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DIVERGENT DEFENSIVE STRATEGIES OF YOUNG LEAVES IN TWO SPECIES OF *INGA*

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Abstract. In the recently radiated genus *Inga* (Fabaceae), few nucleotide substitutions have accumulated among species, yet large divergences have occurred in defensive phenotypes, suggesting strong selection by herbivores. We compared herbivory and defenses of young leaves for *I. goldmanii*, a more derived species that follows a "defense" strategy, and *I. umbellifera*, a more basal species that follows an "escape" strategy. The two species suffered similar rates of herbivory (22% of the leaf area eaten during expansion) but were attacked by different communities of herbivores. *I. goldmanii* relied heavily on extra-floral nectaries and on a diversity of effective secondary metabolites, while *I. umbellifera* minimized damage through rapid leaf expansion and synchronous flushing. The major classes of secondary compounds in both species were flavanoids and non-protein amino acids; however, there were large differences in structure, biosynthetic pathways, and efficacy against herbivores. Growth rates of lepidopteran larvae were significantly lower when fed artificial diets with crude extracts from *I. goldmanii* as compared to *I. umbellifera*. Flavanoids accounted for the majority of growth reduction in both species. *I. umbellifera* had more unusual flavanoids and a non-protein amino acid not reported from plants, but the more common flavanoids found in *I. goldmanii* were more bioactive against herbivores. *I. goldmanii* also had greater ant visitation to extrafloral nectaries, suggesting that there was no trade-off between biotic and chemical defenses. In contrast, young leaves of *I. umbellifera* expanded more rapidly, minimizing the window of vulnerability before toughening. Resources for rapid expansion may have been reallocated from chloroplast development as *I. umbellifera* delayed the greening process until after full leaf expansion. Leaves were also produced synchronously, which can satiate herbivores and reduce damage. These defense differences are reflected in almost completely nonoverlapping herbivore faunas and the more frequent occurrence of generalists on *I. umbellifera*. To understand why defenses have evolved, it is important to view them in light of the herbivore community as well as in the context of the other co-occurring traits. We hypothesize that the effectiveness of chemical defenses determines whether a species follows the evolutionary path of "defense" or "escape" strategies.

Key words: *Barro Colorado Island; bioassays; flavanoids;* Heliothis; *herbivory;* Inga; *leaf development; non-protein amino acids;* Phoebis; *plant defenses.*

INTRODUCTION

Plants and herbivores comprise >50% of the organisms on earth, and their interactions have profound implications for both ecological and evolutionary processes (Ehrlich and Raven 1964, Crawley 1983, Rosenthal and Berenbaum 1992). These interactions have led to the evolution of a staggering diversity of plant defenses, including morphological and chemical traits, extrafloral nectaries, phenological escape, and low nutritional quality. Understanding the macroevolutionary trends, and the selective factors that may have led to different combinations of defensive traits in different species has been addressed by a variety of approaches. To contribute to this discussion, we quantified a broad suite of defensive traits in two congeners in the Neotropical genus *Inga* (Fabaceae: Mimosoideae), with the goal of elucidating constraints and advantages of different trait combinations.

Plant–herbivore interactions are very important in the tropics, where strong reciprocal selection has led to both higher rates of herbivory and greater investment in defenses for tropical as compared to temperate trees (Levin and York 1978, Coley and Aide 1991, Basset 1994, Marquis and Braker 1994). The majority of herbivore damage in the tropics occurs during the short

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TABLE 1. Observed and predicted associations among defensive traits of young leaves.

Characteristic	Escape	Defense
Observed defense syndromes		
Expansion of young leaves	fast	slow
Nitrogen content	high	low
Chloroplast development	delayed	normal
Synchrony of leaf production	high	low
Ant defense	low	high
Predicted chemical defenses		
Amount	low	high
Bioactivity	low	high
Diversity	low	high
Biosynthetic complexity	low	high

window when leaves are young and expanding (Marquis 1991, Marquis and Braker 1994). For example, leaves of shade-tolerant tropical species live for several years, yet 75% of the lifetime damage occurs during the few weeks that leaves are expanding (Coley and Aide 1991). This is due, in part, to the inevitable fact that growing tissue cannot be tough and requires additional enzymes for growth processes, causing all young leaves to be tender and nutritious.

Although young leaves receive the most damage, species vary six-fold, losing from 12–74% of their area during leaf expansion (Coley and Barone 1996). What factors are responsible for this wide range of damage rates to young leaves? A survey of the defensive traits of young leaves from over 200 unrelated species in Africa, Southeast Asia, and the Neotropics has shown that their defenses are organized into suites of traits, strongly suggestive of convergent evolution (Coley and Barone 1996, Kursar and Coley 2003). Although species fall along a continuum, for simplicity we classify them into two "syndromes": those that have well-defended young leaves ("defense" syndrome) and those that rely on rapid expansion to minimize the period when leaves are young and most vulnerable to herbivores ("escape" syndrome; Table 1) (Kursar and Coley 2003).

Analogous to Feeny's (1976) theory of plant defenses, "escape" species allocate energy to rapid leaf expansion, minimizing the period when leaves are tender and vulnerable to herbivores. The most rapid expanders have leaves that double in size in less than a day, an impressive growth rate that presumably requires high levels of enzymes (Coley and Barone 1996, Kursar and Coley 2003). Although the ideal defense combination would be fast expansion and low nitrogen, this is physiologically impossible. "Escape" species also delay chloroplast development until the leaf is full size. Chloroplasts contain high concentrations of proteins and lipid-rich membranes, greatly adding to the energy and nitrogen content of a leaf. We suggest that by delaying chloroplast development until the leaf is fully expanded, these resources could be reallocated to rapid expansion (Kursar and Coley 1992*a*, *b*). Escape species also produce leaves synchronously to satiate specialist herbivores.

