J Chem Ecol (2014) 40:468–475
DOI 10.1007/s10886-014-0434-0

Variation in Cyanogenic Glycosides Across Populations of Wild Lima Beans (*Phaseolus lunatus*) Has No Apparent Effect on Bruchid Beetle Performance

J. Gwen Shlichta · Gaetan Glauser · Betty Benrey

Received: 15 January 2014 / Revised: 4 April 2014 / Accepted: 15 April 2014 / Published online: 27 May 2014

Abstract Cyanogenic glycosides (CNGs) act as feeding or oviposition deterrents and are toxic after enzymatic hydrolysis, thus negatively affecting herbivore performance. While most studies on CNGs focus on leaf herbivores, here we examined seeds from natural populations of *Phaseolus lunatus* in Mexico. The predominant CNGs, linamarin and lotaustralin, were quantified for each population by using ultra-high pressure liquid chromatography-mass spectrometry. We also examined whether there was a correlation between the concentration of CNGs and the performance of the Mexican bean beetle, *Zabrotes subfasciatus*, on seeds from each population. The concentrations of CNGs in the seeds were relatively high compared to the leaves and were significantly variable among populations. Surprisingly, this had little effect on the performance of the bruchid beetles. *Zabrotes subfasciatus* can tolerate high concentrations of CNGs, most likely because of the limited β-glucosidase activity in the seeds. Seed herbivory does not appear to liberate hydrogen cyanide due to the low water content in the seed. This study illustrates the importance of quantifying the natural variation and activity of toxic compounds in order to make relevant biological inferences about their role in defense against herbivores.

Keywords Cyanogenic glycosides · Linamarin · Plant-insect interaction · Plant defense · *Zabrotes subfasciatus* · *Phaseolus lunatus*

Electronic supplementary material The online version of this article (doi:10.1007/s10886-014-0434-0) contains supplementary material, which is available to authorized users.

J. G. Shlichta · G. Glauser · B. Benrey (✉)
Institute of Biology, University of Neuchâtel, Rue Emile-Argand 11, 2000 Neuchâtel, Switzerland
e-mail: Betty.Benrey@unine.ch

Introduction

A variety of chemical defense compounds are part of a plant's arsenal against herbivores. These antiherbivory chemicals or secondary metabolites include terpenoids, phenolics, and nitrogen-containing compounds such as cyanogenic glycosides (CNGs). Cyanogenic plants are found throughout the plant kingdom (Jones 1988; Seigler 1998), and currently over 2500 plant species are known to release toxic hydrogen cyanide from cyanide-containing secondary metabolites (Zagrobelny et al. 2004). Cyanogenic glycosides are stored in vacuoles as inactive glycosylated precursors, *i.e.*, *O*-β-glycosides of α-hydroxynitriles (cyanohydrins) (Gleadow and Woodrow 2002; Vetter 2000). Tissue disruption results in cyanogenesis, a process in which the CNGs are cleaved by β-glucosidases followed by hydrolysis through the action of α-hydroxynitrile lyases (Selmar et al. 1989). Hydrogen cyanide (HCN), a compound that is toxic to many herbivores (Ballhorn et al. 2007; Zagrobelny et al. 2004), is released.

Non-adapted herbivores avoid CNGs (Gleadow and Woodrow 2002; Jones 1988; Nahrstedt 1988), and CNGs can act as feeding deterrents or inhibitors (Ballhorn et al. 2010), as well as oviposition deterrents (Ballhorn and Lieberei 2006; Ballhorn et al. 2007). Furthermore, CNGs can slow down the development of insect herbivores, thereby increasing their risk of predation or parasitism, and thus indirectly defending the plants (Benrey and Denno 1997).

The actual effectiveness of cyanogenic glycosides as a defense mechanism is variable (Gleadow and Woodrow 2002; Puustinen and Mutikainen 2001; Vetter 2000), and in some cases CNGs have minimal or no effect on herbivores (Ferreira et al. 1997). Specialist herbivores can resist the negative effects of CNGs by several metabolic or behavioral mechanisms. For example, the Sara Longwing butterfly, *Heliconius sara*, feeds on the cyanogenic leaves of the passion vine, *Passiflora auriculata*, but prevents the release of cyanide

by metabolizing the CNGs. In fact, the nitrogen that is released is utilized in the insect's primary metabolism (Engler et al. 2000). Other species are able to sequester the toxins and use them as a defense against predators. The larvae of the Mexican fritillary, *Euptoieta hegesia*, sequesters CNGs and is therefore distasteful to *Anolis* lizards (Schappert and Shore 1999). For some specialists, CNGs may even act as feeding or oviposition stimulants (Calatayud and Le Ru 1996; Honda et al. 1997). For instance, larvae of the southern armyworm, *Spodoptera eridania* that are fed a diet containing CNGs have similar or improved growth compared to controls (Brattsten et al. 1983). Calatayud and Le Ru (1996) found that the cassava mealybug, *Phenacoc cuemanihot*, is attracted to the CNGs of cassava plants.

Natural plant populations may vary in CNG concentration (Aikman et al. 1996; Buhrmester et al. 2000; Gleadow and Woodrow 2000a, b). For example, significant quantitative variation in the CNG prunasin was found in two natural populations of the Australian Eucalyptus tree, *Eucalyptus polyanthemos*, with levels in one population ranging from zero to as much as 2.07 mg CN/g dry weight, while levels in the other population were between 0.17 to 1.98 mg CN/g dry weight (Goodger et al. 2002). More extreme variation was found in elderberry, *Sambucus canadensis L*, in which some populations were essentially acyanogenic, while other populations showed high variation in the concentration of CNGs among individuals (Buhrmester et al. 2000). This variability on the plant side adds another layer of complexity and needs to be considered in efforts to understand the role of these compounds in defending plants against herbivory.

