Investigating the Role of Cyanogenic Glycosides as a Potential Defense for *Passiflora incarnata* against *Agraulis vanillae*

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Key words: *Passiflora incarnata*, *Agraulis vanillae*, cyanogenic glycoside, coevolution

Abstract

Plants produce the largest variety of secondary metabolites of any organism on the planet. However, the evolutionary forces that generate this diversity are still unknown. A leading hypothesis is that these metabolites serve as defenses against insect herbivores that utilize plants as a food source and that herbivores evolve to combat these defenses in response. To test the hypothesis that cyanogenic glycoside is a defensive metabolite and therefore is under natural selection by insect herbivores, a common garden experiment was conducted using the plant species *Passiflora incarnata*. Furthermore, a second experiment looking into *Agraulis vanillae* (Gulf Fritillary) performance on *P. incarnata* plants from different regions of the United States was conducted. The results of the common garden experiment suggested that a relationship between cyanogenic glycoside production and fitness was unable to be determined. Furthermore, from the herbivore performance experiment, a significant interaction between toughness and cyanogenic glycoside production on female pupae weight was observed. This work provides further insight into the importance of metabolites in mediating plant-herbivore coevolutionary interactions.

Introduction

Plants produce an extraordinary array of secondary metabolites. However, our understanding of the evolutionary forces that have generated this diversity is still limited. Coevolution is hypothesized to be a major driver of this diversity (Ehrlich and Raven, 1964). Coevolution is defined as the effect that close ecological interactions and genetic compositions of two species have on each other's evolution. Several taxa have been used in order to investigate and better understand coevolutionary interactions in nature. In their seminal paper on coevolution, it was proposed by Paul Ehrlich and Peter Raven that the diversity of secondary metabolites was due to coevolution between plants and the insect herbivores that feed on them (Ehrlich and Raven, 1964). Furthermore, it was theorized that a coevolutionary interaction developed between *Passiflora* and *Heliconius* through the β-glucosidase mechanism (Spencer, 1988). Since then, researchers have found evidence that insect herbivores are selecting on plant chemical defenses. Yet, this has not been investigated in *Passiflora incarnata* and *Agraulis vanillae* (Gulf Fritillary), a specialist herbivore. In this study, the potential coevolutionary interaction that exists between *P. incarnata* and *A. vanillae* was examined and what mechanism mediates this interaction.

Passiflora (*Passifloraceae*), also known as the passionflower, are a family of perennial vine plants which contains 530-700 species and 18-23 genera (Figure 1). Species of this genus can be found throughout the Americas. The genus *Passiflora* can be subdivided in four subgenera (*Strophaea*, *Decaloba*, *Deidamiodes*, and *Passiflora),* which can further be subdivided into 23 subgenera (Cerqueira-Silva *et al.*, 2014). Several interesting adaptions in *Passiflora* suggest that coevolution is occurring in this system. For example, the extreme diversity of vegetative portions in *Passiflora* plants suggests

that evolution of this trait occurred in *Passiflora* in response to coevolution with its primary herbivore, the *Heliconius* butterfly species (Jazen, 1980). As a result, it is possible that coevolution exists between *P. incarnata* and another herbivore species such as *A. vanillae*.

Figure 1. Image of the flower that emerges from the *P. incarnata* plant.

A. vanillae (Gulf Fritillary), is a specialist herbivore that feeds on *P. incarnata* (Figure 2; Copp and Davenport, 1978). This species can be found in many parts of North America, including the United States (Michener, 1942).

Figure 2. Image of the *A. vanillae* in its caterpillar stage.

However, based on the latitudinal region in the United States, there is differential presence and abundance, on a quantitative scale, of *A. vanillae* (personal communication, Nicholas Batora). This differential presence can be used to study if there is a coevolutionary interaction between *P. incarnata* and *A. vanillae*, which is what is being investigated in this study.

