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THE PROCYANIDINS OF DOUGLAS FIR SEEDLINGS, CALLUS AND CELL SUSPENSION CULTURES DERIVED FROM COTYLEDONS

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Abstract—While cotyledons of Douglas fir seedlings contain only 2–3% of their dry weight as procyanidins (mainly in an insoluble form), callus cultures and cell suspension cultures derived from them contain up to 40%. About 70–85% of the procyanidins isolated from these cell suspension cultures are soluble in 70% methanol, but insoluble in ethyl acetate. They can be separated into a minimum of 4 fractions, all of which have apparent molecular weights greater than that of a tetramer. Dimers, trimers or tetramers are absent or present in only trace amounts, but large amounts of catechin, and lesser amounts of epicatechin, are found in the ethyl acetate-soluble fraction.

INTRODUCTION

Procyanidins, or condensed tannins, are polymers of catechin and/or epicatechin (and the corresponding 3,4-diols) of varying molecular weights, and are found in both gymnosperms and angiosperms [1]. An ethyl acetate-insoluble form giving rise to cyanidin upon acid hydrolysis has been recently described from bark of Douglas fir [2]. Although generally considered to be characteristic of mature rather than growing tissues [1], procyanidins have been reported to accumulate in cultures of pine and Juniper [3, 4]. Callus and cell suspension cultures derived from cotyledons of Douglas fir were observed to accumulate large quantities of ethyl acetate-insoluble procyanidins of apparent high molecular weight. Since little is known about these high molecular weight forms, these cultures have been used as a source of procyanidins for purification. Qualitative and quantitative comparisons are made with polymers found in seedling tissues from which the callus and cell suspension cultures were derived. Procyanidins of Douglas fir are easily analysed by determining the absorption at 550 nm, due to cyanidin after acid hydrolysis in *n*-BuOH, of either aqueous MeOH-soluble or -insoluble fractions.

RESULTS

Total procyanidin contents of intact seedlings

Insoluble and aqueous MeOH-soluble procyanidins were found in all parts of 4–6-week-old seedlings (shoots *ca* 6–8 cm high with an epicotyl up to 1 cm in length) (Table 1). The amounts ranged from 2 to 20% of their dry wt. The epicotyl and primary roots contained the largest amount on both a dry and a fr. wt basis. The cotyledons, on the other hand, contained the least amount of procyanidins and of total phenols. The cotyledons also differed in having the greatest percentage of their procyanidins in an insoluble form (80–100% in contrast to *ca* 20–40% for root tissue). Relatively young cotyledons (with the cotyledon whorls still held in a semi-closed 'umbrella-like' position by the seed coat) tended to contain a smaller percentage in the insoluble form. No solvent has been found that will extract these insoluble procyanidins from the residue without degradation. The bulk of the insoluble forms are not associated with the wall *in vivo* since protoplast preparations from mesophyll cells of the cotyledons (kindly supplied by Dr. Ned Kirby) also contained predominantly the insoluble form.

Table 1. Procyanidin and total phenol analysis in seedlings of Douglas fir

Tissue	Fr. wt	Procyanidins			Total phenol
	mg/unit	Total A_{550}/mg fr. wt	% Dry wt*	% MeOH-soluble	A_{650}/mg fr. wt
4-Week-old seedlings-units					
Epicotyl (cluster needles)	30.9	0.24	16	50	3.6
Cotyledons (whorl of 6–7 cotyledons)	31.3	0.03	2	0–20	2.7
Hypocotyl green†	15.1	0.19	13	62–82	3.6
Root primary	6.4	0.30	20	59–77	5.6
2-Week-old seedlings					
Cotyledons 'closed' whorl	9.5	0.11	8	30	1.7

* Based on an average dry wt of 10% and comparison with an $E^{1\%}_{550}$ of 150 (after acid hydrolysis) for a purified procyanidin preparation.

† Red hypocotyls gave similar values after subtraction of the red color due to anthocyanin prior to hydrolysis at 95°.

