Genetic and environmental interactions determine plant defences against herbivores

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Summary

1. Plants express multiple defensive traits, but little is known about the genetic stability and phenotypic plasticity of these traits in nature. To investigate sources of variation and their potential ecological consequences for herbivores, we combined field observations of cyanogenic lima bean with laboratory experiments.

2. Field studies in South Mexico revealed a distinct variability of cyanogenic traits within and among wild lima bean populations. To differentiate among genetic variation and the impact of ambient conditions on plant phenotypes, we used seed-grown plants as well as clones propagated from high- (HC) and low-cyanogenic (LC) wild type plants.

3. In growth chamber experiments, we cultivated plants under three intensities each of drought and salt stress, nutrient supply, and light availability. We consecutively quantified cyanogenesis and total phenolics as chemical defences, soluble proteins as measure of leaf quality, and leaf mass per area and tissue hydration as physical defence-associated traits.

4. Plant traits were genetically stable, as clones propagated from the HC- and LC-mother plants maintained their cyanogenic features and also showed constancy of other leaf parameters tested.

5. In contrast to genetically heterogeneous seed-grown plants, cyanogenesis and soluble protein concentration in clonal plants showed significant variation in response to reduced water supply, increased soil salinity, and fertilization. The other leaf traits, however, showed no significant phenotypic plasticity depending on treatment.

6. Among all traits analysed, genetic and phenotypic variation in cyanogenesis proved the best predictor of herbivore behaviour and development, as LC-plants were preferred by adult Mexican bean beetles and allowed for faster larval development.

7. Synthesis. We demonstrate that (i) functional analyses of plant responses to abiotic factors require methodical separation of genotypic variability and phenotypic plasticity, (ii) different abiotic parameters quantitatively affect the plants’ chemical phenotype and that (iii) changes of plant phenotype can have strong impacts on natural herbivores. Our results suggest that genetic variability and phenotypic plasticity of plant anti-herbivore defences allow plant populations to rapidly respond to changing environmental conditions.

Key-words: cyanogenesis, *Epilachna varivestis*, genotype, global change, *Phaseolus lunatus*, phenolics, phenotypic plasticity, plant–herbivore interactions, resource availability hypothesis, salinity

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Introduction

Plants cannot escape when attacked by pathogens and herbivores or when exposed to unfavourable ambient conditions. However, they are far from being passive organisms. In the last decades, research has demonstrated how plants can actively up-regulate a wide array of defensive traits when attacked by herbivores or pathogens or being subjected to abiotic stress factors (e.g. Hartley et al. 2001; Ballhorn, Heil & Lieberei 2006; Saadatmand et al. 2008). Until now, little is known of the interplay between genetic control and phenotypic plasticity of plant defence traits, although both factors are important for short-term adaptations and the evolution of plant defensive syndromes (Agrawal & Fishbein 2006; Agrawal, Lajeunesse & Fishbein 2008).

Phytochemical changes in response to variation of abiotic conditions may have significant implications for plant palatability and defence (e.g. Hughes & Bazzaz 1997; Stevens, Waller & Lindroth 2007). The extent to which biochemical plant responses to changing abiotic conditions have quantitative effects on natural plant–herbivore interactions is, however, still poorly understood (e.g. Tikkanen & Julkunen-Tiitto 2003; Thylainakis et al. 2008). Given the rapidly accelerating environmental changes on local and global scales, there is strong demand for a better understanding of the consequences of plants’ plasticity on higher trophic levels. Analysing the impact of external factors on plant traits, and consequently on plant–herbivore interactions, is often complicated by the high genotypic variability of plant traits in natural populations (e.g. Donaldson & Lindroth 2007). Thus, functional analyses require experimental separation of genetic variability and phenotypic plasticity to address sources of variation.

Lima bean (Fabaceae: Phaseolus lunatus L.) has been used as an ecological model to study indirect defences via tritrophic interactions (Heil 2008). The elicitation of the production of herbivore-induced volatile organic compounds (VOCs) and extralforal nectar (EFN) benefits this species under natural growing conditions (Heil 2004; Yi et al. 2009). In addition to indirect defences, lima bean expresses direct defensive traits such as cyanogenesis. To date, a defensive function against herbivores could be unambiguously demonstrated for cyanogenesis (Ballhorn & Lieberei 2006; Ballhorn et al. 2009a) and EFN secretion (Kost & Heil 2008), but not for the release of VOCs (but see Heil 2004). Volatile organic compounds are likely to act mainly as within-plant signalling factors in this species and prime rather than induce defences (Heil & Silva Bueno 2007). Interestingly, the anti-herbivore defence of lima bean is subject to various trade-offs, that is, negative correlations among different defensive traits. For example, we found that cyanogenesis is negatively correlated with the release of VOCs (Ballhorn et al. 2008a,b) and the plants’ resistance to pathogens via polyphenol oxidase activity (PPOs; enzymes that oxidise phenolic compounds to reactive quinones) (Ballhorn, Pietrowski & Lieberei 2010b).

In the present study, we combined quantitative analyses of the cyanogenic features of wild lima bean at natural sites in South Mexico with laboratory experiments. We investigated the causes (i.e. genetic variability or phenotypic plasticity) and consequences (on a particular herbivore–plant interaction) of the observed defensive trait variation. To analyse the impact of abiotic factors that purportedly affect plant phenotypes, we experimentally altered water availability, soil salinity, nutrient supply and light intensity under growth chamber conditions. Plants derived from seed material collected in the field (thus exhibiting natural genetic variability) as well as clonal plants propagated from stem cuttings from one high (HC) and one low cyanogenic (LC) mother plant were each included in the analyses. Plants grown from seedlings enabled the assessment of lima bean’s responses to changing abiotic conditions at the intraspecific level, whereas clonal plants allowed for the exclusion of genotypic variability and, thus, quantitative estimations of phenotypic plasticity.

