

THESIS

**The consequences of Squash domestication on
the chemical ecology of plant-insect interactions**

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by

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on the chemical ecology of plant insect
interactions”**

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RÉSUMÉ

La domestication des plantes ainsi que le début de l'agriculture ont été des innovations clés qui ont modelé la société et l'écosystème que nous connaissons aujourd'hui. La plupart des fruits et légumes que nous consommons ont été sélectionnés par l'homme et divergent des plantes sauvages ancestrales en de nombreux aspects que nous nommons syndrome de domestication. Il s'agit en général d'une augmentation de la taille du fruit, d'un complément en nutriment et, dans 60% des cultures, la réduction de la toxicité dans le but de leur consommation. Cette perte de composé chimique est intimement liée à l'augmentation et la prolifération des ravageurs de cultures. Cependant cette affirmation n'est pas ubiquitaire et dépendant de la plante étudiée.

Souvent, la sélection de l'organe d'une plante a lieu en vue de sa consommation alimentaire. D'autres motifs de sélection comme l'utilisation des fibres, fabrication d'huile, décoration comme c'est le cas pour les courges, existent également. Les courges (genre *Cucurbita*) sont d'origine mexicaine et font partie des premières plantes à avoir été domestiquées sur le continent américain avec le maïs et le haricot. Leur domestication daterait d'il y a 10'000 ans selon des preuves archéologiques. Une de leurs premières utilisations a été en tant que récipient car leur chaire était très amère et toxique à cause d'une molécule nommée cucurbitacine. Il existe cinq espèces différentes de courges, toutes ont été domestiquées principalement pour la consommation de leur fruit, mais également pour leur beauté.

La domestication des courges et son impact sur les insectes herbivores n'ayant pas été encore étudié, je me suis lancé le défi de comprendre ses conséquences. J'ai précisément voulu étudier les effets de la domestication sur les défenses physiques (trichomes) et chimiques (cucurbitacines) de la plante de courge et comment ces changements ont impacté les insectes sur plusieurs niveaux trophiques. Pour y répondre, j'ai travaillé sous 4 angles différents :

- 1) J'ai testé l'hypothèse que le but de domestication de la courge (alimentation ou ornementation) influencerait le contenu en cucurbitacines et trichomes des variétés. Pour cela, j'ai utilisé plusieurs espèces de courges par but de domestication. J'ai étudié l'effet de ces changements de défenses sur deux insectes généralistes se nourrissant soit sur les feuilles (*Spodoptera latifascia*), soit sur les racines (*Diabrotica balteata*). J'ai trouvé que les buts de domestication n'expliquaient pas les contenus en cucurbitacines. Cependant, les trichomes ont été diminués sur les variétés sélectionnées pour la consommation de leur fruit. Les cucurbitacines n'ont pas d'impact sur la croissance du stade larvaire des insectes étudiés, en revanche elles attireraient fortement les larves de *D. balteata*. Cette étude a démontré l'importance de la phylogénie lorsqu'on étudie l'impact de la domestication.
- 2) J'ai analysé les différences en termes de défense de la plante, entre les courges sauvages et domestiquées de l'espèce *Cucurbita argyrosperma*. Parmi les courges domestiquées, j'ai gardé le concept de but de domestication en étudiant des variétés sélectionnées pour la consommation du fruit ou l'ornementation. J'ai étudié l'impact de la domestication des courges sur un insecte généraliste (*Diabrotica balteata*) et un insecte spécialiste des courges (*Acalymma vittatum*) en laboratoire et dans leur milieu naturel (Mexique). J'ai trouvé que toutes les courges domestiquées ont perdu les cucurbitacines dans leurs racines. Les cotylédons des variétés sélectionnées pour la consommation du fruit contiennent toujours des cucurbitacines mais beaucoup moins que les

courges sauvages. Le contenu en cucurbitacine des courges sauvages réduit la croissance des insectes généralistes mais pas des spécialistes. La cucurbitacine est un attractif très fort pour les deux types d'insectes. Au Mexique, les dommages sur les plantes domestiquées étaient plus importants.

- 3) Je me suis intéressée à l'impact indirect de la domestication des courges sur le troisième niveau trophique (ennemi naturel de l'herbivore). Pour cela j'ai étudié la survie, la préférence et la performance du prédateur *Atheta coriaria* pour des proies ayant mangé des courges soit sauvages, soit domestiquées. En d'autres termes des larves de *Diabrotica balteata* qui avaient consommé des racines contenant des cucurbitacines versus des larves qui se sont nourries de racines sans cucurbitacines. J'ai trouvé que la cucurbitacine des plantes sauvages n'a pas impacté la survie ou la préférence du prédateur. La séquestration de cucurbitacine par les herbivores ne les protège en rien contre le prédateur étudié.
- 4) J'ai voulu comprendre pourquoi les variétés de courge domestiquées ne contiennent pas de cucurbitacine. Plus précisément, j'ai étudié l'impact de la domestication des courges sur l'expression des gènes liés à la biosynthèse de la cucurbitacine. J'ai voulu savoir si les courges domestiquées avaient perdu le gène ou perdu l'expression du gène permettant la production de cucurbitacines. J'ai commencé par trouver des gènes orthologues aux gènes connus chez le concombre. Puis j'ai découvert un candidat pour le gène de la première enzyme responsable de la production de cucurbitacine (cucurbitadienol synthase) et six gènes du cytochrome P-450 responsable d'oxydation permettant la production de cucurbitacines. Ensuite j'ai analysé l'expression de ces gènes dans les racines, cotylédons et feuilles des courges sauvages et domestiquées. J'ai trouvé que les variétés de courge domestiquées possédaient toujours les gènes responsables de la production de cucurbitacine, mais que c'était leur expression qui était perturbée par la domestication. Dans les racines, l'expression des gènes est plus élevée chez les courges sauvages. L'expression de ces gènes est nulle dans les feuilles et très faible dans les cotylédons.

MOTS-CLÉS : DOMESTICATION, COURGES, CUCURBITACINES, RAVAGEURS

SUMMARY

Since the start of agriculture, crop domestication has induced significant changes in both plants and human societies. Plant domestication has generally resulted in decreased chemical and physical defenses in crop plants compared to their wild ancestors. A reduction in plant defensive traits is often expected to result in increased insect performance. By selecting (modifying the genetics) desirable traits for humans, it may favor the pest insects that we have to face today in agriculture. However, recent studies have shown that the impact of crop domestication on plant-insect interactions is not ubiquitous. Surprisingly, there is still much discussion on the way by which crop domestication has influenced insect pests. In my PhD project, I hypothesize that the outcome of plant-insect interactions may be influenced by the type of organ targeted during domestication, as well as the by purpose of domestication.

Squash (genus *Cucurbita*) is one of the earliest and most important domesticated plants in the Americas, along with maize and the common bean. The genus *Cucurbita* was domesticated on several occasions leading to five different species throughout the Americas, beginning around 10,000 years ago. Wild forms of squash are very bitter and toxic to humans and other mammals because they contain cucurbitacins (toxic secondary metabolites). Squash was domesticated at different times and for different purposes (including soap, oil, music instrument, food and beverage container). As a result, we have great variation on fruits size, color and shape.

The aim of my PhD project was to examine the overall consequences of squash domestication on plant defenses and its impact on plant-insect interactions. To date, no studies with these specific questions were done on this important world-wide crop. To meet the challenge, I had four main axes:

- 1) Study the impact of purpose of domestication on the squash defenses (trichomes and cucurbitacins) and how it altered the interaction with generalist insects (*Spodoptera latifascia* that eat aboveground tissue and *Diabrotica balteata*, whose adults feed on aboveground tissues and larvae feed on belowground tissues). To test that, I had squash varieties from different species selected for consumption (fruit and seeds) and varieties selected for an ornamental purpose. I found that the varietal selection rather than the purpose of domestication explained the differences in cucurbitacin content. However, trichome density was reduced on varieties selected for consumption. The herbivore performance was not negatively affected by high cucurbitacin content nor by trichome density. Instead, the root herbivore *D. balteata* larvae preferred to feed on the varieties with high levels of cucurbitacins. This study highlighted the idea that *D. balteata* is adapted to cucurbits and the importance of phylogeny while studying plant domestication.

- 2) Study the consequences of *Cucurbita argyrosperma* domestication on plant chemical (cucurbitacins) and physical (trichomes) defenses and extent to which altered defenses impacted the interaction with two beetle species, the generalist *Diabrotica balteata* and the squash specialist, *Acalymma* spp. (Coleoptera: Chrysomelidae). These plants and insects have a long co-evolutionary history. To add to the behavioral bioassays in the lab, I tested the susceptibility of *Cucurbita argyrosperma* wild and domesticated varieties in a common garden during a field season in Mexico. I found that cucurbitacins were selected out of the domesticated varieties. Trichome density was not reduced through domestication. Larvae of both insects preferred to feed on roots of wild squash, but this negatively affected the performance of the generalist. In the field, leaves of wild and domesticated plants were mainly attacked by adults of the specialist.
- 3) Study the indirect consequence of *Cucurbita argyrosperma* domestication on the third trophic level. One of the main results from previous axes was the reduction of cucurbitacins in domesticated squash varieties. It was hypothesized that *D. balteata* sequester this compound for its own defense against natural enemies. Then, deprived of cucurbitacin by eating domesticated squash, larvae should be more attractive to soil predators. To test this hypothesis, I used the rove beetle predator (*Atheta coriaria*), which is a generalist soil predator with no coevolutionary history with *D. balteata* or squash. Overall, I found no evidence that cucurbitacins serve as a protection against predators. The assumed lethal and deterrent impacts of sequestered cucurbitacin by the herbivore on the predator were not observed. However, the survival of *Diabrotica balteata* larvae was lower when fed on wild squash populations.
- 4) Study how squash domestication changed the expression of cucurbitacin biosynthesis genes. From *Cucurbita argyrosperma* genome and literature on genes involved in the cucurbitacins pathway in cucumber, I targeted seven candidates for cucurbitacins biosynthetic genes. One candidate for the very first enzyme involved in the cucurbitacin biosynthesis: cucurbitadienol synthase, and six cytochrome P-450 enzyme. I analyzed the expression of those genes in roots, cotyledons and leaves to explain the differences in cucurbitacin concentration among plant tissues and among wild and domesticated squash. Overall, gene expression was higher in roots compared to cotyledons and for some genes, wild squash populations had a higher expression than the domesticated varieties.

KEY-WORDS: DOMESTICATION, CUCURBITA, CUCURBITACINS, HERBIVORES, PREDATORS, GENE EXPRESSION

GRAPHICAL SUMMARY

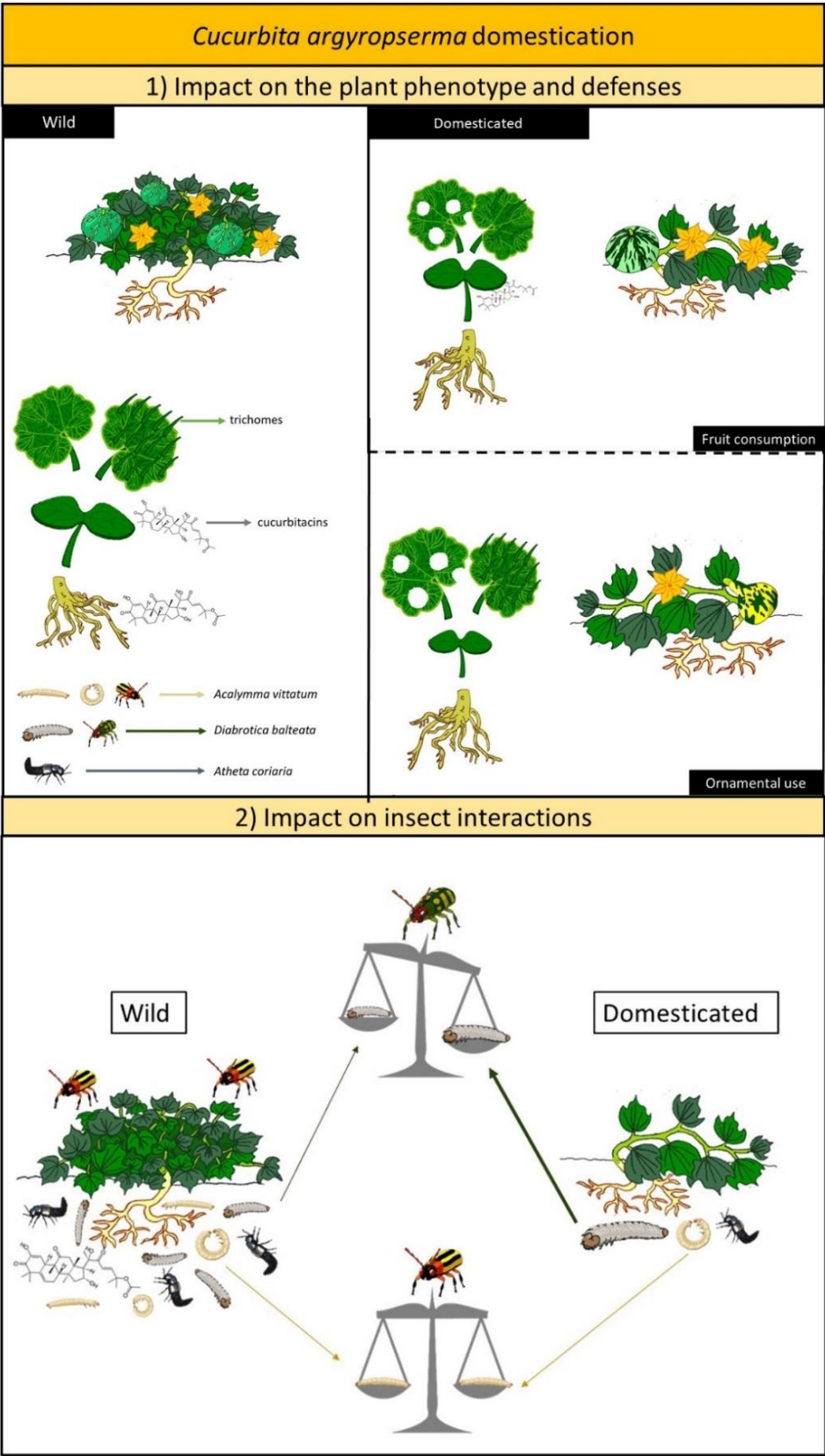


Figure: Summary of the predicted consequences of *Cucurbita argyrosperma* domestication on plant defenses and on the interactions with the second and third trophic level.

Table of contents

General introduction	19
1. <i>Plant defenses</i>	19
2. <i>Crop domestication</i>	20
3. <i>Squash</i>	22
4. <i>Background study</i>	25
5. <i>Aims of the thesis</i>	27
Chapter 1: Squash varieties domesticated for different purposes differ in chemical and physical defense against leaf and root herbivores	33
Abstract	33
Introduction	34
Material and methods.....	36
Results	44
Discussion	53
Author Contributions.....	56
Acknowledgments	56
References	57
Supplementary material	63
Chapter 2: Domestication altered defense traits and gene expression in <i>Cucurbita argyrosperma</i> with differential consequences for a generalist and a specialist herbivore.....	69
Introduction	70
Results:	80
Discussion:.....	90
Author contributions	93
Acknowledgments:	93
References:.....	94
Supplementary data:	100
Chapter 3: The effects of domestication of squash, <i>Cucurbita argyrosperma</i> on a belowground tritrophic interaction	107
Introduction	108
Material and methods.....	110
Results	115
Discussion	122

Author contributions	125
Acknowledgments	125
References	126
Supplementary materials.....	131
Chapter 4: Characterization of cucurbitacin biosynthetic genes associated with reduced bitterness in squash cultivars (<i>Cucurbita argyrosperma</i>)	135
Abstract	135
Introduction	136
Material and methods.....	138
Results	142
Discussion	150
Author contributions	152
Acknowledgments	152
References	153
Supplementary materials.....	157
General discussion and conclusions	165
References	173
Take home messages	179
Future perspectives	181
Appendix.....	183

General introduction

1. *Plant defenses*

Plants produce an enormous array of different chemicals that are usually divided into primary and secondary metabolites¹. Primary plant metabolites refer to chemicals that are essential for plant growth and development, and are commonly produced by most plant species. They generally consist of proteins, carbohydrates, and lipids. These compounds are important for basic physiological processes in plants and are often also essential nutrients for insects^{1,2}. Secondary metabolites often have a more restricted phylogenetic distribution. However, they play an important role in plant interactions with the biotic and abiotic environment.

In nature, plants must deal with a variety of stresses including pathogens, herbivores, and competitors. Plant fitness depends on how they will respond to these stressors. Plants defend themselves against attack by herbivorous insects by utilizing a combination of constitutive and inducible defenses. Constitutive defense refers to defense mechanisms that are continually expressed, whereas induced defenses are defense mechanisms that are increased following herbivory^{3,4}. In addition to the physical barriers such as spines, cuticle or hairs, and trichomes, plants can produce defense proteins and toxic secondary metabolites. Those metabolites are important determinants of plant resistance and herbivore performance in nature. They can deter polyphagous herbivores^{4,5} and in some cases may stimulate oviposition and feeding by specialist herbivores⁶. Plant secondary metabolite content is generally dynamic and varies with biotic and abiotic factors, as well as with plant ontogeny^{7,8}.

However, specialist herbivores have evolved (i. e. feeding on a single or a reduced number of species), presenting another challenge to plants. They can have the ability to avoid these deleterious compounds and feed on well-defended plants. For this to happen specific adaptations are required, such as particular mouthparts, detoxification pathways, sequestration of plant toxins, etc. For instance, many specialized insect species accumulate toxic secondary metabolites and use them for their own defense against predators⁹. For example, *Diabrotica spp.* (Coleoptera: Chrysomelidae, Diabroticites), are able to feed on Cucurbitaceae plants which produce cucurbitacins (i. e. oxygenated tetracyclic triterpenes toxic to most herbivores) and sequester those toxic compounds and become toxic to their predators¹⁰.

All of the plant defense mechanisms described above are considered to be direct defenses because they act upon the attacker directly and reduce the amount of damage inflicted to the plant¹. However, plants can also use indirect defenses, such as volatile compounds and extrafloral nectar¹¹. Those, promote the efficiency of natural enemies to control plant antagonists in order to reduce herbivory¹¹. Some of these indirect types of defenses are mediated by the emission of volatile organic compounds (VOCs). For example, when a caterpillar eats a maize leaf, the attacked plant produces a characteristic volatile blend attracting parasitic wasps that lay eggs in the caterpillar, thereby interrupting the life cycle of the herbivore^{12,13}. This phenomenon is also observed belowground; when roots of maize plants are eaten by a larvae of a

Coleoptera species (*Diabrotica virgifera virgifera*), the plant release (E)-b-caryophyllene which attracts entomopathogenic nematodes, which parasitize the larvae and kill it before it becomes an adult beetle¹⁴. Moreover, some VOCs are used in a phenomenon known as “priming” to prevent herbivory on neighboring plants. For example, indole emitted from maize plants under herbivore attack exhibits a priming effect on surrounding maize plants¹⁵.

Indirect and direct plant defenses are often studied independently of one another, but chemicals associated with indirect defense may also affect herbivore behavior.

2. Crop domestication

Crop domestication was a key historical innovation which enabled the establishment of complex civilizations^{4,16}. All of the major crops around the world were domesticated from a wild progenitor species, primarily between 4000 to 10,000 years ago.

Nicolai I. Vavilov (1887-1943), a Russian agronomist and geneticist pointed out that the centers of origin of most domesticated plants began in botanical areas or active regions, with high diversity. In general, these regions include a significant number of endemic species and a high concentration of genetically related species or wild relatives. Vavilov proposed eight centers of origin of domesticated plants, fundamental and ancient centers of agriculture in the world. Agriculture originated in at least six different areas of the world: Mesoamerica, the Andes of South America, Southwest Asia (The Fertile Crescent), Africa, Southern China and Southeast Asia¹⁶. All the centers are in tropical or subtropical regions generally between 35°N. and 35° S. latitude. Their topography is in general mountainous or hilly. In each of these centers, similar types of crops were domesticated.

The process of domestication had profound consequences on plants. Domesticates and their progenitor species generally differ by a suite of complex physiological, morphological, and genetic changes known as the domestication syndrome^{17,18}. Domestication syndromes may include combinations of several different traits, such as seed retention (no shattering), changes in branching and height, increased fruit or seed size, changes in reproductive strategies and changes in secondary metabolites^{4,18}. The desirable traits are selected for an agricultural environment and with time, they become fixed in the genome. These domestication traits arising through artificial selection are selected in order to satisfy farmer and consumer' needs. Domestication syndromes may evolve over thousands of generations.

Genetic variation tends to be reduced in domesticated organisms compared to their wild progenitors due to genetic bottlenecks associated with the sampling process^{19,20}. The genetic basis of a domestication syndrome comprises protein or regulatory changes in specific genes⁴. Those changes have been extensively studied for divers crops as wheat²¹, maize²², sunflower²³, common bean²⁴, and tomato²⁵. These studies have indicated that a few gene clusters with large effects account for

most of the variation associated with the differences between domesticated forms and their wild progenitors²⁶.

Thus, domesticated plants greatly differ from their wild relatives. For example, domesticated cotton has an increased fiber length and quality²⁷ in comparison to their wild relatives. Teosinte, the maize ancestor, has many lateral branches, while today's maize is unbranched (Figure 1)²⁸. Domesticated tomatoes have a larger fruit size and different shape compared to wild tomatoes²⁹.

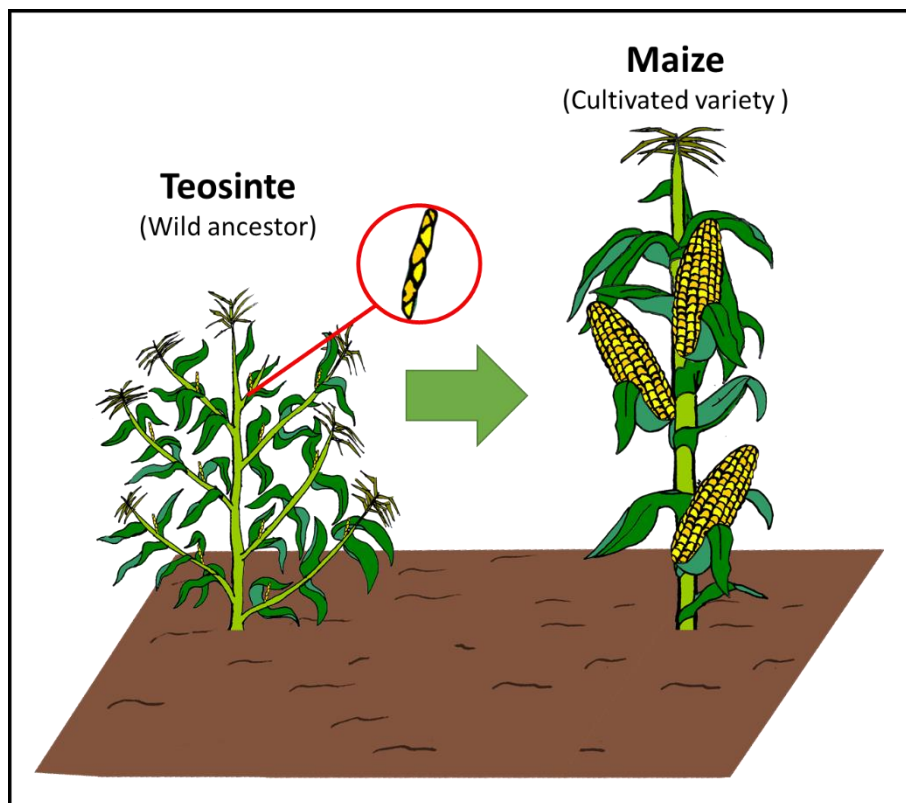


Figure 1. Illustration of domestication syndrome in Maize. Ears of corn are larger and the branches are reduced in the domesticated form. Drawing done by Nicolas Marguier.

Unfortunately, domestication has frequently selected for traits that negatively impact the plant's defensive strategies in the natural environment¹⁶. Indeed, removing toxic compounds from plants used for human consumption, has facilitated other herbivores to feed on these plants. Plant secondary metabolites have been shown to be drastically influenced by the domestication process, especially pertaining compounds that are toxic for herbivores^{18,29}. Meyer, et al. ¹⁸ showed that the most common trait of the domestication syndrome of most crops, is the change in secondary metabolites (loss of bitter or toxic compounds, pigment changes), having occurred in 66% of crops. However, the consequences of these changes on plant/insect interactions are not ubiquitous. Turcotte, et al. ³⁰, studied the impact of domestication on resistance to two generalist herbivores across 29 independent events, and showed that for both herbivores, domestication altered the plant traits that were most strongly associated with herbivore performance, suggesting that artificial selection alters how plants defend themselves against these herbivores. Nevertheless, their results suggest that domestication does not cause predictable reductions in resistance traits against

herbivores as many specific domestication events did not cause differences in resistance to either herbivore. The diversity of outcomes that they observed emphasizes the importance of studying multiple domestication events.

The results of crop domestication can disrupt tritrophic interactions by favoring the success of the herbivore and reducing the efficiency of natural enemies in some systems^{4,30-33}. For example, Chen and Welter³⁴ showed that sunflower domestication has increased larval abundance (*Homoeosoma electellum*), accelerated larval development, and lowered parasitism. However, crop domestication may actually enhance natural enemies' performance in other systems. For example, Benrey, et al.³⁵ found that performance of two herbivores (*Pieris rapae* and *Zabrotes subfasciatus*) and their parasitoids (*Cotesia glomerata* and *Stenocorse bruchivora*) were higher on domesticated plants than on their wild relatives. The impact of crop domestication on the 3rd trophic level cannot be generalized and needs to be investigated for each crop.

Plants have been domesticated for different purposes and different organs have been the target of domestication. We could predict that domestication has led to greater reductions in plant defenses in targeted organs (like for fruit consumption) than in non-targeted tissues because selection directly acts upon defensive traits found only in the targeted tissue^{33,36}. Whitehead, et al.³³ performed a meta-analysis on the impact of domestication on plant-herbivore interactions and found consistent negative effects of domestication only when defense traits were measured in reproductive organs or in the plant organ that was harvested. Moreover, food crops may have experienced additionally direct selection for increased palatability. Thus, it could be expected that domestication will result in reduced defenses and increased herbivore performance on food than non-food crops. However, studies comparing levels of defensive traits for crops and their wild relatives in targeted and non-targeted plant organs remain rare.

3. Squash

3.1 Domestication

Cucurbita (squash and pumpkin species) is among the earliest and most important domesticated plants in the Americas, along with maize and common beans in the genus *Phaseolus*, beginning around 10,000 years ago³⁷. These plants have an incredibly rich and long history of domestication and cultivation. Despite the extinction of their natural dispersers (e.g., mastodons and similar megafauna)³⁸, their distribution was extended by human-guided domestication and breeding. Cucurbits are unrivalled in their wide range of adaptation to cultivation and their fruit morphological diversity. Among all the plants domesticated for consumption of their fruits, cucurbits have the most dramatic increase in fruit size compared to their wild relatives³⁹. Cucurbits represent an interesting system for the study of domestication⁴⁰. With 21 taxa, the *Cucurbita* genus has experienced independent domestication events in five species: *C. pepo*, *C. ficifolia*, *C. moschata*, *C. maxima*, and *C. argyrosperma* (Figure 2)⁴¹. Each domestication event occurred independently, sometimes on more than one occasion⁴⁰. Each *Cucurbita* crop was selected for specific traits, defined at that time by the nutritional and cultural needs of the early human populations in the Americas^{42,43}.

However, many domestication syndromes are common in the domesticated *Cucurbita*, including the loss of various traits such as bitterness (cucurbitacins), physical defense mechanisms (e.g., trichomes), and seed dormancy. The other common traits are the enlargement of fruits and seeds, and the diversification of fruit morphology^{44,45}.

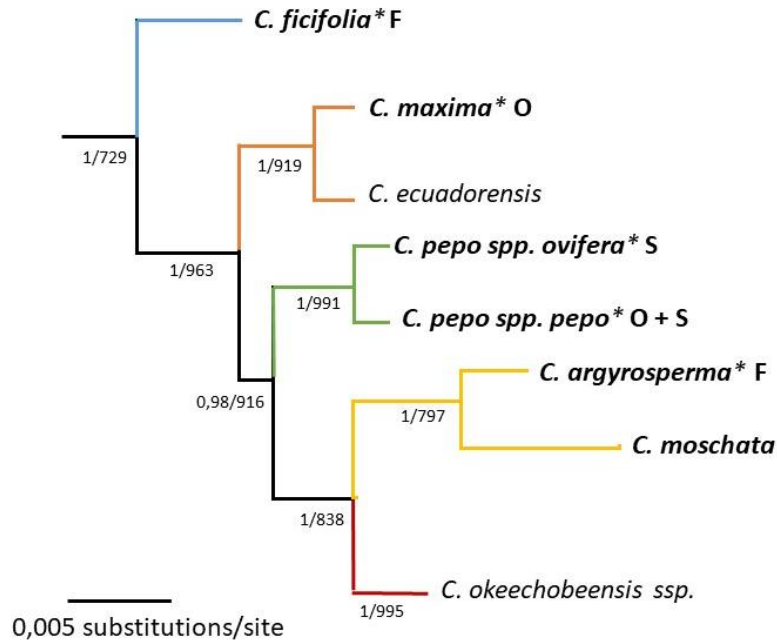


Figure 2. Species used in our experiments based on a *Cucurbita* phylogeny modified from **Castellanos-Morales, et al.**⁴⁶. Color indicates different monophyletic groups: light blue: *Cucurbita ficifolia*, orange: group Maxima, green: group Pepo, Yellow: group *Argyrosperma* and red: group *Okeechobeensis*. Asterisks shows the varieties used in this study. Letters indicate the purpose of domestication: F: fruit consumption, O: ornamental, S: seed oil, used in our study. Domesticated taxa are in bold.

3.2 *Cucurbita argyrosperma*

Among domesticated cucurbits, *C. argyrosperma* subsp. *sororia* (Bailey), the wild cucurbit subspecies of *Cucurbita argyrosperma*⁴⁷, known in Mexico as calabaza pipiana or calabaza mixta, is highly valued for its seeds, which are used in Mexican gastronomy. Fruits are medicinal, and valued for commercial use and as food resources⁴⁸. This species has a cultural and economic importance both locally and worldwide. The oldest evidence of domestication of this species is 8,600 years ago in the Xihuatoxtla shelter, in the modern state of Guerrero (Mexico)⁴⁹. *C. argyrosperma* can be found in tropical and semi-desert regions from the Southeastern United States through Mexico and northern Central America, reaching Nicaragua, from sea level to 1,700 m above sea level⁴⁰. These subspecies have a sympatric distribution in most of their range, except for the Yucatan peninsula, where the wild subspecies is absent⁴⁸. Domestication gave rise to the crop *C. argyrosperma* ssp. *argyrosperma*, commonly named “cushaw pumpkin” and “Japanese pie pumpkin”⁵⁰.

Cucurbita argyrosperma is an important crop in local agricultural systems in Mexico and in other countries in the Americas. It is grown and selected in traditional ways. It is commonly found as a seasonal crop, but irrigation is used in some areas. In

other regions of the world it is not extensively cultivated because of the low quality of its flesh⁴⁸, but there are records of some genetically improved cultivars grown in the United States and Canada. Some improved lines show differences in fruit and seed size, shape, and color, such as “Green Striped Cushaw,” “White Cushaw,” “Magdalena Striped,” “Papago,” “Japanese Pie,” “Hopi,” “Taos,” “Parral Cushaw,” “Veracruz Pepita,” and “Silver Seed Gourd” (Figure 3 and 4).



Figure 3. Fruits of *C. argyrosperma*, (A): wild from Bacocho, (B): Navajo calabacita, (C): wild from Umar, (D): Silver Edge, (E): Veracruz Pepita



Figure 4. Seeds of *C. argyrosperma* wild populations (Wild Bacocho(WB), Wild Umar (WU), Wild Ventanilla(WV)) and varieties selected for fruit consumption (Silver Edge (FSE) and Vera Cruz Pepita (FVP)) and varieties selected for an ornamental use (Cushaw tricolor (OCT) and Navajo calabacita (ONC)).

3.3 Cucurbitacins

Cucurbitacins, as the name suggests, are particularly associated with the family Cucurbitaceae where they have been characterized in at least 30 genera and more than 100 species⁵¹. Wild forms of squash are very bitter to humans and other mammals because they contain cucurbitacins. These molecules constitute a group of diverse triterpenoid substances, which are well known for their bitterness and toxicity. Indeed, cucurbitacins are the most bitter substances identified, and can be detected by humans at dilutions as great as 1 ppb⁵¹. Twenty-two cases of human poisoning after eating *Cucurbita pepo* (zucchini) fruits that contained about 1.1 mg Cuc E glycoside per gram occurred in Queensland, Australia, in 1982. Symptoms included severe cramps, persistent diarrhea and collapse occurring within few hours after eating the zucchini fruits⁵¹.

Cucurbitacin biosynthesis occurs locally in tissues through activation of the first committed step of a single oxidosqualene cyclase (OSC) by leaf, root or fruit-specific transcription factors^{52,53}. Then, cucurbitadienol has to be further modified with a series of oxidation reactions and acetylation, likely catalyzed by cytochrome P-450 enzymes (P450s) and an acyltransferase (ACT)⁵². Cucurbitacins can be found in all parts of the plant in the Cucurbitaceae family: roots, stems, leaves, fruits and occasionally in the seeds.

There is evidence that they were harmless to mastodons (the extinct form of elephants). Interestingly, when the megafauna died off at the end of the last Ice Age, wild *Cucurbita* plants declined⁵⁴. Squash domestication was in part the result of human selection for different traits related to edibility, as well as seed size and rind thickness, but the most important change was the reduction of cucurbitacins^{55,56}.

Cucurbitacins mediate the coevolution between cucurbits and herbivores⁵⁷. In addition to their toxic function, they also serve as feeding attractants for specialist insects and as a defense against generalist insects⁵⁸. Furthermore, the ancestral association between the leaf beetles of the tribe Luperini (Coleoptera: Chrysomelidae: Galerucinae) and the plants in the family Cucurbitaceae seem to have been affected by the presence of the cucurbitacins acting secondarily as kairomone cues for host selection by the beetles. Diabroticina beetles possess sensilla basiconica arranged around the tip of the maxillary palpus that appear to contain cucurbitacin receptors⁵⁸. These are responsible for the high degree of sensitivity to the cucurbitacins and the specificity of behavioral responses induced by proximity to cucurbitacins, such as arrest and compulsive feeding⁵¹.

4. Background study

Previous work on this system during my master's thesis focused on the purpose of domestication. I had different species of *Cucurbita* (same clade); *C. pepo*, *C. maxima*, *C. ficifolia*, and *C. argyrosperma* (Figure 2). The purposes of domestication I tested were for consumption (fruits and seeds) and ornamental uses. I found that cucurbitacins were present only in roots and cotyledons at high concentrations. The

cucurbitacins in leaves were almost undetectable. A small difference in the relative content of cucurbitacins according to the purpose of domestication was found with the tendency for ornamental varieties to contain more cucurbitacins, especially in roots, than plants selected for the consumption of their fruit. However, this did not have an effect on the relative growth rate of the cucumber beetle (*Diabrotica balteata*, Fig. 5) larvae that fed on cucurbit roots.

Physical defense traits were also studied by examining trichome density on the leaves and cotyledons. I observed no trichomes on the cotyledon surface, but a high density on adaxial side of the leaves of all cucurbits. There is a tendency showing that the density of trichomes could be different according to the purpose of domestication, but variation was high among varieties. However, phylogeny, explained better the difference in trichome density than the purpose of domestication. I tested the relative growth rate of a generalist caterpillar (*Spodoptera latifascia*, Fig. 5) on the leaves and cotyledons of different cucurbit varieties in relationship to the defense traits of each plant part (trichomes for leaves and cucurbitacins for cotyledons). The caterpillar growth rate was higher on the leaves of the varieties selected for seed consumption but no difference among purpose of domestication when the caterpillars fed on cotyledons.

A preliminary study was done on gene expression. I collaborated with the plant physiology lab headed by Professor Felix Kessler. I was interested to know if cucurbit cultivars lost the gene responsible for the production of cucurbitacins, or if the genes were under-expressed in plant parts. In particular I examined the cucurbitadienol synthase gene expression in roots. I found that the gene was still present and not silenced, meaning that the biosynthetic pathway of cucurbitacins is more complicated and requires more than one gene. One common garden experiment was also conducted in Puerto Escondido, Oaxaca, Mexico. Varieties selected for ornamental use were more susceptible in field conditions and attacked more often by specialist insects (*Acalymma* spp.).

The results from my master thesis opened many questions. I followed up on some of these during my PhD. I did choice-test experiments with *Diabrotica balteata* larvae that had to choose between roots containing cucurbitacins (ornamental variety) and roots that did not possess cucurbitacin (fruit consumption variety). I demonstrated that *Diabrotica balteata* larvae are significantly attracted to bitter roots. I had the opportunity to repeat the extraction of cucurbitacins with a standard for cucurbitacin B to calculate the real concentration instead of relative content. I could also improve the statistical analyses and wrote a paper on this study. This publication is presented here as the **first chapter** of my PhD thesis.

The hypothesis that the purpose of domestication can explain the differences in physical and chemical defense traits among plants selected for different purposes and the extent to which this has altered the interaction with the herbivores was not fully supported by my previous study. One reason may be that the phylogenetic signals overrides the domestication signal. Thus, this idea led me to further examine this idea by examining purpose of domestication and its relationship with plant defense and insect resistance within one species of squash. I selected a species, *Cucurbita*

argyrosperma with a known wild ancestor and related varieties selected for different purposes. With this design, we can really demonstrate that the differences between wild and domesticated plants or among purpose of domestication are due to domestication. Thus, the first goal of my PhD thesis was to investigate the consequences of *Cucurbita argyrosperma* domestication with wild populations that I collected in Mexico and different varieties from this species that were selected for two different purposes: fruit consumption and ornamental use.

5. Aims of the thesis

The aims of the present thesis are firstly, to investigate the changes on plant defensive traits (cucurbitacins and trichomes) in squash as a result of domestication. Secondly, to test whether these changes may affect interactions with insect herbivores and their natural enemies. Thirdly, to examine how domestication has influenced gene expression for cucurbitacins. Squash has been domesticated for different purposes such as, fruit consumption and as a result, there have been big changes in fruit size and a reduction of toxic compounds in many tissues, but mainly in the fruits. Squash has also been selected for an ornamental purpose, affecting the morphology of the fruits but not necessary the chemical defenses. Consequently, squash is an ideal model for my project. It allowed me to study all the consequences of crop domestication on plant insect interactions. Moreover, using squash as a plant model allowed me to test the hypothesis that the organs domesticated and the purpose of domestication may be the explanation for the inconsistency in the results reported on the effects of plant domestication on insect performance^{30,32,33}.

The first chapter of my thesis is as described above (see Background study section), combines results from my master's thesis and experiments conducted at the beginning of my PhD thesis. This chapter concentrates on the purpose of domestication and how it could explain the differences in chemical and physical defenses among domesticated squash and later on, the impact of those purposes of domestication on plant-insect interactions.

In the second chapter, I used only *Cucurbita argyrosperma* as a squash model. I characterized three wild populations of *Cucurbita argyrosperma* collected in Mexico: subsp. *sororia* (Bailey, 1867), the wild cucurbit subspecies of *Cucurbita argyrosperma*⁵⁹. The diversification of this squash species has led to varieties domesticated for fruit consumption and for ornamental purposes⁶⁰. Thus, I tested six cultivars of *C. argyrosperma*, selected for the consumption or not consumption of their fruits. I hypothesized that cucurbit varieties selected for fruit consumption lost chemical and physical defenses during human selection, but the varieties selected for an ornamental purpose should conserve the ancestral defenses. I assumed that generalist insects (as *Diabrotica balteata*, Fig. 5) should prefer and perform better on varieties selected for food rather than varieties selected for decoration. However, specialist insects (as *Acalymma vittatum*, Fig. 5) should, on the contrary, prefer wild cucurbits or varieties containing cucurbitacins. To test these hypotheses, I used a combination of field studies, as well as behavioral and chemical analyses.

In the third chapter, I hypothesized that the consequences of domestication of *C. argyrosperma* also had an effect on the herbivores' natural enemies. I used domesticated *C. argyrosperma* and wild relatives, *Diabrotica balteata* as the herbivore and *Atheta coriaria* (Figure 5) as the predator. From the literature, I knew that *Diabrotica* can sequester cucurbitacins and it has been hypothesized that it is for their own defense against enemies^{51,61,62}. The first important analysis was to test if this generalist herbivore contained cucurbitacins in its body. I then proceeded to perform several preference and survival tests with the rove beetle predator *Atheta coriaria*, testing the hypothesis that the predator will be repelled by the herbivore that previously ate wild squash containing cucurbitacins.

In the fourth chapter, I wanted to explore what happens at the gene level during domestication. I targeted genes involved in cucurbitacins biosynthetic pathways and found seven good candidates. I compared the expression of these candidate genes in wild and domesticated *C. argyrosperma* for the two different domestication purposes. I also compared gene expression among different plant tissues (roots, leaves, and cotyledons).