The vast majority of studies of CNGs have focused on leaf herbivores. As a consequence, there is little information describing the variation in the concentrations of CNGs in seeds, or the effects of variation on seed-feeding herbivores. Cyanogenic glycoside concentrations in the fruiting structures of plants are important in the context of plant domestication, particularly in cases where fruits and seeds serve as food for humans and/or livestock. Just as for other defensive chemicals, CNGs levels can be expected to be lower in domesticated plants as a result of selective breeding (Poulton 1990).

Phaseolus lunatus, the wild lima bean, appears to be unique in that it is the only *Phaseolus* species reported to contain CNGs (Jones 1988; Poulton 1990; Vetter 2000). Previous studies have focused on CNGs in leaves of *P. lunatus*, and only few have investigated their presence in seeds (Frehner et al. 1990). None of these studies has specifically examined the effect of CNGs on seed-feeding herbivores. The goal of the current study was to examine the potential role of CNGs in the seeds of lima bean as a defense against a seed-feeding herbivore. The bruchid beetle *Zabrotes subfasciatus* Boheman (Coleoptera: Chrysomelidae) attacks the seed of several *Phaseolus* species, including the cyanogenic *P. lunatus*, and it is one of the most important pests of stored beans worldwide (Benrey et al. 1998). It is thought to have evolved in Central America, and initially it used the wild ancestors of *Phaseolus lunatus* and *P. vulgaris* as its hosts (Gonzalez-Rodriguez et al. 2002). *Zabrotes subfasciatus* has a relatively broad host range within the genus *Phaseolus* and may not be specifically adapted to CNG-containing lima beans. Hence, this insect appears ideally suited to test the effects of variation in CNG concentration among lima bean populations on seed-feeding herbivores.

For our study we sampled seeds from natural populations of *Phaseolus lunatus* along the western coast of Oaxaca, Mexico. The dominating CNGs, linamarin and lotaustralin, were quantified for each population using liquid chromatography coupled to mass spectrometry (LC/MS). We further measured the β-glucosidase activity of the beans in order to assess the HCN-capacity, *i.e.*, the release of HCN by the beans when consumed by beetles. Lastly, we quantified the performance of *Z. subfasciatus* on beans from each population by measuring mass, development time, and survival of emerging adult beetles. Our specific objectives were: 1) to characterize wild lima bean populations for variation in cyanogenic glycosides; 2) to assess the performance of bruchid beetles on seeds from each population; and 3) to test whether differences in the concentration of CNGs among populations correlate with bruchid performance.

Methods and Materials

Seed Collection Seed pods were collected from 12 sites in the state of Oaxaca, Mexico from December to February 2010–2011. These sites included 10 populations along the coast from 597 km north to 50 km south of Puerto Escondido, and two populations 586–702 km east of Puerto Escondido (see Table 1, and Online resource 1). Samples were taken from 5 to 10 plants per site, except when less than 10 plants were

Table 1 GPS coordinates and altitude for each site where beans were collected

Population	Latitude	Longitude	Altitude (meters)
HHI	N16 46.626	W99 29.712	80
INK	N15 43.469	W96 39.188	35
ITC	N17 00.675	W100 06.171	25
KM	N15 57.742	W97 20.503	65
MAD	N17 21.158	W101 03.368	35
MAR	N16 35.732	W98 46.102	55
PET	N17 26.118	W101 11.647	7
SMA	N16 47.541	W99 22.688	91
UMA	N15 55.330	W97 09.132	17
WAS	N18 35.938	W98 33.142	1238
YAU	N18 55.191	W99 02.397	1236
YEL	N16 15.071	W97 48.169	240

available. Pods were shelled and subsamples of seeds were separated for chemical extraction and analysis.

Cyanogenic Glycoside Quantification To prepare samples for quantification of CNGs from dry beans, approximately 5–10 beans from each population were submerged in liquid nitrogen and ground with a mortar and pestle to obtain a fine powder for each sample. This process was repeated five times for each population. Approximately 0.020 g of prepared bean powder per sample were stored in a 1.5 ml screw-top plastic tube. Samples were kept cold using liquid nitrogen throughout grinding and weighing of the sample before storing them at −80 °C degrees.

To extract the CNGs from the bean samples, we used a method adapted from two previous studies on CNGs (Franks et al. 2005; Rojas and Morales-Ramos 2010). In the modified method, we added 1 ml of ice cold 70 % methanol to each sample and immediately placed the samples on a heating block at ~90 °C for 10 min. Tubes were removed from the heating block, allowed to cool on ice, and were placed in a Branson 2210 ultrasonic shaker for 10 min and centrifuged at 8000 rpm for 3 min. The supernatants were carefully removed from the tubes, avoiding any particulates, and were stored in a 1.5 ml plastic tubes at −80 °C until analysis. The supernatants were diluted 1:50 with 70 % methanol before analysis.

Cyanogenic glycosides were analyzed using an Acquity ultra-high pressure liquid chromatography (UPLC) system coupled to a Synapt G2 QTOF mass spectrometer (Waters, Milford, MA, USA) controlled by Masslynx 4.1. Separation was performed on a Waters Acquity BEH C18 column (50× 2.1 mm i.d., 1.7 μm particle size) thermostated at 25 °C. Mobile phases consisted of water containing 0.05 % formic acid (solvent A) and acetonitrile containing 0.05 % formic acid (solvent B). The following gradient was applied: 2–30 % B in 1.5 min, 30–100 % B in 1.0 min, hold at 100 % B for 2.0 min, and re-equilibrate with 2 % B for 1.0 min. The flow rate was set to 400 μl/min. Under these conditions, linamarin eluted at 0.83 min and lotaustralin at 1.10 min (Online Resource1). The injection volume was 1 μl. Detection was performed in electrospray negative ionization mode using the $[M+HCOO]^-$ ion. Extracted ion chromatograms at *m/z* 292.113 ±0.02 Da and 306.119±0.02 Da were generated for quantification of linamarin and lotaustralin, respectively. Absolute concentrations of CNGs were determined by external calibration using calibration points at 0.2, 1, 5 and 25 μg/mL prepared from linamarin and lotaustralin standards (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 70 % methanol.

