Allelochemic function for a primary metabolite: the case of L-tyrosine hyper-production in *Inga umbellifera* (Fabaceae)[1]

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Young leaves of tropical forest trees experience far higher herbivory pressure than mature leaves of the same species. Selection on young leaves has led to diverse forms of defense chemical expression. Though most allelochemicals are secondary metabolites, allelochemic function for a primary metabolite remains a possibility. We recently observed this phenomenon in the young leaves of *Inga umbellifera*, which accumulate the protein amino acid L-tyrosine to very high levels. We isolated L-tyrosine from young leaves of trees in Panama and characterized it using spectroscopic and chemical means. We chromatographically quantified leaf L-tyrosine levels across a range of developmental stages, showing that it was present in the youngest leaves and that its concentration increased throughout the period of expansion, reaching an average maximum of ca 10% of leaf dry mass in late-stage young leaves. This chemical phenotype was seen to be highly leaf-age specific: Free tyrosine was only present in mature leaves at very low levels. In bioassays with larvae of the noctuid moth *H. virescens*, L-tyrosine proved to be a potent growth inhibitor when added to artificial diet at 10% of dry mass. This suggests that a rarely observed defense strategy occurs in young *I. umbellifera* leaves, a hyper-produced primary metabolite functioning as an allelochemical.

Key words: 5-amino-4-hydroxy-pentanoic acid; Barro Colorado Island; chemical defense; Fabaceae; *Inga umbellifera*; Panama; primary metabolite; tyrosine.

Secondary metabolites are important mediators of ecological interactions between plants and their environment. One interaction where they play a central role is defense against herbivory. This is particularly true in the expanding young leaves of tropical forest trees. Unlike mature leaves, which following full expansion become highly toughened by structural tissues, young leaves are soft and relatively nutritious. In the absence of toughness, secondary chemistry is commonly the front line of defense against herbivores (Coley and Barone, 1996). Young leaves, therefore, are likely to be focal points of selection on defense chemistry.

Secondary defense metabolites have their biochemical origins in primary metabolism. This implies that, at some point in evolutionary history, the metabolites that straddle the branch points between primary and secondary metabolism assumed derived ecological functions distinct from their basal primary ones. This could occur when mutations in regulatory genes lead to the accumulation of a primary metabolite in excess of physiological sink capacities. If these "hyper-production" phenotypes improve fitness and are heritable, a novel function may become established. But defense strategies based on primary metabolites are almost never observed, possibly because (1) there are few primary metabolites that are sufficiently toxic at realistic concentrations, (2) diversion of large amounts of a primary metabolite away from growth has a considerable fitness cost, or (3)

following any switch from primary metabolic to allelochemic (i.e., ecological) function (sensu Whittaker and Feeny, 1971), selection quickly leads to more active forms of the metabolite. Hence, plant defense chemistry is almost completely dominated by secondary metabolites. Any observation of a hyper-produced primary metabolite having defense function is extraordinary, indicating the occurrence of a rare evolutionary innovation and perhaps an incipient secondary metabolic pathway.

We have recently observed just such an anomalous hyper-production of a primary metabolite. The protein amino acid L-tyrosine accumulates to very high concentrations in the young leaves of the neotropical legume *Inga umbellifera* (Vahl.) Steud. (Fabaceae: Mimosoideae: Ingeae). As part of a study examining correlations between leaf developmental strategy and chemical defense, we are characterizing the full range of potential defense metabolites in several *Inga* species, including *I. umbellifera*. Young shade leaves of *I. umbellifera* accumulate *O*-substituted flavanoids and procyanidins (condensed tannins) to 40–50% of their dry mass, some of the highest concentrations ever observed in leaf tissue (Lokvam et al., 2004; Lokvam and Kursar, 2005). These compounds are potent growth inhibitors of larvae of the noctuid lepidopteran *Heliothis virescens*, a generalist herbivore from tropical and subtropical America. But in addition to phenolic secondary metabolites, young *I. umbellifera* leaves accumulate the phenolic primary metabolite L-tyrosine. In this paper, we present data showing that the hyper-production of this amino acid in *I. umbellifera* is a widespread and highly leaf age-specific chemical phenotype. We also show that this compound is markedly inhibitory to *H. virescens* larval growth when consumed at less than half the mean concentration contained in young leaf tissue. Finally, we contrast tyrosine biosynthesis and bioactivity with that of two other prominent nitrogenous metabolites found in *I. umbellifera* leaves.

MATERIALS AND METHODS

Study species—*Inga* (Fabaceae: Mimosoideae) is a neotropical genus comprised of approximately 300 species (Pennington, 1997). *Inga umbellifera* is a subcanopy tree that is widely distributed in moist forests from southern Mexico to Brazil. Saplings of this species mature in the forest understory. Young leaves flush in large numbers and expand rapidly. Chloroplast development is delayed until leaves have reached full size. Although most species in the genus *Inga* are capable of nodulation, legume seedlings grown at low light or high nitrogen levels are unlikely to do so (McHargue, 1999). Hence, shade-growing *I. umbellifera* trees probably do not associate with nitrogen-fixing soil symbionts.

