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Ectomycorrhizal Fungi: A Phytochemical Survey

The phytochemistry of the higher Basidiomycetes has been extensively reported, especially as it involves identification of secondary metabolites. As seen in the compendia of Karrer (1958), Miller (1961), and Shibata *et al* (1964), which summarize much of this work, the taxa studied seem generally to have been selected either randomly or opportunistically. Inclusion of mycorrhizal fungi has been purely incidental. Relatively few studies have been designed to demonstrate chemotaxonomic principles or establish relationships among taxa in processes such as biosynthesis of secondary metabolites (e.g., Benedict *et al*, 1966; Agurell *et al*, 1966, 1968).

Most studies on ectomycorrhizal fungi *per se* deal primarily with specific fungus-host associations and their nutritional, hormonal, and enzymatic interactions (see reviews by Harley, 1959; Trappe, 1962; Meyer, 1968; and others). A few studies of antifungal activity by ectomycorrhizal fungi have been reported, but only in one has the specific active compound been identified. Marx (1969) determined that the mycorrhizal fungus *Leucopaxillus cerealis* var. *piceina* (Peck) comb. ined. produces diatretyne nitrile, a compound strongly inhibitory to *Phytophthora cinnamomi* Rands.

The hypothesis of Zak (1964), that mycorrhizal fungi protect tree rootlets from attack by at least some pathogens, has been experimentally confirmed by Marx and Davey (1969a, 1969b). Systematic study of the overall metabolic capabilities of mycorrhizal fungi is accordingly timely in order to define the chemical factors involved.

Our initial studies involved a broad chromatographic survey of isolates of selected fungi to determine which types of metabolites would be produced under controlled laboratory conditions, their distribution among these organisms, and a comparison of these data to those obtained from naturally occurring sporocarps similarly examined or to reports in the literature dealing with the same organisms.

Experimental and Results

Isolates were obtained by tissue culture from the mycorrhizal fungi listed in Table I. Data for three nonmycorrhizal fungi under concurrent study are also included. The cultures were maintained on potato-dextrose-yeast agar slants (Benedict and Tyler, 1962).

Isolates were transferred from slants to 125-ml Erlenmeyer flasks containing 30 ml

TABLE I. Species, geographic source, and probable mycorrhizal host tree of fungi included in the phytochemical survey

SPECIES	Trappe collection ¹	Culture no.	Geographic source	Probable mycorrhizal host
Mycorrhizal:				
<i>Amanita citrina</i> (Schaeff.) S. F. Gray	-----	S-6	Georgia	<i>Pinus</i> spp.
<i>A. muscaria</i> (Fr.) Hooker	334	S-80	Oregon	<i>Pinus contorta</i> Dougl.
<i>A. phalloides</i> (Fr.) Secr.	-----	S-16	Georgia	<i>Pinus</i> spp.
<i>A. velosa</i> (Peck) comb. ined.	394	S-92	Oregon	<i>Pinus contorta</i>
<i>A. verna</i> (Fr.) Vitt.	-----	S-32	Georgia	<i>Pinus</i> spp.
<i>Inocybe calamistrata</i> (Fr.) Gill.	385	S-133	Oregon	<i>Pseudotsuga menziesii</i> (Mirb.) Franco
<i>Leucopaxillus cerealis</i> var. <i>piceina</i> (Peck) comb. ined.	-----	S-11	Georgia	<i>Pinus echinata</i> Mill.
<i>Rhizopogon arenicola</i> A. H. Smith	339	S-86	Oregon	<i>Pinus contorta</i>
<i>R. cokeri</i> A. H. Smith	350	S-84	Oregon	<i>Tsuga mertensiana</i> (Bong.) Carr.
<i>R. colossus</i> A. H. Smith	182	S-39	Oregon	<i>Pseudotsuga menziesii</i>
<i>R. nitens</i> A. H. Smith	342	S-83	Oregon	<i>Picea sitchensis</i> (Bong.) Carr.
<i>R. vinicolor</i> A. H. Smith	801	S-156	Oregon	<i>Pseudotsuga menziesii</i>
<i>Suillus luteus</i> (Fr.) S. F. Gray	408	S-101	Oregon	<i>Pinus ponderosa</i> Laws.
<i>S. pseudobrevipes</i> Smith & Thiers	407	S-97	Oregon	<i>Pinus ponderosa</i>
<i>S. tomentosus</i> (Kauffm.) Singer, Snell & Dick	399	S-91	Oregon	<i>Pinus contorta</i>
<i>S. variegatus</i> (Fr.) O. Kuntze	-----	S-14	Finland	<i>Pinus</i> sp.
<i>Xerocomus zelleri</i> (Murr.) Snell	402	S-112	Oregon	<i>Picea sitchensis</i>
Nonmycorrhizal:				
<i>Clitocybe gibba</i> (Fr.) Kummer	-----	S-5	Georgia	-----
<i>Pholiota lubrica</i> var. <i>luteifolia</i> Smith & Hesler	375	S-88	Oregon	-----
<i>Echinodontium tinctorium</i> (Ell. & Everth.) Ell. & Everth.	-----	-----	?	-----

