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DIVERGENCE IN STRUCTURE AND ACTIVITY OF PHENOLIC DEFENSES IN YOUNG LEAVES OF TWO CO-OCCURRING *Inga* SPECIES

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Abstract—The leaves of tropical forest trees are most likely to suffer herbivore damage during the period of expansion. Herbivore selection on young leaves has given rise to a variety of leaf developmental strategies and age-specific chemical defense modes. We are studying correlations between leaf developmental types and chemical defenses in the Neotropical genus *Inga*. We have characterized defense metabolites in *Inga goldmanii* and *Inga umbellifera*, two species that co-occur in the lowland moist forest of Panama. These congeners have markedly different young-leaf developmental phenotypes but suffer approximately equal rates of herbivory. Bioassays of whole and fractionated leaf extracts using larvae of *Heliothis virescens* show that *I. goldmanii* chemical defenses are nearly three times more inhibitory than those of *I. umbellifera*. In both species, most of the inhibitory activity resides in complex mixtures of monomeric and polymeric flavan-3-ols. This group comprises >30% of young leaf dry weight in both *I. goldmanii* and *I. umbellifera*. The species' phenolic chemistry differs markedly, however, both in the structure of the monomeric units and in the distribution of polymer sizes. The differences in chemical structure have pronounced effects on their bioactivities, with *I. goldmanii* flavans being twice as inhibitory to *H. virescens* larvae as *I. umbellifera* flavans, and more than three times more efficient at protein binding. Given the extraordinarily high polyphenol concentrations that are found in the young leaves of these species, protein precipitation could be an important mechanism of growth inhibition. Nevertheless, our data show that another mode of phenolic action, possibly oxidative stress, occurs simultaneously.

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Key Words—*Inga goldmanii*, *Inga umbellifera*, *Heliothis virescens*, epicatechin-4β→8-catechin-4α→8-epicatechin, flavan-3-ol glycoside, procyanidin, condensed tannin, polyphenol, phenolic, protein precipitation, bioassay, bioactivity, chemical defense evolution.

INTRODUCTION

Young leaves of tropical forest trees suffer far higher rates of herbivory than mature leaves of the same species. Surveys of leaf herbivory across a range of tree species in a moist forest in Panama show that nearly 70% of lifetime herbivore damage occurs during the days to weeks of leaf expansion (Coley and Aide, 1991; Coley and Barone, 1996). This is a remarkable finding in that the leaves of tropical moist-forest trees typically last several years. High herbivory pressure on young leaves has led to a variety of developmental and defense chemical phenotypes. We are investigating correlations between developmental pattern, chemical defense, and herbivory in young leaves of the Neotropical genus *Inga* (Fabaceae). As a starting point, we have identified two co-occurring *Inga* species that have markedly different young-leaf development. *Inga goldmanii* is a "normally greening" species in which leaves flush singly, expand slowly, and are photosynthetic. In contrast, *Inga umbellifera* is a "delayed-greening" species: Leaves are deployed in large flushes, expansion is rapid, and full chloroplast development occurs only after leaves have reached full size. Populations of these two species growing in lowland moist forest on Barro Colorado Island, Panama, suffer approximately equal rates of young-leaf herbivory, approximately 22% (Coley 2 et al., in press).We are characterizing the full range of defense metab-olites synthesized in these two species to determine: (1) the relative contributions of chemistry to overall herbivory-avoidance strategies, (2) the structure/activity relationships of defense metabolites, and (3) whether chemical defenses, like developmental strategies, have diverged in these congeners.

We have developed an insect growth assay using larvae of the noctuid lepidopteran *Heliothis virescens*. We used the assay to test whole young-leaf extracts and to guide chemical fractionation. We found pronounced differences between *I. goldmanii* and *I. umbellifera* in the bioactivities of whole young-leaf extracts. Through assays of individual chemical fractions, we showed that, in both species, complex mixtures of phenolic compounds are accumulated at high concentrations and account for the majority of insect growth inhibition. In this paper, we describe the phenolic chemistry in these two species and show that structural divergence within this class of compounds has led to marked differences in bioactivities.

METHODS AND MATERIALS

Collections of Plant Material. Collections from *I. goldmanii* and *I. umbellifera* were made at Barro Colorado Nature Monument (BCNM, 9°10'N, 79°50'W), Republic of Panama. Young leaves, 5–80% of full expansion, were collected from shaded, understory trees during periods of leaf flush between January 2001 and December 2002. Collections were made randomly from trees across BCNM. Leaves were pooled during a given harvesting period, typically 3–4 mo. Tissues were processed shortly after collection by maceration, first in a Waring blender and then a Polytron 3100 (Brinkmann Instruments, Westbury, NY, USA) in 95% EtOH. Suspensions (leaf solids + dissolved extractables) of each species were shipped to the University of Utah on dry ice and then stored at −80°C until processing.

Fractionation of Plant Material, Isolation of Phenolic Components, and Determination of Phenolic Mass. Two sets of extractions were made, one to prepare a whole-leaf extract for bioassay, and one to separate phenolics from leaf solids and all other leaf extractables. The whole-leaf extract contained all soluble metabolites. To prepare this, a portion of leaf material (usually 5–10 g fresh weight) was filtered and extracted repeatedly with 80% EtOH/water, 70% acetone/water, dichloromethane (DCM), and 25 and 70°C water. All solutions were combined and reduced under vacuum to a concentration of 10–20 mg/ml. (Low molecular weight terpenes are not present in the shade phenotypes of these species.) The exact concentration was determined gravimetrically by vacuum-drying 5-ml subsamples.