For the present study, we sampled leaves from two *I. umbellifera* populations, one in Panama and one in Ecuador. The majority of the sampling was carried out at Barro Colorado Nature Monument (BCNM, 79°50′ W, 9°10′ N), Republic of Panama, an area known to have relatively high soil nitrogen (Yavitt, 2000). In the BCNM population of *I. umbellifera*, approximately 18% of the leaf area is damaged by herbivores during the expansion phase, despite the fact that leaves are heavily defended by phenolic metabolites. The largest percentage of young leaf damage is caused by lepidopteran larvae (Coley et al., 2005). We also sampled leaves from an *I. umbellifera* population growing at Parque Nacional Yasuní (77°40′ W, 0°40′ S), Ecuador.

Identification, collection, and processing of plant material—Identification of Panamanian *I. umbellifera* was made by P. D. Coley. For purposes of isolation and structural characterization of the major nitrogenous metabolites, 137 g fresh mass of young leaves, 10–90% expanded, were collected from 40 individual, shaded, understory saplings across BCNM in late 2001 and early 2002. The fresh leaves were macerated in 95% ethanol, first in a Waring blender and then in a Polytron (Brinkmann Instruments, Westbury, New York, USA). The homogenized extracts were pooled and stored at −50°C before shipment on dry ice to the University of Utah.

For tracking ontogenetic changes in nitrogenous metabolite concentrations, one set of leaves was harvested from each of five different shaded understory *I. umbellifera* individuals across BCNM in early 2004. Each set spanned a range of developmental stages, approximately 10, 50, and 90% expanded (designated as early, intermediate and late) and mature leaves. Leaves were individually desiccated in silica gel and returned to Utah for analysis.

For purposes of making qualitative comparisons between two widely separated *I. umbellifera* populations, young leaves were also collected from three individual shade saplings from a population growing at Yasuní, Ecuador. Identifications were made by T. Brenes-Arguedas. In this sampling, leaves were pooled and macerated in 95% ethanol before being returned to Utah for analysis.

Isolation and characterization of major I. umbellifera nitrogenous metabolites—Ethanolic extracts of *I. umbellifera* leaves (see previous section) were filtered, and the marc was repeatedly extracted with aqueous 80% ethanol. The extracts were combined, the organic solvent removed under reduced pressure, and the remaining aqueous portion was defatted with dichloromethane. The defatted aqueous portion was passed onto an octadecylsilane (ODS) column (40 μm, J. T. Baker, Phillipsburg, New Jersey, USA) and washed with water and then 100% methanol. Both the water and methanolic fractions were concentrated to a small volume. The precipitate that formed in each (subsequently determined to be tyrosine) was removed by filtration. The filtered aqueous fraction was passed onto a cation exchange column (Dowex 50WX8-400, Sigma-Aldrich, St. Louis, Missouri, USA) and washed with water to remove non-ionic and anionic species. Cations were eluted with 2 N NH₄OH. The cationic fraction was analyzed by liquid chromatography/mass spectrometry using a Waters 2690 high performance liquid chromatography (HPLC) system (Waters, Milford, Massachusetts, USA) with a 2487 UV detector (recording at 214 nm) configured in tandem with a MicroMass Quattro II mass spectrometer (Waters) operating in the positive-ion electro-spray ionization mode. Separations were carried out using a 4.6 × 250 mm amino-propyl column (Luna 5μ, Phenomenex, Torrance, California, USA) and an isocratic elution of 4 : 1 acetonitrile to aqueous (0.5% v/v) formic acid. Three major components were observed and their masses recorded. One of these was subsequently determined to be tyrosine. The other two components were isolated to purity from the cationic fraction using a 10 × 250 mm amino-propyl column (Luna 5μ, Phenomenex). All three components were structurally characterized from their mass and 1- and 2-dimensional (D) nuclear magnetic resonance (NMR) spectra. The latter were acquired on a Unity iNOVA 500 MHz spectrometer (Varian, Walnut Creek, California, USA). For these analyses, tyrosine was dissolved in D₂O containing ca. 0.3% w/v NaOD; the other nitrogenous metabolites were dissolved in D₂O.