To address the variation of plant defences at natural sites, we focussed on cyanogenic potential (HCNp; concentration of cyanogenic precursors) as characteristic and efficient anti-herbivore defence of wild lima bean (Ballhorn et al. 2008c, 2009a). In laboratory experiments, we included an array of additional traits that potentially affect the palatability to herbivores. Besides HCNp, we quantified total phenolics as a further chemical defensive trait (Ballhorn et al. 2007). While cyanogenesis represents a nitrogen-based direct plant defence against herbivores (Møller & Seigler 1999), phenolics are carbon-based compounds that inhibit the digestion of protein by many herbivores (e.g. Nomura & Itioka 2002; Matsuki, Sano & Koike 2004). Both defences are quantitatively affected by abiotic factors, such as soil moisture (e.g. Foulds 1982), salinity (e.g. Keymer & Ellis 1978) as well as nutrient and light availability (e.g. Schreiner, Nafus & Pimentel 1984; Frehner et al. 1997).

In addition to quantitative or qualitative expression of plant defences, overall attractiveness of plants to herbivores is determined by the plants’ nutritive characteristics (e.g. Awmack & Leather 2002). To consider this point we measured the concentration of soluble (and easily digestible) proteins as an important marker for the nutritious value of leaf tissue (Mattson 1980; Ganzhorn 1992). Previous experiments suggest that the concentration of soluble proteins is correlated with the overall nitrogen content in lima bean leaves (D.J. Ballhorn, unpublished data). Furthermore, we quantified leaf mass per area (LMA) and tissue hydration of plants exposed to different treatments, since these factors mutually affect attractiveness to herbivores (e.g. Goodger, Choo & Woodrow 2007).

Herbivore response to plant defence was quantified using the Mexican bean beetle (Coccinellidae: Epilachna varivestis Muls.), which is a natural herbivore of lima bean. This experimental system has proven to be highly efficient for analysing quantitative effects of chemical plant traits on herbivores (Ballhorn & Lieberei 2006; Ballhorn et al. 2007, 2008c; Ballhorn, Kautz & Lieberei 2010a).

To our knowledge, this is the first comparative approach to analyse the quantitative impact of multiple abiotic factors on different defensive and nutritive plant features at the genotypic and phenotypic level, and to assess quantitative effects.
on performance and choice behaviour of a natural insect herbivore.

**Materials and methods**

**VARIABILITY OF LIMA BEAN CYANOGENESIS IN NATURE**

Different natural lima bean populations in South Mexico (state Oaxaca) were screened for their HCNp in October 2007 (for analysis of HCNp see below). We compared four different populations (Fig. 1). Two populations were located in the vicinity of Puerto Escondido (site PTE1: 15°55’31.80" N, 97°94.68" W, 8 m a.s.l. and PTE2: 15°54’29.40" N, 97°72.58" W, 20 m a.s.l.) near the Pacific coast almost at sea level, another coastal population (site RG: 15°57’45.60" N, 97°20’31.38" W, 106 m a.s.l.) was located on a hill near the village Rio Grande, whereas the fourth population (site MR) was located close to Matias Romero in the Isthmus of Tehuantepec (16°46’53.40" N, 95°12’2.52" W, 202 m a.s.l.). At sites PTE1 and PTE2, lima bean plants were partly shaded by surrounding shrubs and trees, whereas site RG was located on a dry hill with sparse and small vegetation. Sixteen to 26 plant individuals per site were included in the analyses (for number of plants included in this study see Fig. 2). We selected young, fully unfolded leaves (N = 1 trifoliate leaf per plant) inserting three positions down from the apex of the main shoot to reduce uncontrolled variation of HCNp due to ontogenetic variability of leaves (Ballhorn, Heil & Lieberei 2006).

**PLANTS AND INSECTS**

In controlled growth chamber experiments, we used seed-grown and clonal lima bean plants. Seed material was collected at site PTE1 from a large number of mother plants (>50 plants). The testa of seeds was scratched with a razor blade to facilitate water absorption and to ensure homogenous germination of seeds. Plants were cultivated in a growth chamber (Thermotec-Weilburg GmbH & Co.KG., Weilburg, Germany) adjusted to resemble conditions at natural sites in Mexico as recorded for September to October 2007 with light regime of 13:11 L:D under photon flux density of 450-500 μmol photons m⁻² s⁻¹ at table height. Light in the chamber was provided by a combination of light sources.
PLANT CYANOGENIC POTENTIAL

Leaf cyanogenic potential was analysed by complete enzymatic degradation and subsequent quantitative measurement of released cyanide according to Ballhorn, Lieberei & Ganzhorn (2005). For hydrolysis of cyanogenic glycosides in leaf extracts, we used β-glucosidase isolated from rubber tree (Euphorbiaceae: Hevea brasiliensis) according to Ballhorn, Lieberei & Ganzhorn (2005). After 20 min of incubation at 30°C in gas-tight glass vessels (Thunberg-vessels) released cyanide was spectrophotometrically measured at 585 nm (Genesys 20, Thermo Spectronic, Madison, WI, USA) using the Spectroquant® cyanide test (Merck, Darmstadt, Germany). Single leaves and foliage of entire plants were both harvested between 10:00 and 10:45 AM to exclude diurnal effects.

TOTAL PHENOLICS

Total phenolics were analysed following Tikkanen & Julkunen-Tiitto (2003). Homogenates of leaves were extracted three times for 15 min in 5 mL (individual leaves) or 50 mL (leaf material of entire plants) acetone diluted with distilled water (60:40). After each extraction, samples were incubated in an ultrasonic bath (3 min) and finally centrifuged for 10 min at 5000 × g. The supernatant was transferred to 2 mL (20 mL in case of total plants) concentrated acetic acid (Merck KGaA, Darmstadt, Germany), acetone was removed under vacuum (60 mbar) at 40°C, and the residue was quantitatively transferred by using distilled water. Samples were diluted with 2.5% acetic acid, and 1 mL of this solution was mixed with 0.5 mL Folin-Ciocalteu's phenol reagent (Merek). After adding 2 mL 20% Na2CO3, the solution was added to 10 mL with distilled water. Samples were incubated at 70°C and, after cooling, spectrophotometrically quantified at 730 nm against blank containing water instead of sample. Epicatechin (Sigma, Deisenhofen, Germany) at different concentrations served as standard.

