

**CHEMICAL ECOLOGY OF WILD *SOLANUM* SPP AND THEIR INTERACTION
WITH THE COLORADO POTATO BEETLE**

By

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ABSTRACT

CHEMICAL ECOLOGY OF WILD *SOLANUM SPP* AND THEIR INTERACTION WITH THE COLORADO POTATO BEETLE

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To date the Colorado potato beetle (CPB) continues to be an important threat for potato growers world-wide. Wild potatoes are a source of a genetic diversity encoding properties such as resistance to pests, which may provide sustainable alternatives to the use of pesticides. First objective was to investigate the effects of single accessions of three wild *Solanum* species on the growth and development of CPB compared to effects of the cultivated *S. tuberosum* cv. Atlantic. Larvae consumed significantly less foliage of *S. immite* and *S. pinnatisectum* compared to the cultivated potato. Larvae were unable to complete their development on *S. immite* and significantly fewer completed their development on *S. pinnatisectum* compared to the cultivated potato. No significant differences were observed between *S. chacoense* and *S. tuberosum*. Surprisingly, females laid the greatest amount of eggs on *S. immite*, while there were no significant differences among the other species in oviposition preference. My second objective was to analyze chemical defenses in the potato species. *S. immite* and *S. pinnatisectum*, the least preferred by CPB for larval feeding and larval had two volatiles, limonene and terpinolene, which comprised about 90% of the headspace, suggesting that they could be involved in resistance to CPB. There was no significant difference in content of the glycoalkaloid solanine between the least (*S. immite*) and most preferred (*S. tuberosum*) potato species by the CPB. No acyl sugars were found by leaf dip analysis in any of the potato species. This same analysis provided information about glycoalkaloid content with solanine and chaconine present only in *S. tuberosum*, *S. chacoense* and *S. immite* and tomatine only found in *S. pinnatisectum*.

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CHAPTER 1.

History, biology of the Colorado potato beetle and wild *Solanum spp.* chemical ecology

History and importance of the Colorado potato beetle as a crop pest

The Colorado potato beetle (CPB), *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae) is considered the most important defoliator of potatoes world-wide (Alyokhin et al. 2008). The CPB's center of origin is central Mexico, where the main host plants originate from *Solanum rostratum* and *S. angustifolium* (Solanaceae) (Whalen 1979). Currently the CPB can be found in America (Costa Rica, Guatemala, Cuba, Mexico, United States and southern Canada), Africa (Libya), Asia (Iran, China and Japan), and in almost all countries in Europe and in the southern part of the former Soviet Union (Capinera 2001, Walsh 1865). It feeds exclusively on solanaceous plants, including crops and weeds. CPB is an important pest of potatoes, tomatoes and eggplant; it also feeds on peppers, tobacco and weeds such as ground cherry, horse-nettle, nightshade, belladonna, thorn apple and buffalo-bur, the latter being its native host (Capinera 2001). As little as 12.5% defoliation by CPB can significantly reduce yield in potato (Mailloux et al. 1996). Ferro et al. (1985) estimated that larvae can consume an average of approximately 40 cm² of potato leaves per day over 3 to 4 weeks of development, while adults can ingest up to 10 cm²/day. In a controlled field study it was determined that during the middle part of the potato crop growing-period (weeks 4-6), yields were reduced up to 64% when the population of CPB was left uncontrolled (Hare 1980).

In the United States, CPB was first recognized as a pest of potatoes in 1865 (Walsh 1865). Walsh also mentioned that Colorado was the first state where it was reported as a pest and over the next five years the beetle was also found in large numbers in Nebraska and Iowa. It

has been speculated that the beetle moved to the United States from Mexico after seeds of its host plant crossed the border by adhering to the fur of cattle driven to Texas for sale (Lu & Lazell 1996). From there, the CPB dispersed to most of the North American territory where the economic impact is currently estimated at hundreds of millions of dollars in chemical control and crop losses each year (USDA 2009).

Biology of the Colorado potato beetle

CPB overwinter in the soil as adults and emerge from woody areas next to fields where they fed the previous summer. Post-diapause beetle's occurrence matches the location of potatoes in the field in the previous season. Non-rotated potato fields are colonized by adults walking from their overwintering places. Beetles can fly up to several kilometers so rotated fields can be colonized when they locate a new plant host (Alyokhin et al. 2007).

The beetle undergoes complete metamorphosis. Eggs hatch within 4 -10 days, depending on weather. Larvae pass through 4 developmental instars over a 2-3 week period, then pupate in the soil for 5-10 days. Adults may live for a couple of months. The entire life cycle is completed in 14 to 56 days depending on temperature (Ferro et al. 1985).

Wild potato species and plant resistance

Vavilov (1940) pointed out the potential of crop relatives as a source of genes to improve crop plants. The wild tuber-bearing *Solanum* L. species (*Solanaceae* sect. *Petota* Dumort) and outgroup relatives in *Solanum* sect. *Etuberosum* (Bukasov & Kamerz), (hereafter referred to as wild potatoes), are relatives of the cultivated potato (Hawkes 1990). Besides the cultivated potato, *Solanum tuberosum* L., there are six other cultivated species that only grow in the Andes

(Walker et al. 1999). One hundred and eighty seven wild potato species are known apart from these seven cultivated potato species and they are found from the southwestern United States to Chile (CIP 2012), although the most recent taxonomic study identified 232 species (Hawkes 1990). These wild potato species are distributed in 16 countries, yet 88% of them are found in only four countries (Argentina, Mexico, Bolivia and Peru). The highest number of species was found in Peru (93), followed by Bolivia (39) (Hijmans & Spooner 2001).

Wild potato species grow in diverse soils and climates including the coastal desert of Peru and the inter-Andean valleys, up to altitudes of 4,200 meters above sea level. The tubers of these species are much smaller than cultivated potatoes and they are shaped in various forms and colors. Contrasting with cultivated potatoes that are poor in genetic diversity lost during domestication, their wild crop relatives possess a rich source of natural resistance to pests, diseases, and climatic conditions (CIP 2012).

Different studies have been conducted with wild potato species in search of resistant genes to control fungal, viral, nematode, and insect pests. With respect to studies including insect pests, 1,686 potato accessions that represent 100 species in the genus *Solanum* L., subgenus *Potatoe*, section *Petota*, were evaluated for field resistance to one or more of these insect pests: green peach aphid, *Myzus persicae* (Sulzer), Hemiptera: Aphididae; potato aphid, *Macrosiphum euphorbiae* (Thomas), Hemipetra: Aphididae; Colorado potato beetle; potato flea beetle, *Epitrix cucumeris* (Harris), Coleoptera: Chrysomelidae; and potato leafhopper, *Empoasca fabae* (Harris), Hemiptera: Cicadellidae (Flanders et al. 1992). The same study showed that accessions of 36 species were highly resistant to green peach aphid, 24 species to potato aphid, and 10 species to CPB. Other relevant findings were that insect resistance seems to be a primitive trait in wild potatoes. On the other hand, the authors also found that the glycoalkaloid

tomatine was associated with field resistance to CPB and potato leafhopper, and dense hairs were associated with field resistance to CPB, potato flea beetle, and potato leafhopper.

Pelletier et al. (1999) evaluated 7 species of wild *Solanum* in field and laboratory settings to identify the mechanisms of resistance to the CPB. They found that *S. berthaultii*, *S. tarijense*, and *S. capsicibaccatum* have semiochemical-based resistance expressed by reduced oviposition and a low recovery rate when exposed to the plants, and adult and larval mortality was low when feeding on these plants. These authors also mentioned that *S. capsicibaccatum* may have a different set of chemicals involved in the resistance mechanism since this species lacks trichomes. *Solanum jamesii*, *S. polyadenium*, and *S. trifidum* were also included in this research and a toxin/feeding deterrent was found in these plants, causing high mortality of adults and larvae, and low egg production. Finally, larval mortality was high on *S. pinnatisectum* but adult mortality was relatively low, indicating that resistance may be less effective in this species or that it has different effects on adults and larvae.

The resistance of three wild *Solanum* tuber-bearing potatoes to CPB was studied by Pelletier et al. (2001) in the field. *S. okade*, *S. oplocense*, and *S. tarijense* showed high levels of resistance to CPB. The mode of resistance differed among the three species. *S. okade* and *S. oplocense*, affected host acceptance and consumption but in different ways (*S. tarijense* affected adult colonization and oviposition); therefore, the authors assumed that antifeedants varied in quality or quantity among these species.

Control strategies for the Colorado potato beetle

A number of strategies have been used for more than a century in an attempt to reduce economic damage caused by CPB to the potato industry. Insecticides still remain the most important

control tools for growers and new insecticide chemistries are showing good results in the field. The CPB has a remarkable ability to easily adapt to new biotic and abiotic conditions, locally and worldwide (Hare 1990) and has developed resistance to most of the currently used insecticide products (Alyokhin et al. 2008; Bishop & Grafius 1996). The long-term efficacy of new insecticide chemistries is uncertain.

Other control alternatives have been used with different degrees of efficacy. Crop rotation is one approach that can be effective, if there are large distances between the newly planted fields and crops of the previous year. Fields separated by 0.4 km or more from a previous potato field had lower CPB densities than fields that were adjacent to a field planted with potatoes the year before (Hough-Goldestein & Whalen 1996). Planting time delays and trap crops are other cultural modifications that offer some control (Weber et al. 1994). Predators, parasitoids, nematodes, bacteria and fungi are known biological control agents of CPB but with low success rates (Hough-Goldestein et al. 1993, Ferro 1994 and Berry et al. 1997). Transgenic crops including *Bacillus thuringiensis* are promising alternatives, but they are still not universally accepted (Coombs et al. 2003).

Chemical Ecology

Chemical ecology is increasingly being considered for pest control: the role of secondary metabolites in defending plants from insect herbivory and mammalian grazing has recently received more attention than any other aspect of chemical ecology (Harborne 2001).

Plant volatiles.

