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ORIGINAL ARTICLE

Role of cyanogenic glycosides in the seeds of wild lima bean, *Phaseolus lunatus*: defense, plant nutrition or both?

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Abstract

Main conclusion **Cyanogenic glycosides present in the seeds of wild lima bean plants are associated with seedling defense but do not affect seed germination and seedling growth.**

Abstract Wild lima bean plants contain cyanogenic glycosides (CNGs) that are known to defend the plant against leaf herbivores. However, seed feeders appear to be unaffected despite the high levels of CNGs in the seeds. We investigated a possible role of CNGs in seeds as nitrogen storage compounds that influence plant growth, as well as seedling resistance to herbivores. Using seeds from four different wild lima bean natural populations that are known to vary in CNG levels, we tested two non-mutually exclusive hypotheses: (1) seeds with higher levels of CNGs produce seedlings that are more resistant against generalist herbivores and, (2) seeds with higher levels of CNGs germinate faster and produce plants that exhibit better growth. Levels of CNGs in the seeds were negatively correlated with germination rates and not correlated with seedling growth. However, levels of CNGs increased significantly soon after germination and seeds with the highest CNG levels produced seedlings with higher CNG levels in cotyledons. Moreover, the growth rate of the generalist herbivore *Spodoptera littoralis* was lower in cotyledons with high-CNG levels. We conclude that CNGs in lima bean seeds do not play a role in seed germination and seedling growth, but are associated with seedling defense. Our results provide insight into the potential dual function of plant secondary metabolites as defense compounds and storage molecules for growth and development.

Keywords Secondary metabolites · Nitrogen storage · Herbivory · Cyanogenesis · Seed germination

Introduction

Throughout their long evolutionary history with herbivores, plants have evolved direct chemical defenses in the form of secondary metabolites that can have repellent, anti-nutritional or toxic effects on their attackers. However, the limit between primary and secondary metabolism is not always clear-cut, and increasing evidence suggests that secondary metabolites can, in some cases, be involved in signaling and regulation of primary metabolic pathways (Maag et al. 2015a, b; Malinovsky et al. 2017), can work as pigments and thereby influence plant attraction to pollinators or predators (i.e., carotenoids and flavonoids) (Harborne and Williams 2000; Tanaka et al. 2008), or as is the case for phenolic compounds, they can mitigate damage by UV radiation (Dai and Mumper 2010). Cyanogenic glycosides (CNGs) are a group of secondary metabolites for which other functions have been proposed such as being involved in transportation and turnover of nitrogen (Forslund and Jonsson 1997;

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Selmar et al. 1990; Møller 2010; Lechtenberg 2011; Nielsen et al. 2016; Bjarnholt et al. 2018), and in plant development (Swain and Poulton 1994). CNGs are widely distributed in the plant kingdom (Selmar et al. 1988; Vetter 2000). They are stored in vacuoles, and upon damage of plant tissue they come in contact with β-glucosidases that hydrolyze them, causing the release of toxic hydrogen cyanide (HCN) (Zagrobelny et al. 2004). This process is referred to as cyanogenesis (Saunders and Conn 1978; Bak et al. 1998; Vetter 2000; Gleadow and Woodrow 2002) and is known to be toxic for most living organisms (Cork 1996; Zagrobelny et al. 2004). Yet, the presence of CNGs is not always harmful for insect herbivores. Contrary to mammals and chewing insects, which cause tissue disruption and trigger cyanogenesis (Frehner and Conn 1987; Poulton 1988; Ballhorn et al. 2009; Vassão et al. 2018), insects with sucking mouthparts cause minimal tissue disruption and may avoid the defensive reaction (Pentzold et al. 2015), and some specialist chewing insects can cope with CNG toxicity by detoxifying, tolerating, or sequestering CNGs for their own defense (Scriber 1978; Brattsten et al. 1983; Engler-Chaouat and Gilbert 2007; Zagrobelny et al. 2007). The impact of CNGs also depends on their levels within plant tissues, which can be highly variable, not only among plant populations or cultivars (Ballhorn et al. 2008a; Shlichta et al. 2014, 2018), but also within populations and among different plant structures (Dirzo and Harper 1982, Godschalx et al. 2016; Shlichta et al. 2018). Finally, the effectiveness of CNGs as a defense may be dependent on the cyanogenic capacity, which refers to the amount of hydrogen cyanide released resulting from the activation of β-glucosidases (Ballhorn et al. 2005).

Lima bean (*Phaseolus lunatus* L., Fabaceae) is the only reported species within the entire *Phaseolus* genus that contains CNGs (Vetter 2000). This species has been used as a model in studies on direct and indirect defense (Heil 2004; Ballhorn et al. 2008b, 2009; Yi et al. 2009; Shlichta et al. 2014, 2018; Hernandez-Cumplido et al. 2016). High concentrations of CNGs in the leaves and other vegetative tissues have been associated with negative effects on generalist leaf herbivores (Ballhorn et al. 2005; Ballhorn and Lieberei 2006; Godschalx et al. 2016; Shlichta et al. 2018). However, seed feeders appear to be unaffected, despite the considerable higher concentrations of these compounds in seeds as compared to leaves (Shlichta et al. 2014, 2018). A possible reason for this is the lack of moisture in the seeds, which might be needed for the degradation of CNGs by the β-glucosidases, thus preventing the release of toxic HCN (Shlichta et al. 2014).