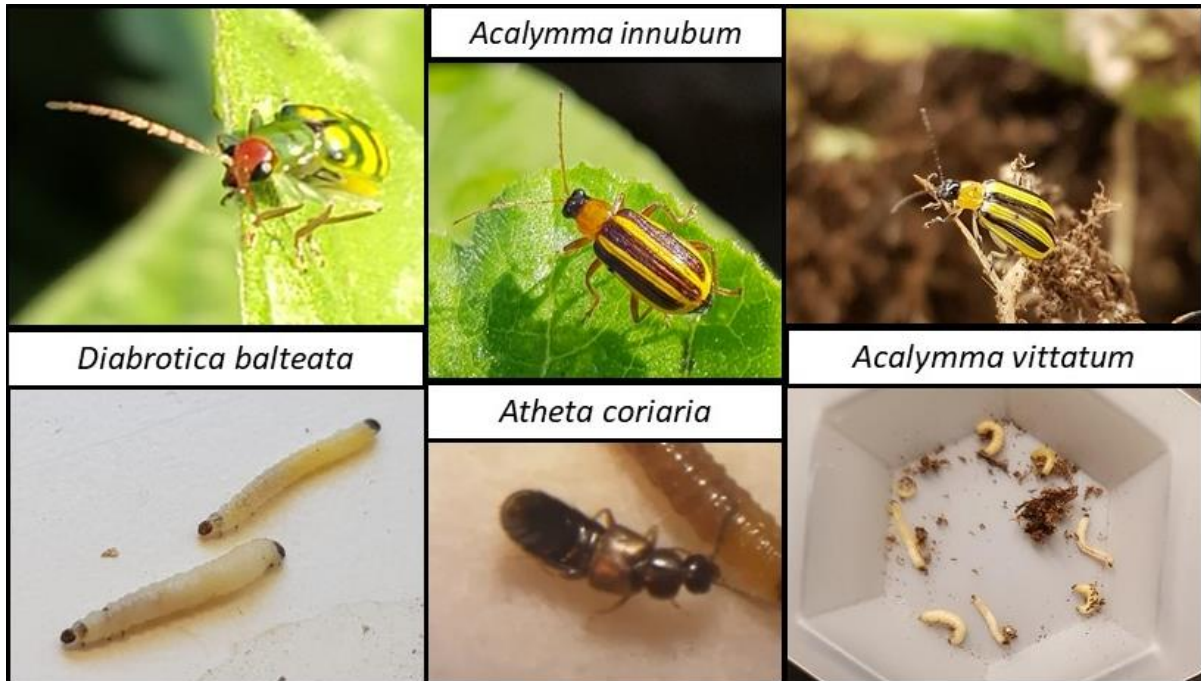


Figure 5. Insects used in the experiments, *Diabrotica balteata* (adults and larvae), *Acalymma innubum* (adults), *Atheta coriaria* (adults), *Acalymma vittatum* (adults and larvae). Pictures taken in the lab and the field

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Chapter 1: Squash varieties domesticated for different purposes differ in chemical and physical defense against leaf and root herbivores

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Abstract

Plant domestication often reduces plant defenses by selection on chemical and physical defense traits. Thus, it is expected that herbivorous insects perform better on crop varieties than on their wild relatives. However, recent studies show that this pattern is not ubiquitous. We examined how varieties of squash (*Cucurbita spp.*) domesticated for different purposes (for consumption or as ornamentals), differ in plant defense traits and in their interactions with a leaf and a root herbivore. Two types of defenses were measured: cucurbitacins, which are toxic phytochemicals, and trichomes that are physical barriers for most herbivores. We addressed the following questions: i) what is the variation in cucurbitacin content and leaf trichome density among varieties? ii) does purpose of domestication explain differences in defense traits among varieties? and iii) are herbivore feeding preferences and performance altered by the defense traits of squash varieties? We found great variation in cucurbitacin content among varieties, but not according to their purpose of domestication. Cucurbitacins were found mostly in cotyledons and roots and in very small quantities in the leaves. In contrast, trichome density was greater on the varieties selected for consumption than on the ornamental varieties. The performance of a leaf herbivore (*Spodoptera latifascia*) and a root herbivore (*Diabrotica balteata*), was not different among squash varieties. Moreover, in a choice experiment, larvae of the root herbivore preferred to feed on squash varieties with high cucurbitacin content. Whereas in the field, native leaf herbivores preferred to feed on varieties selected for consumption. Our results contribute to a better understanding on how varietal selection may differentially affect plant defenses. This knowledge could help in the development of crop varieties with enhanced insect resistance.

KEYWORDS: DOMESTICATION, SQUASH, PLANT DEFENSES, CUCURBITACINS, HERBIVORES

Introduction

Plant domestication is an evolutionary process whereby wild plants are modified to meet human needs^{1,2}. The suite of morphological and physiological traits that distinguish crops from their wild ancestors is known as the domestication syndrome^{3,4}. Domestication traits are common in many crop species and generally include those that facilitate cultivation and harvesting, as well as other desirable traits, such as an increase in fruit size, yield, number of seeds, or plant growth⁵⁻⁷.

A study comparing 203 crops from 68 families showed that in 66% of these crops, the most common trait altered by domestication was the plant's secondary chemistry (loss or reduction of toxic or bitter compounds), followed by changes in plant morphology (e.g. aerial vegetative part or fruit size)⁸. In recent years, there has been a surge in research examining how these domesticated traits have affected the interactions between crop plants and their associated herbivores. Overall, these studies reveal that often a decrease in chemical and physical plant defense results in increased insect performance⁹⁻¹¹. However, these patterns are not ubiquitous. For example, Turcotte, *et al.*¹² compared the performance of a leaf chewing herbivore (*Spodoptera exigua*) and a phloem-feeding aphid (*Myzus persicae*) on 29 pairs of crop plants and their wild relatives. They found that while domestication reduced the levels of morphological and chemical defenses in most of the examined crops, it did not always result in increased insect performance. In another study, Shlichta, *et al.*¹³ found that in lima bean, *Phaseolus lunatus*, domestication has reduced the content of cyanogenic glycosides in seeds but not in leaves. Accordingly, the performance of the seed beetle *Zabrotes subfasciatus*, was better on seeds of domesticated varieties than on seeds of wild plants, whereas the performance of the generalist *Spodoptera exigua*, was not affected by the levels of cyanogenic glycosides¹³. The authors argue that the trade-off between lower levels of defense and increased insect performance is supported for the seeds because this is the organ targeted by the domestication process.

Several other factors might be important to consider regarding the trade-off between decreased plant defense and increased insect performance. These include factors associated with the plant, the insect, or their interaction. For example, patterns of plant resistance in crops may vary across locations, degree of domestication and the organ or life stage attacked by the herbivore^{14,15}. Crop domestication can also have a strong impact on the plant's nutritional content¹⁵⁻¹⁷ and insects may preferentially select and perform better on domesticated crops because of their higher nutritional quality, which may not always correlate with a decrease in defense traits¹⁸. From the insect's side, feeding mode, host range and/or level of specialization are important factors that can also influence their performance^{8,19}.

One factor that has been neglected in studies that examine the relationship between plant domestication and resistance to herbivores is the crop's purpose of domestication. This refers to the particular use for which a crop or a specific variety were domesticated, for example, for human or livestock consumption, fiber, as ornamental or medicinal use, and various other purposes depending on the type of

crop. A large number of our food plants were originally selected for purposes other than food consumption⁶. For example, carrots were first domesticated for their seeds that were used as both spice and medicine²⁰ and saffron was used as a body paint, dye and perfume²¹. Meyer, *et al.*⁶ hypothesized that shifts in the primary used organs of a crop would cause changes in other organs, and further, that there would be a correlation between some organs used for food and nonfood uses of other organs. In such cases, changes in chemical and physical defenses in a crop compared to its wild ancestor may be the result of the original desire for selection and not the current use of that crop.

Squash, *Cucurbita* spp. represents a fascinating system to study the implications of domestication for ecological interactions²². With 21 taxa, the genus has experienced independent domestication events in five species: *C. pepo*, *C. ficifolia*, *C. moschata*, *C. maxima* and *C. argyrosperma*²³. Each domestication event occurred independently, sometimes on more than one occasion²². Each variety was selected for specific traits, defined by the nutritional and cultural needs of early human populations in America^{24,25}. Even though each species underwent a unique selection regime to meet local conditions and preferences, many domestication traits are common. One of the main traits altered with domestication is the content of a class of secondary metabolites called cucurbitacins. These molecules are oxygenated tetracyclic triterpenes, extremely bitter that render plants toxic or unpalatable to many invertebrates and vertebrate herbivores, including humans^{26,27}. However, cucurbitacins can also serve as feeding attractants for a number of specialized phytophagous beetles belonging to the Chrysomelidae family (tribe Luperini, Old World Aulacophora)^{28,29}. The second trait modified during domestication is the density of trichomes^{30,31}. Trichomes can develop on the surface of all plant organs and can be “glandular” or “non-glandular”. Non-glandular trichomes can serve as a physical defense against herbivores by interfering with their movement, causing physical entrapment or in some cases inflicting injury^{32,33}. Whereas glandular trichomes can synthesize and secrete toxic chemicals³⁴⁻³⁷. Indeed, high densities of foliar trichomes increase plant resistance against herbivores^{38,39}.

Although detailed studies exist on the history of domestication of *Cucurbita*^{22,31,40,41}, the extent to which squash varieties selected for different purposes differ in physical and chemical traits associated with defense and affect the interaction with herbivores, is not yet known. In this study we examined how variation in defense traits in several squash varieties affects the interaction with two generalist herbivores, and whether plant resistance against these herbivores can be explained by the variety’s purpose of domestication. To do this, we used several varieties of squash from the *Cucurbita* genus and investigated the relationship between levels of chemical (cucurbitacins) and physical defenses (trichomes), and the preference and performance of a leaf and a root herbivore. We addressed the following questions: i) what is the level of variation in cucurbitacin content and leaf trichome density among varieties? ii) does purpose of domestication (consumption vs. ornamental) explain differences in defense traits among varieties? and iii) are herbivores feeding preferences and performance altered by defense traits of squash varieties? Therefore,

we quantified the content of cucurbitacins in roots, cotyledons and leaves, and density of trichomes in leaves and cotyledons of several squash varieties. Then, we conducted performance bioassays with two generalist herbivores, a leaf-chewing caterpillar (*Spodoptera latifascia*) and a root-feeding beetle (*Diabrotica balteata*). As larvae of *D. balteata* can move freely in the soil where the adult female lays its eggs, we also conducted choice experiments to test whether beetle larvae showed differential preferences for squash varieties. Finally, we carried out a common garden experiment in Mexico, the place of origin of squash and quantified infestation and damage from native generalist and specialist herbivores.

Material and methods

Plants

We selected squash varieties across different species of *Cucurbita* within the same clade and with known wild ancestors²⁹ (Figure S1). This clade includes *Cucurbita* varieties of the species *pepo*, *maxima*, *ficifolia* and *argyrosperma*. Seeds were purchased from KCB-Samen (GmbH, Bottmingen, Switzerland) and Zollinger (biologische Samengartenerei, Les Evouettes, Switzerland). In total, we used nine varieties selected for two different purposes (Table 1). Four varieties were domesticated for consumption, either of their fruits (Chilacayote (Fcf), Silver Edge (Fbg), or their seeds, Seed oil pumpkin (Scpo) and Lady Godiva (Slg)) and three varieties domesticated for ornamental use (Atlantic Giant (Oag), Griemlins (Ocp) and Turkish Turban (Ott)). For the field experiment, two of the consumption varieties (Scpo and Slg) were replaced by two hybrid varieties, also used for consumption, collected in Mexico, Hsc and White Fall F1 (Hwf). For the laboratory experiments, seeds of each variety were individually germinated in 8-cm-diameter plastic pots filled with soil (Sinntal-Altengronau, Germany) mixed with 50% sand. Fifteen-day-old plants with cotyledons and two developed leaves were used for all experiments. Plants were grown under controlled conditions in a greenhouse ($24 \pm 5^\circ\text{C}$, 16:8h L: D) and watered every other day. For the experiments with *S. latifascia* on leaves and *D. balteata* on roots, all the afore-mentioned varieties were used. For the experiments with *S. latifascia* on cotyledons the variety Slg was not used due to low germination rate at the time of the experiment.

Species	Wild ancestor	Common name	Domestication purpose	Abbreviation
<i>Cucurbita argyrosperma</i>	<i>Cucurbita argyrosperma</i>	Silver edge ^{a,b,c,d,e,g}	Consumption	Fbg
<i>Cucurbita ficifolia</i>	<i>Cucurbita ficifolia</i>	Fig-leaf gourd (chilacayote) ^{a,b,c,d,e,f,g}	Consumption	Fcf
<i>Cucurbita pepo</i> var. <i>oleifera</i>	<i>Cucurbita pepo</i> ssp. <i>pepo</i>	Seed oil pumpkin ^{a,b,c,d,e}	Consumption	Scpo
<i>Cucurbita pepo</i>	<i>Cucurbita pepo</i> ssp. <i>pepo</i>	Lady Godiva ^{b,c,e}	Consumption	Slg
<i>Cucurbita maxima</i>	<i>Cucurbita maxima</i>	Atlantic Giant ^{a,b,c,d,e,g}	Ornamental	Oag
<i>Cucurbita maxima</i>	<i>Cucurbita maxima</i>	Turkish Turban ^{a,b,c,d,e,f,g}	Ornamental	Ott
<i>Cucurbita pepo</i>	<i>Cucurbita pepo</i> spp. <i>pepo</i>	Gremlins ^{a,b,c,d,e,g}	Ornamental	Ocp
<i>Cucurbita argyrosperma</i>	<i>Cucurbita argyrosperma</i> spp. <i>argyrosperma</i>	Calabacita [§]	Consumption (hybrid crossed with wild <i>C. argyrosperma</i>)	Hsc
<i>Cucurbita pepo</i>	<i>Cucurbita pepo</i> spp. <i>pepo</i>	White fall F1 [§]	Consumption (hybrid, <i>C. pepo</i> and <i>C. maxima</i>)	Hwf

^a included in the cucurbitacin quantification

^b included in the trichome analysis

^c included in the *Spodoptera* caterpillar performance on leaf

^d included in the *Spodoptera* caterpillar performance on cotyledon

^e included in the *Diabrotica* larvae performance

^f included in the *Diabrotica* larvae preference

[§] included in the common garden experiment

Insects

Spodoptera latifascia, Walker, 1856 (Lepidoptera: Noctuidae), commonly known as the velvet armyworm, is a polyphagous insect whose caterpillars have been reported to feed on, and cause economic damage to cowpea, soybean, cotton and many other crops^{42,43}. In our field site in Mexico, caterpillars are often found feeding on domesticated squash⁴⁴. Female moths lay their eggs in batches on the leaf surface and upon hatching, caterpillars feed gregariously before dispersing and individually feeding on the leaves throughout their later developmental stages. Colonies of *S. latifascia* were established in the lab in Switzerland from caterpillars collected mainly from wild bean or domesticated maize plants in a field site located at the experimental campus of the Universidad del Mar in Puerto Escondido, Oaxaca, Mexico (15°55'27.9"N 97°09'04.3"W). Caterpillars were reared on artificial diet ("beet armyworm diet", BioSery, Flemington, NJ, USA)⁴⁵.

The banded cucumber beetle, *Diabrotica balteata* LeConte, 1865 (Coleoptera; Chrysomelidae), originates from the tropical Americas^{46,47}. In this region it has been associated with wild and domesticated squash for thousands of years^{48,49}. This species is a pest of several agricultural crops including cucurbits, beans and sweet potatoes⁵⁰. Larvae feed on roots and tubers and adults eat leaves, cotyledons and flowers⁵⁰. Eggs of *D. balteata* were obtained from Syngenta (Stein, Switzerland). Upon hatching, larvae were reared on maize roots (hybrid DFI 45321, DSP, Delley, Switzerland) until second instar when they were used for experiments. Both insect rearings were kept under quarantine conditions at the University of Neuchatel, Switzerland (25°C ± 2°C, 16:8h L: D and 60% RH ± 5%).

Adult beetles of the genus *Acalymma* (Chrysomelidae: Galerucinae) frequently visited our squash plants during the field experiments in Mexico. The genus *Acalymma* mainly occurs in the New World^{51,52}, but it is mostly distributed in the tropical regions of Mexico⁵³. Some species are considered oligophagous (they feed on several species of Cucurbitaceae as adults and larvae)⁵⁴, and major pests of cucurbits. Adults feed on pollen, leaves and flowers and larvae are root feeders^{54,55}.

Plant defense measurements

Cucurbitacin content

We measured cucurbitacin content in the plant tissues that are readily eaten by the two herbivore species. Leaves, roots and cotyledons of two-week-old *Cucurbita spp.* were ground separately into powder in liquid nitrogen. We weighed 100 mg of powder on a microbalance to the nearest 0.1 mg (Mettler Toledo XP6, Columbus, Ohio, USA), mixed it with 1 ml of methanol (99.999%)⁵⁶ and added five glass beads in a 1.5 mL Eppendorf tube. Samples were placed in Retsch tissue lyser (Quiagen, Hilden, Germany) at 30 Hz for 4 minutes, then centrifuged during 5 minutes at 14000 rpm, and 700 μ l of supernatant were removed and diluted with 300 μ l of water. The cucurbitacin analyses were performed by UHPLC-QTOFMS using an Acquity UPLCTM coupled to Synapt G2 high-resolution mass spectrometer (Waters, Milford, USA) and an Acquity UPLC BEH C18 1.7 μ m, 2.1 x 50mm (Waters) column. Two mobile phases were used in gradient mode; water plus 0.05% of formic acid and acetonitrile plus 0.05% of formic acid. The injection volume in the machine was 2.5 μ l. Detection was performed in electrospray negative ionization (ESI) using the MS^E acquisition mode (data-independent acquisition alternating between low and high collision energies). The source parameters were capillary voltage -2.0 kV, cone voltage -25 V, source temperature 120 °C, desolvation gas flow and temperature 800 l/h and 350 °C respectively, cone gas flow 20 l/h. The exact mass measurements (< 2 ppm) were ensured by infusing a 500 ng/ml solution of leucine-enkephalin at 15 μ l/min through the LocksprayTM probe. For the acquisition and data processing we used the software MasslynxTM v.4.1 (Waters). Cucurbitacins were identified based on their molecular formula and fragmentation patterns provided by mass measurements. Peaks corresponding to known cucurbitacins were automatically integrated using QuanlynxTM with a 0.1 min chromatographic window centered on the retention time of each component and a 0.02 Da mass window centered on the (M+HCOO) ion.

Quantification of all cucurbitacins was done either in a relative manner or by external calibration using cucurbitacin B as standard. The cucurbitacin concentration was expressed in μ g per g of plant material. In a first step, cucurbitacins were expressed as relative content as we did not have the cucurbitacin B standard (Figure S2). In a second step, we could calculate absolute cucurbitacins concentration using the standard. However, the hybrids seed from Mexico (Hsc, Hwf) and one variety selected for the consumption (Slg) were not available anymore.

Trichomes

A preliminary analysis revealed a significant positive correlation between trichomes present on adaxial and abaxial surfaces of the leaf. We only counted the number of trichomes on the adaxial side of the leaves and cotyledons for all squash varieties using a stereoscopic microscope. For this, 1.14 cm² circles were punched from the middle of the cotyledon and second leaf, at the base of the main vein. We counted the trichomes longer than 1.5mm from seven plants of each variety.

Performance of S. latifascia caterpillars on leaves and cotyledons of squash varieties

To test the effect of plant variety and domestication purpose on insect performance, we measured the relative growth rate of *S. latifascia* caterpillars feeding on leaves and cotyledons of different squash varieties. For the experiment on leaves, individual second instar caterpillars were weighed and randomly placed on adaxial side of the third leaf of each plant, using a 1.13 cm² clip cage (homemade). This method has been used in other studies to maintain caterpillars on a single leaf^{13,57}. For the experiment on cotyledons, we use larger clip cages (4.52 cm²) adapted to the size of the cotyledons, then we placed two second instar caterpillars, previously weighed, inside each clip cage (BioQuip product, USA) on one random cotyledon. Therefore, we divided the weight by two to have a mean weight per caterpillar. After two days on leaves or cotyledons, the caterpillars were weighed again. Mean relative growth rate (RGR) was calculated as, $RGR = (\ln(\text{final weight}) - \ln(\text{initial weight})) / \text{number of days}$ ^{58,59}. These experiments were done once with ten replicates per variety. Dead larvae were removed from the analysis.

Performance of D. balteata larvae on roots of squash varieties

We conducted an experiment to test the effects of squash varieties and domestication purpose on the performance of larvae of *D. balteata*. Five second instars were randomly selected and placed on the roots of 21-day-old squash plants that were placed inside a small plastic bag. In order to calculate the initial weight, the five larvae were weighed together before the experiment and the total weight was divided by five to obtain an estimate of the individual mean weight. As larvae feed in the soil and their development cannot be monitored, they were again weighed only at the end of the experiment. After six days, larvae were collected and the final weight was calculated by weighing all the larvae together for each plant and dividing the total weight by the number of larvae recovered from each plastic bag. Plants were watered with 20 ml of tap water one time in the middle of the bioassay. The number of replicates for each variety was dependent on the availability of plants. This experiment was repeated three times with a total of 13 replicates for Fbg, 14 for Fcf, 10 for Scpo, 14 for Slg, 4 for Oag, 15 for Ocp and 13 for Ott. Larval RGR was calculated as described above.

Choice-experiment with D. balteata on roots of squash varieties

The purpose of this experiment was to test the feeding preference of *D. balteata* larvae when offered squash varieties with different cucurbitacin content. We used five varieties with contrasting cucurbitacin content in the roots: Ott and Scpo with high content and Fcf, Fbg and Ocp with low or no cucurbitacins. We conducted pairwise comparisons (Fcf x Ott, Fbg x Ott and Ocp x Scpo), each time with one variety containing none or low content of cucurbitacins and a second variety with high cucurbitacin content. To avoid any bias in the choice of larvae for a particular variety, newly hatched larvae were reared on maize roots until second instar (approximately 10 days). Larvae were starved for 12h before the experiments. We used squared Petri dishes (12cmx12cm, Sarstedt, Germany) with moistened filter paper and a wet cotton ball. We pierced two holes in one side of the Petri dish to place the roots of the two tested varieties. The Petri dishes were sealed with parafilm. Five *D. balteata* larvae were released in the middle through a hole in the lid. Petri dishes were covered with red cellophane paper to decrease the amount of light, which could disturb larval behavior (Petri dishes, Figure S3). Larval choice, defined by the larval position on the roots was recorded every 10 minutes during the first hour, then every hour for 6 hours and their final choice after 24h. We performed 30 replicates for the combination Fcf x Ott, 10 replicates for the combination Fbg x Ott and 10 replicates for the combination Ocp x Scpo.

Herbivore infestation and plant damage of squash varieties in the field

We conducted a common garden experiment to examine the susceptibility of the different squash varieties to natural infestation by herbivores. The field was located in the coastal area of the state of Oaxaca, Mexico, 15 km northwest of Puerto Escondido in the UMAR (Universidad del Mar) experimental campus (15°55.596'N, 97°9.118'W)^{45,60}. The experimental field area is mainly used for cultivation of maize, and it is surrounded by native vegetation including wild cucurbits and wild lima bean plants⁴⁵. The experiment was performed during the dry season, under a tropical climate from 9 December 2016 to 30 January 2017. At the time of our experiment, the field site was also used for a parallel experiment conducted with bean plants⁴⁵. In this site we could find natural populations of herbivorous insects that feed on squash including Chrysomelidae beetles from the subtribe Diabroticina (*Acalymma* spp. and *Diabrotica balteata*) and lepidopterans (*Spodoptera latifascia* and *Diaphania* spp.)^{45,61,62}.

For this experiment, the varieties Scpo and Slg used in lab experiments did not grow well and were replaced by one Mexican hybrid (local variety crossed with a wild squash of *C. argyrosperma*, Hsc) and a hybrid between two species (domesticated *C. pepo* crossed with domesticated *C. maxima*, Hwf). These two varieties are commonly grown and used for their fruits by locals in the region.

Plants were grown in biodegradable pots (4 cm of diameter) with local soil. Pots were placed inside field tents (Lumite® 2 x 2 m, Bioquip) for two weeks to protect plants from insect's attack. In total, seven varieties (fruit: Fbg, Fcf, ornamental: Oag, Ott, Ocp

and hybrids: Hwf, Hsc; see Table 1) were used for this experiment. Two-week old plants were transplanted directly into the ground, in a common garden setting and randomly distributed among six rows separated by 1 meter. Each row contained one plant of each variety with six plants per variety, 35 plants in total (Figure S4). The number and identity of insects on each plant and the damage level were recorded every 3-4 days. Plant damage was visually estimated in percentage of total surface damage (eaten/removed) for each plant. This experiment lasted 6 weeks until most plants (80%) that survived had produced fruits.

At the end of this period, end of January, very few herbivores remain in the field. Because many Diabroticina species look very similar, adult beetle samples were collected and kept in a solution of 95% EtOH for identification. A DNA barcoding was performed with two legs of five similar adult beetles and one extract from larvae collected on 30 January 2017 at the field site to confirm the identity of the insects. DNA was extracted with a “NucleoSpin Tissue” kit (Macherey-Nagel, Switzerland) following the manufacturer’s protocol. The 658 base-pair-long barcoding fragment of the mitochondrial gene Cytochrome Oxidase I was amplified using the primers LepF and LepR⁶³. PCR products were purified enzymatically using a mix of the enzymes Exonuclease and FastAP Thermosensitive Alkaline Phosphatase (Fermentas, MA, USA). Sequencing was performed bidirectionally by an external company (Microsynth AG, Switzerland) with the primers used for the PCR. Chromatograms were edited and assembled with GeneiousR⁶⁴. The consensus sequences were submitted to Genbank (Genbank accession numbers MT773619 to MT773624).

Statistical analyses

We used the statistical software R (ver. 4.02.2, the R foundation for statistical computing, Vienna, Austria). The amount of cucurbitacins was log transformed before the analysis. The variation in cucurbitacin relative content and concentration in plant parts and the number of trichomes among cucurbit varieties were analyzed with a linear model. Differences for RGR of leaf caterpillars and root beetle larvae were analyzed with linear mixed models with the domestication purpose as fixed factor and plant variety as a random factor (*lme4* package, ver. 0.99999-0). Replicates from the three experiments with *D. balteata* were pooled. Larval mortality was analyzed with a linear model. To test the effect of purpose of domestication, plant defense (cucurbitacins concentration and trichome density) and insect performance were analyzed with linear mixed models with plant variety as a random factor.

Results from the choice experiment with *D. balteata* were analyzed first for the whole experiment with a generalized linear model (*glm*) with time as a factor for quasibinomial data. Then for each timepoint, we constructed a *glm* for quasibinomial data and performed a Chi-squared test.

For the common garden experiment, although other herbivore species were frequently observed foraging and feeding on plants in the adjacent plots of beans and maize (including *D. balteata*, *S. latifascia* and unidentified Chrysomelidae species), 95% of

the insects recorded visiting and feeding on the squash varieties were adults of *Acalymma* spp. Therefore, analyses were performed only with these insect species.

We tested the difference in number of insects and leaf damage among squash varieties with time point as fixed effect and the ID of the plant as random factor, to account for the repeated measurement structure. As the number of insects was not normally distributed, we used a negative binomial distribution. To test the effect of purpose of domestication on the insect abundance, we used a generalized linear mixed model (*glmer*) included 'purpose', 'week' and the interactions between 'purpose' and 'week', varieties, as fixed factors, replicate and time as random factors. FDR-corrected post-hoc tests were carried out in order to check differences among treatments. Finally, to test whether plant damage was associated with *Acalymma* beetles, using a linear mixed model, we performed a regression between amount of damage per plant and the number of *Acalymma* adults.

Results

Cucurbitacin content in the different squash varieties and plant tissues

The type of cucurbitacins found in the squash varieties were identified as putative cucurbitacins B. Cucurbitacins were present in leaves, cotyledons and roots of squash varieties. The concentration in the different plant tissues followed the same trend across varieties; the highest concentration was found in the cotyledons, followed by the roots and a very low concentration was found in leaves (Fig. 1). In leaves, we did not find any significant differences in cucurbitacin content among varieties ($F_{5,11} = 2.259$, $P = 0.121$; Fig. 1A), nor among domestication purposes ($F_{1,4} = 2.733$, $P = 0.173$). Differences were, however, significant among varieties in cotyledons ($F_{5,12} = 116.4$, $P < 0.0001$; Fig. 1B) and roots ($F_{5,11} = 7.742$, $P = 0.002$; Fig. 1C), but not according to the purpose of domestication (cotyledons: $F_{1,4} = 1.866$, $P = 0.243$ and roots: $F_{1,4} = 0.896$, $P = 0.397$). The highest cucurbitacin content in the cotyledons was found in Fcf, and the lowest in Ocp (Fig. 1B). The highest concentration in the roots was found in Ott, and the lowest in Fcf (Fig. 1C). For the Slg and hybrid varieties, we only measured relative contents of cucurbitacins (Figure S2) and not absolute concentrations, as these varieties were not available at the time of the second experiment with the cucurbitacin standard. The relative content of cucurbitacins in cotyledons and roots of the Slg variety was significantly different than Scpo, the other variety selected for seed consumption (Figure S2), but similar to the content in the Ott, Fcf and Hwf varieties. Hybrid fruit varieties (Hsc and Hwf) had similar cucurbitacin content relative to the other fruit consumption (Figure S2).

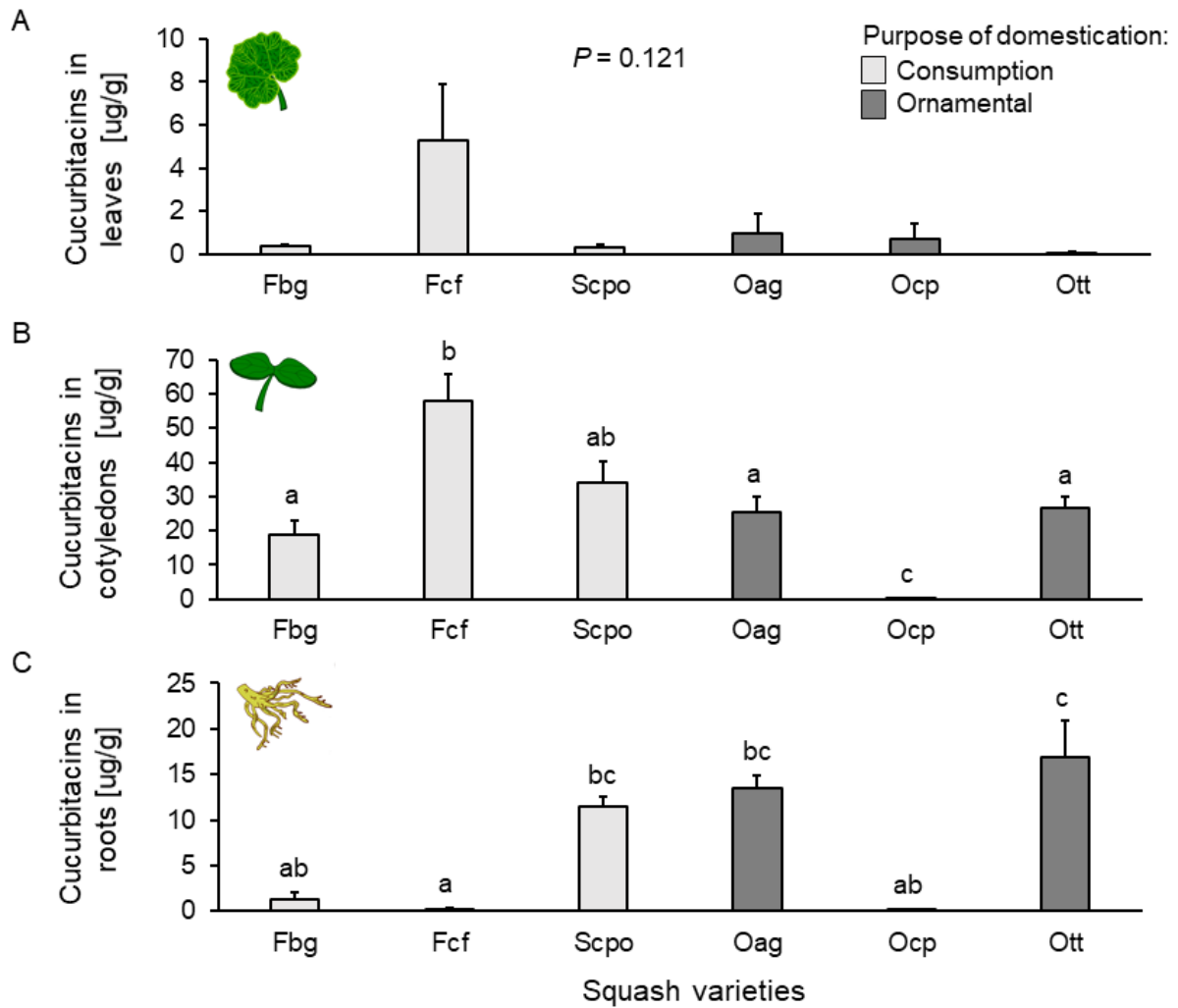


Figure 1. Cucurbitacin concentration in **A** leaves (n=3), **B** cotyledons (n=3) and **C** roots (n=3) of the studied *Cucurbita* spp. varieties. The bars represent means (\pm SE). Light-colored bars are varieties domesticated for consumption and dark-colored bars are varieties domesticated for an ornamental purpose. P values are given for treatment comparisons with log transformed data [linear model], followed by pairwise comparisons of Least Squares Means (LS means).

Number of trichomes

The foliar trichomes identified on all of the tested varieties were simple and non-glandular (Figure S5). No trichomes were found on cotyledons. Leaf trichome density on the adaxial side of the leaf was significantly different among cucurbit varieties ($F_{6,40}=4.379$, $P=0.001$; Fig. 2) and between the two categories of purpose of domestication. Varieties selected for ornamental use had fewer trichomes than varieties selected for consumption ($\chi^2_{(1)}=5.709$, $P=0.01$). The highest trichome density was found in Scpo (variety domesticated for consumption) and the lowest in the ornamental varieties (Oag and Ott).

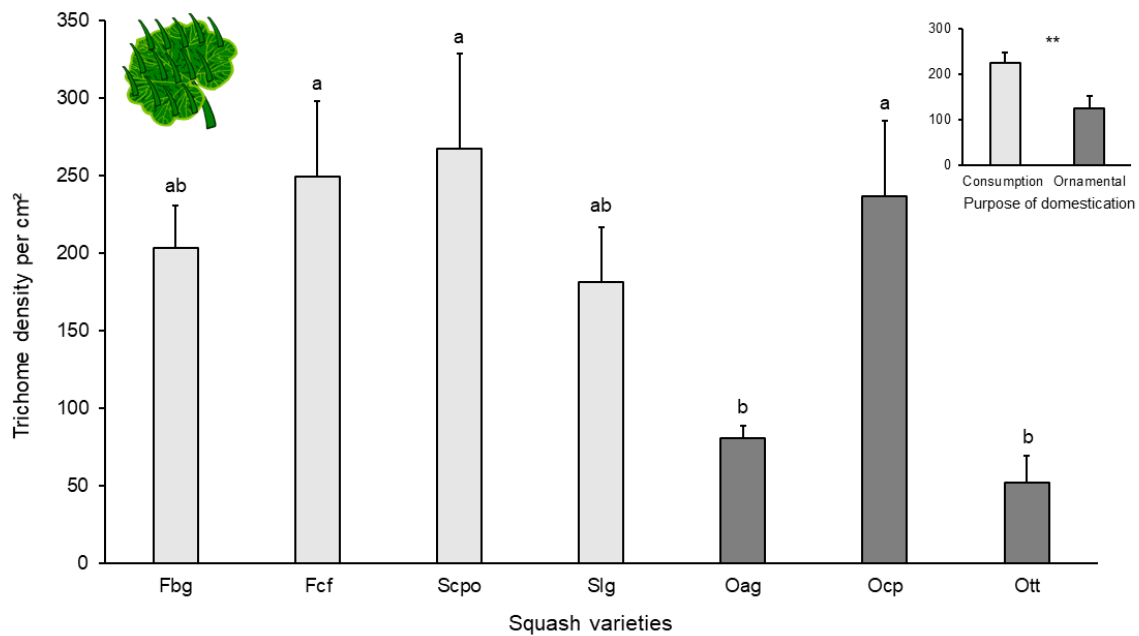


Figure 2. Number of trichomes per cm² on adaxial side of the leaves from different squash varieties (n= 7). Bars represent means (\pm SE). Light-colored bars are varieties domesticated for consumption and dark-colored bars are varieties domesticated for an ornamental purpose. P values are given for treatment comparisons with log transformed data [linear model], followed by pairwise comparisons of Least Squares Means (LS means). Upper graph is number of trichomes per purpose of domestication (consumption or ornamental). P values are given for treatment comparison with *glmer* model with varieties as random factor.

Performance of S. latifascia caterpillars on leaves and cotyledons of squash varieties

Larval survival at the end of the experiment was almost 100%, only 2 larvae died on the Ocp variety and one on Ott. We did not find significant differences among squash varieties in the RGR of *S. latifascia* caterpillars fed on leaves ($F_{6,57} = 1.298$, $P = 0.268$; Fig. 3A). However, when varieties were grouped by purpose of domestication, the RGR was significantly different, with the higher RGR on the ornamental varieties ($F_{1,57} = 5.787$, $P = 0.01$, Fig 3A). Larval survival on cotyledons at the end of the experiment was 100% on all varieties. The RGR on the cotyledons was significantly affected by the plant variety ($F_{5,54} = 3.015$, $P = 0.018$; Fig 3B). Caterpillar growth was higher on Oag compared to the other varieties, but the difference was only significant when compared with Fcf ($P = 0.005$; Fig. 3B). Finally, no significant effect on the RGR of the caterpillars was found when varieties were grouped according to their domestication purpose ($F_{1,60} = 4.328$, $P = 0.106$), likely due to the great variation between varieties of the same group.

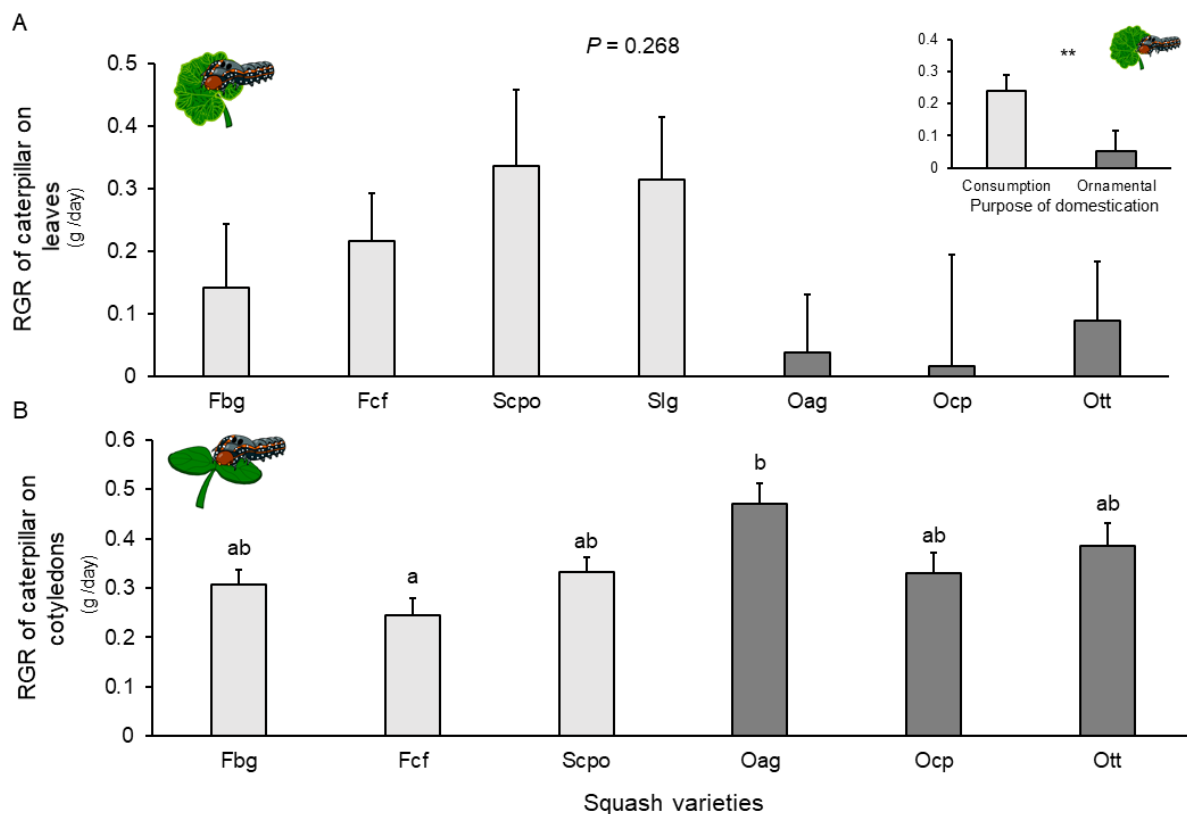


Figure 3. Relative growth rate (RGR) of *Spodoptera latifascia* caterpillars (A) on leaves (n=10) and (B) cotyledons (n=10) of squash varieties (*Cucurbita* spp.). Bars represent means (\pm SE). Light-colored bars are varieties domesticated for consumption and dark-colored bars are varieties domesticated for an ornamental purpose. RGR was calculated for each individual caterpillar as the weight difference between initial and final weight after 48 hours of feeding. P values are given for treatment [linear mixed model], followed by pairwise comparisons of Least Squares Means (LS means). Upper graph is RGR of caterpillars on leaves per purpose of domestication (consumption or ornamental). P values are given for treatment comparison with glmer model with varieties as random factor.

Performance of *D. balteata* on roots of squash varieties with different cucurbitacin content

Diabrotica balteata larvae did not survive on the Oag variety (which had fewer replicates) and was not included in the analysis. For all other varieties, larval mortality at the end of the experiment varied between 40 to 60% with no significant differences among varieties ($F_{6,76} = 0.774$, $P = 0.11$). These percentages reflect the number of larvae recovered from the soil at the end of the experiment and it is possible that mortality was overestimated. Notably, for the analysis of RGR only live larvae were used and all replicates had surviving larvae. No significant differences were found in the RGR of *D. balteata* larvae fed on roots of the different squash varieties ($F_{6,58} = 1.824$, $P = 0.11$; Fig. 4), nor according to their domestication purpose ($F_{1,6} = 0.24$, $P = 0.64$).

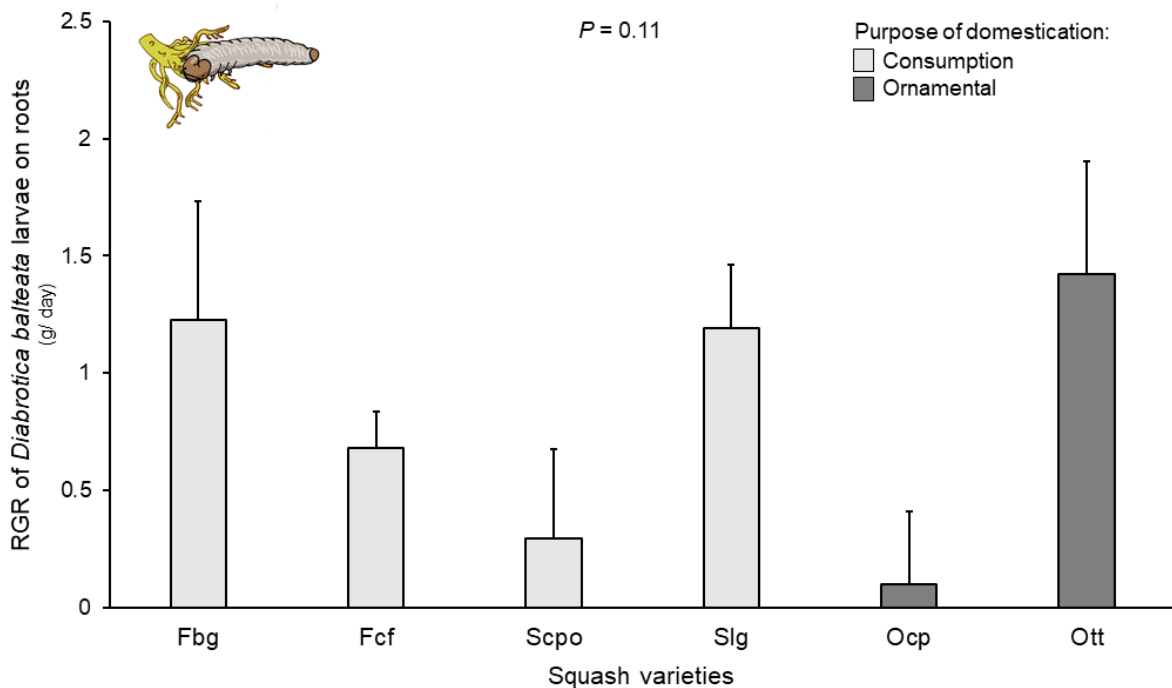


Figure 4. Relative growth rate (RGR) of *Diabrotica balteata* larvae on roots of squash varieties (*Cucurbita* spp.) $n=10-15$. Bars represent means (\pm SE). Light-colored bars are varieties domesticated for consumption and dark-colored bars are varieties domesticated for an ornamental purpose. RGR was calculated for groups of larvae as the weight difference between initial and final weight (\ln) after 6 days of feeding. P values are given for treatment [linear mixed model].

