THE EXTRACTIVES OF THE MYCORRHIZAS AND ROOTS OF *PINUS RADIATA* AND *PSEUDOTSUGA MENZIESII*

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Summary

The amounts of acetone extractives from mycorrhizas and their polyphenolic portion were variable over a 6-month period. During August the amounts of extractives and polyphenols were higher in slow- than in fast-growing *Pinus radiata* seedlings. The amount of extractives in the roots were greater than those in the mycorrhizas but in the latter the polyphenols were concentrated in the outer layer. The composition of the mycorrhizal and root extractives of *P. radiata* were very similar and in addition to resin contained catechin, two components that are very similar to 3,5,3′,4′-tetrahydroxystilbene and one of its glucosides, and leucocyanidin polymers. The mycorrhizas of *Pseudotsuga menziesii* contain 15 components, including catechin, epicatechin, leucocyanidin polymers, and a polyene. With the exception of the latter the roots of *Ps. menziesii* contained the same components and in addition poriolin, poriol, taxifolin, taxifolin-3-glucoside, and quercetin-3-glucoside. Evidence supports the view that the polyphenols are formed *in situ* and appear to be formed in enhanced amounts in the tannin layer of mycorrhizas. Their possible role in the establishment of mycorrhizas is discussed.

I. Introduction

One of the features of mycorrhizas is the presence of a layer of "tannin"-filled cells in the epidermis and the outer cortex of the root (MacDougal and Dufrenoy 1944). The distribution of these cells is characteristic of the type of host–fungal relationship in mycorrhizal and pseudomycorrhizal associations of healthy and stunted trees of *Pinus radiata* D. Don and *Pseudotsuga menziesii* (Mirb.) Franco (Foster and Marks 1966, 1967; Marks and Foster 1967). The accumulation of the tannin which reacted with different stains was considerably less in the inner cortical cells and practically absent in the fungal mantle. Foster and Marks (1966, 1967) also observed that the mycorrhizal fungus showed symptoms of being affected by toxins as it passed through this tannin layer but not when it passed between the inner cortical cells to form the Hartig net.

Mycorrhizas represent special cases of balance between the fungus and the plant and in view of the probable relationship between a satisfactory establishment of mycorrhizas and good growth of some tree species under different soil conditions (e.g. Bowen 1965), it is desirable to know the factors controlling the establishment of them. There are few studies (Lewis and Harley 1965) on the biochemistry of compounds involved in the symbiosis between fungus and host plants and little attention has been given to the tannin layer. For some time, preformed polyphenols

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have been considered to convey resistance to fungal infection (e.g. Farkas and Király 1962; Rohringer and Samborski 1967; Wood 1967*a*). In addition, phytoalexins, some of which are phenolic compounds, have been shown to arise in response to fungal infection (Cruickshank 1963). The toxic polyphenols orchinol and hircinol (Boller *et al.* 1957; Hardegger, Biland, and Corrodi 1963; Hardegger, Schellenbaum, and Corrodi 1963; Urech *et al.* 1963), which could be considered to be phytoalexins, are produced in the tubers of orchids when affected by mycorrhizal fungi.

An examination has been made of the extractives of mycorrhizas and those of the long roots of *P. radiata* and *Ps. menziesii* seedlings grown under different natural conditions. A preliminary report has been made (Hillis *et al.* 1968). The small amounts of material available permitted only a chemical examination of the components and the results are described in this paper. The results show the complex composition of the polyphenolic portion of the extractives, the difference in composition between mycorrhizas and roots of *P. radiata* and *Ps. menziesii*, and, in the case of the latter species, the difference in composition between roots and mycorrhizas. The possible origin and influence of the extractives is discussed also.

II. Materials and Methods

(*a*) *Materials*

Three-year-old seedlings of *P. radiata* and *Ps. menziesii* were collected from three nurseries with different climates and soil types. The samples were collected during late autumn (May) and spring (October). The seedlings of *P. radiata* collected from the Victorian Forests Commission nursery at Narbethong had stem lengths of 30–35 cm and root lengths of *c.* 25 cm and from their nursery at Mt. Macedon had stem lengths of 25–30 cm and root lengths of *c.* 25 cm. The growth and vigour of the seedlings from the "good" nursery at Narbethong were appreciably better than those in the "poor" nursery at Mt. Macedon. The seedlings of *Ps. menziesii* collected from the Victorian Forests Commission nursery at Trentham had stem lengths of 30–40 cm and root lengths of *c.* 30 cm.

