively.

INTRODUCTION

Germ-free callus tissues grown in synthetic media have proven useful in the study of certain biochemical and physiological processes in higher plants. In our previous studies, we have compared the lipid composition of tissue cultures with that of the intact plants and found that, although they are generally similar, certain qualitative and quantitative differences are present (1-3). As part of our continuing studies to characterize the lipid components of higher plant tissue cultures, we have identified and compared the sterol components of slash pine tissue cultures, seeds, and seedlings.

RESULTS AND DISCUSSION

The eleven sterols identified (as acetates) in the pine tissues of this study had GLC relative retention times similar to the corresponding authentic standards prepared in our laboratory and those reported by Patterson (4). Compound I (figure 1) is identified as cholesterol. The mass spectrum of its trimethylsilyl ether compared closely with those of authentic standards and those reported by Brooks et al. (5). Compound II appears to be desmosterol. The base peak is m/e 366 for desmosteryl acetate which corresponds to the loss of the acetate moiety from the parent molecule. The silyl ether derivative of the same compound gave a base peak of m/e 129 with a major fragment (68% relative intensity) at m/e 343 which can be interpreted as the loss of the side-chain and two protons from the parent compound. Other sterol derivatives of the same molecular weight and similar retention values such as zymosterol and 7-dehydrocholesterol can be eliminated on the basis of no major fragments

at m/e 456 in the case of zymosteryl and m/e 351 for 7-dehydrocholesteryl trimethylsilyl derivatives.

Compound III is identified as lophenol (4α-methylcholest-Δ^7-en-3β-ol). The acetate derivative of this compound produced a small molecular ion with a base peak at m/e 327 and major fragments at m/e 370, 329, 269, and 227. The spectrum of the acetate derivative of this compound is in good agreement with that reported previously by Knights (6). The mass spectra of silyl derivatives of Compounds IV and V are identical to authentic standards of campesteryl (M^+472) and stigmasteryl (M^+486) trimethylsilyl ether, respectively. A compound which was only partially resolved and possessed a slightly longer retention time than stigmasterol was present in this fraction (Compound VI). The identity of this compound is not known, but the present evidence suggests that it may be a 4-methyl sterol. Additional unresolved compounds are present in this peak and interfere with the spectral interpretation. The mass spectrum of Compound VII had a small molecular ion at m/e 454 with a base peak at m/e 327 and major fragments at m/e 439, 370, 268, and 227. This spectrum was in general agreement with that published by Knights (6) for 24-methylene lophenol.

The major sterol component in each of the tissues in this study was β-sitosterol (Compound VIII). The spectrum of this compound was the same as that obtained for the acetate and silyl ether derivatives of authentic β-sitosterol and that described by others (5,6). Another compound is associated chromatographically with β-sitosterol and there is some evidence that it may be 28-isofucosterol (Δ^5-avenasterol) or similar compound. In several cases, a peak at m/e 394 was observed which corresponds to the M-60 peak of a diunsaturated isomer of β-sitosterol.

Further evidence for the 24-ethylidene cholesteryl acetate structure is suggested by the presence of a peak at m/e 296 which is the base peak for this compound (7).

Since the remaining sterols were found in low relative concentrations, it was necessary to use trapping techniques to isolate these compounds as they were eluted from the chromagraphic column. The collection trap was made of glass capillary tubing packed with 1% OV-17 on Chromosorb P. Compound IX was collected repeatedly in this manner as the free sterol and analyzed using the solid injection probe of the mass spectrometer. A parent ion of the free sterol is m/e 426 with other prominent peaks at m/e 411, 408, 393, 353, 343, 300, and 283. The mass spectrum of this compound is in general agreement with that reported previously for cycloeucalenol (8,9). Compound X is characterized by a molecular ion at m/e 468 for the acetate derivative with peaks at 453, 408, 393, 365, 339, 297, and 286. The mass spectrum of this compound is in accord with those reported previously for cycloartenyl acetate (10,11).