Preference of D. balteata on roots of squash varieties with different cucurbitacin content

Consistently, significantly more larvae were observed on roots of varieties containing high cucurbitacin content over varieties with low or no cucurbitacins (Fig 5A ($\chi^2_{(1)}= 47.87$ $P<0.0001$), Fig 5B ($\chi^2_{(1)}= 39.46$, $P<0.0001$), Fig 5C ($\chi^2_{(1)}= 16.42$, $P<0.001$). Larval choice was significantly different already after one-hour of the start of the experiment in the first two comparisons and highly significant after 24 hours in all three comparisons.

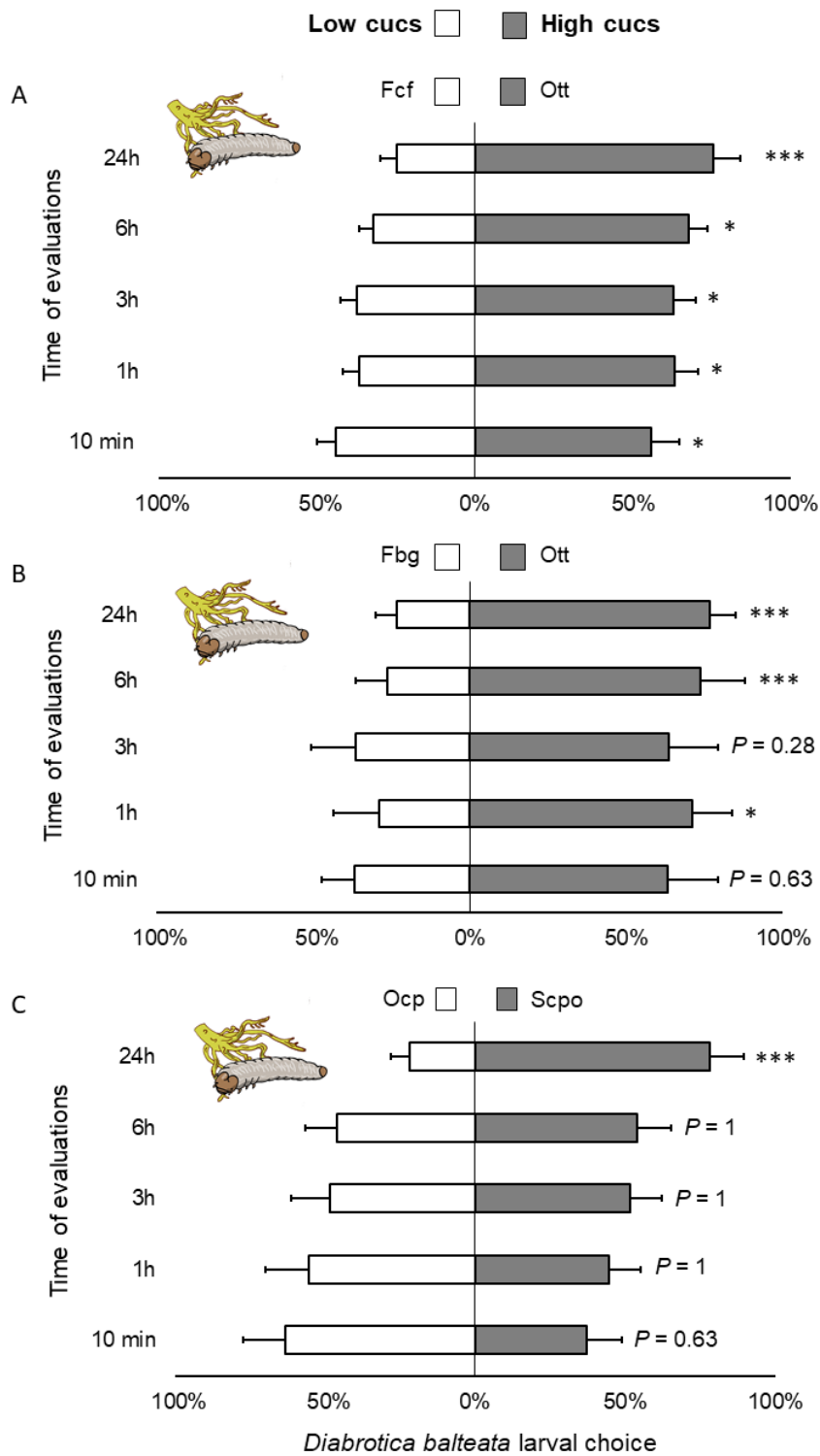


Figure 5. Feeding preference of *Diabrotica balteata* larvae on roots of domesticated varieties of squash with high cucurbitacin content. (A to C) Five larvae of *D. balteata* were allowed to choose between two varieties with low and high levels of cucurbitacins. Larval choice was recorded as number of larvae on roots after 10 minutes, 1, 3, 6 and 24 hours after the start of the experiment. Sample sizes: A, n = 30, B and C, n = 10. Bars represent means (\pm SE) express the percentage of larvae that chose a treatment per time point (x axis). Bonferroni corrected P values are given for treatment comparisons [generalized linear model (family, Binomial)], followed by pairwise comparisons of Least Squares Means (LS means). * P < 0.05, ** P < 0.01, *** P < 0.001.

Herbivore infestation and plant damage of squash varieties in a common garden

Although *D. balteata* was abundant at the field site, it was mainly observed on bean and maize plants adjacent to the squash common garden. Approximately, 95% of the insects that were surveyed visiting and feeding on the squash plants belonged to the genus *Acalymma* spp., which was confirmed by the later sequencing and identification in the lab. Blast searches on GenBank found matches (more than 99% similarity) for *Acalymma blomorum* and *A. innubum*. As the accurate identification of these two species was not possible in the field, for the analysis on abundance we pooled both *Acalymma* species. The number of insects on the plants was different depending on the purpose of domestication ($\chi^2_{(1)}= 7.462$, $P= 0.006$, Fig.6A). Overall, the varieties selected for consumption attracted more *Acalymma* beetles than ornamental varieties, especially after the fourth week ($\chi^2_{(1)}= 15.752$, $P<0.001$, Fig.6A). The variation in *Acalymma* beetle abundance in the common garden was explained by the time ($\chi^2_{(4)}= 204.41$, $P<0.001$, Fig.6A). The population of *Acalymma* spp. peaked in the middle of the season and decreased towards the end.

Leaf damage was significantly different among varieties ($\chi^2_{(5)}= 81.424$, $P<0.001$, Fig.S6B), and only marginally different for purpose of domestication ($\chi^2_{(1)}= 2.71$, $P=0.09$, Fig.6B). However, the analysis revealed some trends; the ornamental varieties suffered the highest herbivore damage and overall, the lowest damage was on the hybrid Mexican varieties suggesting some degree of resistance.

Leaf damage also increased through time ($\chi^2_{(5)}= 81.424$, $P<0.001$, Fig. 6B). After the third week, the percentage of damage was higher for the variety Ott compared to the hybrid varieties Hsc and Hwf ($P= 0.0005$, Fig S6B), but also compared to one fruit variety (Fbg). In the last week of the field census, only the fruit variety (Fbg) and the hybrid variety (Hsc) suffered less than 20% of leaf damage. Plant damage was not correlated with the abundance of *Acalymma* beetles ($\chi^2_{(1)}= 0.193$, $P= 0.660$).

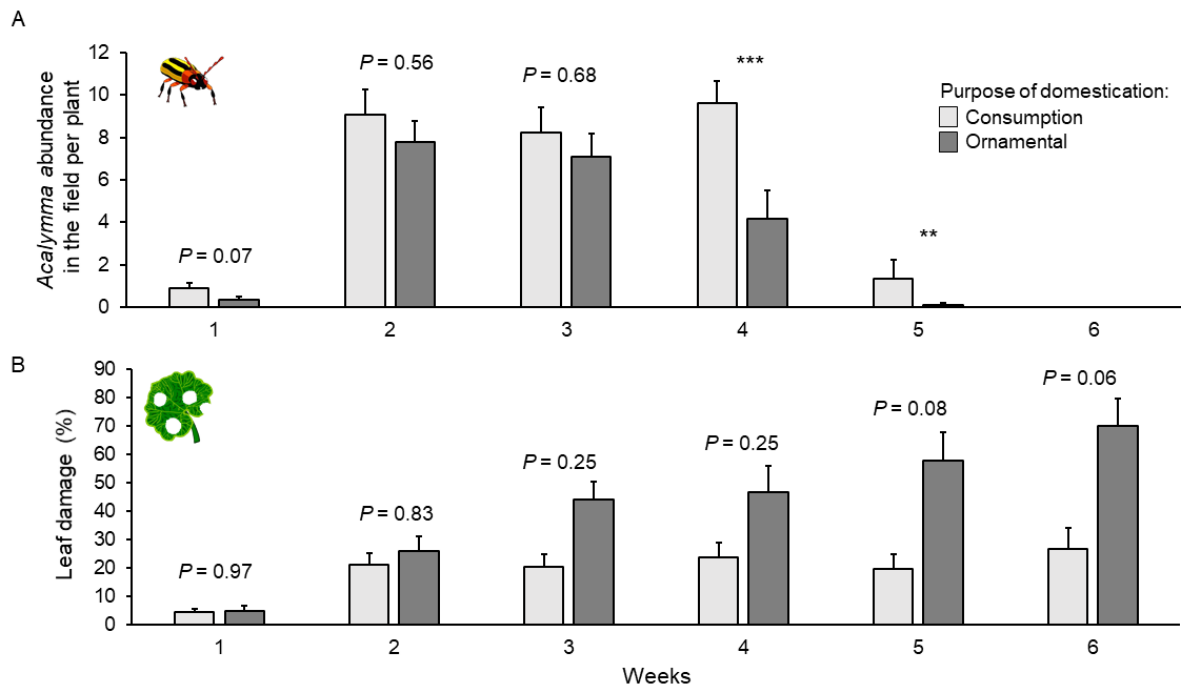


Figure 6. Mean number of *Acalymma* spp. beetles per plant (A) and percent of leaf damage (B) on squash varieties domesticated for different purposes in a common garden field experiment in Puerto Escondido, Oaxaca, Mexico. Data were recorded once per week for 6 weeks. Bars represent means (\pm SE). Light-colored bars are varieties domesticated for consumption and dark-colored bars are varieties domesticated for an ornamental purpose. P values are given for treatment [linear mixed model] per week and followed by pairwise comparisons of Least Squares Means (LS means).

Discussion

We found great variation in cucurbitacin content and leaf trichome density across all squash varieties. Overall, we did not find support for our hypothesis on reduced defenses in consumption varieties. Our results also show that herbivore performance is not negatively affected by high cucurbitacin content nor by trichome density. Instead, larvae of the root herbivore, *D. balteata* preferred to feed on varieties with high levels of cucurbitacins.

The content of cucurbitacins in leaves for all varieties was very low, and relatively high in cotyledons and roots as has been shown in other studies⁶⁵⁻⁶⁸. The highest content was in cotyledons. Greater levels of chemical defense in young tissues are often found in plants^{69,70}. If cotyledons, the first photosynthetic organ, are damaged, the consequences for plant development will be more detrimental than if older organs are damaged^{70,71}. For example, glucosinolates in *Arabidopsis thaliana* vary among plant tissues and the highest concentrations are found in seedlings⁷². Similarly, in lima bean (*Phaseolus lunatus*), cyanogenic glycosides are found in higher quantity in cotyledons than in other plant vegetative tissues⁷³.

The differences in defense traits, cucurbitacins vs. trichomes, between tissues are in line with plant defense theories that predict a trade-off in resource investment for chemical and physical defenses^{74,75}. Similar trade-offs in plant tissues have been found in several wild populations of *C. argyrosperma*, in which roots and cotyledons have a high content of cucurbitacins, while leaves have high trichome densities and low levels of cucurbitacins (Jaccard *et al.* unpublished data). This suggests that although domestication reduced plant defense in squash^{31,41,65,68}, it did not alter the trade-off between physical and chemical defense in different plant tissues. The different concentration of cucurbitacins and trichome density in cotyledons and leaves could indicate that plants switch defense strategies during their ontogeny towards the production of trichomes in leaves^{76,77}. Brzozowski, *et al.*⁷⁸ found that, in one variety of *Cucurbita pepo*, there is a peak of cucurbitacin content in seedlings very early in the development, starting at the radical emergence, but cucurbitacins diminished once cotyledons were fully expanded. However, in a different variety of the same species containing less cucurbitacins, they did not detect changes in cucurbitacin levels throughout plant development. Thus, it would be interesting to further examine changes in defense traits throughout plant ontogeny for different species and domesticated varieties of squash.

Contrary to our expectations, we did not find lower levels of cucurbitacins or fewer trichomes in varieties domesticated for consumption compared to ornamental varieties. Several factors may explain these findings. First, other defense traits not considered in this study could have been reduced or selected during the domestication process. Alternatively, as fruits are the main edible part of the squash plant, it is possible that levels of cucurbitacins in leaves, cotyledons and roots do not reflect the direct target of domestication. In retrospective, it would have been informative to examine cucurbitacin levels in fruits and seeds. But this was not initially considered as we only examined the tissues eaten by the studied insects. However, because of the

extreme bitterness of cucurbitacins, fruits of consumed cucurbit plants usually are void of these compounds. This is due to the original selection of non-bitter plants for food use by our ancestors and later by new breeders^{79,80}. Yet, further studies should examine the levels of cucurbitacins present in the seeds and their effect on seed feeding insects.

Secondly, we found great variation in trichome density and cucurbitacin content among varieties domesticated for the same purpose, particularly among ornamental varieties and this variation may override differences resulting from the domestication purposes. Finally, the varieties used in our study belong to different *Cucurbita* species and it could be that the differences caused by speciation are higher than the putative effect of purpose of domestication. Ideally, to control for a phylogenetic signal, we should evaluate varieties of the same squash species. However, in this study we chose to use different species from the same clade that allowed us to test various uses of squash, which are not readily available within a single species. In the future, we expect to elucidate more questions regarding this aspect as we are currently exploring the relationship between purpose of domestication and plant resistance against herbivores with varieties of the species *C. argyrosperma* that have the same wild ancestor (Jaccard *et al.* unpublished data).

Changes in plant defense as a result of domestication are expected to alter the interaction with the plant's natural enemies^{81,82}. Accordingly, we expected the performance of the two generalist herbivores to be lower on varieties with higher trichome densities and cucurbitacin levels. Cucurbitacins are among the bitterest plant secondary compounds found in plants^{83,84} and their roles are diverse⁸⁵, including protection against plant pathogens^{18,86} and deterrence of generalist insects and other arthropods¹⁸. But they can also act as attractants and feeding stimulants for specialist insects, like *Acalymma*⁸⁶⁻⁸⁸. Interestingly, although *S. latifascia* and *D. balteata* are known to be very polyphagous insects^{42,89}, the content of cucurbitacins in cotyledons and roots did not affect larval performance. Moreover, choice experiments with *D. balteata* revealed that consistently, larvae preferred to feed on roots with high cucurbitacin content, which suggests that this species is adapted to these compounds. A likely explanation may lie in the common origin of genus *Cucurbita* and *D. balteata*, Mexico, where both plants and insects have been interacting for thousands of years probably well before the domestication of squash^{83,90}. Also in this region, squash is commonly grown in traditional mixed cropping with maize and beans (both crops readily consumed by *D. balteata* larvae). Thus, it is possible that during the generalist's long-time association with squash it has evolved to tolerate specific secondary defenses leading to an adaptation to the plant without being specialized on it. In addition, *D. balteata* belongs to the subtribe Diabroticina, which includes the specialist *Acalymma*, thus the preference towards cucurbitacins could be a shared trait among the Diabroticina. Because they can readily tolerate cucurbitacins the plants may provide a nutritional niche for the larvae, without much competition.

We also consider it possible that the larval feeding choices are the consequence of physiological adaptations in the adult *Diabrotica* beetles, which are known to be

attracted to and feed on *Cucurbita* plants. Yet, it is not excluded that feeding on plants with cucurbitacins provides benefits that we have not yet uncovered.

Caterpillars of *S. latifascia* grew better on the consumption varieties, despite the higher density of trichomes. One reason may be the size and form of these trichomes, or the larval instar used in our study. Indeed, non-glandular trichomes only physically impede the movement of small arthropod herbivores on the plant surface^{91,92}. Moreover, like most caterpillars of the genus *Spodoptera*, *S. latifascia* have strong mandibles⁹³ which likely facilitated the chewing of the hairy leaves. Alternatively, selection on these varieties might have increased the nutritional quality of the leaves. This hypothesis remains to be tested.

Due to the low germination rate of the seeds in the field, the number of varieties and of plants of each variety that comprised the common garden experiment was small. Therefore, the conclusions that we can draw from this experiment are limited. Nevertheless, this experiment yielded interesting results. Overall, the ornamental varieties suffered the highest herbivore damage, particularly the variety Ott that did not grow well. However, we believe that these results do not necessarily reflect that insects preferred these plants. Rather, their poor growth might have compromised their ability to resist herbivore damage. In contrast, the Mexican hybrid varieties grew well and were less damaged by the natural herbivore populations, suggesting some degree of resistance, same for one variety selected for the fruit consumption (Fbg). One interesting and unexpected result was the low number of *D. balteata* adults that visited the plants, despite their high numbers in the field site. Instead, they preferred other crops in the site, such as beans, peanuts and maize. Individuals of *Acalymma* spp. on the other hand, were highly abundant in our experimental plots, but their abundance and amount of damage on the plants were not correlated with cucurbitacin levels or trichome density. This is not surprising as species in the genus *Acalymma* are known to be specialists on squash, adapted and attracted to cucurbitacins which they use as kairomones to locate their host plants and capable of sequestering them for their own defense^{90,94,95}. Our results are in accordance with those from Theis, et al.⁹⁶. They found that the abundance of *Acalymma vittatum* in a squash field site in Massachusetts (USA), was not correlated with the cucurbitacin content of 20 cucurbit varieties, including domesticated and wild species. They suggest that the damage could be predicted by other plant traits and not by beetle presence and that floral sesquiterpenoids were driving these interactions. Similarly, in an ongoing study with wild and domesticated plants of *C. argyrosperma*, we found that larvae of the specialist *A. vittatum* were strongly attracted to cucurbitacins present in roots, but cucurbitacin content was not correlated with larval performance (Jaccard *et al.* in prep.). Interestingly, these plants do not contain any cucurbitacins in the leaves which supports the hypothesis that other traits are likely involved in the attraction of adult beetles. Future work should explore the role of leaf and flower volatiles in this attraction.

In conclusion, our study suggests that varietal selection, but not the purpose of domestication in squash has altered cucurbitacin content and leaf trichome density, but the performance of the leaf and root herbivore was not affected by these defense

traits. Further, the preference of *D. balteata* larvae to cucurbitacins may be likely due to the evolutionary history that this species has shared with wild and domesticated squash plants in their region of origin and domestication. Future efforts should be directed at elucidating the effects of domestication of this and other crops on their susceptibility to herbivorous pests. A better understanding of these effects could help in the development of crop varieties with enhanced insect and disease resistance.

Author Contributions

C.J. and B.B. originally formulated the idea. C.J., B.B. and M.A.C.C. designed the experiments. C.J. performed all the experiments and wrote the first version of the manuscript. G.C. developed the cucurbitacin extraction method. C.J., M.A.C.C. and C.B.S. worked in the field in Mexico. C.J. and C.B.S. did the DNA barcoding for field insect determination. L.G. performed the experiment on cotyledons and C.J., C.B.S. and C.C.M.A. analyzed the data. All co-authors contributed to the elaboration of the design and to the writing of the last version of the manuscript.

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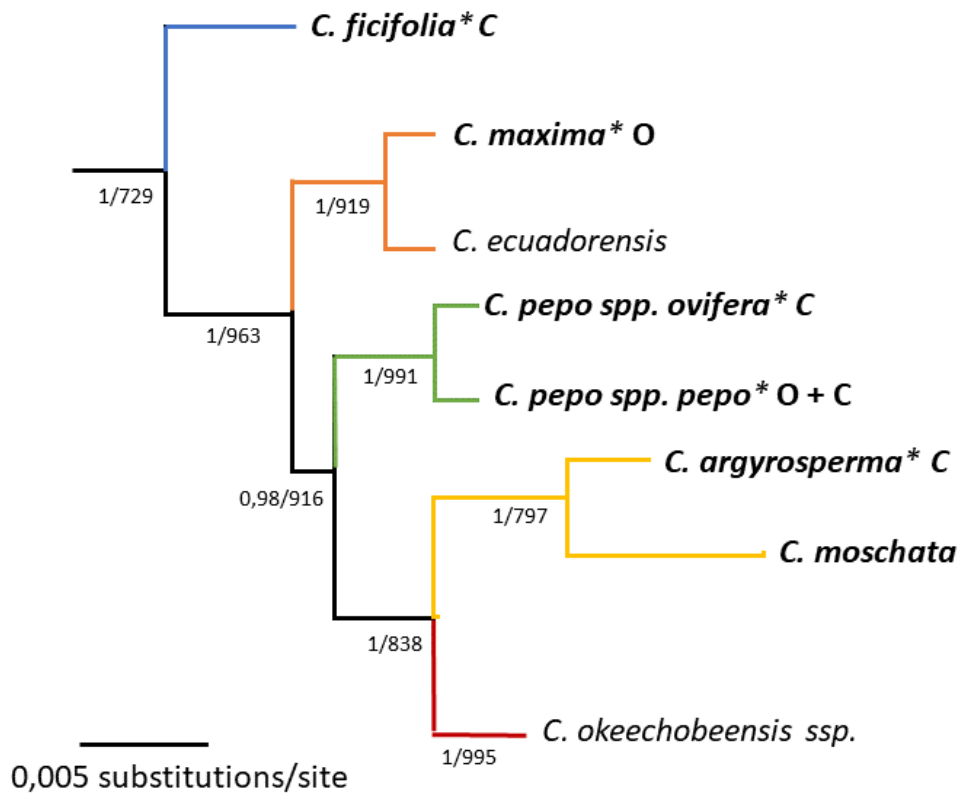
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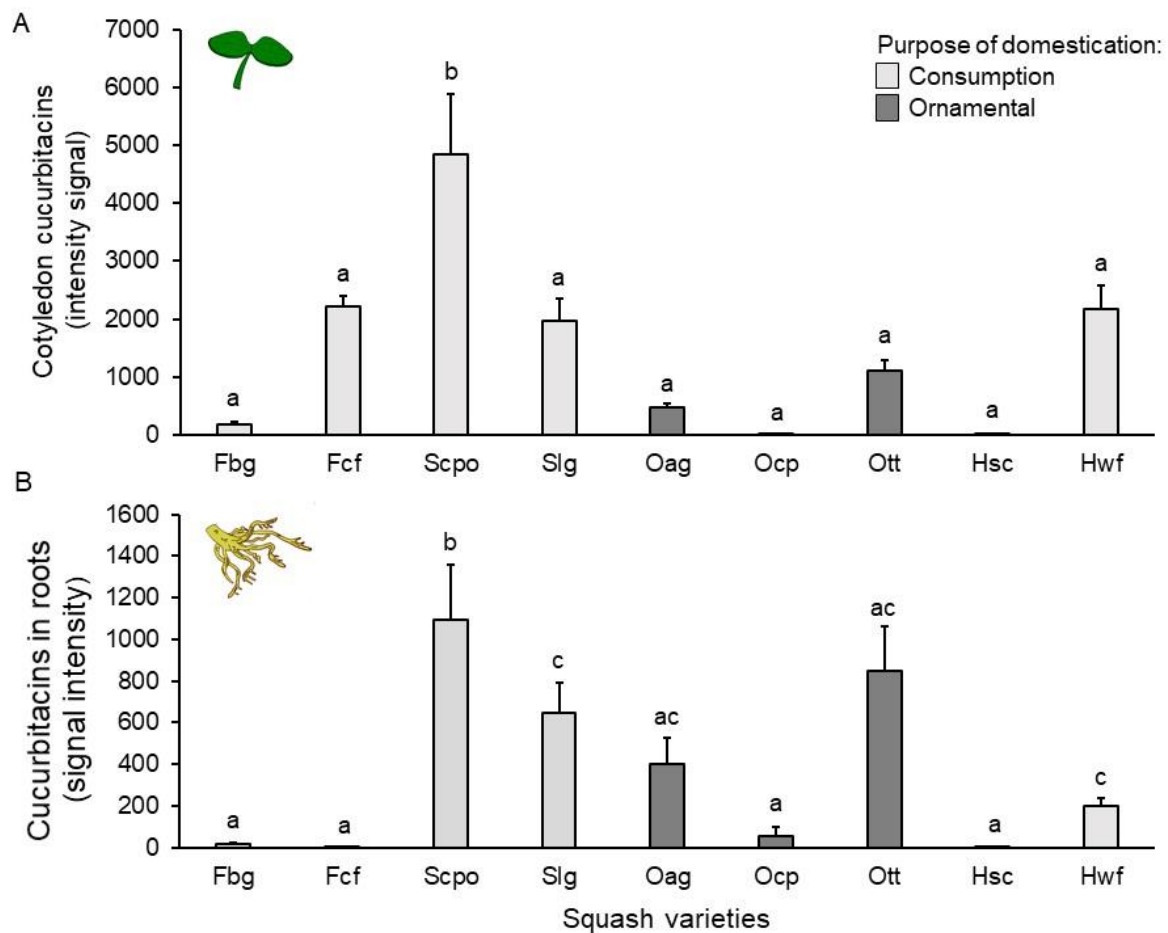
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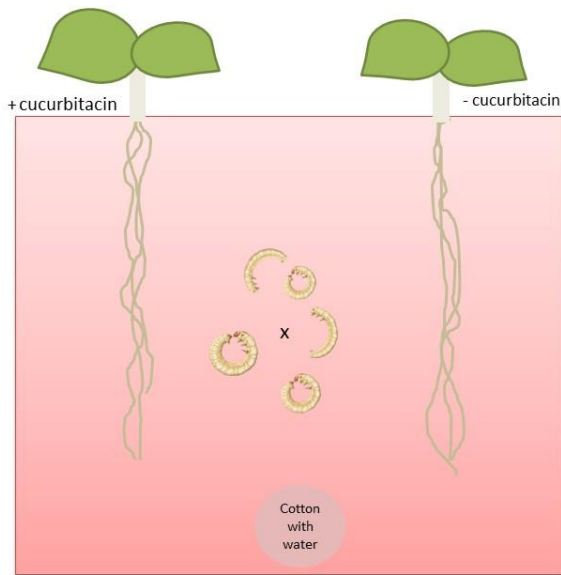
Supplementary material



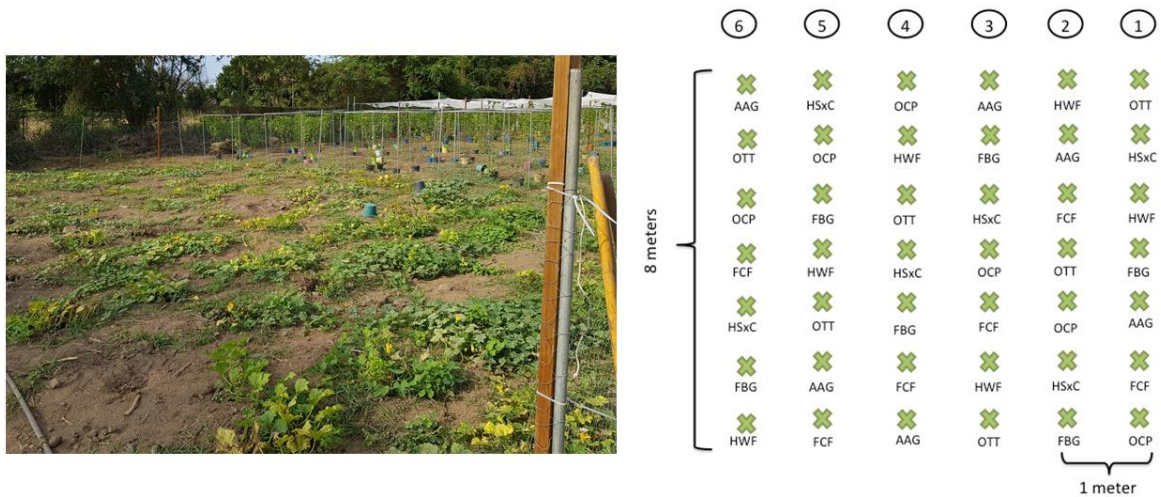
Supplementary Figure 6. Species used in our experiments based on a *Cucurbita* phylogeny modified from Castellanos-Morales *et al.* [32]. Color indicates different monophyletic groups: light blue: *Cucurbita ficifolia*, orange: group Maxima, green: group Pepo, Yellow: group Argyrosperma and red: group Okeechobeensis. Asterisks shows the varieties used in this study. Letters indicate the purpose of domestication: C: Consumption and O: ornamental used in our study. Domesticated taxa are in bold.



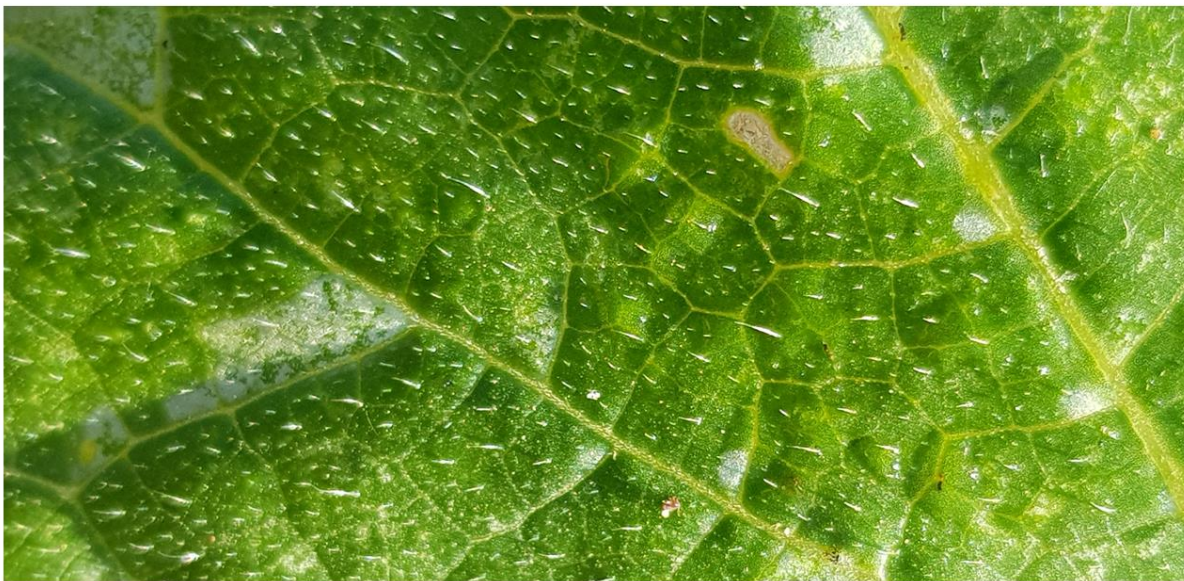
Supplementary Figure 2. Cucurbitacin relative content (signal intensity) in cotyledons (A) and roots (B) of the studied *Cucurbita* spp. varieties. Bars represent means (\pm SE). Light-colored bars are varieties domesticated for consumption and dark-colored bars are varieties domesticated for an ornamental purpose. Different letters indicate significant differences among varieties. P values are given for treatment comparisons with log transformed data [linear model], followed by pairwise comparisons of Least Squares Means (LS means).



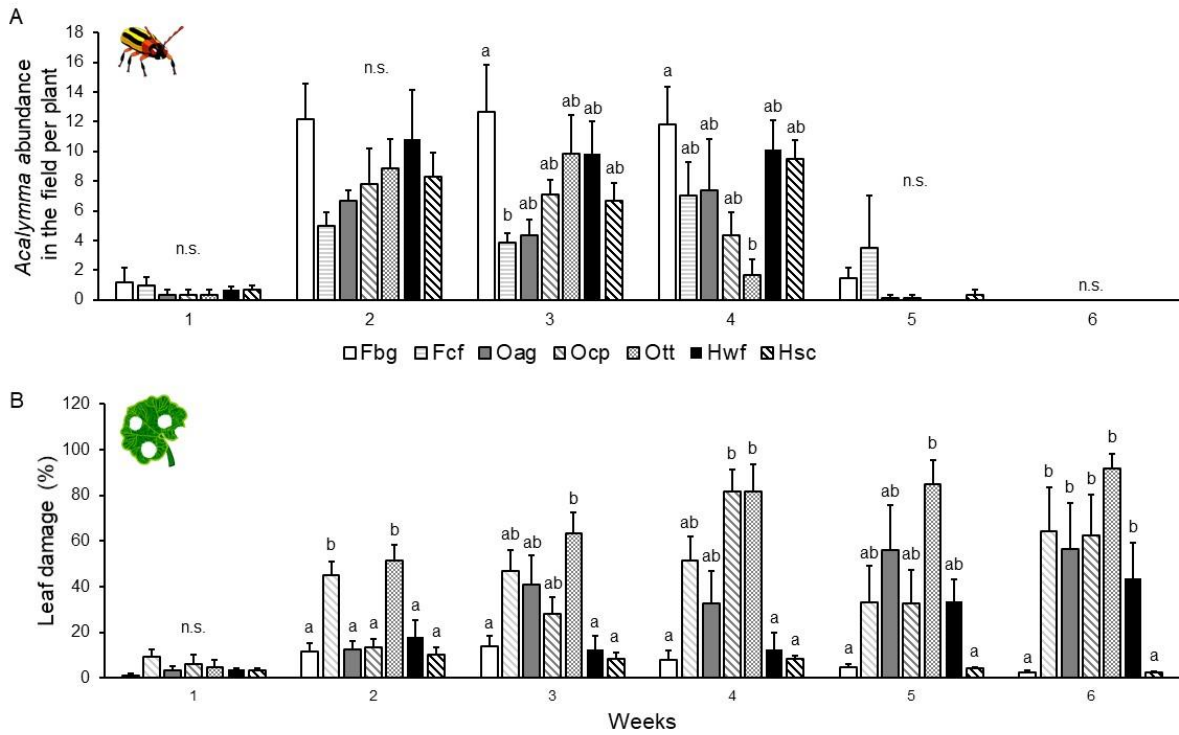
Supplementary Figure 3. Design for the choice-experiment with *D. balteata* on roots of two squash varieties with different cucurbitacin content. Position of the plant varieties in the squared petri dishes were alternated. At each time, five larvae were put in the middle of the petri dish by the hole on the lid. The red cellophane is used to mimic darkness as the UV light cannot go through. N=30.



Supplementary Figure 4. Common garden of squash varieties selected for consumption and ornamental purpose. The field was located in the coastal area of the state of Oaxaca, Mexico, 15 km northwest of Puerto Escondido in the UMAR (Universidad del Mar) experimental campus. The experimental field area is mainly used for cultivation of maize, and it is surrounded by native vegetation including wild cucurbits and wild lima bean plants.



Supplementary Figure 5. Picture of adaxial side of the squash leaf. Trichomes are non-glandular (hairy).



Supplementary Figure 6. Mean number of *Acalymma* spp beetles per plant (A) and percent of leaf damage (B) on squash varieties domesticated for different purposes (n=6) in a common garden field experiment in Puerto Escondido, Oaxaca, Mexico. Data were recorded once per week for 6 weeks. Bars represent means (\pm SE). P values are given for treatment [linear model] per week and followed by pairwise comparisons of Least Squares Means (LS means). n.s. not significant.

Chapter 2: Domestication altered defense traits and gene expression in *Cucurbita argyrosperma* with differential consequences for a generalist and a specialist herbivore

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Abstract

- Plant domestication is assumed to have reduced levels of defensive compounds, thereby increasing the plant's susceptibility to insects. For squash, *Cucurbita* spp., the reduction of cucurbitacins, which are extremely bitter and toxic compounds, has independently occurred several times during the domestication of this lineage. However, the mechanisms underlying these changes and the consequences for insect herbivores are largely unknown.
- Using a combination of chemical, molecular, and behavioral analyses, as well as field experiments, we investigated how the domestication of *Cucurbita argyrosperma* has affected plant defenses and the interactions with two sympatric root herbivores, the generalist *Diabrotica balteata* and the specialist *Acalymma* spp. We compared three wild populations and four domesticated varieties.
- Except for cotyledons, the domesticated varieties contained virtually no cucurbitacins. The expression of genes involved in cucurbitacin synthesis was indeed considerably higher in roots of the wild populations. Larvae of both insects preferred to feed on roots of wild squash, but, surprisingly, this negatively affected the performance of the generalist. In the field, leaves of wild and domesticated plants were mainly attacked by adults of *Acalymma* spp..
- Our findings provide insights into the natural and human-mediated selective pressures that may have shaped the interactions in this system.

KEY WORDS: CUCURBITACIN - GENE EXPRESSION – GENERALIST/SPECIALIST - PLANT DOMESTICATION – ROOT HERBIVORE – WILD RELATIVE

Introduction

Plant domestication is one of the key developments that allowed the establishment of human societies and modern civilizations^{1,2}. Several plant structures including roots, seeds, fruits and leaves have been subjected to human selection³ for the production of food, fabric, oil, medicine, and other purposes. After domestication, crops were transported from their center of domestication to new environments, leading to diversification and local adaptation, which in some cases was achieved through introgression with their wild ancestors or other domesticated relatives⁴. However, genetic variation tends to be reduced in domesticated organisms compared to their wild progenitors due to genetic bottlenecks associated with the sampling process⁵⁻⁷. This loss of diversity varies greatly, depending on the species^{6,8} and the purpose of domestication.

The suite of traits that distinguish a crop from a wild relative is known as the domestication syndrome. The genetic basis of a domestication syndrome can be due to protein and/or regulatory changes in specific genes, transposable elements, structural variation and even genome duplication^{9,10}. Domestication can influence species interactions either by changes in expression of single genes associated with resistance or through selection on quantitative traits⁹. These changes can result in intraspecific variation in defense compounds in crop plants, either with a complete loss of biosynthesis¹¹ or a reduction of secondary metabolites¹² that affect the plant's interaction with herbivores.

In recent years, there has been increased interest in understanding the impact of plant domestication on interactions with herbivores and their natural enemies^{9,13,14}. Studies on insect oviposition^{15,16}, survival^{17,18} and larval development^{19,20} show that an increase in herbivore performance and attraction to domesticated plants is often correlated with a decrease in plant defense traits in domesticated plants^{18,21,22}, however this pattern is not ubiquitous^{13,23-26}.

Several factors may be responsible for a lack of correspondence between decreased plant defense and increased herbivore performance; among these, plant-related traits such as the organ targeted by the domestication process or the purpose^{25,27} and history of domestication²³, as well as herbivore traits, like degree of specialization and mode of feeding^{25,28-30}. In addition, different geographical origins of the tested plants and herbivores may result in unrelated patterns of chemical defense and insect performance²⁶. Such that, the selective pressures that molded the plant's resistance responses and the patterns of herbivore performance are more likely driven by organisms from their own region^{26,31}.

Most studies on plant domestication and insect interactions have focused on aboveground plant structures⁹. Comparative studies that examine the consequences of plant domestication on insects that feed on belowground organs are underrepresented. Only two studies, both with maize, have examined the impact of domestication on belowground herbivores^{30,32}. Although the results of these two studies found that insects tend to perform better on domesticated maize, plant genotype and degree of domestication (modern breeding vs. local landraces) had a

stronger effect. Thus, to date there is insufficient information on the relationship between altered defense traits and the performance of belowground insects that would allow comparisons with patterns reported for aboveground herbivores.

Plants from the genus *Cucurbita* have been subjected to several independent domestication events^{33,34}. There are five domesticated species: *C. argyrosperma* Huber, *C. ficifolia* Bouche, *C. maxima* Duschesne, *C. moschata* Duschesne and *C. pepo*. All of these species display great diversity in fruit morphology, color and purpose of use³³. Archeological records indicate an initial domestication event 10 000 years ago, highlighting that *Cucurbita* is one of the earliest domesticated crops³⁵. Studies on cucurbit domestication date to 1930^{36,37}. Evidence shows that *Cucurbita* was initially domesticated for its use as containers and for seed consumption, while non-bitter flesh and large fruit size were selected later^{34,38}. One of the main traits altered during the process of domestication was the loss or reduction of cucurbitacins, extremely bitter and toxic compounds, even lethal when consumed by many organisms including mammals^{33,39,40}. Cucurbitacins have been independently lost from fruits of all domesticated species⁴¹. The selection for non-bitter fruits occurred initially by our ancestors and later by plant breeders^{42,43}. In cucumber, loss of fruit bitterness was caused by mutations at the *Bt* locus and in homologous regulators at the syntenic regions in watermelon and melon⁴⁴⁻⁴⁶. In a recent study using two independently domesticated lineages of *Cucurbita pepo*, Brzozowski, et al.⁴³ suggested that the genes responsible for cucurbitacin accumulation in cotyledons are in the *Bi-4* locus together with genes relevant for transport and regulation. As yet, changes in gene expression for chemical defense in squash domesticates compared to their wild ancestors are unknown.

In this study we used a combination of chemical, molecular and behavioral analyses to investigate the effect of domestication of *Cucurbita argyrosperma* on plant defenses and interactions with two herbivorous insects. *Cucurbita argyrosperma*, known in Mexico as “calabaza pipiana”, is a species with local cultural and economic importance^{47,48}. Some varieties are highly appreciated for their seeds, which are used in Mexican gastronomy, and for their fruits that are used for consumption and medicinal purposes⁴⁷. We used three wild populations and four domesticated relatives of *C. argyrosperma* selected for two different purposes (fruit consumption and ornamental use) and two beetle species, the generalist *Diabrotica balteata* and the squash specialist, *Acalymma* spp. (Coleoptera: Chrysomelidae). These plants and insects have a long co-evolutionary history. They all originate from Mesoamerica where *C. argyrosperma* was domesticated and where wild and cultivated plants have coexisted for thousands of years, together with their associated organisms^{49,50}.

Specifically, we addressed the following questions: (1) How has domestication of *C. argyrosperma* altered plant resistance traits (e.g., trichome density and cucurbitacin content)? (2) Are the genes in the cucurbitacin metabolic pathway functional in domesticated varieties? and (3) How do altered defense traits in domesticated varieties correlate with patterns of host plant preference and performance in generalist and specialist herbivores?