A second extraction was performed on the remainder of the sample to (1) isolate phenolic components into purified fractions and (2) determine the mass percentage of phenolics of the total extractable metabolites. Leaf samples were submitted to a partitioning process (details in Index), which resulted in five extract fractions plus the marc. Two of these were shown by subsequent high-performance liquid chromatography (HPLC) and mass/nuclear magnetic resonance (NMR) spectral analyses to be composed nearly entirely of phenolic components, and were designated phenolics I and II. Except where noted, these were combined into a single "phenolics" fraction. The remaining three fractions, "lipids", "proteins", and "aqueous", were vacuum-dried along with the marc. The weight of each fraction was recorded to determine total leaf dry weight (DW) and the mass percentage of phenolics. The crude and phenolic fractions were submitted to bioassay (details below). The marc was further processed (see the following section).

Butanol–HCl Digestion of Marc. Samples of *I. goldmanii* and *I. umbellifera* marc were acid digested following the method of Porter et al. (1986). After digestion, the marc was washed with water to remove the acid and then extracted with BuOH until most of the cyanidin had been removed. The marc

was dried, lyophilized, and its mass recorded. Portions of extracted and unextracted marc were subsequently submitted to bioassay (details below).

Chromatographic Separations of Phenolics. Characterization of the phenolic content of *I. goldmanii* and *I. umbellifera* began with the isolation of individual components. Low molecular weight compounds were separated from EtOAc extracts of the phenolics fraction of *I. goldmanii* by flash chromatography on silica gel using dichloromethane/MeOH, 92:8 → 80:20. A similar separation of low molecular weight compounds from *I. umbellifera* is described elsewhere (Lokvam et al., 2004).

A higher molecular weight phenolic compound, a procyanidin trimer (compound **1**, Figure 1a), was isolated from *I. goldmanii* by HPLC. The compound was separated on a 10 × 200 mm polyhydroxyethyl aspartamide (PHA) semipreparative column (5 μm, PolyLC Corp., Columbia, MD, USA) and then purified using a 10 × 250-mm Microsorb ODS semipreparative column (5 μm, Varian Analytical Instruments, Walnut Creek, CA, USA) with a guard column (ODS, 8 μm, Varian). Compound structural characterization was based on one- and two-dimensional NMR spectra (described below) and on the high-resolution matrix-assisted laser desorption initiation time-of-flight (HR-MALDI-TOF) mass spectrum, obtained using a Voyager DE-STR spectrometer (MDS Sciex, Concord, ON, CA) with the sample dissolved in MeOH/CHCA. The separation and characterization of individual procyanidins from *I. umbellifera* are described elsewhere (Lokvam et al., 2004).

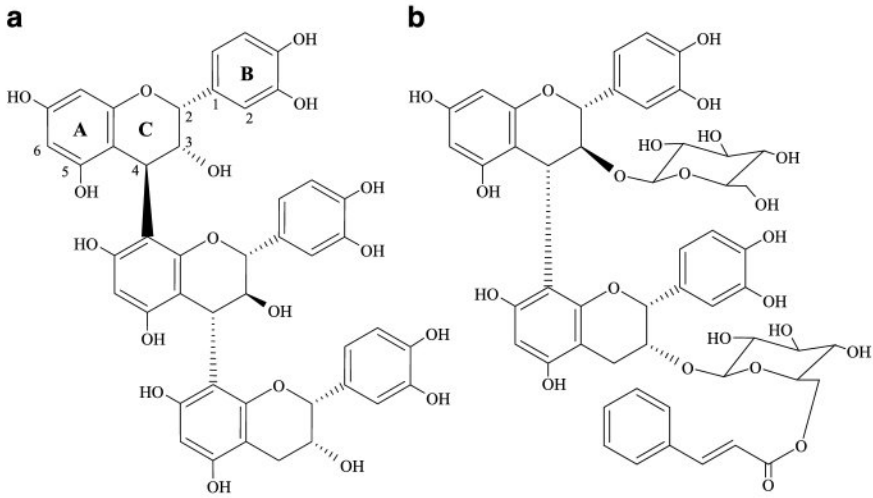

FIG. 1. (a) Trimeric procyanidin (compound **1**) from *I. goldmanii*. (b) Representative substituted procyanidin from *I. umbellifera*.

As a qualitative test of the purity of the phenolics extracts, phenolics from both *I. goldmanii* and *I. umbellifera* were analyzed by HPLC using a diode array detector (DAD) and an evaporative light-scattering detector (ELSD, SEDERE S.A., Alfortville, France). For both sample analyses, comparisons of the ELSD and DAD outputs were made to check for the presence of non-UV-absorbing compounds. *I. umbellifera* phenolics were further analyzed by liquid chromatography/electrospray ionization mass spectrometry (LC/ESI-MS; details below).

NMR. ^{1}H and ^{13}C NMR spectra were acquired on a Unity iNOVA 500-MHz spectrometer (Varian). The *I. goldmanii* trimer was dissolved in CD_3OD. ^{1}H and ^{13}C spectra were referenced to the centerline of the solvent multiplets, 3.31 and 49.15 ppm from trimethylsilyl, respectively. Acquisition temperatures were $-39°C$ (for trimer ^{13}C spectrum) and $-20°C$ (for trimer ^{1}H spectrum), respectively. ^{1}H–^{1}H connectivities as well as short- and long-range ^{1}H–^{13}C correlations were observed with gradient-selected DQCOSY, HSQC, and HMBC pulse sequences.