SOLUBLE PROTEIN CONCENTRATION

Concentration of soluble proteins in leaves and plants was quantified according to Bradford (1976) with modifications following Ballhorn et al. (2007). Bradford reagent (Biorad Laboratories, Munich, Germany) was diluted 1:5 with ddH2O and 20 μL of each homogenized plant sample was combined with 1 mL of diluted Bradford solution. Bovine serum albumin (BSA; Fluka Chemie AG, Buchs, Switzerland) at different concentrations served as standard. After 5 min incubation time, concentration of protein was spectrophotometrically measured at 595 nm. We used the same individual plant extracts for protein measurements as for HCNp analyses, thus, both parameters could be quantitatively attributed to the same sample.

PHYSICAL LEAF TRAITS AND MORPHOLOGICAL PARAMETERS

Leaf mass per area and tissue water content of individual leaves of seed-grown and clonal plants used in feeding trials were measured to assess potential effects of leaf toughness (deduced from leaf dry weight per area) and water content on performance and food plant selection of the Mexican bean beetles. Leaf samples of defined area (leaf discs, 1.2 cm in diameter) were cut from the leaf tissue weighed to the nearest 0.001 g and dried at 45 °C to a constant weight. Leaf samples were consecutively weighed for determination of dry matter.

In addition, we considered total foliage biomass, plant height and number of leaves. All leaves per plant were counted at the end of the experiment and total leaf biomass (fresh weight) was determined. Measuring dry weight of total leaf biomass was not possible, since fresh leaf material was required for chemical analyses. Plant height was determined by cutting plants off at the substrate surface and measuring the outstretched shoot.

CHOICE BEHAVIOUR OF HERBIVORES

Attractiveness of differently treated plants was analysed in experiments where adult Mexican bean beetles were offered a choice between leaf material from seed-grown and clonal plants of all treatments (N = 9 leaf discs per feeding trial, N = 7 feeding trials per group of experimental plants, i.e. seed-grown, HC- or LC-clonal plants). We used leaf discs (16 mm in diameter) to exclude any effects on the beetles’ preference resulting from leaf size or shape. After cutting the leaf discs with a cork borer, we waited for 30 min to allow evaporation of gaseous cyanide from the cutting edges. For feeding trials, the beetles were deprived of food for 2 h prior to the experiment and then leaf discs (one disc from one plant of each treatment) were offered to individual beetles for 2 h. Feeding trials were carried out in Petri dishes (14 cm in diameter) lined with slightly moist filter paper. Order of leaf discs derived from plants of the different treatment groups (G1–G9) was set at random. After the experiment, leaf discs were scanned on a scale and the missing leaf area was quantified using analySIS® software (Olympus, Hamburg, Germany).

LARVAL GROWTH AND PERFORMANCE

Freshly hatched first larval instars of the Mexican bean beetle were used for quantitative analyses of herbivore biomass accumulation and performance on differently treated seed-grown and clonal plants. Individual larvae were placed on one leaflet, which was then bagged with gauze. Each larva was weighed to the nearest 0.001 g before and after the experiment (4 d) and the absolute increase in body weight was considered. In addition, to larval biomass accumulation per time, ecdisis of larvae was recorded to evaluate differences in herbivore performance.

STATISTICAL ANALYSES

To test for significant differences in HCNp among natural lima bean populations we applied one-way ANOVA followed by least significant difference (LSD) post hoc analyses. To analyse effects of plant treatment on plant traits, we applied a one-way ANOVA followed by LSD post hoc analyses for each trait within each group of plants (i.e. seedlings, HC- or LC-clonal plants). Additionally, we used a multivariate generalised linear model (GLM) design to analyse effects of ‘Cyanogenic type’ (i.e. HC or LC), ‘Treatment’ and ‘Cyanogenic type × Treatment’ on plant traits. Analyses were carried out exclusively using clonal plants and separately for the respective treatments (i.e. reduced water supply, salt treatment, fertilization and shading) and for plant material (i.e. ontogenetically defined leaflets and total plants). For the analysis of leaflets, we set ‘Cyanogenic type’, ‘Treatment’, and ‘Cyanogenic type × Treatment’ as factors, while HCNp, protein concentration, concentration of total phenolics, LMA and tissue hydration served as variables. To analyse effects on whole plants, we set total amount of cyanide, phenolics, soluble proteins as well as total biomass, plant height and leaf number as variables. To analyse effects on choice behaviour of adult beetles as well as on larval growth parameters (body mass accumulation and ontogenetic development), we included leaf consumption, larval weight gain and larval performance as variables into a GLM.

To test for trade-offs between cyanogenesis and total phenolics and between cyanogenesis and protein concentration in untreated HC- and LC-plants (control groups; G1), we applied Pearson’s correlations. In addition, to test for correlations of both traits among plants with natural genetic variability (seed-grown plants), we applied a Pearson’s correlation for data on cyanide and total phenolics. All statistical analyses were carried out using spss 13 (SPSS for Windows, SPSS Inc., Chicago, USA).

Results

VARIABILITY OF CYANOGENIC POTENTIAL IN NATURE

Cyanogenic potential (HCNp) of young leaves showed substantial quantitative variability within and among different lima bean populations. In the populations at sites PTE1 and PTE2, HCNp ranged from 5.05 to 84.99 and 8.83 to 83.01 μmol HCN g−1 fresh wt., at site RG values varied between 12.67 and 99.38, while HCNp at site MR ranged from 4.36 to 30.35 μmol HCN g−1 fresh wt. (Fig. 2). Among populations, we found significant differences in HCNp (according to one-way ANOVA: F3,35 = 7.946, P < 0.001). Post hoc analysis (LSD, P < 0.05) revealed that HCNp of lima bean plants at site RG was significantly higher compared to sites PTE1 and PTE2, whereas HCNp at site MR was significantly lower than in the three other populations (Fig. 2). Lima bean plants at sites PTE1 and PTE2 showed no significant differences in HCNp.

VARIABILITY OF TRAITS IN SEED-GROWN AND CLONAL PLANTS

Seed-grown lima bean plants cultivated under growth chamber conditions showed similarly high variability of HCNp
Fig. 3. Responses of seed-grown and clonal plants to variation of abiotic parameters. Nine experimental groups (G1–G9; N=7 plants per group) of seed-grown and clonal (HC = high-cyanogenic and LC = low-cyanogenic) lima bean plants were cultivated under different conditions. Defined leaf stages of plants were analysed for cyanogenic potential (HCNp) (a), concentration of total phenolics (b) and soluble proteins (c). Values shown are means ± SD. Small white circles represent extremes. Among treatments, differences in leaf parameters were analysed separately for each plant type (seed-grown, HC, LC) and significant differences are indicated by different letters (lower-case letters for seed-grown plants, capital letters for HC-plants, and Greek letters for LC-plants) at the box plots [according to least significant difference post hoc analysis (LSD; P < 0.05) after one-way ANOVA].