Based on the above questions, we tested the hypotheses that domestication affected the pattern of gene expression for cucurbitacins resulting in lower levels of chemical defense in domesticated varieties, particularly in varieties used for consumption. Additionally, we predicted that domesticated varieties have lower leaf trichome density. Accordingly, we expected that the specialist herbivores *Acalymma* spp. would be unaffected by domestication-mediated reductions in resistance, while the generalist *D. balteata* would prefer and perform better on domesticated squash.

Understanding the intraspecific genetic basis of defense traits in wild and domesticated plants and its consequences for herbivorous insects, might shed light into the selective pressures that have molded the interactions we see today and greatly enhance our understanding of the action of natural selection in the wild. Moreover, the comparison of species interactions using domesticated plants and their wild relatives allows us to test hypotheses about the evolution of plant defense and resistance to pests.

Materials and Methods

Study system

The oldest evidence of *C. argyrosperma* domestication is around 8,600 years ago from the Xihuatoxla rock shelter in the state of Guerrero, Mexico^{48,51}. This is a highly diverse species in shape, size and color of its seeds and fruits⁴⁷. Both wild and cultivated subspecies can be found in tropical and semi desertic regions in Mesoamerica⁵². *Cucurbita argyrosperma* is an important crop in local agricultural systems in Mexico and in other countries in the Americas⁵⁰. In other regions of the world, it is not extensively cultivated because of the low quality of its flesh. However, some cultivars like the “Green Striped Cushaw”, “Vera Cruz Pepita” and “Silver Seed Gourd” grown in the United States are improved lines with different shapes and colors⁵⁰.

Plants of the Cucurbitaceae family synthesize bitter cucurbitacins, which are oxygenated tetracyclic triterpenes. They are efficient allomones (metabolites that benefit the producer)⁴⁰ and at the same time kairomones (benefiting the receiver)⁴⁰ for specialized insects including the well-studied striped cucumber beetle (*Acalymma vittatum*, Fabieri)⁵³.

Plants

Seeds of wild squash were collected in January 2018 in the region of Puerto Escondido along the Pacific coast in the state of Oaxaca, Mexico where wild *C. argyrosperma* occurs naturally. The climate is hot and humid with an average temperature of 27°C and 84% relative humidity. Fruits were collected from three wild populations along the coast (WU: 15.924929, 97.150977; WB: 15.864463, -97.081142; WV: 15.727992, -96.708606). Based on the literature and fruit shape, the wild plants were identified as belonging to *C. argyrosperma* species^{48,52,54} (Fig. 1A/C). The WV population was used only for cucurbitacin content and the preference bioassays with the insects in the lab

and not for the gene expression, the insect performance and the field common garden because we run out of seeds quickly due to the low germination rate.

We used four domesticated varieties, including: Vera Cruz Pepita (FVP) and Silver Edge (FSE) varieties initially domesticated for fruit consumption (Fig. 1D/E), and the Cushaw Tricolor (OCT) and Navajo calabacita (ONC) varieties selected as ornamentals (Fig. 1B). Seeds were obtained from KCB Samen (<https://www.kcb-samen.ch/>). For lab experiments, plants were grown in 8 cm diameter plastic pots filled with “Einheitserde” classic soil (Sinntal-Altengronau, Deutschland) mixed with 30% sand. In order to enhance germination of wild seeds, seed coats were pierced, scratched and placed between two layers of wet cotton for one week in an incubator at 28°C. The plants grew in a greenhouse at ambient temperatures ($24 \pm 5^\circ\text{C}$), under natural light conditions (16:8h L: D) and were watered as needed. For the experiments we used 15-day-old plants, with two cotyledons and two true leaves. To control for the size difference between plants from different treatments, wild plants were incubated for germination 10 days before domesticated plants, which grow faster.



Figure 7. Seeds and fruits of *Cucurbita argyrosperma*. (A) WB: Wild Bacocho, (B) ONC: Navajo calabacita, (C) WU: Wild Umar, (D) FSE: Silver edge, (E) FVP: Veracruz pepita.

Insects

We worked with two genera of chrysomelid beetles well-known for their association with cucurbits: the generalist *D. balteata* and specialist species of *Acalymma*. These insects originate from Mesoamerica where they have been associated with wild and domesticated squash for thousands of years^{40,55-58}. Larval stages of *D. balteata* and of *A. vittatum* were used in lab experiments, while in the field adults were identified as *D. balteata*, *A. blomorum* and *A. innubum*.

Diabrotica balteata LeConte, 1865 (Coleoptera; Chrysomelidae), commonly known as the banded cucumber beetle, is a pest of many agricultural crops including cucurbits⁵⁹. Adults eat all plant structures including leaves, cotyledons and flowers, while larvae feed exclusively on roots and tubers⁵⁹. Eggs of *D. balteata* were provided by Syngenta (Stein, Switzerland). Upon hatching, larvae were reared on maize roots (hybrid DFI 45321, DSP, Delley, Switzerland) until second instar when they were used for experiments. Rearings were kept in quarantine facilities at the University of Neuchatel (25°C ± 2°C, 16:8h L: D and 60% RH ± 5%).

The striped cucumber beetle, *A. vittatum* Fabricius, 1775 (Coleoptera: Chrysomelidae: Galerucinae) is a member of the tribe Luperini. This tribe is believed to have an association with plants in the family Cucurbitaceae dating back at least 30 million years^{55,60}. *Acalymma vittatum* is considered a specialist as it can consume large quantities of cucurbitacins and is known to metabolize, excrete and sequester these bitter compounds, which can be present in its body and eggs⁶¹. Adults feed on leaves, flowers and pollen, while larvae feed on roots^{62,63}. Lab experiments with this species were conducted with a colony maintained in the Insect Ecology Lab at Purdue University, Indiana, USA. Adult beetles were collected locally on cultivated squash and reared on zucchini plants in an incubator (28°C ± 2°C, 16:8h L: D and 60% RH ± 5%).

Plant defense measurements

Cucurbitacin quantification

Samples of leaves (n=5), roots (n=5) and cotyledons (n=5) of two-week old wild and domesticated plants were ground into fine powder in liquid nitrogen. We weighed 100mg (+/- 20mg) of powder in a microbalance to the nearest 0.1 mg (Mettler Toledo XP6, Columbus, Ohio, USA) and added 1 ml of methanol (99.99%)⁶⁴ with five glass beads in 1.5 mL Eppendorf tubes. The cells were broken with a TissueLyser (Qiagen, Hilden, Germany) at 30 Hz for four minutes. Samples were centrifuged for five minutes at 14000 rpm; 700 µl of surfactant was removed and diluted with 300 µl of MiliQ water. The analysis of cucurbitacins was performed with a UHPLC-QTOFMS instrument (Waters, Milford, USA) with an Acquity UPLC™ coupled to a Synapt G2 high-resolution mass spectrometer. The column used in the chromatograph was ACQUITY UPLC BEH C18 1.7µm, 2.1 x 50mm (Waters, Milford, USA). The temperature was maintained at 25°C. Two eluants were used; A was water and 0.05% formic acid and B was acetonitrile and 0.05% formic acid. The injection volume in the machine was 2.5 µl. We used Electrospray negative ionization (ESI) with MS^t (alternates between low and high

collision energies) as acquisition mode. The source parameters were: capillary voltage -2.0 kV, cone voltage -25 V, source temperature 120°C, desolvation gas flow and temperature 800 l/h and 350°C respectively, cone gas flow 20 l/h. The exact mass measurements (< 2 ppm) were ensured by infusing a 500 ng/ml solution of leucine-enkephalin at 15 µl/min through the Lockspray™ probe. For the acquisition and data processing, we used the software Masslynx™ v.4.1 (Waters, Milford, USA). Cucurbitacins were identified based on their molecular formula and fragmentation patterns provided by mass measurements. Peaks corresponding to known cucurbitacins were automatically integrated using Quanlynx™ with a 0.1 min chromatographic window centered on the retention time of each component and a 0.02 Da mass window centered on the (M+HCOO) ion. Quantification of all cucurbitacins was completed by external calibration using cucurbitacin B as standard. The cucurbitacin concentration was expressed in µg per g of plant material.

RNA Preparation and Real-Time qPCR

Freshly harvested roots and cotyledons were ground in liquid nitrogen, and RNA was isolated using the SV Total RNA Isolation System (Promega) according to the manufacturer's instructions. Each total RNA sample (300 ng) was reverse transcribed using the GoScript™ Reverse Transcription System (Promega). Nine cucumber genes in the pathway for biosynthesis of cucurbitacins⁶⁵ were used to search orthologs in *C. argyrosperma* based on its genome database⁶⁶ by using BLAST. We found one cucurbitadienol synthase (CPQ) gene and six cytochrome P-450 enzyme genes that might be involved in the cucurbitacin biosynthesis pathway (Supplemental Table S1) in the genome database of *C. argyrosperma*. Thus, we measured differences in the relative expression of these seven genes among two wild populations (WB and WU, see *Plants* section above) and four cultivated varieties (FSE, FVP, OCT and ONC). For details on the protocol for the Real-time qPCR see chapter 4.

The *actin* gene (GenBank accession number: HM594170) was used as the reference for the transcript profiling of roots and cotyledons. Primers used for real-time qPCR were designed by using Primer-BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) and are listed in Supplemental Table S2. Five independent biological replicates were analyzed, after which the average threshold cycle (Ct) per sample was calculated. For the expression analysis of each gene, samples from the WB variety were designated as calibrator (for roots, cotyledons and comparison among plant tissue). The relative expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method⁶⁷.

Trichomes

We obtained leaf and cotyledon samples (1.14 cm²), with a leaf hole puncher at the central part of the leaf base. In a previous study we found that the number of adaxial and abaxial trichome was correlated²⁷. Thus, we counted the number of trichomes on the adaxial side of leaves using a stereoscopic microscope. We counted only trichomes longer than 1.5 mm and used among seven to ten replicates per variety and wild population.

Preference and performance of generalist and specialist chrysomelid larvae on Cucurbita argyrosperma roots

Preference and performance experiments with the generalist *D. balteata* were conducted in May 2018 in the quarantine facilities at the University of Neuchatel, Switzerland, whereas the experiments with the specialist *A. vittatum* were conducted in July 2019 in the Insect Ecology Lab at Purdue University, USA.

Choice-experiment with beetle larvae on roots of wild and domesticated plants of C. argyrosperma

Beetle larval preference for domesticated varieties or wild populations of *C. argyrosperma* was tested in two-choice experiments. Newly hatched larvae were reared on maize roots (for *D. balteata*) or zucchini roots (for *A. vittatum*) until second instar (approximately 10 days). Larvae were starved for 12 hours before the experiments. We used square Petri dishes (12 x 12 cm, Sarstedt, Germany) with moist filter paper and a wet cotton ball. We pierced two holes in one side to place the roots of the domesticated varieties OCT or FSE and the wild WB or WV. For the test with *A. vittatum* larvae, only the combination FSE x WB was used, representing the two extremes in cucurbitacin content. The petri dishes were sealed with parafilm. Each time, five *D. balteata* or *A. vittatum* larvae were released in the middle of the petri dish through a hole in the lid. Petri dishes were covered with red cellophane to reduce light and allow observations (Fig. S1). The larvae were able to move freely to feed on the roots. Larval choice, defined as the number of larvae found on the roots (from 0 to five), was recorded every 10 minutes during the first hour, then every hour for 6 hours and then the final choice after 24 hours. We performed 22 replicates for the OCT x WV combination and 12 replicates for the FSE x WB combination for the experiment with *D. balteata* and 12 replicates for the FSE x WB for the experiment with *A. vittatum*. Larval choice was calculated in percentage $((\text{number of larvae on roots} / 5) * 100)$.

Performance of beetle larvae on roots of wild and domesticated C. argyrosperma

The performance of larvae of *D. balteata* and *A. vittatum* was determined on roots of wild and domesticated plants. Five second instars were randomly assigned to a plant of each variety (FSE, FVP, OCT and ONC) and wild population (WB and WU). Larvae were placed on the roots and soil of fifteen to twenty-one-day old squash plants inside a small plastic bag. To calculate the initial larval weight, the five larvae were weighed together before the experiment and the total weight was divided by five to obtain an estimate of the individual mean weight. After six days, larvae were collected. The final weight was calculated by weighing all the larvae together for each plant and dividing the total weight by the number of larvae recovered from each bag. Plants were watered with 20 ml of tap water once in the middle of the bioassay. The experiment with *D. balteata* was repeated three times with five replicates per variety per experiment (15 replicates in total per variety) and two times (10 replicates) with *A. vittatum*. Plants

were randomly distributed in an incubator (28°C, LD 12:12). Mean relative growth rate (final weight divided by initial weight) was calculated for each larva^{68,69}.

Field experiment: Susceptibility of wild and domesticated plants of C. argyrosperma to insect attack in natural field conditions

We carried out a common garden experiment in the field to determine herbivore presence and damage on wild and domesticated plants. The field site was located 15 km northwest of Puerto Escondido (Oaxaca, Mexico, 15°55'33.3" N 97°09'03.0" W). The experimental field site is mainly used for cultivation of maize, and it is surrounded by native vegetation including wild cucurbits and wild lima bean plants. The experiment was performed under a tropical climate, during the dry season, from November 3rd 2018 to January 30th 2019. In this site we can find natural populations of herbivorous insects that feed on squash including Chrysomelidae beetles from the subtribe Diabroticina (*Acalymma* spp., *Diabrotica balteata*) and lepidopterans (i.e. *Spodoptera latifascia* and *Diaphania* spp.)⁷⁰⁻⁷². At the time of our experiment, the field site was also used for experiments conducted with maize, beans⁷³ and chili peppers; these crops are also readily attacked by *D. balteata*.

Seeds of domesticated varieties were individually sown in 8 cm biodegradable pots with a mixture composed of natural soil and peat moss. Pots were placed first inside field tents (Lumite® 2 x 2 m, Bioquip) for three weeks to protect plants from unwanted herbivory until they reached the desired size (2 true leaves). Then, plants were arranged in a common garden in the experimental field (9m x 16m) randomly distributed among 21 plots (each plot 2 x 1 m). Each plot consisted of six plants, each one separated by 1 m, with a total of 21 plots with 1.5 m separation between them. Plant position was alternated to avoid plants of the same domestication status or with the same purpose of domestication neighboring each other (Figure S2). Plants were watered every 2 days with 500 ml of water.

From the 11th of December 2018 to the 23rd of January 2019 plants were monitored once a week between 8 and 11 am. Each time we recorded the number of total leaves, the number of leaves with evidence of insect damage and insect identity (either *D. balteata* or *Acalymma* spp). We were only able to confirm the insect species identity with DNA barcoding upon return to the lab in Neuchâtel. The two species of *Acalymma* identified were *A. blomorum* and *A. innubum*, Genbank accession numbers MT773619 to MT773624. Thus, for the analyses we pooled the number of individuals of both species recorded in the field. We also recorded the number of insects present on the plant, the category of damage per plant (0 = 0%, 1-25% = 1, 26-50% = 2, 51-75% = 3, 76-100% = 4; Fig. S3), and later in the season, the number of flowers and fruits produced by each plant. The first data were collected two days after the transfer of plants into the ground to allow them to acclimate to field conditions.

Statistical analysis

We used the statistical software R (v. 4.02.2, the R foundation for statistical computing, Vienna, Austria) and its complementary console R-studio (Integrated Development for R. RStudio, Inc., Boston, MA) for data analysis. Cucurbitacin concentrations were analyzed separately for each plant tissue (leaves, cotyledons and roots) to test differences among wild populations and domesticated varieties with a Kruskal-Wallis test. Pairwise comparisons were performed using a Wilcoxon rank sum test with a continuity correction. Data were checked for normality with Shapiro-Wilk's test.

To compare the number of trichomes among all treatment (three wild populations and the four domesticated varieties) a generalized linear mixed model was applied. To test the impact of domestication status (wild or domesticated), we included varieties as a fixed factor with a quasi-Poisson distribution.

Insect preference (choice-test) was analyzed for each timepoint and factor combination with a generalized linear model (*glm*) with a binomial distribution. Alpha values were adjusted with Bonferroni correction to correct for multiple comparisons. ANOVA was used to test for differences between wild and domesticated roots. The relative growth rates of *D. balteata* and *A. vittatum* were log-transformed and analyzed with a linear model (*lm*). The larval RGR difference between cucurbit domestication status (wild or domesticated) was analyzed with a generalized linear model (*lmer*) with varieties as a random factor.

We tested the differences in leaf damage (categorized as an ordinal factor) among wild populations and domesticated varieties in a common garden field experiment in Mexico with a cumulative link mixed model (*clmm*) with time as interaction, time point as fixed effect and the ID of the plant and plot number as random factors. A separate test was done for each week of measurement without time as interaction, followed by a pairwise comparison of Least Squares Means (LS means).

We tested the difference in the abundance of *Acalymma* spp. among squash wild populations and domesticated varieties with time point as fixed effect and the ID of the plant and plot as random factors, to account for the repeated measurement structure. We used a generalized linear mixed model with a negative binomial error distribution (*glmer.nb*).

To account for the location of the plot in the field, as the garden was surrounded by diverse vegetation, the field site was divided into three blocks (Fig. S2). To test if the position of the plot explained insect distribution, we used the function *glmer.nb* with time point as fixed effect and the ID of the plant and plot number as random factors. A Kendall correlation at week six of the measurements was used to test if flowers increased the number of insects per plant.

The statistical analysis for the gene expression experiment was conducted with GraphPad Prism 8.4.3. To test the differences in gene expression in roots of wild populations and among varieties, we used the Shapiro-Wilk test to evaluate the data for normality and the Brown-Forsythe test for homogeneity of variance. For non-normally distributed data, Kruskal-Wallis test followed by Dunn's multiple comparisons test were used.

To test the differences in gene expression between plant tissues (roots versus cotyledons) of the same wild population or domesticated variety, we evaluated the data for normal distribution with a Shapiro-Wilk test and an F-test for homoscedasticity. We used unpaired t-tests to evaluate the data for normality and normally distributed data with unequal standard deviation were analyzed by using unpaired t-tests with Welch's correction. For non-normally distributed data a non-parametric (Mann-Whitney) test was used.

Results:

Cucurbitacin content in wild and domesticated plants

Cucurbitacin concentrations in the cotyledons were significantly different between wild populations and domesticated varieties (Chisq=28.408, df=6, $p<0.0001$) (Fig. 2 a). Plants selected for fruit consumption (FVP and FSE) contained lower levels of cucurbitacins in the cotyledons. The Silver Edge variety (FSE) contained more cucurbitacin than the other fruit variety ($p=0.04$). However, cotyledons from the Vera Cruz Pepita (FVP) and from both ornamental varieties, did not contain cucurbitacins. No significant differences in cucurbitacin content in cotyledons were found among wild populations (WB-WU: $p=0.726$, WB-WV: $p=1$, WU-WV: $p=0.129$, Fig. 2 a). Domestication status explained the difference in cucurbitacin content in cotyledons (Chisq=25.655, df=1, $p<0.001$) (Fig. 2 b), with wild plants containing considerably higher levels than domesticated plants.

Cucurbitacin concentrations in the roots were also significantly different among wild populations and domesticated varieties (Chisq=26.805, df=6, $p<0.0001$) (Fig. 2 c). None of the domesticated varieties contained cucurbitacins in the roots. In contrast, cucurbitacins were present at high concentrations (ca. 5-20 $\mu\text{g/g}$) in roots of the wild populations. No significant differences in cucurbitacin content in roots were found among wild populations (WB-WU: $p=0.264$, WB-WV: $p=1$, WU-WV: $p=0.429$, Fig. 2c). Again, when data were pooled as wild or domesticated, domestication status explained the difference in cucurbitacin content in roots (Chisq=26.418, df=1, $p<0.001$) (Fig. 2 d). No cucurbitacins were found in leaves of domesticated varieties or wild plants.

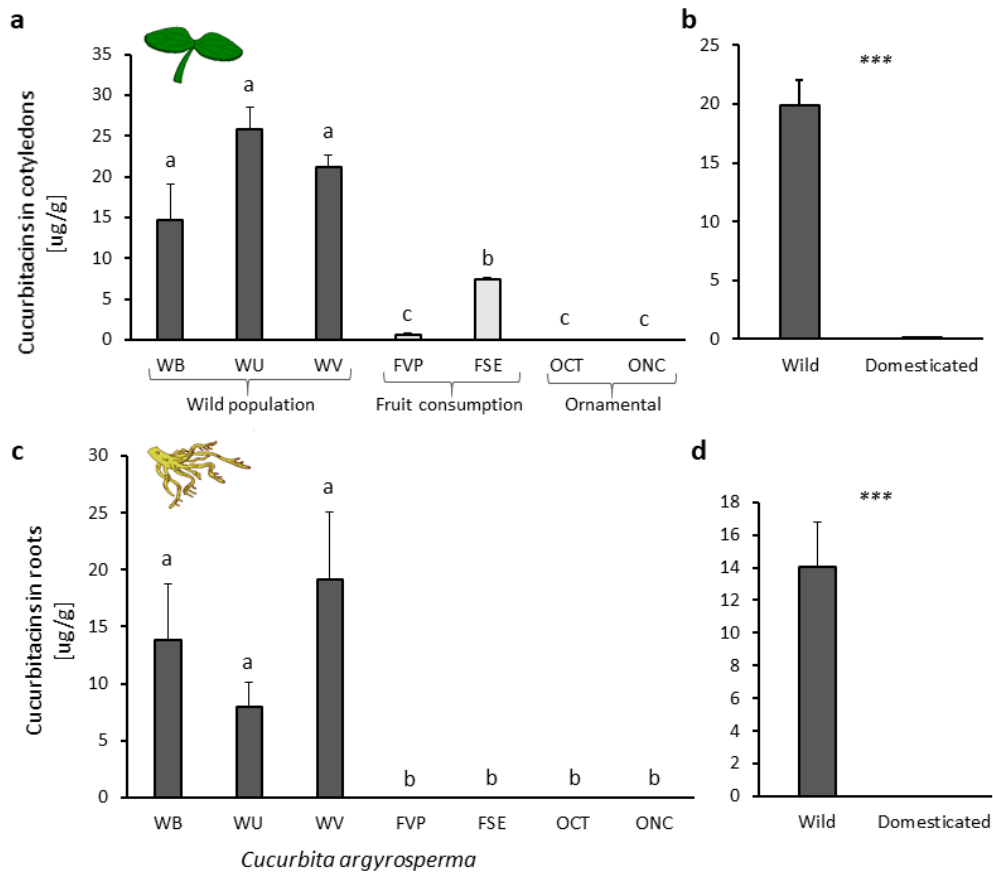


Figure 2. Cucurbitacin concentrations in cotyledons (a and b) and roots (c and d) of three wild populations (WB, WU and WV, in dark grey) of *Cucurbita argyrosperma* and two varieties selected for the fruit consumption (FVP and FSE, in light grey) and two varieties for an ornamental use (OCT and ONC, in medium grey). Bars are means (\pm SE). P values are given for treatment comparisons with a Kruskal-Wallis test followed by pairwise comparisons using Wilcoxon rank sum test with a continuity correction. Letters indicate significant differences among varieties. *** indicate significant differences between wild and domesticated varieties ($P < 0.0001$).

Cucurbitacin biosynthetic gene expression in roots of wild and domesticated plants

Carg11552 is a candidate *C. argyrosperma* ortholog of the *C. pepo* cucurbitadienol synthase gene, potentially responsible for the first step of cucurbitacin biosynthesis^{44,74}. Its expression is higher in roots of the wild population WB compared to the ornamental varieties OCT and ONC (Dunn's test, $p=0.0141$ and $p=0.0427$, respectively), but not compared to the varieties selected for fruit consumption (Fig. 3). The six candidate P450 cytochrome genes (*Carg11550*, *Carg03795*, *Carg03797*, *Carg07313*, *Carg07314*, *Carg06672*) in roots of the wild populations showed a relatively higher transcription level than in the roots of the ornamental varieties (Fig. 3). Variety FSE showed lower levels of expression for genes *Carg03795* and *Carg07313* in roots compared to wild populations. When the data were pooled by domestication status, the relative gene expression were higher in wild population than domesticated varieties for almost all genes tested (Figure S4). Surprisingly, the candidate gene coding for cucurbitadienol synthase gene (*Carg11552*) was more expressed in domesticated varieties (Figure S4). We also tested the relative expression of those seven genes in cotyledons, but the level of expression was almost null in all samples (Fig. S5). Tissue-specific expression pattern analysis revealed that the mRNA levels of *Carg11552*, *Carg11550*, *Carg03797* and *Carg06672* in roots of the wild population WB and the fruit consumption variety FVP were drastically higher than the expression in cotyledons.

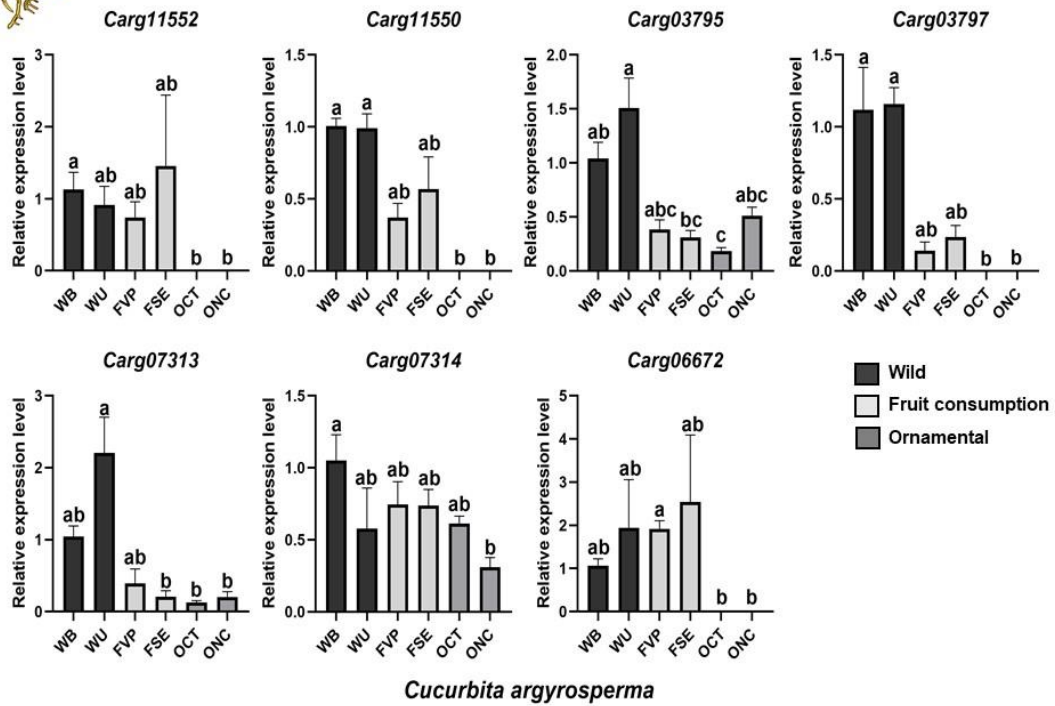


Figure 3. Relative gene expression of seven cucurbitacin biosynthetic genes in roots of two wild populations (WB and WU, in dark grey) of *Cucurbita argyrosperma*, and four related varieties; two selected for fruit consumption (FVP and FSE, in light grey) and two varieties selected for ornamental purpose (OCT and ONC, in medium grey). Shown are means (\pm SE). Letters indicate significant differences among wild populations or varieties ($P < 0.05$, Kruskal-Wallis test followed by Dunn's multiple comparisons test, $n = 4-6$ biological replicates).

Leaf trichome density on wild and domesticated plants

The trichomes identified on the adaxial part of the leaves were simple and non-glandular. No trichomes were found on cotyledons. Overall, we found no significant differences on leaf trichome density among the different squash varieties and wild populations ($F_{5,12}=2.16$, $P=0.12$) (Fig.4 a). Thus, data were pooled by domestication status (wild or domesticated). We found that leaf trichome density was lower on domesticated varieties than on the wild populations ($\text{Chisq}=2.275$, $\text{df}=1$, $p=0.013$) (Fig. 4 b).

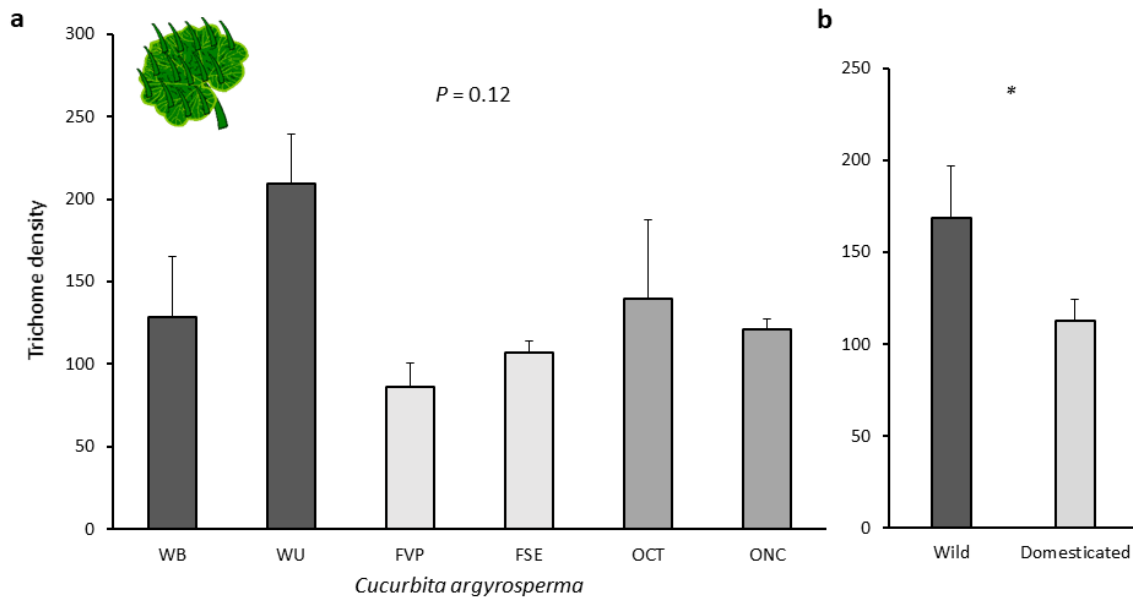


Figure 4. Number of trichomes per cm² on the adaxial side of 2nd leaf of plants from wild populations and each variety of *C. argyrosperma*. In dark grey are the wild populations (WU and WB), in light grey are the varieties selected for fruit consumption and in medium grey are the varieties used for ornamental purpose. Bars are means (\pm SE). P values are given for comparison with generalized linear mixed-effects models (*glmer* function) with a quasi-Poisson distribution, followed by pairwise comparison of Least Squares Means (LS means). Because no significant differences were found within treatment groups, data were pooled by domestication status showed in graph (b). * indicate significant differences between wild populations and domesticated varieties.

Insect preference and performance on wild and domesticated plants

Both beetle species (the generalist *D. balteata* and the specialist *A. vittatum*) highly preferred to feed on wild *C. argyrosperma* (Fig. 5). Results from the two pairwise combinations for *D. balteata* showed similar results (Fig. S6), thus data were pooled. Larval choice was the strongest after 3 hours of the start of the bioassay ($z=4.283$, $p<0.0001$) (Fig. 5 a). The specialist *A. vittatum* showed a strong preference for the roots of wild plants only one hour after the start of the experiment ($z=3.482$, $p=0.00249$) (Fig. 5 b). and after 24 hours ($z=3.222$, $p=0.0007$) (Fig. 5 b).

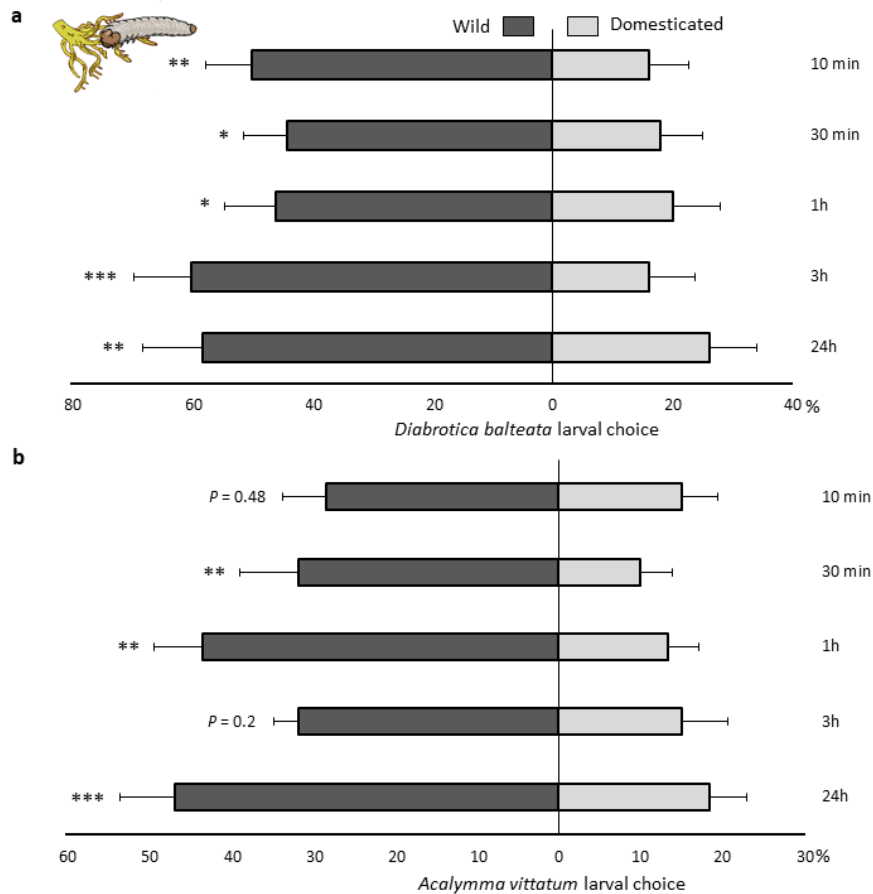


Figure 5. Feeding preference of *Diabrotica balteata* (a), graph represents the mean of the two choice-test combinations and *Acalymma vittatum* (b) larvae when given a choice between roots from wild population containing cucurbitacins and roots from domesticated varieties without cucurbitacins. Larval choice was recorded as number of larvae on roots after 10 minutes, 30 minutes, 1, 3 and 24 hours after the start of the experiment. Bars are means (\pm SE) that express the percentage of larvae that chose a treatment per time point (y axis). Bonferroni corrected P values are given for treatment comparisons [generalized linear model (family, Binomial)], followed by pairwise comparisons of Least Squares Means (LS means). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (a: $p < 0.0005$, ANOVA, $N = 24$, b: $P < 0.05$, ANOVA, $N = 12$).

For the generalist *D. balteata* the growth rate was nearly significantly different among squash varieties and wild populations ($F_{5,54}= 2.14$, $p=0.075$) (Fig. 6 a). However, when pooled by domestication status (wild population or domesticated varieties) larval growth was two-fold faster on domesticated varieties independently of their purpose of domestication (fruit consumption or ornamental use) than on wild plants (Chisq=10.731, $df=1$, $p=0.001$) (Fig. 6 b).

In contrast, the relative growth rate of the specialist *A. vittatum* was not significantly different among the wild populations or cultivars ($F_{5,18}= 0.713$, $p= 0.621$) (Fig. 6 c) nor when pooled by domestication status (Chisq=0.469, $df=1$, $p=0.493$) (Fig. 6 d). However, a trend showed that larvae grew slower in domesticated varieties.

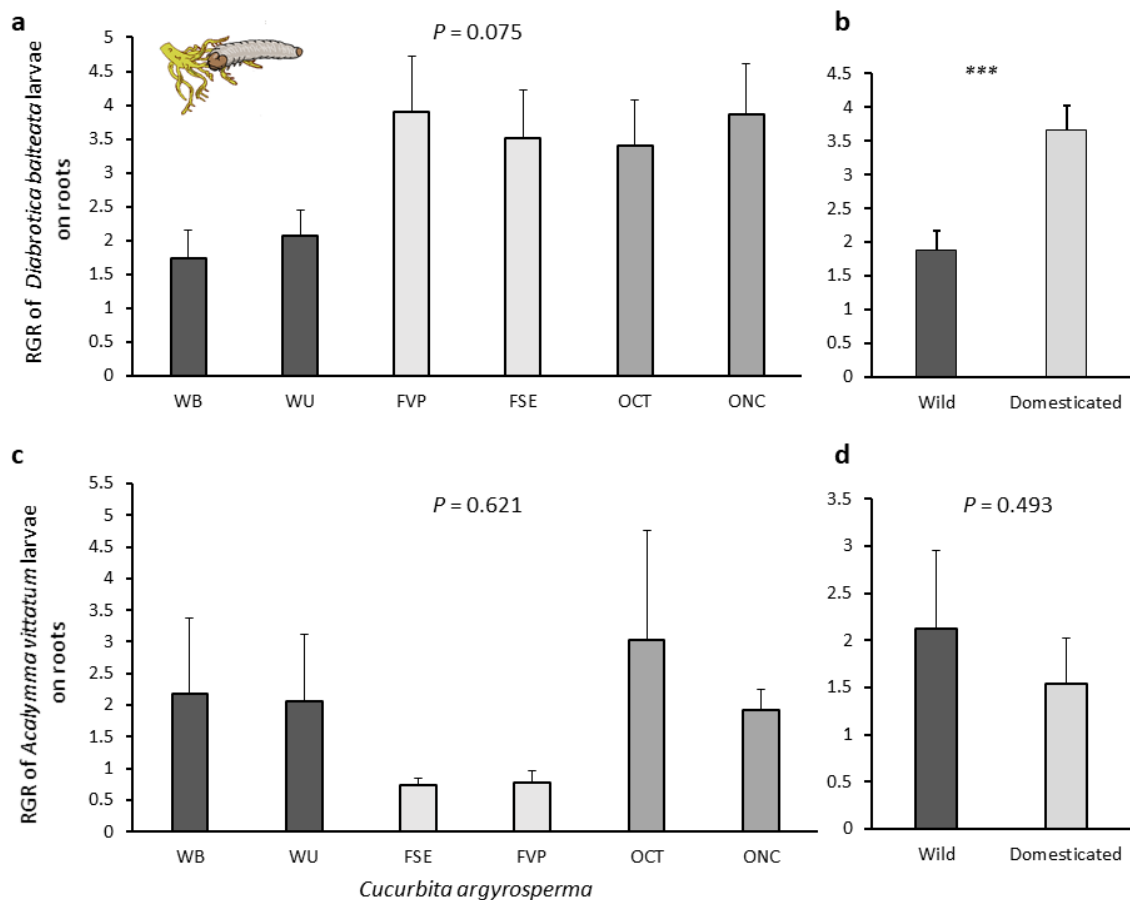


Figure 6. Relative growth rate of *Diabrotica balteata* (a) and *Acalymma vittatum* (b) larvae fed on *C. argyrosperma* wild population (dark grey) and varieties domesticated for fruit consumption (light grey) and ornamental use (medium grey) after 6 days treatment. (a: $p=0.075$, ANOVA, $N=30$ for the wild, $N=20$ for fruit and $N=40$ for ornamental, B: $p>0.05$, ANOVA, $N=5$). Bars are means (\pm SE). P values are given for treatment comparison with linear model. Because no significant differences were found within treatment groups, data were pooled by domestication status showed in the graph (b) and (d).*** indicate significant differences between wild and domesticated varieties.

Field experiment: Susceptibility of wild and domesticated plants to insect attack in natural field conditions

Although *D. balteata* was abundant in the field site and was sometimes observed visiting and feeding on the squash plants, it was mostly present on beans and maize plants adjacent to the squash common garden (Fig. S2). Around 90% of the chrysomelids recorded in the common garden belonged to the species *Acalymma innubum* and *A. blomorum*. As the identification of the two species was impossible in the field, both species were pooled for the *Acalymma* abundance analysis.

Plants from wild populations had on average more leaves than domesticated plants (Fig. S6 a). We found significant differences among wild populations and domesticated varieties in leaf damage (Chisq=19.47, df=5, p=0.01) (Fig. 7 a), but not explained by domestication status. The OCT variety suffered more damaged than plants from the wild populations and the FSE variety in the last week of sampling (z= 3.16, p=0.01) (Fig. 7 a). Damage significantly increased during the season, with the highest damage on week seven (z=3.105, p=0.0019) (Fig. 7 a).

The number of insects on the plants was significantly different among wild populations and domesticated varieties (Chisq=13.379, df=5, p=0.02) (Fig. 7 b) and depended on time (Chisq=46.475, df=1, p<0.0001) (Fig. 7 b). However, the presence of insect was not depending on the domestication status. The abundance of *Acalymma* spp. in the field peaked in the middle of the season and decreased towards the end (Fig. 7 b).

Wild plants started to produce flowers in week 4 which corresponded with an increase in insect abundance at the site (Chisq=45.703, df=1, p<0.0001) (Fig. 7 b). Plot position influenced *Acalymma* spp. distribution (Chisq=44.480, df=2, p<0.0001) in the common garden. Plots in rows close to the maize field (Block III, Fig. S2) had more insects than plots far from the maize field close to the fence (Block I, z= 6.256, p<0.0001).