Electro-Spray Ionization Mass Spectrometry. I. goldmanii phenolics were analyzed with a Finnigan LCQ (Thermo-Finnigan, Bremen, Germany) operated in the negative ion ESI mode. *I. umbellifera* phenolics were analyzed with a MicroMass Quattro II mass spectrometer (Waters, Milford, MA, USA) operated in the positive ion ESI mode. For both analyses, phenolic solutions in MeOH at a concentration of approximately 0.1 μg/μl were introduced into the instrument by direct infusion at a rate of 5 μl/min. *I. umbellifera* phenolics were further analyzed by LC/MS using a Waters 2690 HPLC system with a Waters 2487 UV Detector (recording at 280 nm) configured in tandem with the MicroMass spectrometer described above.

Separation of I. goldmanii Phenolics into Size Classes. Samples of *I. goldmanii* phenolics I (0.75 g) and phenolics II (0.3 g) dissolved in 50% MeOH were each applied to a water-equilibrated LH-20 column and eluted with 50% MeOH and 70% acetone. Solvents were removed under reduced pressure, and all fractions were vacuum-dried. This gave four fractions, designated phenolics I–MeOH, phenolics I–acetone, phenolics II–MeOH, and phenolics II–acetone. (*I. umbellifera* phenolics cannot be separated on LH-20 under the protocol described here.)

Estimation of Degree of Polymerization. The mean degree of polymerization (mDP) and catechin/epicatechin ratios of each of the *I. goldmanii* LH-20 fractions were determined by thiolysis, acid-catalyzed degradation of polymers in the presence of benzylmercaptan (Thompson et al., 1972). This reaction releases lower units as free monomers and extender units as their respective thioethers. Product mass percentages were estimated by comparison of their peak areas (280 nm) to calibration curves made with catechin or epicatechin standards (Sigma, St. Louis, MO, USA) or the purified thioethers. Following

conversion to moles, the mDP was calculated by the formula: (catechin + epicatechin + thioethers) / (catechin + epicatechin). HPLC analyses of derivatized and underivatized phenolics yields the relative masses of catechin and epicatechin as free monomers and as lower or extender units.

The mDP and the monomer/dimer mass percentages of *I. umbellifera* phenolics were estimated using a combination of mass and evaporative light scattering detection following the HPLC separations outlined above. The molecular ion masses of the major ELSD-detected peaks were determined using LC/ESI-MS. From these data, the number of flavanol + pyranose + cinnamate units in each of the major peaks was deduced. The overall monomer/dimer mass percentages were determined by summing the appropriate peak area percentages. Upper and lower bounds for the mDP of *I. umbellifera* phenolics were estimated by mass correction of each major polymer peak, so that area percent value reflected flavan-monomer number. The mDP was calculated from the weighted area percentages of the major polymer peaks.

Procyanidin–Protein Binding. A bovine serum albumin (BSA) precipitation assay (Hagerman and Butler, 1978) was used to assess protein binding by *I. goldmanii* and *I. umbellifera* procyanidins. This method measures the quantity of procyanidin (as absorbance of iron-phenolate at 510 nm) that precipitates with protein from a standard BSA solution over a range of total phenolic concentrations (0.125–3.0 mg/ml). Binding curves are thus obtained. The slopes of the linear portions, reported as absorbance units per milligram total phenolics per milliliter, are used as a relative measure of procyanidin–protein binding capacity. In both cases, r^2 values were >0.99.

Insect Feeding Trials. The bioactivities of whole-leaf extracts, all phenolic fractions, and the pre- and post-butanol-extracted marcs from both species were tested in growth trials using larvae of *H. virescens* (Lepidoptera: Noctuidae). Larvae were fed an artificial diet amended with plant products, and their growth relative to controls was measured. The diet was modified from Chan et al. (1978). Whole-leaf extracts (as the solutions that were described above) and phenolics (as powders) were added at prescribed mass percentages of the total diet DW. For the marc assays, marc was ground to a fine powder using a Retsch MM 200 Mixer Mill (Retsch GmbH, Haan, Germany) and was substituted at the same mass percentage (35% total diet DW) for the cellulose in the diet. Once prepared, the diet was divided into individual portion cups, typically 16 per treatment level. Controls were prepared in the same manner as treatments but without the addition of plant products. (We have verified that following high vacuum-drying there are no effects from solvents used in the whole-leaf extracts, so solvent controls were omitted.) One freshly hatched, unfed *H. virescens* larva was sealed into each cup with a vented lid and kept in a 75% RH controlled growth chamber on a 12–12 h L/D schedule for 8 d. At the end of this period, treatments and controls were weighed. Treatment weights were divided

by the control weight (GRC = growth relative to control) and fitted to the following dose–response function using the NLIN procedure in SAS:

$$\mathrm{GRC} = \frac{a_0 - a_0}{1 - (\log C/b_2)^{b_1}}$$

where a_0 is the response at low concentration, C is the dose in percent, b_1 is the slope, and b_2 is the logarithm of the concentration that inhibits growth by 50%. The antilog of b_2, the GI_{50}, was used to compare the relative inhibitory capacities of given plant chemical classes (Streibig et al., 1993). This statistic is based on the combined results of several assays, such that it can be assigned a 95% confidence interval (CI). If, for a given *I. goldmanii*/*I. umbellifera* assay class (whole-leaf extract or phenolics), there was no overlap in 95% CIs, the GI_{50} values were considered statistically different.