Of the several factors that could create a coevolutionary relationship between *Agraulis vanillae* and *Passiflora incarnata*, host-plant chemistry is the targeted trait in this coevolutionary dynamic that is being investigated in this study. Of the several metabolites by *P. incarnata*, the one that is of most interest are cyanogenic glycosides. Cyanogenic glycosides are sugars that are bound to cyanide via a glycosidic bond. Cyanogenic glycosides, when hydrolyzed in *Passiflora incarnata*, lead to the production of hydrogen cyanide. This compound is diverse in terms of structure and is suggested to be toxic and deter herbivory, which introduces the potential for defense mechanism evolution in *P. incarnata* (Bernays *et al.*, 1977). In other words, the process of cyanogenesis in *P. incarnata* leads to cyanogenic glycosides being broken down to produce the toxic compound cyanide, which can be used as a defense mechanism (Spencer, 1988).

Cyanogen diversity has been determined to be present in *P. incarnata* (Fischer, *et al.*, 1982; Seigler *et al.*, 1982; Spencer and Seigler, 1983). The production of cyanogenic glycosides in *P. incarnata* and its role in *Passiflora* and *Heliconius* defense and coevolution was studied in 1988. Based on research done since then, it has been suggested that cyanogenic glycosides play a role as a defensive metabolite that intermediates the coevolutionary relationship between the two organisms (Gleadow,

2002). In addition, the specialist, *Lepidoptera,* in the *Heliconius* genus has the ability to alter the cyanide group and convert it into a thiol group making it suddenly benign to the herbivore (Engler, 2000). Furthermore, *A. vanillae* has been known to effectively metabolize cyanogenic glycosides while feeding (Engler *et al.*, 2007). However, the crucial role of cyanogenic glycosides in mediating the coevolutionary interaction between *P. incarnata* and *A. vanillae* has not been examined.

Here, we investigated whether or not cyanogenic glycosides are defensive metabolites under natural selection by herbivores and important in mediating coevolution between *P. incarnata* and the *A. vanillae*. A common garden experiment was conducted in the summer of 2015 in Athens, Georgia to test this hypothesis. If herbivores are selecting for cyanogenic glycoside production, then plants that produce the most cyanogenic glycosides should have the highest fitness when exposed to herbivores. In order to further investigate if cyanogenic glycosides are important defensive metabolites for mediating the *P. incarnata* - *A. vanillae* coevolutionary interaction, *P. incarnata* plants from different regions of the United States were grown and exposed to *A. vanillae* during the fall of 2016. If cyanogenic glycosides are defensive metabolites under natural selection, herbivore performance should vary between *P. incarnata* plants that are producing differing levels of cyanogenic glycosides. Therefore, *P. incarnata* plants from various regions should be expressing different levels of cyanogenic glycosides. Herbivore performance was measured through development time, pupae weight, and survival time. This research provides insight into how secondary metabolites in plants be used as a defensive mechanism and mediate a coevolutionary relationship.

Materials and Methods

Common Garden

In order to test if herbivores are selecting on cyanogenic glycoside production, we performed a common garden experiment during the summer of 2015. The University of Georgia Horticulture Farm was used to conduct the common garden experiment. A total of 384 samples were used, with 64 genotypes and six clonal replicates per genotype (3 replicates in each treatment). These plants were planted in the field and grown to maturity. Plants that were protected from herbivores were done so using Acephate 97 UP, a broad-spectrum pesticide, whereas plants that were exposed to herbivores were sprayed with water. It was previously determined that this pesticide does not affect the fitness of the plants or cyanogenic glycoside production. Leaves from the plants were harvested for cyanogenic glycoside analysis and stored in a -80° freezer. Fruits were then harvested from each plant as a measure of fitness. Only the fruits that were fully developed (i.e.- contained large, dark brown seeds) were counted.