Plant volatiles are volatile organic compounds (VOCs) that can diffuse at ambient temperature. These compounds have different functions including attracting pollinators, seed dispersers, and

predators or parasitoids that attack herbivores; repelling or intoxicating herbivores; priming defenses of neighboring plants against imminent attack; conferring antimicrobial properties vital to defense against pathogens, and mitigating oxidative stresses (Qualley & Durareva 2010).

McIndoo (1926) was the first to demonstrate the effect of VOCs from undamaged potatoes on the CPB. Using its olfactory system, the CPB locates food after completing diapause and emerging from soil in the spring (Sablon et al. 2012). Visser & Nielsen (1977) demonstrated that the CPB was attracted to solanaceous species and after contact with the host plant, there are other mechanisms involved in final host acceptance. Weissbecker et al. (1997) confirmed previous findings regarding the capacity of the CPB in detecting green leaf volatiles such as (Z)-3-hexen-1-ol, (E)-2-hexenal, linalool, and some terpenes in gas chromatography-electroantennographic detector (GC-EAD) bioassays. They also identified β -myrcene, benzeneethanol, and several sesquiterpenes (e.g., caryophyllene and germacrene-D) as volatiles that CPB perceives.

Feeding deterrents in the Solanaceae

- a. Glycoalkaloids. *Solanum* species contain glycoalkaloids (Chen and Miller, 2001) that have antimicrobial and pesticidal properties (Tingey 1984). Steroidal glycoalkaloids (SGAs) have been described as nonspecific antifeedants (Sturckhow & Low 1961) or toxins (Smith 1989). It is important that the expression of SGAs be restricted to leaves and be absent in tubers as it can impose health risks to humans (Zitnak & Johnston 1970). Unlike other glycoalkaloids found both in leaves and tubers, the steroidal alkaloid leptine is found only in leaves (Sinden et al. 1986). Leptine glycoalkaloids in leaves of the wild potato species *S. chacoense*, a diploid species, have been shown to reduce feeding by CPB (Rangarajan et al. 2000).

Kowalski (1999) evaluated the effect of five alkaloids (α -tomatine, α -chaconine, α -solanine, leptine I, and steroidal aglycone solanidine) on the development of CPB larvae, from hatching to the prepupal stage. The author found adverse effects of leptine I and solanidine on larval weight gain and molting time.

- b. Acyl sugars. Acyl sugars are exuded by glandular trichomes in several species of the Solanaceae. They are non-volatile metabolites and have shown a defensive role against pests (Smeda et al. 2014). For example, Goffreda et al. (1989) found that settling of the potato aphid, *Macrosiphum euphorbiae*, on feeding membranes was deterred by methanolic leaf rinses of *Lycopersicon pennellii*, or by its F1 cross with tomato, *L. esculentum*. The authors found that the active compounds in the *L. pennellii* rinsates are 2, 3, 4-tri-O-acylglucoses bearing short to medium chain length fatty acids. These compounds are found in the glandular exudate of the type IV trichomes and may accumulate to levels in excess of 400 $\mu\text{g}/\text{cm}^2$. In a different experiment, the feeding and oviposition of leafminer *Liriomyza trifolii* (Burgess) (Diptera, Agromyzidae) was significantly lower on *L. pinellii* and its F1 hybrid with *L. esculentum* than that on the cultivated tomato, *L. esculentum*. The resistance of *L. pennellii* and the F1 was reduced after the foliage was rinsed with ethanol (Hawthorne et al. 1992).
- c. Other feeding deterrents. Apart from glycoalkaloids and acyl sugars, other chemical compounds have also been associated with antifeedant activity including tetranortriterpenes, or limonoids (Alford et al. 1987). The authors evaluated the behavior of fourth-instar CPB larvae when exposed to limonin in choice and no-choice laboratory experiments; potato leaf disks were treated on the upper surface with 50 μl of different solutions of limonin in acetone while control leaves were treated with 50 μl of acetone

only. They found that foliage consumption was reduced by 19.5 - 67.4% compared to the control, with limonin dosages between 10 - 100 ug/cm² in the choice experiments. Consumption was reduced by 64.6-96 % with the same dosages compared to the control in the no-choice tests. Constitutive phenolics in plants are also important compounds with significant roles in plant defense: individual phenolic subclasses are active against specific herbivores, more so than total phenolics or total tannins in a plant (Harborne 2001). Castanera et al. (1996) studied the biological performance of CPB fourth instars on two potato genotypes resistant to CPB, with different levels of the enzyme polyphenol oxidase. They found a positive correlation between polyphenol oxidase in potato leaves and larval mortality and negative correlation between this enzyme's content and larval weight, fecundity and relative larval growth rate.

Antifeedants and resistant cultivars

None of the commercial potato cultivars have a significant level of resistance or tolerance to CPB (Ferro & Ferro 1993). A resistant potato cultivar, even with a moderate resistance level would help potato production immensely, since less insecticide would be required and biological control could be established (Pelletier et al. 1999). Since plant resistance is frequently based on chemical properties of plants and the CPB has developed strategies to detoxify synthetic insecticides, it could also develop tolerance to plant chemicals (Smith 1989). If a plant produces several toxins with different modes of actions, the insect would need selection for several traits which would retard the development of tolerance to the resistant plant (Pelletier et al. 1999). When a toxin and a semiochemical are present in the resistant plant at the same time, it would force the development of behavioral and physiological characters in the insect population, in order to accept the plant. For these

reasons, the mode of action of resistant characters have to be studied as well as the number of traits in the plant, when evaluating tactics to develop a resistant potato variety.

Study objectives

This thesis presents results of the responses of the CPB exposure to single accessions of three wild potatoes species: *S. chacoense*, *S. immite*, *S. pinnatisectum* and *S. tuberosum* cv. Atlantic as a control (Fig. 1.1). These species have exhibited varying degrees of resistance to CPB in initial screening tests. The general purpose of this work focused on locating resistance characters in these wild potato species. Resistant characters in wild potato species can provide useful tools in the development of a breeding germplasm. Chapter 2 aimed to determine how these wild potato species affect the growth and development of the CPB. For this purpose, foliage consumption of the wild species by second instar larvae was quantified and compared with consumption of cultivated potato *S. tuberosum* cv. Atlantic. Also, the survival and development of neonates to pre-pupae on the four plant species was compared in no choice assays. Finally, oviposition preference of gravid CPB females when exposed to the four plants in choice experiments was also compared. In Chapter 3, I present results of the analysis of chemical plant defenses of the four species with the intent to identify compounds potentially responsible for differences in host acceptance. Amounts of the glycoalkaloid α -solanine were evaluated in all four potato species. Headspace analyses were used to compare qualitative differences in volatile compounds among the potato species. Finally, acyl sugar content and glycoalkaloids on the surface of the leaves were compared among species using leafdip analyses.



Figure 1.1. Four *Solanum* species included in this study. A. *Solanum immite* B. *Solanum pinnatisectum* C. *Solanum chacoense* and D. *Solanum tuberosum* cv. Atlantic (cultivated potato). Notice the differences in leaves: while the wild species tend to have narrow leaflets, the cultivated potato has larger, wider leaflets.

CHAPTER 2.

Wild potatoes' effect on Colorado potato beetle growth and development

Introduction

Colorado potato beetle (CPB), *Leptinotarsa decemlineata* (Say) adults and larvae feed on potato leaves and leaves of solanaceous crops, driving insecticide use for their control (Gauthier et al. 1981). The development of insect resistant potato cultivars as an alternative control strategy against the CPB is of interest (Flanders et al. 1992 because of the insect's potential to develop insecticide resistance (Boiteau et al. 1988) and public awareness of the possible health problems linked to pesticide use (Rimal et al. 2001).

The germplasm array of cultivated potato is not extensive, but there is a wide genetic diversity in their wild relatives (Jansky & Rouse 2000). Breeders have been studying wild potatoes for decades and more recently molecular geneticists are also focusing on their rich source of genes for potato improvement (Thieme et al. 2010). Experiments conducted in the laboratory and in the field have uncovered many *Solanum* sp. accessions that are resistant to one or a few potato pests (LeRoux et al. 2007). Understanding the mode of action of plant insect resistance is important in the development of resistant cultivars (Pelletier & Dutheil 2006). Every resistance mechanism is linked to specific genes that breeders can use when generating new crop varieties (Johnston et al. 1980).

Resistance mechanisms in plants have been categorized as either antibiosis or antixenosis (Painter 1958). Antibiosis resistance consists of traits that either poison insects or make feeding difficult for herbivores after they have selected a plant to feed on. Studies on host appropriateness for insect development can help determine the antibiosis mode of resistance in different plant species. For example, Pelletier et al. (2001) evaluated the resistance of three wild

tuber-bearing potatoes for the CPB (*S. okadae*, *S. oplocense* and *S. tarijense*). They found that the mechanism was a reduction in feeding frequency on wild *Solanum* foliage compared to the cultivated cv. Russet Burbank. These authors also looked at the suitability of the same wild *Solanum* species for larval development. Larval mortality varied within plant species and also with larval instars, but the clearest results were seen with *S. oplocense*, on which no larvae survived past the second instar.

In a different study, Pelletier et al. (1999) assessed several fitness parameters of the CPB, when maintained on foliage of seven wild *Solanum* species. Larval survival was one of the parameters evaluated, and the highest mortality of first instar CPB occurred when feeding on *S. pinnatisectum*, *S. polyadenium* and *S. trifidum*. The mortality of second instars was highest on *S. jamesii*, *S. polyadenium* and *S. pinnatisectum*. Third instar mortality was not significantly different among any of the tested *Solanum* species.

Antixenosis involves physical, chemical or phenological characters that make a plant less desirable for feeding or oviposition (Painter 1958). Glandular trichomes may provide antixenosis-type resistance to plants (Gibson 1976). Glandular trichomes of *S. berthaultii* confer both chemical and physical resistance as they affect settling and probing behavior of aphids (Lapointe & Tingey 1984). Glandular trichomes on the foliage of *S. berthaultii* contain oviposition deterring compounds against the potato tuber moth, *Phythorimaea operculella* Zeller (Malakar & Tingey 2000).