RESULTS

I. goldmanii Phenolics. I. goldmanii phenolics are composed almost entirely of monomeric catechin and epicatechin or polymers of these subunits.

TABLE 1. ^1H AND ^{13}C NMR SHIFTS FOR COMPOUND 1 IN CD_3OD AT −20°C (^1H) AND −39°C (^{13}C)[a]

	Upper		Middle		Lower	
C no.	δ ^{13}C	δ ^1H, mult.: Hz	δ ^{13}C	δ ^1H, mult.: Hz	δ ^{13}C	δ ^1H, mult.: Hz
A/C-2	75.5	5.18, br s	84.3	3.77, d: 10.0	79.6	5.09, br s
A/C-3	72.6	3.63, br s	74.2	4.58, m	65.9	4.38, o
A/C-4	36.9	4.44, br s	38.5	4.38, dd: 10.0, 7.5	23.5	2.55, br d: 16.0; 2.17, br d: 16.0
A/C-5	157.3		156.3		156.0	
A/C-6	95.5	5.22, o	97.2	6.12, s	94.6	6.02, s
A/C-7	157.3		155.4		154.7	
A/C-8	95.0	5.66, d: 2.0	110.5		108.6	
A/C-9	155.8		154.7		153.0	
A/C-10	103.9		107.8		100.4	
B-1	133.3		132.7		132.1	
B-2	115.7	6.90, br s	115.3	6.69, br s	112.6	6.35, br s
B-3	145.5		145.4		146.2	
B-4	144.7		146.2		145.3	
B-5	115.3	6.55, d: 8.0	115.3	6.62, o	116.2	6.42, o
B-6	118.9	5.47, o	121.3	6.41, o	118.6	5.46, o

[a]All values relative to the center line of the solvent multiplet (3.31 ppm for ^1H, 49.15 ppm for ^{13}C).

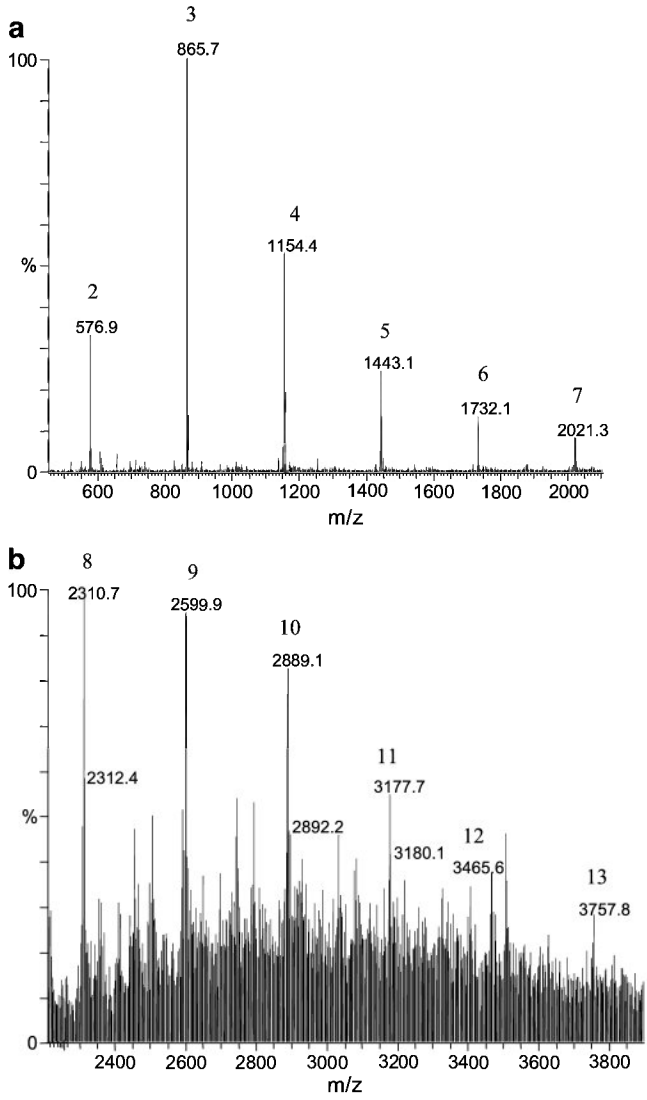

FIG. 2. ESI mass spectra of *I. goldmanii* phenolics. The low mass spectrum (a) shows masses from 500 to 2100 amu; the high mass spectrum (b) shows masses from 2200 to 3900 amu. Peaks are labeled by mass and polymer length.

This conclusion was based on NMR and mass spectral analyses and on thiolytic degradation followed by HPLC separation and diode array detection. Both catechin and epicatechin were isolated as monomers by flash chromatography on silica gel. They were identified by comparison of their ^{1}H NMR spectra to standards (Sigma). In addition, a procyanidin trimer, compound **1** (Figure 1a) was isolated by semipreparative HPLC. The structure of this compound, epicatechin-4β→8-catechin-4α→8-epicatechin, was deduced from 1D (Table 1) and 2D NMR spectra. HR-MALDI-TOF MS gave an $[M + Na]^+$ ion of *m/z* 889.1996, calculated for $C_{45}H_{38}O_{18}Na$ (5.1 ppm error) and consistent with a singly linked trimeric procyanidin. Compound **1** was first isolated but only minimally characterized by (Hsu et al., 1985).