Although some of the higher MW forms of procyanidins in the above aqueous MeOH extracts are water-soluble, over 50% of the procyanidins of an aqueous buffered extract of cotyledons are associated with particulate fractions. When extracts of cotyledons were made in 0.1 M HEPES buffer at pH 7.5 with 0.5 M sorbitol, the procyanidins were associated with all major fractions; the cheesecloth residue plus the pellets obtained after centrifugation at 1000 and 20000 *g* contained *ca* 63% of the total, while the rest was found equally in

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Table 2. Procyanidin synthesis in basal portions of excised cotyledons incubated on agar nutrient medium with and without added hormones

Incubation time after excision	Fr. wt	Chl	Procyanidins		
	mg	A_{663}	Total A_{550}		MeOH-soluble
	/unit*		/unit*	/mg FW	%
Zero time	20.7	1.5	0.52	0.025	< 10
1 week					
− hormones	30.8	1.7	6.8	0.21	54
+ hormones†	26.5	1.9	9.3	0.36	35
2 weeks					
− hormones	40.3	1.0	7.3	0.17	34
+ hormones†	182.5	0.6	25.3	0.14	39
3 weeks					
− hormones	66	1.3	13.1	0.20	48
+ hormones†	246	0.7	50.1	0.20	52

* 1 unit = 6 basal segments 1.2 cm in length; averages of 4–6 analyses.

† 0.5 μM BAP, 5 μM NAA.

an ammonium sulfate precipitate of the supernatant (500 g/l. extract) and the final supernatant.

Procyanidins formed in excised cotyledons on nutrient agar with and without added hormones

Incubation of excised mature cotyledons in the absence of exogenous hormones increased the total procyanidins per cotyledon section *ca* 30 times over that of the zero time controls during the first 1–3 weeks of incubation (Table 2). A limited amount of growth occurred during that period (based on an increase in fr. wt), so that the increase per mg fr. wt was not as great. The increase was dependent on the separation of the cotyledons from the whorl, but there was no further increase if each cotyledon was cut into two pieces.

The addition of the hormones BAP and NAA in concentrations known to induce callus formation increased the total procyanidin content per cotyledon segment *ca* 100-fold or *ca* 4 times that of segments without added hormones (Table 2). Callus growths or swellings were macroscopically visible by two weeks of growth. The increase in procyanidins on a per cotyledon segment basis was approximately linear over a 3 week period, but on a per mg fr. wt basis was relatively constant after the first week. This indicated that the increase in procyanidins eventually kept pace with callus growth. The ratio of added hormones was not important. The varying concentrations shown previously to induce callus, callus-buds or buds [5] gave results similar to those shown in Table 2. Apparently, induction of new growth was the important factor. Both soluble and insoluble procyanidin components were increased, but the soluble increased more rapidly, so that the percentage of soluble portion changed from 10% or less to *ca* 50%. The highest values obtained represent a procyanidin content of *ca* 24% of the dry wt. Substitution of 2,4-D up to 20 mg/l. did not lessen the procyanidin content, in contrast to its effect on the accumulation of phenolics in many dicot tissues [6].

Procyanidin contents of cell suspension cultures

About 5 chloroplast-containing cell lines derived from Douglas fir cotyledons have been isolated. Data for two of these are shown in Table 3. The N.K. cell line has 2–3 times as much procyanidin as line I and actively growing cell suspension cultures contain up to 40% of their dry wt as procyanidins. This is in contrast to the 3–4% present in the original intact cotyledons, and was higher than the 3–4-week-old callus cultures from which the cell lines were obtained. None of the procyanidins appeared in the medium, indicating that these compounds were not secreted from living cells. Dead or dying cells did not release procyanidins to the medium, presumably because they were adsorbed by cellular proteins. The cell walls contained little or no lignin-like materials (based on a phloroglucinol–HCl test).

Table 3. Procyanidins in two different cell suspension cultures *ca* 3 weeks after subculture in the amino acid supplemented medium. Values are averages of duplicate samples

Cell line	Total A_{550} (Procyanidins)				
	/mg fr. wt	/chl unit*	/ml PCV†	% dry wt of cells	% MeOH-soluble
N.K.‡	0.36	73	326	28	86
I§	0.10	28	94	12	82

* $A_{550} \div A_{663}$.

† PCV = packed cell volume determined after grinding in methanol.

‡ Chl. $A_{663\,nm}$ = 0.48/10 mg fr. wt cells; dry wt = 8.5% fr. wt.

§ Chl. $A_{663\,nm}$ = 0.44/10 mg fr. wt cells; dry wt = 5.9% fr. wt.

