THE FORMATION OF POLYPHENOLS IN SMALL BLOCKS OF PINUS RADIATA SAPWOOD WITH AND WITHOUT THE FUNGAL SYMBIONT OF SIREX

by M.P. Coutts*

Summary

Small blocks of sapwood cut from the outer growth ring of Pinus radiata were induced to form polyphenols under controlled humidity and other conditions. The formation of polyphenols was detected histochemically and chromatographically. Exposure of blocks to air at high humidity favoured polyphenol formation. After a few days, the (apparently extracellular) formation of polyphenols in tracheids around resin ducts was followed by intracellular formation of polyphenols in the cells of the ducts and in medullary rays. After two weeks, pinosylvin was the chief polyphenol detected. Inoculation of wood blocks with Amylostereum areolatum (Fries)Boidin, a white rot fungus which is injected into trees by Sirex noctilio while ovipositing, had no stimulating effect on polyphenol in these preliminary experiments.

* * *

Introduction

The two stilbenes, pinosylvin (PS) and pinosylvin monomethyl ether (PSM) were extracted from Pinus sylvestris heartwood and described by Erdtman (1939). Rennerfelt (1943, 1945) has shown that these substances are toxic to wood rotting fungi, and that they account in large measure for the durability of pine heartwood. Jorgensen (1961) found PS and PSM in heartwood of P. resinosa, and, as they were also formed in the sapwood in response to wounding, fungus attack, and desiccation, giving rise to "protection wood", Jorgensen considered that heartwood and protection wood might arise from similar stimuli, such as desiccation or aeration.

In P. radiata zones containing polyphenols form in response to the white-rot fungus Amylostereum areolatum (Fries) Boidin which is injected during oviposition by the wood wasp, Sirex noctilio (Coutts and Dolezal, 1966). Hillis and Inoue (1968) analysed the polyphenols from these zones, and found that PS and PSM were present, the ratio of PS to PSM being greater than that found in heartwood. The heartwood of P. radiata contained the flavonoids, pinocembrin and pinobanksin, in addition to PS and PSM, suggesting that different stimuli might be involved in the formation of heartwood and protection wood. Sapwood

*Formerly at Forest Research Institute, Hobart, Tas.
Aust. For. Res. Vol. 4(1): 29-34

contained traces of PSM, but did not contain the other heartwood polyphenols.

Both PS and PSM have been found to be fungistatic to A. areolatum in nutrient agar at a concentration of 25 p.p.m., PS being more effective than PSM. Although tree resistance to Sirex attack appears to depend on a number of factors, the rate of polyphenol formation is likely to be important. It is well known that vigorous trees are generally more resistant to Sirex attack than suppressed trees, but nothing is known about the relative ability of individual trees in a stand to synthesise PS and PSM. Supply of substrate or triggering substances or general metabolic rate may all be important. The fungus may cause the tree to produce polyphenols by chemical stimuli or by its strong desiccating effect on the sapwood. To investigate these problems, techniques are being developed to induce polyphenol formation under controlled conditions. This account presents the progress of preliminary work with small blocks of P. radiata sapwood.

Method

Blocks of fresh sapwood were cut from the outer growth ring of four vigorous trees felled in July and August. They were taken from about half tree height, and were cut in such a way as to avoid needle traces, which contain quantities of phenolic material. For seven of the eight treatments the blocks were surface-sterilised by dipping in 10% sodium hypochlorite; in the other case the blocks were autoclaved.

The treatments were as follows:-

(1) Blocks about 1 cm x 2 cm x 2 cm were placed on 2% malt agar against the growing margin of A. areolatum culture in closed Petri dishes in an incubator at 24°C.

(2) Similar but unsterilised blocks were autoclaved and treated as in (1).

(3) Similar blocks were treated as in (1) but without any fungus culture on the agar.

(4) Similar blocks were placed in closed Petri dishes in an incubator at 24°C and a 6 mm diameter disc of A. areolatum culture on 2% malt agar was placed on the morphologically-outer, tangential surface of each block.

(5) Similar blocks were treated as in (4) but without any fungus on the agar discs.

(6) Blocks of 1 cm cube were placed with their outer, tangential surfaces resting on a layer of 2% glucose agar in closed Petri dishes in an incubator at 24°C.

(7) 20 blocks of 1 cm cube and 50 blocks measuring 1 cm radially and

longitudinally and 1 mm tangentially were placed in desiccators over water and KOH solutions to maintain relative humidity at the following theoretical levels:- 100%, 95%, 90%, 75% and 40%. The desiccators were kept on the laboratory bench and covered with aluminium foil to reduce the effects of changes in the ambient temperature, but in the large desiccators used it is likely that actual humidities varied with the changing temperature of the laboratory.

(8) Blocks of 1 cm cube were treated as in (7) with an atmosphere of oxygen in the desiccator and a theoretical humidity of 95%.

