Developmental Changes in the Composition of Proanthocyanidins from Leaves of Sainfoin (*Onobrychis viciifolia* Scop.) As Determined by HPLC Analysis

Mohammed R. Koupai-Abyazani,† John McCallum,† Alister D. Muir,*,‡ Bruce A. Bohm,*,† G. H. N. Towers,† and Margaret Y. Gruber‡

Department of Botany, University of British Columbia, Vancouver, British Columbia V6T 1Z4, Canada, and Agriculture Canada Research Station, 107 Science Place, Saskatoon, Saskatchewan S7N 0X2, Canada

Proanthocyanidin (PA) polymers (condensed tannins) were extracted from sainfoin leaves (*Onobrychis viciifolia* Scop.) at different stages of plant development. Analysis of the phloroglucinol degradation products by high-performance liquid chromatography showed that catechin, epicatechin, gallocatechin, and epigallocatechin were present as terminal units at all stages, while gallocatechin and epigallocatechin were the predominant extension units with lesser amounts of epicatechin incorporated at early stages. Catechin was not incorporated as an extension unit. The number-average molecular weight and degree of polymerization increased with leaf development. There was a very distinct change in the isomerization and degree of hydroxylation of the polymer constituents with development. The composition of *cis*-isomers decreased from 83 to 48% and the proportion of trihydroxylated B-rings increased from 60 to 90% with increasing leaf maturity.

INTRODUCTION

Proanthocyanidin (PA) polymers (condensed tannins) have been isolated from the leaves of a number of species from a range of plant families (Horigome et al., 1988; Baldwin et al., 1987; Kumar and Horigome, 1986; Stafford and Lester, 1986; Williams et al., 1983; Reed et al., 1982; Foo and Porter, 1981; Lane and Schuster, 1981; Bate-Smith, 1978), including herbaceous legumes, in particular those with potential for fodder crops (Lowther et al., 1987; Foo et al., 1982; Foo and Porter, 1980; Jones et al., 1976; Sarkar et al., 1976). These polymers exhibit considerable structural diversity in their degree of polymerization (DP), stereochemistry, and hydroxylation pattern of their flavan-3-ol subunits (Porter, 1988, 1984; Czochanska et al., 1980). Despite recent advances in understanding of their chemistry, many aspects of their biosynthesis remain poorly understood, notably the enzymatic processes involved in the establishment of their stereochemical and polymeric properties (Stafford, 1990). Developmental studies of PA polymers are few, and in most cases, colorimetric procedures or chromatographic analysis of low molecular weight (MW) PA oligomers has been employed (Butler, 1982; Czochanska et al., 1979a).

Evidence has been presented to suggest that PA composition can be influenced by environmental and developmental factors (Stafford et al., 1989; Brandon et al., 1982). Earlier studies, utilizing chiroptical methods (Czochanska et al., 1980), ^{1}H and ^{13}C NMR (Cai et al., 1991; Newman et al., 1987; Mattice and Porter, 1984; Czochanska et al., 1979b), gel permeation chromatography (GPC) (Williams et al., 1983; Foo et al., 1982; Karchesy and Hemingway, 1980), and vapor pressure osmometry (VPO) (Porter, 1984), have shown that the major PA of sainfoin (*Onobrychis viciifolia* Scop.) leaves are prodelphinidin (Pd) polymers, for which widely varying MW ranges have been reported. We recently developed a reversed-phase HPLC (RP-HPLC) method for the separation and identification of flavan-3-ol units in the PA of this species (Koupai-Abyazani et al., 1992). In the present study we have examined changes in the content and composition of PA polymers at different stages of development of sainfoin leaves using RP-HPLC and other quantitative procedures.

