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PROTEIN IN FOLIAGE LEACHATES OF *PINUS RADIATA*

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Abstract—Protein, together with polysaccharide, was identified among components of foliage leachates of *Pinus radiata*. The protein separated into two main fractions on Sephadex G-75 when eluted with Tris buffer or with sodium dodecyl sulphate. Electrophoresis of the mixture in polyacrylamide gel revealed seven protein-staining bands, while cellulose chromatography using acidic and basic solvents separated the protein into ten ninhydrin-reactive compounds. Acid-catalysed hydrolysis released fourteen amino acids, together with galactose, mannose, fucose, and galacturonic acid as the main monosaccharides.

INTRODUCTION

The loss of substances from plant parts by leaching with water is a well-documented phenomenon [1]. The inorganic materials leached include all the essential minerals commonly found in plants, including the macro- and micro-elements [2–4]. Organic compounds identified in leachates include sugars, pectic substances, amino acids, carboxylic acids [5, 6], growth regulators [7, 8], alkaloids [9], and phenolic compounds [8, 10]. A polypeptide was found in leachates from bean (*Phaseolus vulgaris*) [2], and protein has been shown to be leached from clover forage upon exposure to rain [11].

The crude leachate obtained from *Pinus radiata* D. Don foliage has been shown to stimulate germination of the spores of the needle pathogen *Dothistroma pini* Hulb. [12]. In New Zealand, young *P. radiata* trees are susceptible to attack by *D. pini* [13], whereas older trees (>15 yr) show a significant resistance to the blight [14]. The degree of infection in young trees depends on duration of leaf wetness periods [15], when substances can be leached from foliage. This paper describes part of an investigation into the organic constituents in leachates of *P. radiata* foliage.

RESULTS AND DISCUSSION

The solutions obtained by briefly washing *P. radiata* twigs and foliage with water were odoriferous, suggesting the presence of terpenes, and were also found to contain polymers in addition to low-MW compounds. From 4 kg fr. wt of foliage, *ca* 1 g of crude leachate was obtained. The materials were divided into high and low MW classes by hollow fibre dialysis. Simple functional group tests on the low MW fraction showed it to contain carbohydrates, amino acids, carboxylic acids, and phenols. The high MW fraction was obtained as a slightly opaque, very viscous solution which freeze-dried to a powder. The material failed to redissolve completely in water to form a concentrated solution, but did so in the presence of a detergent (0.1% sodium dodecyl sulphate, SDS) or an alkaline buffer (Tris–HCl, 0.1 M, pH 8.6) to form a pale-brown solution. There was consistently more high MW material in leachates from old than from young trees. The proportion of high MW material in the total leachate was *ca* 2.0–3.5% for 5- and 10-yr trees, and 9.5–10.0% for 15-, 20- and 40-yr trees. The protein content of the material was *ca* 40% for the young trees and *ca* 50% for the older trees.

The IR spectra of the high MW fractions derived from all trees studied were similar. A typical spectrum showed absorptions due to hydroxyl, carboxyl OH, and amino NH groups, and carbonyl absorption bands due to carboxylic acid ester, and peptide. The UV spectrum showed absorption bands characteristic of protein [16].

The chromatographic and electrophoretic behaviour of the high MW fraction showed that it comprised several protein molecules. Gel filtration indicated two major protein fractions; the first fraction appeared immediately after the void volume, at $Ve/Vo = 1.3$, and the second at $Ve/Vo = 3.0$. The elution profiles were identical using both Tris buffer and SDS as eluants. The MW's of the two main fractions were estimated to be *ca* 200000 and 14000. While electrophoresis on cellulose acetate showed mainly a single broadened band, electrophoresis in polyacrylamide gel in the presence of SDS revealed up to seven bands. There were only small differences in the number and intensity of the bands depending on the age of the tree. The protein migrated slowly in the polyacrylamide gel when electrophoresed in buffer alone. The addition of SDS to the gel greatly facilitated the protein mobility, possibly due to the dissociation of aggregates or the altering of the charge on the molecules.

Cellulose TLC with acidic and basic solvent systems revealed ten spots after the chromatograms were sprayed with ninhydrin. The chromatograms obtained using both solvent systems had almost identical R_f-values for the separated spots, suggesting the neutral, or amphoteric, nature of the compounds.

Acid-catalysed hydrolysis of the high MW fraction, followed by cation-exchange chromatography, yielded *ca* equal weights of carbohydrate and amino acid fractions. Lipid extraction proved negative. In the carbohydrate fraction, galactose, mannose, and fucose were identified by TLC, glucose was absent. The most polar spot on the TLC corresponded to galacturonic acid. The IR spec-

trum of the carbohydrate fraction had, in addition to the OH band, one at 1710 cm^{-1} confirming the presence of uronic acid. In the basic fraction from the ion-exchange chromatography, fourteen amino acids were identified by 2D-TLC. They were (in order of increasing polarity) tryptophan, phenylalanine, leucine, tyrosine, valine, histidine, alanine, threonine, glutamic acid, glycine, serine, aspartic acid, arginine, and lysine; amino sugars were absent.