Nitrogenous metabolite content analysis—For purposes of tracking ontogenetic changes in nitrogenous metabolite abundance in *I. umbellifera*, dried leaves from each of the five expansion sequences were weighed and then individually pulverized using a Retsch MM 200 Mixer Mill (Retsch GmbH & Co., Haan, Germany). A subsample of each leaf (5 mg for tyrosine, 30 mg for the other nitrogenous metabolites) was extracted with 1 mL of aqueous acetic acid (pH 3) for 1 h at 85°C and the mixture was centrifuged. (Repeat extractions showed that this method removes >99% of the extractable nitrogenous metabolites from leaf tissue.) Fifteen microliters of the supernatant was injected directly onto a 4.6 × 250 mm amino-propyl HPLC column (Microsorb 5μ, Varian). Nitrogenous metabolites were chromatographed using a linear gradient (17–23%) of aqueous acetic acid (pH 3.0) in acetonitrile over 25 min. An evaporative light scattering detector (SEDERE S.A., Alfortville, France) was used to measure the mass of solutes in each injection. Leaf nitrogenous metabolite concentrations were determined by reference to a four-point standard curve (0.2–3.0 mg nitrogenous metabolite/mL, $r^2 > 0.99$ in all cases) prepared for each metabolite from purified compounds. The robustness of the analysis was verified by subdividing one high-tyrosine leaf sample (90% expansion level) into three parts and performing three separate extractions/injections (tyrosine: mean = 14.3%, SD = 0.3%; nitrogenous metabolite 1: mean = 1.34%, SD = 0.03%; nitrogenous metabolite 2: mean = 1.7%, SD = 0.3%).

Tyrosine chirality analysis—The stereochemistry of leaf tyrosine was determined following treatment with Marfey's reagent (Marfey, 1984). Extracted tyrosine as well as reagent L- and DL-tyrosine (Sigma-Aldrich, Milwaukee, Wisconsin, USA) were converted to their respective diastereomers and analyzed by HPLC. The derivatives were separated on a 4.6 × 250 mm ODS column (Microsorb 5μ, Varian) operated at 40°C with a mobile phase of 0.1% v/v trifluoroacetic acid/water (A) and acetonitrile (B) at 1 mL /min. Detection was by UV at 350 nm. A linear gradient elution of 90–40% A in B over 40 min resolved the L- and D-tyrosine derivatives, which eluted at 38.5 and 39.8 min, respectively.

Insect feeding trials—The bioactivities of *I. umbellifera* nitrogenous metabolites as well as commercial DL-tyrosine were assayed in growth trials using larvae of *Heliothis virescens* (Lepidoptera: Noctuidae). Two types of assays were undertaken, one an 8-d growth trial involving all the nitrogenous metabolites mentioned and the other a time-to-pupation trial involving L-tyrosine only. For both, larvae were fed an artificial diet (Coley et al., 2005) amended with nitrogenous metabolites. Once prepared, the diet was divided into individual portion cups, 16 per treatment level. Controls were prepared in the same manner as treatments but without the addition of test metabolites. One recently hatched, unfed *H. virescens* larva was sealed into each cup with a vented lid and kept in a 75% relative-humidity-controlled growth chamber on a 12 h/12 h day/night schedule. For the growth trial, nitrogenous metabolite levels ranged between 0.125 and 10.0% of food dry mass. Treatment and control larvae were weighed at the end of 8 days. Treatment masses were divided by the control mass (GRC = growth relative to control) and fitted to the following dose-response function using the NLIN procedure in SAS, version 8.2 (SAS Institute, Cary, North Carolina, USA):

$$GRC = k / \left[1 - (c/b_2)^{b_1}\right],$$

where *c* is the test-metabolite concentration in percentage, *k* is the response at low concentration, b_1 is the slope and b_2 is the concentration (GI₅₀) that inhibits growth by 50%. The GI₅₀ was used to compare the relative inhibitory capacities of assay metabolites (Streibig et al., 1993). For the time-to-pupation trial, a single, 5.0% dry mass tyrosine treatment level was used. Larvae were allowed to feed ad libitum during the growth period, and food was replaced as necessary. At pupation, total time-to-pupation and pupal masses were recorded and then analyzed in GNU R, version 2.1.0 (R Development Core Team, Vienna, Austria), using a Kruskal-Wallis signed-rank test and a one-way ANOVA, respectively.