Therefore, it remains unclear to what extent *P. lunatus* benefits from having high levels of CNGs in its seeds. Especially, because generalist seed beetles account for approximately 95% of pre-dispersal seed mortality (Bustos-Segura et al. unpublished data). One hypothesis is that CNGs get translocated from the seeds to seedlings (Clegg et al. 1979), providing the latter with protection against leaf-feeding herbivores. Alternatively, CNGs in the seeds could serve as transporters of nitrogen and glucose involved in primary metabolism (Møller 2010). However, support for this hypothesis is still scarce (Schilcher and Wilkens-Sauter 1986; Kakes 1990; Selmar et al. 1990; Stensjo and Jonsson 1997; Bjarnholt et al. 2018), and for lima bean this putative role of CNGs in plant growth and development has not yet been investigated.

In the current study, we investigated the role of CNG levels in the seeds of wild lima bean in plant defense and their potential as nitrogen storage compounds, influencing seed germination and seedling growth. Using seeds collected from four different natural populations that are known for their variable CNGs contents (Shlichta et al. 2014), we examined the relationship between seed CNGs levels and seed germination rate, seedling performance, and plant resistance against a generalist herbivore. We tested two non-mutually exclusive hypotheses: 1) seeds with high-CNG levels produce seedlings with high concentrations of CNGs and thus higher resistance to herbivores, and 2) that seeds with high-CNG levels have better germination and produce plants that exhibit better growth than seeds with low-CNG content.

Materials and methods

Seeds were obtained from four wild populations (INK, ITC, KM and HHI) collected in 2013 in the area of Puerto Escondido in the State of Oaxaca, Mexico. Sampling sites were located from 597 km north to 50 km south Puerto Escondido (see Shlichta et al. 2014 for details). These populations were previously characterized for the type and content of CNGs in their seeds (Shlichta et al. 2014) and we had an a priori idea on the main CNGs and their relative content. Nonetheless, to establish a baseline on CNGs content for the subsequent experiments, a sample from each population was analyzed ($N=9$ for INK, $N=10$ for ITC, $N=9$ for KM and $N=10$ for HHI). One replicate consisted of seven seeds. Samples were then analyzed for their content of linamarin and lotaustralin, the two predominant CNGs present in leaves and seeds (Shlichta et al. 2014) and were ranked from low to high according to CNGs levels.

Preparation of plant material

Individual seedling structures

Wild lima bean plants grow as twining vines forming a dense bush. Seeds were selected randomly from two patches of each population, planted in small plastic pots (11 cm of height and 4 cm of diameter) and placed in a phytotron (28/24 °C, 60–80% R.H. and placed under a lamp (550 µmol)). Plants were watered every 3 days or as needed. As seeds germinated and plants developed, we collected the different plant structures: cotyledons ($N = 9$ for INK, $N = 10$ for ITC, $N = 10$ for KM and $N = 8$ for HHI), primary leaves ($N = 14$ for INK, $N = 12$ for ITC, $N = 18$ for KM and $N = 12$ for HHI), trifolia of first ($N = 10$ for INK, $N = 9$ for ITC, $N = 8$ for KM and $N = 10$ for HHI) and third secondary leaves ($N = 11$ for INK, $N = 11$ for ITC, $N = 11$ for KM and $N = 11$ for HHI) (Fig. 1). Only one structure was collected from each individual plant. After removal, samples were directly drowned in liquid nitrogen and stored in a freezer at − 80 °C until CNG extraction.

Seedlings at different developmental stages

A group of 30 plants was used to determine the content of CNGs in young plants throughout seedling development. Here, we did not remove individual plant structures, but rather we analyzed the content of cyanogenic glycosides present in the whole young plant at different stages of development. This procedure allowed us to determine if these compounds are transferred from the seeds to seedlings and/or synthesized de novo. Because of a lack of seeds, only one population (INK) was used to test this.

To prevent any soil particles from contaminating the samples, seeds were germinated in wet cotton (in a phytotron, 28/24 °C, 60–80% R.H. and placed under a lamp (550 µmol)). For the analyses, we used seeds, sprouted seeds (less than 24 h) and full seedlings (3–4 days old) (Fig. 1). One individual plant was used for each developmental stage, ten plants per stage. Samples were submerged in liquid nitrogen and stored in a freezer at − 80 °C until extraction.

Quantification of cyanogenic glycosides

Linamarin and lotaustralin were quantified from seeds (one sample consisted of seven seeds), cotyledons (one sample consisted of two cotyledons from two plants), primary leaves, first and third secondary leaves, 1-day-old whole sprouted seed and 4-day-old whole seedling. We used a method adapted from two previous studies on CNGs (Franks

