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THE FORMATION OF POLYPHENOLS IN TREES—IV.

THE POLYPHENOLS FORMED IN *PINUS RADIATA* AFTER *SIREX* ATTACK

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Abstract—The composition of the polyphenolic extractives of unattacked, damaged and *Sirex*-affected sapwood, heartwood and knotwood of *Pinus radiata* D. Don differ considerably. The sapwood contained vanillin, vanillic, protocatechuic, 4-hydroxybenzoic, salicylic and ferulic acids, hydroquinone, traces of pinosylvin monomethyl ether and unidentified compounds. The amounts of pinobanksin, pinocembrin, pinosylvin and its monomethyl ether are much greater in the knotwood than in the heartwood. Whereas only very small amounts of pinosylvin were found in heartwood, the amount in knotwood was larger than that of its ether. No flavonoids were found in *Sirex*-affected wood but the amount of pinosylvin was much larger than its ether. Damaged sapwood contained pinosylvin monomethyl ether and pinocembrin. A compound with the properties of a flavanonol C-glycoside was isolated from the heartwood and knotwood. It is concluded that wood extractives are formed *in situ* and that the conditions initiating their formation influence their composition. The restriction of the spread of the symbiotic fungus of *Sirex* in trees which resisted attack, appears to be due to the formation of stilbenes in the sapwood after infection.

INTRODUCTION

IN RECENT years, the European wood-wasp *Sirex noctilio* has reached epidemic proportions in the extensive *Pinus radiata* D. Don forests of New Zealand and Tasmania and has killed a large number of trees. Enormous economic loss would probably result if a *Sirex* species became established in the forests on the Australian mainland. Consequently, studies have been made to determine the causes which enable some trees to resist attack.

Under certain conditions, *Sirex* females insert their ovipositors into the sapwood to a depth of about 2 cm. At every insertion, the wood is inoculated with a symbiotic fungus which is a species of *Amylostereum*, probably *A. chailletii*.[1] Subsequently, if the conditions are suitable, rapid drying of the wood adjacent to the tunnels takes place. (The sequence of events has been described in detail by Coutts).[2] With the trees which recover from attack, resin appears first in the dry wood and then polyphenols form, particularly at the periphery. The dry wood zone continues to enlarge up to about the sixth week from attack and in this time it may have advanced radially a distance of 2–3 cm with 1 cm width. Usually, the affected regions do not become much larger in those trees which recover from *Sirex* attack. In trees which do not resist attack, large areas of dried sapwood appear but no sign of polyphenol formation has been detected. Although there is an association between a high amount of resin in the tree, a high resin pressure, and resistance to *Sirex* attack, this association does not always apply.[2]

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[1] J. M. KING, *Australian J. Botany* **14**, 25 (1966).

[2] M. P. COUTTS, *Dep. Nat. Develop., For. Timber Bureau, Bull.* No. 41 (1965), and further work by M. P. COUTTS and J. E. DOLEZAL *For. Timber Bureau*, Leaflet 101 (1966).

Disease resistance in plants is the result of many factors and prominent amongst these is the pre- or post-infection formation of polyphenols.[3] In this present work, the composition of polyphenols, in the sapwood of *P. radiata* attacked by *Sirex* and in other tissues, has been examined in order to assess the possible association between polyphenols and resistance to the *Sirex* wasp and its symbiotic fungus.

RESULTS

The amount of extractives in knotwood and in *Sirex*-affected sapwood was much higher than in the other tissues (Table 1) and this is largely due to the greater amount of resinous material present.

TABLE 1. EXTRACTIVES IN *P. radiata* TISSUES

	Tissue				
	Sapwood			Heartwood	Knotwood
	Unaffected	Damaged	*Sirex*-affected		
Weight of Tissue Examined (g)	37	30	59	100	37
Acetone Extract*	2·34	8·06	18·8	2·22	13·6
Ether insoluble	0·47	0·32	0·18	0·18	0·13
Water soluble	0·05	—	0·10	0·08	0·06
$NaHCO_3$ soluble	0·55	0·29	0·83	0·08	1·33
Na_2CO_3 soluble	0·01	0·08	0·05	0·07	0·46
NaOH soluble					
resinous layer	0·48	4·20	14·10†	1·17	8·75
aqueous layer	0·28	0·36	0·46	0·35	1·37
Neutral fraction	0·26	1·10	1·36	0·35	1·45
Methanol Extract*	0·54	—	2·38	0·60	0·82

* Percentage of air-dried wood.
† Containing 93 per cent material soluble in cold petrol (60–80°).