The mycorrhizas were stripped off the roots along with the adhering soil and collected using a wet sieving method (Marks, Ditchburne, and Foster 1967). The fine soil adhering to them was removed by stirring in water for 1 hr, pouring off the water, and repeating the procedure twice. Within 24 hr after collection, the mycorrhizas were freeze-dried, and subsequently stored in the dark below 0°C.

(*b*) *Extraction of Mycorrhizas and Roots*

The freeze-dried mycorrhizas were extracted thoroughly with acetone at 15–20°C and the extract evaporated to dryness, weighed, and examined. The acetone extract was dissolved in a minimum volume of methanol and extracted repeatedly with n-heptane. Both portions were separately dried, weighed, and tested by means of paper and thin-layer chromatography and spectrally.

The roots remaining after the mycorrhizas had been stripped off were extracted and examined as above.

(*c*) *Chromatographic Examination*

All paper chromatographic examinations were carried out under comparable conditions in a constant temperature room at 20°C, using Whatman No. 1 paper. The following solvent systems were used:

1, n-butanol–acetic acid–water (6 : 1 : 2);
2, 6% aqueous acetic acid;
3, 30% aqueous acetic acid;
4, benzene–acetic acid–water (125 : 72 : 3);
5, n-butanol–ethanol–water (4 : 1 : 5);
6, benzene–acetic acid–water (6 : 3 : 7);
7, acetic acid–hydrochloric acid–water (30 : 3 : 10);
8, n-butanol–hydrochloric acid–water (7 : 2 : 5);
9, ethyl acetate–pyridine–water (12 : 5 : 4);
10, n-butanol–ethanol–water (40 : 11 : 19);
11, n-butanol–pyridine–water (6 : 4 : 3).

Two-dimensional chromatograms were prepared using first solvent 1, then solvent 2. Solvents 1–8 were used in preparation of one-dimensional chromatograms of polyphenols, and solvents 9–11 for sugars.

The chromatograms were examined under ultraviolet light (u.v.) before and after exposure to ammonia vapour. The following sprays were then used to locate polyphenols:

A, diazotized *p*-nitroaniline in 20% sodium acetate (Swain 1952);
B, vanillin–hydrochloric acid (Bate-Smith and Swain 1953);
C, ferric chloride–potassium ferricyanide (Barton, Evans, and Gardner 1952);
D, phosphomolybdic acid (Reio 1960);
E, potassium permanganate (Reio 1960);
F, diazotized *o*-dianisidine followed by exposure to ammonia vapour (Reio 1960) and *p*-toluenesulphonic acid.

Chromatograms were sprayed with alkaline silver nitrate, aniline–citric acid, and anthrone to locate sugars.

Chromatoplates of silica gel [GF 250 (E. Merck A. G., Darmstadt); thickness 0·25 mm] were prepared in a constant-temperature room at 20°C. The following solvents were used for thin-layer chromatography (t.l.c.):

12, methanol–chloroform–petroleum ether (60–80°C) (2 : 4 : 7);
13, chloroform–acetic acid (6 : 1);
14, benzene–methanol (9 : 1);
15, benzene–methanol–acetic acid (10 : 3 : 3);
16, benzene–methanol–acetic acid (45 : 8 : 4);
17, chloroform–ethyl acetate–formic acid (5 : 4 : 1);
18, toluene–ethyl formate–formic acid (5 : 4 : 1).

(*d*) *Large-scale Paper Chromatography and Spectral Examinations*

The samples were streaked onto Whatman No. 3MM papers which had been previously washed with ethanol, and were resolved with the required solvent. After development the different bands were eluted with ethanol and then examined spectrophotometrically in different media. When necessary the purification was repeated using two or more different solvents.

III. Results

(*a*) *Extractives Content of Mycorrhizas and Roots*

The details of the samples collected and the amount of their extractives are listed in Table 1. The number of mycorrhizas formed by *P. radiata* seedlings grown in the Narbethong (good) nursery was greater (1·5–2 times) than that by the seedlings from the Mt. Macedon (poor) nursery. During August the amount of acetone extractives were higher in the mycorrhizas from the Mt. Macedon nursery. All seedlings from this nursery consistently yielded extractives with a higher proportion of n-heptane-insoluble material which contains mainly polyphenols. However, the

heptane-insoluble portion of the extractives from the Narbethong mycorrhizas varied considerably in amount. The seedlings of *Ps. menziesii* which were obtained from the Trentham nursery over a 4-month period showed variations as to the number of mycorrhizas and the amount of their acetone extractives. The amount of heptane-insoluble material was higher in two samples (August 16 and October 19) than that in the *P. radiata* samples. With both species the percentage of mycorrhizal extractives was lower than the root extractives but the percentage of n-heptane-insoluble material was similar in both tissues.

