WOOD TANNINS — ISOLATION AND SIGNIFICANCE IN HOST RESISTANCE TO *VERTICILLIUM* WILT DISEASE*

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Apricot trees often recover from the "black heart" disease incited by the fungus *Verticillium albo-atrum* Reinke & Berthold, and a characteristic of such resistant trees is that the infected wood becomes dark brown to black in colour (Dufrenoy and Dufrenoy 1927). The fungus dies 1–6 months after it has colonized the wood (Taylor 1963). Analysis of total phenolics by the Folin–Denis assay showed an approximate fivefold increase (to about 60 mg/g wood) compared with that of uninfected wood of the same branch, and suggested their involvement in disease resistance mechanisms.

Discoloration of infected plant tissue has been widely attributed to the accumulation of tannins and other phenolics as a resistance response to the pathogen (Farkas and Kiràly 1962; Rubin and Artsikhovskaya 1964). The toxicity of tannins to many microorganisms is thought to be due to their capacity to form complexes with proteins, and consequently to inhibit enzymes (Byrde, Fielding, and Williams 1960; Goldstein and Swain 1965). The latter has been well demonstrated, although most of the investigations have necessarily involved the use of crude tannin extracts or artifically produced polymers (Goldstein and Swain 1965). Also, as the latter authors have pointed out, most polymeric substrates are themselves capable of binding tannins, and the presence of such materials in the bioassay media used by some investigators reduces the value of their findings. Until recently, no means were available for isolation and fractionation of total phenolic constituents, so that there has been no satisfactory demonstration of interaction between a plant pathogen and total extractable phenolics of diseased tissue. A gel-filtration procedure developed for the isolation of wine tannins (Somers 1966) and subsequently modified for the resolution of total phenolic constituents of grape pigment (Somers 1967) has now been applied to the isolation and partial fractionation of apricot wood phenolics. The activities of the various fractions against a fungus pathogen have been assayed in liquid medium.

Extraction and Fractionation of Total Phenolics

Ground shavings (2·0 g) from diseased apricot wood were extracted at room temperature by soaking successively twice in methanol (20 ml, 1 hr) and twice in 50% aqueous methanol (20 ml, 1 hr). Total phenolics were precipitated from the combined extracts by addition of saturated neutral lead acetate solution (4 ml) and adjustment to pH 8·5 with ammonia (sp. gr. 0·880). The yellow precipitate was

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centrifuged, washed once with water, and the lead complex broken by treatment with methanolic 1·0% HCl (40 ml). After removal of lead chloride by centrifugation and washing once with methanol, the combined solution was evaporated to dryness below 35°C. No loss of phenolics during such treatment was indicated by Folin–Denis analyses.

Fractionation of wood phenolics by gel-filtration and quantitative recovery was achieved by use of Sephadex G-25 Fine in 50% aqueous acetone. A column 2·0 by 35 cm was prepared after allowing the gel to swell in this solvent for several days. The extract (from 2·0 g discoloured wood) was dissolved in 50% aqueous acetone (1·0 ml) and applied to the column. Elution with the same solvent, at about 20 ml/hr, gave an intense dark brown band (effluent volume 52 ml, Fig. 1) followed by a lighter brown to yellow trail. Because of the opacity of acetone in the ultraviolet, the elution curve (Fig. 1) was obtained by absorbance measurements, in a 1 mm cell, at 450 mμ. Total colour was eluted from the column within 60 ml and after a volume of 105 ml had been eluted the effluent contained no phenolic material.

The eluants were combined to make fractions A, B, C, and D (Fig. 1), and each was evaporated to dryness below 35°C. The respective weights, from 2·0 g wood, were 49, 24, 22, and 48 mg. All four fractions had absorption maxima (in ethanol) at about 280 mμ, and each gave strong positive reactions with the "leucoanthocyanidin reagent" (Swain and Hillis 1959). A sample of each fraction was examined by two dimensional chromatography in 6% aqueous acetic acid and n-butanol–acetic acid–water (6 : 1 : 2 v/v). The chromatograms were inspected in daylight and in ultraviolet light before and after exposure to ammonia vapour. Fractions A and B were largely immobile with brown trails from the origin in the first solvent, and no discrete spots were seen. Fractions C and D each gave lighter trails from the origin, with 7 and 12 fluorescing spots respectively.