The polyphenols in normal sapwood are present in trace amounts. They were obtained in an amount sufficient for identification, from a large sample of 'unattacked sapwood' from an unattacked tree of comparable age to those trees attacked by *Sirex*.

The polyphenolic compounds observed on the chromatograms of the extract were, in the following order of decreasing amounts: vanillin, vanillic (3-methoxy-4-hydroxybenzoic) acid, protocatechuic (3,4-dihydroxybenzoic) acid, 4-hydroxybenzoic acid, salicylic (2-hydroxybenzoic) acid, ferulic (3-methoxy 4-hydroxycinnamic) acid, hydroquinone and pinosylvin monomethyl ether. A number of these had been previously observed in bisulphite pulping liquor from this species.[4]

[3] G. L. FARKAS and Z. KIRÁLY, *Phytopathol. Z.* **44**, 105 (1962).
[4] W. E. HILLIS, Unpublished data.

The yields of the polyphenols isolated from the different tissues are given in Table 2. The results shown confirmed the conclusions drawn from a visual inspection of two-dimensional chromatograms. The results of the heartwood examinations are in good agreement with those of earlier examinations when pinobanksin (5,7-dihydroxy flavanonol), pinocembrin (5,7-dihydroxy flavanone) and pinosylvin monomethyl ether were each isolated in yields of

TABLE 2. YIELDS (%) OF COMPONENTS ISOLATED FROM *P. radiata* TISSUES

	Tissue				
	Sapwood			Heartwood	Knotwood
	Unaffected	Damaged	*Sirex*-affected*		
Pinosylvin	—	—	0·071†	0·003†	0·151‡
Pinosylvin monomethyl ether	—	0·021	0·005†	0·044†	0·096‡
Pinobanksin	—	—	—	0·063‡	0·162‡
Pinocembrin	—	0·064	—	0·037‡	0·115‡

* From trees grown in Victoria.
† Homogeneous by TLC and spectral examination but crystals not obtained.
‡ Recrystallized from benzene or toluene to give correct m.p. and mixed m.p.

0·08 per cent[5] and pinosylvin (3,5-dihydroxy-stilbene) detected chromatographically.[6] Determinations by a chromatographic-spectrophotometric method[7] would doubtless show that larger amounts are present. Although the amount of neutral fraction previously recorded[5] was about the same, the ether-soluble material (8 per cent) was much greater than in this study. The composition of the heartwood polyphenols of a fast-grown specimen from Australia is very similar to that of a slow-grown sample from England[5] so that apparently composition is independent of rate of growth and environment. Similarly the amount of pinosylvins in *P. sylvestris* heartwood varies only slightly from the south to north of Sweden.[8]

An unidentified material ('Compound X') (Table 3) was observed in the sodium bicarbonate extracts of the heartwood and knotwood extracts. It was indistinguishable from pinobanksin on the two-dimensional chromatograms prepared with BAW and 6 per cent acetic acid, but was appreciably different on TLC using solvents III, IV and VI. The isolated material had a slight yellow colour, an opaque appearance on paper chromatograms under u.v. (254 nm), and gave an orange-yellow colour with diazotized *p*-nitroaniline. It did not give the flavanone colour with sodium borohydride.[9] These properties, the R_f values and spectral properties indicate that the compound is a flavanonol glycoside. The R_f values were unchanged after heating with 2 N hydrochloric acid for 1·5 hr so that Compound X could be a C-glycoside but insufficient material was available to characterize the compound further.