TABLE 1

DESCRIPTION OF THE MYCORRHIZAS AND ROOTS OF *P. RADIATA* AND *PS. MENZIESII*

Collection Date	Number of Seedlings	Dry Weight (g) of Mycorrhizas (M) or Root (R)	Number* of Mycorrhizas per Seedling	Acetone Extractives (%)	Weight (mg) of Heptane-insoluble Part†
		P. radiata (from Narbethong nursery)			
May 2	112	(M) 29·0	2589	3·6	15 (14·6)
Aug 15	78	(M) 9·5	1218	4·2	174 (43·6)
Aug 15	6	(R) 14·1		6·6	410 (43·9)
		P. radiata (from Macedon nursery)			
Aug 3	48	(M) 4·4	917	6·0	146 (55·5)
Aug 24	38	(M) 2·8	737	6·5	108 (59·5)
Oct 19	50	(M) 4·4	880	3·5	72 (47·2)
Aug 24	12	(R) 29·7		8·5	1705 (67·7)
		Ps. menziesii			
July 21	15	(M) 6·0	4000	5·2	144 (46·4)
July 31	15	(M) 6·7	4467	2·6	56 (32·6)
Aug 16	38	(M) 12·6	3316	5·5	587 (85·5)
Oct 19	48	(M) 13·6	2833	2·7	270 (73·7)
Aug 16	13	(R) 49·5		15·2	6489 (86·0)

* Numbers are calculated from 1 mycorrhiza = 0·1 mg.

† Values in parentheses are heptane-insoluble parts expressed as percentage of acetone extractives.

(*b*) *Composition of the Extractives of* P. radiata

The two-dimensional paper chromatograms of the acetone extracts of the mycorrhizas and roots [Figs. 1(*a*) and 1(*b*)] showed two spots (1 and 2) which appeared mauve under ultraviolet light and turned white to cream with ammonia vapour. These spots and the other six gave brown colours with spray A.

(i) *Examination of Components*

The polyphenols were isolated from the heptane-insoluble portion of the acetone extracts. The compounds corresponding to spots 1 and 2 were unstable and purification by paper chromatography using in sequence solvents 1, 3, and 2 resulted in large losses. Hydrolysis of spot 1 with 1N sulphuric acid for 1 hr yielded

glucose (identified chromatographically) and an aglycone which coincided completely with spot 2 (which is identical with 3,5,3′,4′-tetrahydroxystilbene, Table 2) on two-dimensional chromatograms. Spectral examination (Table 2) showed that spot 1 is different from astringin (3,5,3′,4′-tetrahydroxystilbene-3-β-glucoside) (Hillis and Ishikura 1968).

From the extract of the mycorrhizas collected from Narbethong on May 2, the compound corresponding to spot 4 was isolated in a yield of 11·4% by t.l.c. using solvent 12 followed by 15. The colour reactions, R_F values in several solvents, and spectral values in different media were completely identical with those of authentic catechin.

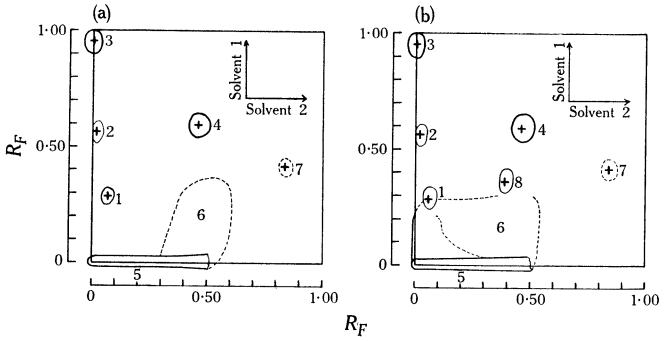

Fig. 1.—Two-dimensional chromatograms of extracts of *P. radiata* mycorrhizas (*a*) and roots (*b*).

The evaporated heptane-insoluble portion of the acetone extract of mycorrhizas from Narbethong (May 2) was extracted with hot water. After filtration, this solution was washed thoroughly with ether and ethyl acetate, and then acidified by addition of 5% hydrochloric acid. After standing for 2 days at 0–4°C a creamy-white, fine precipitate formed, and this compound appeared as spot 5 on the chromatograms. The compound had λ_{max} (nm) 283 in ethanol (λ_{min} 264), 301 in ethanol+sodium ethoxide ($\Delta\lambda$18), 283 in ethanol +$AlCl_3$, and 283 in ethanol+sodium acetate. Its R_F values and colour reactions were consistent with those expected for a leucoanthocyanin polymer. When heated with n-butanol–hydrochloric acid (95 : 5) for 30 min, and the products then examined chromatographically in three solvents, a compound identical with authentic cyanidin was recognized.