The ESI mass spectrum of *I. goldmanii* phenolics showed only molecular masses consistent with monomeric catechin/epicatechin or singly linked polymers thereof (Figure 2). $[M - H]^-$ ions were observed ranging from 576.9 to 3757.8 amu, corresponding to degrees of polymerization 2 to 13. No evidence of multiply charged ions was detected. Thiolytic degradation of *I. goldmanii* phenolics followed by HPLC separation and diode array detection of the products indicated that the great majority of the product mass is catechin/epicatechin and their thioethers. This was corroborated using HPLC separation and detection by evaporative light scattering, a mass-sensitive technique, which showed no other major components.

TABLE 2. MASS PERCENTAGES, PHYSICAL PROPERTIES, AND BIOACTIVITIES OF SOME *I. goldmanii* AND *I. umbellifera* METABOLITE FRACTIONS

	% DW	mDP[a]	PP[b]	GI_{50}[c]	95% CI
I. goldmanii crude extract	48	–	–	1.1	0.87–1.42
I. umbellifera crude extract	61	–	–		2.11–3.64
I. goldmanii whole phenolics	29	7.2[d]	0.44	0.59	0.38–0.8
I. umbellifera whole phenolics	31	2.2–3.4	0.13	1.45	1.31–1.58
I. goldmanii LH-20 fractions					
Phenolics I–MeOH	6	2.0[e]	0.12	1.0	0.3–1.6
Phenolics I–acetone	14	6.1	0.56	1.2	0.7–1.9
Phenolics II–MeOH	2	nd	0.16	nd	–
Phenolics II–acetone	6	15.3	0.72	1.1	0.4–3.0

nd = Not determined.
[a] Mean degree of polymerization.
[b] Procyanidin–protein binding capacity: slope of linear binding curve (absorbance per milligram phenolic per milliliter).
[c] Concentration (% DW) of extract in insect diet that is required to reduce growth by 50% relative to controls.
[d] Weight average, based on LH-20 fractions.
[e] Free monomer content removed.

I. goldmanii phenolics I and II fractions were analyzed for percent monomer composition and degree of polymerization after separation on LH-20 (96 and 94% recovery, respectively). Monomeric catechin and epicatechin in the *I. goldmanii* phenolics were approximately 15.0 and 0.8%, respectively, of the total phenolic mass as estimated by HPLC analysis of underivatized phenolics I–MeOH, the only fraction that contained detectable quantities of free monomer. The mDP of the *I. goldmanii* phenolics was determined by HPLC analysis following thiolysis of the interflavanyl linkage in the presence of toluene-α-thiol (Table 2). Phenolics I–MeOH contained monomeric catechin/epicatechin and low molecular weight procyanidin with an mDP estimated to be 2.0. Phenolics II–MeOH was not amenable to analysis by these methods and gave only low concentrations of the normal thiolysis products, i.e., free monomers and thioethers. Based on its weak protein-precipitation ability, it appears to contain procyanidin. We suspect that this fraction consists of low molecular weight procyanidin/carbohydrate complexes whose chemistry prevents acid hydrolysis under the conditions we used. Phenolics I– and II–acetone contained higher molecular weight procyanidins with mDPs estimated to be 6.1 and 15.3, respectively. Based on the weighted averages of the individual fractions, the mDP of the entire phenolic population was estimated to be 7.2. Catechin–epicatechin ratios of the free monomers and for each of the *I. goldmanii* LH-20 fractions are listed in Table 3. Catechin is the predominant monomeric form and the most common procyanidin initiator unit, particularly in the higher molecular weight polymers. In contrast, epicatechin comprises more than half of the extender units in all *I. goldmanii* fractions.

I. umbellifera Phenolics. I. umbellifera phenolics, like *I. goldmanii* phenolics, are composed entirely of compounds based on catechin and epicatechin. In contrast to *I. goldmanii*, however, *I. umbellifera* flavanols are each substituted at the C3 position with either a simple hexose or a hexose that is mono- or disubstituted with a cinnamoyl ester (see Figure 1b). These conclusions are based on several lines of evidence. Purification and rigorous structural characterization of several *I. umbellifera* flavans (Lokvam et al., 2004) yielded the building blocks of these compounds: catechin/epicatechin, hexose (glucose in each case that was explicitly determined), and cinnamate. ESI mass spectra showed that all observed molecular masses in *I. umbellifera*

TABLE 3. CATECHIN–EPICATECHIN RATIOS OF *I. goldmanii* PHENOLICS

	Free monomer	Extender units	Lower units	Full fraction
Phenolics I–MeOH	18.7	0.9	1.9	1.3
Phenolics I acetone	–	0.7	4.5	1.0
Phenolics II–acetone	–	0.5	4.8	0.6