Estimation of HCN Released Previous studies have established techniques to assess β-glucosidase activity and the release of HCN (Ballhorn et al. 2005; Lieberei 1988). With these techniques, it has been possible to quantify HCN capacity and the rate of HCN release using leaf-feeding herbivores or simulation of feeding on a leaf with mechanical damage. It was not feasible to use these techniques for *Z. subfasciatus* because the beetle larvae are very small (about 2 mm) and feed within the seed over a long period of time (about 35 d). Instead, we quantified the loss of CNG when beans were ground in order to estimate the amount of HCN released from each sample. This provides an indirect assessment of β-glucosidase activity responsible for the hydrolysis of cyanogenic glycosides (HCN capacity or HCN-c). All samples for this analysis came from a random sample of seeds collected from the UMA population. Seeds were ground as a single sample and were used for all tests. Each treatment consisted of 6 samples. We exposed the samples to one of four different treatments that evaluate the constraints on β-glucosidase activity (Ballhorn et al. 2005; Vetter 2000). If there is significant β-glucosidase activity, HCN should be produced when the samples are ground and exposed to air. If there is no activity due to low water content, then adding water to the ground samples should activate the β-glucosidase present. Lastly, if there is a low concentration or absence of β-glucosidase, then the addition of this enzyme to the ground samples should cause hydrolysis. Therefore, we applied the following treatments to ground bean powder; 1) no treatment (bean control); 2) exposure to room temperature air for 60 min at room temperature (bean +air); 3) addition of 100 ul ultrapure water (bean + water) and left for 5 min before further processing; and 4) addition of 100 ul of enzyme extracted from the leaves of the lima bean plant (bean + enzyme) and left for 5 min before further processing. Samples then were processed as described in the CNG quantification section, except that only 900 ul of ice cold 80 % methanol were added to the bean + water and bean + enzyme samples. We processed 6 subsamples for each treatment from the same sample of ground beans. We also extracted CNGs from leaves of a plant from the same population. For comparison, we extracted CNGs from leaves processed normally (leaf control) and from leaves that were ground and exposed to air for 60 min at room temperature (leaf + air). The leaf samples were also replicated 6 times. The CNGs from these samples were analyzed as described above.

Bruchid Performance To examine the performance of bruchid beetles on seeds from the different populations we used our laboratory colony of *Zabrotes subfasciatus* (Coleoptera: Chrysomelidae). This colony was established in the laboratory in 2002 with a mixture of beetles collected from the Mexico City (YAU) area and Puerto Escondido (UMA) (Campan and Benrey 2006). Each year since the initial establishment we have refreshed the colony with beetles collected from the populations near Puerto Escondido (mainly UMA and INK). Beetles were reared on *Phaseolus vulgaris* beans to eliminate possible selection for CNG tolerance.

For the performance experiments, beetles were placed on beans collected from the populations in 2011 and 2012. These

beans had been stored in paper bags at 4 °C and were transferred to small plastic 28×23×5 mm cups with lids (Semadeni AG, A4686). Each cup contained five beans from a single population. These beans had been carefully checked for holes or any sign of previous infestation. A male and female were aspirated from the colony and placed in a cup with the beans. We prepared 10 cups for each population for a total of 25 beans and 5 mating pairs per population for each experimental trial. We repeated the experiment 4 times in 2011. Each cup with a mating pair was placed on a tray and put in a rearing chamber. The chamber was set for 11 hr at 27 °C/13 hr at 25 °C, 11L/13D, and ~80 % humidity. Cups were checked every day for 7 d. Each bean was examined for eggs. If a bean had one or two eggs, the beans were removed and each bean was placed into a 2 ml micro centrifuge tube. Only 2 eggs were allowed to remain on each bean. If additional eggs were laid on a single bean, they were destroyed to prevent competition between beetle larvae in the same bean. A small hole was made at the top of each plastic tube to allow for air exchange. Tubes were returned to the growth chamber and checked daily until adult beetles emerged. Date of emergence and sex were noted, and the adult beetle was weighed. Then the beetle was transferred to a tube with 95 % ETOH for storage. If that bean contained an additional egg, it was returned to the tube and placed in the growth chamber until the second adult beetle emerged.

Statistical Analysis The data from the chemical analyses were processed with Masslynx 4.1. Linamarin and lotaustralin were identified and quantified by measuring peak areas from the corresponding LC/MS extracted ion chromatograms. The concentrations (μg/ml) were calculated using the calibration curve for each standard, and were converted into μg/g or mg/g dry weight (DW) based on the initial amount of dry bean powder. The mean concentrations of linamarin, and lotaustralin, and survival data, were compared using a one-way analysis of variance (ANOVA). Cyanogenic glycoside concentrations were log transformed, and survival data were arcsine transformed. *Post-hoc* pairwise comparisons between populations were examined using Tukey's (Tukey's HSD, $P<0.05$). To examine the differences in adult mass and development time among populations, we used a Kruskal-Wallis test followed by a multiple comparison using Dunn's method when we found significant differences between populations (Dunn's Method, $P<0.05$). SPSS Version 21 (SPPS for Mac, Chicago, IL, USA) was used for all statistical analysis.