While antibiosis and antixenosis resistance are both advantageous for a plant, evolution may not result in plants with high rates of both (Abrahamson & Weis 1997). As an undesirable outcome of selection, antibiosis and antixenosis may end up being negatively correlated. This poor correspondence between herbivore's oviposition preference and performance of its

offspring can be observed in some plant species (Thompson 1988). For example, although *S. nigrum* is accepted by CPB for oviposition, neither adults nor larvae consume it. This leads to the conclusion that oviposition selectivity of the adult is not linked to larval food preference in this species (Hsiao & Fraenkel 1968), and that CPB females have a wider range of hosts for oviposition than larvae can accept as food.

However this may not be the same for all *Leptinotarsa* sp.; *L. undecimlineata* L. is able to make oviposition choices that align with immature performance. Females of this close relative of CPB chose *S. lanceolatum* (their host) leaves significantly more often than the non-host plant (*S. myriacanthum* Dunal), and pupal weight was also significantly higher on *S. lanceolatum* than the non-host plant (Eben & Lopez-Carretero 2008). Curiously larvae showed no significant preferences for either plant species.

In order to identify potato resistance traits and try to elucidate the resistance mechanism, I evaluated the behavior of CPB when exposed to three wild potato species and a cultivated potato in three laboratory experiments. The first experiment quantified the amount of leaf area consumed by second instar CPB in no choice assays. The second experiment measured larval survival of CPB exposing neonate CPB to fully developed plants of the four potato species. The third experiment examined oviposition preference of gravid CPB females in choice experiments.

Materials and Methods

Insect and plant material

CPB larvae and adults used in all the experiments were obtained from a laboratory colony maintained at the Vegetable Entomology Laboratory at Michigan State University (East Lansing,

MI). The colony was kept in continuous culture on *Solanum tuberosum* cv. Atlantic in a rearing room at 25 °C, 40-80% HR, and with 16:8 (L:D) photoperiod.

The wild potato species *Solanum chacoense*, Bitter (PI 123123, selection 3), *Solanum immite*, Dunal (PI 365330 selection 03), and *Solanum pinnatisectum*, Dunal (PI 184774, selection 88) were obtained from the United States Potato Genebank (NRSP-6, Sturgeon Bay, WI). These species are originally from different countries, look different to the commercial potato grown at different altitudes, and studies show different levels of insect and disease resistance for some of them (Table 2.1).

Table 2.1. Some characteristics of wild *Solanum* species used in the current study.

Plant species	Origin	Ploidy	Elevation (m above sea level)	Pest resistance
<i>S. immite</i>	Peru	diploid	80-3700	CPB (Flanders et al. 1992)
<i>S. chacoense</i>	Argentina	diploid	0-3700	<i>Verticillium</i> wilt (Lynch et al. 1997) CPB (Cooper et al. 2009) Potato leaf roll virus (Brown et al. 1993)
<i>S. pinnatisectum</i>	Mexico	diploid	1500-2200	CPB (Flanders et al. 1992)

Once mother plants were established from tissue culture plants, the wild potatoes were propagated by cuttings. The commercial potato *S. tuberosum* cv. Atlantic was used as control in all bioassays conducted in the study and was acquired from Elmira, Michigan. Atlantic seed tubers were used to initiate a group of mother plants and subsequently, propagation was done through vegetative cuttings, similarly to the wild potato species. All plants were grown in a

growth chamber (temperature: 25-27 °C, 75% HR, photoperiod of (16:8) (L: D) in 12 cm diameter plastic pots in a peat/vermiculite soil mix (Suremix , Michigan Grower Products, Inc., Galesburg, MI), weekly supplemented with a 14-10-14 (N-P-K) fertilizer (Scott's, Miracle-Grow Products, Inc. Marysville, OH), adding 1 tablespoon in 3.75L water. All bioassays were conducted under laboratory conditions at the Vegetable Entomology Laboratory (Michigan State University, East Lansing, MI).

Larval foliage consumption

The foliage consumption of the three wild potato species and the commercial potato by CPB larvae was compared in a no choice experiment. The youngest fully expanded leaf from each of the four species was collected from 4-5 weeks old plants. A new plant was used every time to collect one leaf. To prevent desiccation, leaf stems were placed in 1.7 ml water-filled plastic microcentrifuge vials with perforated caps. Prior to placing the leaves into the vials, the leaf area was obtained using a Li-Cor Portable Leaf Area Meter (LI-3000C, Lincoln, NE). Newly ecdysed CPB second instars, fed on cv. Atlantic foliage in Petri-dishes, were starved for four hours preceding the assay. Individual leaves of the three wild potatoes and the Atlantic were placed in plastic Petri dishes (90 x 15 mm) on moist filter paper (Whatman #1, VWR, Radnor, PA). One larva was placed in each dish. Ten Petri dishes per plant species were prepared and held at 22 °C, 70-75% HR and 16:8 (L: D) photoperiod for 48 h. The water in the vials was checked twice a day and refilled when necessary. At the end of the 48 h-period larvae were removed from the leaves, and the remaining leaves were rinsed using tap water to remove excreta, wiped dry and scanned again to record the final leaf area. This experiment was repeated on two different dates using new groups of CPB larvae and new plants every time.

The larval foliar consumption was calculated by subtracting the final leaf area from the initial leaf area. The distribution of the data was tested for conformity to a normal distribution by the Shapiro-Wilk test. Data were evaluated using analysis of variance (ANOVA) and mean comparisons were done by Fisher's least significant difference (LSD) procedure (SAS 9.4 version).

Larval survival test

This test was conducted using 4-5 week-old potted plants in no choice assays, using the three wild potatoes and the cultivated potato species. Egg masses were collected from potato plants with CPB adults from the Vegetable Entomology Laboratory colony. Neonates were collected separating them from egg masses using a fine paintbrush. Five neonates (0-24 h old) from different egg masses were placed on the upper third of every potato plant. Once larvae were in place, plants were carefully covered with mesh (white polyester, 680 μ m mesh aperture, Megaview, Taichung, Taiwan). To create a tent, the mesh was supported by two metal wire hoops that were bent over the plants. The mesh was tied with a string around the pots to stop larvae from escaping.

Infested plants were held in a greenhouse at ~ 25 °C, 70-75 % HR and 16:8 (L:D) photoperiod and arranged in a randomized complete block design with five replications. The numbers and developmental stages of living and dead larvae were recorded at the end of 8 days. The experiment was replicated two times on different dates in 2014 for a total of 10 replications per plant species. Data were tested for normality and homogeneity of variance. The number of larvae that survived after 8 days was analyzed with a two-way ANOVA (plant species and block as factors) using PROC MIXED (SAS version 9.4). The variance of the number of larvae was estimated by the Residual Maximum Likelihood procedure (RELM), from the statistical software

SAS (version 9.4). Fisher's (LSD) procedure was used to determine significant differences among means at a probability level of $\alpha=0.05$.

Oviposition preference

The oviposition behavior of CPB gravid females on the wild potato species and commercial potato was compared in choice tests. One plant of all four species was organized randomly in the four corners of a 0.6 x 0.6 x 0.6 m square collapsible metal cage (BioQuip Products Inc., Rancho Dominguez, CA) (Fig. 2.1). The experiment was replicated in ten cages containing 4-5 weeks old plants in 12 cm diameter plastic pots. The ten cages were organized in a completely randomized design with 10 replications and were kept on lab benches at $\sim 22^{\circ}\text{C}$, 70-75 % HR and 16: 8 (L: D) photoperiod. One female CPB approximately 8-10 days old that was previously seen mating several times was released in the center of each cage. Plants were inspected for new egg masses daily during five days, without removing the egg masses from the plant. At the end of the four-day period the total number of egg masses and number of eggs per egg mass per plant species were counted. Egg masses that were found on places different than plants were counted but were omitted from the analysis. The entire experiment was replicated twice, once in 2014 and once in 2015, using a fresh set of new plants every time, for a total of 20 replications per treatment.

Differences in the number of egg masses and number of eggs per mass laid by CPB females among potato species were compared using a repeated measures analysis of variance to account for the effects of the two years that the experiment was replicated over. For these analyses, blocks were treated as random effects; plant species and year of replication were treated as fixed effects.



Figure 2.1. Oviposition preference test arrangement showing the four *Solanum* species and one Colorado potato beetle adult female.

Results

Larval foliage consumption results

The leaf areas consumed by second instar CPB during 48 hours were significantly different among the potato species (ANOVA: $F = 9.80$; d.f. = 3, 4; $P = 0.03$) and they ranged from a mean of 1.16 to 8.61 cm^2 (Figure 2.2). *Solanum immite* was fed upon the least (mean: 1.16 cm^2) among all the species, followed by *S. pinnatisectum* (mean: 2.73 cm^2). Leaf areas consumed of these two were significantly lower than area consumed of *S. tuberosum*. ($t = -3.88$, d.f. = 4, $P = 0.018$ and $t = -2.92$, d.f. = 4, $P = 0.043$, respectively). Foliar feeding by larvae on *S. chacoense* was similar to feeding on the commercial potato ($t = 0.68$, d.f. = 4, $P = 0.56$). On average, larval

feeding on *S. immite* was 84.53% less than on the commercial potato and 86.53 % less than on *S. chacoense*.