[5] G. LINSTEDT, *Acta Chem. Scand.* **3**, 763 (1949).
[6] G. LINSTEDT and A. MISIORNY, *Acta Chem. Scand.* **5**, 121 (1951).
[7] G. LINSTEDT and A. MISIORNY, *Svensk Papperstid.* **55**, 602 (1952).
[8] H. ERDTMAN, A. FRANK and G. LINDSTEDT, *Svensk Papperstid.* **54**, 275 (1951).
[9] E. EIGEN, M. BLITZ and E. GUNSBERG, *Arch. Biochem. Biophys.* **68**, 501 (1957).

TABLE 3. SOME PROPERTIES OF THE MAJOR COMPONENTS OF *P. radiata* EXTRACTIVES

	R_f values (x100) Chromatographic solvents						
	Paper			TLC			
Compound	BAW	6HA	BeAW	I	II	III	VI
Caffeic acid	69	35	04	63	19	—	—
Ferulic acid	73	37	62	80	54	—	—
Gallic acid	52	40	00	50	25	00	00
Hydroquinone	81	69	07	78	44	06	18
p-Hydroxybenzoic acid	25	59	29	76	47	02	06
Pinobanksin	85	40	45	90	50	28	29
Pinocembrin	85	22	73	95	66	45	57
Pinosylvin	85	03	38	88	49	25	18
Pinosylvin monomethyl ether	85	06	85	93	64	54	60
Protocatechuic acid	73	48	04	68	46	—	—
Salicylic acid	87	65	72	90	62	08	93
Vanillic acid	82	50	57	74	49	08	86
Vanillin	84	66	66	82	54	41	46
'Compound X'	81	37	02	—	48	20	08

	Spectral values λ_{max} (nm)			
	EtOH	NaOAc	NaOEt	$AlCl_3$
Pinobanksin	295	332	298, 332	315, 386
Pinocembrin	292	331	296, 330	312, 380
Pinosylvin	303, 312	—	305, 316	—
Pinosylvin monomethyl ether	302, 309	—	305, 314	—
'Compound X'	292	292, 332	295, 330 (unstable)	316, 385

The areas affected by the *Sirex* fungus had pinkish coloured edges which together with the rays, stain strongly with stains for phenolic compounds. Under u.v. light the affected regions usually had a pronounced blue fluorescence although sometimes the colour is yellow. The polyphenols in the *Sirex*-affected sapwood were markedly different from those in the heartwood and knots in that pinobanksin and pinocembrin were not evident on chromatographic examination. During isolation of the stilbenes, the fractions which corresponded to those containing the flavonoids in other tissues were examined without finding any evidence of the presence of pinobanksin or pinocembrin. The composition of the polyphenols from the *Sirex*-affected wood of trees collected in Tasmania and Victoria was the same. Leucoanthocyanins were absent or present in trace amounts in both unaffected and *Sirex*-affected sapwood. Hydroquinone was found in trace amounts in all tissues of *P. radiata* and its presence has also been recently reported in *Pinus resinosa*.[10]

The composition of the polyphenolic mixture in the damaged sapwood was appreciably different from the different *Sirex*-affected sapwoods. The amount of extractives was less (Table 1), but particularly the amount of pinosylvin was much lower than its monomethyl

[10] E. VON RUDLOFF, *Chem. Ind. (London)* 180 (1965).

ether and also pinocembrin was present (Table 2). Small amounts of unidentified polyphenols, that were not seen in the other tissues, were also present.

The *Sirex*-affected sapwood and the knotwood contained very much higher amounts of resinous material (both alkali soluble and insoluble) than the unaffected sapwood and heartwood. Preliminary studies[4] indicate the composition of this material in unattacked sapwood and *Sirex*-affected wood is very similar. Glucose and arabinose were present in the methanol extracts of all tissues but glucose was the major sugar in both normal and *Sirex*-affected sapwood.

DISCUSSION

Sapwood from unattacked *Pinus radiata* trees contains a very small amount of polyphenols comprising a wide range of components which does not include pinosylvin and the flavonoids of the heartwood. Furthermore none of the acids or vanillin of the sapwood are present as such, or as fragments of the flavonoids, in other tissues. Although qualitatively the extractives of the knotwood have the same composition as those in heartwood they are quantitatively different (Table 2). The composition of the polyphenols from the *Sirex*-affected sapwood is notable for the absence of pinobanksin and pinocembrin and moreover the ratio of the amount of pinosylvin to its methyl ether is very much greater than that in the heartwood and knotwood. The composition of the damaged sapwood lies somewhat between that of the *Sirex*-affected sapwood and heartwood (Table 2). The most notable difference between the unattacked sapwood and the rest of the tissues is that only a very small portion of the polyphenols in the sapwood contains moieties originating from acetate units which are also involved in the energy-producing Krebs' cycle. There is either insufficient acetate units available for incorporation into polyphenols in sapwood[11] or there are different metabolic pathways operating in different tissues.