Results

Population Variation in Cyanogenic Glycoside Content Wild lima bean populations had on average 50 times higher concentration of linamarin than of lotaustralin (Fig. 1). The concentrations varied significantly among populations for both linamarin ($F=13.479$; $df=11, 47$; $P<0.001$) and lotaustralin ($F=33.729$; $df=11, 47$; $P<0.001$). Populations KM, MAR, and SMA had significantly lower concentrations of linamarin compared to the populations MAD and WAS. Lotaustralin concentrations were significantly lower for populations KM, MAR, SMA, YAU, and HHI compared to UMA, YEL, and MAD (Tukey's HSD, $P<0.05$). Overall, KM had the lowest average concentrations and WAS had the highest average concentration of both CNGs.

Estimation of HCN Released In order to assess β-glucosidase activity in the seeds, we estimated HCN release by comparing the concentration of the CNGs linamarin and lotaustralin remaining in seeds and leaves after different treatments. These treatments were compared to the control, in which samples were kept at a constant cold temperature throughout grinding and processing. When we ground beans and exposed them to room temperature for an hour, no loss of CNGs was observed. Instead CNGs average concentration slightly increased (Fig. 2). To assess if β-glucosidases were inactive due to lack of water or due to lack of β-glucosidase activity, we added water (bean + water) or β-glucosidases extracted from a leaf (bean + enzyme) to some samples. There was a large reduction in the concentration of both linamarin and lotaustralin in the beans when water or enzyme was added (Fig. 2). There was no significant difference between samples to which we added water compared to samples to which we added enzyme (Fig. 2). In contrast to dry beans, ground leaves exposed to air at room temperature showed a significant loss of both linamarin and lotaustralin compared to the control (Fig. 2).

Bruchid Performance on Wild Lima Beans from Different Populations The total number of eggs laid by bruchid females on beans from different population ranged from 178 to 314 eggs. Survival to adulthood varied significantly among populations ($F=2.187$; $df=11, 348$; $P=0.015$) (Fig. 3), ranging from 33 % (population ITC) to 63 % (KM, MAD, YEL) (Fig. 3). We found no statistically significant differences among populations for the masses of either the adult male or female beetles. All populations had a similar range of mass for both sexes. Development time from egg to emerging adult differed among populations for female beetles (Kruskal-Wallis, $P=0.019$), but not for males. Multiple pairwise comparisons revealed no difference between pairs of populations (Dunn's Method, $P<0.05$). There was a significant difference among populations in survival to adulthood and female development time, therefore, we used a linear regression to see if these two performance parameters correlated with linamarin or lotaustralin concentration. The concentrations of the two defense compounds did not correlate with survival (linamarin, $R^2=0.0003$, $P=0.953$; lotaustralin, $R^2=0.0168$, $P=0.688$).

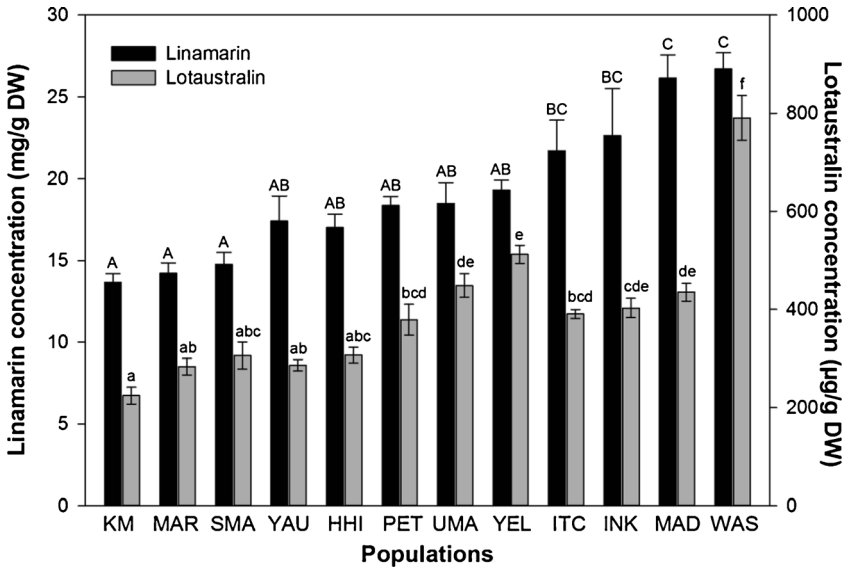

Fig. 1 Mean concentration of the cyanogenic glycoside compound linamarin and lotaustralin quantified from beans collected from each population. Concentration in mg/g dry weight (DW) (*linamarin*) and ug/g DW (*lotaustralin*). Analysis was performed on five samples from five plants in each population. Values shown are means ± standard error. Both linamarin (F= 13.479; df=11, 47; $P<0.001$) and lotaustralin concentrations (F=33.729; df=11, 47; $P<0.001$) were found to be significantly different between populations. Significant differences calculated by a *post-hoc* test (Tukey's HSD; $P<0.05$) after one-way ANOVA are shown as *letters* in figure

Further, neither linamarin concentration (R^2=0.141, P= 0.229), nor lotaustralin concentration (R^2=0.103, P=0.310) correlated with female development time (Fig. 4).