MATERIALS AND METHODS

Materials. Acetone, ethyl acetate, tetrahydrofuran (BDH Inc., Toronto, ON, Canada), and chloroform (Fisher Scientific, Ottawa, ON, Canada) were of analytical grade. Methanol (BDH Canada Inc.) was of HPLC grade. Phloroglucinol was obtained from Aldrich Chemical Co. (Milwaukee, WI) and recrystallized twice from hot water before use. (−)-Epigallocatechin was obtained from Apin Chemicals (Abingdon, U.K.). Bovine serum albumin (BSA), (−)-epicatechin, and (+)-catechin were obtained from Sigma Chemical Co. (St. Louis, MO). (+)-Gallocatechin, (+)-gallocatechin-4-phloroglucinol, (−)-epigallocatechin-4-phloroglucinol, (−)-epicatechin-4-phloroglucinol, and (+)-catechin-4-phloroglucinol adduct standards were kindly supplied by L. Y. Foo (DSIR, Petone, New Zealand). These compounds were used without further purification.

Plant Material. Sainfoin leaves (*O. viciifolia* Scop. cv. Melrose) were obtained from plants grown in a growth chamber (16/8 h, 18/13 °C day/night). A bulk sample of leaves used for polymer characterization contained a mixture of young and old leaves which were freeze-dried after collection. Sainfoin leaves used for the developmental studies were all collected at the same time sequentially down the stems at the following stages: stage 1, youngest leaves (leaflets not separated); stage 2, leaflets separated but folded; stage 3, leaflets partially unfolded; stage 4, leaflets fully unfolded; and stage 5, older mature leaves. Leaves were pooled from several shoots from the same plant, and leaflets were removed from petioles. These samples were stored at −80 °C until analysis. The different plant stages were used as a measure of time since it was not possible to sample the leaves and then let them mature to the next stage to obtain rate measurements.

Isolation and Purification of PA Polymers. Isolation of a bulk sample of PA polymer was carried out as described previously (Koupai-Abyazani et al., 1992). For developmental studies, sainfoin leaves from each developmental stages (1–6 g, fresh weight) were frozen in liquid nitrogen and finely ground in a mortar and pestle. Each sample was extracted with 3 × 50 mL of 75% aqueous acetone containing 0.1% w/v ascorbic acid. The

* Author to whom correspondence should be addressed.
† University of British Columbia.
‡ Agriculture Canada.

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Table I. Degree of Polymerization (DP), $\bar{M}_n$, and Dp:Cy and *Cis:Trans* Ratios of Sainfoin Leaf PA Polymer Sample Obtained from a Mixed Age Leaf Sample Determined by Different Methods

method	DP	$\bar{M}_n$[a]	Dp:Cy	*cis:trans*
HPLC	6–7	1800–2100	88:12	67:33
^{1}H NMR	8	2400	92:8	86:14
GPC	8	2500		

[a] Assuming a unit molecular weight of 300.

acetone was removed under reduced pressure (<30 °C) and the aqueous solution extracted with 150 mL of chloroform. The chloroform extract was discarded and the aqueous phase extracted with ethyl acetate (2 × 50 mL). The remaining aqueous fraction was applied to a C_{18} solid-phase cartridge (Adsorbex RP-18, E. Merck, Darmstadt, Germany) previously equilibrated with 1% aqueous acetic acid. The cartridge was washed with 2 mL of 1% aqueous acetic acid, and the bound PA polymers were eluted with 1 mL of 70% aqueous methanol.

Hydrolysis of PA Polymers. The 70% aqueous methanol fraction obtained above was evaporated to dryness under dry N_2. The residue and 0.8 mg of phloroglucinol were dissolved in 150 μL of 1% HCl in ethanol in a capped vial (Koupai-Abyazani et al., 1992). The mixture was shaken and allowed to stand at room temperature overnight. The solvent was then evaporated under dry N_2 and the residue dissolved in 50 μL of distilled water and extracted with ethyl acetate (2 × 150 μL). The ethyl acetate fractions were recovered, combined, evaporated to dryness under N_2, dissolved in 100 μL of 70% aqueous methanol, and subjected to HPLC analysis.