In contrast, young leaves of "defense" species have a different suite of defensive traits. They expand slowly and have low concentrations of nitrogen, green normally, and produce leaves continuously (Kursar and Coley 2003). Extra-floral nectaries, which are only active on young leaves, are more common in "defense" species because continuously produced young leaves are a more reliable food source for ants (Coley and Kursar 1996). Although chemical defenses appear to be critical in determining the amount of herbivory and the suite of associated traits, little is known about the secondary metabolites of young tropical leaves.

Here we present data from *I. goldmanii* and *I. umbellifera*, two shade-tolerant tree species in the Fabaceae (subfamily: Mimosoideae) that are widespread in the Neotropics (see Plate 1). By quantifying the traits outlined in Table 1, we can see if patterns in *Inga* parallel those seen in general surveys (Coley and Kursar 1996, Kursar and Coley 2003), and, because both species are in the same genus, comparisons of chemical defenses can be made. We also present a preliminary phylogeny of *Inga*, allowing us to place the defensive traits within an evolutionary context.

MATERIALS AND METHODS

Study site

The study was conducted on Barro Colorado Island (BCI; 9°09′ N, 79°51′ W) in the Republic of Panama, a field site administered by the Smithsonian Tropical Research Institute (STRI). The forest is moist lowland forest (Croat 1978, Leigh and Windsor 1982, Leigh 1999) and receives 2600 mm of rain during an eight-month rainy season (Windsor 1990). Both study species are abundant and co-occur across the island.

Field measurements

Herbivory, phenology, and ant visitation data were collected monthly from March 2001 through November 2004 on 1–3 m tall saplings growing in the shaded understory. To quantify herbivory, young leaves were marked as they emerged from the bud and the percentage of area eaten was measured at the end of expansion (Kursar and Coley 2003). Missing leaves were scored as 100% damage. Plants or leaves that were obviously damaged by falling debris were not included. Fifty individuals of each species were marked and monitored monthly for leaf production (scored as presence/absence). The coefficient of variation (CV) for the percentage of individuals leafing each month was used to quantify synchrony in leaf production.

Censuses were conducted to determine the herbivore–host associations. The number of young leaves censused, and the number and morphospecies of any herbivore were noted. Where possible, distinctive damage patterns were also assigned to morphospecies or

PLATE 1. (Left) Young leaves of *Inga goldmanii* with ants visiting the extrafloral nectaries. (Right) Young leaves of *Inga umbellifera* (approximately 50% of full size) that hang limply and have low levels of chlorophyll and delayed greening. Photo credits: (left) Keryn Bromberg and Kathleen Rudolph; (right) Tara Sackett.

groups (e.g., leaf-cutting ants, gall type). Twenty-five percent of the damage events to leaf flushes could be assigned to a particular morphospecies.

Ant visitation to extra-floral nectaries was noted in censuses conducted between 10:00 a.m. and 2:00 p.m. Nectaries are only active on young leaves. For each leaf flush encountered, we noted the number of leaflets, number of nectarines, and the number of ants. Ants were classified as either effective or ineffective at defending the leaf against herbivores (K. Rudolph and K. Bromberg, *personal observation*). The following genera were classified as effective or active defenders: *Azteca*, *Ectatomma*, *Odontomachus*, and large-bodied Pseudomyrmecinae. *Ectatomma ruidum* comprised 55% of the ant visitors. Ineffective ants were *Wasmannia*, *Crematogaster*, *Pheidole*, *Camponotus*, *Solenopsis*, Myrmicinae, Dolichoderinae, and Formicinae.

Leaf expansion and chlorophyll

To determine how quickly young leaves expanded, we measured changes in leaf size with time and calculated the number of days required for the leaf to double in size (Kursar and Coley 1991). Many species of tropical plants delay chloroplast development until the leaf is full size (Kursar and Coley 1992*a*, *b*, *c*). Chlorophyll content provides an excellent measure of chloroplast development, as chlorophyll is highly correlated with the amount of rubisco, light-harvesting proteins, and photosynthetic capacity of leaves (Baker and Hardwick 1973, Kursar and Coley 1992*a*). Chlorophyll content was measured for young leaves that were between 50% and 70% of full size.

Leaf extractions

Extracts of leaves were made to test in bioassays and to characterize major secondary metabolites. Young leaves (5–80% of full expansion) were collected throughout BCI from non-census understory trees during periods of leaf flush between January 2001 and December 2003. To minimize sampling effects, no more than 30% of any given flush was removed from an individual tree in a single harvest. The fresh leaves were processed shortly after collection by maceration in first a Waring blender and then a Polytron (Brinkmann Instruments, Westbury, New York, USA) in ~4 ml of 95% EtOH per gram fresh mass of leaf. Suspensions (leaf solids + dissolved extractables) of each species were stored at −80°C until they were processed.

We also extracted leaves in water to more closely mimic the conditions found in caterpillar mouths and guts and to see if this would allow enzymatic reactions that might alter the toxicity of the extracts. In this procedure, leaves were cut in half and one half was processed in EtOH as described in the previous paragraph, and the other extracted similarly, but in water. After 30 min, 95% ethanol was added to the aqueous extract. This process was repeated for two separate leaf collections for each species and tested at four concentrations in our bioassays. There was no effect of treatment (ANOVA, $P = 0.28$) and no interaction of treatment with concentration ($P = 0.57$). Thus, we exclusively used the EtOH extraction for results presented in this paper.