Discussion

Our results show that the concentration of cyanogenic glycosides in the seeds of *P. lunatus*, is highly variable among populations. This is not entirely surprising because for several plant species it is known that the non-reproductive parts can vary in CNG concentration across populations, as well as with plant age, and environmental conditions (Gleadow and Woodrow 2000a). However, in the current study we examined the variation in CNGs in seeds across plant populations. High variation within populations has been found in other species of plants that contain CNGs, including *Trifolium repens L.* (Hughes 1991), many vascular plant species (Aikman et al. 1996), *Sambucus canadensis L.* (Buhrmester et al. 2000), *Eucalyptus* spp. (Goodger et al. 2002; Woodrow et al. 2002), and *Clerodendrum grayi* (Miller et al. 2006). In contrast, significant variation in CNG concentration among populations rarely has been found (Goodger et al. 2002), except in a few cases, which appear to be largely driven by the acyanogenic individuals in some populations (Aikman et al. 1996). Cyanogenic glycoside concentrations and cyanogenic potential are largely genetically determined (Goodger et al. 2004; Woodrow et al. 2002). This variation among plant populations offers unique opportunities to understand the different selective forces that have favored the role of these compounds in the defense against leaf- and seed-feeding herbivores. Further, this study shows that just as CNGs play a role in defending lima bean leaves against herbivores (*i.e.*, Ballhorn et al. 2005; Gleadow and Woodrow 2000a), CNGs may play a much less important role in defending the seed against seed-feeding herbivores.

To our knowledge this is the first study to compare the level of CNGs in seeds from different plant populations and to correlate these levels to the performance of a seed-feeding insect. We measured high levels of CNGs in the seed, but these supposedly toxic compounds are apparently not the decisive factor for the performance of the bruchid beetle. Although the data show a trend of poor growth on seeds with higher concentration of the two CNGs, there are a number of outliers, and the correlation was not significant, with linamarin or with lotaustralin concentration. Nevertheless, we found significant differences among populations in the development time of the female beetles (Fig. 4; Kruskal-Wallis, P= 0.019). This indicates that, perhaps in addition to CNG concentration, there are other key factors determining female development time on lima bean seeds. These could be other defense chemicals or seed characteristics, such as hardness, moisture content and volume, and seed proteins (Moraes et al. 2000; Zaugg et al. 2013). For example, with respect to the seed protein, it recently has been shown that seeds of wild populations of *P. vulgaris* collected in Mexico contain high amounts of arcelin, which affects development time, emergence rates, and adult mass of *Z. subfasciatus* and *Acanthoscelides obtectus* Say (Zaugg et al. 2013).

Gleadow and Woodrow (2002) proposed some possible explanations for variability in the effectiveness of CNGs; (1) the concentrations of CNGs may be below the threshold level of toxicity; (2) specialist herbivores may be adapted to CNGs; (3) herbivores may have a mixed diet and thus not received a

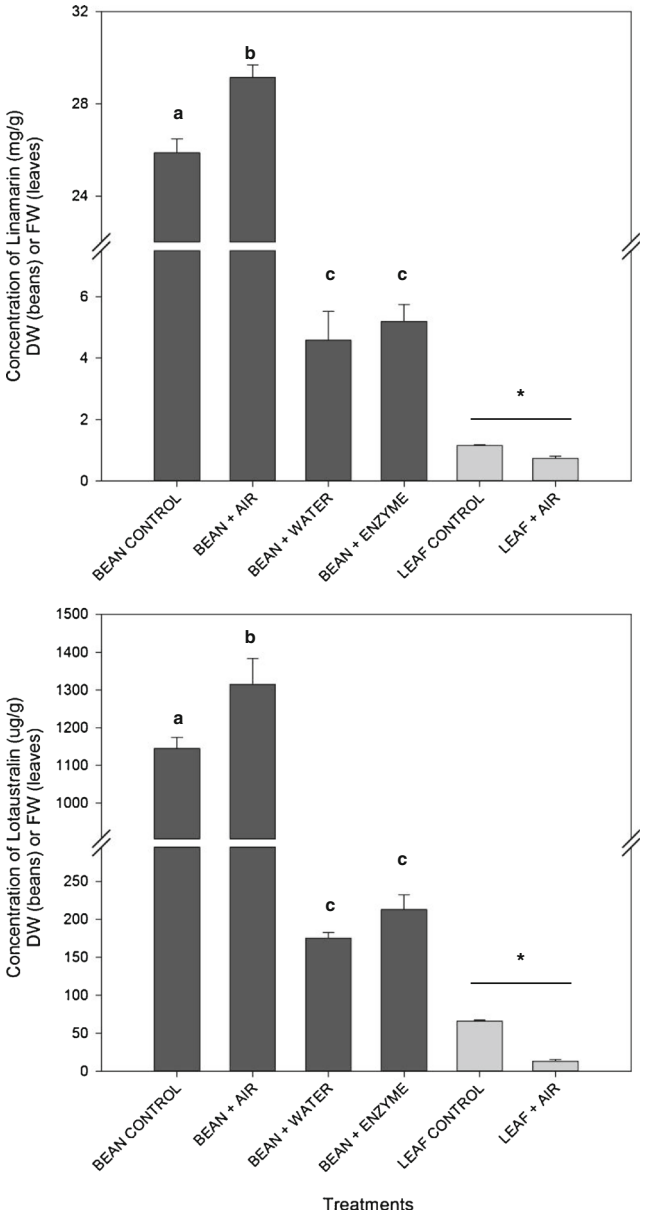

Fig. 2 Analysis of β-glucosidase activity in beans and leaves. Graph shows the mean concentration of the cyanogenic glycoside compound linamarin and lotaustralin quantified from bean dry weight (DW) and leaf fresh weight (FW). Concentration in mg/g dry weight (*linamarin*) and ug/g (*lotaustralin*). Analysis was performed on six samples from leaves or beans from plants from the same population. Values shown are means ± standard error. Both bean (linamarin: F=375.78; df=3, 23; $P<0.001$; lotaustralin: F=245.31; df=3, 23; $P<0.001$) and leaf concentrations (linamarin: df=11, t=5.76, $P<0.001$; lotaustralin: df=11, t=20.07, $P<0.001$) were significantly different. Significant differences calculated for beans by a *post-hoc* test (Tukey's HSD; $P<0.05$) after one-way ANOVA and for leaves by a *t*-test. Significant differences between bean treatments are shown as *letters* in figure