RESULTS

Inga umbellifera leaf tyrosine—Leaf tyrosine was structurally characterized following its isolation as a high purity
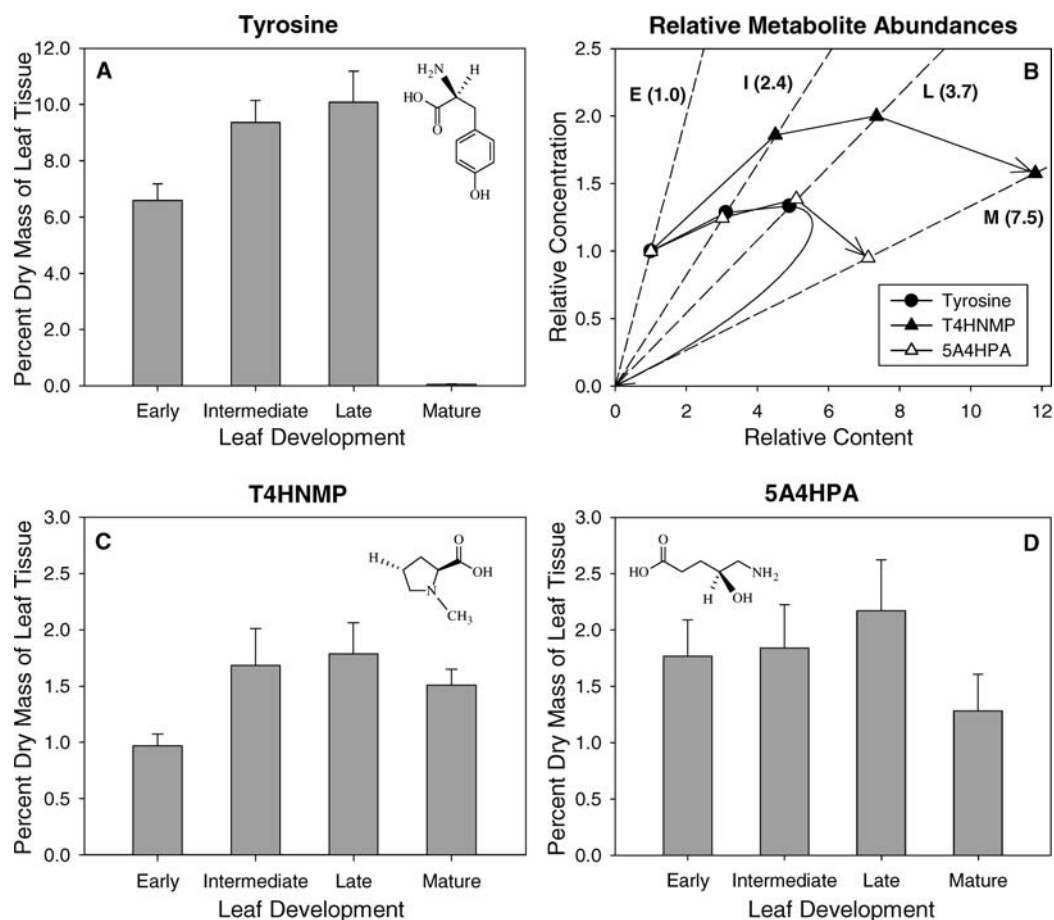
Fig. 1. (A), (C), and (D). Absolute concentrations (% DM) of tyrosine, *trans*-4-hydroxy-*N*-methyl-L-proline (T4HNMP) and 5-amino-4-hydroxy-pentanoic acid (5A4HPA) in *I. umbellifera* leaves through development. (B) Vector diagram showing changes in leaf investment in each metabolite during leaf development: For each metabolite, the relative concentration (*y*-axis) is shown as a function of relative total leaf content (*x*-axis), with all measurements being normalized to the value of the youngest measured leaf. Diagonals represent total leaf dry biomass relative to the youngest measured leaf at each sampling event. The slopes of the diagonals are the inverse of the biomass increase relative to the youngest measured leaf. Diagonal labels refer to leaf developmental stage (early [E], intermediate [I], and late [L] expansion and mature [M]) and are followed in parentheses by the biomass increase relative to the youngest measured leaf. Point 1,1 indicates the youngest measured leaf; arrows indicate the inferred trajectories of metabolite concentration and content between sampling events: a slope $>0°$ implies positive allometry of synthesis with respect to growth; a slope of $0°$ implies neutral allometry; a slope between $0°$ and $-90°$ implies negative allometry, and a slope $<-90°$ implies resorption. See text (Results: *Inga umbellifera* leaf tyrosine) for discussion.

precipitate from concentrated aqueous alcoholic extracts of leaf tissue. It was positively identified by comparison of its mass and proton-NMR spectra to those of commercial tyrosine (Sigma-Aldrich). The enantiomeric composition of the plant metabolite, determined by HPLC analysis after treatment with Marfey's reagent, showed that *I. umbellifera* young leaf tyrosine was essentially 100% the L enantiomer.

Chromatographic analysis of leaf extracts from numerous widely scattered trees at Barro Colorado Nature Monument (BCNM), Panama, indicated that accumulation of tyrosine in young leaves is a widespread phenomenon in this Central American population of *I. umbellifera*. In addition, HPLC analysis of a pooled sample from shaded, understory *I. umbellifera* saplings in Yasuní National Park, Ecuador, showed that tyrosine also accumulates in the young leaves of that Amazonian population.

HPLC was used to analyze *I. umbellifera* leaf tyrosine content across a range of developmental stages in five individual saplings (Fig. 1A) from BCNM. These analyses showed that the very youngest leaves contained considerable amounts of tyrosine (average 6.6% of leaf dry mass, SE = 0.6%) and that the concentration of this amino acid increased in leaf tissue throughout leaf expansion. Tyrosine content reached an average maximum of 10.1% dry mass (SE = 1.1%) in late-expansion young leaves. The concentration of free tyrosine in mature *I. umbellifera* leaves was 0.05% (SE = 0.01%).