Among all treatment groups of seed-grown plants, HCNp of young leaves was significantly negatively correlated to concentration of total phenolics (according Pearson’s correlation: \( r = -0.867, P < 0.001 \)) and to soluble protein concentration (\( r = -0.776, P < 0.001 \)). Concentration of both phenolics and protein was higher in LC- than in HC-plants (Fig. 3b,c). Generalized linear model (GLM) revealed significant effects of ‘Cyanogenic type’ (i.e. HC or LC) on phenolics and protein in young leaves (see Table S1 in Supporting Information). Consequently, phenolics concentration was significantly correlated to concentration of proteins (\( r = 0.700, P < 0.001 \)).

Patterns observed for young leaves held true at the entire plant level (see Table S2). Total production of cyanide was negatively correlated with total amounts of phenolics (\( r = -0.338, P < 0.01 \)) and protein (\( r = -0.309, P < 0.05 \)) per plant, while phenolics and protein were significantly correlated (\( r = 0.559, P < 0.001 \)).

In contrast to covariation of chemical traits, physical leaf traits showed no significant correlation with the leaf chemical parameters. Leaf mass per area and tissue hydration were homogenous among seed-grown and clonal plants (see Table S3). However, morphological parameters such as total plant biomass, plant height and leaf number were affected by ‘Cyanogenic type’ (according to GLM; see Table S4). Among all treatments, LC-plants showed higher values than HC-plants for all three parameters (Table 1).
In seed-grown plants, we found no significant treatment effects on chemical and physical traits – neither at the level of young leaves nor at the whole-plant level [according to one-way ANOVA (HCNp of leaves): $F_{8,54} = 0.367$, $P = 0.934$; (total cyanide in plants): $F_{8,54} = 0.220$, $P = 0.986$; (phenolic concentration in leaves): $F_{8,54} = 2.054$, $P = 0.057$; (amount of phenolics in plants): $F_{8,54} = 0.191$, $P = 0.991$; (protein concentration in leaves): $F_{8,54} = 0.444$, $P = 0.889$; (total protein per plant): $F_{8,54} = 1.896$, $P = 0.058$; (leaf mass per area): $F_{8,54} = 0.300$, $P = 0.963$; (tissue hydration): $F_{8,54} = 0.156$, $P = 0.995$].

**TREATMENT EFFECTS ON SEED-GROWN PLANTS**

In seed-grown plants, we found no significant treatment effects on chemical and physical traits – neither at the level of young leaves nor at the whole-plant level [according to one-way ANOVA (HCNp of leaves): $F_{8,54} = 0.367$, $P = 0.934$; (total cyanide in plants): $F_{8,54} = 0.220$, $P = 0.986$; (phenolic concentration in leaves): $F_{8,54} = 2.054$, $P = 0.057$; (amount of phenolics in plants): $F_{8,54} = 0.191$, $P = 0.991$; (protein concentration in leaves): $F_{8,54} = 0.444$, $P = 0.889$; (total protein per plant): $F_{8,54} = 1.896$, $P = 0.058$; (leaf mass per area): $F_{8,54} = 0.300$, $P = 0.963$; (tissue hydration): $F_{8,54} = 0.156$, $P = 0.995$].

**TREATMENT EFFECTS ON CLONAL PLANTS**

In clonal plants significant treatment effects were observed (Fig. 3). Variation of water supply, salinity, and nutrient availability quantitatively affected HCNp and protein concentration in both HC- and LC-plants as well as in young leaves and in whole plants. Variation of light availability, in contrast, had no significant effect on any of the plant parameters measured.

With focus on HCNp of young leaves, separate analysis of HC- and LC-plants revealed significant differences among treatment groups [according to one-way ANOVA (HC-plants): $F_{8,54} = 7.32$, $P < 0.001$; (LC-plants): $F_{8,54} = 6.77$, $P < 0.001$; Fig. 3a]. Reduced water supply (G3), increased salinity (G5), or enhanced nutrient supply (G7) resulted in significantly increased HCNp in young leaves of both clonal lines (Fig. 3a). For LC-plants, even slightly reduced water availability resulted in a significantly increased HCNp (G2) [according to LSD post hoc analysis ($P < 0.05$) after one-way ANOVA]. Plant cyanide-production responses at the level of young leaves were also confirmed for entire plants (according to ANOVA (HC-plants): $F_{8,54} = 3.532$, $P = 0.002$; (LC-plants): $F_{8,54} = 4.077$, $P < 0.001$; see Table S2). For both clonal lines, GLM revealed significant treatment effects on the total amount of cyanide accumulated per plant (see Table S4). Strictly reduced water supply (G3), high salt treatment (G5) and increased nutrient supply (G7) significantly enhanced total cyanide in HC- and LC-clonal plants compared to the controls [according to post hoc analysis (LSD, $P < 0.05$) after one-way ANOVA; see Table S2].

Soluble protein concentrations in young leaves of HC- and LC-plants showed significant differences among treatment groups [according to one-way ANOVA (HC-plants): $F_{8,54} = 20.143$, $P < 0.001$; (LC-plants): $F_{8,54} = 22.706$, $P < 0.001$; Fig. 3c]. In HC-clones, protein concentration significantly decreased in response to strictly reduced water supply (G3) and salt treatment at higher concentration (G5), significantly decreased in response to strictly reduced water supply (G3) and salt treatment at higher concentration (G5),
whereas it significantly increased in both nutrient-supplied groups (G6–7). In leaves of LC-plants, protein concentration decreased significantly in response to the lower salt concentration treatment (G4) and increased in both nutrient-supplied groups (G6–7) [according to LSD post hoc analysis (P < 0.05) after one-way ANOVA; Fig. 3c]. At the level of whole plants protein accumulation also showed significant differences depending on treatment [according to one-way ANOVA (HC-plants): F$_{6,54}$ = 2.429, P = 0.025; (LC-plants): F$_{6,54}$ = 2.170, P = 0.044; see Table S2]. The amount of protein produced per plant increased in response to nutrient supply (G6–7) and decreased in plants growing under water or salt stress. However, with exception of increased protein production following the high nutrient treatment (G7), these differences were not significant compared to the controls [according to post hoc analysis (LSD, P < 0.05) after one-way ANOVA; see Table S2].