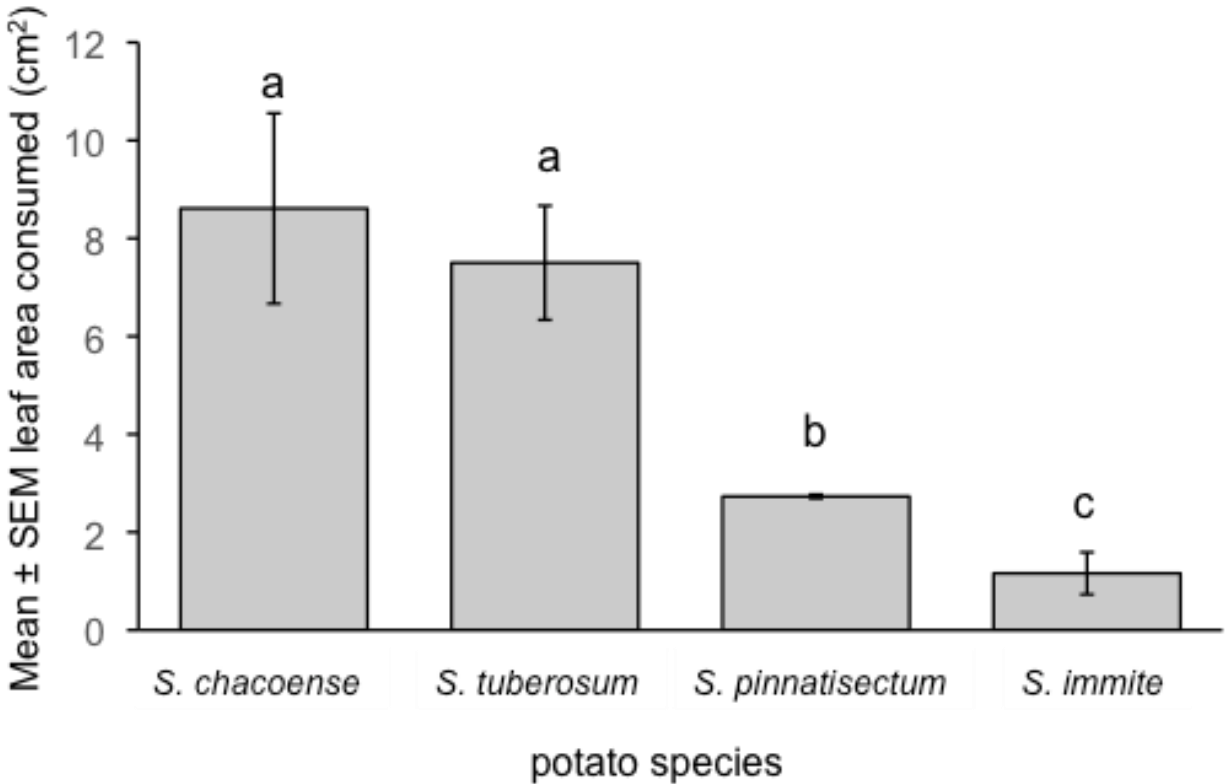


Figure 2.2. Mean (\pm SEM) surface area (cm²) of foliage of three wild *Solanum* species and commercial potato consumed by Colorado potato beetle second instars in a no-choice bioassay during 48 hours. Means followed by the same letter are not statistically different (LSD test, $\alpha = 0.05$). N=20 per plant species.

At the end of the evaluation period larvae that fed on *S. chacoense* and *S. tuberosum* molted to third instar, whereas larvae feeding on *S. immite* and *S. pinnatisectum* remained in the second instar. Larvae on *S. immite* and *S. pinnatisectum* were observed most of the time resting underneath the leaf, along the central vein. Larvae on *S. tuberosum* and *S. chacoense* were constantly feeding on foliage. No larval mortality was observed during the 48 hours evaluation period in any of the potato species.

Larval survival test results

CPB larvae confined on four potato species during 8 days had significantly different survival and growth ($F = 29.43$; d.f. = 3, 25; $P < 0.001$; Figure 2.2). None of the five larvae survived on *S. immite*, while on average 3 larvae survived out of five on the susceptible control, *S. tuberosum* ($t = -7.48$, d.f. = 25, $P < 0.001$). Just one larva was found alive on *S. pinnatisectum* after the experimental period. There were no differences in the number of larvae that survived on *S. tuberosum* and *S. chacoense* (average of 3 larvae in both species) ($t = -0.21$, d.f. = 25, $P = 0.83$). At the end of the evaluation period, live larvae had differences in larval instar depending on the species. All larvae in *S. pinnatisectum* were second instars and larvae in *S. chacoense* and *S. tuberosum* were fourth instars (Table 2.2).

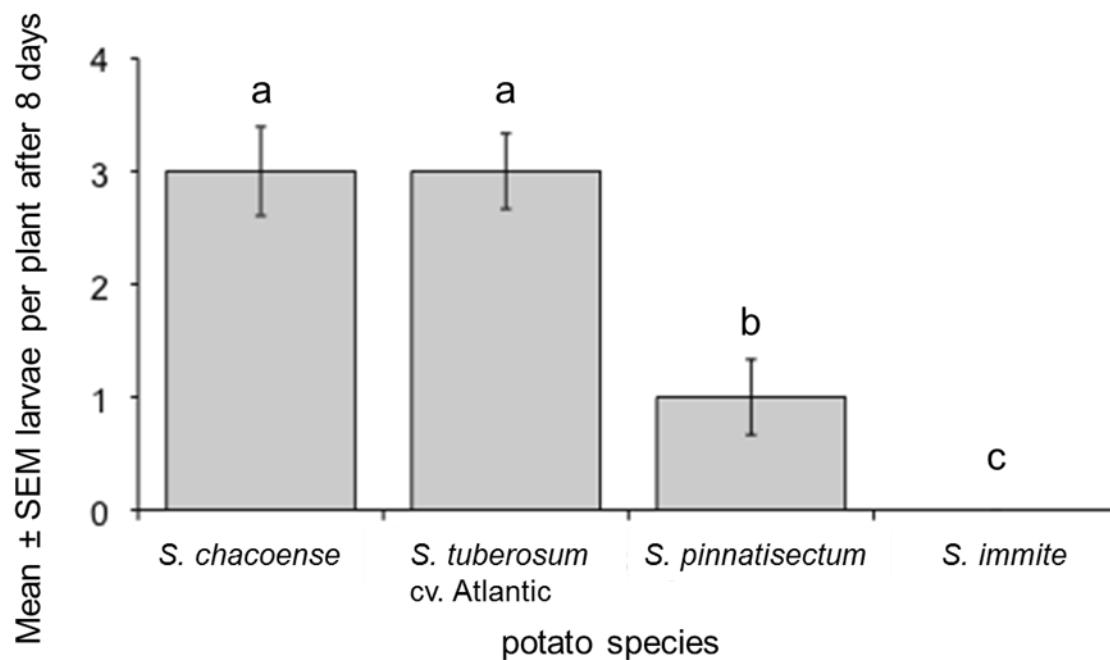


Figure 2.3. Mean (\pm SEM) survivorship of Colorado potato beetle larvae on four potato species after eight days out of five larvae. Differences among means followed by the same letter are not statistically significant (LSD test, $\alpha = 0.05$). $N = 10$ per plant species.

Table 2.2. Final instar and percent mortality of Colorado potato beetle larvae confined on four *Solanum* species for eight days. Five first instars were caged on individual plants at the beginning of the experiment. N=10 per plant species.

Plant species	Larval instar after 48 h	Percent mortality
<i>S. chacoense</i>	4th	40
<i>S. tuberosum</i>	4th	40
<i>S. pinnatisectum</i>	2nd	80
<i>S. immite</i>	-----	100

Oviposition preference results

The number of CPB eggs per mass was significantly different among plant species ($F = 3.65$; d.f. = 3, 72; $P = 0.02$; Figure 2.3). In contrast, when the number of egg masses laid on the potato species was analyzed, no significant differences between treatments were obtained ($F = 1.10$; d.f. = 3, 72; $P = 0.36$). Analysis of variance with replication over years, and with plant species and block as factors, showed no significant effect of repetition over two years on number of egg masses and number of eggs within egg masses.

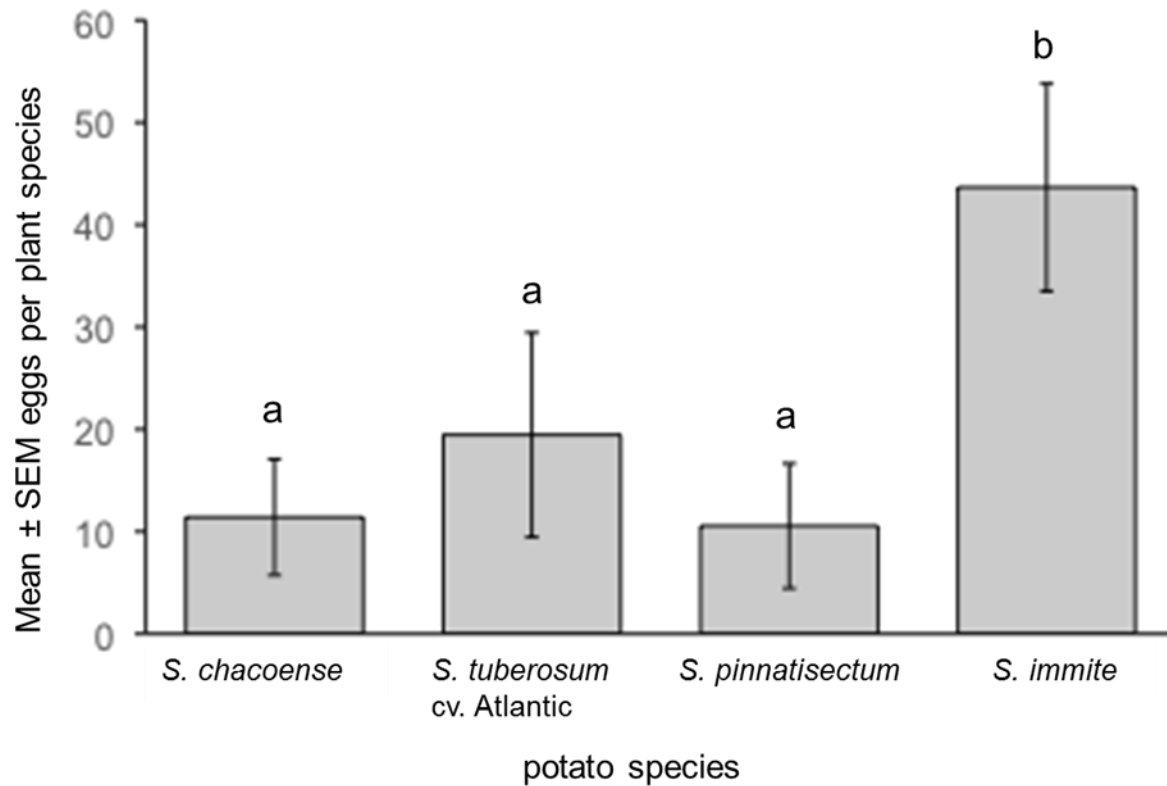


Figure 2.4. Mean (\pm SEM) eggs laid by Colorado potato beetle females on three wild *Solanum* species and the commercial potato during 4 days. Means followed by the same letter are not statistically different (t- test, $\alpha = 0.005$). N= 20 per plant species.