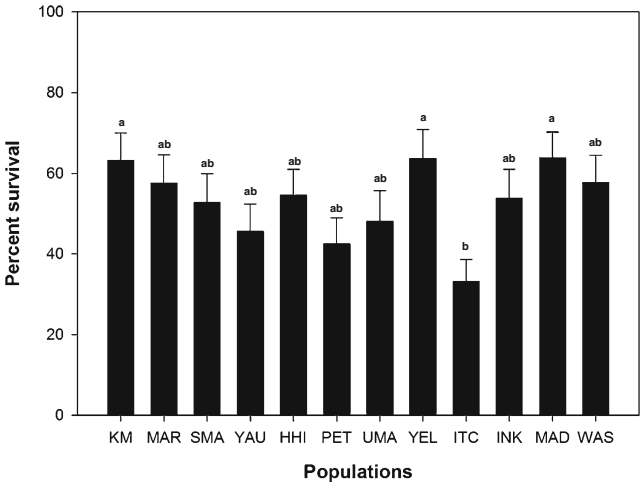

Fig. 3 Percent survival of adult beetles from total eggs laid. Significant variation was found between the populations (F=2.187; df=11, 348; P=0.015). Significant differences were found between population ITC and populations KM, YEL, and MAD (Tukey's HSD; $P<0.050$) calculated by a *post-hoc* test after one-way ANOVA indicated significance. Values shown are means ± standard error

full exposure to CNG toxicity, and lastly; (4) herbivores may cause minimal damage to the leaf (or other plant part) and not trigger the release of toxic HCN. We reflect here on each of these possibilities as an explanation for the fact that bruchid beetles were apparently minimally affected by the CNGs in the seeds.

We can exclude option one and three, as each *Z. subfasciatus* larva exclusively feeds on the content of one seed and therefore receives the full dose of the CNGs contained in this seed. Also, the concentrations of CNGs that we measured in the seeds, although highly variable, were higher than the concentration in the leaves (Fig. 2). The herbivores eating dry seeds are thus exposed to particularly high concentrations of CNGs. Such concentrations are sufficiently high to affect the oviposition, performance, and survival of various leaf feeding insects (Ballhorn et al. 2005; Ballhorn and Lieberei 2006; Shlichta et al. unpublished data).

Leaf chewing insects extensively damage tissues, causing CNGs to come into contact with β-glucosidases and resulting in the release of high levels of toxic HCN (Pentzold et al. 2013). *Zabrotes subfasciatus* is a chewing insect, which utilizes a large portion of an individual bean for development, therefore, it causes a great amount of tissue damage to the bean. Moreover, it is not a specialist on *P. lunatus* and feeds upon the seeds of many plants in the genus *Phaseolus*, none of which contain CNGs, including a closely related species, *P. vulgaris*. The beetle also has been found to feed on other legume species that are CNG free. When given the choice, *Z. subfasciatus* prefers to lay eggs on cultivated *P. lunatus* seeds that are low in CNGs (Shlichta et al. unpublished data). The selection of seeds with lower CNGs suggests that these beetles are able to detect differences in CNG

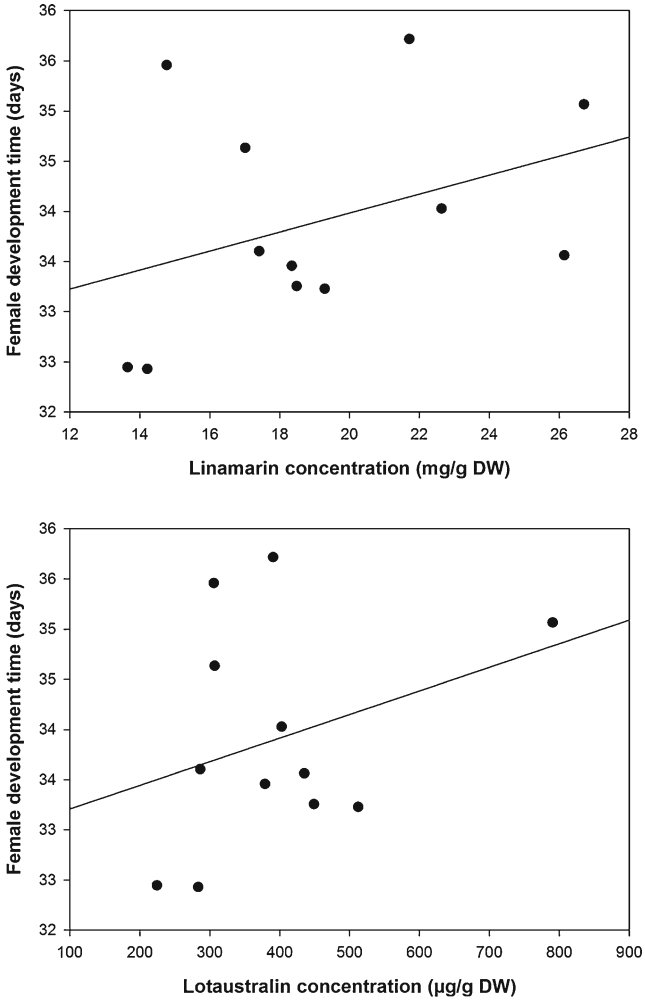

Fig. 4 No correlation between female development time and concentration of linamarin and lotaustralin. Neither linamarin concentration (R^2=0.141, P=0.229), nor lotaustralin concentration (R^2=0.103, P=0.310) correlated with female development time

concentrations in the seed. The ability to discern the different concentrations of CNGs *in leaves* has been shown in other insect herbivores (Ballhorn et al. 2010). This ability to recognize or detect CNG concentrations could be an adaptation of the beetles to cyanogenic plants (Pentzold et al. 2013). However, since this beetle was relatively unaffected by CNGs in our performance tests, it is unlikely to be a CNG plant specialist or to have evolved specific adaptations such as detoxification enzymes to deal with CNGs (Pentzold et al. 2013). The evidence suggests that the beetle is specifically adapted to CNGs in the plant, therefore our data suggest that the fourth explanation proposed by Gleadow and Woodrow (2002) may apply to *Z. subfasciatus*.