HPLC Analysis of Phloroglucinol Adducts. This was carried out on an SP8800 ternary pump HPLC (Spectra-Physics, San Jose, CA) and the elution profile monitored at 280 nm using a Model 111B UV detector (Gilson, Middleton, WI). The detector signal was recorded and integrated using a Spectra-Physics SP4290 integrator. A prepacked analytical column of Lichrospher 100 RP-18 (4 × 250 mm) (E. Merck) protected by a 4 × 4 mm guard cartridge packed with the same packing was used for all experiments. The phloroglucinol adducts were analyzed using the following gradient of 1% aqueous acetic acid (solvent A) and methanol (solvent B): T = 0 min, A = 100%; T = 30 min, A = 85%; T = 45 min, A = 40%; T = 50 min, A = 40%. All gradients were linear. Chromatography was conducted at ambient temperature at 1 mL/min. All values are a mean of three replicated injections (20 μL) with quantitation using purified phloroglucinol adducts and flavan-3-ol standards.

TLC. All two-dimensional TLC (2D TLC) separations were carried out on cellulose plates (E. Merck) developed in TBA [*tert*-butyl alcohol/acetic acid/water (3:1:1)] and 6% acetic acid. Compounds were visualized by spraying with vanillin–HCl [4% vanillin in methanol/concentrated HCl (4:1 v/v)] (Koupai-Abyazani et al., 1992).

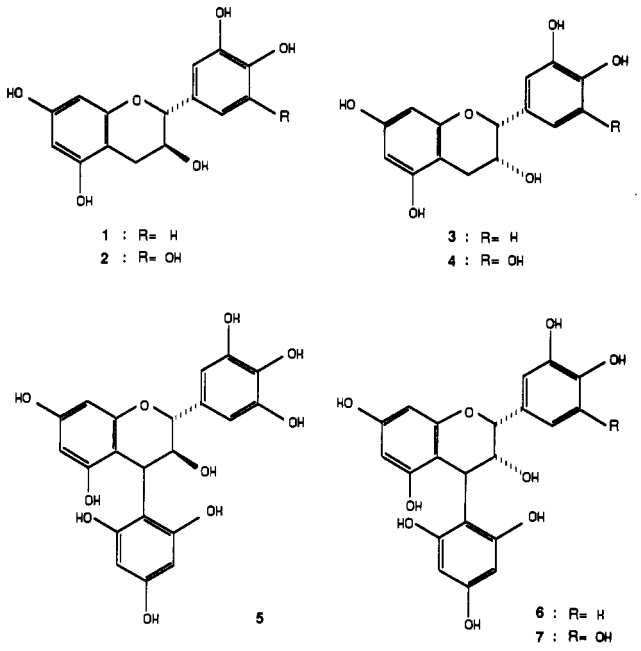
Gel Permeation Chromatography–HPLC. GPC–HPLC was carried out on a Waters M600E system equipped with an M700 autosampler and a 991 photodiode array detector (PDA) [Millipore (Canada) Ltd., Mississauga, ON, Canada]. The PDA software was used to integrate the signal. Peracetate derivatives (Williams et al., 1983) were chromatographed on an Ultrastyragel linear (7.8 × 300 mm) and a 10^3A columns (7.8 × 300 mm) [Millipore (Canada) Ltd.] in series, eluted with tetrahydrofuran at 1.1 mL/min (25 °C). Molecular weights were calculated after calibration with polystyrene MW standards (MW 687, 2000, 4136, 9000, and 32 660) (Aldrich Chemical Co.) and phloroglucinol and catechin peracetates.

NMR Analysis. ^{1}H NMR spectra of the acid-catalyzed thiolytic degradation products of polymers were obtained on a Bruker WH-400 (400 MHz) spectrometer. The signal assignments were made according to the results of Cai et al. (1991).

Quantitative Determination of PA Polymer Content. Determination of PA polymer concentrations in leaf samples was performed by three different methods: an enzyme assay using BSA bound to polystyrene microtiter plates to trap the polymer and then alkaline phosphatase to assay the amount of tannin bound (Ittah, 1991); the standard butanol–HCl hydrolysis assay as modified by Porter et al. (1986); and a modified dimethylaminocinnamaldehyde (DAC) assay (Nagel and Glories, 1991). Purified sainfoin PA polymer was used as a standard in all assays.