The absence of a number of the sapwood polyphenols in the heartwood; the presence in the latter of a number of components not found in the sapwood; the presence in relatively large amounts of polyphenols on the pith side of the *Sirex*-affected regions, when the cambial side of the region was blocked by necrotic tissue; and the difference in composition of the polyphenols in heartwood, damaged sapwood, *Sirex*-affected sapwood and knotwood all support the view that the polyphenols are not translocated in the wood but are formed *in situ* in response to certain stimuli.[12]

A marked drop in the moisture content of *P. radiata* sapwood occurs before the formation of the heartwood extractives[13] and a similar sequence occurs in the regions affected by the symbiotic fungus of *Sirex*.[2] The reduction of water content of branch stubs would presumably be the first stage in the formation of knots. The loss of water, which is common to all cases, cannot be the only factor concerned in polyphenol formation, or the composition of the polyphenol mixture would be the same in all cases (see also Ref. 14).

The ages of the sapwood cells transformed to heartwood and to *Sirex*-affected wood differ by more than 12 yr but this age difference is unlikely to be responsible for the difference in composition. The differences in these and other tissues are more likely due to the nature of the unknown factors which determine or influence the direction of metabolism, before polyphenols are formed in increased amounts. The flavonoids are notably absent in the

[11] W. E. HILLIS and T. INOUE, *Phytochem.* 5, 483 (1966).
[12] W. E. HILLIS, In: *Wood Extractives* (edited by W. E. HILLIS), p. 59. Academic Press, New York (1962).
[13] J. M. HARRIS, *New Zealand Forestry Serv. Forest Res. Inst. Rotorua Tech. Paper* No. 1 (1954).
[14] W. E. HILLIS, *Proc. Sect.* 41 *Intern. Union Forestry Res. Organizations*, Vol. 1. Committee on Heartwood Formation, Div. Forest Products, Melbourne (1965).

Sirex-affected wood, whereas they are present in appreciable amounts in the heartwood and knotwood. The symbiotic *Amylostereum* fungus apparently altered the metabolism of the host cells so that the stilbenes were formed preponderantly and a cytological examination has shown that the ray cells of the host disorganize in advance of the fungal hyphae.[15] The marked difference in composition of *Sirex*-affected wood may be a specific host-pathogen interaction.

Other more marked differences in composition of polyphenols have been found in the comparison of fungal-affected wood with surrounding tissues in the case of *Prunus domestica*,[16] *P. jamasakura*,[17] etc.[12] The wood of *P. domestica* affected by *Stereum purpureum* contained large amounts of scopoletin that was absent elsewhere (scopoletin is also found in other tissues after infection[18, 19]). When *P. jamasakura* sapwood was affected by the fungus *Coriolus versicolor* the major and another flavonoid present in the heartwood were absent and were replaced by a lignan. There is evidence in other cases that different fungi have the same effect on the host as in carrot root[20] and in pea pods.[21] In this regard, there is an association between the formation of pinosylvin and its methyl ether and the infection of the sapwood in roots and stems of *Pinus resinosa* with *Fomes annosus*, which is claimed to be non-specific.[22]

Jorgensen[22] has concluded that slow desiccation is a major factor in the formation of the pinosylvins after damage by *Fomes annosus* or by mechanical means. So far, under our conditions, we have not been able to form pinosylvins by mechanical injury except for a very narrow band around the hole (see also Refs. 11, 14). Examination of 'damaged' sapwood from a deformed tree, in which a large portion of the cambium had been killed four years previously, showed that a relatively large amount of pinosylvin monomethyl ether was present (Table 2). Although this particular aspect is in agreement with Jorgensen's view, the different composition and behaviour (see Experimental) when compared with other dead tissues shows that loss of water is not the primary factor in the initiation of polyphenol formation.