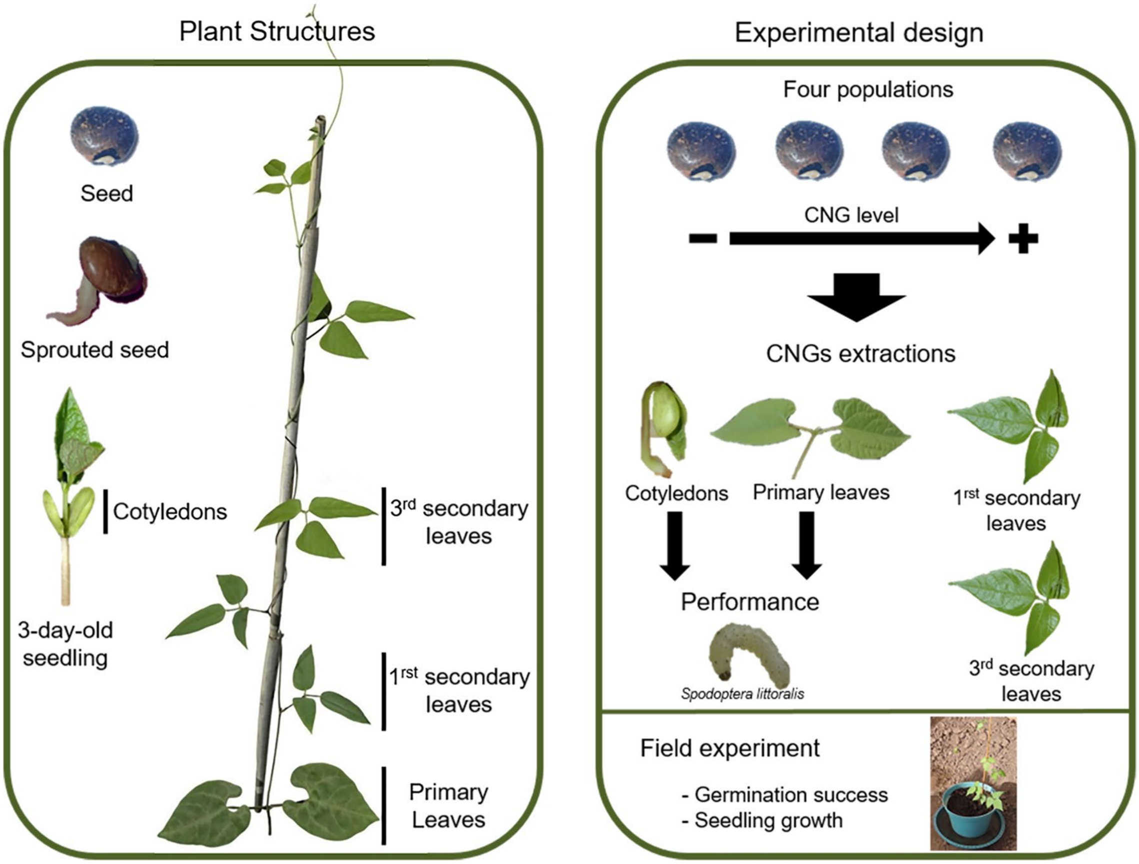

Fig. 1 Schematic illustration of **a** the developmental plant stages and tissues of lima bean, *Phaseolus lunatus* used during the study, and **b** the experimental protocol followed for cyanogenic glycosides' (CNGs) extractions, herbivore performance assays and field experiment. Extractions and experiments were conducted with seeds with low and high-CNG content

et al. 2005; Rojas and Morales-Ramos 2010) and fully described in Shlichta et al. (2014). Briefly, samples were taken out of the freezer and immediately submerged in liquid nitrogen and milled with a mortar to obtain a thin powder. Each sample (approximately 20 mg of powder) was then weighed in a microbalance to the nearest 0.1 mg (Mettler Toledo XP6, Columbus, Ohio, USA), put in an Eppendorf tube with 1 ml of 70% methanol and immediately placed on a heating block at ~ 90 °C for 10 min. The tubes were removed from the heating block, cooled for 5 min and placed in a Branson 2210 ultrasonic shaker for 10 min. Then they were centrifuged at 12000 rpm for 10 min and supernatants were collected. Finally, as CNG concentration is higher in the seeds, we diluted seed and cotyledon samples at 1:25 with 70% methanol.

The analysis of CNGs was performed using an Acquity UHPLC (ultra-high-performance liquid chromatography) system coupled to a Synapt G2 QTOF mass spectrometer (Waters, Milford, USA) controlled by Masslynx 4.1 (see Shlichta et al. 2014 for details).

CNGs expressed in fresh and dry weight

CNG concentrations can be measured in fresh or dry weight. From an ecological point of view, concentrations expressed in fresh weight seem to reflect more accurately the amount of CNGs that herbivores face in nature. However, in fresh weight, the amount of water, which can greatly vary according to the plant tissues, is not considered. Consequently, plant tissues with higher water content (e.g., leaves) tend to have a lower concentration of CNGs due to a dilution effect, while tissues with low water content (e.g., seeds) display higher concentrations. Thus, to follow the variation in CNG content from seed to the early seedling stages, the water content in the different seedling structures has to be considered.

Seeds of the four plant populations were planted and the different seedling structures were collected (five samples per structure for each population) following the same protocol as in the previous experiment. Samples were weighed to the nearest 0.1 mg and oven-dried at 40–60 °C for 48 h, after which, they were weighed again and the percent of water content in each sample was calculated (Online resource 1).

Herbivory experiment

This experiment was done to test our first hypothesis on the role of CNGs as defense compounds against a generalist herbivore. We tested the performance of *Spodoptera littoralis* larvae on cotyledons and primary leaves of plants from the four populations. Egg clutches of *S. littoralis* were provided by Syngenta (Stein, Switzerland) and reared to second instar on artificial diet ("beet armyworm diet", produced by BioServ, U.S.A). Plants used in this experiment were grown under the same conditions as described in the section on preparation of plant material.

To determine larval performance on cotyledons, each second instar of *S. littoralis* was offered one whole cotyledon from young (2-day-old) lima bean seedlings (only one plant was used per larva) of the four populations. This experiment could not be done directly on the plant, because cotyledons are very fragile. Consequently, they were set up in petri dishes on wet cotton. Twenty-two instar larvae were randomly chosen and assigned to cotyledons from the four plant populations. The relative growth rate (larval weight gain divided by the initial larval weight), was calculated by individually weighing larvae before placing them on the cotyledons and 24 h later. Larval growth rate has been generally considered a reliable estimate of plant resistance against insect herbivores (Kempel et al. 2011; Lariviere et al. 2015).