At the time when the trees were felled some blocks were cut into 25 micron thick radial, tangential and transverse sections on a sliding microtome and examined for polyphenols and the presence of fungal hyphae. At intervals thereafter sample blocks from each treatment were similarly sectioned and examined. Polyphenols were stained with diazotised benzidine, made to the recipe of Koch and Krieg (1938), and fungal hyphae with lactophenol-cotton blue and starch with iodine-potassium iodide.

For the extraction and chromatography of polyphenols some of the thin blocks 1 cm x 1 cm x 1 mm used in treatment 7 were, as soon as they had been prepared, separated into inner and outer parts by trimming off with a scalpel a 1 mm layer from the four narrow surfaces. This outer part and the remaining inner part of the blocks were kept separate, quickly dried under a warm fan, and then ground to pass a 40 mesh sieve. Treated blocks, which had been kept for two weeks at relative humidities of 100%, 95% and 90%, were similarly prepared. Acetone extracts of 1 gm samples of the ground wood were concentrated, then made up in acetone to 1 ml. Chromatograms of .05 ml and 0.1 ml of this extract were run in Lindstedts' (1950) standard solvent, a water saturated mixture of benzene and ligroin containing traces of methanol. The remaining extract was concentrated to a few drops, which were also chromatographed. After drying, the chromatograms were examined in ultraviolet light and then treated with diazotized benzidine. A mixture of PS, PSM, pinobanksin and pinocembrin from P. radiata heartwood was chromatographed simultaneously.

Results

General. At the time of felling, polyphenols were detected in only 1 to 8 percent of the medullary rays of the four trees used. Starch was abundant in parenchyma cells.

Treatments 1 to 6. Blocks in Petri dishes. All blocks on agar remained very wet. After 12 to 13 days, A. areolatum had grown only 2 mm longitudinally and about 1 mm radially and tangentially into the surface-sterilised blocks, although at this stage the fungus had completely overgrown the blocks. It had penetrated the autoclaved blocks to about the same distance. After 25 days it had penetrated the surface-sterilised blocks 2 to 3 mm, and the autoclaved blocks 5 to 6 mm, longitudinally. Radial and tangential penetration was the

same as at 12 days. At 12 days polyphenols were present in very small amounts in medullary rays in the outer 1 to 2 mm of the surface-sterilized blocks, except on the side resting on the agar.

Blocks on both glucose and malt agar without fungus produced polyphenols in amounts similar to those in the inoculated blocks.

Blocks in Petri dishes without a layer of agar had dried out around the edges, so that, when cut through the middle, they appeared white except for a moist part in the centre. Those which had been inoculated with Amylostereum discs contained no polyphenols, or traces only, after four weeks, but in those which had not been inoculated, orange-staining polyphenols had formed in 40 to 60 percent of medullary rays in the outer, drier part, but no increase in polyphenols was apparent in the moist central part. Starch was still abundant in medullary rays in the dry outer part, but there was less in the moist inner part.

Treatments 7 and 8. Blocks in desiccators. Blocks in R.H. of 40 and 75 percent dried out quickly and no increase in polyphenols was detected histochemically. Daily examination of blocks in RH of 90, 95 and 100 percent, showed that polyphenols first appeared at day four. The tracheids around resin ducts stained pink in benzidine reagent, but epithelial cells and associated cells of the resin ducts did not stain. The intensity of the staining reaction of the tracheids increased until about day seven, by which time polyphenols were present in about 100 tracheids, as seen in transverse sections, around each longitudinal resin duct in the outer 2 to 3 mm of the blocks, but still no polyphenols could be detected in the cells of the ducts or in medullary rays. At 10 days, orange-staining polyphenols were detected in the epithelial cells of resin ducts and in medullary rays, so the blocks were approaching the condition of those which had been incubated in dry Petri dishes for four weeks (treatment 5). There was no obvious difference in the quantities of polyphenols formed between blocks kept at R.H. of 90, 95 or 100 percent, or between those kept in air or in oxygen. In a later replication, using blocks of wood from a different tree in R.H. 100%, a similar result was obtained, but the polyphenols took longer to appear, the pink-staining substance being first detected in tracheids around resin ducts after seven days.

The nature of polyphenols formed in blocks one millimeter thick. Blocks of wood 1 mm thick behaved like the outer 1 mm of larger blocks. On the chromatograms no PS or PSM could be found in extracts of control blocks, dried on the day the tree was felled, but PS was present in quantity in the blocks kept at 100% R.H. for two weeks. Judging by the intensity of the spots on the chromatograms, PS had formed in rather smaller amounts in blocks kept at 95 and 90% R.H. There was no apparent difference between extracts from the outer edges of these thin blocks, and the inner parts. No PSM was detected, but a substance with similar fluorescence, staining reaction, and R_F to pinobanksin, was present in small quantities, but was not characterized further.