RESULTS AND DISCUSSION

In our initial investigation of the identification of the constituent flavanoid units in sainfoin leaf PA polymers by RP-HPLC (Koupai-Abyazani et al., 1992), it was shown that (+)-catechin (1), (+)-gallocatechin (2), (−)-epicatechin (3), and (−)-epigallocatechin (4) were present. Further



investigations in the present study by HPLC, 2D TLC, and ^{1}H NMR revealed that compounds 2–4 are both extension and terminal units, while 1 is a terminal unit only. The PA polymers isolated from sainfoin leaves (bulk sample) were purified and then hydrolyzed in the presence of phloroglucinol (Koupai-Abyazani et al., 1992), yielding extension units as flavan-3-ol–4-phloroglucinol adducts (5–7) and terminal flavan-3-ols (1–4). The degree of polymerization (DP) calculated by dividing the concentration (nanomoles) of extension units by that of terminal units, the number-average MW ($\bar{M}_n$), and the delphinidin:cyanidin (Dp:Cy) and *cis:trans* ratios of sainfoin polymers derived from the RP-HPLC data are shown in Table I.

The values obtained for the proanthocyanidin sample by RP-HPLC were compared with those obtained by GPC–HPLC and ^{1}H NMR analytical methods. Similar values were observed for the Dp:Cy ratio by RP-HPLC and ^{1}H NMR, while ^{1}H NMR indicated a higher *cis*-isomer content than was determined by RP-HPLC (Table I). These values are also similar to those reported previously for this species (Foo and Porter, 1980; Foo et al., 1982).

Although a very high and wide MW range (17 000–28 100) has been reported for PA polymers from sainfoin (Jones et al., 1976) using ultracentrifugation analysis, the results obtained in the present study as well as other reported data (Foo and Porter, 1980; Foo et al., 1982) are in the range 1800–3300. However, the MW of polymers isolated from sainfoin may vary considerably depending on the season and cultivar (Foo et al., 1982). There is also an inherent variability in the estimates of MW as was demonstrated by the analysis of polymer samples by different methods, e.g., ^{13}C NMR, VPO, and GPC (Porter, 1984; Mattice and Porter, 1984). The contributions of solvation shell (in VPO) and globularity (in GPC) of

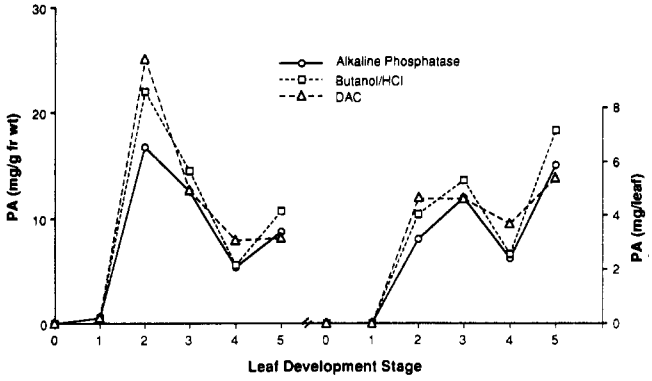


Figure 1. Proanthocyanidin (PA) content of sainfoin leaves at five stages of development as determined by alkaline phosphatase assay (○), butanol/HCl assay (□), and dimethylaminocinnamaldehyde assay (△).