Individual CPB females preferred *S. immite* over *S. tuberosum* for oviposition ($t = 2.11$; d.f. = 72; $P = 0.04$). *Solanum immite* had the greatest number of eggs oviposited of all potato species, more than twice the number of eggs found on *S. tuberosum*. Although, *S. tuberosum* had slightly higher number of eggs oviposited than *S. pinnatisectum* and *S. chacoense*, the difference was not significant ($t = - 0.78$; d.f. = 72; $P = 0.44$ and $t = - 0.70$; d.f. = 72; $P = 0.48$, respectively).

Discussion

The wild potato species included in this study had different levels of responses to the CPB performance parameters evaluated here. In the larval feeding and survival experiments, *S. immite* and *S. pinnatisectum* were significantly more resistant than the commercially cultivated *S. tuberosum* cv. Atlantic. The accession of *S. chacoense* evaluated in this study revealed no significant difference compared to the cultivated potato for the evaluated CPB parameters.

Foliage from *S. immite* and *S. pinnatisectum* reduced development of 2nd instar CPB during 48 hours of feeding, compared to the cultivated potato, indicating that these plants may have resistance against CPB. *S. immite* has not been studied extensively, however, the only two studies I found reported high levels of resistance against CPB. Although *S. immite* belongs to the Tuberosa series, in which resistance to CPB is not common, this species expresses resistance to CPB (Flanders et al. 1992). A study evaluating the resistance to defoliation by CPB larvae in 156 accessions of 41 wild *Solanum* species found that a single accession of *S. immite* and four accessions of *S. pinnatisectum* had no defoliation, while *S. tuberosum* cv. Red Norland experienced 85-95% defoliation levels in untreated field experiments (Jansky et al. 2009). These two potato species were considered the most resistant in this study.

The larval survival study confirmed that *S. immite* had a negative effect on CPB growth and development. None of the neonates released on whole plants developed into large (3-4th instar) larvae. Plants in these experiments were found intact at the end, with no evident feeding damage. *S. pinnatisectum* had significantly lower larval survival than the commercial potato, but contrary to *S. immite*, some neonates were able to reach second instar by the end of the evaluation period and some feeding damage was observed in plants with live larvae. CPB reduced feeding and larval survival when exposed to *S. immite* indicating the presence of

substances interfering with the physiology of the insect, such as a feeding deterrent. These are the characteristics of the antibiosis type of resistance. Lethal antibiosis effects can be acute affecting young larvae. Chronic effects of antibiosis include mortality in older larvae and an inability to pupate or eclose (Smith 2009).

Similarly to Pelletier et al. (1999), first and second CPB instars larval mortality was high when feeding on *S. pinnatisectum*. The study also looked at adult performance and in this case, adult mortality was relatively low. The authors stipulated that the high mortality in larvae could have been due to toxic semiochemicals, but this effect was less evident on adults. In different studies, Pelletier and Tai (2001), and Lu & Yang (2006) also reported that resistance due to undetermined mechanisms has been identified in *S. pinnatisectum*. Grafting experiments have been used to study the mechanisms of plant resistance (Ansari et al. 1989; Lambert & Kilen 1984). Pelletier & Clark (2004) used reciprocal grafts to explain the mode of resistance to CPB and potato aphid in six wild *Solanum* species, including *S. pinnatisectum*. Commercial potato foliage grafted with *S. pinnatisectum* acting as scion, reduced the beetle's development. This result proved that *S. pinnatisectum* has chemical factors that are present in the vascular system of the plant and consequently, these factors can be translocated. Since resistance was found in *S. tuberosum* foliage when *S. pinnatisectum* was positioned as scion, this indicates that the resistance factors are mainly moving through the phloem, the tissue that translocates chemicals from leaves to the rest of the plant.

When CPB females were simultaneously offered the four potato species to make oviposition choices in this study, it was surprising to find that *S. immite*, the least suitable species for larval feeding and survival, was the species that received the highest number of eggs. This suggest that no antixenosis type of resistance occurs in this species. The other three species

received similar numbers of eggs, but *S. immite* had over twice the eggs than *S. tuberosum*.

Similar results of negative correlation between herbivore oviposition preference and performance have been reported before; possible causes are nutritional quality of the host plant, risk of predation, level of competition from other herbivores, and exposure to abiotic stresses (Mayhew 1997; Craig & Ohgushi 2002).

Oviposition on *S. pinnatisectum* was similar to that observed on commercial potato, indicating that this accession is not resistant to CPB egg laying. Although foliage consumption was low and larval mortality was high in this species, both of these parameters were less pronounced than those observed in *S. immite*. This suggests that *S. pinnatisectum* may also have antibiosis-based resistance, but factors responsible for the resistance are probably different from those in *S. immite*.

S. chacoense was as susceptible as *S. tuberosum* cv. Atlantic to CPB feeding, larval survival and oviposition preference. Previous studies conducted with this wild potato species reported resistance against other pests of potatoes, like *Verticillium* wilt (Lynch et al. 1997), CPB (Cooper et al. 2009) and potato leaf roll virus (Brown et al. 1993). However resistance levels in *S. chacoense* are highly variable between different accessions (Sinden et al. 1986).

In summary, out of the four tested species, only *S. immite* provided clear resistance to CPB performance based on feeding and larval survival tests and thus, this species warrants further studies to examine traits that can be included in potato breeding.

CHAPTER 3.

Chemical plant defenses in wild potatoes

Introduction

Plants have developed a variety of mechanisms to fend off herbivores; these defenses can be either physical or chemical (Boulter 1993). Physical mechanisms include morphological changes in the plant providing barriers to insects in their attempt to reach the plant. Some examples of physical barriers include spines, wax, and trichomes. Chemical defenses are represented by secondary metabolites in plants and they can be constitutive or induced after attack. These chemicals are considered direct when they affect the herbivore itself (toxic, repellent, or anti-nutritive activity), or indirect when they draw in other organisms that attack the herbivore (Deverall 1979).

Chemical defenses in plants include substances in different chemical classes, among the most important are the isoprene derived terpenoids (mono-, sesqui-, di-, and triterpenoids), steroids; N-containing alkaloids; and phenolic compounds including flavonoids (Mithofer and Boland 2012). These compounds have different effects on herbivores indicating different modes of actions including membrane disruption, inhibition of nutrient and ion transport, inhibition of signal transduction processes, inhibition of metabolism, or disruption of hormonal control of physiological processes (Wittstock & Gershenzon 2012).

An important group of plant compounds with a defense role are volatile organic compounds (VOCs) (Unsicker et al. 2009). VOCs are defined as any organic compound with vapor pressures high enough under normal conditions to be vaporized into the atmosphere (Dicke & Loreto 2010). VOCs are involved in a variety of ecological functions such as

herbivore disruption, indirect plant defense against insects, pollinator attraction, plant-plant communication, plant-pathogen interactions, reactive oxygen species removal, thermo-tolerance and environmental stress adaptation (Spinelli et al. 2011). The most important groups of VOCs in plants are terpenoids (compounds with an isoprenoid structure), and green leaf volatiles (GLVs). GLVs primarily consist of C6-aldehydes, C6-alcohols, and their acetates derived from lipoxygenase cleavage of fatty acids (Kegge et al. 2015).

Dickens (2000) demonstrated the importance of CPB sexual maturity in plant recognition and attraction to VOCs. He grouped VOCs in 5 categories based on the development and magnitude of electro-antennography (EAG): 1) chemicals with a strong response and a weak variability during sexual maturation such as (Z)-3-hexen-1-ol and (E)-2-hexen-1-ol; 2) chemicals with an intermediate response and slightly increasing response with maturity such as methyl salicylate, nonanal, and (Z)-3-hexenyl butyrate; 3) chemicals with a low response and a slight variation with maturation, including indole, (+)-linalool, and decanal; 4) chemicals with a weak response and slightly increasing reaction during the maturation such as β -caryophyllene and β -selinene; 5) chemicals with a weak response and a decreasing activity with maturation, including 1, 8-cineole, (R)-(+)- α -pinene, (1S)-(-)- α -pinene, α -humulene, and (+)-longifolene.

Mitchel & McCashin (1994) also found that CPB responds to GLVs. In their investigation, CPB was attracted to primary alcohols (e.g. hexanol and heptanol) and other components found among the GLV mixture such as, the monounsaturated (Z)-and (E)-isomers of hexen-1-ol, and six-carbon aldehyde analog, (E)-2-hexenal. Desjardins et al. (1995) studied 46 potato cultivars and breeding selections derived from *S. tuberosum ssp. andigena* CPC 1673. They evaluated their sesquiterpenes composition and abundance identifying rishitin, lubimin, and solavetivone as the main sesquiterpenes. Seven of these genotypes produced significantly higher

sesquiterpene concentrations than the control, Russet Burbank. The high ratios of solavetivone to total sesquiterpenes were strongly correlated with cultivars originated from crosses with *S. tuberosum* ssp. *andigena* CPC 1673, which has resistance to the golden nematode, *Globodera rostochiensis*.

