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RAPID COMMUNICATION

Quantitative Variability of Direct Chemical Defense in Primary and Secondary Leaves of Lima Bean (*Phaseolus lunatus*) and Consequences for a Natural Herbivore

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Abstract Ontogenetic variability in chemical plant defenses against herbivores is a common phenomenon, but the effects of this variability on herbivore–plant interactions are little understood. In a previous study on lima bean (*Phaseolus lunatus*), we found a trade-off between cyanogenesis, a direct defense, and the release of herbivore-induced volatile organic compounds (VOCs; mainly functioning as an indirect defense). Moreover, the expression of these two defenses could change during plant ontogeny. The present study aimed at elucidating whether such ontogenetic changes in plant defense can affect herbivore–plant interactions. We quantified feeding rates of a natural insect herbivore, the Mexican bean beetle (*Epilachna varivestis*), on primary and secondary leaves of individual lima bean plants. These insects strongly preferred low cyanogenic primary leaves over high cyanogenic secondary leaves. Although weakly defended by cyanogenesis, lima beans' primary leaves showed protein concentrations and photosynthetic activities that did not differ significantly from secondary leaves at the time of analysis. Based on our findings, we suggest that lima beans' long-lived primary leaves function as efficient source organs, even beyond the stage of seedlings. This hypothesis may explain why primary leaves express a strong indirect defense by the release of herbivore induced VOCs.

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Introduction

Analyses of different lima bean (*Phaseolus lunatus* L.) accessions revealed strong trade-offs between cyanogenesis (a direct defense) and the release of herbivore-induced volatile organic compounds (VOCs; Ballhorn et al. 2008). Such volatile organic compounds are generally regarded as indirect plant defense (Heil 2008), but lima bean VOCs may also act as direct repellants to herbivores (Heil 2004). We found that this trade-off can change early during plant ontogeny, i.e. from primary to secondary leaves (Ballhorn et al. 2008). Younger secondary leaves showed a high cyanogenic potential (HCNp; the total amount of cyanide that can be released from a given tissue), whereas primary leaves contained almost no cyanide. The reverse pattern was found for VOCs, since primary leaves showed higher VOC release than secondary leaves (Ballhorn et al. 2008). This substantial release of VOCs by primary leaves was surprising, since primary leaves are regarded to be transient organs that are functionally replaced by secondary leaves early in plant development. Extensive allocation of any type of defense to the primary leaves should, thus, incur costs that are likely not outweighed by the benefit of protecting these specific organs (Mauricio 1998).

However, functional aspects of ontogenetic variability in plant–herbivore interactions are still elusive. Here, to elucidate whether ontogenetic variability of plant defensive traits affects plant–herbivore interactions, we quantified the ontogenetic variation in cyanogenesis and in the concentration of soluble (and easily digestible) leaf proteins

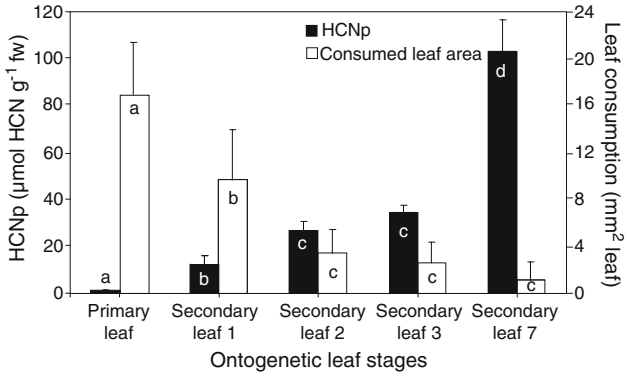

Fig. 1 Cyanogenic potential (*HCNp*) of different *P. lunatus* leaf developmental stages and preferences of herbivorous beetles. In choice experiments presenting all leaf stages simultaneously, attractiveness of leaf material to Mexican bean beetles was quantified. Values shown are means±standard deviation. Letters in/at the columns indicate significant differences in HCNp [according to LSD *post hoc* analysis ($P<0.01$) after one-way ANOVA]

(Mattson 1980). In addition, we measured the feeding rates of a natural herbivore, the Mexican bean beetle (Coccinellidae: *Epilachna varivestis* Mulsant) on these different leaf stages. This combined analysis of a defensive and a nutritive plant feature contributes to understanding the intra-individual variation of plant defensive traits in the interaction with herbivores.