The properties of spot 6 suggested that this compound is a leucocyanidin of lower molecular weight than the compound of spot 5. R_F values of spot 7 were 0·75 in solvent 3 and 0·15 in solvent 4, and it became red-brown with spray A. Compounds represented by spots 6–8 were too small in amount to identify.

The heptane-soluble portion of the acetone extracts appeared as spot 3 on the two-dimensional chromatogram and, by using the solvents 12, 13, 14, and 15, the material was separated into 24 compounds on t.l.c. plates. The majority of these had λ_{max} 270–278 nm. When the material was saponified with 10% NaOH in nitrogen for 1·5 hr and then chromatographed, resinous material and three blue fluorescent spots were apparent, the colour of the latter changing to blue–green with ammonia

vapour. Whereas untreated spot 3 reacted strongly with spray C, the saponified material which occupied the same position did not react. The three blue-fluorescing compounds gave a faint red colour with spray A and blue with spray C, and 100 times the R_F values in solvent 1/solvent 2 of the compounds (listed in the order of amount present) were α 89/51, β 84/35, and γ 90/72. These spots have similar properties to coumarins but did not coincide with umbelliferone or scopoletin. Compound α has λ_{max} (nm) 329 (main) and 300 (minor) (λ_{min} 271) in ethanol; 387 and 319 in ethanol+sodium ethoxide; 329 and 302 in ethanol +$AlCl_3$; and 328 and 304 in ethanol+boric acid+sodium acetate.

TABLE 2

PROPERTIES OF SPOTS 1 AND 2 IN *P. RADIATA* EXTRACTIVES AND OF RELATED COMPOUNDS

Compound	Spectral Properties: λ_{max} (nm) in EtOH			
	Alone	+NaOEt	+$AlCl_3$	+H_3BO_3 and NaOAc
Spot 1	322	304	322	322
	310sh	297sh	310sh	310sh
	(λ_{min} 262)			
Spot 2	328	Decomposed	328	344
	307		307	309
	296sh		296sh	296
	263*		263	264
	(λ_{min} 274)			

Compound	$10^2 \times R_F$ in Solvent				Colour Reactions on Paper with		
	2	3	4	5	Spray C	Spray D+NH_3	Spray E
Spot 1	7	26	2	38	Blue	Violet	Positive
Spot 2	2	11	7	70	Faint blue	Purple	Positive
Astringin	7	25	3	38	Blue	Violet	Positive
3,5,3′,4′-Tetra-hydroxystilbene	2	12	7	71	Faint blue	Purple	Positive

* Possibly due to a decomposition product.

In both the acetone and the methanol extracts of mycorrhizas of *P. radiata*, glucose and fructose were detected chromatographically in small amounts with glucose being the main spot.

(ii) *Relative Amounts of Components*

On the chromatograms, catechin, leucoanthocyanin, and the two stilbenes appeared as the main spots (except for the resinous substances). The extractives of the mycorrhizas from both the Narbethong and Mt. Macedon nurseries were almost the same qualitatively. Catechin was relatively twice as strong in the mycorrhizas from Mt. Macedon nursery as in those from Narbethong. Leucoanthocyanin (spot 6) was present in larger amount in the roots than in the mycorrhizas, otherwise the

composition of the extractives was about the same. These aspects were unchanged throughout the samplings at different times.

(c) *Composition of the Extractives of* Ps. menziesii

Twenty-two spots were detected on the two-dimensional chromatograms of the acetone extracts of mycorrhizas and roots of *Ps. menziesii* (Fig. 2). They were detected either by fluorescence under ultraviolet light or by reaction with spray A.

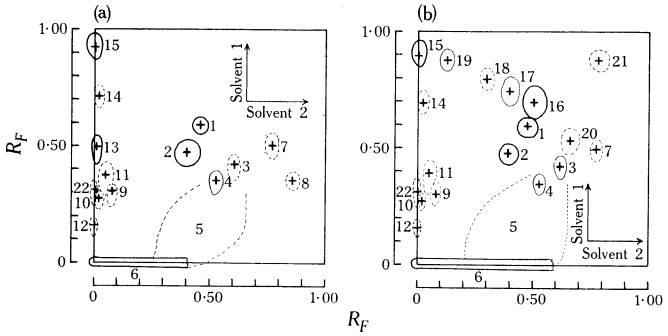

Fig. 2.—Two-dimensional chromatograms of extracts of *Ps. menziesii* mycorrhizas (*a*) and roots (*b*).