The procyanidin contents of the N.K. cell line increased under two conditions: one was a doubling during the first week after sub-culture with a very small inoculum (less than 300 mg fr. wt). A similar dilution effect has been reported in other cultures [7]. The other was an increase per flask during the subsequent growth of the culture. A relatively constant ratio of procyanidin content based on fr. wt, chlorophyll content or PCV was obtained after 3 weeks. The cultures of the N.K. line grew relatively slowly, with only *ca* a two-fold increase in dry or fr. wt per week. This was similar to that reported by Durzan *et al.* for spruce cultures [4]. The growth pattern tended to be linear during the first 3 weeks. There did not seem to be any further increase in procyanidin content upon cessation of net growth of a culture as in the case of many dicot preparations [8]. Inorganic nitrogen did not appear to limit growth nor did additional nitrates decrease the accumulation of tannins. Organic nitrogen, on the other hand, did limit growth, but the additional amino acid supplement did not alter the amount of procyanidins per g fr. wt. As in the case of callus cultures, the amounts or ratios of BAP to NAA had little apparent effect on the procyanidin content since changes in ratio of BAP to NAA from 10:1 to 1:10 had no detectable effect. Although the procyanidin content increased upon growth, it is not known whether recently divided and elongating cells produced the tannins or whether only subsequently differentiating cells accumulated these phenolics. The cell line cultures are not homogeneous, and a tendency toward changes in chlorophyll content and the degree of aggregation have been noticed.

Microscopic examination

Although the N.K. cell line was a darker green color than that of line I, the chlorophyll contents per g fr. wt were similar. There were at least two cell types in the

N.K. line in terms of numbers and sizes of chloroplasts and therefore greenness per cell. Aggregates were present, plus hyphal-like files of cells 'free' or as projections radiating from the aggregates. Some small brown cytoplasmic vacuoles or vesicles were generally present, and occasionally brown pigments filled the large central vacuole. These are probably oxidation products of procyanidins, since the isolated procyanidins themselves are almost colorless with no detectable absorption beyond the 280 nm peak. It is not clear whether healthy cells contain oxidized or brown pigmented vesicles. Poorly growing cultures showed a great increase in the number of these brown deposits, but the cause and effect relationships between cessation of growth and browning of cells are not known.

Spectral and chromatographic characteristics of the major procyanidins isolated from cell suspension cultures

Two-dimensional PC of a 70% methanolic extract in BAW and 5% HOAc solvents indicated the presence of 4–5 major phenolic compounds detectable by the blue color of the $FeCl_3$–$K_3Fe(CN)_6$ spray. The major component was a procyanidin (R_f in BAW of 0 and a streak from 0 to 0.5 in 5% HOAc). Catechin was the major monomer; epicatechin was present in *ca* 1/4 the amount. The other two phenolic compounds have not been identified.

The EtOAc-soluble fraction contained the catechin, epicatechin, and *ca* 3% of the total methanol-soluble procyanidins. The latter migrated to an R_f value of 0.32 in BAW on TLC with cellulose. The remaining 97% of the MeOH-soluble procyanidins were in the lyophilized powder obtained from the non-EtOAc soluble fraction. R_f values for the major procyanidin in BAW on TLC-cellulose were 0, and for the two minor bands 0.32 and 0.12. About 1–5% of the procyanidin at R_f 0 could be recovered as a white or tan powder after precipitation by EtOAc from a MeOH-soluble fraction of the lyophilized powder [2]. The UV spectrum of this major procyanidin was similar to that for catechin, with a long wavelength peak at 280 nm ($E^{1\%}_{280}$ of *ca* 42). This was considerably lower than the value of 260 reported by Porter for *Pinus* bark procyanidins [10]. Since the Douglas fir product showed no detectable absorption in the visible part of the spectrum, in contrast to the sample of Porter, this lower $E^{1\%}$ value may be interpreted as an indication of a purer or less modified product. The $E^{1\%}_{550}$ extinction value for the acid hydrolysis product, cyanidin, was *ca* 150. No extinction values were reported by Karchesy *et al.* for the Douglas fir bark procyanidin [2]. A similar product, in terms of its spectral and chromatographic properties, was found in a fraction that was insoluble in 100% MeOH, but soluble in 70% MeOH; this represented approximately 25% of the total procyanidin of the 70% MeOH extract. The remaining procyanidin of the lyophilized product (*ca* 69%) remained in the supernatant of the MeOH-soluble fraction after precipitation of the purest procyanidin fraction with EtOAc. All the three fractions derived from the lyophilized powder contained the same major type of procyanidin that did not migrate in BAW on PC or TLC-cellulose. Similar R_f values were reported for hexameric procyanidins [11]. Severe streaking occurred in either 5% HOAc (from 0 to 0.5) or EtOH–HOAc–H_2O (from 0.4 to 0.8). These non-migrating, presumably high MW forms could be separated into at least two major solubility groups via HPLC. With a reversed phase C_{18}-μ Bondapak column, they were eluted at 1.8 (V_0) and 2.5 ml with MeOH–H_2O (70:30) (unpublished data). The aqueous MeOH-soluble procyanidins, therefore, could be separated into a minimum of 4 forms.