Fractionation and characterization of secondary metabolites

The EtOH leaf suspension was further processed in Utah, USA, to obtain either a crude extract or specific chemical fractions. The chemical fractions were marc (cell walls, starch, and other insolubles) and five classes of extractables that were given general descriptive names based on their chemical properties: non-protein amino acids, flavanoids, organic acids, lipids, and proteins (Appendix A).

Non-protein amino acids of both *I. umbellifera* and *I. goldmanii* were purified by semi-preparative HPLC

and were structurally identified using 1 and two-dimensional nuclear magnetic resonance (NMR) spectroscopy (Unity iNOVA 500 MHz spectrometer, Varian Instruments, Palo Alto, California, USA) and electrospray mass spectrometry (ESI-MS; Finnigan LCQ, Thermo-Finnigan, Bremen, Germany). The flavanoid profiles of the two species were characterized using a combination of techniques. Flash chromatography on silica gel and semi-preparative reverse-phase (C_{18}) high-pressure liquid chromatography (HPLC) were used to purify monomeric and oligomeric components from flavanoids of both species. These were subsequently identified by their mass and NMR spectra. ESI-MS was used to characterize the polymeric structures of both species. Acid-catalyzed thiolysis in 5% toluene-α-thiol in MeOH mixed with an equal volume of MeOH adjusted to 0.2 N HCl was followed by HPLC analysis of the products was used to identify the monomeric units and the average polymer lengths for the *I. goldmanii* flavanoids. HPLC separation with diode array, evaporative light scattering, and ESI-MS detection were used to characterize the suite of *I. umbellifera* flavanoids.

Bioassays

We compared larval growth on artificial diets with and without the addition of plant extracts using larvae of *Heliothis virescens* (Lepidoptera: Noctuidae), a generalist herbivore of tropical origin, and *Phoebis philea* (Lepidoptera: Pieridae). *P. philea* feeds on Fabaceae, especially *Cassia* species, but not on *Inga* species, and is found in the southern United States and much of the Neotropics, including Panama (Appendix A). For *Heliothis*, neonate larvae were grown for 8 d on the artificial diet. Because neonate *Phoebis* will not eat artificial diet, they were reared on *Cassia* until they were larger (90 mg or 20% fresh mass of the final instar), and then moved to artificial diet for 3 d. Growth reductions could arise from repellent substances that reduce consumption or from direct toxicity. As we were interested in the combined effect on insect performance, we refered to metabolites that have a more negative impact on growth as being more bioactive.

Phylogenetic analyses

To gain some preliminary insights into the phylogenetic relationships of *I. goldmanii* and *I. umbellifera*, DNA sequences of the chloroplast *trnL-F* intron and spacer were generated and added to the data matrix of Richardson et al. (2001). This contains accessions of 31 species of *Inga*, representing 10 of the 14 sections of the genus and its full geographic range. DNA extraction, polymerase chain reaction (PCR), DNA sequencing, and parsimony analysis protocols followed Richardson et al. (2001).

Because of the low levels of sequence divergence for *trnL-F* between *Inga* species, and the consequent poorly resolved phylogeny, a second phylogenetic approach was adopted. For 14 accessions representing 13 *Inga* species, including *I. goldmanii* and *I. umbellifera*, DNA sequences were generated for three non-coding chloroplast regions, *trnL-F*, *atpB-rbcL*, and *trnD-T*. Species of *Hesperalbizia* and *Pithecellobium* were included in the study as an outgroup to root phylogenetic trees. In total, ~3130 base pairs of sequence were gathered for each accession. PCR and sequencing protocols are detailed in Appendix D. PCR primers and sequencing primers for *trnD-T* followed Grivet et al. (2001), and followed Chiang et al. (1998) for *atpB-rbcL*. DNA sequence alignment was carried out manually and was unambiguous. Because of the low number of terminal taxa in the resulting data matrix, parsimony analysis used an exact exhaustive search in PAUP* (Swofford 2000).

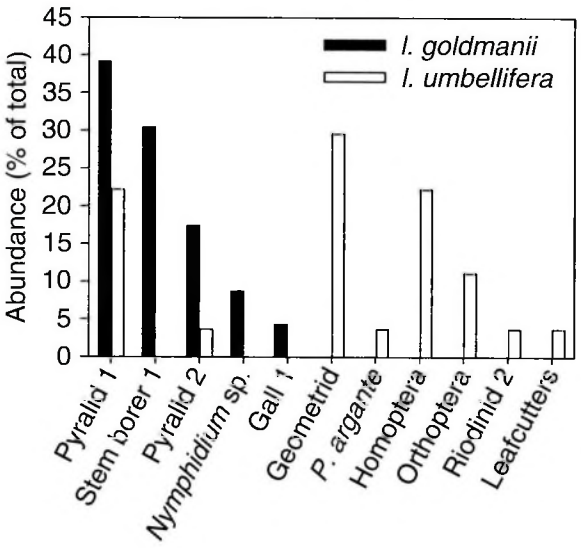

FIG. 1. Herbivore communities on *Inga goldmanii* and *Inga umbellifera*. Data include only those herbivores that were identified to morphospecies, or to order in the case of Homoptera and Orthoptera ($n = 50$). Abundance is the percentage of the total observations assigned to a particular herbivore morphospecies (e.g., sums to 100% for each plant species).