(i) *Examination of Components*

The R_F values, colour reactions, and spectrophotometric properties of the components corresponding to spots 1 and 2 were identical with catechin and epicatechin respectively.

Spot 3 from the roots became faint red with spray F and its R_F values in solvents 4 (0·12), 7 (0·83), and 3 (0·75) coincided with those of taxifolin-3′-β-glucoside (c-f. Barton 1967*a*). The identity of spot 3 from the mycorrhizas could not be confirmed because of the unavailability of sufficient amounts.

Compounds corresponding to spots 5 and 6 turned purplish red on heating with n-butanol–hydrochloric acid (95 : 5). Spot 6 formed cyanidin (identified chromatographically) and another red compound with R_F values of 0·94 in solvent 1 (on acid-washed paper) and 0·99 in solvent 8. Spot 8 was not detected in the roots.

The n-heptane-insoluble portion from the mycorrhizas (Trentham, July 21) was dissolved in ether, filtered, and washed several times with water. The ethereal solution was evaporated to dryness, dissolved in a minimum volume of ether, two volumes of freshly distilled chloroform added, and the precipitate washed with petroleum ether. More than 90% of the precipitate consisted of the purple-fluorescing compound represented by spot 13. It was further purified by large-scale chromatography using solvent 3, when 10 mg (1·2%) of material were obtained. The R_F values were 0·75 in solvent 6, 0·67 in solvent 5, 0·04 in solvent 3, 0·68 in 50% ethanol, and 0·54 in n-butanol (on acid-washed paper). The compound gave a positive Molisch test (pink) and negative ninhydrin, Benedict, anthrone, ferric chloride, and 2,4-dinitrophenylhydrazine reactions, and it was also negative to the

colour reactions for polyphenols. The spectrum (Fig. 3) of this compound showed four sharp maxima (λ_{max} 260, 328, 342, and 359 nm) giving a characteristic "finger effect" which is peculiar to polyene antibiotics (Nayler and Whiting 1955; Oroshnik *et al.* 1955; Ball, Bessell, and Mortimer 1957; Cope *et al.* 1962). It occurs for example in filipin, fungichromin, and lagnosin. The R_F value in n-butanol of spot 13 (0·54) was different from those of filipin (0·85) and fungichromin (0·70) (Whitfield *et al.* 1955), although the colour reactions were the same as those of filipin. Rapid degradation of compound 13 occurred when concentrates were allowed to stand at room temperature and the spectral maxima disappeared simultaneously. The degradative product fluoresced brick-red under ultraviolet light and had R_F 0·97 using the solvent methyl isopropyl ketone–methanol–water (50 : 1 : 0·4) (Cope *et al.* 1962).

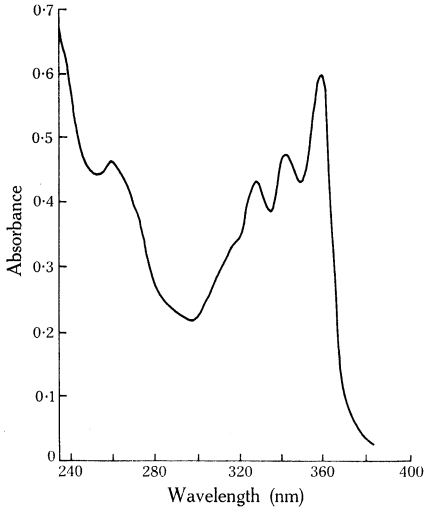

Fig. 3.—Ultraviolet absorption spectrum of compound 13 in *Ps. menziesii* extractives (see text).

The heptane-soluble portion ("resin") is represented by spot 15.

Spot 16 is the largest on the two-dimensional chromatogram of the root extracts but is absent in the mycorrhizas. It has been identified as poriolin, the 7-glucoside of 4′,5,7-trihydroxy-6-methylflavanone (Hillis and Ishikura 1969).

The compound corresponding to spot 17 was isolated in small amounts from the roots and purified by means of paper chromatography using solvent 3 and subsequently solvent 4. It behaved identically with authentic taxifolin on chromatographic examination using several solvents and colour reactions.