The composition of the resin in the affected regions is similar to that in the rest of the wood, and presumably it was forced into the empty cells after the water moved out of them[2, 23] or as the permeability of the tissue increased. There is no record of the toxicity of resin components to fungi, and it is unlikely that resin would restrict fungal spread significantly though it could act as a mechanical barrier.

The phenolic compounds formed after the sapwood was infected with the *Sirex* fungus could play an important role in restricting the spread of the fungus. There are numerous instances in which varietal resistance of plants to fungal attack has been associated with the post-infectional content of polyphenols.[3, 24–27] Flavonoids of the type found in *Pinus* woods

[15] R. C. FOSTER, personal communication.
[16] W. E. HILLIS and T. SWAIN, *J. Sci. Food Agr.* **10**, 533 (1959).
[17] M. HASEGAWA and T. SHIRATO, *J. Japan Forestry Soc.* **41**, 1 (1959).
[18] L. SEQUEIRA and A. KELMAN, *Phytopathology* **52**, 439 (1962).
[19] J. C. HUGHES and T. SWAIN, *Phytopathology* **50**, 398 (1960).
[20] P. CONDON and J. KUĆ, *Phytopathology* **52**, 182 (1962).
[21] I. A. M. CRUICKSHANK and D. R. PERRIN, *Australian J. Biol. Sci.* **14**, 336 (1961).
[22] E. JORGENSEN, *Can. J. Botany* **39**, 1765 (1961).
[23] J. M. HARRIS, *Proceedings Section* 41 *International Union of Forestry Research Organizations*, Vol. 1. Committee on Heartwood Formation, Div. Forest Products, Melbourne (1965).
[24] H. OKU, *Phytopathol. Z.* **44**, 39 (1962).
[25] S. WAKIMOTO and H. YOSHII, *Ann. Phytopathol. Soc. Japan* **23**, 78 (1958).
[26] Z. KIRÁLY and G. L. FARKAS, *Phytopathology* **52**, 657 (1962).
[27] I. A. M. CRUICKSHANK, *Ann. Rev. Phytopathol.* **1**, 351 (1963).

have very little fungal toxicity.[28] Consequently it is noteworthy that pinosylvin was by far the major polyphenol biosynthesized in the *Sirex*-affected region (Table 2) as this stilbene and its methyl ether have strong fungicidal activity[29] (see also Ref. 30). Pinosylvin was found to be rather more toxic in most cases and a more universal poison[31] than its monomethyl ether; they are inhibitors in 0·01–0·02 per cent concentrations. (Pinosylvin monomethyl ether has been shown to be toxic to the *Sirex* fungus.)[32] Lyr[33] found that pinosylvin monomethyl ether inhibited cellulase and proteinase at about the same concentration and xylanase and pectinase less effectively and from his work concluded that the stilbenes are uncoupling agents. Consequently, in addition to being fungitoxic, stilbenes could confine the spread of the fungus by inactivating the extra-cellular enzymes.

It is unlikely that resistance of some *P. radiata* trees to *Sirex* is due to one factor alone. Resistance could be due to a combination of physiological and biochemical factors. For example, the higher incidence of damage caused by *Sirex* in the Tasmanian and New Zealand forests compared with the European forests, might be due to a higher degree of water stress. Under such conditions the amount of water in the affected region could decrease so quickly that there is insufficient time for the cells to form protective extractives after the original stimulus was given. A number of biochemical factors may be involved. The restriction of fungal growth to a relatively small area could be due to the availability of the stored or translocated carbohydrates required for the formation of polyphenols, at a suitable rate and in sufficient quantity in the affected region. It appears from our work that a suitable stimulus must be given and a suitable reaction obtained before a characteristic polyphenolic mixture is formed. On this basis, the greater pathogenicity of a fungal strain or the decrease in resistance of a race of *Pinus radiata* would be due mainly to the lack of suitable stimuli or triggers to alter the course of metabolism in the required direction. As the heartwood and knotwood contain both stilbenes and flavonoids, it would appear that the *Sirex* fungus blocks the pathway to flavonoids at the divergence point. Several *Eucalyptus* species have been found[34] in which, owing to some unknown factor, the normal pathway to flavonoids is partly, at least, diverted to stilbenes.[35] This factor in the eucalypt leaves may be similar to that involved in *Sirex*-resistant tissues of *P. radiata* and investigations to define this factor are underway. If a *Sirex*-resistant variety of *P. radiata* exists, as some observers suspect, this unknown factor which triggers off the formation of stilbenes could be the fundamental cause of resistance.