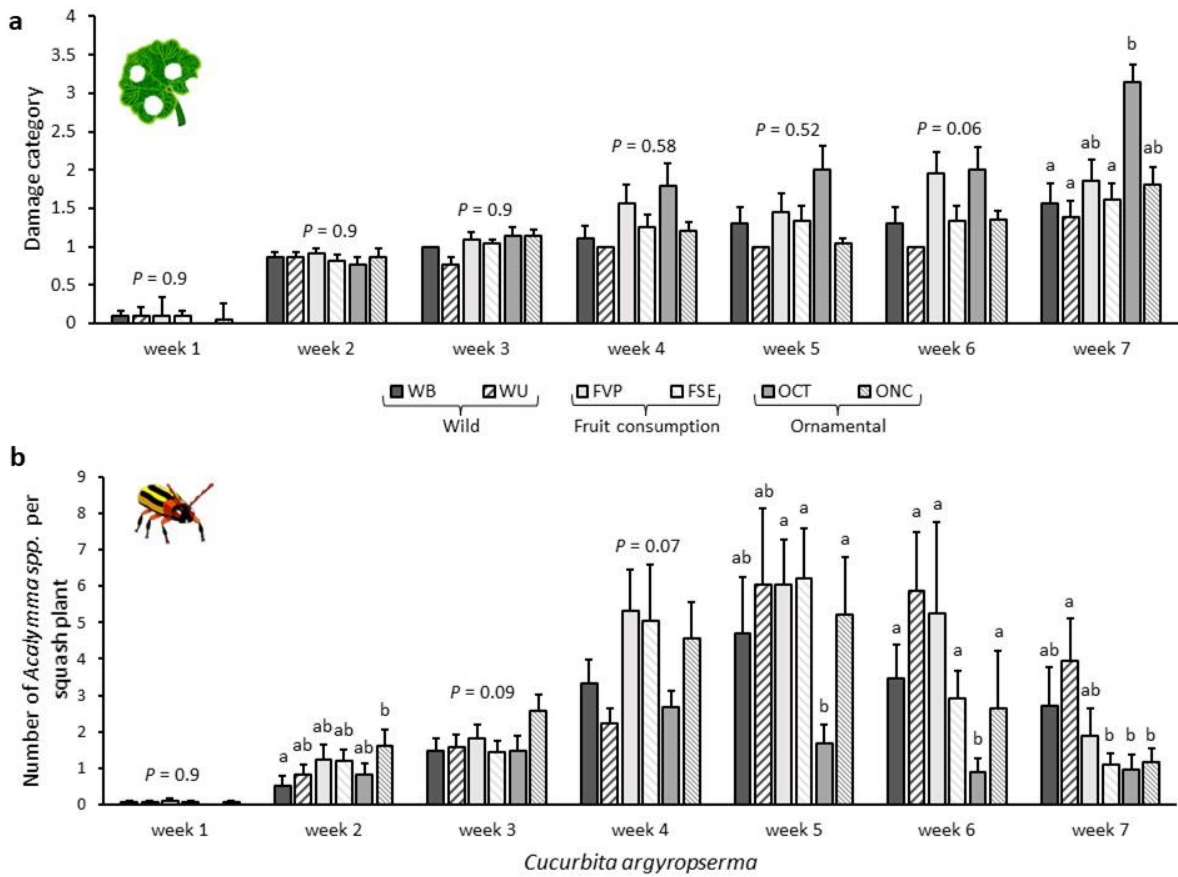


Figure 7. Common garden experiment in natural field conditions in Puerto Escondido (Oaxaca, Mexico). **(a)** Damage categories for *C. argyrosperma* varieties and wild populations through time. **(b)** Number of individuals of all *Acalymma* spp. per plant of *C. argyrosperma* varieties and wild populations through time. In dark grey are the wild populations, in light grey are the varieties selected for fruit consumption and in medium grey are the ornamental varieties. Bars are means of 21 plots (\pm SE). Letters indicate significant differences among different wild populations and domesticated varieties ($p < 0.05$, pairwise comparison of Least Squares Means (LS means)).

Discussion:

In this study we examined the extent to which domestication of *C. argyrosperma* altered its chemical and physical defense traits and the consequences for resistance against herbivorous insects. Accordingly, we expected that the pattern of gene expression related to cucurbitacin synthesis would be down regulated in plants from domesticated varieties because of the selection for non-toxicity and predicted that a reduction in defense in domesticated plants should increase the performance of the generalist insect but would not affect that of the specialist.

We found support for some of our hypotheses as well as unexpected results. Roots and cotyledons from wild cucurbits have significantly higher (10-20 times more) cucurbitacin levels than domesticated plants. Thus, decreasing concentrations of bitter and toxic compounds appears to be an intended goal of the selection process³³. As predicted, gene expression for cucurbitacin levels was higher in the roots of the wild populations. However, while varieties selected for an ornamental purpose showed no expression of most of the genes linked to cucurbitacin production resulting in no cucurbitacin synthesis, varieties selected for fruit consumption that also do not have cucurbitacins in roots, do express genes involved in cucurbitacin production. These results imply the cucurbitacin biosynthesis in roots of ornamental varieties might have been inactivated or suppressed during domestication.

The reduction of chemical defenses in squash cultivars compared to their wild relatives agrees with most studies on crop domestication^{23,24,30,75,76}. The fact that cotyledons of domesticated varieties still contain some cucurbitacins, is supported by the “optimal defense theory”, which predicts that highly valuable plant parts should be better defended than older tissues^{13,77}. If cotyledons, the first photosynthetic organ⁷⁸ are damaged, the consequences for the plant would be more detrimental than leaf damage. Moreover, cotyledons in squash are long-standing tissues that can tolerate a certain amount of herbivory, and where the continuous production of secondary compounds could represent an important investment⁷⁹. Alternatively, a reduction of cucurbitacins in aboveground plant parts can also be explained by the biogeographical origin of the plant. Cultivars of *C. argyrosperma* are bred exclusively in the American continent⁷ within the range of the cucurbitacin-sequestering specialists *Acalymma* spp., whose species are important pests of squash crops^{80,81}. Thus, the plants were potentially subject to strong selection against high cucurbitacin content in breeding programs in order to reduce damage by these beetles. This idea remains to be tested.

The number of genes and mutations required for a critical domestication transition has been addressed in several studies^{44,45,82}. It has been suggested that in many crops, a single gene played a pivotal role in moving the population over the trajectory of a key domestication transition⁸². The gene expression of cucurbitacins in cucumber was studied by Shang, et al.⁴⁴. They identified nine cucumber genes in the pathway for cucurbitacins biosynthesis and discovered transcription factors that regulate this pathway in leaves and fruits and found traces of genomic signatures, indicating that domestication led to selection of non-bitter cucumber from their bitter ancestor. In our study, we identified ortholog genes of cucurbitadienol synthase and

cytochrome P-450 from the study of Shang, et al.⁴⁴ and we found the same pattern as in cucumber. The level of expression of the genes involved in the cucurbitacin pathway was higher in the wild populations than in the domesticated varieties. The lower expression of cucurbitacin C (CuC)-related genes in most of the cultivars is linked to the lower production of cucurbitacins compared to wild populations. This is in agreement with recent results from Brzozowski, et al.⁷⁴. They found that in *C. pepo*, biosynthetic genes were expressed in all tissues where cucurbitacins accumulated and not in tissues lacking accumulation and gene expression was undetectable once the cotyledons were already expanded, even if cucurbitacin was present⁷⁴. We found that gene expression is higher in roots than in cotyledons (Figure S5). Since the cotyledons used in our study were already expanded, it is likely the cucurbitacins were synthesized while the cotyledons were developing and may explain the expression levels. Thus, cucurbitacins must be accumulated after they are synthesized, and the gene expression is activated mainly during seedling development and is transient along plant development. An alternative explanation is that there is a transport of enzymes or cucurbitacins from roots to upper plant parts, as observed for alkaloids in other plant species⁸³. Whether this transport occurs would be worth testing in future studies.

In contrast to cucurbitacin content, trichome density does not appear to have been reduced during domestication, as overall, no significant differences were found on the number of trichomes between wild and domesticated plants. Turcotte, et al.¹³ compared 29 pairs of crop plants and their wild relatives and found that the trend of reduced trichome density in cultivars is inconsistent. Similarly, Bellota, et al.¹⁶ examined trichome density in wild maize populations and commercial hybrids and found that trichome density did not significantly decline with domestication. Moreover, they found that trichome density was even increased through modern breeding. The selection for trichome-less plants may depend on where the trichomes grow. If they grow on non-edible parts, trichomes are still useful for breeders as a physical defense against herbivores.

After uncovering how domestication altered chemical (cucurbitacins) and physical (trichomes) defenses in this plant species, we asked how these altered traits impacted the host preference and performance of one generalist (*D. balteata*) and one specialist (*A. vittatum*) root herbivore that commonly attack these plants in the field. We hypothesized that larvae of the specialist herbivore should prefer bitter plants as it has been reported that adult beetles are attracted by cucurbitacins^{62,63}. Our choice experiment confirmed that the larvae of the specialist *A. vittatum* were more attracted to the bitter roots of wild plants. However, we also found that despite the lower performance of larvae of *D. balteata* on high-cucurbitacin roots of wild plants, they preferentially chose these over the roots of domesticated varieties. This result reveals that with squash, *D. balteata* behaves like a specialist even if this is only one of the multiple plant species included in its host range⁵⁹. The preference for bitter roots has been previously explained by the hypothesis that Chrysomelidae seek cucurbitacins to accumulate the compounds for their own defense⁶¹. However, evidence for this idea is still inconclusive (Jaccard et al., Bruno et al., unpublished). An alternative and to date the most plausible explanation for the attraction of *D. balteata* to cucurbitacins is that

these secondary compounds act as compulsive feeding stimulants⁵⁶. Adult cucumber beetles have been shown to be stimulated to feed on cucurbit varieties with relatively high levels of cucurbitacins in the leaves^{81,84}. Moreover, it was demonstrated that when pure cucurbitacin B was added to the surface of soybean leaves, *Diabrotica* beetles displayed compulsive feeding behavior on this non-host plant⁸⁵. Whether cucurbitacins can act as phagostimulants also for *Diabrotica* larvae, remains to be investigated.

In addition to the laboratory experiments, we asked if cultivars of *C. argyrosperma* were more susceptible to natural herbivores than wild plants. Interestingly, although *D. balteata* was highly abundant in our field site, we mainly found it feeding on leaves of beans and maize, while plants in the squash common garden were predominantly visited and damaged by adults of *A. innubum* and *A. blomorum*. In Mesoamerica, squash has been grown for thousands of years together with maize and beans in the traditional agricultural system known as the Milpa^{86,87}. Thus, it is possible that squash is not the original host plant of *D. balteata*, but this beetle species has been selected to feed on it, and uses the cucurbitacins as reliable host plant cues to locate its maize and bean hosts.

Results from the common garden did not reveal that the domestication status of the plants (wild or domesticated), explained the differences on leaf damage or beetle abundance. As squash leaves, wild and domesticated, do not contain cucurbitacins, adult beetles likely use other cues to locate and select the plants, both for feeding and oviposition. Theis, et al.⁸⁸ also found that cucurbitacins did not predict leaf damage by *Acalymma vittatum* on 20 varieties from 12 diverse Cucurbitaceae species. Instead, they found that sesquiterpenoids were the chemical signal driving the distribution of this species. Thus, even if they act as attractants, the presence of cucurbitacins in other plant tissues might not explain adult abundance in the field.

In conclusion, we show that domestication of *C. argyrosperma* selected for a decrease in cucurbitacins in the roots and cotyledons via the lower expression of cucurbitacin-related genes. As predicted, lower levels of cucurbitacins in domesticates differentially affected the performance of the generalist and specialist herbivores. Interestingly, both herbivores are highly attracted to high-cucurbitacin plants revealing that at least for the generalist insect there may be opposing selective pressures driving patterns of host plant selection by the adults and the performance of the larvae. Most studies examining the impact of plant domestication on insect herbivores have focused on insects that feed aboveground. To our knowledge this is the first study specifically designed to examine the consequences of plant domestication on insects that feed belowground. Moreover, by studying these plants and insects in their region of origin and domestication, our study provides unique insights into the evolutionary trajectories of wild and domesticated plants, as well as the natural and human-mediated selective pressures that have molded the interactions we see today.

Author contributions

C.J and B.B originally formulated the idea and designed the experiments. C.J performed lab experiments. C.J. and C.B.S. performed the field experiment. G.C developed the cucurbitacin extraction method. C.J and C.B.S analyzed the data. W.Y designed the primers and performed gene expression experiments. I.K. provided logistic support and guidance during experiments conducted at Purdue university. C.J. and B.B. wrote the first versions of the manuscript and all co-authors contributed to the final versions.

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Supplementary data:

Table S1. Genes involved in the cucurbitacins pathway in *Cucumis sativus* and orthologs found in *Cucurbita argyrosperma* with their functions.

Query gene in cucumber	Orthologs in squash	Putative Function in cucumber
<i>Csa6G088690</i>	<i>Carg11552-RA</i>	oxidosqualene cyclase (ortholog of cucurbitadienol synthase)
<i>Csa6G088160</i>	<i>Carg03795-RA</i>	cytochrome P-450 enzymes (P450s)
<i>Csa6G088170</i>	<i>Carg11550-RA</i>	cytochrome P-450 enzymes (P450s)
<i>Csa6G088710</i>	<i>Carg03797-RA</i>	cytochrome P-450 enzymes (P450s)
<i>Csa3G698490</i>	<i>Carg07313-RA</i>	cytochrome P-450 enzymes (P450s)
<i>Csa3G698490</i>	<i>Carg07314-RA</i>	cytochrome P-450 enzymes (P450s)
<i>Csa1G044890</i>	<i>Carg06672-RA</i>	cytochrome P-450 enzymes (P450s)

Table S2. Primers used for qRT-PCR.

Genes	Forward Primer	Reverse Primer
<i>Carg11552-RA</i>	AGATGGAGGGTGGGGTCTAC	TCTCCAAGCAGCCTTAGTGC
<i>Carg03795-RA</i>	GCCCACTTCTACTGCCACAT	CATCCCCACCCTCAAACCTC
<i>Carg11550-RA</i>	CCCTTCTCGCAAATGGGTCT	CTAAACATGGCGTGGCGAAG
<i>Carg03797-RA</i>	AGCCGTCCGTCTGTTGAAAT	TTCAACGGGACCGCCATAAA
<i>Carg07313-RA</i>	CGAAATTCGCGTTCGGAGAC	TGATCGCTCAACTTGTCGCT
<i>Carg07314-RA</i>	CGCGCATTATCGCAAACAGA	AGGAGTGTCTCCGGCTGTAT
<i>Carg06672-RA</i>	CGCTGGCGGTTGCTTATTAC	AGCCAACTGAAGCGTCTCTC



Figure S1. Design for the choice-experiment with *D. balteata* on roots of two squash varieties with different cucurbitacin content. Position of the plant varieties in the squared petri dishes were alternated. Always five larvae were put in the middle of the petri dish by the hole on the lid. The red cellophane is used to mimic darkness as the UV light cannot go through.

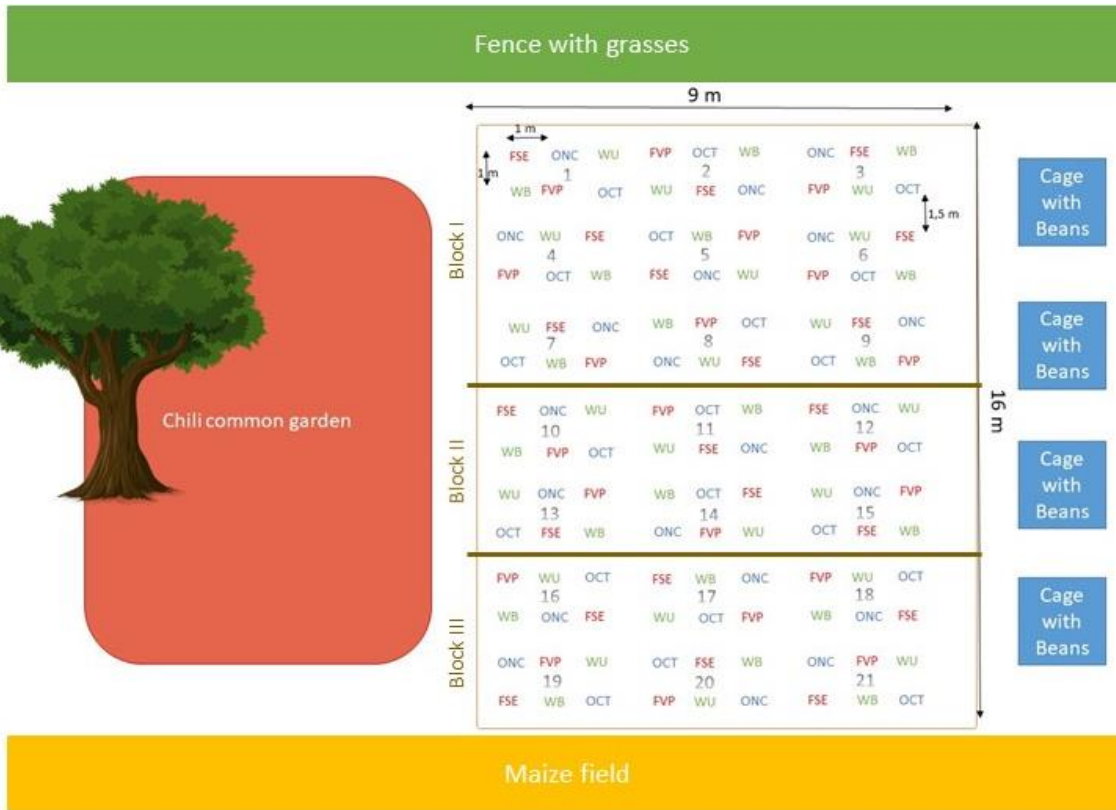


Figure S2. Design for field experiment in Puerto Escondido, Oaxaca, Mexico. Twenty-one plots containing each plant ID (wild populations or cultivars of *C. argyrosperma*). The plots were surrounded by grasses, chili common garden, maize field and beans.





	
<p>1-25% (Cat. 1)</p>	<p>26-50% (Cat. 2)</p>
	
<p>51-75% (Cat. 3)</p>	<p>76-100% (Cat. 4)</p>

Figure S3. Category of leaf damage.

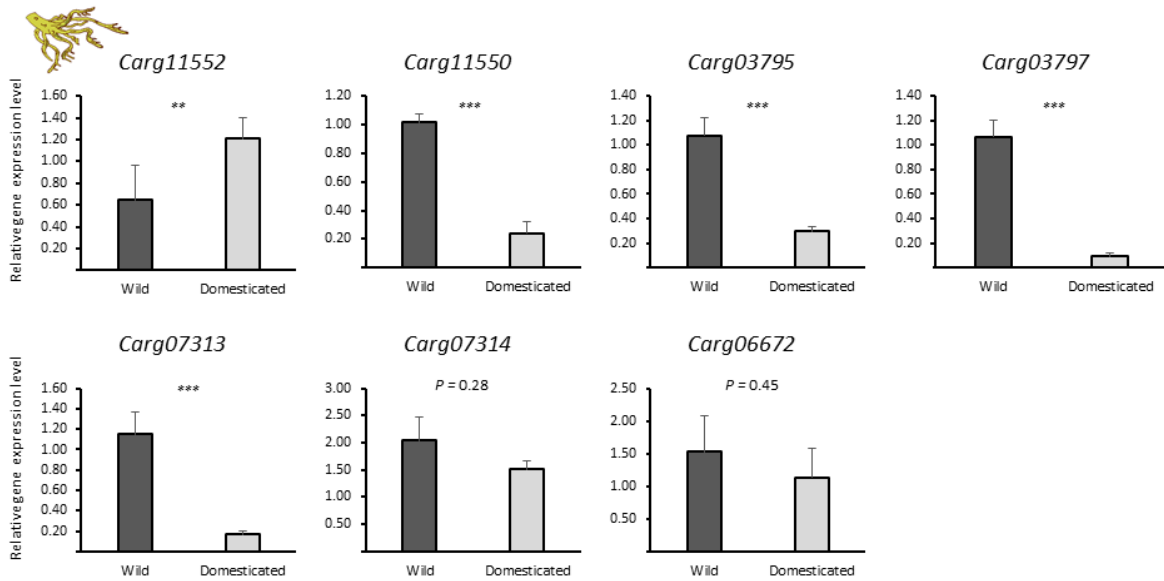


Figure S4. Relative gene expression of seven cucurbitacin biosynthetic genes in roots of two wild populations (WB and WU, in dark grey) of *Cucurbita argyrosperma*, and four related domesticated varieties (FVP, FSE, OCT and ONC in light grey). Shown are means (\pm SE). Letters indicate significant differences among wild populations or varieties ($P < 0.05$, Kruskal-Wallis test followed by Dunn's multiple comparisons test).

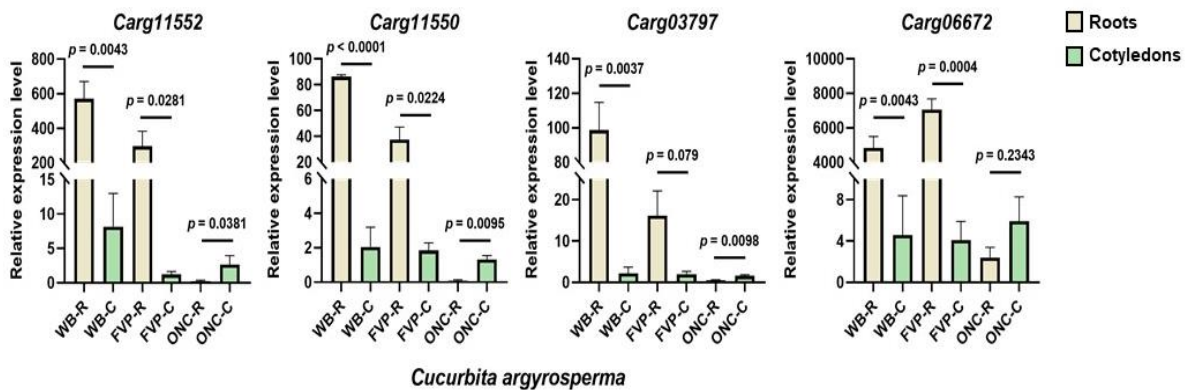


Figure S5. Relative gene expression levels of four cucurbitacin biosynthetic genes in roots (-R) and cotyledons (-C) of one wild population of *Cucurbita argyrosperma* and two related varieties, one selected for the fruit consumption (FVP) and one selected for an ornamental purpose (ONC). Shown are means \pm SE. P-value indicate the difference in gene expression between the two-plant tissue (roots and cotyledons) of the same wild population/variety.

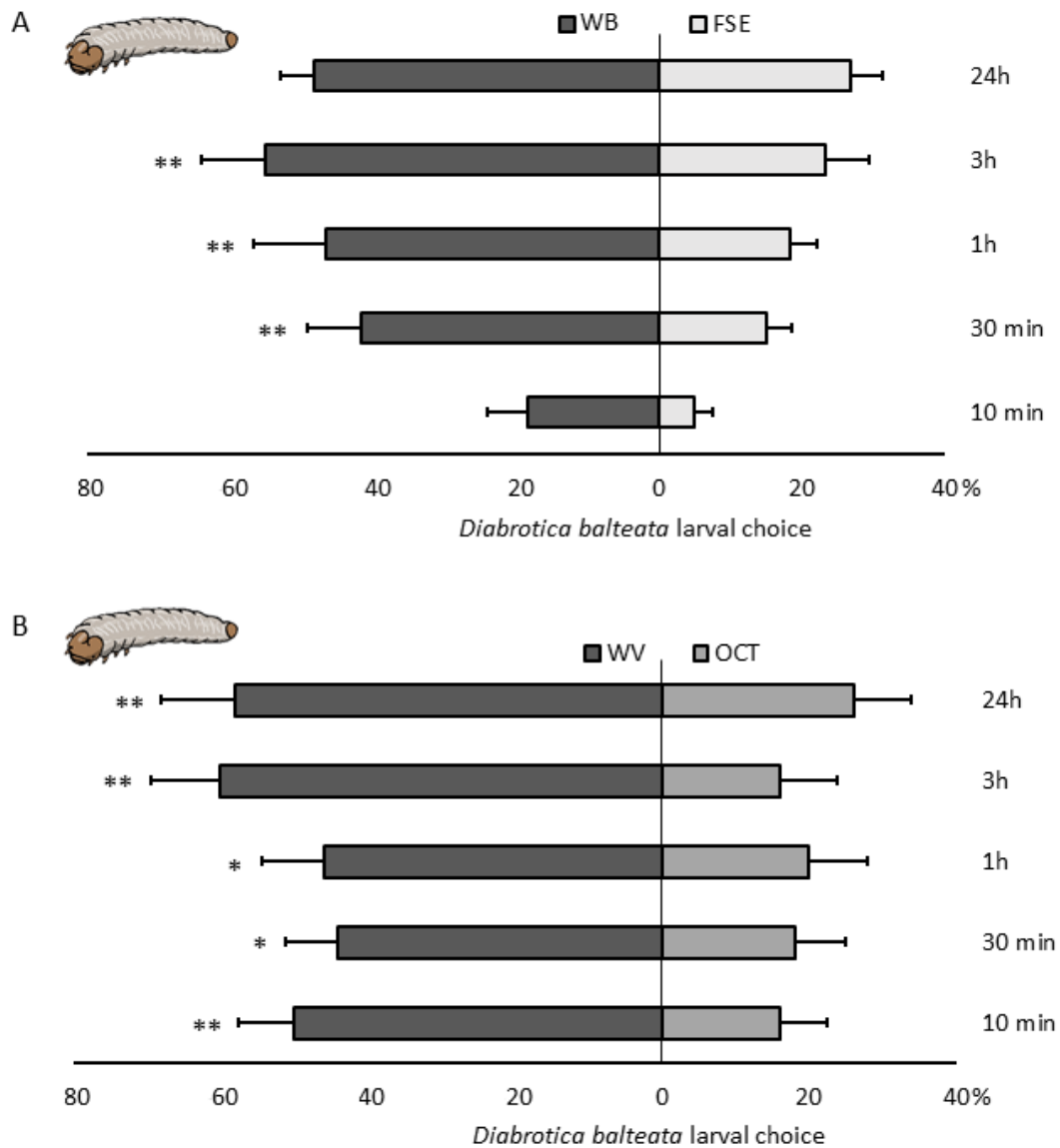


Figure S6. Feeding preference of *Diabrotica balteata* larvae when given a choice between roots from wild population (WB or WV) containing cucurbitacins and roots from domesticated varieties for fruit consumption (FSE) or ornamental use (OCT) without cucurbitacins. Larval choice was recorded as number of larvae on roots after 10 minutes, 30 minutes, 1, 3 and 24 hours after the start of the experiment. Bars are means \pm SE express the percentage of larvae that chose a treatment per time point (y axis). Bonferroni corrected P values are given for treatment comparisons [generalized linear model (family, Binomial)], followed by pairwise comparisons of Least Squares Means (LSM). * P < 0.05, ** P < 0.01, *** P < 0.001 . (A: P < 0.0005, ANOVA, N=12, B: P < 0.05, ANOVA, N=22).

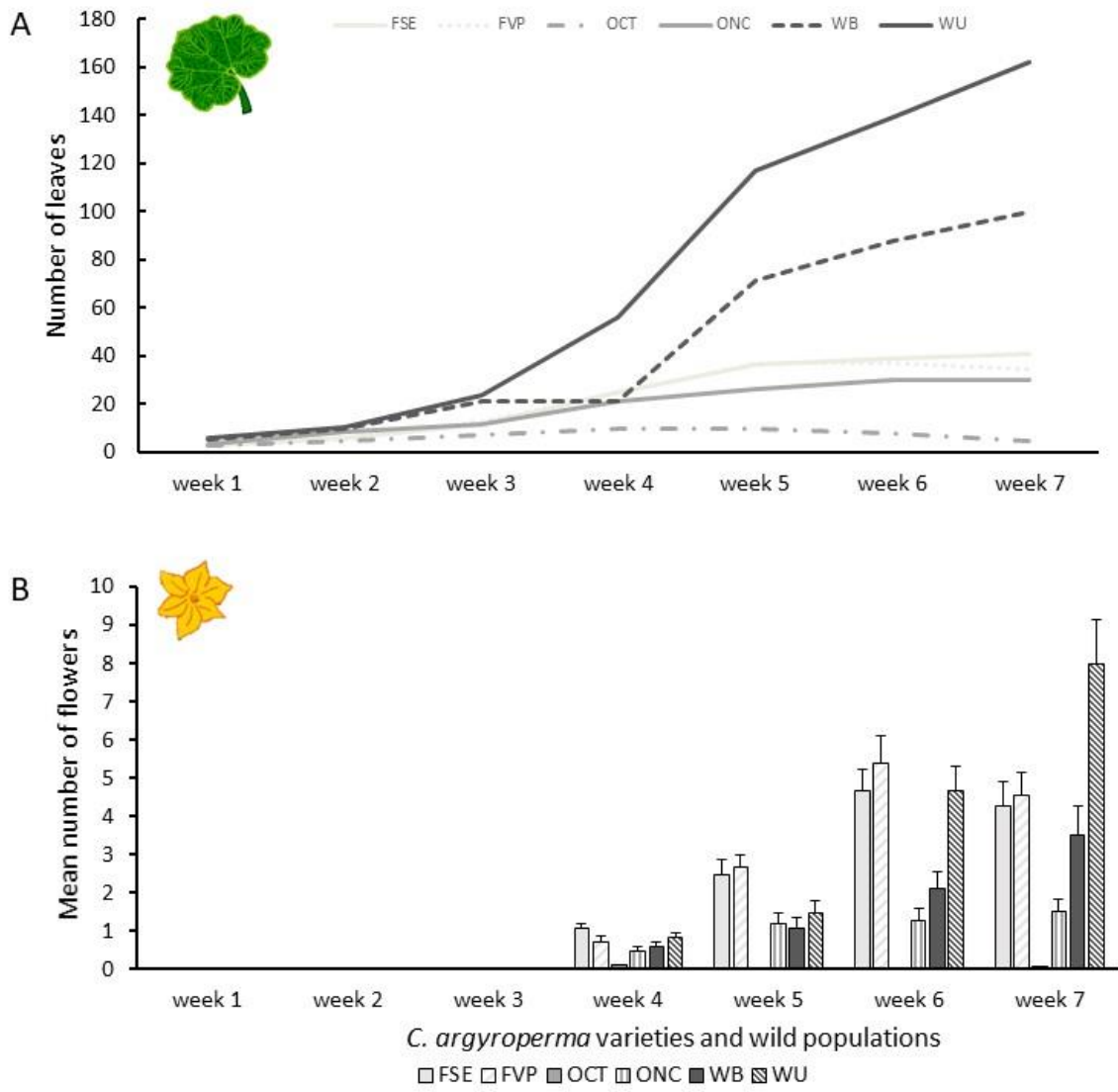


Figure S7. Mean number of leaves (A) and flowers (B) per *C. argyrosperma* varieties and wild populations in common garden through seven weeks.

Chapter 3: The effects of domestication of squash, *Cucurbita argyrosperma* on a belowground tritrophic interaction

Charlyne Jaccard, Nicolas Marguier, Carla C. M. Arce, Pamela Bruno, Gaëtan Glauser, Ted C. J. Turlings and Betty Benrey

Abstract

The domestication of squash started 10'000 years ago and has led to the loss of specialized secondary compounds called cucurbitacins. Cucurbitacins are toxic triterpenes that are considered to be part of the plant's natural defense against herbivores. The banded cucumber beetle (*Diabrotica balteata*), a generalist herbivore, is adapted to feed on plants from the Cucurbitaceae and is known to sequester cucurbitacins, supposedly for its own defense. However, the evidence for this is inconclusive. In this study we tested the impact of squash domestication on the chemical protection of *D. balteata* larvae against the predatory rove beetle (*Atheta coriaria*). We found no evidence for our hypothesis that cucurbitacins play a role in larval defense against this soil dwelling predator. In fact, *D. balteata* larvae were less attacked when they fed on cucurbitacin-free roots of domesticated varieties compared to high-cucurbitacin roots of wild plants. This study appears to be the first to look at the consequences of plant domestication on belowground tritrophic interaction. Our results challenge the generalized assumption that sequestered cucurbitacins protect this herbivore against natural enemies, and instead reveals an opposite effect that may be due to a tradeoff between coping with cucurbitacins and resisting predation.

Key words: Cucurbitacins₁, Diabrotica₂, domestication₃, predator₄, squash₅, sequestration₆

Introduction

For thousands of years, humans have imposed strong selection on domesticated crops. This has drastically altered crop phenotypes as compared to their wild relatives^{1,2}. Domesticated plants have larger plant structures, are more nutritious and have greater yields. In addition, crop selection has reduced plant architectural complexity^{3,4} and plant specialized metabolite diversity^{5,6}. Empirical evidence indicates that domestication has unintentionally decreased plant defenses against herbivores⁷⁻¹⁰. Indeed, one of the most important consequences of plant domestication is the loss or reduction of plant specialized compounds that are toxic for herbivores^{8,11-13}, therefore making plants more vulnerable to herbivory. For different crops, domestication has a direct impact on the herbivore's oviposition¹⁴⁻¹⁷, growth^{15,18,19} and survival^{3,15,20} and consequently has an indirect impact on the third trophic level^{8,21}. This, because natural enemies of herbivores obtain their nutrients from their hosts which in turn get theirs from the plant. Thus, the quality of a host for a natural enemy (predators and parasitoids) is directly linked to the plant's chemistry and nutritional content²².

Studies that have investigated the effect of plant domestication on natural enemies show that, overall, they perform better on herbivores that feed on domesticated crops than on their wild counterparts^{19,20,23-25}. However, most of these studies have focused on aboveground herbivores and their associated natural enemies⁸. Little is known on the impact of plant domestication on belowground plant-insect interactions, particularly on how altered chemical traits in domesticated plants have influenced the susceptibility of belowground herbivores to soil predators.

One classical example of reduction in chemical defense as a result of crop domestication involves species of squash, *Cucurbita* spp.. There is detailed knowledge on the history of domestication of *Cucurbita*²⁶⁻²⁹ but the consequences of squash domestication for the interaction between herbivores and their natural enemies have hardly been explored. *Cucurbita* plants were domesticated during different independent events in the American continent³⁰⁻³². The oldest evidence for squash domestication dates to about 10'000 years ago in Mesoamerica along with maize and beans³³. One of the main traits altered during the domestication of squash is the loss of cucurbitacins in the different plant organs²⁷. These defense metabolites are oxygenated tetracyclic triterpenes, are extremely bitter and render plants toxic or unpalatable to many invertebrates and vertebrate herbivores, including humans^{34,35}. However, cucurbitacins can also serve as kairomones (semiochemicals that benefit certain receivers)³⁶ for a number of specialized phytophagous chrysomelid beetles of the Old World tribe Luperini, in the genus *Aulacophora*³⁷⁻⁴⁰. Both squash and Diabroticine beetles share origins in Mesoamerica and have coevolved over an extended period of time^{36,41,42}. Earlier studies have shown that those beetles have overcome the chemical defense of *Cucurbita* via physiological adaptations to bitter and toxic cucurbitacins and use them as attractants and possibly as feeding stimulants⁴¹. Moreover, it has been hypothesized that species of *Diabrotica* actively seek bitter squash plants to accumulate cucurbitacins in their body and use them as a defense against natural enemies^{35,43}. This hypothesis was first supported by Ferguson and

Metcalf ³⁵, who showed that a significant proportion of laboratory-reared adult beetles of *Diabrotica* species (*D. balteata*, *D. undecimpunctata howardi* and *D. virgifera virgifera*) and of *Acalymma vittatum* fed on varieties of bitter squash, were rejected by Chinese praying mantis. Cucurbitacins were also shown to be efficient against pathogenic fungi that infect eggs and larvae of *Diabrotica undecimpunctata howardi* ^{44,45}. However, the evidence that cucurbitacins effectively protect herbivores against natural enemies is scarce and has been challenged ⁴⁶⁻⁴⁹. Indeed, while several studies showed that herbivores can sequester cucurbitacins from their host plants, they did not find evidence for their protective role against a diverse array of natural enemies (arthropods, birds, mice and toads) ^{45,46}. For example, Barbercheck, et al. ⁴⁹ found that infection by entomopathogenic nematodes was not different between rootworm larvae (*D. undecimpunctata howardi*) fed on bitter or non-bitter squash, although the nematodes' fecundity was lower when their hosts fed on bitter squash compared to corn, peanuts or non-bitter squash. Other studies have mostly examined the role of herbivore-sequestered cucurbitacins against natural enemies in adult beetles, that feed on aboveground tissues, and/or their eggs. However, for most squash species, the content of cucurbitacins is higher in the roots than in aboveground tissues ³⁷. Moreover, the earlier studies exclusively used domesticated varieties of cucurbits (either cucumber or squash) with varying levels of cucurbitacin but that in general contain very low levels of these compounds ^{27,32} as compared to their wild counterparts (Jaccard et al unpublished results).

In this study, we tested the hypothesis that the reduction of cucurbitacins in the roots of domesticated *Cucurbita argyrosperma* affects the susceptibility of a root larval-herbivore to a generalist predator. To do this, we compared the preference and performance of a generalist soil predator (*Atheta coriaria*) when offered larvae of *Diabrotica balteata* fed on roots of wild *C. argyrosperma* with expected high cucurbitacin content or on roots of related domesticated varieties where the cucurbitacins ought to be nearly absent.

Material and methods

Plants

Cucurbita argyrosperma, known as “calabaza pipiana” is an important crop in local agricultural systems in Mexico⁵⁰. The oldest evidence of its domestication is ~8,600 years old from the Xihuatoxtla shelter, in the state of Guerrero⁵¹. Plants can be found in tropical and semi desertic regions from the Southeastern United States through Mexico and Northern Central America³³. This is a diverse species in form, color and size of its seeds and fruits⁵².

Fruits of two wild *C. argyrosperma* populations (WU and WB) were collected in the region of Puerto Escondido along the Pacific coast in the state of Oaxaca, Mexico in January 2018, and their seeds were harvested (WU: 15.924929, 97.150977; WB: 15.864463). The weather conditions are hot and humid with an average temperature of 27°C and 84% relative humidity. In this region, domesticated plants coexist side by side with their wild relative³³. Seeds of domesticated varieties were purchased from the KCB-Samen GmbH Bottmingen, Schweiz. We used four varieties selected for two different purposes, two varieties selected for fruit consumption: Vera Cruz Pepita (FVP) and Silver Edge (FSE) and two varieties selected as ornamentals: Cushaw Tricolor (OCT) and Navajo calabacita (ONC). These varieties had been previously used in a related study in which we found differences in their cucurbitacin content (Jaccard et al. unpublished results).

Depending on the experiment, either one individual seed or seven seeds of domesticated varieties were germinated in one individual plastic pot filled with a mixture of soil:sand 70:30 (Einheitserde, Sinntal-Altengronau, Germany). As wild seeds take longer to germinate, they were planted 10 days before the domesticated seeds. Also, wild seeds were subjected to a specific germination procedure to enhance their germination rate: seed coats were pierced, scratched on both sides and placed in groups of 10 in a square Petri dish with wet cotton for one week in an incubator at 28 °C degrees in the dark. Germinated wild seeds were transferred following the same procedure as seeds from domesticated varieties. Plants with two cotyledons and two fully developed leaves (15 day-old) were used for all the experiments. All plants were grown under controlled conditions in a greenhouse (24 ± 5°C, L: D 16:8h) and watered every other day.

Insects

The banded cucumber beetle (*Diabrotica balteata* (LeConte, 1865, Coleoptera: Chrysomelidae)) originates from the tropical Americas⁵³⁻⁵⁵ and is considered a pest of agricultural crops including beans, sweet potatoes and cucurbits. Larvae feed belowground on roots, while adults eat leaves and flowers^{53,55}. Eggs used to establish our *in-house* colony were supplied by Syngenta (Stein, Switzerland). Emerging larvae were maintained in soil (Sinntal-Altengronau, Deutschland), fed on roots of four-day-old maize seedlings (Hybrid DFI 45321, DEFI genetics AG, Switzerland), and kept at 25 ± 2°C, 60% RH, and 16:8 h L/D cycles. First and second instar larvae were used in all experiments.

The rove beetle (*Atheta coriaria* (Kraatz, Coleoptera: Staphylinidae)), is a soil-dwelling predator used as a biological control agent for certain greenhouse pests⁵⁶. Both larvae and adults feed on various arthropod pests⁵⁷. It is a generalist predator that did not coevolve with chrysomelids and is not expected to be adapted to the potentially deterrent and toxic effects of cucurbitacins⁵⁸. Eggs were purchased from Andermatt Biocontrol (AG, Switzerland) and a laboratory rearing was established. Predators were kept in controlled conditions (24°C, 60% R.H. and L:D 16:8h) with coconut fiber and vermiculite as a substrate and fed on oat and dog pallets. Adults of *A. coriaria* were used in all experiments.

Cucurbitacin content in roots of wild and domesticated plants and in larvae of D. balteata

To verify the cucurbitacin content in the squash roots of wild accessions and domesticated varieties and in the larvae that fed on these roots, we quantified the cucurbitacin content in the roots of the different plants ($N= 4$ plants/host plant treatment) and in the larvae of *D. balteata* ($N= 3$ per time point) fed on roots of either wild or domesticated plants. The cucurbitacin content in the larvae was measured with dead larvae, since the predation bioassays were performed with frozen larvae.

Roots of two-week-old plants of *C. argyrosperma* were harvested, immediately flash frozen and ground to a fine powder in liquid nitrogen. Then 100 mg of sample powder was weighed with a microbalance (Mettler Toledo XP&, Columbus, Ohio, USA) and put in a 1.5 mL Eppendorf tube. We added one mL of MeOH 100% and five glass beads per tube.

Larvae of *D. balteata* fed on roots for seven days were collected and immediately frozen at -80°C. Larvae were taken out of the freezer and we quantified the content of cucurbitacins in the larval body after 2h and 24h. Twenty mg (12 larvae in average) per sample was weighed with a microbalance (Mettler Toledo XP&, Columbus, Ohio, USA) and put in a 1.5 mL Eppendorf tube to which 250µl of MeOH 100% was added. The samples were ground with a pellet pestle, then another 250µl of MeOH 100% were added (total of 500 µl in the tube per sample).

All samples (roots and larvae) were vortexed and centrifuged at 4°C (10 min, 9000 rpm). The supernatants from the larvae samples were filtered (13 mm Syringe filter, PTFE hydrophilic, 0.22 µm, BGB, CHE) before the analysis. Supernatants were used for analysis by liquid chromatography-mass spectrometry according to³⁷

Briefly, the analysis of cucurbitacins was performed by UHPLC-QTOFMS using an Acquity UPLC™ coupled to a Synapt G2 high-resolution mass spectrometer (Waters, Milford, USA). The column used for chromatography was an Acquity UPLC BEH C18 1.7µm, 2.1 x 50mm (Waters, Milford, USA). Data acquisition was performed with the software Masslynx™ v.4.1 (Waters, Milford, USA). Cucurbitacins were identified based on their molecular formula and fragmentation patterns provided by accurate mass measurements, and using available databases such as the Dictionary of Natural Product (CRC Press). In some cases, the presence of several possible cucurbitacin isomers prevented full identification. Peaks corresponding to

known cucurbitacins were automatically integrated using TargetLynx XS™ with a 0.1 min chromatographic window centered on the retention time of each component and a 0.02 Da mass window centered on the (M+HCOO) ion. Quantification of all cucurbitacins was done by external calibration using cucurbitacin B solutions at 0.02, 0.08, 0.4, 2, 5 and 10 µg/ml. The cucurbitacin concentration was expressed in µg per g of plant or larva material.