Spot 19 was identical with 4′,5,7-trihydroxy-6-methylflavanone. The amount in the roots was very small and it was not detected in mycorrhizas. Spots 20 and 18 were very similar in properties to spots 16 and 19 respectively.

Glucose and fructose were detected (in smaller amounts) in the acetone and methanol extracts of the mycorrhizas.

(ii) *Relative Amounts of Components*

Catechin, epicatechin, and leucoanthocyanin appeared as the main components in the mycorrhizas [Fig. 2(*a*)]. On the other hand, the roots contained flavanones (poriolin, poriol, taxifolin, taxifolin-3′-β-glucoside) in comparatively large amounts

in addition to the above [Fig. 2(*b*)]. Compared with the extractives of the roots, the mycorrhizas contained a relatively large amount of the purple-fluorescing compound (spot 13) and very small amounts of spot 8.

The amount of epicatechin (spot 2) in the mycorrhizas was approximately three times greater than that of catechin (spot 1). In the roots, however, the ratio of both compounds was approximately the same, although they were present in smaller amounts than in the mycorrhizas.

IV. Discussion

The mycorrhizas were collected from seedlings growing in established commercial nurseries so as to provide basic information on the extractives formed under natural conditions. The availability of material from such a source does, however, restrict the scope of the work to microtechniques.

Chromatographic and staining reactions showed that the heptane-insoluble portion of the acetone extractives of the tissues consisted largely of polyphenols. The amounts of both the extractives and its polyphenolic fraction in the mycorrhizas of slow-growing *P. radiata* (from Mt. Macedon) were higher than those in the vigorous seedlings (from Narbethong). In both cases these amounts were lower than those of the long roots (Table 1). However, one of the distinctive features seen on microscopic examination (Marks 1965; Foster and Marks 1966, 1967) of mycorrhizas was the accumulation of strongly staining material in a peripheral tannin layer. Such a layer was not apparent in the long roots. In the mycorrhizas and roots of both species the polyphenols present included compounds such as stilbenes, catechins, leucoanthocyanins, etc. which react strongly with permanganate, osmic acid, and tannin stains. Although the amount of extractives was higher in the roots, they were dispersed throughout the roots with some concentration in the endodermis (G. C. Marks, personal communication). In mycorrhizas the tannin layer is situated where it can act most effectively as a protective barrier. It is evident that in a discussion of the relation of extractives to fungal association, consideration should be given not only to their amount but also to their location in the tissues. It is also possible that only the most deeply staining polyphenols are present in the tannin layer.

Chromatographic examination showed that the constituents of the extractives in the mycorrhizas of *P. radiata* were very similar to those of the extractives in the roots (Fig. 1) so that the host–fungus relationship had no qualitative effect on composition. On the other hand, the composition of *Ps. menziesii* mycorrhizas was appreciably different from that of the root extractives (Fig. 2). The production of some constituents, especially the large amounts of flavanones, in the roots did not take place in the ectotrophic mycorrhizas, but a considerable proportion of a polyene-like compound (spot 13) has been formed in the mycorrhizas.

Detailed examination of the isolated components showed that, except for compound 13 in the *Ps. menziesii* mycorrhizas, all the compounds examined are normal plant constituents. The unstable compound 13 had properties similar to those of polyene antibiotics (see Section III) but the existence of this type of compound in plant tissues is uncommon. A compound with such a high degree of unsaturation

would react strongly with stains but there was no sign (Foster and Marks 1966, 1967) of accumulation of such a compound in the fungal mantle of the mycorrhizas. After the completion of this study we saw the work of Marx (1967), who isolated from the culture media of a mycorrhizal fungus grown *in vivo* three diatretynes with strong antibiotic properties. In view of these findings it is concluded that compound 13 is dispersed in low concentration through the mantle, and is probably produced by the fungus and not by the host.