EXPERIMENTAL

Plant Material

The 'unattacked' sapwood was obtained from logs (containing eight growth rings) collected from the Yarrawe plantation (Victoria) in early summer. *Sirex* wasps have not been detected in this plantation.

The '*Sirex*-affected' sapwood samples were obtained from two 6–8 yr-old trees (diameter 12–13 cm) growing near Traralgon, Victoria. Discs cut through the trees showed that the trees had been repeatedly attacked during one season (eight times at one level alone) without appreciably affecting subsequent growth. The resinous fluorescent areas were cut out of the discs. The 'unaffected' sapwood samples were obtained

[28] P. RUDMAN, *Holzforschung* **17**, 54 (1963).
[29] E. RENNERFELT and G. NACHT, *Svensk Botanisk Tidskrift* **49**, 419 (1955).
[30] H. ERDTMAN and E. RENNERFELT, *Svensk Papperstid.* **47**, 45 (1944).
[31] E. RENNERFELT, *Svensk Botanisk Tidskrift* **39**, 311 (1945).
[32] E. W. B. DA COSTA, Personal communication.
[33] H. LYR, *Enzymologia* **23**, 231 (1961).
[34] W. E. HILLIS, *Phytochem.* **5**, 541 (1966).
[35] W. E. HILLIS and K. ISOI, *Phytochem.* **4**, 905 (1965).

from the same growth rings but as far away as possible from the affected regions. Two other lots of '*Sirex*-affected' sapwood samples were collected from a 4- and an 8-year-old tree growing in the Pittwater plantation, Tasmania. These trees were more severely attacked than the Victorian ones, but subsequent growth rate was not appreciably affected. All *Sirex*-affected sapwood samples were clearly defined, resinous and blue fluorescent under u.v. light, with a size of about $0{\cdot}5 \times 0{\cdot}7 \times 3$ cm vertically.

The 'damaged' sapwood samples were collected from two deformed trees in the Pittwater plantation. Both trees had been damaged by an undefined agency and the relevant areas were cut from the trees. The damage extended over a vertical length of more than 60 cm and had occurred when that portion of the trees was 4 yr old and 6 cm in diameter. More than 75 per cent of the cambial region in a cross-section had been killed or badly damaged and in the following 4 yr the wood cells formed by the remaining patches of cambium had overgrown part of the trunk to form vertical ribs about 3·5 cm wide. Between the damaged cambium and the pith a continuous diffuse blue fluorescent band, with yellow fluorescent edges, occupied the outer two growth rings. The band had the same shape over a distance of more than 20 cm. Some of the fungal hyphae present might be those of *Amylostereum* spp. but identification was not possible.[32] The area was less resinous than *Sirex*-affected sapwood and only a few oviposition tunnels could be found. It is possible that fungal infection occurred after the cambium had been damaged.

The two outer heartwood growth rings were obtained from cross-sections of a 25–30-year-old tree in which heartwood occupied nine growth rings. The knotwood was obtained from knots in the inner 3–10 growth rings of the same cross-section.

Chromatographic Examination

All samples were examined by the following methods. Two-dimensional paper chromatograms were prepared using first, *n*-butanol:acetic acid:water (6:1:2, 'BAW') then 6 per cent acetic acid ('6 HOAc'). One-dimensional chromatograms were also prepared using 6 HOAc and benzene: acetic acid:water (6:7:3, 'BeAW'). The sugars were identified chromatographically with BAW, pyridine:*n*-butanol:water (3:3:1) alongside authentic sugars.

The chromatograms were examined under short (254 nm) and long (365 nm) wave u.v. light before and after exposure to ammonia vapour, and then sprayed with diazotized *p*-nitroaniline in 20 per cent sodium acetate for polyphenols or aniline phosphate[36] for sugars.