We followed a similar procedure to test larval performance on primary leaves. We used 1-week-old plants and as soon as the surfaces of primary leaves were large enough, single second instars of *S. littoralis* were placed directly on one of the two primary leaves of the seedling and confined with a plastic clip cage covered with mesh (diameter 1.5 cm, Köhler et al. 2015; $N = 15$ per population). Caterpillar growth rate was also measured by weighing larvae at the start of the experiment and 24 h later. After this period, larvae had eaten a very small amount of leaf, thus they were placed on the second primary leaf of the same plant for an additional 24-hour period. Larvae were not observed while feeding on the plants. Thus, adverse effects of CNGs on larval behavior, such as slower locomotion, were not recorded.

Field experiment: germination and seedling growth

A field experiment was conducted to test the second hypothesis and examine the relationship between CNG levels and seed germination and early plant development. This experiment was performed in January of 2015 on our field station 15 km northwest of Puerto Escondido, Mexico (15°55′27.4″N, 97°09′03.0″W). Seeds from the same four populations representing a gradient of high and low-CNG levels ($N = 30$ for INK, $N = 30$ for KM, $N = 30$ for ITC and $N = 28$ for HHI) were sown in individual pots (4 cm, 11 cm high). Pots were placed in a randomized common garden experiment towards the end of the field season (mid-January) when most leaf herbivores are no longer present in the field (Cuny et al. 2018). We recorded the time to germination (number of days between sowing and germination), the germination success and the time to appearance of the first trifolium (number of days between germination and production of the first trifolium).

Statistical analyses

Linear regressions (PROC REG) were used to test the relationship between the initial CNG levels in seeds from the four populations and the following plant traits: CNG levels in cotyledons, primary leaves, first and third secondary leaves, *S. littoralis* performance on cotyledons and primary leaves, seedling performance.

Ordinal logistic regressions (PROC LOGISTIC) were used to test correlations between different plant structures ordered according to their time of production and their CNG levels, in fresh and dry weight. A logistic regression with a binary distribution was used to test correlation between CNG levels in the seeds and seed germination rate.

The CNG levels (dry weight, INK population) for different early stages of the whole seedling were analyzed using a generalized linear model (PROC GENMOD) following a gamma distribution.

Correlation tests between linamarin and lotaustralin were done using Pearson correlation test for the primary leaves, first and third secondary leaves and Spearman for the seeds and cotyledons (PROC CORR).

All statistical analyses were performed with SAS 9.2 software (Littell et al. 2006, SAS Institute, Cary, North Carolina).

Results

We found small traces of linustatin and dhurrin. Yet their concentration was extremely low compared to that of lotaustralin and around 1000 times lower than linamarin. Concentrations of linamarin and lotaustralin were highly positively correlated for all plant structures (Online resource 2, $r > 0.7$). Linamarin was on average 20 times more concentrated than lotaustralin. Thus, we only report results for linamarin.

See online resource 3–6, for representative chromatograms of the different plant structures for the four populations.

CNGs in seedling structures from seeds with high and low levels

Fresh weight

We found a significant positive correlation between the initial CNG level in the seeds and the CNG level in the cotyledons from seedlings produced by seeds from the same populations (Fig. 2a, $F_{1,35} = 5.04$, $p = 0.0312$, $R^2 = 0.13$). However, no significant correlation was found between the initial CNG level in the seeds and the CNG level in primary leaves ($F_{1,54} = 1.77$, $p = 0.19$, $R^2 = 0.032$), first ($F_{1,35} = 0.14$, $p = 0.71$, $R^2 = 0.004$) and third secondary leaves ($F_{1,40} = 2.03$, $p = 0.16$, $R^2 = 0.048$). Finally, we found a significant negative correlation between the order of production of different plant tissues and their CNG level (Fig. 3a, $\chi^2_{1,205} = 70.64$, $p < 0.001$, $R^2 = 0.34$).

Dry weight

We found a significant polynomial regression between the order of production of different plant tissues and their CNG level expressed in dry weight (to remove possible bias from the dilution effect due to the amount of water in the different tissues) (Fig. 3b, $\chi^2_{2,205} = 6.19$, $p = 0.039$, $R^2 = 0.34$). CNG level was higher in primary leaves, intermediate in cotyledons and secondary leaves and lower in seeds. These results suggest that there is an increase in CNG level relative to dry plant mass when the plant starts to produce leaves.

CNGs levels (in dry weight) throughout seedling development

This experiment was conducted not only to confirm the results from the previous experiment on whole seedling structures, but also to estimate the increase in CNG levels during plant development. Here we only used one population, INK. Twenty-four hours after germination, the sprouted seed already had two times more CNG compounds (in dry weight) than dry seeds. In addition, 3–4 days later, the total amount of CNG compounds in the entire small seedling (cotyledons and seed coat, small roots, stem, small primary leaves) had tripled compared to the sprouted seed (Fig. 4, $\chi^2_{2,27} = 66.41$, $p < 0.001$).

CNGs effect on the performance of *Spodoptera littoralis*

Cotyledons

The relative growth rate of the second instar larvae was negatively correlated with the initial CNG level in the seeds (Fig. 5a, $F_{1,62} = 6.06$, $p = 0.017$, $R^2 = 0.09$; $N = 18$ for INK, $N = 14$ for ITC, $N = 17$ for KM and $N = 15$ for HHI).

Primary leaves

No significant correlation was observed between the relative growth rate of *S. littoralis* larvae and the CNG level of the primary leaves with which they were fed (Fig. 5b, $F_{1,23} = 0.05$, $p = 0.83$, $R^2 = 0.002$; $N = 13$ for INK, $N = 4$ for KM and $N = 8$ for HHI). Due to high larval mortality, we did not have enough replicates for ITC and was excluded from the analysis.