DISCUSSION

A minimum of 4 soluble forms of procyanidins have been isolated from cell suspension cultures of Douglas fir; three forms were separated by PC, while the major one of these was further resolved into two peaks by HPLC. The major forms may be similar to those isolated from bark tissue by Karchesy *et al.* [2] who reported them to consist of catechin and epicatechin units in an undetermined sequence. The major forms appear to have MWs greater than that of a tetramer. The relative scarcity of the lower MW and EtOAc-soluble forms studied by Haslam is surprising, especially if the polymers are mainly formed non-enzymatically as postulated [9, 12].

The aqueous MeOH-insoluble forms of procyanidins, ranging from 80–100% of the total in the intact cotyledons to 20–40% in callus or cell suspension cultures could be due to precipitation occurring during isolation procedures; e.g. upon grinding in aqueous MeOH or upon release of vesicular or vacuolar constituents in aqueous buffer extracts. Alternatively, the insoluble forms might be present *in vivo* and characteristic of fully matured tissues, possibly as a secondary modification. Since the roots, hypocotyl and epicotyl still have active meristems, the procyanidins may remain more soluble in these tissues. Bate-Smith has studied a variety of plants which have varying amounts of insoluble procyanidins [13–15]. An *in vivo* attachment to insoluble proteins or carbohydrates might be expected because tannins appear to be compartmented in vesicles, possibly even when they are in the large central vacuole [16, 17]. Since protoplast preparations from cotyledons contained similar high concentrations of the insoluble forms, the procyanidins do not appear to be major constituents of cell walls. The data presented do not distinguish between the above two possibilities.

No quantitative data for $E^{1\%}_{550}$ (after acid hydrolysis) were presented for the isolated Douglas fir bark procyanidins [2]. Condensed tannins isolated from *Pinus* in 50% acetone were claimed to have a MW of 25000 to 35000, and the reported $E^{1\%}_{280}$ value of 260 was 6 times that observed here for the EtOAc precipitable form. In addition, the *Pinus* preparations were red-brown with considerable absorption at 440 and 550 nm in non-hydrolyzed extracts [10]. Presumably considerable secondary modifications must have occurred in the bark tissue or during the preparation.

The best basis for quantitative analysis of Douglas fir procyanidin content of seedlings or tissue cultures is measurement of the absorbancy at 550 nm, due to the formation of cyanidin, upon oxidative acid hydrolysis in *n*-BuOH. Absorbancy measurements at 280 nm in crude aqueous extracts were not useful due to the presence of other interfering compounds. The use of the $A_{550\,nm}$ value, however, has several drawbacks. One is the non-linearity at high concentrations [18]. This was essentially an unavoidable disadvantage in the analysis of the insoluble procyanidins because they could not be effectively subdivided prior to hydrolysis. Values for the insoluble forms, therefore, are minimal. A second drawback is the assumed variation in amount of cyanidin

product due to varying degrees of polymerization [14, 15]. The data represent, therefore, only average values. This is true also of the $E^{1\%}_{550}$ value of 150 for the EtOAc-precipitable procyanidin used to determine the % procyanidins of the dry weight of tissue. According to Bate-Smith [14], oligomers higher than tetramers give values greater than 150, but this claim was based only on dicot procyanidins, and the values apparently decrease with a very high degree of polymerization. The EtOAc-precipitable form had similar solubility characteristics according to PC, TLC and HPLC as the major form(s) found in the original aqueous MeOH extract.

Condensed tannins, mainly procyanidins, have been detected in other gymnosperm tissue cultures, but little quantitative data have been presented [3, 4, 16, 17]. The significance of the increase in procyanidins upon induction of new growth during callus induction in cotyledon tissue is not clear. They would not be expected to be necessary for growth. There is also no proof that the procyanidins are formed in dividing or even elongating cells. Instead, they might be accumulating only in the surrounding differentiating cells [19]. The increase in the callus tissues was not just an injury response, because further cutting of the cotyledons into segments did not increase the amounts formed. Instead, the increase appeared to require new cell divisions, followed by the differentiation into special tannin synthesizing or accumulating cells. This might be expected if the physiological role of procyanidins in seedling tissue is to protect young seedlings from animal and microorganism predators [18].