None of the treatments had a significant effect on total phenolics in either leaves or entire plants (according to GLM; see Tables S1 and S4). Among all treatment groups of HC- and LC-plants, no significant differences in total phenolics concentration in young leaves were detected [according to one-way ANOVA (HC-plants): F$_{6,54}$ = 1.845, P = 0.085; (LC-plants): F$_{6,54}$ = 2.054, P = 0.057; Fig. 3a]. For entire plants, no differences in absolute accumulation of phenolics were observed [according to one-way ANOVA; HC-plants: F$_{6,54}$ = 0.301, P = 0.999; LC-plants: F$_{6,54}$ = 0.191, P = 0.991; see Table S2].

Furthermore, for both clonal lines no treatment effects on normalized LMA and tissue hydration were observed (according to GLM; see Table S1). Among all treatment groups, we found no significant differences in LMA [according to one-way ANOVA (HC-plants): F$_{6,54}$ = 0.829, P = 0.580; (LC-plants): F$_{6,54}$ = 1.387, P = 0.223] or tissue hydration [(HC-plants): F$_{6,54}$ = 0.894, P = 0.528; (LC-plants): F$_{6,54}$ = 0.280, P = 0.952].

**MORPHOLOGICAL PLANT PHENOTYPE**

Among all plant types (seed-grown, HC- and LC-plants) evaluation of total leaf biomass, plant height and number of leaves revealed the highest values for fertilized, seed-grown and clonal plants (G6–7), whereas water-deprived and salt-treated plants tended to show lower values (Table 1). However, per plant type, treatments applied here resulted in no significant differences of plant morphology for all considered parameters at the time of analysis (Table 1).

**CHOICE BEHAVIOUR OF BEETLES**

The attractiveness of leaf material from seed-grown plants to adult Mexican bean beetles (i.e. leaf consumption per time) was different among plant treatments (Fig. 4a). However, differences in leaf area consumption were not significant (according to one-way ANOVA: F$_{6,54}$ = 1.347, P = 0.241). Among plants of all treatments, (N = 63) leaf consumption by beetles was significantly negatively correlated to HCNp (according to two-tailed Pearson’s correlation: $r = -0.868$, P < 0.001) and positively correlated to leaf protein concentration ($r = 0.695$, $P < 0.001$).

For clonal plants, ‘Cyanogenic type’ had a significant effect on beetle-leaf consumption (according to GLM, see Table S5). In addition, among all treatment groups with exception of plants growing under reduced light availability ‘Treatment’ of plants significantly affected feeding of insects (see Table S5). For both clonal lines, the amount of consumed leaf material was significantly different among the treatment groups [according to one-way ANOVA (HC-clones): F$_{6,54}$ = 2.624,
$P = 0.016$ (LC-clones): $F_{8,54} = 5.450$, $P < 0.001$. Water-deprived (G2–3), salt-treated (G4–5) and extensively fertilized plants (G7) of both clonal lines were significantly less consumed than the controls (Fig. 4a). Leaf consumption was negatively correlated to HCNp [according to two-tailed Pearson’s correlation (HC-plants): $r = -0.313$, $P < 0.05$; (LC-plants): $r = -0.385$, $P < 0.05$, each $N = 63$ feeding trials] but showed no significant correlation to any of the other leaf parameters tested.

**LARVAL BIOMASS ACCUMULATION**

Larvae developing on water-deprived and salt-treated seed-grown plants (G2–5) as well as on extensively fertilized seed-grown plants (G7) showed lower biomass accumulation than larvae feeding on control (G1) or shaded plants (G8–9) (Fig. 4b). Among plant treatments, however, differences in larval weight gain were not significant (according to one-way ANOVA: $F_{8,54} = 1.256$, $P = 0.286$). Insect biomass accumulation among all treatments ($N = 63$) was significantly negatively correlated to HCNp in leaves (according to two-tailed Pearson’s correlation: $r = -0.766$, $P < 0.001$; $N = 63$ feeding trials) and positively correlated to leaf proteins ($r = 0.621$, $P < 0.001$).

For clonal plants, GLM revealed a significant effect of ‘Cyanogenic type’ on larval biomass accumulation (see Table S5). Among all treatments, weight gain of larvae feeding on HC-clones was lower than that of those feeding on LC-clones. In addition, to ‘Cyanogenic type’, ‘Treatment’ of plants quantitatively affected feeding of insects with the exception of experimentally varied light intensity (see Table S5). Differences in larval weight gain were highly significant among plant treatments [according to one-way ANOVA (HC-plants): $F_{8,54} = 18.967$, $P < 0.001$; (LC-plants): $F_{8,54} = 11.998$, $P < 0.001$; Fig. 4b]. For both HC- and LC-clonal lines, larvae that were feeding on strictly water-deprived (G3) and salt-treated plants (G4–5) showed significantly lower biomass accumulation than on the controls, whereas slightly increased nutrient supply (G6) resulted in enhanced larval weight gain that was significant for LC-plants (Fig. 4b). Slightly reduced water supply (G2) and extensive fertilization (G7) resulted in reduced yet not significant biomass accumulation.

Within clonal lines, larval weight gain was significantly negatively correlated to HCNp [according to Pearson’s correlation (HC-plants): $r = -0.552$, $P < 0.001$; (LC-plants, HCNp): $r = -0.570$, $P < 0.001$], and positively correlated to protein concentration in HC-plants ($r = 0.458$, $P < 0.001$) yet not in LC-plants ($r = 0.157$, $P = 0.220$; $N = 63$). For both clonal lines, biomass accumulation of larvae and leaf consumption of adult beetles in choice-feeding trials were significantly correlated (HC-clones: $r = 0.255$, $P < 0.05$; LC-clones: $r = 0.510$, $P < 0.001$).