While beetle larvae are chewing insects and are continuously exposed to CNGs present in the bean, our data suggest that even after severe tissue damage, the CNGs in the beans are not hydrolyzed to release toxic HCN. The beetle attacks the dry seed of the plant, which contains little of the water that is necessary for hydrolysis to occur. Our assessment of β-glucosidase activity, estimated *via* HCN released, indicates that in all likelihood there is little or no β-glucosidase activity in the dried bean due to the low water content (Fig. 2) and thus CNG is not hydrolyzed to toxic HCN in the lima bean. The low water content would mean that the CNGs remain intact and do not yield HCN regardless of the concentration of CNGs in the bean. It also is noteworthy that dry beans contain effective β-glucosidases since the addition of leaf β-glucosidases to the extract did not increase the consumption of CNGs compared to the addition of water alone (Fig. 2). It is not known whether a beetle larva introduces enough moisture through its saliva when feeding on the seed to activate the release of small quantities of HCN. Although at a small scale, this might add enough water to release HCN and be toxic to the beetle.

Additionally, trade-offs may exist between CNGs and other defenses, limiting our ability to evaluate the effect of CNGs. For instance, a trade-off between cyanogenesis and fungal resistance has been reported for the rubber tree, *Hevea brasiliensis* (Lieberei et al. 1996). As well, there are trade-offs between the concentration of CNGs in secondary leaves and volatile emissions in *P. lunatus* (Ballhorn et al. 2008). The emission of volatile organic compounds provides an indirect defense for the plant by alerting third trophic level predators or parasitoids to the presence of herbivores (Ballhorn et al. 2008). Although the concentration of CNGs in the beans appears to have no effect on the bruchid beetle, there may be a difference in parasitism rates of larvae in seeds with high or low CNGs due to a negative correlation with emissions of parasitoid-attracting volatile organic compounds.

Our results indicate that CNGs do not play an important role in defending the *P. lunatus* seeds from the bruchid *Z. subfasciatus* even though the insect larvae are confined to the seed for their entire development and therefore are exposed to high concentrations of potentially toxic CNGs. It is highly unlikely, however, that this non-specialized seed beetle is fully resistant to the effects of CNGs. We speculate that unidentified factors are of key importance for the development of the larvae, and that a lack of moisture in the seeds prevents the release of toxic HCN.

Acknowledgments We are grateful to Johnattan Hernández Cumplido, Geraldine Mudavadi, and John Riordan for their assistance with collections, experiments, chemical analysis, and general support throughout this study. We also thank Stefan Pentzold for advice and guidance on β-glucosidase activity and Ted Turlings and two anonymous reviewers for feedback on the manuscript. We express our gratitude to the Universidad del Mar Campus in Puerto Escondido for providing logistic support and infrastructure during the field work in Mexico. This research was financially supported by the Swiss National Science Foundation (Project No. 31003A_127364) awarded to BB.

References

Aikman K, Bergman D, Ebinger J, Seigler D (1996) Variation of cyanogenesis in some plant species of the Midwestern United States. Biochem Syst Ecol 24:637–645

Ballhorn DJ, Lieberei R (2006) Oviposition choice of Mexican bean beetle (*Epilachna varivestis*) depends on host plants cyanogenic capacity. J Chem Ecol 32:1861–1865

Ballhorn DJ, Lieberei R, Ganzhorn JU (2005) Plant cyanogenesis of *Phaseolus lunatus* and its relevance for herbivore-plant interaction: the importance of quantitative data. J Chem Ecol 31:1445–1473

Ballhorn DJ, Heil M, Pietrowski A, Lieberei R (2007) Quantitative effects of cyanogenesis on an adapted herbivore. J Chem Ecol 33: 2195–2208

Ballhorn DJ, Kautz S, Lion U, Heil M (2008) Trade-offs between direct and indirect defences of lima bean (*Phaseolus lunatus*). J Ecol 96:971–980

Ballhorn DJ, Kautz S, Lieberei R (2010) Comparing responses of generalist and specialist herbivores to various cyanogenic plant features. Entomol Exp Appl 134:245–259

Benrey B, Denno RF (1997) The slow-growth-high-mortality hypothesis: a test using the cabbage butterfly. Ecology 78:987–999

Benrey B, Callejas A, Rios L, Oyama K, Denno RF (1998) The effects of domestication of Brassica and Phaseolus on the interaction between phytophagous insects and parasitoids. Biol Control 11:130–140

Brattsten LB, Samuelian JH, Long KY, Kincaid SA, Evans CK (1983) Cyanide as a feeding stimulant for the southern army worm, *Spodoptera eridania*. Ecol Entomol 8:125–132

Buhrmester RA, Ebinger JE, Seigler DS (2000) Sambunigrin and cyanogenic variability in populations of *Sambucus canadensis* L. (Caprifoliaceae). Biochem Syst Ecol 28:689–695

Calatayud PA, Le Ru B (1996) Study of the nutritional relationships between the cassava mealybug ant its host plant. Bull Soc Zool Fr 121:391–398

Campan EDM, Benrey B (2006) Effects of seed type and bruchid genotype on the performance and oviposition behavior of *Zabrotes subfasciatus* (Coleoptera: Bruchidae). Insect Sci 13:309–318

Engler HS, Spencer KC, Gilbert LE (2000) Preventing cyanide release from leaves. Nature 406:144–145