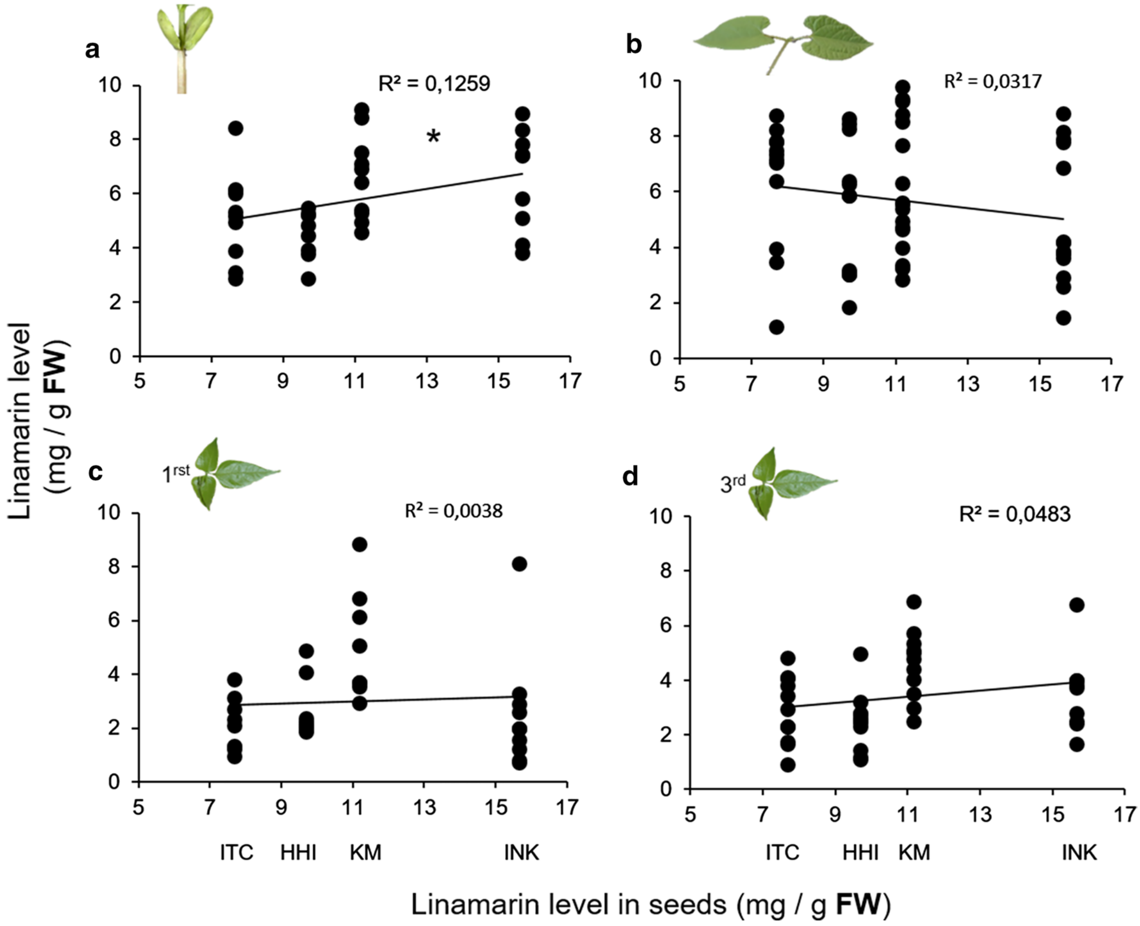

Fig. 2 Mean levels of linamarin expressed in fresh weight in individual plant structures produced by all seeds sampled from each of the four plant populations with varying levels of CNGs: **a** cotyledons, **b** primary leaves, **c** first trifolium and **d** third trifolium

CNGs effect on seed germination and seedling growth in the field

Germination success

A weak significant negative correlation was found between the CNG level in seeds and their germination success (Fig. 6a, $F_{1,114} = 5.97$, $p = 0.015$, $R^2 = 0.052$). This correlation seems to be mainly driven by one population (INK), for which the germination success was about 30% lower than the three other populations (mean germination rates: $\mu = 0.65$ for INK, $\mu = 0.9$ for ITC, $\mu = 0.87$ for KM and $\mu = 0.86$ for HHI).

Germination speed

A weak significant negative correlation was found between the CNG level in seeds and their germination speed (Online resource 7, $F_{1,95} = 8.97$, $p = 0.0035$, $R^2 = 0.086$). This correlation seems to be mainly driven by one population (ITC), for which the germination success was about 50% higher than the three other populations (mean germination speeds: $\mu = 4.68$ for INK, $\mu = 6.89$ for ITC, $\mu = 4.69$ for KM and $\mu = 4.83$ for HHI).

Seedling growth

No significant correlation was found between the time of appearance of the first trifolium in seedlings from different populations and the CNG level in the seeds from which they germinated (Fig. 6b, $F_{1,62} = 1.01$, $p = 0.32$, $R^2 = 0.025$; $N = 16$ for INK, $N = 21$ for ITC, $N = 25$ for KM and $N = 19$ for HHI).

Discussion

Overview

Seeds of *P. lunatus* with higher CNGs levels produced seedlings with higher levels of CNGs in cotyledons, but not in primary leaves and secondary leaves, than low-CNG seeds.