A vector analysis (Koricheva, 1999, and references therein) of leaf tyrosine content was used to place leaf tyrosine concentration in the context of whole plant investment in this metabolite (Fig. 1B). Normalized to the youngest measured leaf (ca. 10% expansion), *I. umbellifera* leaves underwent a greater than seven-fold increase in leaf dry mass during maturation. Tyrosine synthesis showed positive allometry with respect to biomass increase as accumulation of this metabolite exceeded growth during the expansion phase. Sometime following full expansion, resorption, and/or conversion became
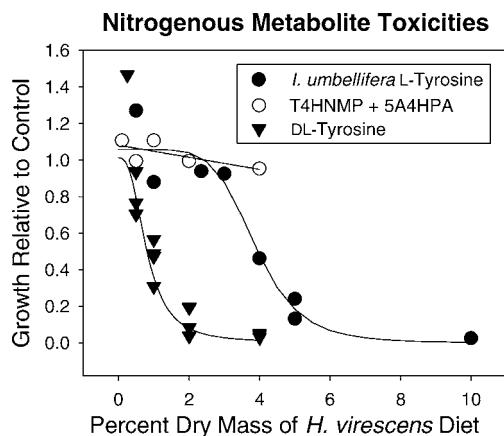
Nitrogenous Metabolite Toxicities

Fig. 2. Insect-growth inhibitory effects (measured as growth relative to controls) of *I. umbellifera* young leaf L-tyrosine, of the *I. umbellifera* nitrogenous metabolites *trans*-4-hydroxy-*N*-methyl-L-proline (T4HNMP) and 5-amino-4-hydroxy-pentanoic acid (5A4HPA) and of commercial DL-tyrosine in feeding trials with larvae of *H. virescens*. For L- and DL-tyrosine, the fitted lines were generated from the GRC (growth relative to control) equation explained in the text (Methods: Insect feeding trials). For T4HNMP + 5A4HPA, the fitted line is the linear regression. The data are illustrative of the fact that at subtoxic concentrations, these nitrogenous metabolites stimulate insect growth compared to controls, i.e., GRC > 1.

the dominant processes and caused leaf tyrosine concentration to fall to very low levels.

Inga umbellifera leaf tyrosine was bioassayed across the range of concentrations at which it occurs in leaf tissue. These experiments showed that it is a moderately potent growth inhibitor for larvae of the noctuid moth *H. virescens* (Fig. 2). Larval growth was reduced to 50% of controls (GI_{50}) over the 8-d growth period with the addition of 3.8% dry mass ($P < 0.01$, SE = 0.3%) of the plant metabolite. When added at 10% of assay food dry mass (the mean maximum tyrosine concentration in *I. umbellifera* young leaf tissues), *H. virescens* larval growth was reduced to ca. 2% of controls ($P < 0.01$, SE = 0.1%). Introduction of the D enantiomer (Fig. 2) into the diet in the form of racemic tyrosine caused substantially greater reduction in growth rates compared to the L enantiomer, and gave a GI_{50} of 0.8% ($P < 0.01$, SE = 0.1%). Time-to-pupation was significantly longer (19.3 ± 0.6 vs. 17.1 ± 0.5 days, $P < 0.01$) and pupal masses significantly lower (273 ± 6 vs. 331 ± 7 mg, $P < 0.001$) than controls for larvae reared on diet containing 5% L-tyrosine.

Nitrogen-containing secondary metabolites—From the young leaves of *I. umbellifera,* we isolated two nitrogenous secondary metabolites in relative abundance. Both were purified by HPLC and structurally characterized. The first, *trans*-4-hydroxy-*N*-methyl-L-proline (T4HNMP) was positively identified by comparison of its 1-D-^{1}H and ^{13}C NMR spectra to literature values (Figliuolo et al., 1987). This conclusion was supported by the ESI-mass spectrum, which gave a molecular ion $(M + H)^+$ of 146.1 amu, consistent with the nominal mass of T4HNMP, 145.159 amu. The absolute stereochemistry was determined by optical rotation: $[\alpha]_D^{22} = -64.2° \cdot g^{-1} \cdot mL^{-1} \cdot dm^{-1}$, $c = 0.015$ g/mL, consistent with literature values for the L enantiomer (Sciuto et al., 1983; Figliuolo et al., 1987).

We structurally characterized a second major nitrogenous metabolite by analysis of 1- (Table 1) and 2-D NMR spectra.

TABLE 1. ^{13}C/^{1}H nuclear magnetic resonance shifts and ^{1}H splitting patterns for *Inga umbellifera* 5-amino-4-hydroxy-pentanoic acid in D$_2$O at 23°C. All values are referenced to the methyl singlet of 3-(trimethylsilyl)-1-propane-sulfonic acid, 0.0 ppm. m = multiplet, dd = double doublet.