Plants in the Solanaceae produce a group of secondary metabolites called glycoalkaloids that are associated with plant defenses. They are potentially toxic compounds to bacteria, fungi, viruses, insects, animals and humans (Friedman 2006). Glycoalkaloids, also called steroidal glycoalkaloids, differ from other compounds in their aglycon structure (Osbourne 1996). They are considered a major cause of antibiosis in leaves of *Solanum* species (Sturckow & Low 1961; Gunther et al. 1997). Glycoalkaloids such as α -solanine and α -chaconine can disrupt cellular membranes and inhibit acetylcholinesterase activity in insects (Friedman et al. 1997). In the case of the CPB, the effect of glycoalkaloids varies depending on the specific glycoalkaloid, its concentration, and the insect's life stage (Tingey 1984). Leptine I, a foliar glycoalkaloid (acetylated forms of α -chaconine and α -solanine), inhibits CPB feeding at a concentration of 1 mM (Lorenzen et al. 2001). Other glycoalkaloids like tomatine and dimissine have milder activity on CPB. Solanine and chaconine are even less effective than the two previous glycoalkaloids in reducing CPB populations, but at a concentration of 6 mM has been reported to reduce CPB feeding by about 50 % (Sturckow and Low 1961). Commersonine and dehydrocommersonine glycoalkaloids showed better response than solanine, chaconine or the combinations of the two in controlling the CPB (Sinden et al. 1981). The reason for this better performance was attributed to the sugar group, which in the case of dehydrocommersonine, differs in size and composition from those in solanine and chaconine. The authors explained that

the number of sugar groups, i.e., a tetrasaccharide vs. a trisaccharide may be as important or more, than the presence of a specific aglycone, or the presence or absence of a particular sugar. Some wild relatives of potatoes also contain glycoalkaloids. For example, *S. pinnatisectum* Dunal and *S. polyadenium* Greenm. have high α -tomatine content, which negatively affects CPB growth (Kowalski et al. 2000). Dimock et al. (1986) found that the resistance of *S. neocardenasii* to CPB may be associated with glycoalkaloids and not with glandular trichomes. In their study, total oviposition rates and number of eggs per mass were significantly reduced when adults reared on *S. tuberosum* were transferred to *S. neocardenasii* plants. Larvae fed on *S. neocardenasii* had low relative consumption and growth rates. When trichome exudates were removed from the leaves the responses of larvae and adults did not change.

Leaf surface compounds can affect the resistance of plants to herbivores and also herbivore behavior (Muller & Hilker 2001). In the case of commercial potatoes, the leaves are covered with cuticular waxes (Szafranek & Synak 2006), and trichomes (Gibson & Turner 1977). The main components of potato waxes are long chain n-alkanes, 2-methylalkanes and 3-methylalkanes, primary alcohols, fatty acids and wax esters, which can vary quantitatively among potato varieties and species. In a study conducted with methylene chloride leaf rinses of the wild potato species, *S. berthaultii* (PI 473334), the nonvolatile fraction was highly deterrent to CPB adults, while the volatile fraction reduced consumption, but not significantly compared to the controls (Yencho & Tingey 1994). In a similar way, experiments with the volatile and nonvolatile fractions of leaf surface rinses from another wild *Solanum* species, *S. tarijense*, identified a phago-deterrent effect in the volatile fraction (Pelletier & Dutheil 2006).

The objective of this chapter was to analyze different chemical compounds that may be present in the four different *Solanum* species that might be the cause for differences in growth

and development of the CPB. General qualitative differences among commercial potatoes and three wild potato species were investigated in VOC emissions. The amount of the glycoalkaloid α -solanine was compared among the *Solanum* species. The last analysis aimed to identify chemical compounds located on the plants' leaf surface, especially the presence of acyl sugars and glycoalkaloids.

Materials and Methods

Plant material

The plant material used in the current experiments is described in Chapter 2.

Headspace analysis

VOC collection from the three wild potato species and the commercial potato was conducted in the same growth chamber where plants were growing (temperature: 25-27 °C, 75% RH, photoperiod 16:8, L: D). Plants used for VOC collection were 4-5 weeks old. The soil was covered with aluminum foil to minimize odor contamination emanating from this part. The above-ground plant parts were enclosed in a 50 by 30 cm Tedlar (TTR20SG4, DuPont, Wilmington, DE) plastic bag and the bottom of the bag was secured to the top of the pot using rubber bands. The volatiles in the bag were collected on Super Q traps (50/80 mesh; 30 mg in a glass tube) for 24 hours (1 l/min) using a battery- operated pump (Model 8R1110-101-1049, Gast Manufacturing, Benton Harbor, MI) to pull air out of the bags through the traps. Volatiles were extracted from the Super Q using 150 μ l of methylene chloride. Ten plants per *Solanum* species were used for this experiment. The extractions were kept at -20°C before GC-MS analyses. The volatile extracts were analyzed on an Agilent 7890A gas chromatograph (GC) equipped with a HP-5MS Agilent J&W GC column (30 m length, 250 μ m diameter and 0.25 μ m

film thickness, He as the carrier gas at constant 50 ml/min flow) coupled with an Agilent 5975C inert XL mass spectrometer (MS). Compounds were separated by injecting 1 µl of sample into the GC/MS. The program consisted of 35°C for 1 min followed by 10°C min⁻¹ to 260°C for 6.5 min. After a solvent delay of 3 min, mass ranges between 50-550 atomic mass units were scanned.

Compounds were identified by comparison of spectral data with those from the NIST library and by GC retention index and confirmed by comparing their retention times and mass spectra with those of commercially available compounds run on the same column. Finally, the contribution of each compound to the total headspace was calculated. Headspace composition of *S. tuberosum* and *S. chacoense* were compared using principal component analysis. The other two species were not included in this analysis due to the low number of compounds present in their headspace.

Tissue analysis

Glycoalkaloids were extracted from 4-5 week old plants of the three wild *Solanum* species and the commercial potato (cv. Atlantic). A total of forty samples (10 per plant species) were processed for glycoalkaloid extraction. One leaflet on the top third part of the plant was collected for extraction, 100mg of each leaf was pulverized with liquid nitrogen, and 1ml of extraction solvent (water, methanol and acetic acid, 49:49:2 v/v/v) was added. The samples were then heated in a water bath at 60°C for 30 minutes. After this, the samples were centrifuged at 15,000 RPM for 20 min and the supernatant was transferred to a clean and labeled 2ml GC vial. Vials were stored at -20°C and solanine was analyzed by Liquid Chromatography/Mass Spectrometry (LC/MS) at the Michigan State University Mass Spec Facility (see Appendix S1 for LC/MS methods).

The distribution of the data was tested for conformity to a normal distribution by the Shapiro-Wilk test. Data were evaluated using one way-analysis of variance (ANOVA) and mean comparisons using Tukey Honest Significant Test (HSD) (SAS 9.4 version).

Leafdip analysis

Leaves for rinses were obtained from wild potato species and cultivated potato. One leaflet from the top third part of 3-4 week old plants was used for this purpose. The leaflet was removed from the plant using fine-point stainless steel forceps (Bioequip Products, Inc. Rancho Dominguez, CA). Forceps were washed between samples using acetonitrile and wiped dry with tissue paper. Leaflets were dipped into 5ml of an extraction solvent (isopropanol, acetonitrile and water (3:3:2 v/v/v) containing 0.1% formic acid and 10 μ M of propyl-4-hydroxybenzoate (Sigma P53357) as internal standard). Leaflets were gently rocked manually for 2 minutes in the extraction solvent. After this, approximately 1ml of the rinsate was transferred to 2ml GC vials, which were stored in a -20°C freezer until they were taken to the Michigan State University Mass Spec Facility for acyl sugars and glycoalkaloid analysis on an LC/MS (Appendix S2).

Pairwise Bray-Curtis similarities were calculated between treatments, and non-metric multi-dimensional scaling (NMDS) was used to visualize the differences. Stress values for NMDS procedure were <0.1, indicating that good interpretation was possible. To test significance of differences, an analysis of similarity (ANOSIM) with 1,000 permutations was employed. The 95% confidence ellipses based on the centroid of each treatment was calculated. These statistical tests and all others, except where otherwise noted, were carried out in R Software (R Team 2013) with $\alpha = 0.05$.

Results

Headspace analysis results

Volatile compounds from healthy plants of four potato species were identified in headspace analysis. Twenty-four compounds were identified in the headspace of *S. tuberosum*, 10 in *S. chacoense*, and only 2 compounds were identified in each of the other two wild potato species, *S. pinnatisectum* and *S. immite* (Table 3.1).

Table 3.1. Mean percent contribution of each compound to total headspace of four *Solanum* species. Headspace was collected for 24 hours and each species was replicated 16 times.

Compound name	<i>S. chacoense</i>	<i>S. immite</i>	<i>S. pnnatisectum</i>	<i>S. tuberosum</i>
Terpinolene	0	0	87.1	0
Limonene	0	98.8	0	0
Ocimene<(E)-beta->	0	0	0	0.16
Methyl benzoate	0.9	0	0	0
Linalool	0	0	0	3.5
Nonanal<n->	5.9	1.2	12.8	8.3
4,8-Dimethyl- 1,3(E),7-nonatriene	12.3	0	0	0
Methyl salicylate	26.5	0	0	6.7
Cubebene<beta->	0	0	0	0.2
Copaene<alpha->	0.9	0	0	66.6
Germacrene A	0	0	0	0.2
Caryophyllene<Z->	39.6	0	0	5.2
Copaene<beta->	0.9	0	0	1.1
Sesquisabinene	0	0	0	2.4
Selinene<beta->	0	0	0	3.3
Farnesene<(E,E)- alpha->	0.5	0	0	0
Germacrene-D	5.8	0	0	0
g- Gurjunene	0	0	0	0.78
Farnesol isomer a	0	0	0	0.2
Nerolidol<E->	6.5	0	0	0.9
Total number of compounds	10	2	2	24

Alpha-copaene was the most abundant compound, comprising 66.6% of the headspace of *S. tuberosum*. The second most abundant compound in the headspace of this species was nonanal (8.3%). The remaining 22 identified compounds comprised less than 27% of the *S. tuberosum* headspace. Methyl salicylate (26.5%) was the second most abundant headspace compound in *S. chacoense*, but in this species the most abundant compound was caryophyllene (39.6%). The two wild species, *S. immite* and *S. pinnatisectum*, were similar in that both of them had nonanal in the headspace as the second most abundant compound, but the most abundant compound was terpinolene for *S. pinnatisectum* (87.1%) and limonene (98.8%) for *S. immite*. These two wild potato species had only two compounds in their headspaces. The only compound that was detected in the headspace of all four species was nonanal; limonene was only present in *S. immite*, and terpinolene was only present in *S. pinnatisectum*. There were five compounds (methyl salicylate, alpha-copaene, caryophyllene, beta-copaene, E-nerolidol) that were present in the headspace of both *S. chacoense* and *S. tuberosum*, but the remaining compounds were different (Figure 3.1). In the principal component analysis, the first two Eigen-values accounted for 65.7% of the variation, with *S. tuberosum* and *S. chacoense* headspace different from each other.