The chromatographic behaviour of the above fractions showed that gel-filtration of wood tannins had occurred on the column with, however, considerable adsorptive tailing of the tannins. This was confirmed by separate treatment of each fraction on the same column. Fractions A and B then emerged in identical elution volumes (of 52 ml) with little other material present in either one. Fractions C and D each contained relatively small quantities of the dark tannin (elution volume 52 ml), and the major portions were eluted in effluent of progressively decreasing colour up to 105 ml as before (Fig. 1). By combination of appropriate fractions and one further gel filtration of each, the total wood phenolics were divided into a "main" tannin fraction (dark brown), which was excluded from the gel, an "intermediate" tannin fraction (light brown), and a largely monomer fraction (pale yellow), as judged by chromatography and behaviour on the gel column. The proportions of each were approximately 7 : 2 : 3 by weight.

Microanalysis of the "main" tannin fraction gave the following percentages: C, 58·8; H, 6·0; O, 34·8%. The absorption spectrum of this fraction, in ethanol, had a peak at 282 mμ ($E^{1\%}_{1\,cm}$, 154) with a smaller less definite peak at 450 mμ ($E^{1\%}_{1\,cm}$, 14·5). Both tannin fractions precipitated gelatin from 1% aqueous solution, whereas the monomer fraction did not.

Bioassay Procedure

Bioassays of phenolics fractions from infected apricot wood against *V. albo-atrum* were conducted aseptically in Petri dishes of 4 cm diameter by 1 cm, in the lids of which a 1 mm hole had been eccentrically drilled. Each dish was placed on a 9 cm filter paper disk within a 9 cm Petri dish. The test materials were each dissolved in an aqueous medium (0·5 g KCl, 0·5 g $MgSO_4$, 1·0 g KH_2PO_4, 2·0 g $NaNO_3$, and 10·0 g sucrose in 5 litres of distilled water) to give a concentration of 0·5 mg/ml. The test solution (1·5 ml) was injected by hypodermic syringe into the smaller dish through the hole in its lid, sterilization being effected by use of a Millipore filter (GS) in a Swinny adapter. A spore suspension in aqueous medium

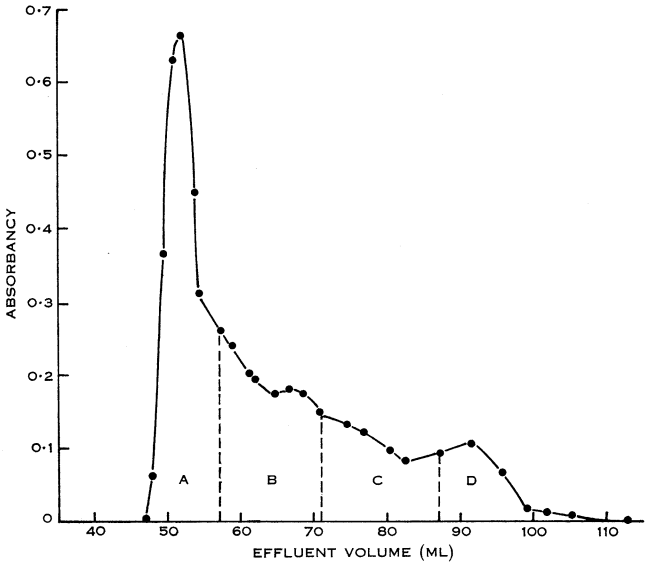

Fig. 1.—Elution curve for apricot wood phenolics from Sephadex G-25 in 50% aqueous acetone. Absorbance measurements were made at 450 mμ in a 1 mm cell.

was prepared by flooding the surface of a 2-weeks-old culture of a microscelerotial isolate of *V. albo-atrum* from apricot on yeast agar (the above solutes plus 0·25 g Difco yeast extract and 10 g agar in 1 litre tap water). Approximately 500 spores were added by injection in 0·05 ml to each assay dish. A humid atmosphere was created in the outer Petri dish by addition of distilled water (1 ml) to the filter paper. After incubation at 22·5±1°C for 24 hr, the spores were killed by the addition of methanol (0·2 ml) to each dish, and a few drops of cotton blue in lactophenol added to stain the hyphae and spores. Measurements of the total length of hyphae produced by each spore were made for 200 spores per treatment under ×570 magnification. Most of the spores with hyphae not exceeding 200 μ in length rested on the bottom of the dish, facilitating measurements.