**LARVAL DEVELOPMENT**

Among plant treatment groups of seed-grown plants, we observed differences in larval development (Table 2). On seed-grown plants, all larvae feeding on strictly water-deprived (G3) and slightly salt-treated plants (G4) failed to develop past the first larval stage, while those feeding on slightly water-deprived (G2) as well as on strongly salt-treated and nutrient-supplied plants (G5, G7) reached the second instar (at a frequency of one in seven larvae). On extensively shaded plants and on the controls (G9, G1) two larvae each reached the second stage, while on slightly nutrient-supplied and shaded plants three individuals each reached this stage (G6, G8). For seed-grown plants, differences in larval development among treatment groups were not significant (according to one-way ANOVA: $F_{8,54} = 1.113$, $P = 0.369$). Independent of plant treatment, larval development was significantly negatively correlated with plant HCNp (according to Pearson’s correlation: $r = -0.664$, $P < 0.001$), while it was positively correlated with soluble protein concentration ($r = 0.657$, $P < 0.001$).

For clonal plants, GLM revealed a significant effect of ‘Cyanogenic type’ and ‘Treatment’ on larval development (see Table S5). While ‘Treatment’ was a source of variation exclusively in differently nutrient-supplied plants, all other

<table>
<thead>
<tr>
<th>Plant type</th>
<th>Treatment group</th>
<th>Treatment</th>
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<th>Percentage of molting larvae</th>
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<td>-</td>
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<td>1(^a)</td>
<td>14.3</td>
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<tr>
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<td>G3</td>
<td>H(_2)O\ [-]</td>
<td>0(^a)</td>
<td>0.0</td>
</tr>
<tr>
<td>-</td>
<td>G4</td>
<td>NaCl\ [+ ]</td>
<td>0(^a)</td>
<td>0.0</td>
</tr>
<tr>
<td>-</td>
<td>G5</td>
<td>NaCl\ [+ +]</td>
<td>1(^a)</td>
<td>14.3</td>
</tr>
<tr>
<td>-</td>
<td>G6</td>
<td>Fertilizer\ [+ ]</td>
<td>3(^a)</td>
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<tr>
<td>-</td>
<td>G7</td>
<td>Fertilizer\ [+ +]</td>
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<tr>
<td>-</td>
<td>G8</td>
<td>Light\ [-]</td>
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<td>-</td>
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<td>0.0</td>
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<tr>
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<td>Control</td>
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</tr>
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<tr>
<td>-</td>
<td>G9</td>
<td>Light\ [- -]</td>
<td>3(^ab)</td>
<td>42.9</td>
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treatments showed no significant effects (according to GLM, see Table S5). Limited treatment effects on larval development as predicted by GLM were due to different performance on HC- and LC-clonal plants. This can be explained by the lack of developmental progression beyond the first developmental stage of all larvae feeding on HC-plants of all treatment groups (Table 2). For LC-plants, differences in development were significant among treatment groups [according to post hoc analysis (LSD, $P < 0.05$) after one-way ANOVA; Table 2]. Larvae feeding on plants exposed to reduced water supply (G3), high salt treatment (G5) and fertilization (G7) showed significantly slower development than larvae feeding on plants fertilized at lower intensity (G6). These differences were not significant, however, when compared to the controls (G1). For all treatment groups during the experimental period, 23, 13 and 0 larvae reached the second instar on LC-clones, seed-grown plants and HC-clones, respectively (Table 2). Among LC-plants, development of larvae was significantly negatively correlated to HCNp (according to Pearson’s correlation: $r = \frac{-0.570}{}, P < 0.001$), while the other leaf parameters tested showed no significant correlation to the performance of larvae.

**Discussion**

In natural systems, analyses of plant responses to abiotic conditions at small and larger scales and analyses of the consequences of these responses for higher trophic levels are complicated by various factors (Ballhorn et al. 2009b). Among these, overlapping effects of the plants’ genetic variability (i.e. genotypic variation), ontogenetic variability (variation depending on developmental stages of plants and/or specific plant organs) and phenotypic plasticity (here defined as variation depending on external factors) are major sources of variation that have to be considered – and separated – in functional analyses of plant traits (Schlichting & Pigliucci 1998; Busk & Møller 2002; Ballhorn, Kautz & Rakotoarivelo 2009c; Ballhorn et al. 2009b).

Lima bean plants in nature (Fig. 2) as well as seed-derived experimental plants (Fig. 3a) showed high genetic variability in all traits investigated. At the same time, the variation of traits was genetically based as clonal propagates retained the same traits as the mother plants. Genetic basis of cyanogenesis has also been found for other plant species, as for example tropical *Prunus turneriana* (Miller, Gleadow & Woodrow 2004). The natural variation of HCNp among lima bean individuals was particularly extensive, with the highest values being 16.8 fold higher than the lowest values. Under controlled growth chamber conditions, experimentally changed abiotic factors such as nutrient supply, drought stress, salinity and light had no significant effects on plant traits in seed-grown plant material (Fig. 3). By contrast, highly significant treatment effects on plant traits such as HCNp and soluble protein were found once genetic variability had been excluded by using clones. Interestingly, the differential effect of water and salt stress and fertilization on HC- and LC-lines increased with increasing stress intensity, pointing towards a dose-depen-

dency in the complex interplay among abiotic factors and plant resistance expression (Fig. 3). All these effects became apparent only when using clonal plants as they were masked by the genetic variability in the wild plant populations (Fig. 3).

Changing chemical traits in plants growing under environmental stress conditions have been attributed to concentration effects resulting from reduced plant size or weight (e.g. Rice & Bazzaz 1989; Koricheva 1999) rather than to increased production of the chemical in question. In this study, physical leaf traits (LMA and tissue hydration; see Table S3) and morphological phenotypes (total plant fresh weight, plant height, and leaf number, Table 1) were not significantly affected by the treatments when compared within the genetically defined plant groups (HC and LC clones). Investments in different chemical components were similarly affected in entire plants and in individual leaves (Fig. 3; see Table S2), demonstrating that traits such as HCNp and protein concentration can be quantitatively up- or down-regulated at the phenotypic level in response to ambient conditions.