EXPERIMENTAL

Douglas fir seedlings were grown for 3–4 weeks in a 1:1 mixture of peat moss and perlite at 25° under 20 klx of cool white fluorescent lamps for a 16 hr photoperiod.

Callus cultures. Cotyledon whorls, including a small epicotyl and the upper 2 mm of hypocotyl, were surface-sterilized by washing briefly in a 1% (w/v) detergent soln (Alconox), then 8% (v/v) Chlorox for 8 min. followed by several sterile H_2O rinses. The cotyledons were excised and incubated directly as intact cotyledons, or the entire whorl was preconditioned on basal medium for Douglas fir [5, 19] plus 0.8% agar without added hormones for 2–5 days prior to excision as above. Callus or bud inducing incubation mixtures contained ratios of NAA to BAP as indicated in the tables (see ref. [5]). The cultures were kept at 25° under 10 klx from fluorescent lights for a 16 hr photoperiod.

Cell suspension lines were isolated from some of the above callus cultures derived from cotyledons and were grown in the light on a gyrotary shaker at 100 rpm in 125 ml Erlenmeyer flasks containing 25 ml nutrient medium. One cell line (N.K.) was isolated by Dr. Ned Kirby in a basal salt medium for Douglas fir containing 6% sucrose, 5 μM BAP and 0.5 μM NAA. It was finally maintained in basal medium containing 3% sucrose, 0.5 μM BAP, 5 μM NAA plus 1 mM glutamine and 1 mM arginine. A second cell line (I) was isolated and maintained in the above amino acid supplemented medium. Subcultures were generally made every 3 weeks, with inocula of *ca* 500–1000 g fr. wt. Cultures were constantly selected for good growth, greenness and the smallest aggregates of cells. Fr. wt values were based on cells washed and filtered briefly under suction. Because of inaccuracies due to air spaces between aggregates, packed cell volumes (PCV) were determined after grinding in MeOH. Chlorophyll was expressed as A, ml^{-1}, cm^{-1} at 633 nm.

Soluble procyanidins of cotyledons and callus cultures could be extracted only in aq. MeOH or Me_2CO. A MeOH–H_2O (70:30) soln was routinely used and 1 ml was sufficient to extract most of the procyanidins from a 200 mg fr. wt sample with a stirring rod as a grinding tool. In order to extract the chlorophyll, it was necessary to add 1 ml 100% MeOH to the residue obtained by centrifugation. The 70 and 100% extracts were combined for analysis of the soluble procyanidins. Analytical data from such combined extracts were similar to those analysed separately. The residue after centrifugation was retained for analysis of the insoluble procanidins.

Procyanidin analyses were based on the formation of cyanidin upon acid hydrolysis [18]. A 0.1 ml aliquot containing an appropriate dilution of a 70% MeOH extract of cells or tissues plus 1 ml of BuOH–HCl reagent (5% conc HCl in *n*-BuOH, v/v) was heated in a boiling water bath (95°) in dim light for 30 min. If significant amounts of chlorophyll were present, freshly made unheated blanks containing the same aliquot were subtracted. Estimates of the MeOH-insoluble form(s) were obtained by direct hydrolysis of the insoluble residue in 1 ml BuOH–HCl reagent. The resulting mixture was diluted after hydrolysis for absorbing (A) determinations if necessary. Since the undiluted values were frequently in the non-linear range of this colorimetric assay, these values are only minimal ones. Absorbancy measurements were made at 550 nm (1 cm), the long wavelength peak of the cyanidin hydrolysis product in BuOH. Chromatographic evidence indicated that no other anthocyanidin was present. Values are expressed as $A\ ml^{-1}$. Estimates of the procyanidins as % dry wt of the tissue were made by comparison of the $E^{1\%}_{550}$ values [14] with that obtained for the purest isolated procyanidin (150) (see below).