Herbivore Performance

In order to assess if cyanogenic glycosides affect herbivore fitness, clones of the *P. incarnata* plants were re-generated and taken from three states in the United States: Georgia, Virginia, and Florida. The clones were then transplanted with the soil mixture including 25x 5-gallon buckets of composted pine bark, 2x 4-cu. ft. bags of vermiculite, 2 cups superphosphate, and 1 cup of each of the following: calcium nitrate, potassium nitrate, micronutrients, gypsum. Plants were grown at the University of Georgia Botanical Greenhouses under ambient light conditions. The *A. vanillae* were from eggs

laid in the growth chamber. These eggs came from adult butterflies caught at the greenhouse. The conditions in the chamber were 24 °C all day, 16 hour days, 70% humidity, and a light intensity of 4. A completely randomized design was then conducted. Each plant then received a *A. vanillae* neonate on the third fully expanded leaf and small mesh bag was placed over them. Four days later, caterpillar mortality was recorded. The small mesh bags were subsequently removed and a large bag was placed on the entire plant. After the large bags were placed on each plant, the date of pupation was recorded. Plant leaves were harvested from the plants with the *A. vanillae* butterflies on them for analysis of cyanogenic glycoside levels. The first two fully expanded leaves from the top of the stem of the plant were harvested. Plant leaves that were harvested were then weighed and the mass was recorded in grams.

Cyanogenic Glycoside Quantification

Cyanogenic glycosides were quantified through modifying previously established methods (Brinker & Seigler, 1992). Leaves were first placed in a 5 mL solution of toluene. A small well containing a 1 mL of a 0.1 M solution of NaOH was placed inside the flask with the toluene. The NaOH is responsible for capturing the volatile CN that is released from the leaf due to toluene exposure. A rubber stopper was placed on the flask to trap any released gases. After 24 hours, a 0.1 mL aliquot of NaOH + CN was taken and added to 0.9 mL of ddH_2O. The following reagents were then added to the ddH_2O + NaOH + CN solution: 0.5 mL of 1.0 M acetic acid, 5 mL of succinimide/N-chlorosuccinimide, and 1 mL of barbituric acid/pyridine. An aliquot of the final solution is then added onto a plate, which is read by a plate reader at absorbance 580 nm.

Standards were created in order to convert absorbance values to cyanogenic glycoside values.

Statistical Analysis

In order to test if cyanogenic glycosides are under natural selection from herbivores, we performed a selection analysis using methods developed by Lande & Arnold (1983). We Z-transformed cyanogenic glycoside production and calculated relative fitness to the population mean fitness. We investigated both linear (directional selection) and quadratic (stabilizing selection) regression analyses.

In order to test if cyanogenic glycoside production impacted herbivore performance, we also performed selection analyses developed by Lande & Arnold (1983). Development time, pupae weight, and survival were the three parameters measured in order examine herbivore performance. Analysis of development time and pupae weight was done with a Partial Least Squares regression. Butterfly gender was analyzed separately for pupae weight because males and females weigh different amounts (personal communication, Nicholas Batora). In short, we standardized performance to the mean and investigated both linear and quadratic relationships of herbivore performance to plant cyanogenic glycoside production.

Results

Natural selection on cyanogenic glycosides

Through the common garden experiment, we saw that the genotype source of variation of the *P. incarnata* plants is statistically significant (Table 1A and 1B; $P < 0.0001$). The treatment result indicates that the use of pesticide affected whether or not the herbivores impacted *P. incarnata* fitness (Table 1A and 1B; $P < 0.0001$). In both the linear and quadratic terms, Treatment X Cyanogenic Glycoside was not statistically significant (Table 1A and 1B; $P = 0.9072$, $P = 0.1655$ respectively). We did not find evidence for natural selection on cyanogenic glycosides.

Table 1. ANOVA for relative fitness of *P. incarnata*. The type III sums of squares are used to remove any possible effect from any other variables in the model before testing the variable being looked at in this study. Both the (A) linear and (B) quadratic terms are included.