Can we regard the changes in plant phenotypes observed in this study as being adaptive? First, the observed changes in biochemical traits clearly affected the development of the herbivore used in this study: larvae of Mexican bean beetle grew faster on the high-protein and low-cyanogenic lines than on the low-protein and highly cyanogenic lines (Fig. 4). The biochemical responses to altered abiotic conditions (Fig. 3) were generally reflected in the behavioural and developmental responses of the herbivore. Thus, protein content and cyanogenic potential showed both genetically fixed and phenotypically plastic components, and affected herbivore performance. Consequently, herbivores can exhibit a selective pressure on these traits.

Several theories have been formulated to predict adaptive patterns of plant investment in resistance. The resource-availability hypothesis (RAH) assumes that plant species adapted to grow at resource-poor sites are characterized by inherently slow growth rates, low maximum photosynthetic rates and low turnover rates, and predicts that these species should be intensively defended by metabolically irreversible, ‘quantitative defences’ such as lignin or tannins (Coley, Bryant & Chapin 1985; Herms & Mattson 1992; Arendt 1997). By contrast, species adapted to grow at resource-rich sites should possess flexible responses to pulses in resource supply and thus should exhibit high morphological and biochemical plasticity (Bryant, Chapin & Klein 1983; Coley, Bryant & Chapin 1985; Lim & Turner 1996). Although we compared plants of a single species showing different degrees of genetic variability rather than different species, our findings are consistent with the basic assumptions of the RAH. Lima bean possesses the characteristics of the latter group of plants, showing low investment in immobile defences (Ballhorn et al. 2007) but extensive allocation of resources to mobile defences such as cyanogenic precursors (Baudoin, Barthélémy & Ndungu 1991), VOCs and EFN (Heil 2004), defensive traits which due to their inducibility show a particularly high phenotypic plasticity. Wild type lima bean appears adapted to high-nitrogen conditions, because the seeds germinate after local fires (thus, at sites rich in mineral...
nutrients) and most individuals are colonized by nitrogen-fixing bacteria (rhizobia) under natural conditions (D.J. B. and M. H., personal observation). Strains of these bacteria (GenBank accession numbers EU842032-33, EU842040-41, EU842048) isolated from lima bean roots at natural sites were found to be highly efficient in nitrogen fixation (D.J. Ballhorn, unpublished data). In the present study, the plants were cultivated in the absence of rhizobia and responded strongly to experimental increase in nutrient availability. This observation is in accordance with assumptions of the RAH.

Our findings suggest that within natural lima bean populations different genotypes exhibit different investment strategies. This is exemplified by the fact that low-cyanogenic plants showed higher growth rates (i.e. biomass production, plant height and number of leaves) and higher soluble protein concentration than high-cyanogenic plants (Table 1,S2).

Such a – genetically partly fixed – variability of investment in defence or growth should substantially enhance the plasticity by which lima bean populations respond to variations in external factors. Although cyanogenogenesis appears to be the major direct defence of lima bean (Ballhorn et al. 2008c, 2009a), defence-associated phenolic compounds also varied depending on genotype. Low-cyanogenic and fast-growing plants showed higher concentrations of phenolics than high-cyanogenic (and slower-growing) plants. Thus, concerning these carbon-based defence compounds we observed no constraints in allocation of resources in growth or defence as predicted by theory (Hermes & Mattson 1992; Googder, Gleadow & Woodrow 2006). This finding suggests that genetic variation of cyanogenogenesis is associated with constraints on growth, while enhanced production of carbon-based phenolics in low-cyanogenic genotypes did result in growth-affecting allocation costs. The apparent trade-off between nitrogen-based defence (cyanogenogenesis) and carbon-based defence (phenolics) indicates the existence of considerable variation in the lima bean’s overall defence. Different investment strategies and/or trade-offs between lima bean traits that have been observed in the laboratory for cyanogenogenesis and release of herbivore-induced Vocs (Ballhorn et al. 2008a, 2008b) as well as for cyanogenogenesis and polyphenol oxidase activity (Ballhorn, Pietrowski & Liebelt 2010b), which suggests a complex defence syndrome in this plant species.

There is continuously growing literature on the impact of specific environmental factors on quantitative expression of cyanogenogenesis. However, studies showed different responses of plant species towards changing environmental conditions (Pederson, Fairbrother & Greene 1996). In our study, water stress resulted in increased cyanide production. This is in line with data on 
Manihot esculenta
plants, which showed increased cyanide concentrations when subjected to drought (Bokanga, Ekanayake & Dixon 1994; Aikman et al. 1996). Furthermore, Caradus et al. (1990) found that the frequency of cyanogenic 
Trifolium repens
was higher in areas with low rainfall in New Zealand. However, Foulds & Grime (1972) found the opposite being true in a study of 
T. repens
in England. In lima bean, soil salinity at higher intensities resulted in increased cyanide levels (Fig. 3). Similar results have been reported for 
Lotus corniculatus
and 
Lotus australis
(Keymer & Ellis 1978; Foulds 1982), but it remains elusive whether salt directly impacts on plant cyanogenesis or whether salt-mediated changes in water availability caused the observed effects (Stockmal & Olesebek 1997).

In our study we found no significant effects of light availability on cyanide concentration (Fig. 3). This finding is supported by our recent study on lima bean cyanogenesis at natural sites in Mexico, in which no effects of shade on cyanide accumulation in lima bean could be observed (Ballhorn et al. 2009a). However, as for water availability and soil salinity, previous studies of this issue have yielded conflicting results (Burns, Gleadow & Woodrow 2002). Studies on bracken fern 
Pteridium aquilinum
(Schreiner, Nafus & Pimentel 1984) and on 
T. repens
(Vickery, Wheeler & Mulcahy 1987) showed that plant individuals from areas with low light availability were more cyanogenic than those growing in open sites, whereas Niedźwiedz-Siegien & Gierasimiuł (2001) found a decrease in cyanogenic glycoside content with shade in flax (Linum usitatissimum). Webber & Woodrow (2009) found no effects of light on cyanogenesis in the tropical rainforest tree 
Ryparosa kurrangii
. There is increasing evidence that there are interacting effects of different environmental conditions on cyanogenesis. Supporting the importance of nitrogen availability for plant investment in cyanogenic compounds demonstrated in our present study (Fig. 3), studies on the highly cyanogenic tree 
Eucalyptus cladocalyx
showed that water stress enhances cyanogenesis by 30% under nitrogen-limited conditions, but resulted in an increase of 70% when plants were grown under enhanced nitrogen availability (Gleadow & Woodrow 2002). Furthermore, Gleadow & Woodrow (2002) showed that different co-occurring plant defences are affected in different ways by the same abiotic factors as, in contrast to cyanogenic glycosides, total phenolics and condensed tannins in 
E. cladocalyx
decreased with increasing nitrogen supply while they were unaffected by water stress. In this line, experimental shading of 
E. cladocalyx
affected a decrease in the concentration of cyanogenic glycosides, while carbon-based secondary metabolites (total phenolics and condensed tannins) were little or not affected by shading (Burns, Gleadow & Woodrow 2002).