Total phenolic contents were estimated by the reaction with the $FeCl_3$–$K_3Fe(CN)_6$ reagent. To the sample in 0.1 ml 70% methanol was added 1.5 ml of a freshly prepared soln of 1% $FeCl_3 \cdot H_2O$ (w/v) followed by 1.5 ml 1% $K_3Fe(CN)_6$. Blanks made with 0.1 ml 70% MeOH were used to zero the spectrophotometer. Readings at 650 nm were taken at 10 min (the absorption in both blank and sample curvettes increases with time). Values are expressed in arbitrary A units ml^{-1}, cm^{-1}, but a gallic acid standard was routinely made with each set of analyses. A 2 μg gallic acid standard gave an A of *ca* 0.28 ml^{-1} cm^{-1}.

Chromatography. Two-dimensional chromatography on Whatman No. 1 was done in BAW (*n*-BuOH–HOAc–H_2O, 6:1:2) followed by 5% HOAc. TLC-cellulose was developed in the above BAW mixture. Spots were detected visually under UV (260 nm) or after spraying with an equimolar mixture of a freshly made 1% mixture of $FeCl_3$–$K_3Fe(CN)_6$ reagent used in the above spectrophotometric assay. R_f values for standards on TLC-cellulose in BAW solvent were: catechin, 0.66; epicatechin, 0.60; B-2 (epicatechin) dimer, 0.55; B-3 (catechin) dimer, 0.51; trimer, 0.45 (the latter 3 procyanidins were kindly supplied by Dr. E. Haslam). For procyanidin analyses on TLC-cellulose, bands were detected under UV or arbitrary subdivisions of a plate were scraped off and hydrolysed and analysed as above with the BuOH–HCl reagent. HPLC was done with a Waters Assoc. Model 440, with a C_{18}-μ Bondapak column (4 mm × 30 cm), with 254 and 280 nm detectors.

Isolation of the major procyanidin fractions from cell suspension cultures. A 70% MeOH extract of a washed cell suspension culture was evapd *in vacuo* to remove the MeOH. The resulting aq. extract was subsequently exhaustively extracted with EtOAc, and then the EtOAc-insoluble fraction was lyophilized. The lyophilized powder was exhaustively extracted with 100% MeOH. Upon the addition of equal vols. of EtOAc, a white ppt. was formed which corresponds to the fraction obtained by

Karchesy *et al.* from bark [2]. This contained the purest preparation of the major procyanidin. The $E^{1\%}_{550}$ of 150 for the BuOH–HCl hydrolysis product was used to estimate the % dry wt values in Table 1. Continued extraction of the remaining residue of the lyophilized powder finally removed no further EtOAc-precipitable product. This final tan or almost white powder was soluble in 70% MeOH or Me_2CO (MeOH-insoluble fraction).

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REFERENCES

1. Swain, T. (1977) *Annu. Rev. Plant Physiol.* **281**, 479.
2. Karchesy, J. J., Loveland, P. M., Laver, M. L., Barofsky, D. F. and Barofsky, E. (1976) *Phytochemistry* **15**, 2009.
3. Constabel, F. (1969) *Planta Med.* **17**, 101.
4. Durzan, A. J., Chafe, S. C. and Lopushanski, S. M. (1973) *Planta* **113**, 241.
5. Cheah, K. and Cheng, T. (1978) *Am. J. Botany* **65**, 845.
6. Phillips, R. and Henshaw, G. G. (1977) *J. Exp. Botany* **28**, 785.
7. Hahlbrock, K. (1976) *Eur. J. Biochem.* **63**, 137.
8. Parkham, R. A. and Kaustinin, H. M. (1977) *Bot. Gaz.* **138**, 465.
9. Haslam, E. (1977) *Phytochemistry* **16**, 1625.
10. Porter, L. J. (1974) *N.Z. J. Sci.* **17**, 213.
11. Gupta, R. K. and Haslam, E. (1977) *J. Chem. Soc. Perkin Trans.* 1, 892.
12. Thompson, R. S., Jacques, D., Haslam, E. and Tanner, R. J. N. (1972) *J. Chem. Soc. Perkin Trans* 1, 1387.
13. Bate-Smith, E. C. (1975) *Phytochemistry* **14**, 1107.
14. Bate-Smith, E. C. (1973) *Phytochemistry* **12**, 907.
15. Bate-Smith, E. C. (1978) *Phytochemistry* **17**, 267.
16. Baur, P. S. and Walkinshaw, C. H. (1974) *Can. J. Botany* **52**, 615.
17. Chafe, S. C. and Durzan, D. J. (1973) *Planta* **113**, 251.
18. Swain, T. and Hillis, E. W. (1959) *J. Sci. Food Agric.* **10**, 63.
19. Cheng, T. (1977) *Plant Sci. Letters* **9**, 179.