The mass spectrum of the acetate derivative of Compound XI had a molecular ion at M^+482. Additional peaks were observed at m/e 467, 422, 407, 379, 300, and 297 which corresponds to the fragmentation pattern of 24-methylene cycloartanyl acetate (10,11). The acetate derivative of this compound co-chromatographed with an authentic standard. This compound was also collected as the free sterol by the methods described above and analyzed by the direct probe of the mass spectrometer. The mass spectrum contains a significant peak at m/e 440 with major ion fragments at m/e 425, 422, 407, 379, 353, 300, and 175 with a characteristic base peak

at m/e 107. These findings are in agreement with those reported by Aplin and Hornby (12) for 9,19-cyclosterols like 24-methylene cycloartanol. Both cycloartenol and 24-methylene cycloartanol have been recently confirmed by NMR (13) in the tall oil from southern pine.

Compound XII, 24-ethylidene lophenol was present in sufficient quantities to obtain an identifiable spectrum only in the seedlings. A molecular ion was observed at m/e 468 as the acetate derivative with the base peak at m/e 327 and significant fragments at m/e 370 and 227. The fragmentation pattern was in agreement with spectra reported by Knights (6) and others (10). The presence of this compound has recently been verified in tall oil from southern pine by Rowe using NMR (13).

In a review on phytosterols, Bean (14) reported that approximately forty-four sterols have been isolated and identified from plant tissues. β-sitosterol has been identified from extracts of pollen from P. sylvestris (15), whereas both sitosterol and campesterol were identified from the bark of P. monticola (16).

This report represents the most thorough investigation of the sterol components of a member of the Pinaceae, but with the exception of cholesterol, desmosterol, stigmasterol, and lophenol the sterols reported for slash pine tissues in this study have also been identified for the leaves of the deciduous conifer Larix decidua (17). The sterols identified in the slash pine tissues represent potential intermediates in the biosynthesis of predominant phytosterols such as β-sitosterol (10, 17, 18). Several sterol components are present in these tissues whose structure is unknown and are under investigation at this time.

With the exception of the lophenol compounds, the identified sterols are qualitatively the same in each of the tissues studied (Table 1). Lophenol was found in the seed and seedling tissues while 24-ethylidene was detected in only the seedlings. These compounds were probably present in the tissue cultures but at undetectable concentrations with the available quantities of tissue, since 24-methylene lophenol was detected in these tissues.

As might be expected when tissues in different stages of development are compared chemically, considerable variation was present in the relative sterol concentrations of these tissues. β-sitosterol is the predominant sterol in each of these tissue types i.e. 80.78, 38.07, and 47.83 percent for the tissue cultures, seeds, and seedlings respectively. Perhaps the most significant difference in the sterol distributions of these tissues and a possible explanation for the differences in β-sitosterol concentrations is that 24-methylene lophenol is found in only trace concentrations (<0.1%) in the tissue cultures while this compound represents a significant portion of the seed and seedling sterols (31.85 and 16.13 percent, respectively). By the nature of its structure, 24-methylene lophenol appears to be an intermediate in the formation of 4-desmethyl phytosterols such as β-sitosterol and may be more readily converted enzymatically in the tissue cultures.

EXPERIMENTAL

The slash pine tissues employed in this study were grown and treated according to the methods described by Laseter et al. (3) and Brown and Lawrence (19). The lipids were extracted and prepared for analysis by the methods previously described (3).

Sterol Separation and Identification: Chromatographic separation was achieved by use of a Hewlett-Packard Model 5750 gas chromatograph equipped with a 10 ft x 2 mm I.D. glass column with 1% SE-30 (GE) on 80/100 mesh Gas-chrom Q. operating isothermally at 270°C. The injector and hydrogen flame detectors were maintained at 290°C. Approximately 90% of the chromatographic effluent was allowed to simultaneously enter a duPont 21-491 double focusing mass spectrometer by means of a jet-type separator. Separator and transfer lines were held at 260°C. The ion source was at 210°C with a filament current of 350 μA. All spectra were obtained at 70 eV with scan speeds of 4 sec./decade.

Trimethylsilyl ether derivatives were prepared by treating the sterols (0.1-1mg) at 50°C with 30-40 μl of N,O-BIS- (trimethylsilyl)-acetamide (Pierce Chemical Company, Rockford, Illinois) in a dry pyridine solvent. Sterol acetates were prepared by heating the sterols with acetic anhydride for 30 min. at reflux temperature.