C	δ^{13}C	δ^{1}H (no.)	Mult.	Hz
1	184.0	—	—	—
2	36.1	2.48 (2)	m	—
3	28.0	1.91 (2)	m	—
4	55.7	3.36 (1)	m	—
5	63.5	3.82 (1)	dd	12.5, 3.7
		3.64 (1)	dd	12.5, 6.6

Single bond ^{1}H–^{1}H (COSY), ^{1}H–^{13}C (HMQC), and multiple bond ^{1}H–^{13}C (HMBC) correlation experiments analyzed together with the low-resolution mass spectrum, yielded the planar structure of 5-amino-4-hydroxy-pentanoic acid (5A4HPA) with a predicted molecular formula of $C_5H_{11}NO_3$. High resolution matrix-assisted laser desorption mass spectrometry gave a molecular ion $(M + H)^+$ of 134.0800 amu (9 ppm error), thus confirming the predicted formula. The absolute stereochemistry of this compound was determined by optical rotation: $[\alpha]_D^{22} = +4.2° \cdot g^{-1} \cdot mL^{-1} \cdot dm^{-1}$, $c = 0.022$ g /mL, consistent with literature values for the 4-(S) enantiomer (Herdeis, 1986).

Inga umbellifera leaf content of T4HNMP and 5A4HPA was measured by HPLC. We analyzed leaf expansion sequences from five widely scattered trees in the BCNM population. As with tyrosine, accumulation of T4HNMP and 5A4HPA in *I. umbellifera* leaves was a general phenomenon. Our analysis showed that both metabolites had already begun to accumulate in the youngest measured leaves and that their concentrations increased throughout leaf expansion. But in contrast to tyrosine, mature *I. umbellifera* leaves contained appreciable concentrations, 1.0–1.5%, of T4HNMP and 5A4HPA. A vector analysis of *I. umbellifera* investment in these metabolites (Fig. 1B) shows that there was positive allometry for the production of both metabolites throughout leaf expansion. During the leaf toughening phase following full expansion, synthesis continued to increase, though dilution effects shifted the allometry to negative.

5A4HPA and T4HNMP were bioassayed together (Fig. 2) in the proportions at which they were extracted from leaf tissue, 1 : 1.5. The combined metabolites had no significant effect on *H. virescens* larval growth at a concentration of 4.0% of food dry mass, a concentration greater than that found in leaf tissue ($P = 0.5$). A GI_{50} for these metabolites could not be experimentally determined owing to lack of material.

DISCUSSION

Hyper-production of the protein amino acid L-tyrosine is a widespread chemical phenotype in young, shade leaves of *I. umbellifera* growing at Barro Colorado Nature Monument, Panama. Tissue concentrations of this metabolite reach a mean maximum of ca. 10% leaf dry mass (Fig. 1A) in late expansion young leaves, with some individual leaves reaching as high as 14% leaf dry mass tyrosine. The hyper-production of tyrosine in *I. umbellifera* is apparently not a localized, ecotypic phenomenon: Extracts of young shade leaves from trees

growing in Amazonian Ecuador also contained appreciable amounts of free tyrosine.

Hyper-production of this metabolite would appear to have considerable fitness value for young leaves. A vector analysis of leaf concentration vs. leaf total content (Fig. 1B; Koricheva, 1999) shows that tyrosine synthesis has positive allometry with respect to increasing leaf biomass, resulting in ever higher concentrations of the amino acid as development proceeds. Tyrosine sequestration is a highly leaf-age-specific phenomenon, however. Young leaves accumulate this metabolite to extraordinarily high concentrations, then, sometime following full expansion, resorption processes reduce tyrosine concentrations by ca. 200-fold. Free tyrosine concentration in mature leaves averages just 0.05% of the dry mass.

To determine if tyrosine hyper-production could potentially impact herbivores, we tested the effects of high dietary tyrosine on growth and development of the noctuid moth *Heliothis virescens*. While there is no perfect bioassay organism for studies of plant chemical defense, we chose *H. virescens* because, even though it is not known to feed on *Inga*, it is a generalist lepidopteran that does feed on several fabaceous genera. Thus, we view it as representative of the class of generalist lepidopteran herbivore that has been a major driving force in the evolution of *Inga* chemical defenses. From these bioassays, we conclude that the fitness benefits of high tyrosine concentrations in young *I. umbellifera* leaves likely result from its growth inhibitory effects on herbivores. When larval food is amended with 10% dry mass of L-tyrosine, the concentration at which it occurs in leaf tissue, 8-d larval growth was reduced by 98% compared to controls. Moreover, time-to-pupation was significantly longer and pupal weights significantly lower when 5% tyrosine was added to larval diet. These effects can have serious impacts on insect survival and future fecundity. Slowed growth rate has been shown to increase the probability of mortality due to parasitism in the pierid moth *Pieris rapae* (Benrey and Denno, 1997) and low pupal mass is strongly correlated with reduced egg production in various Lepidoptera (Haukioja and Neuvonen, 1985; Awmack and Leather, 2002).