Chromatoplates of Silica Gel GF254 (E. Merck, A.-G., Darmstadt) ('SiO_2') with thicknesses of 0·25 or 0·75 mm were prepared in a constant temperature room at 20°. The following solvents were used: I, chloroform:ethyl acetate:formic acid (5:4:1); II, toluene:ethyl formate:formic acid (5:4:1); III, benzene:methanol (9:1); IV, chloroform:methyl ethyl ketone (9:1); V, chloroform:acetic acid (8:2); VI, chloroform:ethyl acetate (9:1).

Examination of Pinus radiata *Sapwood*

Air-dried shavings (3·6 kg) from logs of unattacked sapwood were extracted with acetone and then methanol.

The concentrated acetone extracts (1·4 per cent) were mixed in a large volume of ether and the insoluble material removed. The ethereal solution was extracted with aqueous KOH (5 per cent) and after washing with water, it was dried (Na_2SO_4) and evaporated to leave the neutral constituents (0·9 per cent). The aqueous alkaline solution and washings were acidified, extracted with ether and the residue (0·5 per cent) from the evaporated ether resolved into acids and phenols by counter-current distribution by the method of von Rudloff and Sato[37] using 70 per cent aqueous ethanol equilibrated with petrol (b.p. 60°–70°), and a 30 tube apparatus. The phenolic portion (21 per cent) of the residue remained in the aqueous layer of the first 3 tubes and most of the fraction moved with the upper phase to the end 7 tubes.

Phenolic Fraction from Unattacked Sapwood of P. radiata

The phenolic fraction (3·27 g) from the countercurrent apparatus was concentrated and extracted with ether to leave residue A (0·81 g). The evaporated ether extract was extracted with heptane to remove a yellow viscous mixture B (0·26 g). The heptane insoluble fraction was extracted with hot water to remove a soluble fraction C (0·47 g) and leave a brown residue D (1·72 g). Phenolic materials were absent from residue A. The heptane soluble fraction B was separated on Whatman No. 3 papers using 6 HOAc into bands I (origin–VIII (R_f 0·75–1·0). The methanol extracts from bands I and II (R_f 0–0·075) were further separated on thick layers of SiO_2 using solvent III, and the resultant blue fluorescent band extracted with methanol. The evaporated extract was resolved again using solvent IV, and the methanol extract of the blue fluorescent band was shown chromatographically to contain pinosylvin monomethyl ether. Pinosylvin was absent. Band III (R_f 0·075–0·22) contained an orange yellow fluorescent component R_f 0·85/0·17 (BAW/6 HOAc). Band

[36] A. S. F. ASH and T. M. REYNOLDS, *Australian J. Biol. Sci.* **7**, 435 (1954).
[37] E. VON RUDLOFF and A. SATO, *Can. J. Chem.* **41**, 2165 (1963).

IV–VI (R_f 0·22–0·55) yielded very little material. Band VII (R_f 0·55–0·75) contained salicylic and vanillic acids and vanillin.

The water-soluble fraction C was separated on Whatman No. 3 papers using 6 HOAc into Bands 1 (origin)–11 (R_f 0·75–0·9) on the basis of different colours under u.v. light. Bands 1, 2 and 4 yielded only polymeric material. Band 3 (R_f 0·075–0·15) contained a distinctive orange yellow fluorescent component (R_f 0·85/0·17 BAW/6 HOAc) identical with that in Band III of fraction B. Band 6 (R_f 0·35–0·45) and Band 7 (R_f 0·45–0·5) yielded ferulic and caffeic acids and traces of an unknown compound and Band 8 (R_f 0·5–0·6) protocatechuic acid and traces of an unknown compound. Band 9 (R_f 0·6–0·72) contained vanillin, protocatechuic, *p*-hydroxybenzoic and ferulic acids, hydroquinone and an unknown compound. Band 10 (R_f 0·70–0·75) and Band 11 (0·75–0·90) contained protocatechuic acid, vanillin and hydroquinone. Bands 9–11 were also resolved on Whatman No. 3 paper using BeAW and colourless needles (m.p. and mixed m.p. 169–171°) of hydroquinone were obtained from the extract of the appropriate band.