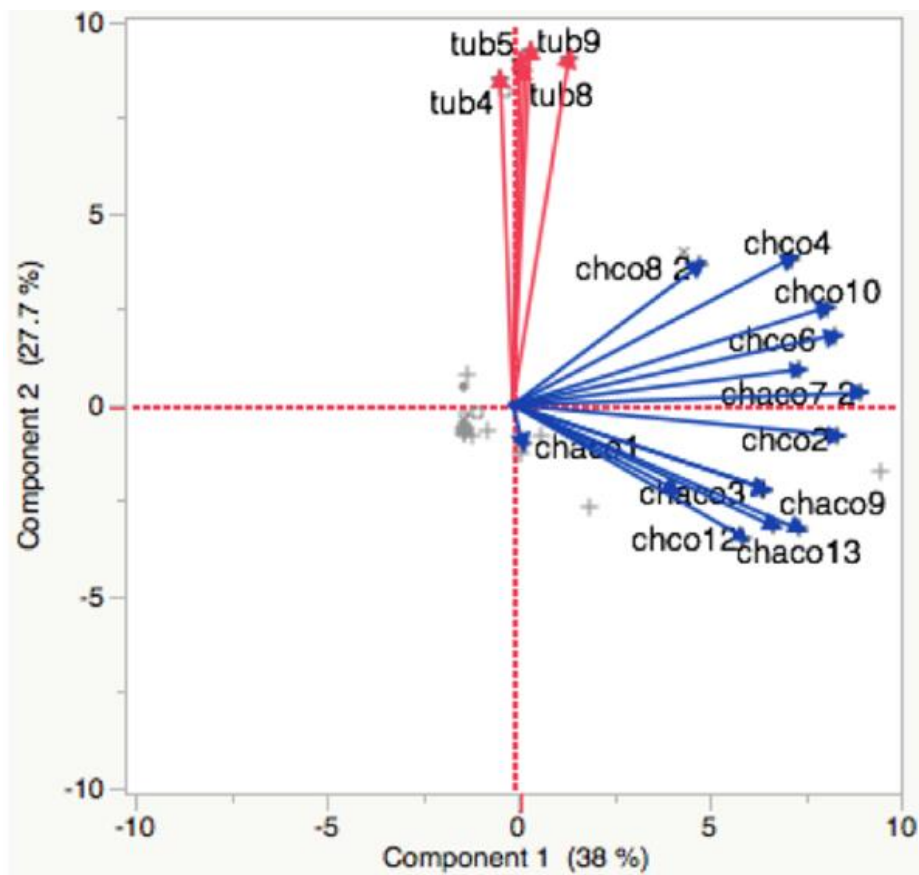


Figure 3.1. Principal component analysis with *Solanum tuberosum* (n=6, tub) and *S. chacoense* (n=13, chaco) headspace, using the percent each compound contributed to the total headspace. The first two Eigen-values account for 65.7% of the variance.

Tissue analysis results

Concentrations of the glycoalkaloid α -solanine were significantly different in the four plant species (ANOVA: $F = 23.81$, d. f. = 3, 36, $P < 0.001$; Figure 3.3). Solanine concentration was highest in *S. tuberosum* and was not significantly different from the concentration in *S. immite*. Solanine concentrations were significantly higher in *S. tuberosum* and *S. immite* than in *S. chacoense* or *S. pinnatisectum* which had the lowest solanine concentration of the four species. The concentration in *S. pinnatisectum* was almost 1000 times lower than the concentration in *S. tuberosum* extracts and about 400 times lower than in *S. chacoense*. The concentration of

solanine in *S. chacoense* was significantly different from concentrations found in the other three species, being the second lowest in the group.

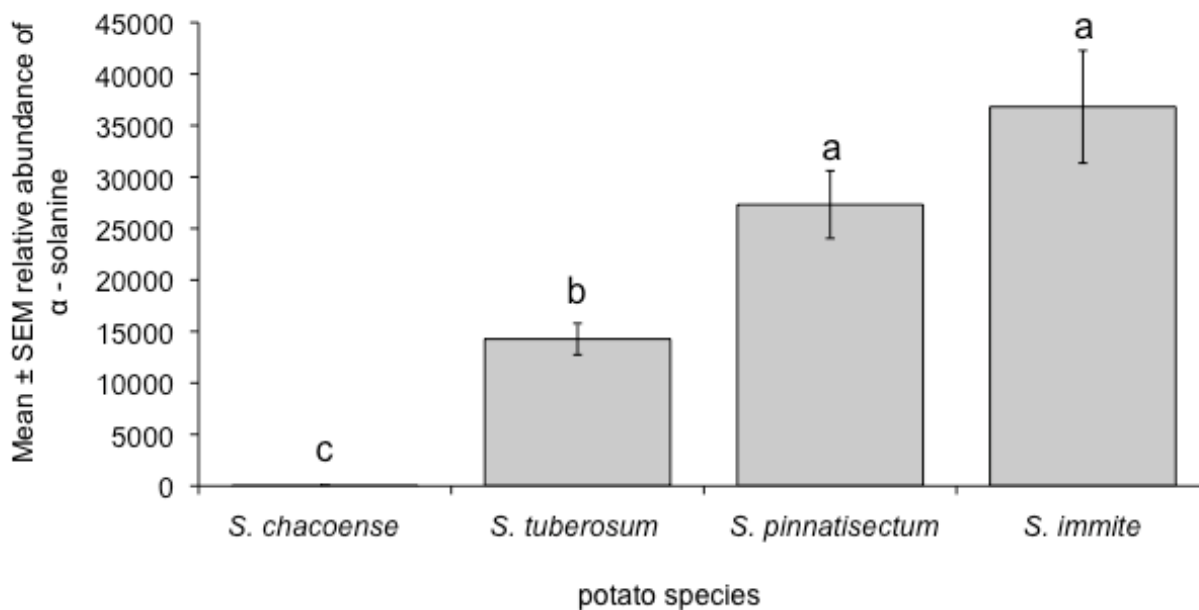


Figure 3.2. Mean (\pm SEM) α - solanine concentration (relative abundance in sample) extracted from foliage of three wild *Solanum* species and *S. tuberosum* cv. Atlantic (cultivated potato). Means followed by the same letter are not statistically different (Tukey test, $\alpha = 0.05$). N= 10 per plant species.

Leafdip analysis results

The leafdip analysis revealed that there were no acyl-sugars present on any of the tested leaves. However the LC/MS analysis was able to detect measurable amounts of different glycoalkaloids in the leafdip samples. The glycoalkaloids, solanine and chaconine were present in *S. chacoense*, *S. tuberosum* and *S. immite*, but not in *S. pinnatisectum*. On the other hand, *S. pinnatisectum* samples were the only ones that contained tomatine. The sample composition was significantly

different among the four plant species (ANOSIM: $R=0.73$, $n=40$, $P < 0.01$; Figure 3.4).

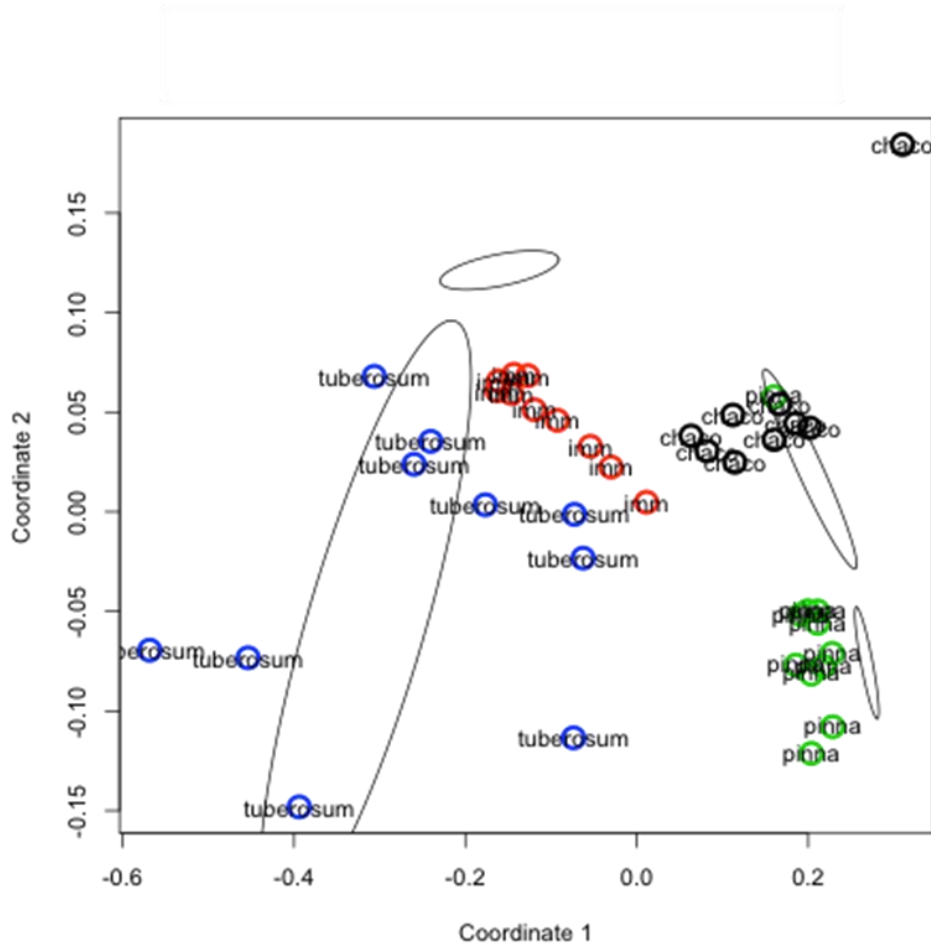


Figure 3.3. Non-metric multi-dimensional scaling plot of the differences in the leafdips of the four *Solanum* species ($n=10$ per species). Plants were *S. tuberosum* cv. Atlantic (blue), *S. immite* (red), *S. pinnatisectum* (green), and *S. chacoense* (black). The ellipses of the treatment colors indicate the 95% confidence interval around the centroid of each group. Sample composition of the four species was significantly different from one another.