A

Source of Variation	*df*	*Type III Sum of Squares*	*F*	*P*
Genotype	61	5.91342	3.1997	**<.0001**
Genotype Region of Origin	2	0.04022	0.3964	0.6731
Treatment	1	1.34615	44.4313	**<.0001**
Cyanogenic Glycoside	1	0.04505	1.487	0.2236
Treatment X Cyanogenic Glycoside	1	0.00041	0.0136	0.9072
Block	2	0.13246	2.1861	0.1142
Error	296	8.96805		

B

Source of Variation	*df*	*Type III Sum of Squares*	*F*	*P*
Genotype	61	5.88599	3.2414	**<.0001**
Genotype Region of Origin	2	0.04296	0.7216	0.4868
Treatment	1	0.81693	27.4427	**<.0001**
Cyanogenic Glycoside	1	0.25343	8.5135	0.0038
Cyanogenic Glycoside^2	1	0.11263	3.7836	0.0527
Treatment X Cyanogenic Glycoside^2	1	0.05754	1.933	0.1655
Block	2	0.13444	2.2581	0.1064
Error	295	8.78168		

Impact of host defense on *Agraulis vanillae* performance

We did not find evidence that herbivore performance varied between *P. incarnata* plants that are producing differing levels of cyanogenic glycosides for two of the three parameters measured. Through the herbivore performance experiment, we saw that there was no interaction between toughness and cyanogenic glycoside production on the development time (Table 2; $P = 0.5520$). A similar model was implemented to analyze the interaction between leaf toughness and cyanogenic glycoside production on the pupae weight of the *A. vanillae* butterflies. Butterfly gender was analyzed separately because males and females weighed very different amounts. We saw that there was no interaction between toughness and cyanogenic glycoside production on the pupae weight of the male *A. vanillae* butterflies (Table 3; $P = 0.8419$). However, we saw that interaction between toughness and cyanogenic glycoside production on the pupae weight of female *A.*

vanillae butterflies is statistically significant (Figure 5; $P = 0.0135$). The interaction between toughness and cyanogenic glycoside production on survival was unable to be determined since only one of the caterpillars survived.

Table 2. ANOVA for interaction between leaf toughness and cyanogenic glycoside production on the development time of *A. vanillae* in *P. incarnata*. Cyanogenic glycoside data was transformed by the following equation to create a normal distribution: ln(CN+.0001). The .0001 value was added to the CN value because there were zeros in the data set. Both the linear and quadratic terms are included.

Source of Variation	***df***	***Type III Sum of Squares***	***F***	***P***
Cyanogenic Glycoside	1	1.12870	0.9469	0.3334
Average Toughness	1	1.91286	1.6048	0.2088
Average Toughness X Cyanogenic Glycoside	1	0.42506	0.3566	0.5520
Error	82	97.73998		

Table 3. ANOVA for interaction between leaf toughness and cyanogenic glycoside production on the pupae weight of the male *A. vanillae* butterflies in *P. incarnata*. Cyanogenic glycoside data was transformed by the following equation to create a normal distribution: ln(CN+.0001). The .0001 value was added to the CN value because there were zeros in the data set. Both the linear and quadratic terms are included.

Source of Variation	***df***	***Type III Sum of Squares***	***F***	***P***
Cyanogenic Glycoside	1	0.01909	30.58	0.1248
Average Toughness	1	0.00320	1.9622	0.1433
Average Toughness X Cyanogenic Glycoside	1	0.22735	1.50	0.8419
Error	36	0.05208		

Table 4. ANOVA for interaction between leaf toughness and cyanogenic glycoside production on the pupae weight of the female *A. vanillae* butterflies in *P. incarnata*. Cyanogenic glycoside data was transformed by the following equation to create a normal distribution: ln(CN+.0001). The .0001 value was added to the CN value because there were zeros in the data set. Both the linear and quadratic terms are included.