L-Tyrosine is not a potent toxin, however, as was emphasized by bioassays with racemic tyrosine (Fig. 2), which contains 50% the nonprotein amino acid D-tyrosine. In these assays, larval growth was reduced by 98% compared to controls with the addition of only 4% of the amino acid mixture. This experiment demonstrates the defense advantage that would accompany the evolution of a tyrosine isomerase. A similar innovation has been observed in the spotted knapweed, *Centaurea maculosa* (Asteraceae) (Bais et al., 2002), a virulent invasive species in North America whose success is attributed to chemically mediated allelopathy. The roots of *C. maculosa* secrete racemic catechin: The (+) form of this very common flavanoid is non-allelopathic while (−)-catechin is a rare and potent phytotoxin.

In *I. umbellifera*, the lack of potency of L-tyrosine appears to be compensated for by its extremely high concentration. To the best of our knowledge, the occurrence of a concentrated protein amino acid in leaf tissue has never been reported. Nevertheless, several have proven to be moderately toxic in bioassays. Janzen et al. (1977) showed that eight of the 20 protein amino acids significantly reduced the probability of survival to adulthood in larvae of the bruchid beetle *Callosobruchus maculatus* when they were incorporated into artificial diet at 1%. When the amendment was increased to 5%, an additional seven amino acids produced significant effects. L-Tyrosine belonged to this latter group.

Though the mechanism of L-tyrosine toxicity was not explicitly investigated, certain physical properties of the free amino acid may shed light on its mode of bioactivity. In the pH range 3–10, tyrosine solubility in water (25°C) is slight and nearly constant at ca. 2.5 mM (Carta and Tola, 1996). *Inga umbellifera* leaves are approximately 70% water by mass with a whole-leaf average pH of ca. 5.9. Assuming optimal solubilization conditions, leaf water would be saturated at leaf-tyrosine concentrations of ca. 0.1% dry mass. Barring the occurrence of supersaturation or of profoundly acidic or basic vacuolar environments, tyrosine would likely be sequestered in *I. umbellifera* leaves as a solid. If this is the case, crystalline tyrosine may act as a surrogate mechanical defense in the absence of secondary cell walls in expanding leaves. But until we develop a confirmatory experiment, using scanning electron microscopy for example, we cannot discount the possibility that a chemically labile and highly soluble tyrosine derivative is present in *I. umbellifera* leaves, one which readily yields free tyrosine when tissues are disrupted.

Ingestion of large amounts of dietary tyrosine likely poses several challenges to an insect herbivore. The lepidopteran mid-gut is strongly alkaline, with the pH in some noctuid guts exceeding 11.5 (Dow, 1992). In such an environment, tyrosine solubility is three-fold greater than in neutral conditions. Presumably, high mid-gut tyrosine concentrations would lead to increased transport through the gut wall. High hemolymph tyrosine has been implicated in increased fluid secretion from the Malpighian tubules of both *Rhodnius prolixis* (Hemiptera) and *Drosophila melanogaster* (Hazel et al., 2003). A high tyrosine diet, then, may disrupt an insect herbivore's water balance. Continuous exposure would likely have other deleterious effects as well, including saturation of broadly selective amino acid transporters and quenching of mid-gut alkalinity. A decrease in gut pH would lead to increased precipitation of certain dietary proteins by procyanidins (Hagerman and Butler, 1978), which are also present in *I. umbellifera* leaves at very high concentrations.

The pattern of tyrosine synthesis in the young, shade leaves of *I. umbellifera* indicates the presence of a rarely observed chemical defense strategy, a hyper-produced primary metabolite functioning as an allelochemical. This phenomenon has been reported at least twice before. Rehr et al. (1973) found L-dopa (3-hydroxy-tyrosine) concentrations up to 9% in the seeds of six species of *Mucuna* (Fabaceae). L-Dopa is an intermediate in the pathway leading from tyrosine to the catecholamines (e.g., dopamine, epinephrine, norepinephrine), compounds that may have primary metabolic activity in plants (Kuklin and Conger, 1995). In insects, catecholamines play an essential role in exo-skeletal sclerotization where, under enzymatic mediation, they form the cross-links between protein layers, thereby hardening and dehydrating the cuticle (Hopkins and Kramer, 1992). Bioassays using *Prodenia* (*Spodoptera*) *eridania* reared on artificial diet showed that severe pupal/adult cuticular deformities resulted from the addition of only 0.25% dry mass of L-dopa (Rehr et al., 1973). Bioassays performed in our lab showed that this is probably not the mechanism by which tyrosine acts on *H. virescens* larvae. With the addition of 5% dietary tyrosine, just two of 16 larvae had cuticular deformities and failed to eclose.