Discussion

Jorgensen (1961) considered that slow dying of cells under the influence

of desiccation stimulated the formation of polyphenols in P. resinosa branches. In this investigation exposure of small sapwood blocks of P. radiata to air at high humidity was the most suitable of the conditions tried. No stimulation of polyphenol formation was caused by the presence of A. areolatum. Aeration seemed a more important condition than desiccation. Polyphenols did not form in blocks which remained very wet on agar, and the failure of A. areolatum to penetrate beyond a few millimetres into such blocks suggests that poor aeration was the limiting factor rather than biological activity of the blocks. The fungus also made little penetration in autoclaved blocks in contrast with the growth rate of 14 mm per day recorded in P. radiata logs (King, 1966).

In conditions of high humidity the restriction of polyphenols to the outer edges of the blocks also suggests that aeration was a controlling factor, but then it is surprising that the blocks in oxygen did not produce more polyphenols than those in air. The effects of wounding, other than of allowing exposure to air, may be important.

Since the fungus on 2% malt agar developed strongly around the blocks (particularly the surface-sterilised ones), it would appear that no diffusible, inhibiting substance was produced within the blocks.

The appearance of polyphenols in tracheids around resin ducts, at a time when none could be detected in the cells of the ducts, suggests that they were being formed extracellularly. Polyphenols always appeared around resin ducts before forming in medullary rays. Although polyphenols formed to a depth of about 4 mm in blocks drying out in Petri dishes, the pink-staining polyphenols which appeared in tracheids formed mostly in the outer 2 mm, and may thus have been stimulated by wounding rather than by desiccation. It is not known whether formation of polyphenols in a wounded tree commences at the resin ducts. The substance forming around resin ducts in the blocks may differ chemically from that which appears later inside the cells of the ducts and rays, because of its different staining reaction in benzidine reagent. On chromatograms, PS and pinobanksin stain pink or red, whereas PSM stains red-orange. Extracts for chromatography were made at a time when the orange-staining substances were present in very small amounts. Extracts made at later intervals might give interesting information on the sequence of synthesis of the various polyphenols.

Hillis and Inoue (1968) found traces of PSM in healthy P. radiata sapwood. The absence of this substance from the material extracted in this investigation may be accounted for by the fact that only the outer growth ring was used, the proportion of rays containing polyphenols being lower in the outer than in the inner growth rings; and also because needle traces, which contain a quantity of orange-staining phenolic substance, presumably PSM, were excluded.

In the preliminary experiments reported here, insufficient work was done to confirm the presence of pinobanksin in uninoculated blocks kept in high humidity. If this substance does form, it would indicate that such conditions can give rise to the synthesis of one of the two stilbenes and one of the two flavonoids which occur in P. radiata heartwood.

Although heartwood is the part of the tree most useful for the timber industry, relatively little is known about the processes leading to its formation. Similarly, little is known about the formation of protection wood. By a more varied and precise control of physical conditions than has been achieved so far; by introducing suitable chemical stimuli; and by regulating substrate, it may be possible to induce various sequences and combinations of polyphenol formations in wood blocks, and thus to gain a better understanding of these aspects of the tree's metabolism.

References

Coutts, M.P., & Dolezal, J.E. (1966) - Polyphenols and resin in the resistance mechanism of Pinus radiata attacked by the wood wasp, Sirex noctilio, and its associated fungus. Forestry and Timber Bureau, Leaflet No.101.

Erdtman, H. (1939) - Tallvedkärnans Extraktivämnen och deras inverkan på Uppslutningen enligt Sulfitmedtoden, Svensk Papperstidn. 42, 344-349.

Hillis, W.E. & Inoue, T. (1968) - The formation of polyphenols in trees - IV. The polyphenols formed in Pinus radiata after Sirex attack. Phytochem., 7, 13-22.

Jorgensen, E. (1961) - The formation of pinosylvin and its monomethyl ether in the sapwood of Pinus resinosa Ait. Can. J. Bot. 39, 1765-1772.

King, J.M. (1966) - Some aspects of the biology of the fungal symbiont of Sirex noctilio. Aust. J. Bot., 14, 25-30.

Koch, J. & Krieg, W. (1938) - Neues Verfahren zur Unterscheidung von Kern und Splint in Kiefernholz. Chem. Ztg. 62, 140-141.

Lindstedt, G. (1951) - Constituents of pine heartwood XXVI. A general discussion. Acta. Chemica. Scandinavica 5, 129-138.

Rennerfelt, E. (1943) - Die Toxizität der phenolischen Inhaltsstoffe des Kiefernkernholzes gegenuber einigen Fäulnispilzen. Svensk Botan. Tidskv. 37, 83-93.

Rennerfelt, E. (1945) - The influence of the phenolic compounds in the heartwood of Scots pine (Pinus sylvestris L.) on the growth of some decay fungi in nutrient solution. Svensk Botan. Tidskv. 39, 311-318.