Ferreira C, Parra RP, Terra WR (1997) The effect of dietary plant glycosides on larval midgut β-glucosidases from *Spodoptera frugiperda* and *Diatraea saccharalis*. Insect Biochem Molec 27:55–59

Franks TK, Hayasaka Y, Choimes S, van Heeswijck R (2005) Cyanogenic glucosides in grapevine: polymorphism, identification and developmental patterns. Phytochemistry 66:165–173

Frehner M, Scalet M, Conn EE (1990) Pattern of the cyanide-potential in developing fruits - implications for plants accumulating cyanogenic monoglucosides (*Phaseolus-lunatus*) or cyanogenic diglucosides in their seeds (*Linum usitatissimum*, *Prunus amygdalus*). Plant Physiol 94:28–34

Gleadow RM, Woodrow IE (2000a) Temporal and spatial variation in cyanogenic glycosides in *Eucalyptus cladocalyx*. Tree Physiol 20:591–598

Gleadow RM, Woodrow IE (2000b) Polymorphism in cyanogenic glycoside content and cyanogenic beta-glucosidase activity in natural populations of *Eucalyptus cladocalyx*. Aust J Plant Physiol 27:693–699

Gleadow RM, Woodrow IE (2002) Constraints on effectiveness of cyanogenic glycosides in herbivore defense. J Chem Ecol 28: 1301–1313

Gonzalez-Rodriguez A, Benrey B, Callejas A, Oyama K (2002) Inter- and intraspecific genetic variation and differentiation in the sibling bean weevils *Zabrotes subfasciatus* and *Z. sylvestris* (Coleoptera : Bruchidae) from Mexico. Bull Entomol Res 92:185–189

Goodger JQD, Capon RJ, Woodrow IE (2002) Cyanogenic polymorphism in *Eucalyptus polyanthemos* Schauer subsp vestita L. Johnson and K. Hill (Myrtaceae). Biochem Syst Ecol 30: 617–630

Goodger JQD, Ades PK, Woodrow IE (2004) Cyanogenesis in *Eucalyptus polyanthemos* seedlings: heritability, ontogeny and effect of soil nitrogen. Tree Physiol 24(6):681–688

Honda K, Nishii W, Hayashi N (1997) Oviposition stimulants for sulfur butterfly, *Colias erate poliographys*: cyanoglucosides as synergists involved in host preference. J Chem Ecol 23:323–331

Hughes MA (1991) The cyanogenic polymorphism in *Trifolium repens* L. (white clover). Heredity 66:105–115

Jones DA (1988) Cyanogenesis in animal-plant interactions. In: Harnett DES (ed) Cyanide compounds in biology. Wiley, Chichester, pp 151–165

Lieberei R (1988) Relationship of cyanogenic capacity (HCN-C) of the rubber tree *Hevea-Brasiliensis* to susceptibility to *Microcyclus ulei*, the agent causing South American leaf blight. J Phytopathol 122:54–67

Lieberei R, Fock HP, Biehl B (1996) Cyanogenesis inhibits active pathogen defence in plants: inhibition by gaseous HCN of photosynthetic CO2 fixation and respiration in intact leaves. Angew Bot 70:230–238

Miller RE, Simon J, Woodrow IE (2006) Cyanogenesis in the Australian tropical rainforest endemic *Brombya platynema* (Rutaceae): chemical characterisation and polymorphism. Funct Plant Biol 33:477–486

Moraes RA, Sales MP, Pinto MSP, Silva LB, Oliveira AEA, Machado OLT, Fernandes KVS, Xavier-Filho J (2000) Lima bean (*Phaseolus lunatus*) seed coat phaseolin is detrimental to the cowpea weevil (*Callosobruchus maculatus*). Braz J Med Biol Res 33:191–198

Nahrstedt A (1988) Cyanogenesis and the role of cyanogenic compounds in insects. Ciba Found Symp 140:131–150

Pentzold S, Zagrobelny M, Rook F, Bak S (2013) How insects overcome two-component plant chemical defence: plant β-glucosidases as the main target for herbivore adaptation. Biol Rev. doi:10.1111/brv.12066

Poulton JE (1990) Cyanogenesis in plants. Plant Physiol 94:401–405

Puustinen S, Mutikainen P (2001) Host-parasite-herbivore interactions: implications of host cyanogenesis. Ecology 82:2059–2071

Rojas MG, Morales-Ramos JA (2010) Tri-trophic level impact of host plant linamarin and lotaustralin on *Tetranychus urticae* and its predator *Phytoseiulus persimilis*. J Chem Ecol 36:1354–1362

Schappert PJ, Shore JS (1999) Effects of cyanogenesis polymorphism in *Turnera ulmifolia* on *Euptoieta hegesia* and potential Anolis predators. J Chem Ecol 25:1455–1479

Seigler DS (1998) Cyanogenic glycosides and cyanolipids. In: Seigler DS (ed) Plant secondary metabolism. Kluwer Academic Press, Boston, pp 273–296

Selmar D, Lieberei R, Biehl B, Conn EE (1989) α-Hydroxynitrile lyase in *Hevea brasiliensis* and its significance for rapid cyanogenesis. Physiol Plant 75:97–101

Vetter J (2000) Plant cyanogenic glycosides. Toxicon 38:11–36

Woodrow IE, Slocum DJ, Gleadow RM (2002) Influence of water stress on cyanogenic capacity in *Eucalyptus cladocalyx*. Funct Plant Biol 29:103–110

Zagrobelny M, Bak S, Rasmussen AV, Jorgensen B, Naumann CM, Moller BL (2004) Cyanogenic glucosides and plant-insect interactions. Phytochemistry 65:293–306

Zaugg I, Benrey B, Bacher S (2013) Bottom-up and top-down effects influence bruchid beetle individual performance but not population densities in the field. PLoS ONE 8:e55317