RESULTS

Herbivory

Herbivory to young leaves was similar, with both species losing ~20% of their leaf area during expansion (*I. goldmanii* lost 22.8% and *I. umbellifera* lost 21.4%; ANOVA, $P = 0.3$; Appendix B). However, the damage was done by different communities of herbivores (Fig. 1). Of the 11 morphotypes that we encountered in our surveys, only two species, both Pyralidae leaf-rollers, were found on both *Inga* species. Three herbivore species were restricted to *I. goldmanii* and six to *I. umbellifera*. Homoptera, Orthoptera, and leaf-cutting ants were found on *I. umbellifera*, but were never recorded from *I. goldmanii*. Coleoptera were occasionally found

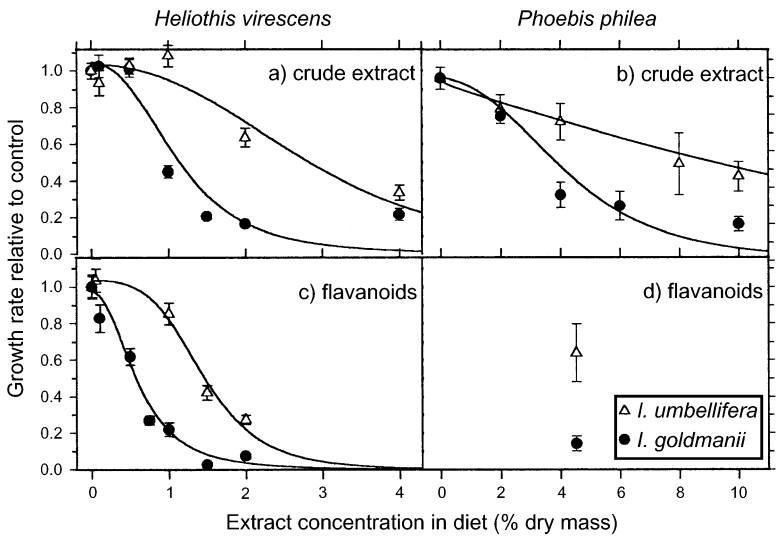

FIG. 2. Effects of total leaf extracts and flavanoids on growth of *Heliothis virescens*, a generalist, and *Phoebis philea*, a tropical legume feeder. Values are the growth rates relative to controls (mean ± SE). From these data, a GI_{50} value is calculated for the concentration of extract in the diet that reduces growth by 50% relative to the controls.

on both species, but because they were not separated into morphospecies, they were not included in the analysis. As *I. goldmanii* and *I. umbellifera* co-occur in the same range of microhabitats, differences in herbivore communities most likely reflect differences in defensive traits.

Feeding trials

Despite similar herbivory in the field, extracts from young leaves of *I. goldmanii* were significantly more bioactive in feeding trials with insect herbivores. When crude extracts were incorporated into artificial diets, growth rates of larvae were dramatically reduced (Fig. 2a). For the generalist, *Heliothis virescens*, extracts from *I. goldmanii* were more bioactive, causing a 50% reduction in growth (relative to the control) at only 1.1% of the diet. In contrast, a diet of 2.8% extract for *I. umbellifera* was required to reduce growth by 50%. The legume specialist tested in Panama, *Phoebis philea*, could tolerate much higher concentrations of extracts in the diet (Fig. 2b). Because *Phoebis* was tested at a larger size, we cannot determine if size or diet breadth caused its greater tolerance. Nonetheless, results were similar to the generalist, with *I. goldmanii* being approximately twice as bioactive as *I. umbellifera*.

We fractionated the crude extracts into a number of different classes of compounds in order to evaluate relative investments and efficacy in feeding trials (Table 2). Although we have found saponins in other *Inga* species, none were detectable in these two focal species. Dry mass investments in flavanoids, non-protein amino acids, proteins, organic acids/carbohydrates, and lipids were very similar between the two species. The major difference was that *I. umbellifera* had varying amounts of the essential amino acid, tyrosine.

Flavanoids were the most abundant and bioactive of the secondary metabolite classes (Table 2). For *Heliothis*, the GI_{50}'s for flavanoids were more than twice as high for *I. umbellifera*, requiring concentrations of 1.4% as compared to 0.6% to reduce growth by 50% (Fig. 2c). As with the crude extracts, *Phoebis* could tolerate higher concentrations than *Heliothis*, but the flavanoids from *I. goldmanii* were significantly more bioactive ($t = 3.17$, df = 12, $P < 0.01$; Fig. 2d).

Non-protein amino acids comprised 2–3% of the leaf dry mass (Table 2). They are generally considered to be defensive compounds (Rosenthal and Bell 1979); however, they had only marginal deterrence in our bioassays. When tested at 4% dry mass of the diet, a concentration higher than in the plant, growth was reduced to 72% of the control for *I. umbellifera* ($t = 2.4$, df = 30, $P < 0.025$) and to 70% of the control for *I. goldmanii* ($t = 2.33$, df = 30, $P < 0.025$).

In addition to the groups of secondary metabolites, we also found anomalously high concentrations of a primary metabolite, tyrosine, in the young leaves of *I. umbellifera*. The amino acid ranged from negligible amounts in some extracts to 10% of the dry mass of other extracts. Although tyrosine was not as bioactive as the flavanoids ($GI_{50} = 4.0$; Table 2), larval growth was reduced to <5% of controls when fed concentrations similar to those found in the young leaves.