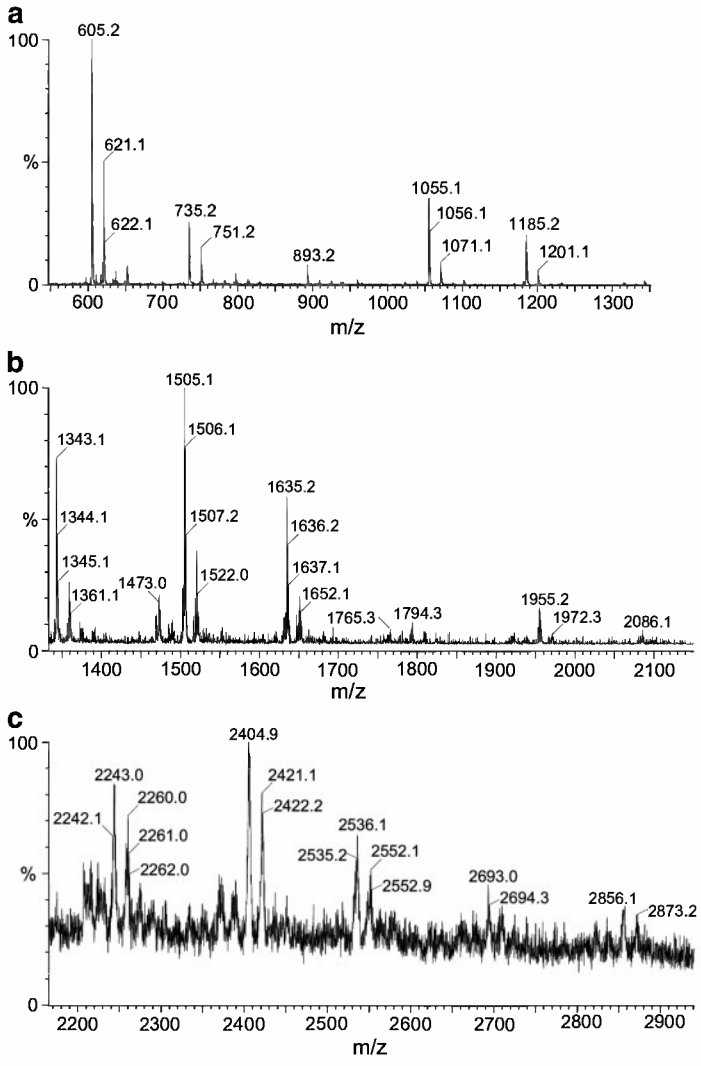

FIG. 3. ESI mass spectra of *I. umbellifera* phenolics. (a) Shows masses from 550 to 1350 amu, (b) shows masses 1350 to 2150, and (c) shows masses from 2150 to 2950 amu. In the positive ion mode, M + Na and M + K are the dominant molecular ions observed in spectra of *I. umbellifera* phenolic extracts. Peaks are labeled by mass (M + Na ions). For structural inferences, see Table 2.

TABLE 4. STRUCTURAL INFERENCES BASED ON MASS SPECTRA OF *I. umbellifera* PHENOLICS

$(M + Na)^+$	Flavan no.	Pyranose no.	Cinnamate no.
605.2	1	1	1
735.2	1	1	2
893.2	2	1	1
1,055.1	2	2	1
1,185.2	2	2	2
1,343.1	2	2	3
1,473.0	3	2	2
1,505.1	3	3	1
1,635.1	3	3	2
1,765.2	3	3	3
1,794.3	4	3	1
1,955.2	4	4	1
2,086.1	4	4	2
2,243.0	5	3	3
2,404.9	5	4	2
2,536.1	5	5	2
2,693.1	6	5	1
2,856.1	6	6	1

phenolics can be accounted for by combinations of the known monomeric subunits (Figure 3, Table 4, and the following section). *I. umbellifera* flavanols, unlike mixtures of unsubstituted *I. goldmanii* procyanidins, separate cleanly by ODS HPLC. DAD chromatograms of *I. umbellifera* phenolics showed over 40 (mostly minor) peaks having UV absorbance spectra nearly identical to the purified monomeric and dimeric compounds already structurally characterized. In addition, evaporative light scattering detection showed only minor contributions to the phenolics fraction mass from non-UV-absorbing components.

The ESI mass spectrum of *I. umbellifera* phenolics (Figure 3, Table 4) was recorded between 200 and 4000 amu. It showed monomer molecular masses of 605.2 and 735.2, and polymer masses between 893.2 and 2856.1 amu. These latter correspond to polymer sizes ranging from dimer (monoglucosyl, monocinnamoyl) to hexamer (hexaglucosyl, monocinnamoyl). As with *I. goldmanii*, no evidence of multiple charging was observed. The monomer and dimer concentrations and mDP of *I. umbellifera* phenolics were estimated following separation by HPLC and detection by complementary modes, mass and evaporative light scattering (Figure 4). Monomeric forms comprise approximately 30% of the total phenolic mass, and dimeric forms an additional 37%. Following mass correction of each peak to molar flavanol equivalents, the mDP of the *I. umbellifera* polymer population was estimated to be between 2.2 and 3.4.

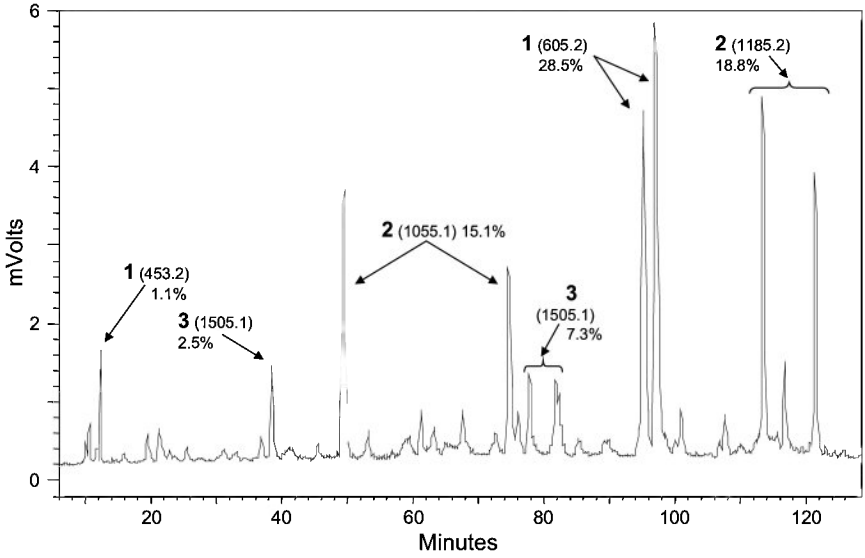

FIG. 4. HPLC chromatogram of *I. umbellifera* phenolics as detected by evaporative light scattering. Main peaks are labeled by flavanol number, mass (M + Na ions), and area percent of the full integrated chromatogram. For structural inferences, see Table 2.