Predation of Diabrotica balteata larvae fed on different wild populations and varieties of squash

With this experiment, we tested the hypothesis that predators will be repelled when exposed to high-cucurbitacin larvae of *D. balteata*. For this, we evaluated the preference of *A. coriaria* adults for *D. balteata* larvae fed on roots either of wild populations or domesticated varieties in a choice test. Second instars were fed on high-cucurbitacin roots of two wild populations of *C. argyrosperma* (WB and WU) and no-cucurbitacin roots of four domesticated varieties (FVP, FSE, OCT and ONC) for seven days. Then, larvae were frozen at -80°C. The predation assay was performed with dead-frozen larvae to allow us to distinguish among the different treatments (high-cucurbitacin larvae versus no-cucurbitacin larvae). The experiment was performed in a red Petri dish to simulate darkness (as in the soil) (Sarstedt, Ø 16mm, Germany). To maintain humidity, 850 µl of water was added on the filter paper placed in the Petri dish. Adults were starved for 12 h prior to use. One *D. balteata* larva fed on roots of wild squash (either WB or WU) with cucurbitacins was placed on one side on the Petri dish, and on the opposite side (approximately 7 cm distant from each other), one larva fed with no-cucurbitacin roots (either FSE, FVP, OCT or ONC). One adult rove beetle was released in the center of the arena. Petri dishes were sealed with parafilm to prevent the predator from escaping. A total of eight combinations were performed, always one high-cucurbitacin larva against one no-cucurbitacin larva: WB x ONC, WB x OCT, WB x FSE, WB x FVP, WU x ONC, WU x OCT, WU x FSE, WU x FVP with 20 replicates per combination and the experiment was performed twice. The predation rate was recorded after 24 hours. We considered predation when the hemolymph of the larva was leaking from the body or when body parts were removed (Figure S1). If the larva had one of these visible injuries, it was recorded as a predation event. Otherwise, larvae without any signs of damage/attack were considered not predated. Petri dishes without a predator were kept as comparison (control) for the predation damage/attack, to account for natural deterioration of the dead larvae.

Predator survival on Diabrotica balteata larvae fed on different wild populations and varieties of squash

We tested the hypothesis that cucurbitacins sequestered by *D. balteata* larvae can be deleterious for the predators. Thus, we evaluated *Atheta coriaria* adult's survival when fed on dead *D. balteata* larvae previously reared on wild populations (high-cucurbitacin roots) or domesticated varieties (no-cucurbitacin roots) of *C. argyrosperma* for seven days. The predators were individually placed in a tray and fed with one of seven diet

treatments (n=32; see Supplementary Fig 2): *D. balteata* larvae that fed on either roots of two wild populations (WB and WU) or roots of four domesticated varieties (FVP, FSE, OCT and ONC) and one control diet consisting of frozen eggs of *Ephestia* sp. (Pyralidae) (Andermatt Biocontrol, Switzerland). Transparent stickers were used to cover the top of each cell to prevent predator escape. We added a moist filter paper on the bottom of each cell as a source of water and to maintain humidity. For seven days, every 24 hours, predator survival was evaluated and the diet was replaced. The trays were kept in the dark to simulate soil darkness. If a predator escaped the cell, the replicate was removed from the analysis (between one to five replicates were lost per treatment).

Performance and survival of Diabrotica balteata larvae on roots of wild populations and varieties in the presence of a predator

In the previous experiments the predators were exposed to *D. balteata* larvae after they fed on the different plant treatments. In this experiment, we wanted to examine the role of the plant on the predator's response and mimic more closely the natural environment. Thus, larvae were exposed to the predator while still feeding on the plant. We tested two different hypotheses. First, that larvae reared on roots of wild populations will suffer lower predation than larvae fed on domesticated varieties and secondly, that the predator survival will be higher on larvae fed with roots of domesticated varieties. In contrast, to the previous experiments, here we used live larvae. We carefully placed the root system of two-week-old squash plants (n=20, Figure S3) from all plants (two wild populations and four domesticated varieties) into a plastic bag. The plants were left for three days under laboratory conditions (L: D 16:8h) to acclimate to the new environment. Then, we added ten, second instars of *D. balteata* in each plastic bag and allowed them to move and feed freely on the roots. Bags were closed under the cotyledons with elastic film (Parafilm; Pechiney Plastic Packaging, Menasha, WI, USA) to prevent larvae from escaping (Figure S2). Larvae were left in the bags for five days, then on day 6, we added 20 predators (*A. coriaria*) in half of the bags of each plant treatment. The bags without predators were used as a control for survival and growth of *D. balteata* in the absence of predators. Bags were maintained in the lab at room temperature ($22^{\circ}\text{C} \pm 2^{\circ}\text{C}$) and natural light conditions (L: D 16:8h) for four days. To calculate *D. balteata* larval relative growth rate (RGR), the total weight of the ten larvae was recorded at the start of the experiment and then nine days later. For both time points, the individual mean larval weight was calculated by dividing the sum of the weight of the initial (start of the experiment) or recovered larvae (end of the experiment) by the total number of larvae present. At the end of this period, the presence and survival of both *D. balteata* larvae and predators was recorded. The four-day period was chosen to allow enough time for predation and before the pupation of *D. balteata* larvae.

Statistical analyses

Statistical tests were carried out with R statistical software (v. 4.0.0; R Development Core Team, 2020) and its complementary console R-studio (<http://www.rstudio.com>) using Analysis of Deviance (ANODEV; a maximum likelihood equivalent of ANOVA), followed by residual analysis to verify suitability of distributions of the tested models.

Kruskal-Wallis models followed by a pairwise comparison using Wilcoxon rank sum test with continuity correction were used to analyze the total amount of cucurbitacin content in the plant's roots. The cucurbitacin concentration in larval bodies was first log transformed before the analysis and then analyzed with a Linear Model (LM) followed by Least Squares Means (*LSMeans*) to compare treatments for the two different time points and the difference among wild populations and domesticated varieties. A partial least squares discriminant analyses (PLS-DA) and hierarchical clustering heatmap were carried using MetaboAnalyst 5.0 to check for differences in cucurbitacin profile among plants and larvae of the wild populations.

Generalized linear model (*glm*) with binomial distribution was used to evaluate the effects of treatment on predator preference. and Least Squares Means (*LSMeans*) were used to compare differences among treatments.

Relative growth rate (RGR) of *D. balteata* larvae in the presence or absence of the herbivore was first log-transformed, then analyzed with a Linear Model followed by Least Squares Means (*LSMeans*) to compare among host plant treatments. The RGR difference among squash domestication purposes (consumption or ornamental) was analyzed with a generalized linear mixed model (*lmer*) with varieties as a random factor.

Larval survival of *D. balteata* was evaluated with a Linear Model (*lm*) followed by Least Squares Means (*LSMeans*) to compare among host plant treatments. The survival difference among squash domestication purposes was analyzed with a generalized linear mixed model (*lmer*) with varieties as a random factor.

The survival of the predator on *D. balteata* larvae previously fed on different squash roots was analyzed using Kaplan-Meier estimator by log-rank method in the Sigma Plot software (v. 11; Systat Software Inc., San Jose, CA, USA).

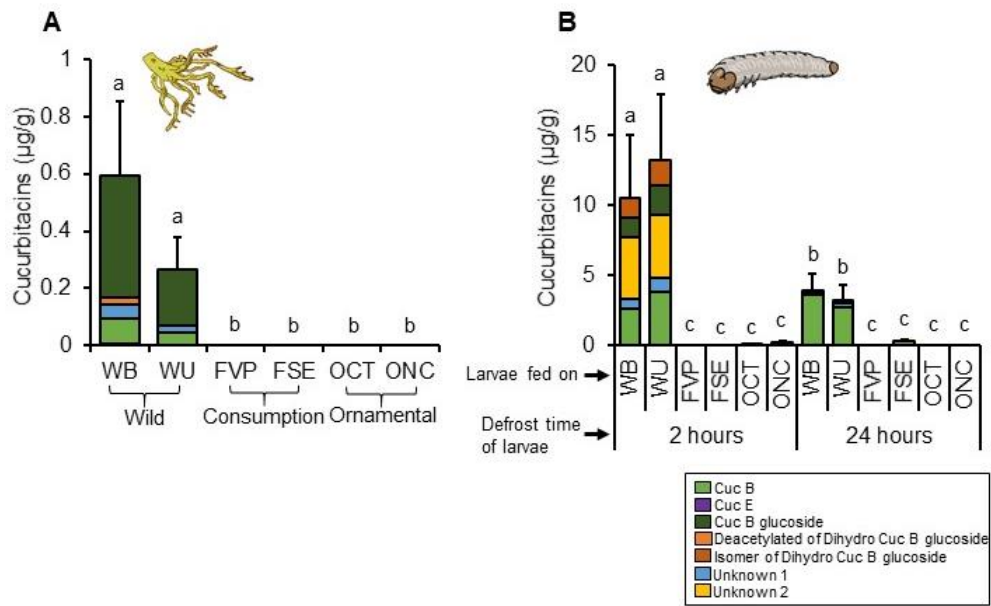
Results

Cucurbitacin content in roots of wild and domesticated plants and in larvae of D. balteata

The total amount of cucurbitacins in the roots was significantly different between wild populations and domesticated varieties ($\chi^2_{(5)} = 26.805$, $P = 0.0001$, Fig. 1A). Roots of domesticated varieties both for consumption and as ornamentals, did not contain cucurbitacins, whereas cucurbitacins were present in high concentrations ($\mu\text{g/g}$) in roots of the wild populations. No significant differences in cucurbitacin content were found between the two wild populations (WB-WU: $P = 0.264$, Fig. 1A).

Accordingly, after two hours of defrosting, larvae that fed on roots of wild plants contained cucurbitacins in their body, while larvae that fed on the domesticated varieties did not ($F_{3,12} = 10.61$, $P = 0.001$; Fig. 1B). No significant difference was found in cucurbitacin content in the body of larvae that fed on roots of the two wild populations ($t = 0.615$, $P = 0.925$, Fig. 1B). After 24 hours out of the freezer, the cucurbitacin content in the larvae drastically decreased and the difference between larvae fed on wild plants and domesticated varieties was smaller ($F_{3,12} = 3.481$, $P = 0.038$). Results clearly show that the larvae actively sequester and accumulate the cucurbitacins since the levels in their body are much higher compared to the amount found in the roots (Figure 1A and 1B). Interestingly, the cucurbitacin profiles differed between the roots and the larvae that fed on these roots (Fig. 1C and 1D). Two cucurbitacins (Cucurbitacin E and deacetylated dihydro cucurbitacin B glucoside (or isomer) were exclusively found in the roots (Table S1).

In the larvae after 2h out of the freezer, we found in total five compounds and after 24h only three. The main difference observed between roots and larvae were after 2h, the larvae had high amounts of an isomer of dihydro form of Cuc B glucoside and an unknown Cuc (Fig. 1A-D). However, these compounds disappeared after 24 h. This difference could be due to either degradation or transformation during the defrosting process.



Wild Plant versus Larvae

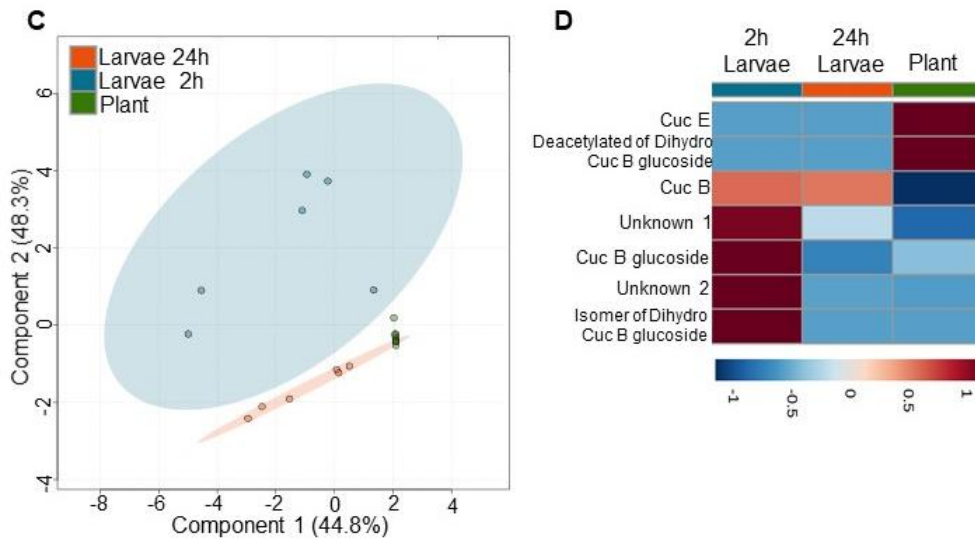


Figure 1. Differences in cucurbitacins profiled in *C. argyrosperma* roots and *D. balteata* larvae. **(A)** Cucurbitacin levels in squash roots (n=4) of 2-week-old plants of wild populations (WU and WB), consumption (FVP and FSE) and ornamental varieties (OCT and ONC). **(B)** Cucurbitacin content in 2 and 24 hours defrosted larvae (n=3) that fed on roots of wild plants, consumption and ornamental varieties for 7 days. Bars represent the mean (\pm S.E.). **(C)** Results of a discriminant analysis (PLS-DA) and **(D)** hierarchical clustering heatmaps of the cucurbitacins present in roots of squash and larvae that fed on these roots. Different letters indicate significant differences between treatments within each time point (P values are given for treatments [generalized linear model (family, Gaussian)] followed by pairwise comparisons of Least Squares Means (LSMeans). ** $P < 0.01$, *** $P < 0.001$).

Cucurbitacin content in Diabrotica balteata larvae does not affect predation by Atheta coriaria

The predator *Atheta coriaria* did not show any preference for *D. balteata* larvae that fed on high-cucurbitacin roots (WB and WU) or no-cucurbitacin roots (FSE, FVP, OCT and ONC) (Fig. 2). Also, predators were not repelled by the cucurbitacins present in the larvae. Predator preference was recorded as significant for two different combinations, both including ornamental varieties and the wild population WU. Nevertheless, the choice appears to be random as in one case predators significantly chose larvae fed with domesticated roots (OCT, no-cucurbitacin roots) over the larvae that ate wild squash roots (WU) ($P= 0.04$) and in the other case they showed the opposite pattern, larvae that fed on wild (WU, high-cucurbitacin roots) over larvae that fed on domesticated roots (ONC) ($P=0.03$).

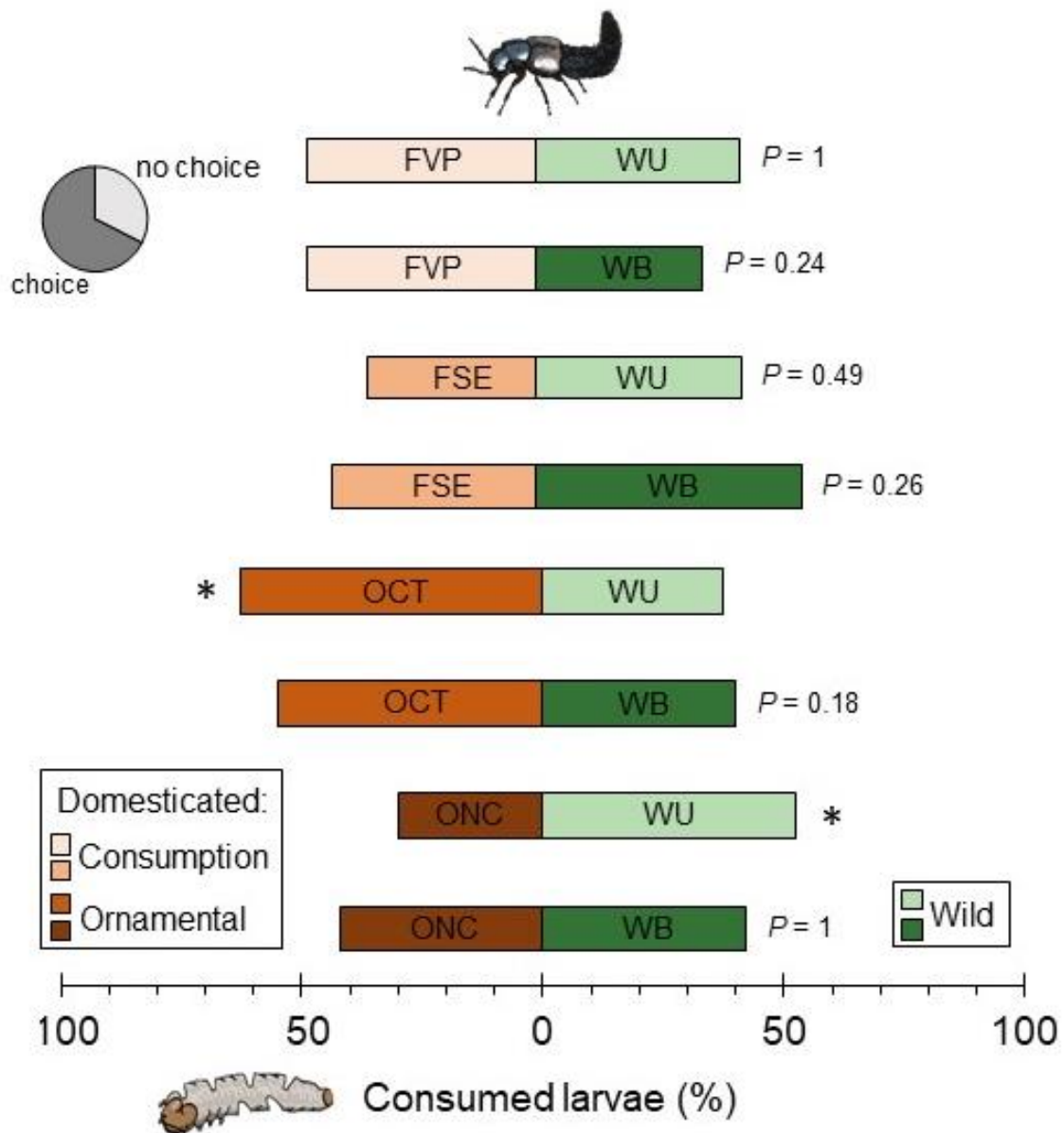


Figure 2. Predation preference by *A. coriaria* for *D. balteata* larvae that fed on roots of wild populations (n=40, WU= light green and WB= dark green) with cucurbitacins and larvae that fed on domesticated varieties (fruit consumption: FVP and FSE and ornamental: OCT and ONC, shades of orange, n=40) without cucurbitacins. Predation rate was evaluated after 24 hours. Bars are the percentages of predated larvae. Pie chart indicate the overall proportion of predators that ate or not during the assay. Bonferroni corrected P values are given for treatment comparisons (generalized linear model (family, Binomial)), followed by pairwise comparisons of Least Squares Means (LSM). * P < 0.05.

Predator survival is not affected by the content of cucurbitacins in Diabrotica balteata larvae

The survival of the predator (*A. coriaria*) was not affected by the content of cucurbitacins in *D. balteata* larvae fed with roots of wild or domesticated plants ($P=0.77$; Fig. 3). After seven days, overall predator survival was between 76% and 93% and no significant differences were found among plant treatments.

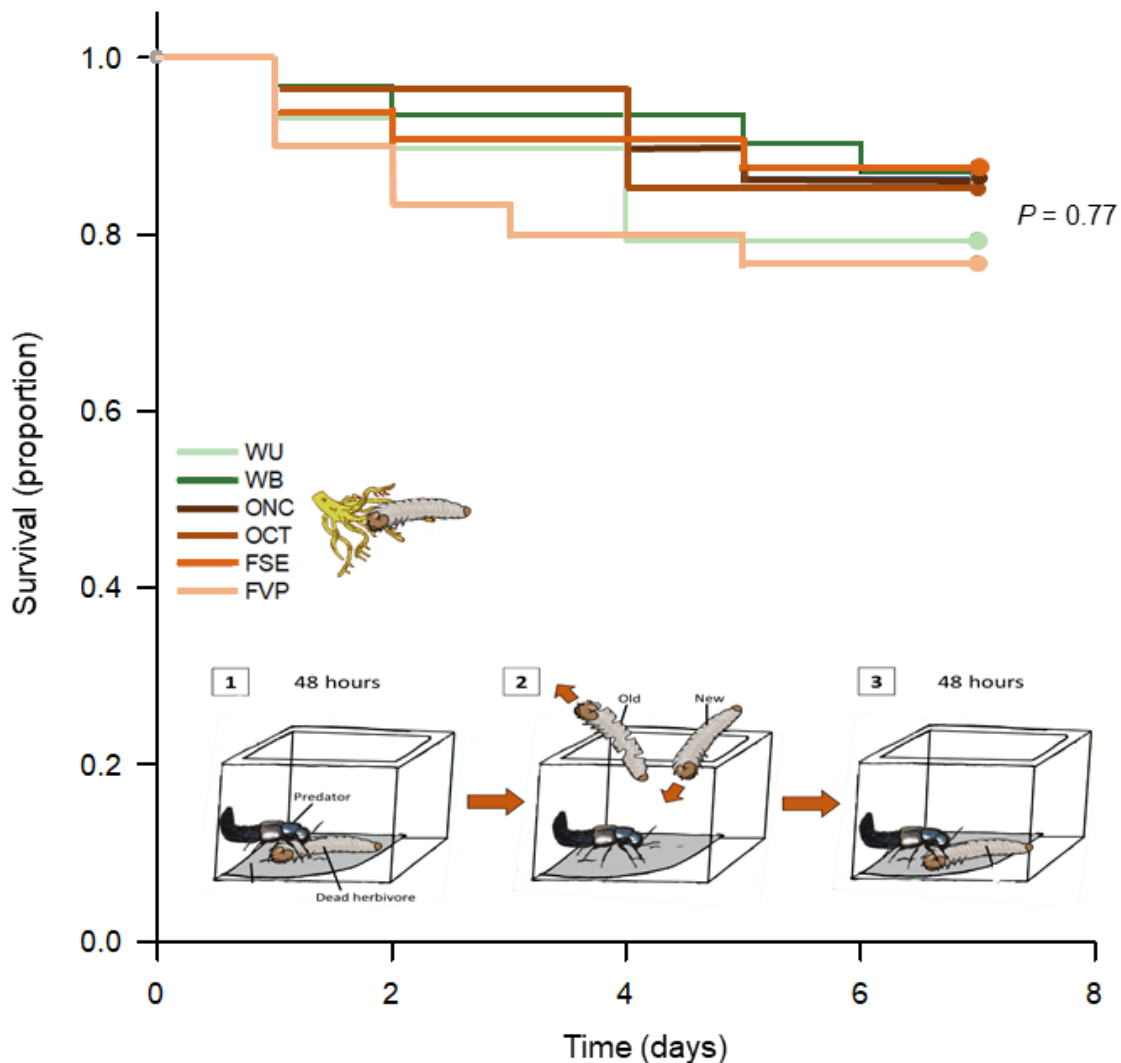


Figure 3. Survival of *Atheta coriaria* exposed to *Diabrotica balteata* larvae fed with roots of wild populations (WU= light green, WB= dark green n= 32) and domesticated varieties (fruit consumption: FVP and FSE and ornamental: OCT and ONC, shades of orange, n=32). The y axis indicates the proportion of survived predators. The x axis represents the time in days. Larvae were frozen 2 hours after collection from the squash roots and defrosted 30 minutes before the start of the experiment. The effect of treatment on predator survival was analyzed using Kaplan-Meier estimator by log-rank method.

Predation of Diabrotica balteata by Atheta coriaria is affected by larval plant-diet

The survival of *Atheta coriaria* was optimal when placed in the bags with the *Diabrotica balteata* larvae. Between 18 and 20 predators were consistently recovered from the bags at the end of the experiment. As the predators did eat the larvae of *D. balteata* (Figure S4), this indicates that the cucurbitacins present in the roots of wild plants did not affect their survival.

The RGR of *D. balteata* larvae was not significantly different among wild populations and domesticated varieties with or without predators (with predator: $F_{5,46}=0.75$, $P=0.59$, Fig. 4A, without predator: ($F_{5,49}=0.82$, $P=0.53$, Fig. 4B). Similarly, when pooled by domestication status or purpose of domestication (wild, fruit consumption and ornamental use), we did not find significant differences on larval RGR ($F_{2,49}=1.664$, $P=0.199$).

Conversely, predator presence had a significant effect on larval survival across plant treatments ($F_{5,48}=4.23$, $P=0.002$, Fig. 4C). When pooled by domestication status and purpose, survival of *D. balteata* larvae was lower when feeding on roots of wild populations than on roots of domesticated varieties selected for consumption ($F_{2,51}=10.506$, $P=0.0001$, Fig. 4C), but not different from larvae feeding on ornamental plants. However, in the absence of predators in the bag, no significant differences in larval survival were found among host plant treatments ($F_{5,50}=1.059$, $P=0.394$, Fig. 4D) nor purpose of domestication ($F_{2,53}=2.428$, $P=0.09$).

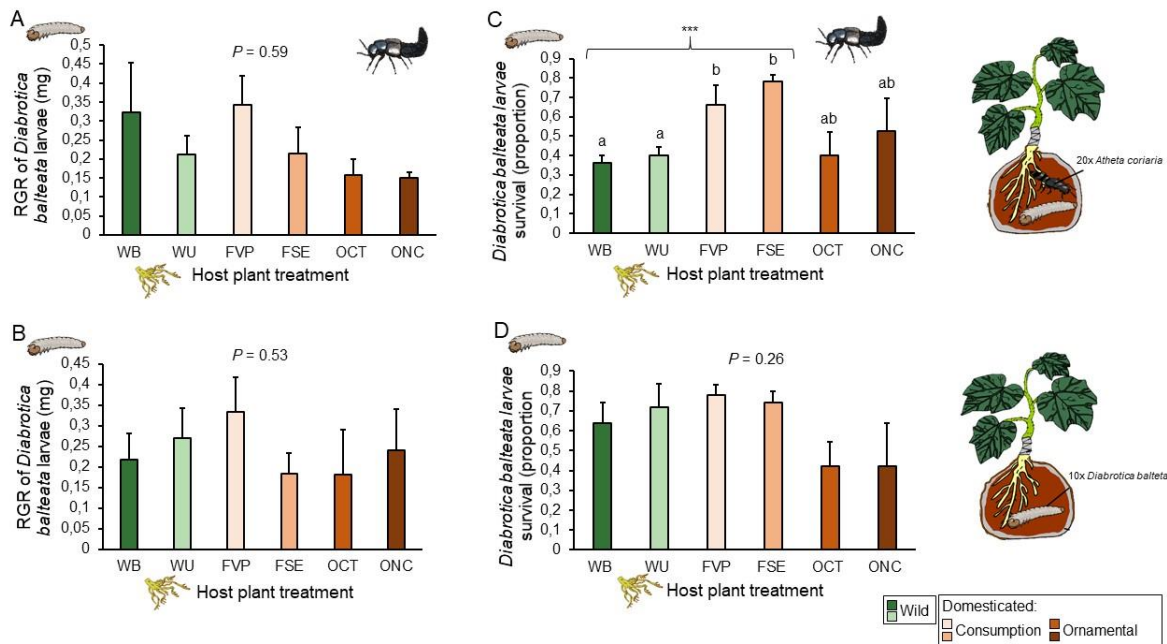


Figure 4. Relative growth rate of *Diabrotica balteata* larvae feeding on roots of wild populations of *C. argyrosperma* (WB= dark green, WU= light green, n=10) or domesticated varieties (FVP and FSE selected for fruit consumption, OCT and ONC selected for an ornamental use, shades of orange, n=10) with **(A)** and without **(B)** predators after nine days. Survival of *D. balteata* larvae with **(C)** and without **(D)** predators. Larvae fed on roots of wild populations of *C. argyrosperma* (WB= dark green, WU= light green, n=10) or with domesticated varieties (FVP and FSE selected for fruit consumption, OCT and ONC selected for an ornamental use, shades of orange, n=10) after nine days. Bars are RGR **(A and B)** or the proportion of survived larvae **(C and D)** (\pm S.E.). *P* values are given for treatment comparisons [generalized linear mode]. Letters indicate the difference on herbivore survival among host plant treatments with predator presence. *P*-values are given for differences among host plant treatments. *** indicates significant differences between wild populations and domesticated varieties domesticated for fruit consumption.

Discussion

The most important trait selected during the domestication of the genus *Cucurbita* (squash) is the absence of cucurbitacins in the fruits^{59,60}. For *C. argyrosperma*, cucurbitacins were also selected out from the roots of domesticated varieties. *Diabrotica balteata*, as many other species of Chrysomelidae in the subfamily Galerucinae is capable of sequestering cucurbitacins through the ingestion of plant tissue^{36,41,42}. The behavior of *Diabrotica* beetles toward cucurbitacins has been described as pharmacophagous because the benefits derived from eating these compounds are assumed to be ecological and not nutritional^{61,62}. Therefore, it has been commonly assumed that by sequestering these compounds, both larvae and adults avoid being eaten by natural enemies (Castellanos-Morales et al., 2018). Yet, the evidence for this assumption is inconclusive^{45,49}. To test this, we hypothesized that by feeding on high-cucurbitacin roots of wild plants, larvae of *D. balteata* would be more protected from the rove beetle predator *A. coriaria*, compared to larvae feeding on no-cucurbitacin roots of domesticated varieties. More precisely we predicted that by sequestering the cucurbitacins present in the host plant, the predators would be repelled and their survival negatively affected. As a result, we expected lower predation of *D. balteata* larvae fed with roots of wild plants than larvae fed with roots of domesticated varieties.

Contrary to our prediction, the domestication of *C. argyrosperma* did not have a negative effect on the studied predator. We also did not find evidence for cucurbitacins playing a role on the protection of *D. balteata* larvae against *A. coriaria*. The analysis of cucurbitacin content in the larvae confirmed that they do sequester these compounds when feeding on the roots of wild plants. The sequestration ability of *D. balteata* larvae was recently confirmed in a recent study with bitter and non-bitter cucumber plants (Bruno et al unpublished results). This study showed that *D. balteata* larvae fed on bitter cucumber plants, containing cucurbitacins, accumulated cucurbitacins in their bodies. Despite the sequestration ability of *D. balteata* larvae, predator survival was not different when exposed to beetle larvae fed with high-cucurbitacin roots (wild populations) or no-cucurbitacins roots (domesticated varieties). This was further confirmed by the results from the choice-test in which larvae from the different plant treatments were equally attacked by the predator. It should be noted that here we used adult predators; it would be interesting to examine the behavior and survival of the predator larvae when exposed to *D. balteata* larvae with and without cucurbitacins.

Of interest is the fact that cucurbitacin profiles were different between plants and larvae fed on these plants (Table S1). *Diabrotica balteata* larvae had high amounts of an isomer of dihydro form of Cuc B glucoside and an unknown cucurbitacin and these two compounds were not found in the plants. This finding highlights the fact that the cucurbitacins sequestered by *D. balteata* larvae are certainly metabolized into different compounds. Moreover, the quantity of cucurbitacins in the larvae was higher than in the roots. At the beginning of the predator survival and choice bioassays, the larvae from wild squash contained at least 50% more cucurbitacins than larvae that fed on

domesticated squash (Fig. 1B). After 24 hours, the trend was the same, but cucurbitacin content degraded, most likely due to larval decomposition.

One unexpected result of this study was that the RGR of *D. balteata* larvae was similar when fed on roots of wild plants and of domesticated varieties. In a different study using the same plant treatments we found very large differences in RGR rate; *D. balteata* larvae grew slower on wild roots than on roots of domesticated varieties (Jaccard et al unpublished results). We do not know the reason for these different results. The only plausible explanation is the difference in the duration between this experiment and the previous one, nine and six days, respectively. It may be that there is an effect of plant treatment only at the early stages of development and then these differences level off. Differences in development time, even for short periods, could have important consequences on the risk of predation, as the window of vulnerability to predators could be extended⁶³. Thus, it would be important to examine the effect of cucurbitacins throughout the whole duration of larval development. Unfortunately, this represents a big challenge as larvae complete their development inside the soil and cannot be observed and weighed without disturbing them.

Interestingly, when larvae were exposed to the predators by simulating the natural conditions in the soil (bag experiment), predation was higher on larvae feeding on the wild plants. This further supports the findings that the predator was neither deterred, nor harmed by the cucurbitacins present in the herbivore's body. In fact, it could even suggest that cucurbitacins may have a phagostimulant effect on the predator as it has been shown to be the case for *D. balteata*^{43,62,64,65}. For example, when pure cucurbitacins B was added to soybean leaves, *Diabrotica* beetles exhibited compulsory feeding on this non common host plant⁶⁶. Similarly, Kim and Mullin⁶⁷ found that adding Cuc B (0.2 nmol/disk) on a cellulose disk stimulated feeding of the western corn rootworm. In a more recent study, Lang, et al.⁶⁸ found that for another cucurbit specialist, the coccinellid *Epilachna paenulata*, the phagostimulation properties of cucurbitacins were due to only one type of cucurbitacin among 28 tested. *Diabrotica* beetles share similar thresholds of sensitivities to various cucurbitacins irrespective of their degree of Cucurbitaceae specialization, with cucurbitacin B (Cuc B) believed to be the most phagostimulatory compound^{69,70}. Yet, in order to prove whether cucurbitacins might also act as phagostimulants for the predators, further experiments are needed.

Based on the random preference of the predator for larvae from the high or free-cucurbitacin treatment, their similar survival on the two types of larvae and the higher predation on *D. balteata* larvae from high-cucurbitacin roots, we can convincingly reject the idea that cucurbitacins provide protection against this predator. Evidence from other studies corroborates this conclusion^{35,36,71}. Moreover, it has never been shown that cucurbitacins have a lethal effect on an invertebrate and the only indication of their toxicity is the increased mortality in some adult chrysomelids when consuming these compounds at high doses (Castellanos-Morales et al., 2018) and in vertebrates such as, cows and sheep^{36,41,42}. Thus, to date the earlier study by Ferguson and Metcalf³⁵ is the only one showing a negative effect of herbivore-sequestered cucurbitacins on its predator. In a recent study Bruno et al. (unpublished results) tested a wide range of

natural enemies including entomopathogenic nematodes, insect predators (also *A. coriaria*), and pathogens (fungi and bacteria) and found that sequestered-cucurbitacins by *D. balteata* larvae fed on bitter cucumber varieties did not provide any protection against any of these natural enemies.

In this context, Gould and Massey⁴⁶ offered an alternative hypothesis for the role of cucurbitacins and suggested that sequestered cucurbitacins may have an antibiotic effect against entomopathogens. Tallamy, et al.⁴⁴ found partial support for this hypothesis as they showed that sequestering host-derived cucurbitacins reduced the pathogenicity of fungi (*M. anisopliae*) towards both the eggs and larvae of spotted cucumber beetles when reared on bitter plants. However, the size of the larvae was different between treatments (bitter or no-bitter plants) and could mislead their results. In the case of the western corn rootworm (*Diabrotica virgifera virgifera*) specialized on maize, Robert, et al.⁷² found that the sequestration of secondary metabolites (benzoxazinoids) from maize roots, protects the herbivore larvae against the attack of the third (entomopathogenic nematode) and even the fourth (entomopathogenic symbiotic bacteria) trophic level. Therefore, it is possible that the sequestration of cucurbitacins is only efficient against obligate parasites or entomopathogens, because they are more directly or severely influenced by the internal chemical environment of the host insect⁴⁵. There is strong evidence of the effect of sequestered compounds against other natural enemies including predators⁷³.

In conclusion, our results show that for *C. argyrosperma*, the dramatic reduction of cucurbitacins as a result of domestication, does not increase the susceptibility of root-feeding larvae to *A. coriaria*. Instead, larvae feeding on high-cucurbitacin roots suffered higher predation. Thus, our results do not support the earlier hypothesis suggesting that cucurbitacins confer protection against natural enemies. Our study represents an important step towards the understanding on how crop domestication affects the interactions between herbivores and their natural enemies, particularly pertaining belowground organisms that until now have been particularly neglected. This knowledge can help identify specific plant traits that could enhance crop resistance against insect pests.

Author contributions

C.J, C.C.M.A and B.B. originally formulated the idea. C.J., B.B. C.C.M.A and N.M. designed the experiments. C.J. and N.M performed all the experiments and wrote the first version of the manuscript. G.C and P.B developed the cucurbitacin extraction and analysis method and identified the cucurbitacins. N.M, C.C.M.A and C.J analyzed the data. N.M did the drawings. All co-authors contributed to the writing of the last version of the manuscript.

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Supplementary materials

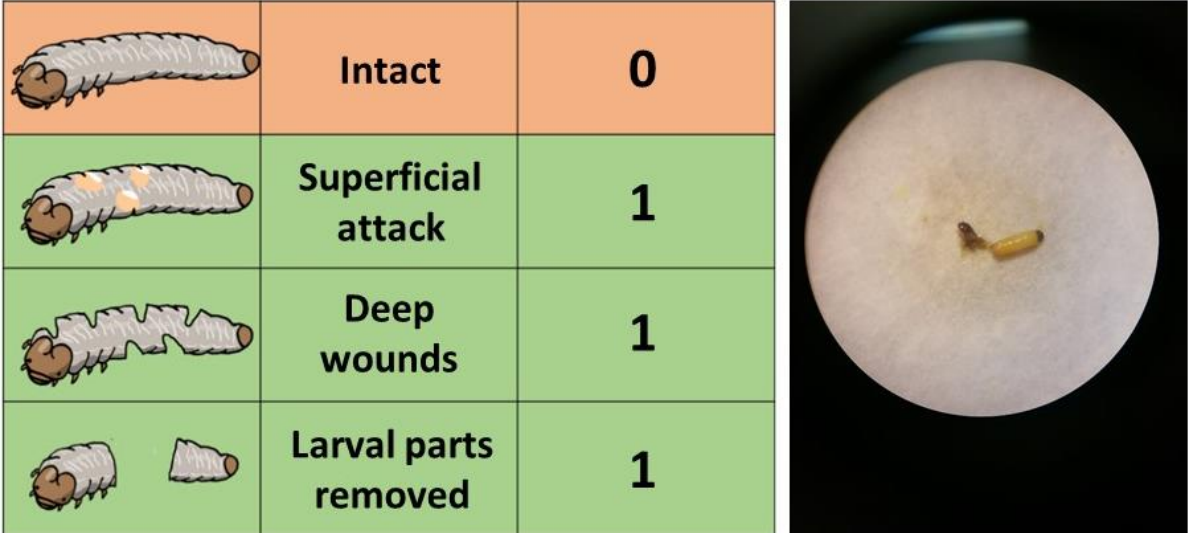


Figure S1. Predation indicators. When the type of damage illustrated in the greens squared was observed, the *Diabrotica balteata* larvae were considered as predated.

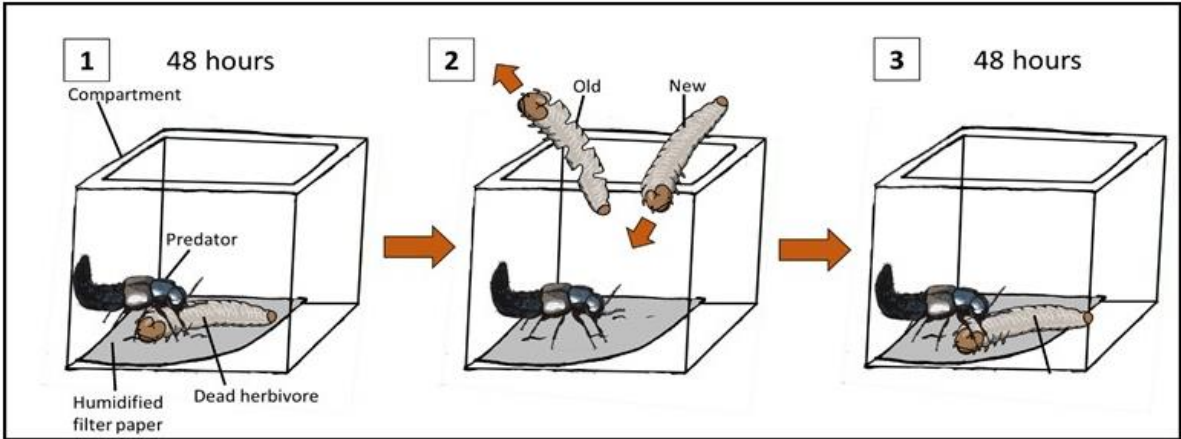


Figure S2. *Atheta coriaria* survival experiment in trays with 32 compartments. One dead *Diabrotica balteata* larvae, one *Atheta coriaria* were placed in each compartment with humidified paper filter as source of water. The dead larvae were replaced every two days to avoid degradation.

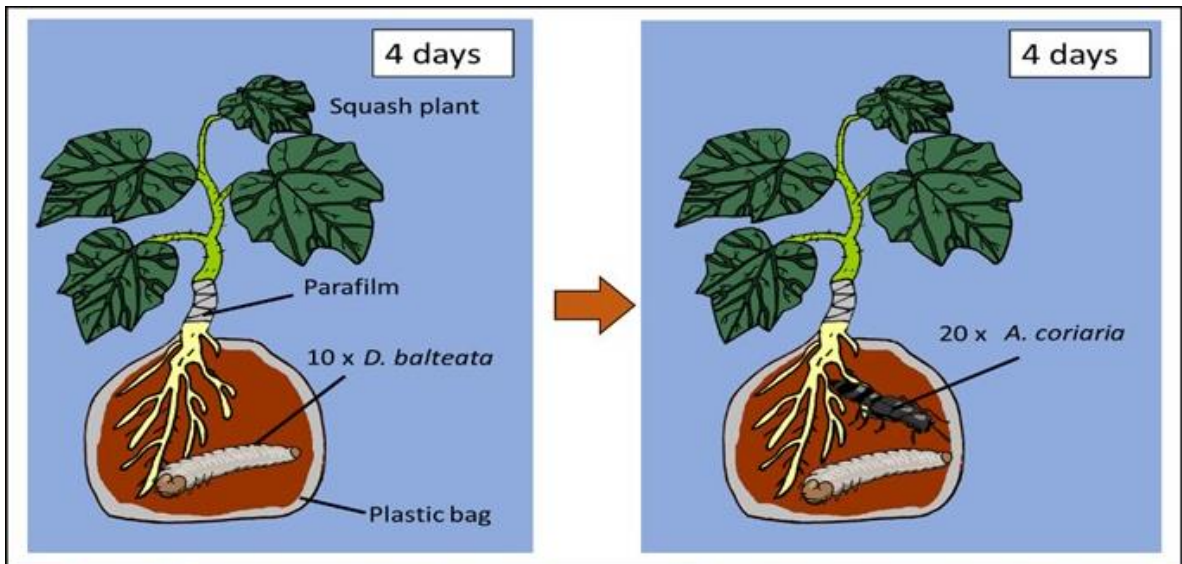


Figure S3: Individual squash plant enveloped with plastic bag containing roots, 10 *Diabrotica balteata* larvae and 20 *Atheta coriaria* soil.

Table S1: Putative identification of cucurbitacins found in roots of *C. argyrosperma* and *Diabrotica balteata* larvae. Cucurbitacins were putatively identified based on their exact masses (allowing for molecular formula determination) and retention times and compared with those of the standard Cucurbitacin B as well as with available databases such as the Dictionary of Natural Product (CRC Press).