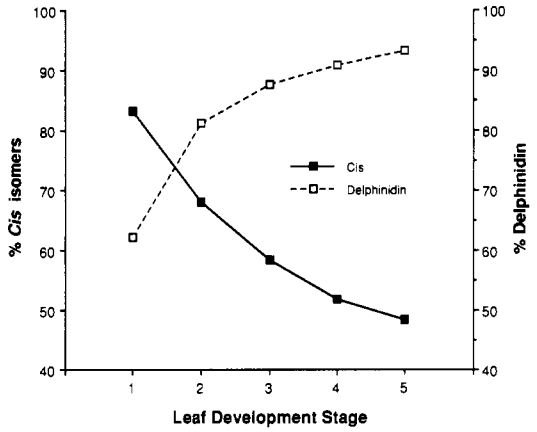


Figure 2. *Cis*-isomer content and proportion of extension flavan-3-ol units with trihydroxy B-rings (delphinidin) found in PA polymers isolated from sainfoin leaves at different stages of development.

polymers are thought to affect the results in the measurement of MW of PA polymers (Porter, 1984).

To investigate the influence of leaf age on the concentration of polymer units, MW range, and other characteristics of the PA polymers, leaf samples at five developmental stages were collected. The total PA content at each stage of leaf development was determined using three different assays (Figure 1). The values obtained by these methods were similar except for stage 2, where the enzymatic assay method resulted in a lower estimate of the PA content than the other two methods. These results indicate that PA synthesis occurs at an early stage in leaf development both in terms of the concentration of PA polymer per gram of fresh weight and in terms of the total amount per leaf. It would appear that PA synthesis is essentially complete by the time the leaf unfolds (leaf stage 3). The variation observed after this stage could be attributed to a lower rate of synthesis when these mature leaves were at the earlier leaf stages.

The concentration of extension and terminal units was determined for each developmental stage by RP-HPLC analysis of the phloroglucinol hydrolysis products. Calculation of the *cis:trans* ratio and the percentage of delphinidin-producing residues in the extension units indicated a significant change in the mean polymer composition with leaf development (Figure 2). The *cis*-isomer composition declined from 83 to 48%, while the proportion of delphinidin-producing extension units increased from 62 to 93% in the mature leaf. This change can be seen in more detail by examining the changes in the flavan-3-ol composition during leaf development. Of the four flavan-3-ol monomers present, catechin (1) was the least abundant (Figure 3) and was only present as a terminal unit (Figure 4). The other three monomers were found both as terminal units and as 4-phloroglucinol adducts of the extension units (Figures 4 and 5). These results are in disagreement with those observed by Foo et al. (1982), who did not detect gallocatechin terminal units. When the concentratioins of flavan-3-ols in the extension and terminal units are considered together, a distinct pattern of polymer synthesis is apparent. Catechin (1) was incorporated only into terminal units and only in young leaves (Figure 4). The other *trans*-isomer, gallocatechin (2), was the next least abundant monomer in the very young leaves; however, in the mature polymer it is the most abundant monomer (Figure 3), and this is reflected in the increase in the *trans*-isomer content (Figure 2). Gallocatechin (2) was incorporated as both terminal and extension units, with most of it incorporated into extension units (Figure 5).