Lipids, organic acids, and proteins showed little bioactivity in either species in concentrations exceeding what was found in the plant (Table 2). However, the organic acid fraction of *I. goldmanii* reduced growth to 70% of the controls ($t = 2.32$, df = 30, $P < 0.05$).

TABLE 2. Percentage of dry mass investments in different classes of compounds and bioactivity (GI_{50}, GRC) in bioassays with *Heliothis virescens*.

A) GI_{50} Compound	Dry mass (%)		*I. goldmanii*			*I. umbellifera*		
	I. goldmanii	*I. umbellifera*	Mean	r^2	SE	Mean	r^2	SE
Water (% of fresh mass)	73.5	80.6						
Total metabolites (crude)	48.2	61.3	1.11	0.80	0.13	2.77	0.75	0.36*
Flavanoids	29.4	31.2	0.59	0.88	0.09	1.45	0.97	0.06*
Tyrosine	...	0–10	...	...	...	4.00	0.99	0.09

B) GRC Compound	Dry mass (%)		*I. goldmanii*			*I. umbellifera*		
	I. goldmanii	*I. umbellifera*	Mean	Max con. (%)	*P*	Mean	Max. con (%)	*P*
Non-protein amino acids	3.3	2.3	0.70	4	<0.05	0.72	4	<0.05
Water soluble proteins	1.6	3.8	1.13	5	NS	2.03	5	NS
Organic acids and carbohydrates	5.1	5.2	0.70	5	<0.05	0.77	5	NS
Lipids	5.9	7.7	0.73	7.4	NS	1.09	7.4	NS
Marc + bound phenolics	51.8	38.7	0.12	35	<0.05	0.12	35	<0.05
Marc − phenolics	35.2	18.2	0.69	35	<0.01	0.87	35	NS

Notes: Bioactivity is expressed as either the GI_{50}, the percentage of dry mass of compound in the diet that reduces growth by 50% relative to the control, or as GRC, the growth relative to the control at the highest concentration tested (max. con.). For example, growth was 0.70 relative to the control for larvae fed diet containing 4% dry mass of *Inga goldmanii* non-protein amino acids. NS indicates that there was no significant growth reduction. An asterisk indicates that there was a significant difference ($P < 0.05$) between the GI_{50} values of *I. goldmanii* and *I. umbellifera*. No tyrosine was detected in *I. goldmanii*.

In the initial extraction protocol, the marc (cell walls, starch, and other insolubles) was quite bioactive in both species. This was apparently due to flavanoids that were tightly bound to the cell wall material. When the marc was thoroughly extracted to remove all bound flavanoids, there was still a slight growth inhibition for *I. goldmanii*, but not for *I. umbellifera* (Table 2).

Divergence of secondary metabolites

Chemical characterizations demonstrate that *I. umbellifera* and *I. goldmanii* young leaf extracts have pronounced structural differences in two classes of secondary metabolites, the non-protein amino acids and the flavanoids (Fig. 3). *I. umbellifera* has two forms of non-protein amino acids: N-methyl-4-hydroxy proline and 5-amino-4-hydroxy-pentanoic acid. *I. goldmanii* has four major non-protein amino acids: two mono-hydroxylated and two di-hydroxylated pipecolic acids.

Similar evidence for divergence in secondary metabolite expression is observed in the flavanoids (Fig. 3). Though the flavanoids of both species are based on catechin and epicatechin, they assume markedly different forms. NMR and mass spectral analyses of purified *I. goldmanii* flavanoids show that they are monomers of catechin and epicatechin and their polymers (condensed tannins; J. Lokvam and T. A. Kursar, *unpublished manuscript*). Analysis of the full *I. goldmanii* flavanoid fraction shows that the polymer length ranges up to 13, with a mass-averaged degree of polymerization of 7.2. Complete structural characterization of the *I. umbellifera* flavanoids showed that this fraction is composed of 3-O-(cinnamoyl)glucosides of catechin and epicatechin in both monomeric and polymeric forms (Lokvam et al. 2004). ESI-MS analyses showed the presence of polymers up to hexamers. However, the *I. umbellifera* flavanoids are dominated by monomers and dimers, with a mass-averaged degree of polymerization estimated at 2.2 to 3.4. Hence, the masses of the *I. goldmanii* condensed tannins are much less than for *I. goldmanii*.

Biotic defenses

In addition to having more effective chemical defenses, *I. goldmanii* also has higher levels of biotic defenses (Table 3, Plate 1, and Appendix C). It has more extrafloral nectaries per leaflet, but fewer per square meter of leaf area than *I. umbellifera*. However, in *I. goldmanii*, almost five times as many ants are attracted to each nectary, which results in twice as many ants per square meter of leaf ($P < 0.001$). If only the most aggressive species of ants are considered (see *Methods* for species list), the patterns are similar, with almost twice as many ants patrolling per square meter in *I. goldmanii* (Table 3).

Phenological defenses

The synchronous production of young leaves can satiate populations of herbivores and has thus been considered a phenological defense (Aide 1988, 1993). Young leaves of *I. umbellifera* were produced in fewer, more synchronous flushes than those of *I. goldmanii* during the three-year survey (Fig. 4). In both species, ~15% of the individuals produced leaves each month; however, the coefficient of variation, a measure of synchrony, was significantly higher for *I. umbellifera* (Table 3).