The different compositions of the *Ps. menziesii* tissues (Fig. 2) indicate that the polyphenols are formed *in situ*, otherwise the composition in both the roots and mycorrhizas would be identical. In addition neither poriolin, which is the major component in the root tissues, nor its aglycone (compare Barton 1967*a*, 1967*b*) have been detected in the stem wood of this species; also the catechins are weak or absent in the stem sapwood, where taxifolin is a major component. Similarly, the stilbenes and catechin of *P. radiata* root and mycorrhizas are absent in the stem sapwood, and the components in the latter (Hillis and Inoue 1968) are lacking in the roots, showing that translocation of polyphenols is unlikely. It can be concluded that the composition of the polyphenols is determined by the nature of the cells (mycorrhizas involve only the primary xylem cells), by their environment, or in the case of mycorrhizas, by the effects of fungi. The formation of the tannin layer could be initiated by mechanical injury, but if mycorrhizal fungi can cause a considerable shift in the carbohydrate physiology of the roots of *Fagus sylvatica* Linn. (Lewis and Harley 1965) it seems more likely that they would cause blockage of the biosynthesis of the flavanones found in the root tissues of *Ps. menziesii* (Fig. 2). The composition of the polyphenols was the same in *P. radiata* from two nurseries where the type of mycorrhizal fungi differs (Marks, personal communication). In these cases, the increased synthesis of polyphenols in the tannin layer could be due to a local enhancement of metabolism and be independent of the species of fungus causing it. In support of this view the compositions of the polyphenols of *P. radiata* roots and mycorrhizas were very similar, and the root of a 5-week-old germinated seed grown in culture contained small amounts of extractives which were similar to those in Figure 1 except that they lacked the leucoanthocyanin spots 5 and 6.

The tannin layer could play a significant part in the establishment of a satisfactory mycorrhizal association, by providing a physical barrier to prevent the fungi entering the root to establish the Hartig net, or by weakening the fungi in the association and excluding parasitic fungi. The morphology of the fungus changes as it passes through the tannin layer (Foster and Marks 1966, 1967).

The shortage of material available prevented testing the antifungal activity of the components but an indication of their possible influence in the control of symbiosis can be gauged from their known properties. The class of stilbenes in *P. radiata* mycorrhizas and roots has strong fungicidal activity (Rennerfelt 1945; Rennerfelt and Nacht 1955) and inhibits cellulase, proteinase, and, less effectively, xylanase and pectinase (Lyr 1961). The toxic components orchinol and hircinol, produced in orchid tubers as a result of mycorrhizal formation (Boller *et al.* 1957; Hardegger, Biland, and Corrodi 1963; Hardegger, Schellenbaum, and Corrodi 1963; Urech *et al.* 1963) have been identified as dihydrophenanthrenes. It is noteworthy that orchinol at least has the same carbon skeleton and positioning of hydroxyl or

methoxyl groups as the naturally occurring stilbenes. Leucoanthocyanins in small amounts have been associated with cellulase inhibition in several materials (Mandel and Reese 1963, 1965) and together with catechin may also inhibit pectinase and polygalacturonidase (Byrde and Whitaker 1963; Wood 1967*b*). The resin could provide a physical barrier and a correlation has been shown between the resistance of conifers to *Fomes annosus* and their ability to mobilize resin (Gibbs 1968). However, conclusions cannot be drawn without information on the amount of different components and their location in the tissues.

In cases where polyphenols have been associated with resistance to pathogens, the integrity of the plant cells is lost. In the case of mycorrhizas, however, the fungus does not enter the cells of the tannin layer and there is no visible injury to the cells (Foster and Marks 1966, 1967). In some cases it is possible that some cells in the tannin layer rupture and their contents migrate to form a continuous layer and barrier to fungi. Further study is required to determine how the polyphenol-filled cells prevent the passage of hyphae between the cells in certain cases and how the polyphenols affect the hyphal morphology and cytoplasmic fine structure when the hyphae do penetrate the layer (Foster and Marks 1966).

This study has defined many of the polyphenols in the extractives of the mycorrhizas and roots of seedlings grown under natural conditions. It is evident from the heterogeneity of composition as shown in this study and the unequal distribution of the extractives as shown by microscopic examination (Foster and Marks 1966, 1967) that attention should be directed to the chemical and biochemical conditions of individual cells and the determination of amounts of biologically active components.

V. Acknowledgments

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VI. References

Ball, S., Bessell, C. J., and Mortimer, A. (1957).—*J. gen. Microbiol.* **17**, 96.

Barton, G. M. (1967*a*).—*Can. J. Bot.* **45**, 1545.

Barton, G. M. (1967*b*).—*Can. J. Chem.* **45**, 1020.

Barton, G. M., Evans, R. S., and Gardner, J. A. F. (1952).—*Nature, Lond.* **170**, 249.

Bate-Smith, E. C., and Swain, T. (1953).—*Chemy Ind.* **1953**, 377.

Bhate, D. S. (1962).—In "Chemistry of Natural and Synthetic Colouring Matters". (Eds. T. S. Gore *et al.*) p. 341. (Academic Press, Inc.: New York.)