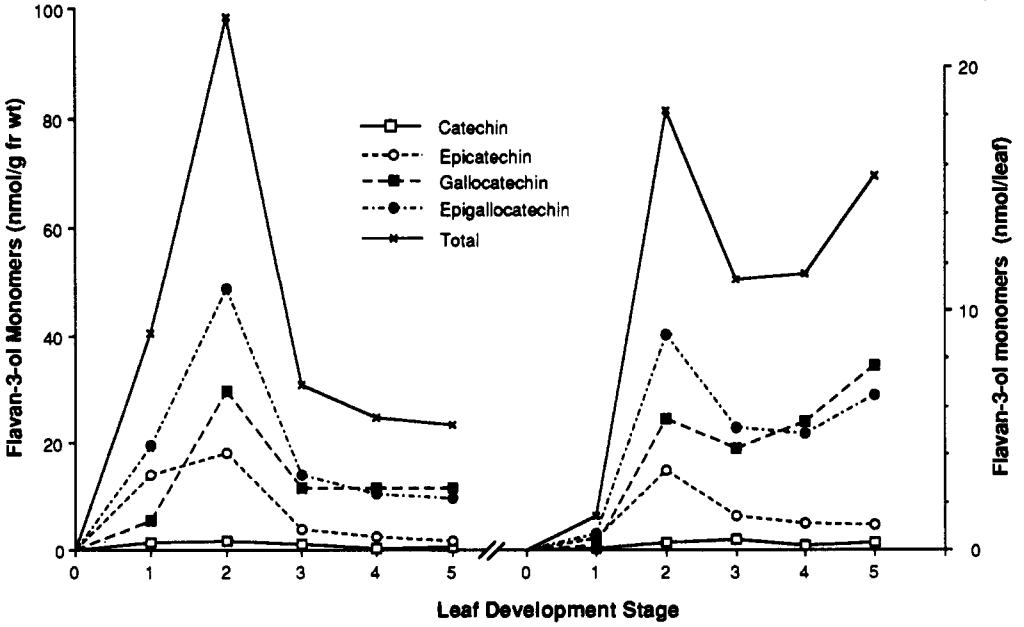


Figure 3. Changes in the concentration of the four flavan-3-ol monomers found in proanthocyanidin polymers isolated from sainfoin leaves at different stages of development.

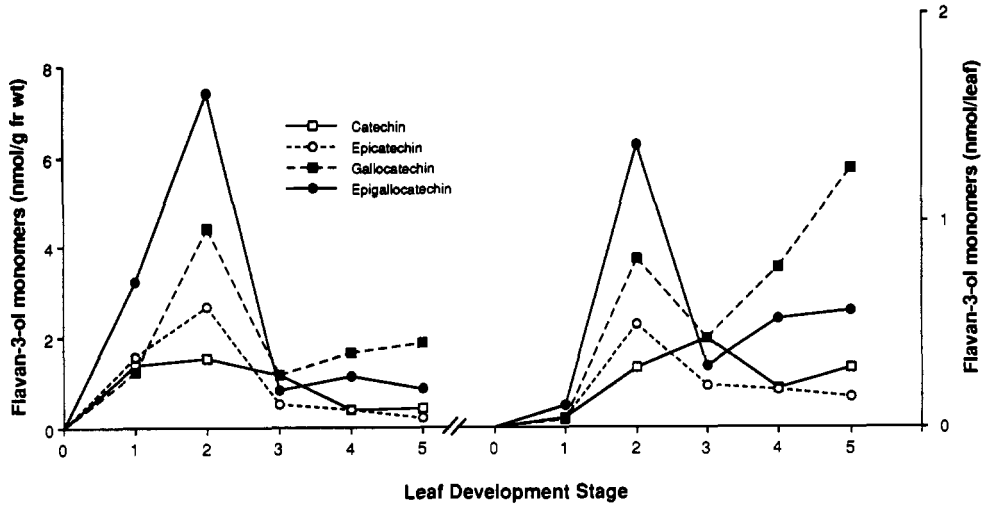


Figure 4. Changes in the relative abundance of the four flavan-3-ol monomers found as terminal units in proanthocyanidin polymers isolated from sainfoin leaves at different stages of development.