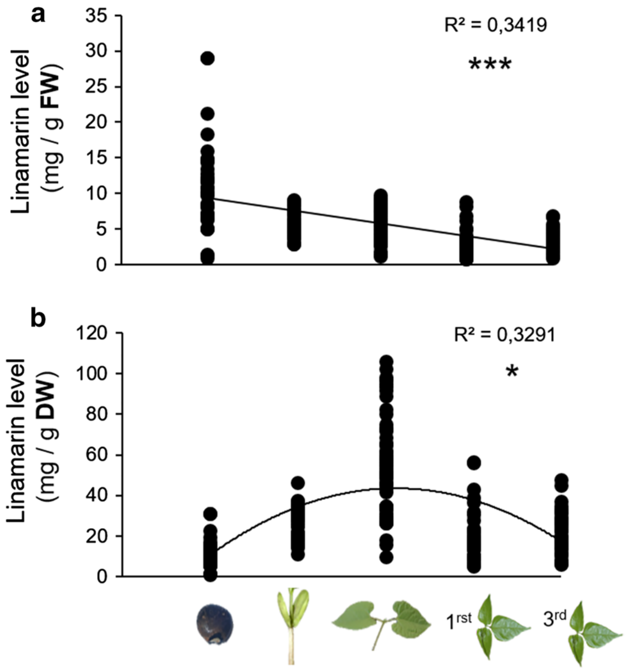

Fig. 3 Logistic regression between different plant structures ranked according to their order of production by the plant and their linamarin levels, expressed in **a** fresh weight and **b** dry weight

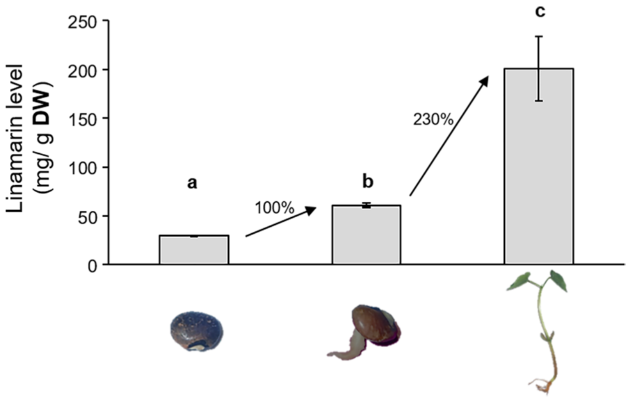

Fig. 4 Linamarin levels expressed in dry weight in seeds, sprouted seeds and 3–4-day-old seedlings. Seeds from only one population, INK were used for this experiment. Bars (mean ± SE) with different letters are significantly different from each other. Linamarin level increased 100% from seeds to sprouted seeds, and 230% from sprouted seeds to young seedlings

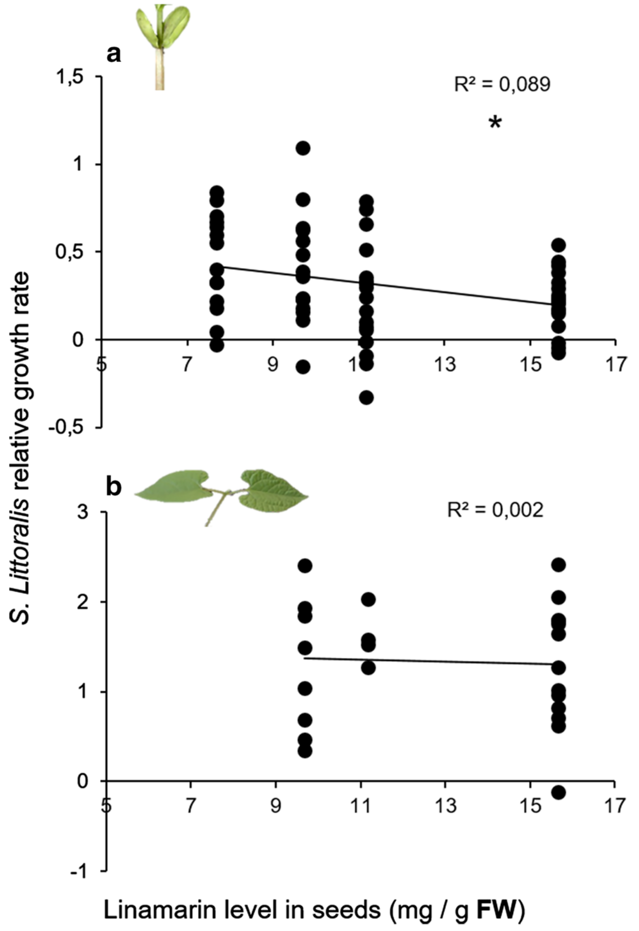

Fig. 5 Relative growth rate of *S. littoralis* fed on **a** cotyledons and **b** primary leaves produced by seeds with different levels of linamarin. Relative growth rate was calculated for each individual larva as the mass difference between initial and final mass divided by the initial mass. Negative values correspond to larvae that lost weight during the experiment

Moreover, cotyledons with high-CNG levels were better protected against the generalist herbivore *S. littoralis* than low-CNG cotyledons. However, when comparing the CNGs levels between different seedling tissues (measured in dry weight to standardize for differences in water content), we found that regardless of the initial level in the seed, primary leaves had higher levels of these compounds. Further, CNG levels increased significantly soon after germination. Taken together, these results show that initial level of CNGs in the seeds is positively correlated with the levels in cotyledons but not in leaves, indicating de novo production in quantities compared to the initial seed level.

Finally, we found weak negative correlations between the initial CNG level in the seeds and their germination rate and speed. Additionally, no significant relationship was found between the initial CNG level in the seed and seedling growth. Therefore, more CNGs in seeds do not appear to increase seed germination or seedling growth.