Boller, A., Corrodi, H., Gäumann, E., Hardegger, E., Kern, H., and Winterhalter-Wild, N. (1957).—*Helv. chim. Acta* **40**, 1062.

Bowen, G. D. (1965).—*Aust. For.* **29**, 231.

Byrde, R. J. W., and Whitaker, D. R. (1963).—Bull. Conn. agric. expl. Stn No. 663. p. 34.

Cope, A. C., et al. (1962).—*J. Am. chem. Soc.* **84**, 2170.

Cruickshank, I. A. M. (1963).—*A. Rev. Phytopath.* **1**, 351.

Farkas, G. L., and Király, Z. (1962).—*Phytopath. Z.* **44**, 105.

Foster, R. C., and Marks, G. C. (1966).—*Aust. J. biol. Sci.* **19**, 1027.

Foster, R. C., and Marks, G. C. (1967).—*Aust. J. biol. Sci.* **20**, 915.

Gibbs, J. N. (1968).—*Ann. Bot.* (N.S.) **32**, 649.

Hardegger, E., Biland, H. R., and Corrodi, H. (1963).—*Helv. chim. Acta* **46**, 1354.

HARDEGGER, E., SCHELLENBAUM, M., and CORRODI, H. (1963).—*Helv. chim. Acta* **46**, 1171.
HILLIS, W. E., and INOUE, T. (1968).—*Phytochemistry* **7**, 13.
HILLIS, W. E., and ISHIKURA, N. (1968).—*J. Chromat.* **32**, 323.
HILLIS, W. E., and ISHIKURA, N. (1969).—*Aust. J. Chem.* **22**, 483.
HILLIS, W. E., ISHIKURA, N., FOSTER, R. C., and MARKS, G. C. (1968).—*Phytochemistry* **7**, 409.
KEEVES, A. (1966).—*Aust. For.* **30**, 51.
LEWIS, D. H., and HARLEY, J. L. (1965).—*New Phytol.* **64**, 224, 238, and 256.
LYR, H. (1961).—*Enzymologia* **23**, 231.
MACDOUGAL, D. T., and DUFRENOY, J. (1944).—*Pl. Physiol., Lancaster* **19**, 440.
MANDEL, M., and REESE, E. T. (1963).—"Advances in Enzymic Hydrolysis of Cellulose and Related Materials." (Ed. E. T. Reese.) p. 115.
MANDEL, M., and REESE, E. T. (1965).—*A. Rev. Phytopath.* **3**, 85.
MARKS, G. C. (1965).—*Aust. For.* **29**, 238.
MARKS, G. C., DITCHBURNE, N., and FOSTER, R. C. (1967).—*Aust. For.* **32**, 26.
MARKS, G. C., and FOSTER, R. C. (1967).—*Aust. For.* **31**, 193.
MARX, D. H. (1967).—Proc. 14th Congr. Int. Un. Forest Res. Org., Munich. Vol. V. p. 172.
NAYLER, P., and WHITING, M. C. (1955).—*J. Chem. Soc.* **1955**, 3037.
OROSHNIK, W., VINING, L. C., MEBANE, A. D., and TABER, W. A. (1955).—*Science, N.Y.* **121**, 147.
REIO, L. (1960).—In "Chromatographic Reviews". (Ed. M. Lederer.) Vol. 1. p. 39. (D. Van Nostrand Co., Inc.: New York.)
RENNERFELT, E. (1945).—*Svensk bot. Tidskr.* **39**, 311.
RENNERFELT, E., and NACHT, G. (1955).—*Svensk bot. Tidskr.* **49**, 419.
ROHRINGER, R., and SAMBORSKI, D. J. (1967).—*A. Rev. Phytopath.* **5**, 77.
SWAIN, T. (1952).—*Biochem. J.* **53**, 200.
URECH, J., FECHTIG, B., NUESCH, J., and VISCHER, E. (1963).—*Helv. chim. Acta* **46**, 2758.
WHITFIELD, G. B., BROCK, T. D., AMMANN, A., GOTTLIEB, D., and CARTER, H. E. (1955).—*J. Am. chem. Soc.* **77**, 4799.
WINTERHALTER-WILD, N., CARRODI, H., GÄUMANN, E., HARDEGGER, E., and KERN, H. (1957).—*Helv. chim. Acta* **40**, 1062.
WOOD, R. K. S. (1967*a*).—"Physiological Plant Pathology." p. 413. (Blackwell Scientific Publications: Oxford.)
WOOD, R. K. S. (1967*b*).—"Physiological Plant Pathology." p. 458. (Blackwell Scientific Publications: Oxford.)