Source of Variation	***df***	***Type III Sum of Squares***	***F***	***P***
Cyanogenic Glycoside	1	0.02469	29.85	0.9529
Average Toughness	1	0.24987	3.20	0.8619
Average Toughness X Cyanogenic Glycoside	1	0.18348	2.59	**0.0135**
Error	39	0.07410		

Discussion

Numerous taxa have been used in order to investigate the role of defensive metabolites in coevolution between herbivores and plants, but the important role of cyanogenic glycosides in mediating the coevolutionary interaction between *P. incarnata* and *A. vanillae* has not been examined. In this study, we investigated the crucial role of cyanogenic glycosides in mediating the coevolutionary interaction between *P. incarnata* and *A. vanillae*. In order to study this, a common garden and herbivore performance experiment was done to determine whether or not cyanogenic glycosides are defensive metabolites under natural selection by herbivores and important in mediating coevolution between *P. incarnata* and the *A. vanillae*.

Through the common garden experiment, we saw that the genotype was a significant predictor of cyanogenic glycoside production, implying that this variation in *P. incarnata* is genetically based (Table 1A). Furthermore, we saw that the treatment was also significant, indicating that the use of pesticide affected whether or not the herbivores impacted *P. incarnata* fitness (Table 1A). The fact that interaction between the treatment and cyanogenic glycoside was not significant suggests that the relationship between fitness and cyanogenic glycoside production was not different between the two treatments, not supporting the hypothesis. We could not determine relationship between cyanogenic glycoside production and fitness, preventing us from detecting natural selection by herbivores on *P. incarnata*. One possible reason we did not detect natural selection on cyanogenic glycoside production among treatments is that plants were heavily fertilized and grew substantially larger than what is normally observed in nature. Thus, any selection by herbivores on plant defenses would be more difficult to detect.

It was seen through the herbivore performance experiment that the interaction between toughness and cyanogenic glycoside production on the development time and male pupae weight was not significant. These results suggest that development time and male pupae weight do not vary when feeding on *P. incarnata* plants that are producing differing levels of cyanogenic glycosides. However, the interaction between toughness and cyanogenic glycoside production on the pupae weight of the female *A. vanillae* butterflies was significant (Table 4). These results suggest that female herbivore performance varied when feeding on *P. incarnata* plants that are producing differing levels of cyanogenic glycosides and leaf toughness. Furthermore, these results indicate that female herbivore performance could potentially be a trait that is impacted by cyanogenic glycosides, suggesting that this defensive metabolite is possibly mediating a coevolutionary interaction between *P. incaranta* and *A. vanillae*.

Due to the short nature of the common garden experiment (one growing season), further research is needed to see the impacts of herbivory by specialist *A. vanillae* on fitness of *P. incarnata* for longer periods due to the perenniality of *P. incarnata*. Also, research should be done to identify other possible herbivores that potentially have a coevolutionary relationship with *P. incarnata* so that the evolutionary ecology of this plant species can be further understood. Additionally, future research can be geared towards identifying other defensive metabolites produced by *P. incarnata*. This research could potentially provide further insight into the defense mechanisms that mediate coevolutionary interactions in this species.

Several aspects of the herbivore performance should be addressed and improved in future studies. We did not detect a relationship between survival of *A. vanillae*

butterflies and cyanogenic glycoside production. This is possibly due to the fact that this species is a specialist that feeds on this species and we know it utilizes the cyanogenic glycosides as it feeds (Engler and Gilbert, 2007). In addition, the results suggest that development time is not a factor of herbivore performance that is affected by cyanogenic glycosides (Table 2). However, the results indicate that female pupae weight can be a target trait for future studies in order to better understand this coevolutionary dynamic between *P. incarnata* and *A. vanillae*.

It has been previously stated that secondary metabolism results in the production of defensive compounds that organisms can use to deter herbivory (Berenbaum, 1995). Furthermore, it has been observed that insects like *Lepidoptera* evolve physical resistance to defensive compounds such as coumarins, resulting in the formation of a coevolutionary interaction between the two species (Berenbaum, 1983). This study was another investigation into chemical mediation of coevolution between plants and herbivores. Though the research in this study as a whole needs to be further expanded on, it provides a basis for understanding if cyanogenic glycosides are an important defensive metabolite in *P. incarnata* and if there is an importance for defensive metabolites in mediating plant-herbivore evolutionary interactions.

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