Developmental defenses

Because young leaves are particularly attractive to herbivores, the longer a young leaf takes to reach full

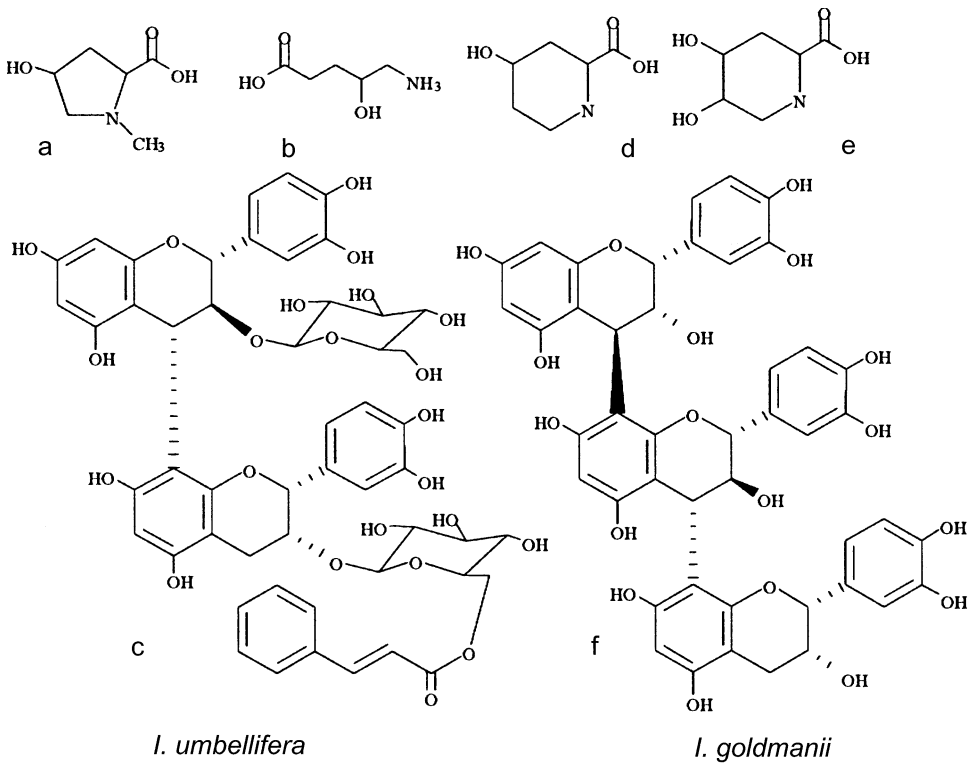

FIG. 3. Representative secondary metabolites. For *Inga umbellifera*, non-protein amino acids include (a) N-methyl-4-hydroxy-proline and (b) 5-amino-4-hydroxy-pentanoic acid; a representative flavanoid is (c) catechin-3-*O*-β-glucopyrano-(4α → 8)-epicatechin-3-*O*-β-gluco(6-cinnamoyl)pyranoside. For *I. goldmanii*, non-protein amino acids include (d) 4-hydroxy-pipecolic acid and (e) 4,5-dihydroxy-pipecolic acid; a representative flavanoid is (f) epicatechin-4β → 8-catechin-4α → 8-epicatechin.

size and toughen up, the longer it is vulnerable to herbivores. Thus, rapid leaf expansion should reduce the amount of herbivory suffered while leaves are young (Aide and Londoño 1989, Ernest 1989). Young leaves of *I. umbellifera* expand significantly faster than those of *I. goldmanii*, taking 2.07 days to double in size as compared to 2.4 days, respectively (Table 3 and Plate 1).

Rapid expansion must require high concentrations of nutrients and energy to fuel this growth (Kursar and Coley 1991). One function from which resources can be reallocated is development of the photosynthetic apparatus (Kursar and Coley 2003). *I. umbellifera* appears to be following this strategy, as chlorophyll content in young leaves, an indicator of chloroplast development (Kursar and Coley 1992*a*), is significantly lower than in *I. goldmanii* (Table 3 and Plate 1).

Phylogenetic relationships

Both phylogenetic analyses indicate that *I. goldmannii* and *I. umbellifera* are distantly related within *Inga*, and are not sister species (Appendix D). This

TABLE 3. Ant visitation to extrafloral nectaries, synchrony of leaf production, leaf expansion rates, and chlorophyll content for young leaves of *Inga goldmanii* and *Inga umbellifera*.

Species	No. ants/ m^2 leaf	Plant flushing/mo		Doubling time (d)	Chlorophyll (mg/dm^2)
		Mean ± SE (%)	CV		
I. goldmanii	3.55	15.1 ± 2.0	90.8	2.40 ± 0.04	1.31 ± 0.07
I. umbellifera	1.65	13.4 ± 2.8	138.3	2.07 ± 0.15	0.58 ± 0.04
P	<0.001	NS	<0.001	<0.001	<0.001

Notes: For additional ant data, see Appendix C. Phenology of young leaf production was censused monthly for 3.75 years, and we calculated the percentage and coefficient of variation (CV) of plants flushing each month. The rate of young leaf expansion was calculated as the number of days for a leaf between 20% and 80% of full size to double in size. Chlorophyll content was determined on young leaves at 50–80% of full size. Values are means ± SE. A *Z* test was used to compare the CV values. Other significant differences between species were determined by a *t* test.