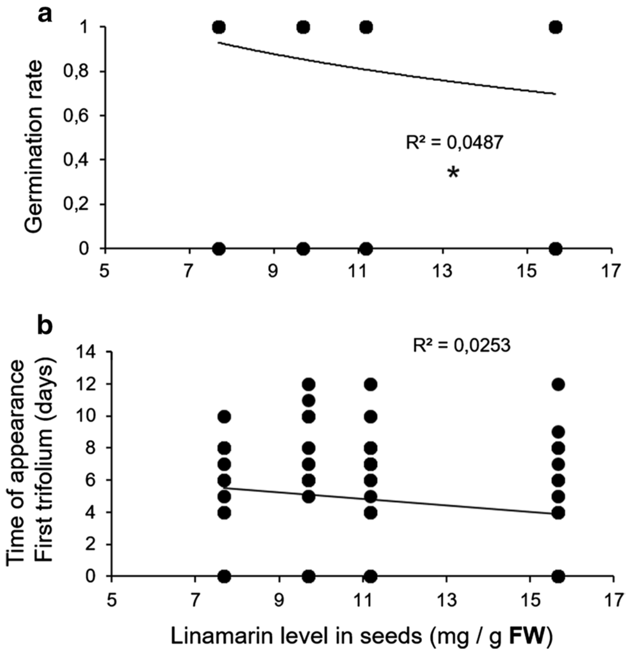

Fig. 6 **a** Binary logistic regression between the initial CNG level in the seeds of four plant populations and seed germination and **b** regression between the initial CNG level in the seeds from four plant populations and appearance of the first trifolium. Number of seeds that germinated: ITC:27 HHI:24 KM:26 INK:20. Number of seeds that did not germinate: ITC:3 HHI:4 KM:4 INK:10

Variation in CNG levels among seedling structures according to seed content

High-CNG seeds produced cotyledons that had higher levels of CNGs and that were better defended against herbivore damage than cotyledons from low-CNG seeds. For leaves, this pattern was not so clear. When expressed as fresh weight, which reflects the level of CNGs that the herbivore will face upon plant damage, CNG levels in primary leaves and secondary leaves (first and third trifolium) were not different between leaves from high- and low-CNG seeds. Ontogenic changes in plant defense have been widely reported for several plant species (Boege and Marquis 2005; Goodger et al. 2007; Barton and Koricheva 2010; Desurmont et al. 2015; Ochoa-López et al. 2018). A variable combination of multiple defense mechanisms in lima bean plants (direct and indirect) throughout ontogeny could provide plants with the best strategies to cope with different types of herbivores and other antagonists.

The higher levels of CNGs in cotyledons may be the result of two non-mutually exclusive processes. First, CNG compounds from the seeds may be directly transferred to the sprouted seed and then cotyledons. This is supported by cotyledons from high-CNG seeds having higher levels of CNGs than those from low-CNG seeds (Fig. 2b). Alternatively, or additionally, higher levels of CNGs in the seed could provide more of the precursors needed for the production of new CNGs in later plant structures. Further, the correlation between the different seedling structures and their CNG level expressed in fresh weight showed that the younger tissues, cotyledons and primary leaves had overall higher levels of linamarin (Fig. 3a). This is not an unexpected result. Godschalx et al. (2016) measured the cyanogenic potential (HCNp; the total amount of CNG compounds) of different plant structures from wild lima bean plants from Costa Rica mainly, flower buds, flowers, seed pods and leaves and found that young leaves had the highest potential.

CNG levels and seedling development

To better understand the variation in CNG concentrations during early plant ontogeny, we converted concentrations from fresh to dry weight. Concomitant with the levels of CNGs in the seeds, the total amount of CNGs compounds following germination began to increase in the young seedling. The level of CNGs was doubled after 24 h (from seed to cotyledon), and then tripled after 3–4 days (from cotyledon to primary leaves). These results indicate that most of the CNG compounds present in the young seedling are produced de novo, starting as soon as the seed germinates. Yet, future work would be needed to be able to separate the effect of cyanogenic glycosides present in seeds from the effect contributed by de novo synthesis in seedlings to seedling defense.

In their study, Ballhorn et al. (2008b) showed that primary leaves had a lower CNG concentration than secondary leaves. The difference between their results and ours may be due to the age of the plant examined. They used older lima bean plants, 6-week-old, compared to 1-week-old plants in our study. It may be that primary leaves are first defended by CNGs which then get transferred to other plant tissues, possibly switching the defense strategy towards the production of volatile organic compounds (Ballhorn et al. 2008a). Yet, in a different study, and in agreement with our findings, this same group reported that plants characterized by high cyanogenic potential in their young leaves showed consistently higher HCNp in later developmental stages than plants with low HCNp in young leaves (Ballhorn et al. 2005).

Other studies with this and other plant species have also suggested a transfer of CNG compounds from younger to older plant tissues (Selmar et al. 1987; Frehner et al. 1990). For instance, Selmar et al. (1987) showed that in seeds of the rubber tree *Hevea brasiliensis*, 90% of the CNGs are stored in the endosperm as linamarin and lotaustralin. Upon seed germination, linamarin the dominating CNG is transformed into linustatin which is transferred to the seedling. Similarly, in a study with wild lima bean plants in Costa Rica, Clegg et al. (1979) found that linamarin content remained constant throughout plant development, but observed a drop in the total CNG content in the plant when the cotyledons were removed. They suggested that the plant transfers the linamarin intact from the seeds to the growing seedling. These findings run counter to our study, as we detected a dramatic increase in linamarin levels during early plant development.