In addition to GC-MS analyses of the intact sterol fraction, each fraction was further fractionated into the 4,4-dimethyl, 4-monomethyl, and 4-desmethyl sterols by the method of Goad and Goodwin prior to forming the derivatives. Most chromatographic standards were obtained from Applied Science Laboratories (College Park, Pennsylvania). Other standards have been graciously supplied by: Drs. L. J. Goad, and Gonzales Gonzales.

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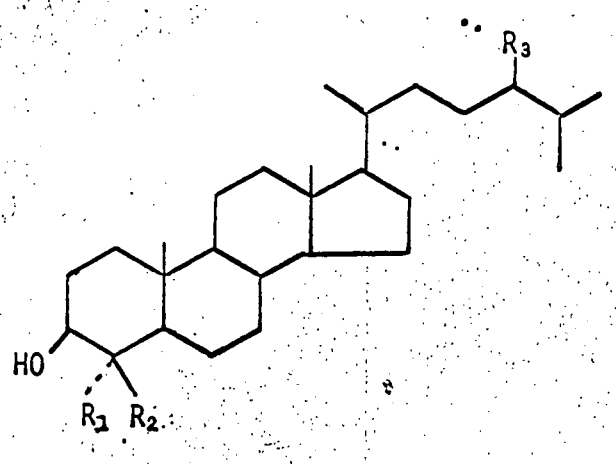
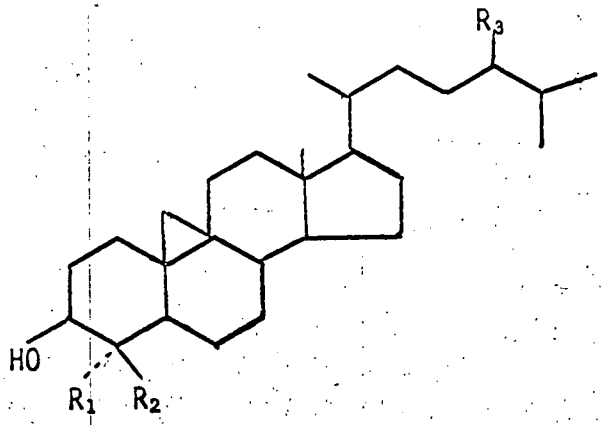
FIGURE 1. Sterol structures identified from slash pine tissue cultures, seeds, and seedlings. Compounds are listed in their order of elution from the 1% SE-30 chromatographic column.



I.	CHOLESTEROL:	Δ^5, $R_1 = R_2 = R_3 = H$
II.	DESMOSTEROL:	$\Delta^{8,24}$, $R_1 = R_2 = R_3 = H$
	LOPHENOL:	Δ^7, $R_1 = R_3 = H$; $R_2 = CH_3$
IV.	CAMPESTEROL:	Δ^5, $R_1 = R_2 = H$; $R_3 = CH_3$
V.	STIGMASTEROL:	Δ^5, $R_1 = R_2 = H$; $R_3 = CH_2CH_3$
VI.	UNKNOWN 4-MONOMETHYL STEROL	
VII.	24-METHYLENE LOPHENOL:	$\Delta^{7,24(28)}$, $R_1 = H$; $R_2 = OH_3$; $R_3 = CH_2$
VIII.	β-SITOSTEROL:	Δ^5, $R_1 = R_2 = H$; $R_3 = CH_2CH_3$
XII.	24-ETHYLIDENE LOPHENOL: (CITROSTADIENOL)	$\Delta^{7,24(28)}$, $R_1 = H$; $R_2 = CH_3$; $R_3 = CH\ CH_3$



IX. CHCLOEUCALENOL $\Delta^{24(28)}$, $R_1 = R_3 = H$; $R_2 = CH_3$

X. CYCLOARTENOL: Δ^{24}, $R_1 = R_2 = CH_3$; $R_3 = H$

XI. 24-METHYLENE CYCLOARTANOL: $\Delta^{24(28)}$, $R_1 = R_2 = CH_3$; $R_3 = CH_2$