Materials and Methods

Plants Lima bean plants (Fabaceae: *P. lunatus* L.) were derived from seed material collected in a natural population in Oaxaca, South Mexico (15°55′ N, 097°09′ W). Plants were cultivated in a climatic chamber adjusted to mimic conditions as recorded for the natural site in August–October 2007 (13:11 h light to dark ratio; 30:23°C; 60–70% air humidity and a photon flux density of 450–500 µmol s^{-1} m^{-1} at table height). Plants were cultivated in standard substrate (TKS®-1-Instant, Floragard, Oldenburg, Germany) mixed 1:3 with sand (grain size of 0.3–0.7 mm). Plant containers were 80 mm in diameter. We fertilized plants twice a week with an aqueous solution (0.5%) of a NPK-fertilizer (Flory-3®, EUFLOR GmbH, Munich, Germany). Plants were used for experiments when they had developed 9 to 10 leaves (6 weeks after germination; $N=8$ plants). In addition to primary leaves, we selected four secondary leaf stages for analyses. We defined the ontogenetically oldest secondary leaf as 'secondary leaf 1', while 'secondary leaf 7' was the youngest one, inserting seven positions above the primary leaves.

Cyanogenic Potential HCNp was quantified by complete enzymatic degradation of cyanogenic precursors by using specific β-glucosidase isolated from cyanogenic *Hevea brasiliensis*. Concentration of released cyanide was quantified spectrophotometrically (at 585 nm) by using the Spectroquant®Cyanide kit (Merck KGaA, Darmstadt, Germany) according to Ballhorn et al. (2007).

Protein Concentration We quantified soluble protein contents in leaves according to Bradford (1976). Polyvinylpolypyrrolidone (Sigma-Aldrich, Buchs, Switzerland) was added to leaf extracts to avoid potential interference of plant phenolics with protein.

Electron Transport Rate Photosynthetic activity of leaves was measured *in situ* in the climatic chamber. For nondestructive measurements, a chlorophyll fluorometer (Junior PAM, Walz GmbH, Effeltrich, Germany) was used. After 10 min of shading, leaf spots were illuminated with blue light at 400 µmol photons m^{-2} s^{-1} for 7 min and steady state photosynthesis was quantified as electron transport rate (ETR) values (µmol electrons m^{-2} s^{-1}).

Feeding Trials The feeding rates of adult Mexican bean beetles on leaves of different ontogenetic stages were analyzed in Petri dishes (9.5 cm) lined with moist filter paper. Beetles were starved for 4 h before leaf discs (16 mm in diameter; representing each one of the five leaf

Table 1 Photosynthetic activity and soluble protein concentration in different ontogenetic leaf stages of *P. lunatus*

Physiological characteristics of leaves used in feeding trials	Leaf developmental stages				
	Primary leaf	Secondary leaf 1	Secondary leaf 2	Secondary leaf 3	Secondary leaf 7
ETR [µmol electrons m^{-2} s^{-1}]	98.9±3.5a	101.4±2.0a	99.2±2.7a	96.6±2.6a	92.4±3.5a
Soluble protein [mg g^{-1} fw]	14.2±0.3a	14.0±0.7a	14.3±0.6a	14.5±0.4ab	15.2±0.8b

Values shown for electron transport rate and soluble protein concentration of different ontogenetic leaf stages are means±standard deviation ($N=8$ plants). Letters indicate significant differences among leaf stages according to *post hoc* analysis (LSD; $P<0.01$) after one-way ANOVA
ETR Electron transport rate

developmental stages) were offered to individual animals for 2 h. Position of leaf discs was set at random. After the experiment, leaf discs were scanned and the missing leaf area was quantified with Analysis® software (Olympus, Hamburg, Germany).