Results and Discussion

Preliminary precipitation of total phenolics with basic lead acetate, which has long been used for such purposes, again seemed necessary in this investigation because of uncertainty as to the nature of materials in the crude extract, and the desire to exclude non-phenolics from the gel-filtration system. However, a similar gel-filtration curve to Figure 1 was obtained without prior use of lead acetate, indicating that the treatment had produced no gross changes in the phenolic content of the extract.* Although the possibility of changes in the tannins during their isolation cannot so easily be discounted, there was no evidence of chemical instability in the samples obtained. These, in contrast to the monomer fraction, which was a buff-coloured powder, were brittle brown materials which appeared to have consistent chromatographic and gel-filtration properties.

TABLE 1

INTERACTION BETWEEN FRACTIONS OF TOTAL EXTRACTABLE WOOD PHENOLICS AND *V. ALBO-ATRUM*

Fraction (concn. 0·5 mg/ml)	Average Length of Hyphae Produced by Spores (μ)	Percentage Germination of Spores
Control	137	99
"Main" tannin	6** } *	63**
"Intermediate" tannin	87** } *	96**
Monomer	152	99·5

* Mean values for main and intermediate tannins are significantly different from each other at 5% level.

** Significantly different from control at 1% level.

Although all phenolic eluates from the gel column (to 105 ml, Fig. 1), which together yielded 143 mg material from 2·0 g diseased wood, were coloured, a colourless non-phenolic fraction (37 mg), which was not entirely organic, was eluted from 105 to 130 ml. However, the chromatographic and other data suggested that the coloured fractions were entirely phenolic, the tannins containing condensed pro-anthocyanidin structures. Some measure of the probable chemical heterogeneity of the tannins was indicated by the large number of constituent phenolics in the accompanying monomer fraction.

The "main" tannin fraction is considered to have molecular weights greater than 2000, this value being based on the swelling of Sephadex G-25 in 50% aqueous acetone relative to that in water in which the exclusion limit (for polysaccharides) is about 4000. No better estimate of minimum molecular weights of this heterogeneous polymer fraction is at present possible, but the "intermediate" tannin would, in any case, certainly be of smaller average molecular size as it was not excluded from the gel.

* Subsequent to the work described, it has been found, in the analysis of grape and wine phenolics by similar gel-filtration, that the lead precipitation can be omitted without detectable qualitative or quantitative changes in the tannin fractions thus isolated. Non-phenolics are apparently excluded by the solvents used in preparation and analysis of the extract.

Most of the unidentified constituents of the monomer fraction could be expected to have molecular weights in the range of 200–500.

As noted previously, the absence of polymeric substrate material in the bioassay medium is an important feature of the method used to obtain a comparative measure of interaction between the pathogen and the various fractions of total extractable wood phenolics. The results presented in Table 1, together with the relative gel-filtration and chromatographic properties of the three fractions, indicate that activity against the fungus is a function of molecular size of the polyphenols. The "main" tannin fraction, of greatest average molecular size, was markedly inhibitory to spore germination and to hyphal growth of *V. albo-atrum* at 0·5 mg/l, whereas the monomer fraction was ineffective at this concentration.

It is perhaps significant that inhibition of spore germination and germ tube growth by tannins has been demonstrated at a dilution level well below that actually present in the infected wood. Much of the tannin which accumulates may not be available for interaction with the fungus due to adsorption on cell wall components, or to distribution within arbitrary compartments of the host cell structure.

Although there is much evidence that polyphenolics are involved in disease resistance in plants, the importance of the molecular size of these heterogeneous materials has not previously been demonstrated in this fashion. Farkas and Ledingham (1959) have shown that short-term *in vitro* oxidation of certain phenolics having low toxicity to fungal spores can cause striking increases in toxicity. Our results suggest that high molecular weight tannins, probably formed *in situ* by oxidative condensation of monomeric precursors, are of primary significance in plant resistance to wilt disease, and lend support to the theory of their general involvement in disease resistance mechanisms.

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