¹ Specimens deposited in Oregon State University Herbarium.

of the following medium: *Gm per liter*: glucose, 20.0; neopeptone, 2.5; yeast extract, 2.5; calcium chloride, 0.5; and the trace mineral composition of Benedict and Tyler (1962). Subsequent shaken-flask fermentations were carried out as previously described (Catalfomo and Tyler, 1964). For the present study, fermentation flasks were uniformly harvested after seven days.

At the end of the fermentation period, contents of the flasks were separated by suction filtration. The mycelium was dried in a forced-air dryer at 50°C; the medium was concentrated to dryness in a flash evaporator, *in vacuo*, at 40°C. The residue was redissolved in a minimum amount of methanol and stored in a refrigerator until used for chromatographic analysis. The dried mycelium was pulverized to a number 40 powder in a laboratory model Wiley mill and extracted in semi-micro Soxhlet apparatus with petroleum ether (30-60°) followed by methanol. These extracts were concentrated to a lesser volume in a flash evaporator, as described earlier, and used for subsequent chromatographic examination. The results of our broad thin-layer chromatographic screening for steroids/triterpenes, amines, indole derivatives, and phenolic constituents in mycelial extracts, are summarized in Table II. Routine analysis of media extracts revealed little of interest and these data are not reported here.

The compounds most frequently encountered appeared to be ergosterol and choline. To establish, unequivocally, the presence of these compounds, more refined preparative techniques were employed. In order to accumulate sufficient mycelium to attempt

TABLE II. Chromatographic analysis*

SPECIES	Steroids ^a			
	Triterpenes	Amines ^b	Indoles ^c	Phenols ^d
Mycorrhizal:				
<i>Amanita citrina</i>	+	+	+	+
<i>A. muscaria</i>	+	+	+	+
<i>A. phalloides</i>	+	±	+	±
<i>A. velosa</i>	+	-	+	-
<i>A. verna</i>	+	+	+	±
<i>Inocybe calamistrata</i>	+	+	+	nt
<i>Leucopaxillus cerealis</i> var. <i>piceina</i>	nt	+	+	nt
<i>Rhizopogon arenicola</i>	+	+	+	nt
<i>R. cokeri</i>	+	-	±	±
<i>R. colossus</i>	+	+	+	nt
<i>R. nitens</i>	+	-	+	+
<i>R. vinicolor</i>	+	-	-	nt
<i>Suillus luteus</i>	+	-	+	+
<i>S. pseudobrevipes</i>	+	+	-	±
<i>S. tomentosus</i>	nt	-	+	nt
<i>S. variegatus</i>	+	+	+	+
<i>Xerocomus zelleri</i>	±	+	±	±
Nonmycorrhizal:				
<i>Clitocybe gibba</i>	nt	-	+	nt
<i>Pholiota lubrica</i> var. <i>luteifolia</i>	+	+	+	+
<i>Echinodontium tinctorium</i>	+	+	+	+

* Silica-gel-G plates according to Stahl.

Solvent systems and spray reagents—*a.* benzene-ethylacetate (4:1), antimonytrichloride in chloroform (sat. solution); *b.* butanol-acetic acid-water (4:1:5), Dragendorff reagent; *c.* propanol-ammonium hydroxide (5:1), paradimethylaminobenzaldehyde (2% in conc. HCl); and *d.* same as *b* but sprayed with phosphomolybdic acid (1% in methanol).

+ = present; - = absent; ± = equivocal; nt = not tested.

isolation procedures, the level of fermentation was scaled up to 2800-ml Fernbach flasks containing 300 ml of medium. The mycelial contents of 24 flasks were pooled and processed as previously described. *Amanita verna* and *Suillus variegatus* were chosen as model organisms. The petroleum extract of each organism was subjected to column chromatography over alumina. Elution with chloroform removed the sterol subsequently identified as ergosterol. Recrystallized from methanol, the homogeneous compound melted at 160-162°C, which was undepressed when mixed with reference ergosterol. Characteristic ultraviolet spectra (273, 283, and 294 m μ) and superimposable infrared spectra further verified the isolated material as ergosterol.