The brown residue D contained resinous materials and a trace of vanillic acid.

Fractionation of Extracts from Different Tissues

Samples of unaffected, damaged and *Sirex*-affected sapwood, heartwood and knotwood (30–100 g) were finely ground (−60 mesh), air dried and extracted in a soxhlet-type apparatus with acetone for 20 hr and then methanol for 12 hr.

To the concentrated acetone extract a large volume (× 15) of ether was slowly added and after removal of the ether-insoluble portion, the ether solution was extracted successively with water, (three times), saturated $NaHCO_3$ solution, (four times), saturated Na_2CO_3 solution (four times) and finally 4 per cent NaOH (six times). Three layers were formed by the latter extractant—an upper ether, middle oily and lower aqueous layers. Immediately after separation the alkali extracts were extracted with ether to remove unreacted material, acidified and again extracted with ether. The latter ether extracts were dried (Na_2SO_4) and evaporated. The ether solutions (neutral fraction) remaining after alkali extraction were washed with water, dried and evaporated.

The acetone extracts of the *Sirex*-affected tissues from Tasmania were very similar to those from Victorian trees.

The damaged sapwood extract behaved differently in that a gelatinous sludge formed on alkali extraction and it was insoluble in ether and alkali. In addition, the NaOH extract after acidification and ether extraction was dark coloured. These two materials probably account for the low recovery of the different fractions.

Isolation of Components from the Acetone Extracts

Pinosylvin and its methyl ether were obtained from the 'aqueous' layer of the alkali extracted material by streaking on thick layers (0·75 mm) of SiO_2 and developing with solvents III or IV. The blue fluorescent bands were cut out quickly after development, extracted with methanol and the extracts purified in the same way but using the alternate solvent. The stilbenes were identified spectrally and chromatographically, and pinosylvin was recrystallized from benzene as scaly crystals m.p. 153–154° and its methyl ether from benzene m.p. and mixed m.p. 119–120°. Pinosylvin monomethyl ether was also present in the middle oily layer of the sodium hydroxide extract.

Pinobanksin was largely found in the sodium carbonate extract of the heartwood and knotwood extracts with smaller amounts in the aqueous layer of the sodium hydroxide extract and traces in the sodium bicarbonate extract. Pinocembrin was very largely found in the aqueous layer of the sodium hydroxide extract. Both were separated on thick layers of SiO_2 using solvents III or IV. The compounds were recrystallized from toluene with m.p. and mixed m.p. of 174–176° and 197–199° respectively.

Vanillin was found in the sodium carbonate extracts and hydroquinone in the sodium bicarbonate extracts.

'Compound X' (Table 3) was separated from the sodium bicarbonate extract of the heartwood and knotwood by using solvent III and thick layers of SiO_2 on which it had a yellow colour under u.v. After heating with 2 N hydrochloric acid in a sealed tube at 100° for 1·5 hr, no change in R_f values was observed. It gave an orange-brown with diazotized *p*-nitroaniline but no colour after spraying with sodium borohydride followed by exposure to HCl vapour.

The oily middle layers obtained on extraction with 4 per cent NaOH, was neutralized and repeatedly extracted with cold petrol (60–80°) to remove an almost colourless resinous material, leaving a small amount of reddish brown residue. The residues from all tissues except the sapwood contained pinosylvin and its methyl ether. Almost all the resinous material had R_f values at about 0·40 and 0·26 in Solvents III and IV respectively.

Methanol Extracts

The evaporated extracts were extracted with hot water which removed glucose and arabinose, identified chromatographically. Glucose was present in much greater amounts than arabinose in the unaffected and *Sirex*-affected sapwoods and present in equal amounts in the other tissues.

Chromatographic examination of the aqueous insoluble methanol soluble extracts showed that traces of pinosylvin and its ether was present in all extracts (except that of sapwood) and in addition pinobanksin and

pinocembrin in the heartwood and knotwood extracts. Small amounts of an unidentified compound were present in all extracts with R_f 0·86 (6HA) and 0·15 (BeAW).

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