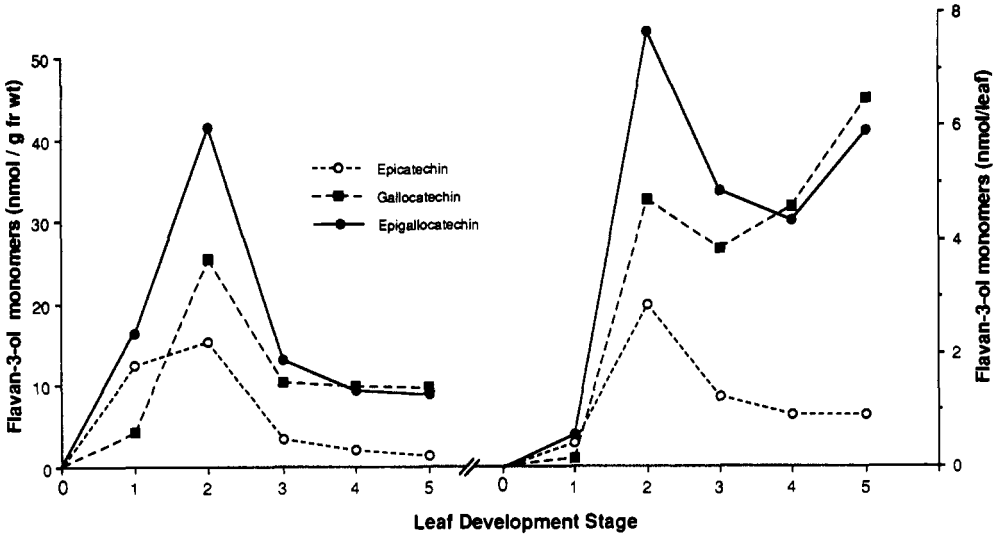


Figure 5. Changes in the relative abundance of the three flavan-3-ol monomers found as extension units in proanthocyanidin polymers isolated from sainfoin leaves at different stages of development.

Of the *cis*-isomers present, epicatechin (3) was the second most abundant monomer in the early leaf polymer and was present as both terminal and extension units (Figures 3–5). As leaf development proceeded, epicatechin declined rapidly in abundance. The other *cis*-isomer, epigallocatechin (4), was the most abundant monomer in the young leaf polymer and continued to be the most abundant monomer until eclipsed by the other trihydroxylated B-ring monomer, gallocatechin (2), in the mature leaf polymer (Figure 3).

As the leaf development proceeded, there was a small increase in the degree of polymerization (5.4 to 6.9) with a peak of 8.3 in the newly unfolded leaf, which coincided with a sharp reduction in the rate of polymer synthesis (Figure 6). These results demonstrate that proanthocyanidin polymer formation is a dynamic process and that polymer composition undergoes significant changes as synthesis proceeds. This is especially noticeable in the case of epicatechin incorporation. As leaf development proceeds from stage 1 through stage 3, epicatechin ceases to be incorporated into new polymer. Since there are no dramatic changes in polymer size as indicated by the DP, particularly between leaf stages 1 and 2, the doubling of the monomer concentration which occurs at this time must be due to new synthesis of polymer. In the mature leaf, the proportion of gallocatechin in both terminal and extension units increases (Figures 4 and 5), suggesting

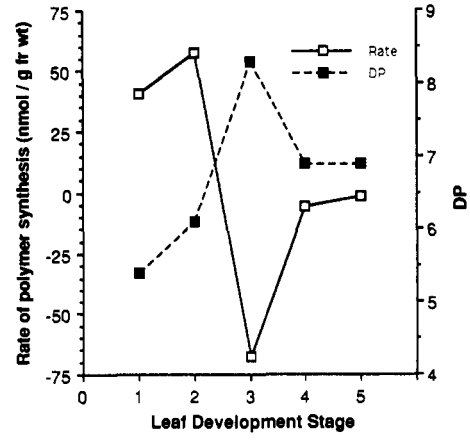


Figure 6. Changes in the rate of proanthocyanidin polymer synthesis and degree of polymerization (DP) in polymers isolated from sainfoin leaves at different stages of development.

that there is still some new synthesis or metabolism of polymeric material even in the mature leaf.

The steady increase in the proportion of trihydroxy-flavan-3-ol units and decline in *cis*-isomer content which occur with increasing leaf age (Figure 2) also suggest that polymer synthesis or catabolism is occurring even though the fresh weight polymer concentration declines with leaf maturity (Figures 1 and 3). These results also demonstrate

that the sainfoin polymer is a heterogeneous complex involving a large number of molecules of different monomer composition. Since there are only relatively small changes in the mean DP, the increase in gallocatechin content in the mature leaves could be attributed to the *de novo* synthesis of new polymer composed largely of gallocatechin.

These results indicate that care must be taken to define the growth stage of plant material extracted for polymer analysis. It also suggests that, metabolically, polymer synthesis is subject to a complex regulatory mechanism.

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