Ontogenic changes in CNG levels have also been reported for other plant species (e.g., *Sorghum*, Schappert and Shore 2000; *Eucalyptus*, Gleadow and Woodrow 2000; Neilson et al. 2011; and several species in the genus *Prunus*, Dicenta et al. 2002; Sendker et al. 2016; Del Cueto et al. 2017). For example, in the tropical shrub *Turnera velutina* (Passifloraceae), hydrogen cyanide is present only in cotyledons and early seedling stages, while in later plant stages, the plant relies on other type of direct and indirect defenses such as trichomes and extrafloral nectar (Ochoa-López et al. 2015, 2018).

For lima bean, contrasting results from the different studies on differential levels of CNG across plant tissues could also be the result of natural variation among plant populations, particularly taking into consideration that studies have been conducted with populations from different geographical regions (i.e., from Costa Rica and Mexico), likely subjected to different abiotic and biotic conditions and selective pressures.

Defense or nutrition?

For lima bean, the great majority of studies concerning CNGs focus on their role in defense, while their role as nitrogen storage compounds that might be used during germination and seedling establishment is less understood (Seigler 1998; Møller 2010). Our results showed that seeds with high levels of CNG content produced better protected cotyledons, and indicate rather a defensive than nutritional role.

These findings point to a defensive role of CNGs and add to those by numerous studies that have documented how this and other generalist herbivores are affected by the CNG content in their host plants (Jones 1988; Schwarz et al. 1996; Ferreira et al. 1997; Gleadow and Woodrow 2002; Pentzold et al. 2013; Vassão et al. 2018). For example, Stauber et al. (2012) found higher mortality of first instars of *S. littoralis* on transgenic *Arabidopsis* plants containing the CNG, dhurrin than on the wild type. Specifically for lima bean, field and lab studies have shown that CNGs effectively protect plants against generalist herbivores (Ballhorn et al. 2007, 2008b, 2009; Rojas and Morales-Ramos 2010; Shlichta et al. 2018). When exposed to wild and cultivated leaves of lima bean, larvae of *S. exigua* ate less and developed slower on leaves of cultivated varieties with higher content of linamarin and lotaustralin, than on leaves of wild plants with a lower content (Shlichta et al. 2018). Most of the studies cited above (as well as the current study) did not investigate the mechanism that lead to the herbivore's decreased performance on plants with high-CNG levels. In a recent study, however, (Vassão et al. 2018) showed that β-glucosidases resist digestion and are active in the gut of *S. littoralis* which results in the release of toxic HCN. Thus, our findings on the slower larval growth rate on high-CNG plants are presumably the combination of larvae eating less, probably due to the bitterness associated with CNG levels and the toxic effects of these compounds.

Conversely, we did not find a positive relationship between the level of CNGs in the seeds and the success of germination and early seedling development in the field. As cyanogenic glycosides are nitrogen-based compounds, it has been suggested that in addition to their defensive role, they may also provide a storage place for nitrogen and sugar needed for plant growth and development (Clegg et al. 1979; Lieberei et al. 1985; Selmar et al. 1988; Forslund and Jonsson 1997; Møller 2010). Evidence for this idea is growing and comes mostly from lab studies on different plant species that examine the biosynthetic pathway of CNGs during plant development. These studies have identified the intermediate products and the enzymes responsible for their catalyzation, and examine how and if these products are translocated and supply nitrogen to latter plant tissues (Forslund and Jonsson 1997; Busk and Møller, 2002; Sánchez-Pérez et al. 2008, 2009; Jørgensen et al. 2011; Kadow et al. 2012; Bjarnholt et al. 2018). While some of these additional molecules are still CNGs, for example, in *Eucalyptus*, prunasin is replaced by amygdalin and eucalyptosin (Neilson et al. 2011), others are derivatives or products of pathways (Pičmanová et al. 2015; Sendker et al. 2016) that may serve to recover nitrogen from the CNGs (Del Cueto et al. 2017). For example, Sendker et al. (2016) using leaves of *Prunus laurocerasus*, showed that during leaf senescence, decomposition of the cyanogenic glycoside prunasin is followed by an increase of 36 senescence-associated compounds, including several benzoic acid derivatives. In our study, we did not follow the biosynthetic pathway of CNGs from seed to seedling, therefore we cannot exclude the possibility that some of the intermediate and end products formed during the metabolic pathway serve as nitrogen sources for latter tissues. Yet, by using seeds with high- and low-CNG levels, we were able to test the ecological value of CNG variation in the seeds on germination and early seedling development.

Conclusion and future directions

In conclusion, results from this study reveal that cyanogenic glycosides present in the seeds of wild lima bean plants are likely associated with seedling defense but do not affect seed germination and early plant development. We found support for our first hypothesis by showing that seeds with more CNG produced seedlings that contain more CNG in cotyledons, which had a significant negative impact on the performance of a generalist herbivore. In addition, we found a significant increase in CNG compounds as soon as the seed germinates. Conversely, we did not find support for our second hypothesis that states that higher levels of CNGs in the seeds would result in higher germination and better seedling growth.

To further understand the varying defense strategies and ecological functions of CNGs in this species, we need detailed studies that contemplate different selective pressures by herbivores and other antagonists (i.e., pathogens, fungi, small vertebrates) across lima bean populations coupled with plant ontogeny.

Author contribution statement BB conceived the idea. BB, MC, DL designed the research. MC and DL conducted the experiments. MC and GD analyzed the data, and GG carried out the chemical analyses. MC, DL, GD and BB wrote the manuscript. All authors contributed critically to drafts and gave final approval for publication.

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Compliance with standards

Conflict of interest The authors declare that they have no conflict of interest.

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