From an ecological perspective, the active up-regulation of cyanogenic glycosides under stress conditions such as reduced water availability and soil salinity we report here can be interpreted in the light of the resource-availability hypothesis (Coley, Bryant & Chapin 1985) as plants under stressful environmental conditions are limited in replacing leaves lost due to herbivory.

Considering dramatically changing environmental conditions on local and global scales, functional studies on the ecological consequences of variation of toxic and nutritive plant traits are of great ecosystemic importance (reviewed by Bidart-Bouzat & Imeh-Nathanial 2008). In the present study we showed that the treatment-dependent up-regulation of HCNp significantly affects natural herbivores when genetic variability was excluded from the experimental plant system (Fig. 4). In genetically heterogeneous seed-grown plants the individual plants’ HCNp was the most important predictor.
for Mexican bean beetle biomass accumulation (Fig. 4b) and larval development (Table 2), while different plant growth conditions had no significant effect on herbivores. In contrast, larval performance on clonally propagated plants was significantly reduced in treatment groups with phenotypically increased HCNp (Fig. 4b). For larva feeding on HC-clones, we observed no variation of development among treatment groups, since all clonal plant individuals showed HCNp values sufficient to slow down larval development (Table 2). According to the ‘slow-growth-high-mortality hypothesis’ (Lill & Marquis 2001), lower body weight accumulation per time and slower development are important constraints of herbivore fitness, since decelerated herbivore development increases exposure time to natural enemies. However, we could demonstrate that changes of HCNp in response to variation of abiotic factors also directly affected herbivory. While in seed-grown plants choice behaviour of herbivores was negatively correlated to genetic variability of HCNp, in both clonal lines phenotypically enhanced cyanide concentrations increased resistance to herbivores (Fig. 4a).

Besides chemical defensive traits, physical traits, such as leaf resilience and tissue water content, and nutritive plant compounds strongly affect the outcome of plant–herbivore interactions (e.g. Fowler 1983; Cooper, Owen-Smith & Bryant 1988; Robbins 1993). Thus, the attractiveness of plant tissues to herbivores is often strongly affected by covariation of defensive and nutritive traits (e.g. Palo, Summerhein & Theander 1985; Belovsky & Schmitz 1994).

When consuming cyanogenic plant material, protein content in host plants is essential for herbivores (Ballhorn et al. 2008c). The major mechanism of cyanide detoxification in mammals is the conversion of cyanide to thiocyanate by activity of rhodanase (e.g. Nahrstedt 1985). This enzymatic reaction requires presence of sulfur-containing amino acids, such as methionine or cysteine (Montgomery 1969). In insects and plants detoxification of cyanide mainly occurs by activity of β-cyanoalanine synthase (Castric, Farnden & Conn 1972; Urbańska et al. 2002). This enzyme requires cysteine and free cyanide to form β-cyanoalanine, which is then converted to asparagine and returned to the amino acid pool. Thus, for efficient detoxification of cyanide by both enzymes the amount of available protein, i.e. amino acids, can be limiting.

In the present study, however, the observed effects on herbivores are rather attributed to variation of HCNp than to variation of protein content or other plant parameters. While total phenolics and physical leaf features remained unaffected by treatments, the increase in protein concentration in fertilized clonal plants (i.e. a potential increase in plant palatability) could not compensate for detrimental effects of simultaneously increased cyanide levels (Figs. 3 and 4). Genetic variability and phenotypic plasticity of cyanogenesis in lima bean crucially affect resistance to herbivores.

Conclusions

Our study shows that variations in abiotic factors can quantitatively change the defensive and nutritive features of lima bean. These shifts affect performance and choice behaviour of a natural insect herbivore. Furthermore, we show the need for experimental differentiation of genotypic and phenotypic plasticity in functional analyses of the ecological consequences of biochemical plant responses to a changing environment. The high variability of plant features such as cyanogenesis, even within a single plant species, represents a major characteristic of these systems that generally complicates functional analyses of herbivore–plant interactions (e.g. Buhrmester, Ebinger & Seigler 2000; Goodger, Capon & Woodrow 2002; Ballhorn et al. 2009b). Our combined approach including genetically heterogeneous plants and clones with different biochemical characteristics enabled us to link the observed effects on herbivores to distinct traits – and to different sources of variation, i.e. plants’ phenotypic plasticity resulting from different ambient conditions and genetically based variability. The results of our study suggest that genetic within-population variability of plant anti-herbivore defence and phenotypic plasticity allow plant populations to rapidly respond to changing environmental conditions.

Acknowledgements

We thank Monika Fillippek and Tina Meyer for maintenance of beetles. This work was financially supported the University of Duisburg-Essen and by the DFG (grants Ba 3966/1-1 and He 3169/4-2). SK is supported by a postdoctoral fellowship from the German Academic Exchange Service (DAAD).

References


Received 4 March 2010; accepted 15 September 2010
Handling Editor: David Gibson

Supporting Information
Additional Supporting Information may be found in the online version of this article:
Table S1. Treatment effects on leaf traits.
Table S2. Total amounts of cyanide, soluble protein and total phenolics per plant.
Table S3. Physical leaf traits. Leaf mass per area and tissue water content of leaves were measured among different treatments.
Table S4. Treatment effects on level of entire plants.
Table S5. Treatment-mediated effects on leaf consumption and biomass accumulation of Mexican bean beetles.

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