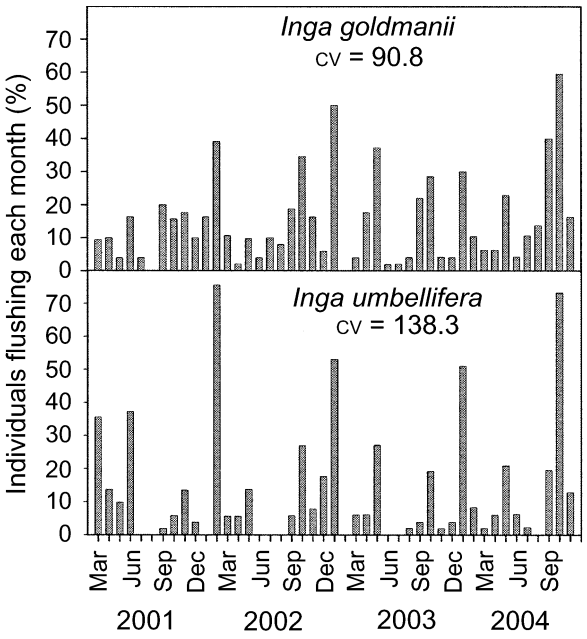

FIG. 4. Monthly production of young leaves of *Inga goldmanii* ($n = 50$) and *Inga umbellifera* ($n = 50$) censused monthly from March 2001 through November 2004.

conclusion is clear despite the inadequacies of both analyses: the *trnL-F* tree suffers from a lack of phylogenetic resolution because of few informative characters, and the tree based upon multiple loci sampled very few species. Both phylogenetic hypotheses suggest that *I. umbellifera* belongs to a more basally divergent lineage than *I. goldmanii*.

DISCUSSION

Selection by herbivores apparently can have rapid and profound effects on plant defensive strategies. In the recently radiated genus *Inga*, few nucleotide substitutions have accumulated among species (Richardson et al. 2001), yet large divergences have occurred in defensive phenotypes. *I. goldmanii* relies heavily on extra-floral nectaries and on a diversity of effective secondary metabolites, while *I. umbellifera* minimizes damage through rapid leaf expansion and synchronous flushing. Furthermore, the classes of secondary metabolites that are present in both species, flavanoids and non-protein amino acids, show large differences in structure, biosynthetic pathways and efficacy. These defense differences are reflected in almost completely nonoverlapping herbivore faunas and the more frequent occurrence of generalists on *I. umbellifera*.

Divergence in chemical defenses

In this study we attempted to examine all major classes of compounds in order to completely characterize the chemical defense profiles. The major classes of secondary metabolites for both species were flavanoids and non-protein amino acids, and investments were similar (Table 2). In *I. goldmanii*, the majority of the flavanoids are relatively large polymers of unsubstituted catechin and epicatechin (Fig. 3). This is in contrast to *I. umbellifera*, in which the flavanoids occur as relatively small polymers of 3-O-(cinnamoyl)glucosides of catechin and epicatechin, the majority of which are unpolymerized. The non-protein amino acids also appear to be synthesized by distinct pathways in the two species. *I. umbellifera* makes two non-protein amino acids that are based on five-carbon backbones and are likely associated with proline biosynthesis. *I. goldmanii* synthesizes four compounds based on a six-carbon skeleton likely associated with lysine biosynthesis (Meyer and Grobelaar 1986). As predicted (Table 1), *I. goldmanii* has a higher diversity of non-protein amino acids (four vs. two).

The non-protein amino acids of our focal species were only marginally bioactive in our bioassays with *Heliothis*. This is surprising, as most non-protein amino acids are considered to be defensive (Rosenthal and Bell 1979). However, *Heliothis* tolerates diets of 40% canavanine, a non-protein amino acid that is highly bioactive against most other Lepidoptera (Coromoto et al. 1997). Perhaps the mechanisms that allow it to consume such high concentrations of canavanine may provide some protection against other non-protein amino acids. In other studies, combinations of pipecolic acid and its mono- and dihydroxy derivatives are inhibitory to the blackbean aphid, *Aphis fabae*, at concentrations as low as 10^{-4} mol/L (Simmonds et al. 1988) and to fall armyworm, *Spodoptera frugiperda*, at concentrations as low as 0.1% of dry mass (Romeo 1984). In addition, Bernays and Chapman (1977) showed that sucrose diets containing pipecolic acid are bioactive against *Locusta migratoria* nymphs ($GI_{50} = 1\%$). These data suggest that the absence of a pronounced effect of the *Inga* non-protein amino acids on *Heliothis* may not be typical of their effects on other herbivores.

Tyrosine, an essential amino acid was present in concentrations up to 10% dry mass in some of our *I. umbellifera* extracts (Table 2). Tyrosine had a GI_{50} of 4.0% suggesting that the over-expression of this primary metabolite may play a defensive role. In bioassays with grasshoppers, Bernays also found bioactive effects of alanine ($GI_{50} = 2\%$) and glutamine ($GI_{50} = 7\%$), as well as some other essential amino acids, although tyrosine was not tested (Bernays and Chapman 1977; E. A. Bernays, *personal communication*).

Extra-floral nectaries

Both species have active extrafloral nectaries on the young leaves that effectively attract ants (Table 3 and Appendix C). However, *I. goldmanii* attracts more ants per square meter of leaf, particularly species thought to be aggressive. This results from greater ant attraction per nectary, perhaps due to better nectar rewards (not measured) and/or to more continuous young leaf production (Fig. 4). *I. goldmanii* also has more effective chemical defenses, so there does not appear to be a

trade-off between investment in chemical and biotic defenses for this species. Although a negative correlation between extrafloral nectaries and other defenses has frequently been reported (Koptur 1985, Folgarait and Davidson 1994, Dyer et al. 2001, Eck et al. 2001), this trade-off is not universal (Heil et al. 2002, Rudgers et al. 2004).