Total Phenolics Mass. The extraction procedure used removed approximately 30% of young leaf DW as phenolics from both *I. goldmanii* and *I. umbellifera* (Table 2). In both species, approximately 70% of the extractable phenolics dissolved into aqueous EtOH. A further 30% of the extractable phenolics was removed using aqueous acetone. In addition to soluble leaf phenolics, butanol/HCl extraction of the marc produced a dense cyanidin solution, the result of acidolysis and oxidation of carbohydrate-bound procyanidins (Shen et al., 1986). The extraction removed approximately 32 and 52%, respectively, of the starting mass of the marcs of *I. goldmanii* and *I. umbellifera* (Table 5). The exact

TABLE 5. PRE- AND POST-ACID DIGESTED MASSES AND BIOACTIVITY OF *I. goldmanii* AND *I. umbellifera* MARC

	% Leaf DW	GRC[a]	*P*
I. goldmanii marc (+) phenolics	52	0.12	<0.05
I. goldmanii marc (−) phenolics	35	0.69	<0.01
I. umbellifera marc (+) phenolics	39	0.12	<0.05
I. umbellifera marc (−) phenolics	18	0.87	n.s.

[a] Growth relative to the control at a single concentration (35% of dry weight).

proportion of the extracted mass that was phenolic was not determined, but this proportion was likely considerable.

Procyanidin–Protein Binding. Procyanidin–protein binding curves were obtained for phenolics from both species. The linear portions were 0.125–1.0 mg phenolics/ml for *I. goldmanii*, and 0.125–2.5 mg phenolics /ml for *I. umbellifera*. In both cases, r^2 values were >0.99. The slopes of the linear portions of the binding curves were used to compare procyanidin–protein binding between the two species (Table 2). In comparison to *I. umbellifera* phenolics, approximately 3.5 times more *I. goldmanii* phenolics were involved in precipitating protein from a standard BSA solution. A similar analysis of *I. goldmanii* phenolics I–MeOH/acetone and phenolic II–acetone (Table 2) having mDPs of 2.0, 6.1, and 15.3, respectively, showed that protein-binding capacity increases sharply as a function of polymer length.

Bioactivities of I. goldmanii and I. umbellifera Young Leaf Metabolites. Whole-leaf extracts of the young leaves of *I. goldmanii* and *I. umbellifera* gave GI_{50} values (Table 2) of 1.1 and 2.8%, respectively, in the *H. virescens* larval growth assay. Assays of specific fractions (see "Methods and Materials") showed that phenolics accounted for more than 50% of the growth-inhibiting activity observed in the whole-leaf extracts of each species. The *I. goldmanii* phenolics fraction, however, was more than twice as inhibitory as that from *I. umbellifera* (GI_{50} = 0.59 and 1.45%, respectively, 95% CIs nonoverlapping). The marcs from both species were assayed before and after digestion with BuOH/HCl to remove tissue-bound phenolics (Table 5). When substituted into the insect diet at the mass percentage of cellulose (35%), marc (+) phenolics reduced insect growth by more than 80% relative to controls in both species. In contrast, the growth reduction caused by marc (−) phenolics was not significant for *I. umbellifera* and was approximately 30% for *I. goldmanii*.

DISCUSSION

Feeding trials with larvae of the noctuid moth *H. virescens* show that whole-leaf extracts of young leaves from *I. goldmanii* and *I. umbellifera* have markedly different growth-inhibitory capacities. Overall, the suite of extractable metabolites synthesized by the normally greening species, *I. goldmanii*, is nearly three times more inhibitory than those synthesized by the delayed-greening species, *I. umbellifera*. Isolation and bioassay of the phenolic portion of the extractable metabolites shows that most of the observed activity is caused by metabolites in this fraction. Detailed chemical characterization indicates that, in both species, the phenolic fraction consists of a complex mixture of monomeric and polymeric flavan-3-ols.

In *I. goldmanii*, flavan-3-ols are synthesized as monomeric catechin/epicatechin and oligomeric to highly polymerized procyanidins. The polymeric forms have an average length of approximately 7.2. In *I. umbellifera*, like *I. goldmanii*, catechin and epicatechin are the only phenolics that were detected. In this species, they occur exclusively as their 3-*O*-glycosyl or 3-*O*-(cinnamoyl)-glycosyl derivatives. Moreover, in contrast to *I. goldmanii*, *I. umbellifera* flavan-3-ols are dominated by lower molecular weight monomeric and dimeric forms.