Isolation of choline from the methanol fraction as the insoluble reineckate salt was achieved by using the method of Hogg *et al* (1961). Subsequent identification was accomplished according to the chromatographic procedure of Sullivan and Brady (1965) and comparison of infrared spectra.

Similar extracts of other species of mycorrhizal fungi were prepared from freshly harvested mycelium which was grown in 125-ml Erlenmeyer flasks as described earlier. Comparative thin-layer chromatography was employed to determine the occurrence of these compounds in additional species. The results are summarized in Table III.

These studies were extended to a similar analysis of herbarium specimens from the same collections from which isolates were obtained, as available. In all cases, the results were comparable.

TABLE III. Occurrence of ergosterol and choline

Species	Ergosterol ^a	Choline ^b
MYCORRHIZAL:		
<i>Amanita citrina</i>	+	+
<i>A. muscaria</i>	+	+
<i>A. phalloides</i>	+	±
<i>A. velosa</i>	+	-
<i>A. verna</i>	+	+
<i>Inocybe calamistrata</i>	+	+
<i>Leucopaxillus cerealis</i> var. <i>piceina</i>	nt	+
<i>Rhizoglyphus arenicola</i>	+	±
<i>R. cokeri</i>	+	-
<i>R. colossus</i>	+	±
<i>R. nitens</i>	+	-
<i>R. vinicolor</i>	+	-
<i>Suillus luteus</i>	+	-
<i>S. pseudobrevipes</i>	+	-
<i>S. tomentosus</i>	nt	-
<i>S. variegatus</i>	+	+
<i>Xerocomus zelleri</i>	+	-
NONMYCORRHIZAL:		
<i>Clitocybe gibba</i>	nt	-
<i>Pholiota lubrica</i> var. <i>luteifolia</i>	+	±
<i>Echinodontium tinctorium</i>	+	+

^a Silica-gel plates developed in Benzene-ethylacetate (4:1), sprayed with a saturated solution of antimonytrichloride in chloroform.

^b Aluminum oxide-G plates developed in methanol-carbon tetrachloride-acetic acid (28:12:1) and sprayed with Dragendorff reagent.

+ = present; - = absent; ± = equivocal; nt = not tested.

Summary and Conclusions

Ectomycorrhizal fungi from the genera *Amanita* and *Rhizopogon* and the family Boletaceae were grown in artificial culture and examined for presence of secondary metabolites with emphasis on terpenes, amines, indole derivatives, and phenols. Of particular interest was the distribution of ergosterol and choline. The former appears to be nearly ubiquitous according to the literature (Cochrane, 1958), and our findings do not deviate from this trend. Choline, although quite widespread, was identified in only one bolete.

It is not possible yet to generalize about the overall metabolism and the distribution of metabolites of these mycorrhizal fungi, although this would seem possible since all of them form symbiotic associations with the Pinaceae. One consistent aspect is indicated by the occurrence of products derived from acetate metabolism, ergosterol, and other steroids not yet identified. This is readily understandable, because most host plants, especially the Pinaceae, are rich in terpenic metabolites and precursor materials. Steroids do not exhibit antibiotic activity, and their possible role in any protective mechanism of mycorrhizae cannot be ascribed to this quality. These compounds do become an integral part of cell wall material (Bloch, 1965), and choline has been implicated similarly (Tomasz, 1967). It is possible therefore, that these materials might in some way enhance the effectiveness of cell walls as effective barriers. This would support Zak's contention (1964) that the fungal mantle characteristically present on the surface of ectomycorrhizae performs a physical, protective function.

Nitrogen metabolism must be considered also since complex nitrogenous materials (alkaloids) are rarely found in higher fungi in general (Tyler and Stuntz, 1962, 1963) as well as being absent in mycorrhizal fungi (Worthen *et al*, 1965) and their host plants. On the other hand, the presence or absence of certain simple nitrogen metabolites in specific taxa of mycorrhizal fungi is noteworthy: for instance, the occurrence of simple indoles and isoxazole derivatives reported in *Amanita* species (Tyler and Gröger, 1964; Benedict *et al*, 1966) or the apparent absence of simple amines in bolets: choline as herein reported or urea as indicated by Tyler *et al* (1965).

Probably no one factor is responsible for the suspected protective role of mycorrhizal fungi. The overall metabolism of these organisms must be systematically determined and appropriate test systems established before the complex chemical associations between these organisms and their host plants can be assessed fully.

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