Zou and Cates (1994) found that D-(+)-galactose was the predominant carbohydrate (ca. 78%) in current-year growth of Douglas fir (*Pseudotsuga menziesii*), which can contain up to 18% sugar (Clancy, 1992). In feeding trials with the spruce

budworm (*Choristoneura occidentalis*: Lepidoptera: Tortricidae), galactose significantly increased larval mortality in comparison to other simple sugars present in fir foliage. While hyper-production of a simple sugar is consistent with the traditional ecological concept of a constitutive, carbon-based defense, tyrosine deployed as a defense metabolite is more difficult to reconcile with current theories of plant defense. Like galactose in fir, tyrosine accumulation in *I. umbellifera* conforms to the concept of a "quantitative" chemical defense (sensu Feeny, 1976): Its deterrent effects are dosage dependent and it is present at high concentration. The fact that a quantitative defense is based on a nitrogenous primary metabolite is an unprecedented observation, however. In most ecosystems, nitrogen (or phosphorous) is a limiting nutrient, such that nitrogen-based defenses tend to be present at low concentration and to be potent toxins (e.g., many alkaloids, cyanogenic glycosides and glucosinolates). The implication from our *I. umbellifera* data is that in the low-light habitat of the tropical forest understory, a quantitative defense based on a nitrogenous metabolite may be an evolutionarily favored outcome, provided that soils contain sufficient nitrogen to support the demands of both growth and defense. One might be tempted to say that a nitrogen-based defense occurring in a carbon-poor environment is consistent with the carbon–nutrient balance hypothesis (Bryant et al., 1983) were it not for the fact that the young leaves of *I. umbellifera* also accumulate very high concentrations of flavanoid metabolites, purely carbon-based defense chemicals. The extreme nature of defense chemistry in the young leaves of tropical forest trees is well illustrated by *I. umbellifera* where 10% leaf dry mass tyrosine is complemented by at least 40% flavanoid metabolites (Lokvam and Kursar, 2005).

In addition to tyrosine, *I. umbellifera* accumulates two other nitrogenous metabolites to appreciable concentrations (Fig. 1C, D). The first, *trans*-4-hydroxy-*N*-methyl-L-proline (T4HNMP) is an imino acid with a fairly wide distribution among plants. It is known from at least seven angiosperm families. The second, 5-amino-4-(*S*)-hydroxy-pentanoic acid, is a δ-amino acid. To the best of our knowledge, it has not been described as a natural product before, regardless of source. The presence of these compounds contrasts with that of tyrosine in that (1) they have no significant deterrent effect on *H. virescens* larval growth (Fig. 2) at the concentration they occur in the plant, and (2) they persist in mature leaf tissue (Fig. 1C, D). These data suggest that these metabolites have evolved under selection by a non-lepidopteran herbivore or possibly a pathogen. For example, Figliuolo et al. (1987) showed that T4HNMP had profound effects on the probability of survival to adulthood in the bruchid beetle *Callosobruchus maculatus*.

What we may be observing in the case of *I. umbellifera* tyrosine hyper-production is a rare evolutionary event, one where regulatory gene mutations have released a primary metabolic pathway from the "normal" controls that are in place during the leaf expansion phase and have fortuitously conferred increased resistance to herbivory. The example of *I. umbellifera* presented here is illustrative of two important points regarding chemical defense expression in tropical trees. First, tyrosine metabolism in *I. umbellifera* shows the precision with which chemical defenses in tropical trees can be controlled, that their expression can be very finely tuned to a given tissue's developmental stage. And second, while structural gene mutations are the engine that drives the long-term diversification of defense metabolites, regulatory gene

mutations are equally important. Not only are they the gateway to long-term defense chemical evolution, they also provide a mechanism that allows for very rapid shifts in defense chemical expression, thus providing long-lived tree species with a potentially critical evolutionary response to insect herbivores whose generation times are one to two orders of magnitude shorter. In the case of tyrosine, regulatory gene mutations followed by shifts from primary to allelochemic function appear to have happened several times before. Selection following these events has given rise to both a large number of phenolic secondary metabolites in the Poaceae and to the diverse families of tyrosine-derived alkaloids (e.g., isoquinoline, papaverine, ipecac) that occur in numerous plant taxa.

With respect to tyrosine hyper-production in *I. umbellifera*, our observation of what appears to be a transitory evolutionary state may simply be a matter of good fortune. But rather than witnessing a rare event, it is also possible that primary metabolites are commonly mediators of ecological interactions. In the study of plant–herbivore interactions, this concept is certainly not new. Its discussion up to this point, however, has almost exclusively focused on the heritable diminution of nutritious primary metabolites, protein in particular, and the resulting ecological consequences of lowered food quality (Berenbaum, 1995). But as more and more studies examine the full range of potential defense metabolites in the most vulnerable tissues and at the most vulnerable developmental stages, we may find that hyper-produced primary metabolites function as allelochemicals more often than previously thought.

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