Flavan-3-ols are a primary line of chemical defense in *I. goldmanii* and *I. umbellifera*. This class of compounds is synthesized at extraordinarily high concentrations in the young leaves of these species. The phenolic extraction protocol used in this study was not exhaustive, yet approximately 30% of the dry weight of both species was recovered as soluble flavans. In addition, an acid–butanol digestion of the marc showed the presence of appreciable amounts of tissue-bound flavans such that flavanol content may approach 50% of the leaf dry weight. In the *H. virescens* assay, the GI_{50} values for the whole phenolics extracts were 0.6% for *I. goldmanii* and 1.3% for *I. umbellifera*, concentrations that are far below what *Inga* herbivores encounter in the wild. Assays of pre- and post-acid-digested marc indicate that tissue-bound phenolics also contribute substantially to the overall phenolic toxicity. Bioassays such as these, against a naive, generalist herbivore, demonstrate the potency.

Structural differences in the phenolic metabolites expressed by *I. goldmanii* and *I. umbellifera* produce differentiable effects on the growth of *H. virescens* larvae. *I. goldmanii* phenolics are composed of unsubstituted procyanidin polymers. On a mass basis, this set of compounds is more than twice as inhibitory as the substituted forms produced by *I. umbellifera*. Similar results were observed in bioassays conducted in Panama with *Phoebis philea*, a lepidopteran that specializes on mimosoid legume leaves (Lokvam and Kursar, unpublished data).

Phenolics are among the most widely distributed and abundant secondary metabolites in plants (Bate-Smith, 1962, 1968), but their mode of action and ecological function have proven difficult to define, particularly with respect to insects. Following Feeny's (1969) observation that polyphenols could interfere with herbivore digestion by precipitating enzymes and/or dietary proteins, considerable attention was focused on the defensive role of high molecular weight phenolics (Feeny, 1976; Rhoades and Cates, 1976; Swain, 1979; Zucker, 1983). Their mechanism of action, however, remains obscure. The "tannin–protein binding" mode of action has been questioned since (1) the guts of many herbivorous lepidoptera, with their high pHs and detergent content, are well adapted to prevent formation of polyphenol/protein complexes (Martin and Martin, 1984; Blytt et al., 1988), and (2) this mechanism has never been demonstrated *in vivo*. Martin et al. (1987) showed that in *Manduca sexta* gut fluid hydrolyzable tannin–RUBISCO ratios must be at unity or greater for appreciable protein precipitation to occur. They suggest that such high

polyphenol/protein ratios would only rarely be encountered in nature, and that protein precipitation is, therefore, probably not an ecologically relevant process. The extraordinarily high procyanidin content that we observe in young *Inga* leaves, however, argues against this point. At concentrations of 25–30% leaf dry weight, procyanidins could inhibit digestion through protein binding.

Simple phenolics do not precipitate protein, yet have been shown to have strong negative effects on insect herbivores. For instance, functioning as pro-oxidants, the *o*-diphenols, caffeic and chlorogenic acid, were shown to alter gut chemistry in *Helicoverpa zea* (Summers and Felton, 1994), and are correlated with low larval growth rates in that species. These compounds function as toxins by degrading the gut epithelium. A distinction can be made then between the activity of *o*-diphenols (which include procyanidins) as outright toxins and high molecular weight phenolics as "digestibility reducers" (Duffey and Stout, 1996). In a chemically complex group like the flavan-3-ols, the distinction must allow for considerable functional overlap (Butler et al., 1986; Blytt et al., 1988), but it is likely that there are multiple modes of activity that occur simultaneously.

Using LH-20, we separated *I. goldmanii* procyanidins into size classes having mean degrees of polymerization of 2.0, 6.1, and 15.3. The BSA precipitation assay showed that there is a strong positive correlation between protein-binding capacity and procyanidin molecular weight, a finding that is in agreement with earlier studies (Kumar and Horigome, 1986; Horigome et al., 1988). Bioassays showed, however, that both the low and high molecular weight fractions were equally inhibitory to *H. virescens* larvae. Similarly, comparisons of the BSA binding characteristics of *I. goldmanii* and *I. umbellifera* phenolics showed a pronounced difference in binding efficiency. The simple, unsubstituted *I. goldmanii* phenolics were approximately four times more efficient than the substituted forms from *I. umbellifera*. This is almost certainly due to the presence in *I. goldmanii* phenolics of high molecular weight procyanidins. Nevertheless, *I. umbellifera* phenolics are potent growth inhibitors for larvae of *H. virescens*. Assuming that *in vitro* BSA precipitation approximates the *in vivo* interaction between procyanidin and dietary protein, our data are consistent with a multiple-mode view of phenolic bioactivity in *Inga*: oxidative stress due to the presence of *o*-diphenols, for example, and protein precipitation due to high molecular weight procyanidins. *H. virescens* is not a suitable assay organism for testing the multiple-mode concept. A rigorous bioassay will require a specialist herbivore, one that can tolerate diets containing the high levels of phenolics found in *Inga* leaves.

Simple procyanidins are likely the primitive form in this class of compounds. It appears that a novel set of selection pressures has led to a derived form of phenolic metabolites in *I. umbellifera*. The evolution of the delayed-greening/rapid expansion phenotype in the young leaves of *I. umbellifera* has likely imposed physiological constraints on the production

and storage of phenolics at the high concentrations seen in these species. The derived phenolics in *I. umbellifera* may have diverged from the ancestral form in parallel with the divergence in developmental strategy (Kursar and Coley, 2003). A more thorough knowledge of the phenolic chemistry of other delayed-greening *Inga* species, as well as an understanding of the whole-genus phylogenetic relationships, will certainly shed some light on this question.

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