CU C	Tissue, Organism	RT (min)	(M+HCO O) ⁻	(M-H) ⁻	MF	PI
1	Roots & larvae	3.10	767.383 4	721.37 79	C38H58O 13	Dihydrocucurbitacin B glucoside (or isomer)
2	Roots & larvae	3.02	765.369 4	719.36 4	C38H56O 13	Cucurbitacin B glucoside (or isomer)
3	Roots & larvae	3.79	603.317 4	557.26 24	C32H46O 8	Cucurbitacin B
4	Roots	4.06	601.301 6	555.29 61	C32H44O 8	Cucurbitacin E (or isomer)
5	Roots & Larvae	2.52	723.365 1	677.36 01	C36H54O 12	Unknown Cucurbitacin 1
6	Larvae	2.16	725.373 0	679.37 20	C36H56O 12	Unknown Cucurbitacin 2
7	Roots	2.95	763.353 0	717.34 89	C38H54O 13	Deacetylated dihydro cucurbitacin B glucoside (or isomer)

CUC : cucurbitacins, RT : retention time, MF : molecular formula, PI : putative identification

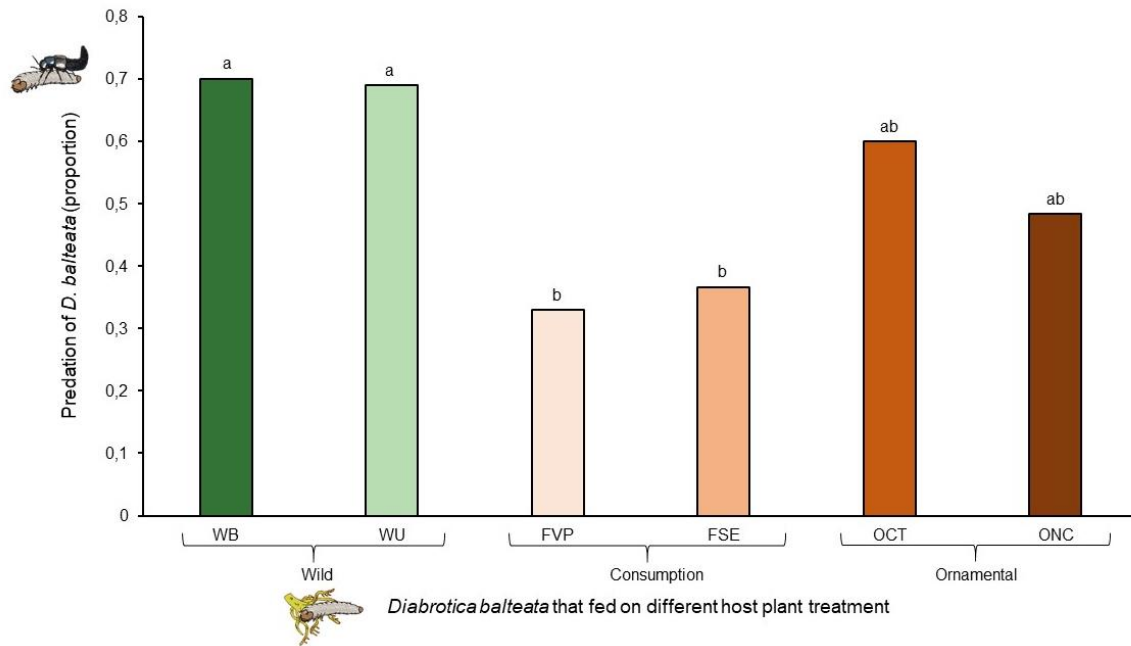


Figure S4: Predation of *Diabrotica balteata* larvae by *Atheta coriaria* while herbivore feed on either wild population of *Cucurbita argyrosperma* (WB and WU in green, n=10) or domesticated varieties (selected for fruit consumption (FVP and FSE) and selected as ornamental (OCT and ONC, n=10). Bars indicate proportions. Letters indicate predation differences among treatment (P<0.05).

Chapter 4: Characterization of cucurbitacin biosynthetic genes associated with reduced bitterness in squash cultivars (*Cucurbita argyrosperma*)

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⁺Equal contributions

Abstract

Wild squash (*Cucurbita argyrosperma*) contains characteristic triterpenoids called cucurbitacins which confer bitter tasting. However, domesticated cultivars do not contain cucurbitacins in leaves and roots, suggesting that domestication has selected for less toxic plants. Surprisingly, cotyledons of some cultivars still contain cucurbitacins. To understand the differences in defense expression between wild and domesticated squash and between plant tissues, we examined the gene expression involved in the cucurbitacins biosynthesis.

We first identified one OSC gene candidate involved in triterpene biosynthesis and six P450 genes candidates responsible for skeletal modifications of cucurbitacin. To examine the regulation of cucurbitacin biosynthesis, we studied the relative expression levels of these candidate cucurbitacin biosynthetic genes in roots and cotyledons of two wild populations and four cultivars (two varieties selected for the fruits consumption and two varieties selected for an ornamental purpose) of *C. argyrosperma*.

Their distinct expression pattern based on quantitative RT-qPCR showed how the characteristic triterpenoids were synthesized in each tissue. We found high expression of the OSC and P450 genes in the roots of wild squash. Although cucurbitacins were not accumulated in roots of the fruit and ornamental varieties, we observed that the genes were highly expressed in roots of the fruit varieties. On the other hand, the studied genes in ornamental squash cultivars were down regulated in roots. The relative gene expression in cotyledons was highly variable and did not allow to draw any conclusions. Between plant tissue of the same plant, gene expression was higher in roots than cotyledons.

This study proposes candidates genes for OSC and P450 in *C. argyrosperma* and highlights the impact of domestication at the transcription level.

Keywords: Cucurbitadienol synthase, Cucurbitacins, Domestication, Squash

Abbreviations: OSC, oxidosqualene cyclase; P450s, cytochrome P450s; UHPLC-QTOFMS, Ultra high performance chromatography coupled with a mass spectrometer

Introduction

Plant domestication is a continuous evolutionary process driven by human selection^{1,2}. All major crops around the world were domesticated from a wild progenitor species, primarily between 4000 and 10'000 years ago³. Domestication has moderately reduced the genetic diversity relative to the wild ancestor across the genome and severely decreased diversity for genes targeted by domestication^{4,5}. All crop species went through genetic bottlenecks throughout domestication³.

Domestication fixed alleles associated with favorable traits for human consumption and cultivation conditions. However, this can reduce the fitness of plants growing under natural conditions. As a result, the majority of domesticated crop are now dependent on humans⁶ such that they are no longer capable of propagating themselves in nature.

The molecular mechanism that leads to the domestication of a crop from wild relatives is of great interest for plant breeders as well as for evolutionary biologists. The suite of traits associated with the genetic change to a domesticated form of an organism from a wild progenitor form is called domestication syndrome⁷. The genetic basis of the domestication syndrome has been extensively studied in wheat⁸ and several other crop plants, including maize⁹, sunflower¹⁰, common bean¹¹ and tomato¹². These studies have indicated that a few gene clusters with large effects account for most of the variation associated with the differences between domesticated forms and their wild progenitors¹³.

Widely consumed as a vegetable, squash was domesticated from their wild ancestor that has extremely bitter and toxic fruits^{14,15}. Squash domestication was characterized by the selection for shape, less bitter flesh, larger and fewer seeds and larger fruit^{16,17}. Selection for non-bitter fruit was a key step in the domestication of this crop¹⁸. Bitterness comes from cucurbitacins, a group of oxygenated tetracyclic triterpenes that are produced in the Cucurbitaceae family and other plant families¹⁹. In addition to their toxic function, cucurbitacins also serve as feeding attractants for specialist insects and as a defense against generalist insects²⁰. Thus, it was hypothesized that they mediate the coevolution between cucurbits and herbivores²¹.

In addition, cucurbitacins have for centuries been exploited for their anti-inflammatory effects in traditional herbal medicine²² and have been shown to have antitumor properties²³. To date, 18 cucurbitacins (named by letters A-T) have been identified and most of them from cucurbit plants²⁴. Their abundance varies within and among species, tissues²⁵ and plant developmental stages²⁶. In melon, watermelon and cucumber, cucurbitacin biosynthesis occurs locally in tissues through activation of the first committed step of a single oxidosqualene cyclase (OSC) by leaf, root or fruit-specific transcription factors^{23,27}. Zhao, et al.²⁸ found that the loss of fruit bitterness in *Cucumis* is due to the loss of the OSC function.

Shang, et al.²⁹ identified nine cucumber (genus = *Cucumis*) genes in the pathway for biosynthesis of cucurbitacin C (CuC). They discovered transcription factors *B1* (*Bitter leaf*) and *Bt* (*Bitter fruit*) that regulate this pathway in leaves and fruits, respectively. They found traces of genomic signatures indicating that selection

imposed on Bt during domestication led to selection of non-bitter cucurbits from their bitter ancestor. Cucurbitadienol (Figure S1), a triterpene synthesized from oxidosqualene cyclase (OSC), is the first precursor of cucurbitacins produced by a specialized OSC named cucurbitadienol synthase^{19,30}. To catalyze the formation of cucurbitacin C, cucurbitadienol has to be further modified with a series of oxidation reactions and acetylation, likely catalyzed by cytochrome P-450 enzymes (P450s) and an acyltransferase (ACT)²³.

Building upon the work of Shang, et al.²³ and Brzozowski, et al.³¹, we studied another cucurbit species: *Cucurbita argyrosperma*. We compared two wild populations and four related varieties selected for fruit consumption or ornamental use. In the edible varieties, cucurbitacins were always selected out or reduced in the fruits during the breeding process³². But, cucurbitacins were not removed from all plant tissues; they can still be found in cotyledons of some domesticated varieties (chapter 2). Based on these findings, we explored how the expression of genes involved in the cucurbitacins biosynthesis is affected by the domestication of *C. argyrosperma*.

In this chapter we first identified and characterized candidate genes of OSC related to cucurbitacins biosynthesis in *C. argyrosperma* and studied their expression patterns in different tissues. Specifically, we examined the extent to which those genes are expressed in wild populations of *C. argyrosperma* and several related varieties. We hypothesized that the level of gene expression will be higher in roots and cotyledons of the wild populations as they contain high amounts of cucurbitacin, particularly in roots. As fruit varieties contain cucurbitacins in cotyledons, we expected that the genes will be expressed at least in this plant tissue. We predicted lower or no expression of those selected genes in ornamental varieties as they do not contain cucurbitacins.

Material and methods

Plant materials

Cucurbita argyrosperma was domesticated in Mesoamerica according to archaeological and genetic evidence^{33,34}. It is a monoecious species and gene flow has been previously described between the domesticated and wild subspecies³⁵. This Mexican squash is consumed primarily for its seeds rather than fruit flesh. This makes it a good model to study *Cucurbita* domestication, as seeds were an essential component of early Mesoamerican diet and likely the first targets of human-guided selection in cucurbits³⁶.

Fruits of wild *C. argyrosperma* were collected in January 2018 at three locations in the region of Puerto Escondido along the Pacific coast in the state of Oaxaca, Mexico (15°55'33.3" N 97°09'03.0" W), where this species occurs naturally. The climate is hot and humid with an average temperature of 27°C and 84% relative humidity. Fruits were collected in two sites along the coast. One population was found near our field site in the experimental campus of the UMAR University (15.924929, 97.150977)³⁷. The second wild population was collected in Bacocho, Puerto Escondido, Oaxaca (15.864463, -97.081142). The fruits were sun-dried before harvesting the seeds. Each wild (W) population was named according to the location where it was found, namely Bacocho (WB) and Umar (WU). Based on the literature and fruit shape, the wild plants were identified as belonging to *Cucurbita argyrosperma*^{16,38}. Cultivars of *C. argyrosperma* with different domestication purposes were selected to compare wild and domesticated cucurbits. We used two varieties of *C. argyrosperma* domesticated for their fruit, Vera Cruz Pepita (FVP) and Silver Edge (FSE) and two varieties selected as ornamentals; Cushaw Tricolor (OCT) and Navajo calabacita (ONC). Seeds of these four cultivars were purchase from the KCB Samen website (<https://www.kcb-samen.ch/>). Plants were grown in 8 cm diameter plastic pots filled with einheitserde classic soil (Sinntal-Altengronau, Germany) mixed with 30% of sand. To enhance the germination of wild seeds, seed coats were pierced, scratched at both sides and placed between two layers of wet cotton for one week in an incubator at 28 degrees. To control for the size difference between plants from different treatments, wild plants were germinated 10 days before the domesticated varieties that grow faster. All plants grew in a greenhouse at ambient temperature (24 ± 5°C) and under natural light conditions (16:8h L: D). Plants were watered as needed. We used 15-day-old plants, with two cotyledons and two true leaves for all analyses.

Characterization of cucurbitadienol synthase gene

Nine cucurbitacin genes listed in Shang, et al.²³ were used to find potential cucurbitacin biosynthetic genes in *Cucurbita argyrosperma* based on its genome database³⁹ by using BLAST search. The molecular weight and isoelectric point of the predicted proteins were determined using Computer pI/Mw tool (https://web.expasy.org/compute_pi/). Amino acid sequences of cucurbitadienol synthase of other Cucurbitaceae (*Cucumis melo*, *Cucurbita pepo*, *Cucumis sativus*,

Citrullus colocynthis, *Siraitia grosvenorii*) downloaded from the TriForC database⁴⁰ and NCBI (National Center for Biotechnology Information) were aligned using Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>). This analysis was not performed for the six genes encoding for P-450 enzymes.

Phylogenetic tree construction and analysis of cucurbitadienol synthase gene

Sequence alignments were carried out with the Clustal W program. The phylogenetic tree was constructed from the amino acid sequences of known plant OSCs (oxidosqualene cyclases, obtained from TriForC database, <http://bioinformatics.psb.ugent.be/triforc/#/home>) by the Neighbor-joining method⁴¹ and created by using MEGAX program. The number of bootstrap replications was 1,000. Accessions of OSCs are listed in supplementary Table S2. We aligned sequences from the OSCs only.

RNA Preparation and Real-Time Quantitative Reverse Transcription PCR (RT-qPCR)

Freshly harvested roots and cotyledons were ground in liquid nitrogen, and RNA was isolated using the SV Total RNA Isolation System (Promega) according to the manufacturer's instructions. Each total RNA sample (300 ng) was reverse transcribed using the GoScript™ Reverse Transcription System (Promega). Nine cucumber genes in the pathway for biosynthesis of CuC²³ were used to search orthologs in *Cucurbita argyrosperma* based on its genome database³⁹ by using BLAST. Six cytochrome P-450 enzymes (P450s) genes and one cucurbitadienol synthase (CPQ) gene (Supplemental Table S1) have been found in the genome database of *C. argyrosperma*, and the differences in the relative expression of these seven genes among two wild populations and four domesticated varieties were measured per tissue (roots and cotyledon). We selected one wild and 2 domesticated samples to test the relative expression between plant tissue and among varieties. Real-time qPCR was performed on the Rotor-Gene™ 6000 (Corbett Research) platform. The qPCR mix consisted of 10 µl GoTaq® qPCR Master Mix (Promega), 8.2 µl H₂O, 0.4 µl each primer (10 µM) and 1 µl of cDNA sample. The qPCR was performed using 50 cycles with the following temperature curve: 10s at 95°C, 20s at 65°C and 2s 72°C. The melt curve was obtained by ramping from 72°C to 99°C, rising by 1°C each step and wait for 5s for each step afterwards. The *actin* (GenBank accession number: HM594170) was used as the reference gene for the transcript profiling of roots. Primers used for real-time qPCR were designed by using Primer-BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) and listed in Supplemental Table S3. Five independent biological replicates per wild populations or domesticated varieties were analyzed, after which the average threshold cycle (Ct) per sample was calculated. For the expression analysis of each gene, samples from WB variety were designated as calibrator. The relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method⁴².

Cucurbitacin analysis

Roots and cotyledons of wild and domesticated *C. argyrosperma* were ground to a fine powder in liquid nitrogen. We weighed 100mg (+/- 20mg) of powder and added 1ml of methanol (99.999%)⁴³ with five glass beads in 1.5 mL Eppendorf tubes. The cells were broken with a Retsch MM300 at 30 Hz for four minutes. The samples were centrifuged during five minutes at 14000 rpm. 700 µl surfactant were removed and diluted with 300 µl of MiliQ water. A second centrifugation was performed before transferring into 2ml NO Screw Vial w/WOP 32x12 mm. The analysis of cucurbitacins was done by UHPLC-QTOFMS instrument (Waters, Milford, USA) made up of Acquity UPLC™ coupled to Synapt G2 high-resolution mass spectrometer. The column used in the chromatograph was precisely ACQUITY UPLC BEH C18 1.7µm, 2.1 x 50mm (Waters, Milford, USA). The temperature was maintained at 25 degrees. Two eluants were used; the A was water and 0.05% of formic acid and B was acetonitrile and 0.05% of formic acid. The injection volume in the machine was 2.5 µl. The source for the mass spectrometer was Electrospray negative ionization (ESI) with MS^t (alternates between low and high collision energies) as acquisition mode. The source parameters were capillary voltage -2.0 kV, cone voltage -25 V, source temperature 120 °C, desolvation gas flow and temperature 800 l/h and 350 °C respectively, cone gas flow 20 l/h. The exact mass measurements (< 2 ppm) were ensured by infusing a 500 ng/ml solution of leucine-enkephalin at 15 µl/min through the Lockspray™ probe. For the acquisition and data processing, we used the software name Masslynx™ v.4.1 (Waters, Milford, USA). Cucurbitacins were identified based on their molecular formula and fragmentation patterns provided by accurate mass measurements. Peaks corresponding to known cucurbitacins were automatically integrated using Quanlynx™ with a 0.1 min chromatographic window centered on the retention time of each component and a 0.02 Da mass window centered on the (M+HCOO) ion. Quantification of all cucurbitacins was done by external calibration using cucurbitacin B as standard. The peak height threshold was 161.802. The cucurbitacin concentration was expressed in µg per g of plant material. Since many different cucurbitacins were isolated from wild populations of *C. argyrosperma*, with different molecular mass, we pooled them to show total cucurbitacin content.

Statistics

Statistical analysis was conducted with GraphPad Prism 8.4.3. To test for differences in gene expression in roots and cotyledons among varieties and wild populations, we used the Shapiro-Wilk test to evaluate the data for normal distribution and the Brown-Forsythe test for homogeneity of variance. For non-normally distributed data Kruskal-Wallis test followed with Dunn's multiple comparisons test was used. To test for differences in gene expression between plant tissues of the same wild population or related varieties, we used the Shapiro-Wilk test to evaluate the data for normal distribution and the F-test for homoscedasticity. For normally distributed data groups with the same standard deviation, we applied unpaired t-test. The normally distributed data groups with unequal standard deviation were analyzed with unpaired t-tests with

Welch's correction. For non-normally distributed data a non-parametric test (Mann-Whitney test) was used.

For the cucurbitacin content analysis in roots and cotyledons see details in chapter two (Statistics section).

Results

Identification and characterization of cucurbitadienol synthase gene in Cucurbita argyrosperma

Using nine cucurbitacin C biosynthetic genes from cucumber (*Cucumis sativus*) as queries, BLAST searches of the genome of *Cucurbita argyrosperma* identified seven putative cucurbitacin biosynthetic genes, including one candidate for cucurbitadienol synthase gene and six for P-450 enzymes (P450s) genes (table S1). Among all seven cucurbitacin biosynthetic genes identified from *C. argyrosperma*, the putative cucurbitadienol synthase gene (CuGenDB ID: *Carg11552*) is particularly interesting due to its potential function in the first step of cucurbitacin biosynthesis. The *Carg11552* contains an open reading frame (ORF) of 2,295 bp that encodes a 764-amino-acid protein with a predicted molecular weight of 86.64 kDa and a pI of 6.70. The predicted amino acid sequence of *Carg11552* shares high similarity with that of cucurbitadienol synthases (97.91% with CpCPQ from *C. pepo*, 87.13% with CsBi from *C. sativus*, 91.10% with CcCDS2 from *C. colocynthis*, 89.66% with SgCbQ from *S. grosvenorii*, 88.60% with CmBi from *C. melo*). In addition, the predicted amino acid sequence of *Carg11552* contains a highly conserved DCTAE motif (presumed to be responsible for the initiation of the cyclization reaction⁴⁴), six conserved QW motifs (stabilizer of the carbocation intermediates during cyclization⁴⁵) and a MWCHCR motif (presumed to be important for stabilization of the protosteryl cation intermediate⁴⁶). Therefore, *Carg11552* might play a key role in the cucurbitacins biosynthesis of *C. argyrosperma* by encoding cucurbitadienol synthase.

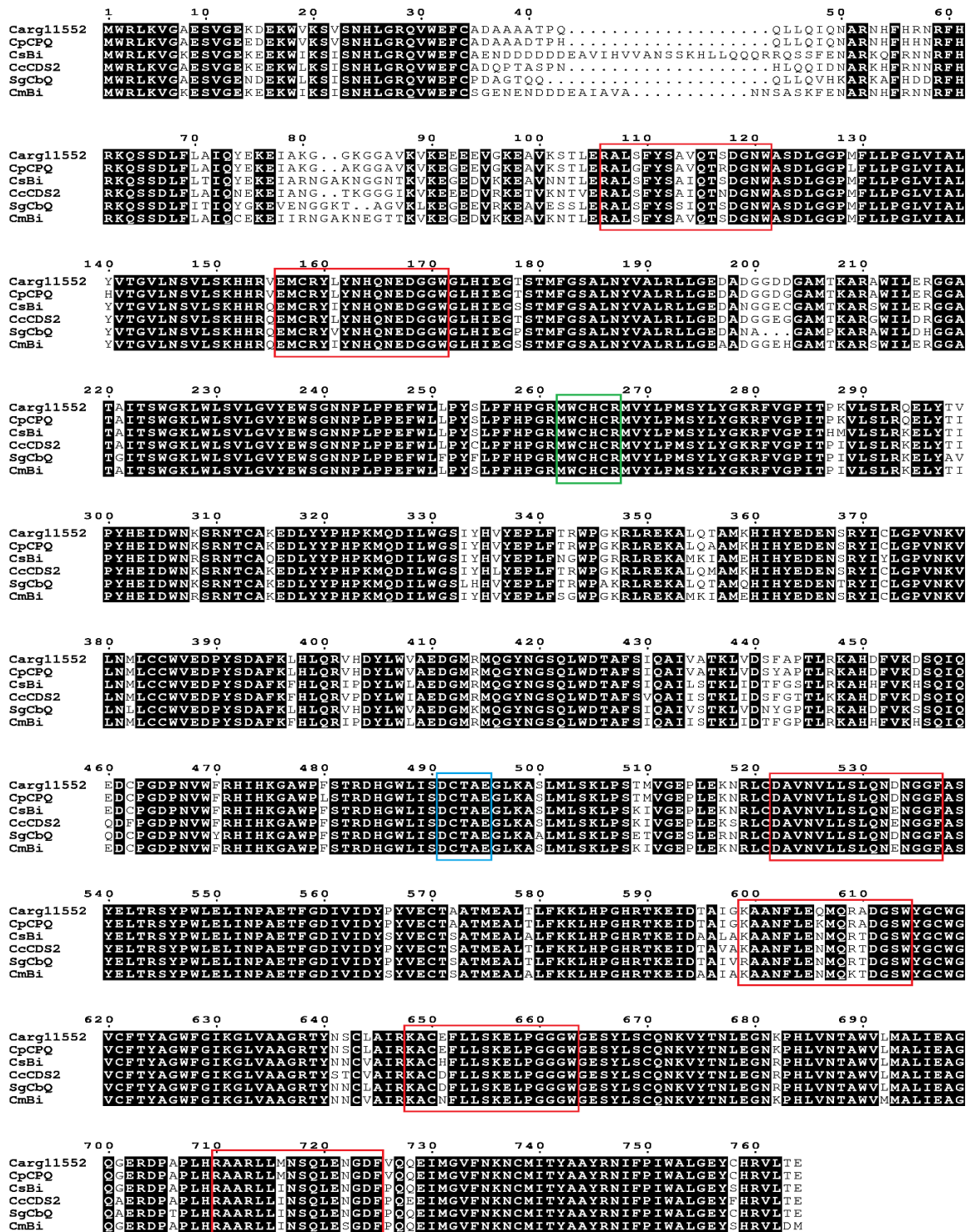


Figure 1. Alignment of the deduced amino acid sequence of Carg11552 from *C. argyrosperma* with known cucurbitadienol synthases. Amino acid residues identical in six protein sequences are shaded in black. Selected protein GenBank accession numbers: CpCPQ (*Cucurbita pepo*, BAD34645), CsBi (*Cucumis sativus*, AIT72030), CcCDS2 (*Citrullus colocynthis*, AJR21210), SgCbQ (*Siraitia grosvenorii*, AEM42982). CuGenDB accession number: CmBi (*Cucumis melo*, MELO3C022374). Motifs are indicated as follows: DCTAE motif, blue box; QW motif, red box; MWCHCR motif, green box.

Phylogenetic analyses of the cucurbitadienol synthase gene

To analyze the phylogenetic relationships between the putative cucurbitadienol synthase of *Cucurbita argyrosperma* and OSCs genes from other plant species, we generated an unrooted phylogenetic tree based on OSC genes from different plant species listed in Supplementary Table 2. According to the phylogenetic tree (Figure 2), Carg11552 is clustered with the other cucurbitadienol synthase from the other Cucurbitaceae plants (*Citrullus colocynthis*, *Citrullus lanatus*, *Cucumis melo*, *Cucurbita pepo*, *Cucumis sativus*, *Cucurbita maxima*, *Cucurbita moschata*). This data indicates that Carg11552 belongs to the triterpene synthase superfamily and might function as a cucurbitadienol synthase.

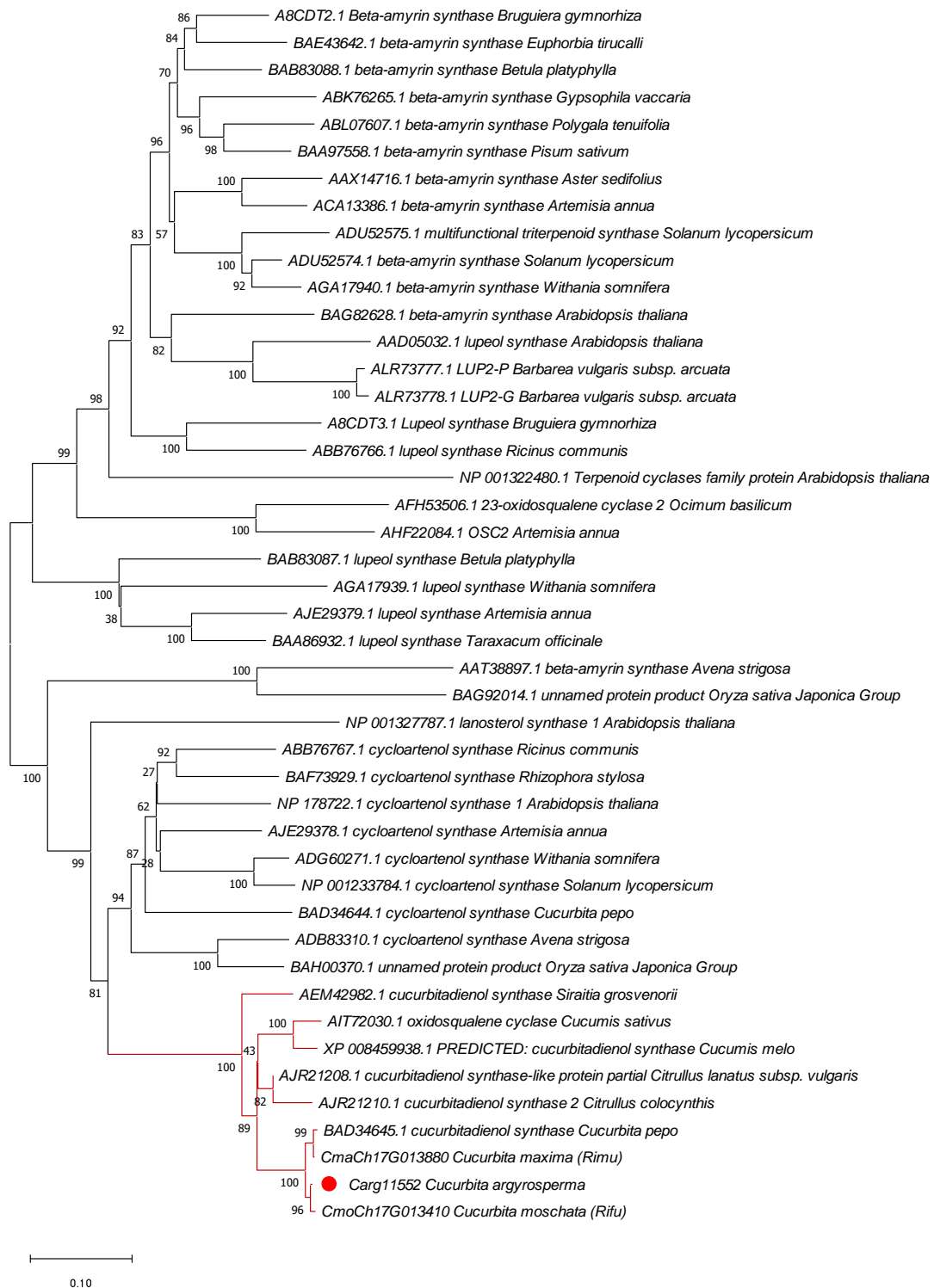


Figure 2. Unrooted phylogenetic tree of Carg11552 from *Cucurbita argyrosperma* (red dot) with previously characterized OSCs from other plant species. The tree was constructed using the Neighbor-joining method and created by using MEGAX program with 1,000 bootstrap replicates. The unit for the scale bar displays branch lengths. The red branches represent the clade with Cucurbitaceae plants. Gene accessions for phylogenetic analysis are given in Supplementary Table S1.

Cucurbitacin biosynthetic gene expression in roots

The expression level of *Carg11552* (candidate for cucurbitadienol synthase ortholog) is higher in roots of one wild population WB compared to the ornamental variety OCT (Dunn's test, $p=0.0141$; Fig. 3) and to the other ornamental variety ONC (Dunn's test, $p=0.0427$; Fig. 3) but not compared to the varieties selected for fruit consumption. The six P450 cytochrome genes (*Carg1150*, *Carg03797*, *Carg07313*, *Carg07314*, *Carg06672*) in roots of both wild populations are significantly more expressed than in the roots of the ornamental varieties (Fig. 3). This graph is also presented in Chapter 2.

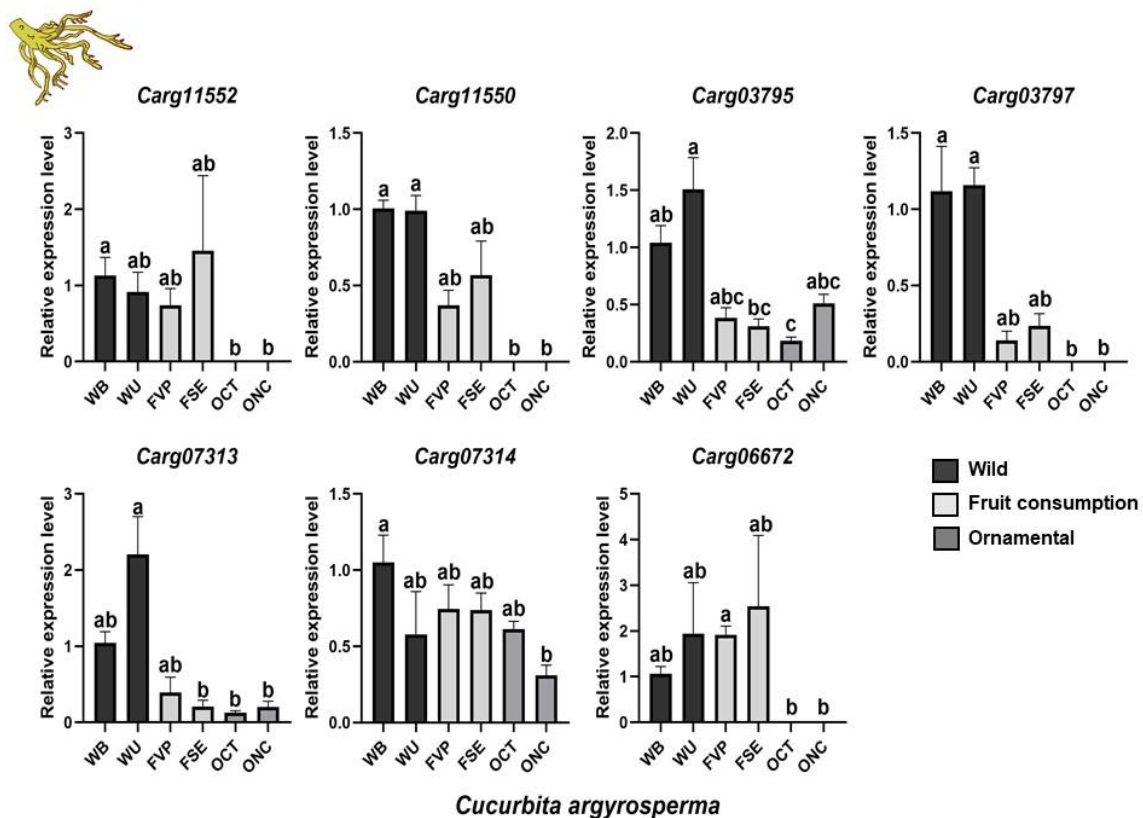


Figure 3. Relative gene expression of seven cucurbitacin biosynthetic genes in roots of two wild populations (WB and WU) of *Cucurbita argyrosperma*, and four related varieties; two selected for the fruit consumption (FVP and FSE) and two varieties selected for an ornamental purpose (OCT and ONC). Shown are means \pm SE. Letters indicate significant differences among wild populations or varieties ($P < 0.05$, Kruskal-Wallis test followed by Dunn's multiple comparisons test, $n = 5$ biological replicates).

Cucurbitacin biosynthetic gene expression in cotyledons

No significant differences in gene expression in cotyledons were found for six of the seven genes studied among the wild *C. argyrosperma* populations and varieties. The variation among samples might be due to low expression levels of genes (relatively high Ct values for tested genes in cotyledons of plants were recorded in the raw data of qRT-PCR, which implied low transcription levels). The P450 cytochrome (*Carg03797*) gene showed higher expression in the ornamental variety than in the wild *C. argyrosperma* WU (Dunn's test, $P = 0.015$; Fig. 4).

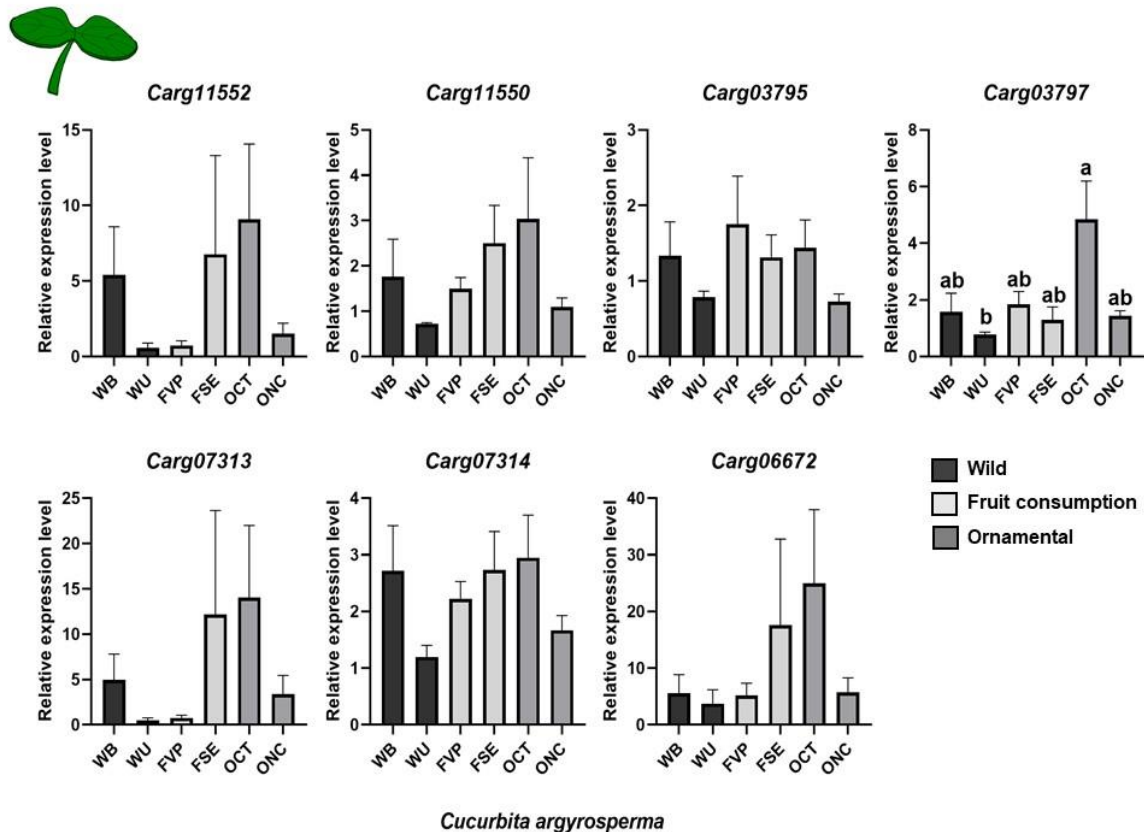


Figure 4. Relative gene expression of seven cucurbitacin biosynthetic genes in cotyledons of two wild populations (WB and WU) of *Cucurbita argyrosperma*, and four related varieties; two selected for fruit consumption (FVP and FSE) and two varieties selected for an ornamental purpose (OCT and ONC). Shown are means \pm SE. Letters indicate significant differences among wild populations or varieties ($p < 0.05$, Kruskal-Wallis test followed by Dunn's multiple comparisons test, $n = 4-6$).

Cucurbitacin biosynthetic gene expression in plant tissues

The bitterness reduction as a result of squash domestication might be caused by the suppression of expression of key genes in the cucurbitacin biosynthesis pathway. The transcription of four cucurbitacin biosynthetic genes (*Carg11552*, *Carg11550*, *Carg03797*, *Carg06672*) were detected in the roots of wild populations and fruit consumption cultivars but barely detected in ornamental plants (Fig. 3). To further test for variation in tissue-specific expression patterns of cucurbitacin biosynthetic genes with squash cultivars, we compared the transcript levels of these four cucurbitacin biosynthetic genes in roots and cotyledons from three, one wild squash populations and two varieties (WB, FVP, and ONC). Only these three genotypes were tested due to capacity (machine) limitations. The gene expression in roots were drastically higher than the expression in cotyledons of the wild population ($P=0.0043$; Fig. 5) and in the variety selected for fruit consumption ($P=0.0281$; Fig. 5) at least for the *Carg11552* gene. However, in the variety selected for an ornamental use, the expression of the different tested genes was higher in cotyledons than in roots due to a very low general expression ($P=0.0381$; Fig. 5). This graph is also presented in Chapter 2.

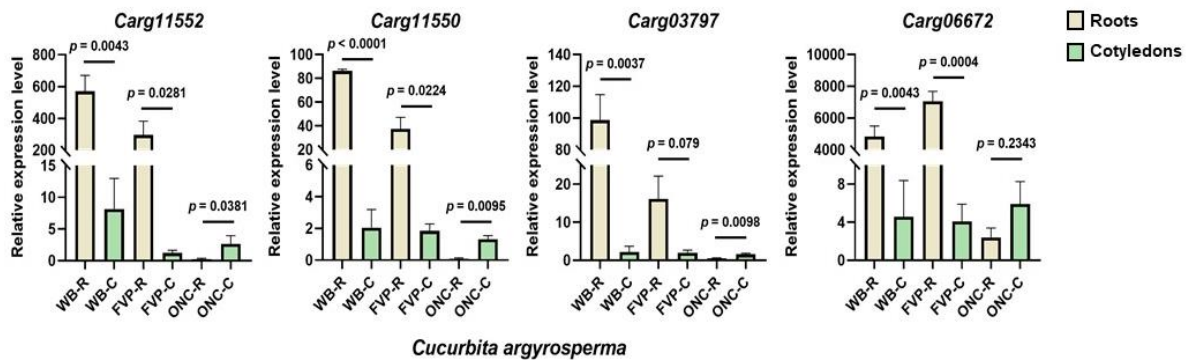


Figure 5. Relative gene expression levels of four cucurbitacin biosynthetic genes in roots (-R) and cotyledons (-C) of one wild population of *Cucurbita argyrosperma* and two related varieties, one selected for fruit consumption (FVP) and one selected for an ornamental purpose (ONC). Shown are means \pm SE. P-value indicate the difference in gene expression between the two-plant tissue (roots and cotyledons) of the same wild population/variety.

Cucurbitacin content in wild and domesticated *C. argyrosperma*

In order to see if the observed expression patterns among different squash plants (wild populations and varieties) and between different tissues correlated with metabolite profile, cucurbitacin concentration was analyzed with UHPLC-QTOFMS. Since many different cucurbitacins were isolated from wild populations of *C. argyrosperma*, with different molecular mass, we pooled them together to show total cucurbitacin content.

The cucurbitacin concentration in the roots was significantly different between domesticated and wild plants ($\chi^2_{(6)}=26.805$, $P=0.0001$, Fig. 6A). Domesticated varieties did not contain cucurbitacins in roots whether they are used for fruit consumption or as ornamentals. Cucurbitacins were present at high concentrations ($\mu\text{g/g}$) in roots of the wild populations. No significant differences in cucurbitacin content in roots were found between the two wild populations (WB-WU: $P=0.264$, Fig. 6A).

The cucurbitacin concentration in the cotyledons was significantly different between domesticated and wild plants ($\chi^2_{(6)}=28.408$, $P < 0.0001$, Fig. 6B). Varieties selected for fruit consumption (FVP and FSE) contained less cucurbitacin in cotyledons. The Silver Edge variety (FSE) contained more cucurbitacin than the other fruit variety ($P=0.04$). However, the Vera Cruz Pepita (FVP) variety did not contain significantly more cucurbitacins than the ornamental varieties which did not have cucurbitacins in the cotyledons. No significant differences in cucurbitacin content in cotyledons were found between wild populations (WB-WU: $P=0.726$, Fig. 6B).

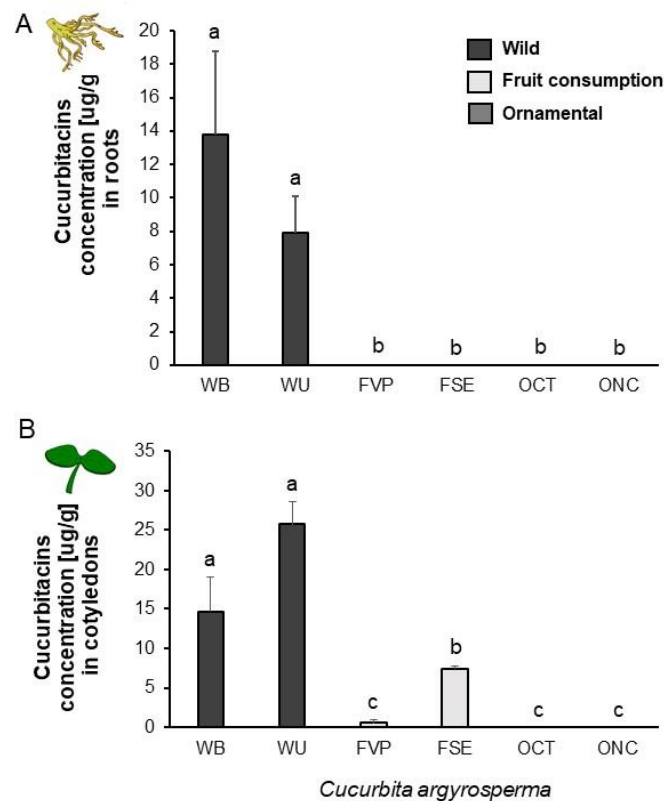


Figure 6. cucurbitacin concentration in roots (A) and cotyledons (B) of 3 wild populations (WB and WU) of *Cucurbita argyrosperma* and 2 varieties selected for fruit consumption (FVP and FSE) and 2 varieties for ornamental use (OCT and ONC). Bars are means \pm SE. Letters are pairwise using pairwise Wilcoxon-Test.