Escape and satiation

I. umbellifera is clearly less effectively defended by secondary metabolites or protective ants, yet rates of herbivory are similar to those on *I. goldmanii*. We suggest this is because of greater synchrony and rapid leaf expansion, two phenological traits that have been shown to reduce herbivore damage (Tables 3 and 4; Aide and Londoño 1989, Aide 1993).

As with secondary metabolites and biotic defenses, there are presumably costs associated with synchrony and rapid leaf expansion. Synchrony implies that resources are stored until there are enough to produce a flush of many leaves. Thus, compared to continuous leaf production, there is the opportunity cost of lost photosynthesis due to the delay associated with periodic flushing. Furthermore, stored reserves incur additional costs if they are actively defended.

The high energy and nutrient demands of rapid leaf expansion also represent costs. In multi-species surveys we have found a negative correlation between expansion rate and chlorophyll content ($r^2 = 0.57$, $n = 51$, $P < 0.001$; Fig. 4 in Kursar and Coley 2003). Because rapid expansion requires additional resources, construction of the photosynthetic machinery is delayed until after the leaf stops expanding (Kursar and Coley 1992*a*, *b*, *c*). This pattern was also seen in this study, as *I. umbellifera* had more rapid leaf expansion, but a lower chlorophyll content (Table 3).

Trade-offs and the evolution of defense syndromes

We propose that "defense/escape" syndromes evolved because of constraints due to the effectiveness of secondary metabolites against both specialist and generalist herbivores as well as the trade-offs in resource allocation to leaf growth or defense (Kursar and Coley 2003). One possible evolutionary outcome that would lead to a defense strategy is an "arms race" between herbivores and plants wherein continual selection on plant secondary metabolites maintains effective defense against herbivores (Ehrlich and Raven 1964, Berenbaum et al. 1986, Bowers 1990, Kareiva 1999, Thompson 1999, Rausher 2001). Over evolutionary time, we would expect to see increased diversity and bioactivity of compounds and perhaps more biosynthetically derived compounds if plants are elaborating on existing chemical backbones (e.g., Berenbaum 1978, Berenbaum et al. 1986, Laue et al. 2000). Alternatively, if resistance evolves in herbivores (i.e., secondary metabolites become ineffective), then selection will favor the escape syndrome, similar to the defense theory proposed by Feeny (1976). In the escape strategy plants shift resources from other functions to increase leaf expansion rates and shorten the period of vulnerability. One possibility is to delay investment in the photosynthetic machinery. Another is to shift resources from the ineffective secondary metabolites to growth. Once reductions in secondary metabolism have evolved, they may be less likely to reverse if they involve loss of function of structural genes and more likely to reverse if they involve changes in gene expression, such as has been shown for genes that determine symmetry (e.g., Hileman et al. 2003). Thus, the escape strategy seems to be a mechanism for species to minimize damage given the failure of their secondary metabolites to protect them from herbivores.

How well do the data from *I. goldmanii* and *I. umbellifera* follow the above predictions for "defense" and "escape" strategies (Table 1)? As predicted, *I. goldmanii* had more effective secondary metabolites as well as greater densities of protective ants, while *I. umbellifera* relied on escape by synchronously producing rapidly expanding young leaves. However, the predictions that secondary metabolites of *I. goldmanii* should be more diverse and biosynthetically derived were only partially supported. For example, although *I. goldmanii* had four non-protein amino acids, one of the two found in *I. umbellifera*, 5-amino-4-hydroxypentanoic acid, had never before been reported from any plant. Interestingly, *I. umbellifera* is the more basal species (Appendix D). In addition, although the flavanoids of *I. goldmanii* were more bioactive, as predicted, they are not more diverse, nor more biosynthetically derived. In fact, the class of flavanoids found in *I. umbellifera*, acyl-glucosides of catechin or epicatchin, is rare, having been reported from only two other plants (Morimoto et al. 1985, Murakami et al. 1985), and the specific compounds are known only from *I. umbellifera*. But the form of flavanoids in *I. goldmanii*, polymers of catechin and epicatechin, is extremely widespread, occurring in all classes of vascular plants (Swain 1979).

The different suites of defensive traits of *I. goldmanii* and *I. umbellifera* parallel those seen in surveys of many unrelated species (Kursar and Coley 2003), underscoring that these combinations are adaptive and that trade-offs and constraints powerfully shape the defense options. The investment patterns in secondary metabolites match many of the predictions for "defense/escape" trade-offs, but questions regarding their evolution and interplay with other defensive traits require further exploration. Nonetheless, we suggest that the effectiveness of secondary metabolites may play a key role in shaping the observed "defense/escape" syndromes of young leaves, particularly in biomes where biotic interactions are strong, such as tropical rain forests.

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APPENDIX A

A description of methods for extraction and fractionation of secondary metabolites and for artificial diets used in bioassays is presented in ESA's Electronic Data Archive: *Ecological Archives* E086-140-A1.

APPENDIX B

An ANOVA table showing herbivore damage to young leaves of *Inga goldmanii* and *I. umbellifera* is presented in ESA's Electronic Data Archive: *Ecological Archives* E086-140-A2.

APPENDIX C

A table showing the densities of extra floral nectaries and ants on young leaves of *Inga goldmanii* and *I. umbellifera* is presented in ESA's Electronic Data Archive: *Ecological Archives* E086-140-A3.

APPENDIX D

A figure showing phylogenetic relationships of *Inga* species based upon chloroplast *trnL-F* DNA sequences is presented in ESA's Electronic Data Archive: *Ecological Archives* E086-140-A4.