It appears that the high MW fraction in the leachates of *P. radiata* is composed of polysaccharide and protein. The polysaccharide fraction may be composed of hemicelluloses (which release mannose and galactose on hydrolysis), as well as pectic substances (polygalacturonate). It is possible that the mixture may contain glycoprotein, as the electrophoresed material reacted with both periodic acid-Schiff reagent and protein stains, and also contained mannose, galactose, and fucose, monosaccharides commonly found in glycoprotein [17].

That high MW compounds are so readily leached suggests that the leaf cuticle is very permeable cf. [18], and nutrient for fungal growth is therefore available at the needle surface during periods of wet weather. It is probable, however, that the protein in leachates may be derived partly from the pine foliage and partly from the phylloplane microflora, composed of bacteria, yeasts, and moulds [19].

EXPERIMENTAL

Foliage samples were collected during early summer from *P. radiata* trees aged 5, 10, 15, 20 and 40 yr (5 replicates per age), growing in Kaingaroa State Forest, New Zealand. All trees were growing at the edge of north-facing sites with similar soil types. Only foliage of healthy appearance was used, and it was collected from *ca* mid-height in the green crown which was exposed to full sun. Branches and twigs with needles attached were placed in a large polythene bag with 2 l. H_2O and agitated for *ca* 1 min. Care was taken to keep the cut end of the branch outside the bag. H_2O washings were filtered through Whatman GF-B to remove particulate material, and then through a membrane filter stack (8, 0.45 and 0.2 μm) to remove cellular material. The clear solns were dialysed using a hollow fibre apparatus (Amicon Model DC2, MW cut-off 10000) to separate and concentrate the high MW compounds. The concentrated soln was shell-frozen and freeze-dried to yield a light, tan powder, a portion of which was analysed for protein content [20].

Chromatography and electrophoresis. Gel filtration chromatography was carried out on columns of Sephadex G-75 and G-200 at a controlled flow rate of 0.28 ml/min. Columns were calibrated using Dextran Blue, serum albumin, and lysozyme. The fractions were analysed for protein content [20]. Electrophoresis was carried out on cellulose acetate membranes (Titan III, Helena Laboratories) at 120 V for 30 min. The samples were applied in 0.1% SDS soln, and the electrolyte was barbitone buffer, 0.1 M, pH 8.5. The bands were visualized using three staining procedures: (1) Ponceau S in 10% TCA; (2) Coomassie blue (Brilliant Blue R) in 10% TCA; (3) periodic acid-Schiff reagent [21]. Polyacrylamide gel electrophoresis was carried out on 7.5% slab gels (containing 0.1% SDS) at 120 V, 30 mA for 2 hr. The electrolyte was Tris–glycine, 0.05 M, pH 8.6. The protein was precipitated by immersion of the gel in 12.5% TCA for 30 min, and the bands were visualized by staining the gel with 0.01% Coomassie blue in 10% TCA [22]. TLC was carried out on cellulose (Eastman Chromagram Sheet) in vapour-saturated tanks using two solvent systems: (1) *n*-BuOH–HOAc–H_2O (4:1:2); (2) *n*-PrOH–25% NH_3–H_2O (8:1:1). The chromatograms were visualized using ninhydrin spray reagent.

Spectroscopy. UV spectra were recorded using H_2O solns, and IR spectra using KBr discs. λ_{max} (nm): 210, 280. ν_{max} (cm^{-1}): 3400 (hydroxyl, carboxyl OH, amino NH), 1730 (carboxylic acid ester C=O), 1660 (peptide C=O).

Acid-catalysed hydrolysis of high MW fraction. The material (10 mg) was dissolved in redist. 6 N HCl (7.5 ml) and heated in sealed tubes at 110° for 24 hr. Hydrolysate was cooled, filtered, and concentrated under vac. at 40°. Residue was dissolved in a minimum of 0.1 N HCl and transferred to a column of Dowex 50W X8. Carbohydrate fractions were eluted with H_2O, and basic fractions with 7 N NH_3. Carbohydrates were identified by TLC on cellulose and Si gel using *n*-PrOH–H_2O (4:1) and EtOAc–Py–H_2O (2:1:2, upper phase). Visualization sprays were PhOH–H_2SO_4, *p*-anisidine–HCl, and $KMnO_4$–NaOH. Amino acids were identified by 2D-TLC on cellulose using $CHCl_3$–MeOH–25% NH_3 (8:1:1), followed by PhOH–H_2O (3:1). Visualization spray was ninhydrin.

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