Discussion

Breeding crops over millennia for increased productivity and desirable traits has led to reduced genetic diversity⁴⁷. As a result, depending on the crop, beneficial traits of wild species, such as those that confer resistance against diseases or pests may have been lost⁴⁸. Changes in plant secondary metabolites through domestication is one of the main traits shared across crop species⁴⁹. For example, in domesticated Brassicaceae there was selection for high and low glucosinolates concentration in multiple tissues⁵⁰ and in maize, an alteration of expression of benzoxazinoid biosynthetic genes was observed⁵¹.

The reduction or even loss of cucurbitacins in squash is a typical example of the consequences of domestication on plant chemical defense. The production of cucurbitacins is linked to several genes including the cucurbitadienol synthase gene (CPQ for *C. pepo*) and cytochrome P450 genes for example^{23,27,31}. We were interested in understanding how the regulation of the genes involved in the cucurbitacin biosynthesis are affected during the domestication of *C. argyrosperma*.

With this study, we aimed at identifying the genes involved in the cucurbitacin biosynthesis in *C. argyrosperma*. Based on the study of Shang, et al.²³, we propose one OSC gene encoding for putative cucurbitadienol synthase (Carg11552) and six genes encoding for putative cytochrome P-450 enzymes (Carg11550, Carg0395, Carg03797, Carg07313, Carg7314, Carg06672). We suggest from our sequence alignment and phylogenetic analysis that the *Carg11552* gene may be involved in the production of characteristic triterpenoids in *C. argyrosperma*. Indeed, the close relationship of *Carg11552* to each of the homologues from *C. pepo* (CpCPQ), *C. sativus* (CsBi), *C. colocynthis* (CcCDS2), *S. grosvenorii* (SgCbQ) and *C. melo* (CmBi), respectively, indicates that these genes are conserved among Cucurbitaceae and that *Carg11552* may encode for cucurbitadienol synthase. Further experiments are needed to verify the identity and characterization of these genes.

Secondly, we investigated the expression of genes involved in the cucurbitacin biosynthesis in both wild and domesticated *Cucurbita argyrosperma* in two different tissues, roots and cotyledons. We hypothesized that gene expression will be higher in plants from wild populations than domesticated varieties as they contain high amounts of cucurbitacins, especially in roots (see chapter 2). As fruit varieties contain cucurbitacins in cotyledons, we expected that genes would be expressed at least in some tissues of these plants. We predicted lower or no expression of those selected genes in ornamental varieties as they do not contain cucurbitacins.

We found strong support for our hypotheses. The expression level of the candidate genes in roots was higher in wild populations compared to the varieties selected for an ornamental purpose, and fruit varieties showing intermediate levels of expression. This correlates well with the patterns of cucurbitacin content found among the varieties in roots and cotyledons. Gene expression in cotyledons is very low and highly variable among squash wild populations and varieties. In wild plants and fruit varieties, gene expression in roots is higher by two or more orders of magnitude compared to cotyledons of the same population/variety in the same replicate. However,

for ornamental variety tested, the expression is very low in both tissues, but higher in cotyledons than in roots. As cucurbitacins were not detected in ornamental varieties, it is likely that the expression levels detected in cotyledons do not contribute to significant cucurbitacin synthesis and accumulation. In *Cucurbita pepo*, expression of genes associated to cucurbitacin production is undetectable once the cotyledons are already expanded even if cucurbitacins are present³¹. Since the cotyledons used for the present study were already expanded, it is likely that cucurbitacins in cotyledons were synthesized when they were developing when expression levels are higher. Thus, cucurbitacins must be accumulated after they are synthesized, and the gene expression is activated mainly during seedling development and is transient throughout plant development. This idea remains to be tested.

Even if the *Carg11552* gene is expressed in the roots of the varieties selected for fruit consumption, there is no cucurbitacin production in their roots. As the P450s genes are also expressed, it is possible that they have changed the carbon skeleton of the cucurbitadienol by oxidation into other molecules rather than cucurbitacins. Indeed, the OSCs can give more than two cyclization products (Figure S1)³⁰. The possibility of the presence of a single enzyme giving two products cannot be excluded⁵². An alternative explanation could be that there is transport of enzymes or cucurbitacins from roots to upper plants of the plants, as observed for alkaloids in other plant species⁵³. Whether this transport occurs would be worth examining in future studies.

The variation in cucurbitacin content in cotyledons among squash species (*C. pepo* and *argyrosperma*) could be due to the biogeographical domestication origin of these species. The species origin is Mesoamerica for all *Cucurbita*, but then there was a split in the cultivar development. *C. pepo* was mainly domesticated in the US, while the domestication of *C. argyrosperma* occurred in the South-East of Mexico^{33,54}. Differences between these two regions in local human preferences and taste, in the purpose of selection of cultivars and in biotic and abiotic selective pressures, may explain the differences in cucurbitacin content in these two species⁵⁵. For example, Rasmann, et al. ⁵⁶ showed that most North American maize lines do not release (E)- β -caryophyllene, whereas European lines and the wild maize ancestor, teosinte, do so in response to the specialist insect (*Diabrotica virgifera virgifera*). They explained that North American maize lines must have lost the signal during the breeding process. However, European lines release this sesquiterpene in response to feeding by the specialist larvae that has recently invaded Europe⁵⁶.

The extent to which anthropogenic and environmental pressures have contributed to varying cucurbitacin levels in squash species and varieties has not been explored and may be of interest for future research.

Our study confirmed that the trait for non-bitterness has been selected and fixed during the domestication process. The loss of bitterness was caused by mutations occurring at homologous fruit bitterness regulators in cucumber, melon and watermelon²⁷. Zhou, et al. ²⁷ found that the major gene clusters for cucurbitacin biosynthesis are highly conserved in these three cucurbits. Our results suggest that selective pressures may have been imposed on cucurbits to retain these biosynthetic

gene clusters, thereby maintaining effective biosynthesis of specialized metabolites in the wild plants.

Given that useful alleles can be transferred into modern cultivars, native genetic variation can be used in breeding programs. Native variation crossed into modern cultivars from wild relatives via traditional breeding methods offers an alternative to genetically modified (GM) crops in countries where GM crops are not accepted. Native genetic variation could then provide alternative strategies to enhance plant resistance against insects and diseases. For example, the corn root worm (*Diabrotica virgifera virgifera*) has evolved resistance to multiple Bt transgenic events⁵⁷. Identification and incorporation of native host plant resistance genes for corn rootworm resistance could slow the rate at which these insects can adapt to commercial transgenic events⁴.

In conclusion, we propose one candidate gene for cucurbitadienol synthase in *Cucurbita argyrosperma*, but further experiments to test the specific role of this gene on the coding of cucurbitadienol synthase, are required. We found that the cucurbitacin biosynthetic genes domain are conserved among Cucurbitaceae. Squash domestication did not remove the genes from the genome of *C. argyrosperma* but altered the gene expression in roots of ornamental varieties that are cucurbitacin free. We believe that domesticated plants and their wild relatives provide ideal models to understand how selection on plant genomes can influence plant resistance traits against insect pests. Ultimately, this knowledge can guide breeding efforts and determine the feasibility of reintroducing ecologically important genes back into commercial agricultural germplasm.

Author contributions

C.J, B.B and W.Y originally formulated the idea and designed the experiments. W.Y designed the primers. C.J and W.Y performed lab experiments. C.J, W.Y and C.B.S analyzed the data. C.J. wrote the first version of the manuscript and all co-authors revised the subsequent versions.

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Supplementary materials

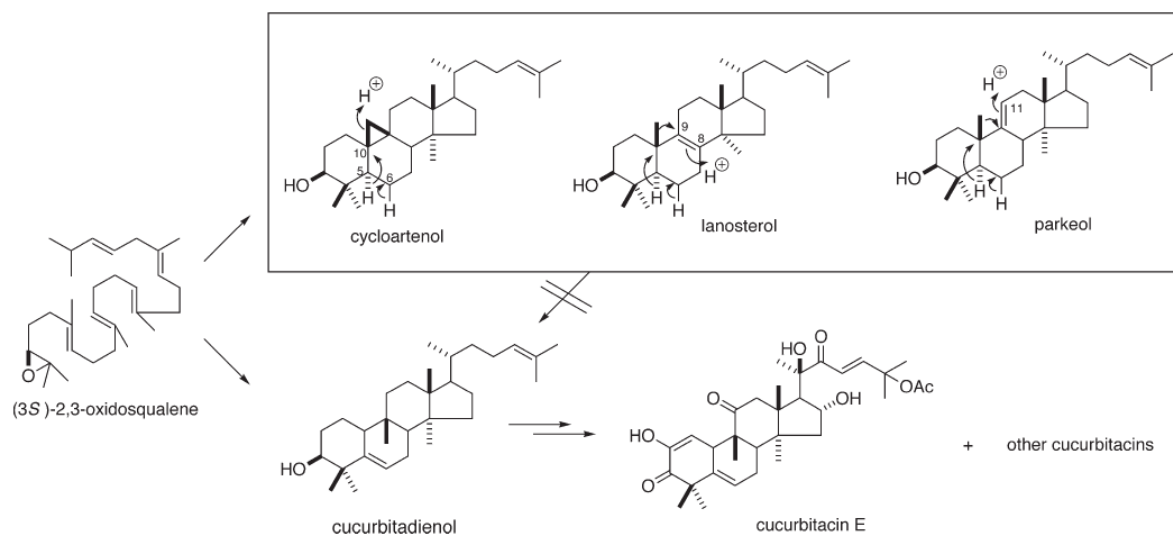


Figure S1: Biosynthesis of cucurbitacins from Shibuya, et al. ³⁰. Cucurbitadienol are biosynthesized from a precursor 2,3-oxidosqualene by OSC. The subsequent modification steps involve oxidation and glycosylation.

Table S1: genes involved in the cucurbitacins pathway in *Cucumis sativus* and orthologs found in *Cucurbita argyrosperma* with them functions.

Query gene in cucumber	Orthologs in squash	Function in cucumber
<i>Csa6G088690</i>	<i>Carg11552-RA</i>	oxidosqualene cyclase (ortholog of cucurbitadienol synthase)
<i>Csa6G088160</i>	<i>Carg03795-RA</i>	cytochrome P-450 enzymes (P450s)
<i>Csa6G088170</i>	<i>Carg11550-RA</i>	cytochrome P-450 enzymes (P450s)
<i>Csa6G088710</i>	<i>Carg03797-RA</i>	cytochrome P-450 enzymes (P450s)
<i>Csa3G698490</i>	<i>Carg07313-RA</i>	cytochrome P-450 enzymes (P450s)
<i>Csa3G698490</i>	<i>Carg07314-RA</i>	cytochrome P-450 enzymes (P450s)
<i>Csa1G044890</i>	<i>Carg06672-RA</i>	cytochrome P-450 enzymes (P450s)

Table S2: Amino acid sequences of plant oxidosqualene cyclase genes used for the phylogenetic analysis. References are listed in TriForC database

Name	Type	Plant	Description	Genbank	Genbank Protein ID
AaBAS	OSC	<i>Artemisia annua</i>	<i>Artemisia annua</i> beta-amyirin synthase (BAS) mRNA, complete cds.	EU330197	ACA13386
AaCAS	OSC	<i>Artemisia annua</i>	<i>Artemisia annua</i> cycloartenol synthase mRNA, complete cds.	KM670093	AJE29378
AaLUS	OSC	<i>Artemisia annua</i>	<i>Artemisia annua</i> lupeol synthase mRNA, complete cds.	KM670094	AJE29379
AaOSC2	OSC	<i>Artemisia annua</i>	Multi-functional OSC	KF309252	AHF22084
AsbAS1	OSC	<i>Avena strigosa</i>	<i>Avena strigosa</i> mRNA for beta-amyirin synthase (bAS1 gene)	AJ311789	AAT38897
AsCS1	OSC	<i>Avena strigosa</i>	<i>Avena strigosa</i> mRNA for cycloartenol synthase (cs1 gene)	AJ311790	ADB83310
AsOXA1	OSC	<i>Aster sedifolius</i>	<i>Aster sedifolius</i> beta-amyirin synthase (OXA1) mRNA, complete cds.	AY836006	AAAX14716
AtBAS	OSC	<i>Arabidopsis thaliana</i>	<i>Arabidopsis thaliana</i> AtBAS mRNA for beta-amyirin synthase, complete cds.	AB374428	BAG82628
AtCAS	OSC	<i>Arabidopsis thaliana</i>	<i>Arabidopsis thaliana</i> cycloartenol synthase 1 (CAS1), mRNA.	NM_126681	NP_178722
AtLAS1	OSC	<i>Arabidopsis thaliana</i>	<i>Arabidopsis thaliana</i> lanosterol synthase 1 (LAS1), mRNA.	NM_114382	NP_001327787

AtLUP1	OSC	<i>Arabidopsis thaliana</i>	<i>Arabidopsis thaliana</i> lupeol synthase mRNA, complete cds.	U49919	AAD05032
AtPEN6	OSC	<i>Arabidopsis thaliana</i>	Multi-functional OSC	NM_106497	NP_001322480
BgBAS	OSC	<i>Bruguiera gymnorhiza</i>	<i>Bruguiera gymnorhiza</i> BgbAS mRNA for beta amyirin synthase, complete cds.	AB289585	A8CDT2
BgLUS	OSC	<i>Bruguiera gymnorhiza</i>	<i>Bruguiera gymnorhiza</i> BgLUS mRNA for lupeol synthase, complete cds.	AB289586	A8CDT3
BpOSCBPW	OSC	<i>Betula platyphylla</i>	<i>Betula platyphylla</i> OSBPW mRNA for lupeol synthase, complete cds.	AB055511	BAB83087
BpOSCBPY	OSC	<i>Betula platyphylla</i>	<i>Betula platyphylla</i> OSBPY mRNA for beta-amyirin synthase, complete cds.	AB055512	BAB83088
BvLUP2G	OSC	<i>Barbarea vulgaris</i>	lupeol synthase	KP784688	ALR73778
BvLUP2P	OSC	<i>Barbarea vulgaris</i>	lupeol synthase	KP784687	ALR73777
Carg11552	OSC	<i>Cucurbita argyrosperma</i>	putative cucurbitadienol synthase	NA	NA
CcCDS2	OSC	<i>Citrullus colocynthis</i>	multi-functional OSC	KM821405	AJR21210
CIBi	OSC	<i>Citrullus lanatus</i>	cucurbitadienol synthase	KM821403	AJR21208
CmBi/CmC PQ	OSC	<i>Cucumis melo</i>	cucurbitadienol synthase	XM_008461716	XP_008459938
CpCPQ	OSC	<i>Cucurbita pepo</i>	<i>Cucurbita pepo</i> CPQ mRNA for cucurbitadienol synthase, complete cds.	AB116238	BAD34645

CpCPX	OSC	<i>Cucurbita pepo</i>	<i>Cucurbita pepo</i> CPX mRNA for cycloartenol synthase, complete cds.	AB116237	BAD34644
CsBi/CsCPQ	OSC	<i>Cucumis sativus</i>	cucurbitadienol synthase	KM655855	AIT72030
SgCBQ	OSC	<i>Siraitia grosvenorii</i>	<i>Siraitia grosvenorii</i> cucurbitadienol synthase mRNA, complete cds.	HQ128567	AEM42982
CmaCPQ	OSC	<i>Cucurbita maxima</i>	NA	NA	CmaCh17G013880
CmoCPQ	OSC	<i>Cucurbita moschata</i>	NA	NA	CmoCh17G013410
EtAS	OSC	<i>Euphorbia tirucalli</i>	β -amyrin synthase	AB206469	BAE43642
ObAS2	OSC	<i>Ocimum basilicum</i>	multi-functional OSC	JQ809437	AFH53506
OsOSC2	OSC	<i>Oryza sativa</i>	cycloartenol synthase	AK121211	BAH00370
OsOSC8	OSC	<i>Oryza sativa</i>	multi-functional OSC	AK070534	BAG92014
PsoSCPSY	OSC	<i>Pisum sativum</i>	β -amyrin synthase	AB034802	BAA97558
PtbAS	OSC	<i>Polygala tenuifolia</i>	β -amyrin synthase	EF107623	ABL07607
RcCAS	OSC	<i>Ricinus communis</i>	<i>Ricinus communis</i> cycloartenol synthase mRNA, complete cds.	DQ268870	ABB76767
RcLUS	OSC	<i>Ricinus communis</i>	<i>Ricinus communis</i> lupeol synthase mRNA, complete cds.	DQ268869	ABB76766
RsCAS	OSC	<i>Rhizophora stylosa</i>	<i>Rhizophora stylosa</i> RsCAS mRNA for cycloartenol synthase, complete cds.	AB292608	BAF73929
SICAS	OSC	<i>Solanum lycopersicum</i>	cycloartenol synthase	NM_001246855.2	NP_001233784.1

SITTS1	OSC	<i>Solanum lycopersicum</i>	β -amyirin synthase	HQ266579	ADU52574
SITTS2	OSC	<i>Solanum lycopersicum</i>	multi-functional OSC	HQ266580	ADU52575
ToTRW	OSC	<i>Taraxacum officinale</i>	<i>Taraxacum officinale</i> TRW mRNA for lupeol synthase, complete cds.	AB025345	BAA86932
VhBS	OSC	<i>Vaccaria hispanica</i>	<i>Vaccaria hispanica</i> beta-amyirin synthase (BS) mRNA, complete cds.	DQ915167	ABK76265
WsOSC/BS	OSC	<i>Withania somnifera</i>	<i>Withania somnifera</i> cultivar WS-Y-08 beta-amyirin synthase mRNA, complete cds.	JQ728553	AGA17940
WsOSC/CS	OSC	<i>Withania somnifera</i>	<i>Withania somnifera</i> cultivar WS-Y-08 cycloartenol synthase mRNA, complete cds.	HM037907	ADG60271
WsOSC/LS	OSC	<i>Withania somnifera</i>	<i>Withania somnifera</i> cultivar WS-Y-08 lupeol synthase mRNA, complete cds.	JQ728552	AGA17939

Table S3 : Designed primers for cucurbitacin biosynthetic genes

Genes	Forward Primer	Reverse Primer
<i>Carg11552-RA</i>	AGATGGAGGGTGGGGTCTAC	TCTCCAAGCAGCCTTAGTGC
<i>Carg03795-RA</i>	GCCCACTTCTACTGCCACAT	CATCCCCACCCTCAAACCTC
<i>Carg11550-RA</i>	CCCTTCTCGCAAATGGGTCT	CTAAACATGGCGTGGCGAAG
<i>Carg03797-RA</i>	AGCCGTCCGTCTGTTGAAAT	TTCAACGGGACCGCCATAAA
<i>Carg07313-RA</i>	CGAAATTCGCGTTCGGAGAC	TGATCGCTCAACTTGTCGCT
<i>Carg07314-RA</i>	CGCGCATTATCGCAAACAGA	AGGAGTGTCTCCGGCTGTAT
<i>Carg06672-RA</i>	CGCTGGCGGTTGCTTATTAC	AGCCAACCTGAAGCGTCTCTC

General discussion and conclusions

Plant domestication is a process of artificial selection by which populations are adapted to the conditions of cultivation and human taste. In other words, plant domestication can be defined as evolution under human management. In general, domesticated plants tend to possess more exaggerated physical traits, simpler morphologies and altered nutritional content compared to their wild ancestors¹. However, the process of plant domestication has often led to the decrease of plant resistance against herbivorous insects^{2,3}. A reduction of plant defense compounds could be the result of the allocation of resources towards traits that increase the yield at harvest, but also by active selection against these compounds to improve suitability for human consumption⁴.

*The mechanism behind the loss of cucurbitacins in domesticated *C. argyrosperma**

Cucurbitacins are tetracyclic triterpenes, very toxic for mammals⁵ and they were selected out or reduced during squash domestication^{6,7}. The results presented in my thesis support the hypothesis and common findings that cucurbitacins, are reduced or lost during domestication. We observed that domestication significantly decreased the concentration of cucurbitacins in roots and cotyledons of the varieties of *Cucurbita argyrosperma* (Chapter 2). We attempted to explain this reduction of cucurbitacins by analyzing the expression of genes involved in their biosynthetic pathway. Indeed, the accumulation of plant secondary metabolites relies on functional biosynthetic genes and therefore their regulation, their transport, or storage but they are also dependent on the developmental stage of the plant. In Chapter 4, we proposed seven gene candidates responsible for cucurbitacin production by comparing the genome of *C. argyrosperma* with other Cucurbitaceae. Even though domesticated varieties did not have cucurbitacin in their roots, the expression of the genes was still high in this organ. This may be explained by various factors. First, those genes may not be the ones responsible for cucurbitacin production, or even if the gene is expressed, it does not imply that the final molecules produced will be cucurbitacins. Putative P-450 genes that we found are still expressed in domesticated squash varieties and could potentially oxidize the molecule into another triterpene. Cucurbitacin gene expression in cotyledons was very low and highly variable. This result is similar to Brzozowski, et al.⁸ observation. They did not find evidence for cucurbitacin biosynthetic gene expression for full expanded cotyledons of *C. pepo*, but radical emerging cotyledons do. This highlights the need for plants to trigger chemical defense early in their development, which may switch afterwards for less costly defense strategies. The cotyledons I collected for the experiment may have been already too old (two weeks old, with true leaves) and therefore we did not detect high gene expression.

Moreover, Brzozowski, et al.⁸ suggested that the divergence of cucurbitacins in cotyledons is due to its regulation and its transport and not to the disruption or the biosynthetic gene function. The transport of cucurbitacin through different plant tissues was acknowledged by the study of Zhang, et al.⁹ in which they demonstrated that a

non-bitter *Cucumis melo* (melon) grafted into *Cucurbita maxima* (squash) containing cucurbitacins rendered the fruit bitter. As roots of multiple *Cucurbita* species contain cucurbitacins¹⁰, it can be hypothesized that cucurbitacins are produced mostly in the roots and are then carried to the other plant tissues for protection purposes.

Trade-off between the allocation of trichomes and the production of cucurbitacins

The fact that cotyledons stop producing cucurbitacins could be explained by plant defense theories that predict a trade-off in resource investment for chemical and physical defenses^{11,12}. Another and related explanation may also be that plants switch defense strategies during their ontogeny towards the production of trichomes in leaves^{13,14}. In Chapters 1 and 2, we observed a trade-off in physical and chemical defense among plant tissues. In wild populations of *C. argyrosperma*, roots and cotyledons had a high content of cucurbitacins, while leaves had high trichome densities and low levels or no cucurbitacins. This strongly suggests that even though domestication has reduced plant defense in squash^{6,7,15,16}, it did not alter the trade-off between physical and chemical defense among the different plant tissues.

To further investigate this phenomenon, it would be interesting to assess the induction of both cucurbitacins and trichomes in the context of plant domestication. Plants commonly use both constitutive and inducible defenses that are often not independent of each other. Cucurbitacins are constitutive defenses, although the literature on this subject is not very consistent. For example, Agrawal, et al.¹⁷ showed cucurbitacin induction at the different developmental stages of *Cucumis sativus* (cucumber) by a generalist mite. In contrast, Milano et al.¹⁸ with the specialist beetle *Acalymma vittatum*, refuted the cucurbitacin induction feeding on cucumber did not find any evidence for induction. In a recent study from our group, Di Caprio et al. (unpublished results), examined the induction of cucurbitacins and trichomes by adults and larvae of *D. balteata* on wild and domesticated plants of *C. argyrosperma*. They found that while herbivory by root larvae did not induce the production of cucurbitacins, leaf herbivory by adults of *D. balteata* induced the production of trichomes in plants of a wild population of *C. argyrosperma*.

The consequences of squash domestication on plant-insect interactions

One of the main hypotheses in the field of plant domestication and insect interactions, although the results are mixed, is that a reduction on defense compounds may lead to increase performance and reduced mortality of the herbivores feeding on crop plants^{1-3,19}. Indeed, the increased susceptibility of crop plants to herbivores is not an inevitable consequence of domestication³. Indeed, not all domesticated plants suffer from reduced insect resistance^{3,20}. What is clear is that in order to be able to generalize on the relationship between reduced plant defense and increased insect performance, more studies of individual crop systems are needed¹. The impact of domestication on plant-insect interactions has been studied for different, but few important crops such as maize²¹⁻²³, beans²⁴⁻²⁶, tomato²⁷⁻²⁹, sunflowers^{30,31}, berries^{32,33}, cabbage³⁴, potatoes³⁵, and cotton³⁶. Surprisingly squash is not one of them. Moreover, most of the

existing studies were done on aboveground plant tissues and the insects that feed on these¹⁻³.

Although root feeders play a crucial role in natural and agricultural ecosystems^{37,38}, as pointed out by Hunter³⁹, being “out of sight” has kept them largely “out of mind” from entomologists and plant biologists³⁹. In my thesis, I decided to examine mainly interactions between squash roots and root herbivores (larvae of *Diabrotica balteata* and *Acalymma* spp.). One of the reasons for this was that, for *C. argyrosperma* domestication led to the loss of cucurbitacins in the roots. I conducted two types of experiments, one designed to assess whether domestication increased herbivore performance with the assumption that the detoxification of cucurbitacins has a metabolic and ecological cost for the insect. The second experiment was to assess whether domesticated varieties are preferred by the insects, with the hypothesis that cucurbitacins drive the host preference of root herbivores. We found that the so-called generalist insect *Diabrotica balteata* grows bigger while feeding on roots of domesticated varieties compared to wild relatives (Chapter 2) but not among domesticated varieties of different squash species (Chapter 1). On the other hand, as we had predicted, the specialist *A. vittatum* did not grow differently on wild populations or domesticated varieties (Chapter 2).

We believe that the impact of cucurbitacins on the metabolism of herbivores might be dose-dependent. Indeed, no consequence on herbivores’ performance was observed either for the leaf-feeding herbivore (*Spodoptera latifascia*) or root-feeding herbivores (*Diabrotica balteata*) (Chapter 1). Both insects were forced to eat on bitter plant tissue (cotyledons and roots respectively) and their growth was not impacted by the cucurbitacin content. Yet, in Chapter 1, only domesticated varieties were used in the experiment. We assume that the cucurbitacin content either on roots or cotyledons was not comparable to the ones present in the plants of the wild populations (Chapter 2). Because no difference in RGR of both herbivores was observed, we hypothesized that the level of cucurbitacins was too small to affect the growth of both herbivores. Many studies on pesticide efficiency have demonstrated that toxic compounds are dose-dependent in the way that they affect insect physiology⁴¹⁻⁴⁴. If we compare the results found in Chapter 1 and Chapter 2, the RGR of *D. balteata* larvae feeding on wild *C. argyrosperma* roots was lower than that for larvae feeding on the roots of domesticated varieties (Chapter 2). However, when we compared the larval RGR on roots of different squash varieties containing different levels of cucurbitacins, we did not find any differences (Chapter 1). We suggest that future experiments should examine the performance of the generalists *S. latifascia* and *D. balteata*, and of the specialist *A. vittatum*, on diets with added levels of cucurbitacins. Another possibility could be that there is another compound in plants of wild populations that is responsible for the observed results.

Yet, the dose-dependent hypothesis presented above does not explain why *Diabrotica balteata* larvae are highly attracted to cucurbitacins (Chapter 1 and 2), even at the lower concentrations present in domesticated varieties (Chapter 1). The strong preference for bitter squash has been interpreted as evidence for the evolutionary relationship between *Cucurbita* and Diabroticites⁴⁵. A likely explanation may lie in the

common origins of those organisms. Species of the genus *Diabrotica* are currently distributed in the United States but have broadened their host range in the native habitat in southern Mexico and Central America, where squash has been cultivated since pre-colombian times in polycultures with corn and beans, in the traditional agricultural system known as the milpa or with other crops such as, peanuts⁴⁶. Thus, it is possible that during this long-time association with squash, *D. balteata* has evolved to tolerate specific secondary defenses leading to an adaptation to the plant without being specialized on it. The attraction to cucurbitacins for Diabroticites was already demonstrated^{45,47,48}. However, Eben, et al. ⁴⁷ found that when they offered the choice between corn roots or bitter squash roots, *D. balteata* and *A. blomorum* significantly preferred corn roots over roots with cucurbitacin B. Yet, beetles preferred bitter roots when offered the choice between bitter or non-bitter roots. But their experiment demonstrates that squash is not the preferred host of *D. balteata* when compared to maize.

The importance of field studies in the context of plant domestication

In addition to the laboratory experiments, we conducted two different common garden experiments in Puerto Escondido, Oaxaca (Mexico). This region is the place of origin of *C. argyrosperma*. The seeds of the wild populations were collected around the field site and in a village less than 10 km apart. For the first field experiment, we had different squash species domesticated for two purposes (consumption and ornamental). The goal was to observe the interaction between squash plants and insects that share evolutionary history and current agricultural associations endemic to the Americas. At the time of both common gardens, there were surprisingly few *D. balteata* adults visiting the squash plants despite the high numbers of beetles in the field site. However, *D. balteata* was found nearby on bean, maize, and chilis plants. In contrast, plants in both common gardens heavily attracted *Acalymma* spp. It was not a surprise as they are true specialists of Cucurbitaceae. Species of *Acalymma* are adapted and attracted to cucurbitacins which they use as kairomones to locate their host plants and can sequester them⁴⁹⁻⁵¹. However, as squash leaves (wild and domesticated), do not contain cucurbitacins, adult beetles likely use different cues to locate and select the plants, both for feeding and oviposition. Indeed, the ornamental varieties (Chapter 1 and 2) were always the most damaged in the field.

Overall, results from the laboratory did not match the results from the field. Indeed, even if cucurbitacins act as an attractant, at least for the root-feeding larvae, the presence of cucurbitacins in other plant tissues did not explain *Acalymma* spp. abundance. We did not find more *Acalymma* on wild populations (Chapter 2) or bitter varieties (Chapter 1). The presence of the flowers increased their abundance in the field. Even though cucurbitacins are known to act as feeding stimulants¹⁵, we did not observe that the wild plants were more damaged than the domesticated varieties. A possible explanation is that there is no cucurbitacin in the leaves (Chapter 1 and 2). In the study of Theis, et al. ¹⁰, they also found that cucurbitacins did not predict leaf damage by *Acalymma vittatum* on 20 varieties from 12 diverse Cucurbitaceae species.

Instead, they found that sesquiterpenoids were the chemical signal driving the distribution of this species. They also found that beetle abundance was not correlated with leaf damage, suggesting that damage may be better predicted by plant resistance than by beetle presence across varieties. Thus, even if they act as attractants, the presence of cucurbitacins in other plant tissues does not explain adult abundance. Instead, Brzozowski, et al.⁵² found that the spatial distribution of *A. vittatum* was due to vittatolactone, a pheromone produced by the male beetles. .

Role of sequestered cucurbitacins as protection against the herbivore's natural enemies

In the earlier literature (50 years ago)^{5,53}, It was always implied that herbivores sequester cucurbitacins that in turn will protect them against their natural enemies. Indeed, the fact that beetles sequester the bitter compounds at different life stages (e.g., in eggs, as demonstrated by Arce *et al.* (in prep) and larvae as shown in Chapter 3 and Bruno *et al* (unpublished) supports this hypothesis. Studies showed that cucurbitacins protected Diabroticina beetles against praying mantis⁵ and nematodes⁵⁴. In Chapter 3, we investigated the consequences of squash domestication on the third trophic level. We hypothesized, that because domesticated squash lost the cucurbitacins (Chapter 2), herbivores such as *Diabrotica balteata* larvae will not be able to sequester these compounds from the roots of domesticated varieties and therefore, should suffer greater predation by a generalist predator. We did not find support for this hypothesis. Predation was not higher on larvae fed with roots of domesticated plants. Moreover, the survival and host preference of the predatory rove beetle *Atheta coriaria* were not impacted by sequestered cucurbitacins. Interestingly in the “bag experiments”, where roots were exposed to the herbivore larvae and the predators in the same environment, *A. coriaria* preferred to feed on larvae that were feeding on the wild squash that contained cucurbitacins. Cucurbitacins in roots and possibly in larvae could be responsible for the attraction of the predator and therefore, cucurbitacins could play a role of phagostimulant rather than repulsive. Future research should further explore this idea.

The impact of the purpose of domestication in squash on plant-insect interactions

Throughout my thesis, I tested the hypothesis that the purpose for which the plants were domesticated could explain the differences among squash species (Chapter one) and between wild and domesticated varieties (Chapter 2,3, and 4). I used domesticated varieties selected for human consumption of the fruits and varieties domesticated for ornamental purposes. We hypothesized that squash varieties selected for consumption will be less defended either by chemical (cucurbitacins) or physical (trichomes) defenses against herbivores. As cucurbitacins are very bitter and toxic, they have to be removed at least from the fruits to be edible. I found partial support for this hypothesis. For trichome density, I found a tendency for varieties selected for consumption to have less hairy trichomes on the adaxial side of their leaves. However, for cucurbitacin content, we observed the opposite results than

expected. The ornamental varieties did not contain cucurbitacins in any of the tissues (Chapter 2) or negligible amounts depending on the species (Chapter 1) and the gene expression for cucurbitacins supported these results (Chapter 4). The purpose of domestication did not differentially impact the behavior of the generalist and specialist herbivores. I believe that a plausible explanation is that for both of the studied purposes, consumption or ornamental, artificial selection targeted the fruits. Archeological evidence shows that the first purpose of squash domestication was as receptacle or medicine rather than consumption^{6,56}. It is likely that selection and breeding for the fruits as ornamentals came after the purpose of consumption. Thus, some of the ornamental varieties could be edible and this would explain why I did not find differences between varieties selected for consumption and varieties with ornamental use.

Together, the results from Chapter 1 and Chapter 2 highlight the importance of taking into account the phylogeny of the studied organisms. It would be interesting to further investigate the relationship between chemical defense, insect performance and domestication purpose in other squash species (*C. pepo*, *C. maxima* and *C. ficifolia*) by comparing, as we did, for *C. argyrosperma*, the wild ancestors and the domesticated varieties within each species.

The role of volatile compounds in plant domestication

One aspect that was not studied in my thesis is how domestication altered the indirect defenses of squash, typically the emission of plant volatile. Roots emit specific volatile blends when attacked by herbivores⁵⁷. These volatiles can diffuse through the soil and alter the behavior of herbivores and natural enemies⁵⁸. The emission of herbivore-induced plant volatiles (HIPVs) is triggered with herbivore damage, saliva, or egg deposition⁵⁸. HIPVs can attract natural enemies and repel herbivores⁵⁹. It is known that volatile signaling can differ between crops and wild plants resulting in a change of the interaction with natural enemies. Indeed, if through domestication, the plant has reduced or lost its ability to release specific volatiles, it could affect the densities of natural enemies responding to herbivore attack^{57,60}. However, this is not ubiquitous, Rowen and Kaplan⁶¹ in a meta-analysis that included 236 experiments examining the volatiles emitted by herbivore-damaged and undamaged plants, showed stronger indirect defense responses (volatile emissions) in domesticated species compared to their wild relatives. Moreover, they showed that in general, the inducibility of plant volatiles increased with domestication. For example, the inducibility of chemical defenses against an herbivore was lower in domesticated cabbage (*Brassica oleracea*)⁶². In contrast, there are few reports of reductions in herbivore-induced plant volatiles through domestication^{1,60}. Paudel, et al. ²⁷ found that while all tomato genotypes exhibited increased volatile organic compound (VOCs) emissions in response to herbivory, the domesticated variety responded with generally higher levels of VOCs²⁷.

Compare volatile emissions between wild *C. argyrosperma* and related domesticated varieties could be the following step of research following my thesis. It could be of great interest, especially to explain how *Diabrotica* finds their host at first, but also to investigate if it also affects the attraction of the third trophic level⁶³. This knowledge could be exploited in biological control programs⁶⁴.

As cucurbitacins are not volatiles compound^{65,66}. The attraction of beetle larvae to bitter plants (Chapter 1 and 2) is most likely the response to taste. I observed during the bioassays that larvae were moving around in the Petri dishes, suggesting tried the different types of roots (cucurbitacin-containing or cucurbitacin-free). This is in accordance with the results; the preference for bitter roots was higher after 24h hours. Moreover, this supports again the hypothesis that cucurbitacins are compulsive feeding stimulants⁶⁷.

Conclusions

The aim of this thesis was to further our understanding of the consequences of plant domestication and its impact on plant-insect interactions at different trophic levels. We focused on squash a crop of worldwide importance for which information on this subject is still limited. Taken together, the results from my thesis showed that domestication of *C. argyrosperma* selected for a decrease in cucurbitacins in the roots and cotyledons via the lower expression of cucurbitacin related genes. In our experimental set up, the insects were not negatively impacted by the cucurbitacins. Interestingly, cucurbitacins act as an attractant to the larvae of Chrysomelidae, even though I did not find evidence for a benefit for the insect. Indeed, throughout the thesis It becomes clear that there is no evidence that cucurbitacins are beneficial for the plant as a defense against insect herbivores, nor for the insects that sequester them. The hypothesis that the purpose of domestication (consumption or ornamental) differentially altered plant defenses in roots and leaves and the interactions with insects was not supported. By studying these plants and insects in their region of origin, Mexico, our study provides unique insights into the evolutionary trajectories and selective pressures that have likely molded the interactions we observe today. However, new results led to new questions which could benefit from additional research.

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Take home messages

- Squash domestication y reduced the content of cucurbitacins in different plant parts, particularly in the roots.
- Candidate genes for cucurbitacin biosynthesis are less expressed in roots of the domesticated varieties that contain less or no cucurbitacins. However, the expression of these genes in cotyledons is highly variable. Globally, gene expression is higher in roots than in cotyledons.
- The density of leaf trichomes tends to be lower in varieties selected for fruit consumption than in varieties selected for an ornamental use.
- The performance of *Spodoptera latifascia* is similar on leaves and cotyledons among domesticated squash varieties.
- Generalist (*D. balteata*) and specialist (*A. vittatum*) root-feeders are highly attracted to cucurbitacins.
- The preference of *D. balteata* larvae to cucurbitacins may be likely due to the evolutionary history that this species has shared with wild and domesticated squash plants in their region of origin and domestication.
- Squash domestication increased the relative growth rate of larvae if the generalist root-feeder (*D. balteata*) but not of the specialist insect (*A. vittatum*).
- Varietal selection rather than the purpose of domestication has altered cucurbitacin content and leaf trichome density.
- Squash domestication did not indirectly affect the survival and preference behavior for the rove beetle predator (*A. coriaria*).
- Sequestered cucurbitacins by root herbivores do not provide protection against predators.

Future perspectives

1. Test the role of the putative cucurbitacins biosynthetic genes, to be sure they code for the studied enzymes.
2. Test the interactions between plant and insects, with herbivores with a different feeding mode (e.g., piercing-sucking) and from different origin than Mesoamerica.
3. Test the dose-dependent effect of cucurbitacins
4. Test the phagostimulation effect of cucurbitacins
5. Test the protection role of cucurbitacins against pathogens
6. Examine the relationship between chemical defense and herbivore performance with different species of squash, the wild ancestor and domesticated varieties
7. Test the impact of domestication on squash indirect defense (volatile emission)

Appendix

Publications published and in preparation:

Charlyne Jaccard, Maximilien AC Cuny, Carlos Bustos-Segura, Carla Arce, Lorenzo Giollo, Gaëtan Glauser, Betty Benrey. Squash varieties domesticated for different purposes show distinct changes in their chemical defense against leaf and root herbivores (Published in *Frontiers in Agronomy*).

Charlyne Jaccard, Carlos Bustos-Segura, Wenfeng Ye, Gaetan Glauser, Ian Kaplan, Betty Benrey. The domestication of *Cucurbita argyrosperma* altered plant defense and differentially affected the performance of a specialist and a generalist herbivore. (In preparation)

Charlyne Jaccard, Nicolas Marguier, Gaëtan Glauser, Pamela Bruno, Carla C. M. Arce, Ted Turlings, Betty Benrey. Loss of cucurbitacins in domesticated *Cucurbita argyrosperma* does not increase predation of a root feeding herbivore. (In preparation)

Charlyne Jaccard, Wenfeng Ye, Carlos Bustos-Segura, Ted Turlings, Betty Benrey. Characterization of cucurbitacin biosynthetic genes associated with reduced bitterness in squash cultivars (*Cucurbita argyrosperma*). (In preparation)

Pamela Bruno, Carla CM Arce, Ricardo AR Machado, Gaia Besomi, Anna Spescha, Gaëtan Glauser, **Charlyne Jaccard**, Betty Benrey, Ted CJ Turlings. *Diabrotica balteata* sequester cucurbitacins from cucumber plants, but this provides no protection against common natural enemies. (In preparation)

Master thesis co-supervision

Nicolas Marguier 2019-2020: *Impact of squash domestication on the insect soil community*

Leandro Di Caprio 2020-2021: "constitutive and induced defenses of wild *C. argyrosperma* populations"

Attended Meetings (Poster and Oral Presentations)

Annual PhD Student Meeting 2018 (Neuchâtel, Switzerland), Poster + Oral Presentation

Biology18 Meeting 2018 (Neuchâtel, Switzerland), Poster

International Society of Chemical Ecology 34th Annual Meeting 2018 (Budapest, Hungary), Poster

Biology20 Meeting 2020 (Fribourg, Switzerland), Oral Presentation

Extension/Vulgarisation

Fête des Sciences, Ecole Moser S.A 2018 (Nyon, Switzerland), Oral presentation: « La communication chez les plantes »

Fête de la Samin, Jardin Botanique, 2018 (Neuchâtel, Switzerland), Oral presentation: « Au Secourge »

MT180, April 2021 (Neuchâtel, Switzerland), 2ème prix de la sélection Neuchâteloise

MT180, May 2021 (Lausanne, Switzerland), swiss final

Events organization

Biology18, committee member

SeeDS 2018, flyer design

Teaching

Travail de maturité, Xenia Berger 2016

APP (apprentissage par problème), 3rd year of Bachelor in Biology, 2017, 2018,2019, 2021

TP Ecology of population, 2nd year of Bachelor in Biology, 2018,2019,2020

Internship, Célia Ruiz 2018: “*Investigating the impact of domestication on the constitutive level of defense in Cucurbita argyrosperma*”

Internship, Lorenzo Giollo 2020: “*The effect of squash domestication purposes on plant defenses against a generalist herbivore*”

Assistant Workshop Sustainable Agriculture, 1st Msc in Biology + PhD courses Organismal biology, 2020