Statistical Analysis We used SPSS 16.0 (SPSS for Windows, SPSS, Chicago, IL, USA) for all statistical analyses.

Results

Cyanogenic Potential HCNp in primary leaves was significantly lower than in secondary leaves [according to least significant difference (LSD) *post hoc* analysis ($P<0.01$) after univariate analysis of variance (ANOVA); Fig. 1]. Among secondary leaves, the youngest leaves showed highest HCNp values (Fig. 1).

Protein Concentration The youngest secondary leaves contained significantly more soluble proteins than the primary leaves (one-way ANOVA with 'ontogenetic stage' as factor: $F_{4,35}=5.70$, $P<0.01$), whereas protein concentrations in older secondary leaves and primary leaves were not significantly different (Table 1).

Electron Transport Rate Chlorophyll fluorescence measurements revealed that plants showed high ETR in all leaf developmental stages analyzed. The ETR values were not significantly different among all leaf stages (Table 1).

Feeding Trials Beetles significantly preferred primary over secondary leaves (Fig. 1). Young secondary leaves were less consumed by beetles than relatively older secondary leaves. Among all leaf stages, consumed leaf area and HCNp in leaves were significantly negatively correlated (according to two-tailed Pearson correlation: $r=-0.638$, $P<0.001$).

Discussion

Primary leaves of wild-type lima bean plants were long living and showed high photosynthetic activity for a period of time that exceeded early seedling stages (>6 weeks after germination). In addition, protein concentrations in primary leaves were similar to those of the three following secondary leaves, indicating that no strong reallocation of resources from primary to the secondary leaves occurred as part of a beginning senescence of primary leaves during the considered time span.

Although presumably being valuable source organs for carbohydrates, primary leaves were poorly defended by cyanogenesis—and, consequently, Mexican bean beetles significantly preferred them over higher cyanogenic secondary leaves. Higher protein concentrations in the youngest secondary leaves ('secondary leaf 7') did not compensate for low attractiveness due to higher HCNp (Fig. 1; Table 1). In accordance with a previous study, cyanogenesis proved to be highly efficient in deterring the Mexican bean beetle (Ballhorn and Lieberei 2006).

In the present study, we focused on ontogenetic variation of cyanogenesis as a direct defense against herbivores, whereas indirect defense by VOCs was not measured. However, we demonstrated previously that the primary leaves of lima bean are not without defense, as they released high amounts of VOCs, whereas high cyanogenic secondary leaves emitted much lower amounts of VOCs (Ballhorn et al. 2008). Why do different leaf developmental stages express different types of defense? It appears to be a general phenomenon that plants do not rely on a single defense mechanism but rather express multiple defenses (Agrawal and Fishbein 2006). Nevertheless, despite beneficial effects, co-occurrence of defenses might be costly for a plant (Mauricio 1998), since investment in defensive traits is assumed to reduce the resource availability for growth and reproduction (Herms and Mattson 1992). Simultaneous resource allocation to growth and expensive nitrogen-based defenses (such as cyanogenesis) likely is constrained, particularly during the first weeks of plant development. Thus, carbon-based indirect defenses (VOCs) may be a good alternative for these early leaves, particularly when regarding their high photosynthetic capacities that were found in the present study. Although individual leaves of lima bean apparently could not express more than one type of defense at high quantities, plants may defend different leaf stages via different strategies. We suggest that multiple defense syndromes also may emerge at the 'plant level', not only at the level of individual leaves. Future studies should focus on effects of ontogenetic variation in defense mechanisms against herbivores and pathogens.

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