Discussion

Wild potato species are a source of a diversity of genes that encode chemical properties such as the ones associated with resistance to pests (Pelletier 2007). The headspace analysis showed strong differences among the four *Solanum* species. An important difference among the four species was that *S. immite* and *S. pinnatisectum*, the two least preferred species by CPB for

feeding and larval survival, had just two compounds in their headspace, while *S. tuberosum* and *S. chacoense* had 24 and 10 compounds, respectively. The latter two species were equally accepted for feeding by CPB and larvae successfully developed on them. Another difference among the headspace of the four species was in their chemistry. Limonene was only found in *S. immite*, and terpinolene was only found in *S. pinnatisectum*. The most abundant compound found in *S. tuberosum* was copaene and for *S. chacoense* it was caryophyllene, while the second most abundant compounds were nonanal for *S. tuberosum* and methyl salicylate for *S. chacoense*. Close to 99 % of the volatile profile of *S. immite* was limonene, and terpinolene occupied 87 % of the *S. pinnatisectum* headspace. These two compounds could have negatively affected CPB when exposed to these potato species. Limonene and terpinolene are both monocyclic monoterpenes and closely related compounds. Terpinolene is the most abundant product of limonene isomerization at high temperatures (Comelli et al. 2005). Limonene has been reported to have anti-microbial, insecticidal and repellent properties (Ibrahim et al. 2008). Limonene emitted by the holm oak (*Quercus ilex*) is considered an allelochemical because it inhibits seed germination of other plant species (Singh et al. 2006). Some of the properties attributed to terpinolene are anti-bacterial and anti-fungal (Brookes et al. 1987).

S. tuberosum and *S. chacoense* were well accepted by the CPB in this study. *Solanum tuberosum* volatile collection showed a diverse mix of compounds, five of them were also found in *S. chacoense*. The most important compound found in *S. tuberosum* volatile profile was copaene. This sesquiterpene was also found in several other potato varieties using Column Chromatography, Gas Chromatography, Mass Spectrometry and Nuclear Magnetic Resonance Spectra (Szafranek 2005). The second most important compound in *S. tuberosum* was nonanal. In the case of *S. chacoense* caryophyllene and methyl salicylate were the first and second most

important compounds in the headspace analysis, respectively. The aldehyde nonanal and organic ester of methyl salicylate have both been found attractive to the CPB in electroantennograms Dickens (2000). Weissbecker et al. (1997) found in gas chromatography-electroantennographic detector (GC-EAD) bioassays that CPB is attracted to caryophyllene.

The tissue analysis determined that solanine concentrations were higher in *S. tuberosum* and *S. immite*, showing no correspondence with acceptance of these species by the CPB. Previous experiments in this study (Chapter 2) indicated that *S. tuberosum* and *S. immite* were the most and least preferred species by CPB, respectively. *S. chacoense* and *S. pinnatisectum* have lower levels of solanine than the previous species, and there was no relationship between these results and the result of the CPB feeding and survival trials. The LC/MS analysis of leafdips determined that the species don't have acyl-sugars on the leaf surface, but also provided extended information on the presence of glycoalkaloids. This analysis identified solanine and chaconine in *S. tuberosum*, *S. chacoense* and *S. immite*, while tomatine was only found in *S. pinnatisectum*. Although solanine and chaconine can reduce CPB feeding, their concentrations have to be very high and exceed 6 mM (Tingey 1984). This suggests that levels of solanine and chaconine in *S. tuberosum* and *S. chacoense* are not high enough to dissuade CPB from feeding on them.

Tomatine was the only glycoalkaloid found in *S. pinnatisectum*. Tomatine is the principal foliar glycoalkaloid of *S. pinnatisectum* (Denno & Roderick 1990). Gregory et al. (1981) also identified α -tomatine in *S. pinnatisectum*, corresponding to 100 % of its total glycoalkaloid composition. Using synthetic diets supplemented with increasing concentrations of α -tomatine Kowalski et al. (2000) demonstrated retarded growth and delayed development of CPB from egg to pre-pupal stage. The aglycone of α -tomatine, tomatidine, was found to have no effect on CPB

thus, the insecticidal activity was attributed to the tetrasaccharide moiety of the glycoalkaloid, which has a membrane-lytic mechanism of action. Other studies showed that the negative effect of tomatine on CPB is dose-dependent (Sinden et al. 1981, Sturkow & Low 1961). Taking into account the results from the headspace and leafdip analyses for *S. pinnatisectum*, it can be speculated that both, terpinolene and tomatine play a role in the low performance of the CPB on this potato species.

Gregory et al. (1981) studied the glycoalkaloid content in 16 wild potato species and some commercial cultivars of potato. Their results, like ours, recognized the presence of solanine and chaconine in *S. tuberosum*, and *S. chacoense*. These authors used three different accessions of *S. chacoense* and in two of them, they found leptine I and leptine II (acetyled forms of solanine and chaconine, respectively) in addition to solanine and chaconine. Leptines are a group of glycoalkaloids that only occur in potato foliage and not in tubers, for this reason they are safer than other glycoalkaloids (Aylokin et al. 2013). This group of glycoalkaloids have been found in only few accessions of *S. chacoense* (Tingey & Yencho 1994), and the resistance of these genotypes to the CPB seem associated with the high content of leptines in their leaves (Sturkow & Low 1961). More evidence about the strong negative effect of leptines on CPB is shown by Lorenz et al. (2001), who examined hybrid populations of *S. chacoense* x *S. tuberosum* and noted that only levels of leptines but not other glycoalkaloids were correlated with resistance to CPB. This could be the reason why the beetle fed and survived on the *S. chacoense* accession used in this study, which lacks leptines.

Considering all the results of the chemical analyses for the wild potatoes, the volatile compounds in the monoterpene class play an important role in the negative effect on growth and

development of CPB larvae. The close relationship of volatile emissions with herbivory strongly suggests that these substances are involved in plant defense (Unsicker et al. 2009).

CHAPTER 4.

Conclusions and future research

To date the Colorado potato beetle (CPB) continues to be an enormous threat for potato growers; its geographical expansion to new regions of the world has not stopped (Alyokhin et al. 2013). In the continuing search for sustainable alternatives to the use of pesticides, evaluation of wild potatoes species with resistance traits to insects remains a hopeful option. *Solanum immite* is an Andean wild potato species that has been little studied but with important results. The Mexican wild potato *S. pinnatisectum* has shown clear results in studies against late blight (Ramon & Hanneman 2002; Chen et al. 2004) and *S. chacoense* is considered a rich source of genes for resistance to the CPB, but the accession selected in this study behaved as the control *S. tuberosum* cv. Atlantic. To assess the effect of these wild potatoes on the growth and development of the CPB, I conducted laboratory experiments (Chapter 2) that revealed important potential in *S. immite* as a candidate for breeding programs. CPB larvae not only did not feed on *S. immite* but the percentage of survival after 8 days was zero, demonstrating a strong antibiosis effect in the foliage. The fact that adult females deposited significantly more eggs on *S. immite* as on the control indicates either that the female cannot discriminate between hosts for oviposition or that they may perceive a chemical attractant for oviposition. Choice oviposition tests with newly emerged females and males caged together could help to better understand the adult host and oviposition selection.

Solanum pinnatisectum also showed significant differences compared to *S. tuberosum* in feeding quantification and larval survival, but lower than those observed in *S. immite* in Chapter 2. Additional laboratory experiments to better understand the mechanism of resistance in *S.*

immite and *S. pinnatisectum* including all the larval instars are recommended. Third and fourth instars require larger amounts of plant tissue and it would be beneficial to test them in no-choice settings to distinguish between pre-ingestion behavioral effects (i.e., deterrence) and post-ingestion physiological effects (i.e., toxicity). Complete life cycle studies would show more information in factors affecting the physiology of the CPB, for example pupal or adult malformations or lack of adult emergence. Including insect weight or other measurable parameter in feeding assays would help in assessing direct effects on insect growth. Pelletier & Smilowitz (1990) suggested ethological observations of the insect when in contact with the plant tissue to better understand the resistance of a plant species. Lastly, the influence of these wild species on beetle growth and development needs to be confirmed under field conditions.

The study of the chemical defenses contributed to a better understanding of the resistance mode of *S. immite* and *S. pinnatisectum* (Chapter 3). Headspace analysis showed limonene as almost the only volatile compound in *S. immite* with 99 % of its composition. Terpinolene is present in *S. pinnatisectum* also in high amounts (close to 90%). The effect of limonene on CPB in the case of *S. immite* requires further investigation in order to completely attribute the resistance of the beetle to this compound, especially since it has volatile properties and oviposition was not affected. I first suggest quantitative headspace analysis to determine the amounts of these chemicals in both potato species. Next, controlled limonene and terpinolene dose-dependent tests with artificial diets including larval and adult CPB would pinpoint other effects on the CPB.

Leaf tissue and leafdip analyses (Chapter 3) showed presence of solanine and chaconine for *S. tuberosum*, *S. chacoense* and *S. immite*, and tomatine for *S. pinnatisectum*. However,

quantitative analysis in this case too are necessary to draw conclusions about the involvement of these glycoalkaloids in the resistance of the beetle to *S. immite* and *S. pinnatisectum*.

Additionally, plant nutrient analysis should be conducted, to determine the interactions of nutrients with glycoalkaloids. Lyytinen et al. (2007) argued that the effect of plant deterrents can be altered by the presence of nutrients. For example, nitrogen content in plants above certain levels can counteract the negative effect of glycoalkaloids on other organisms (Hare 1987).

Finally, as recommended for Chapter 2, chemical analysis of these species when growing in field conditions are important, especially in agricultural environments since the mode of action of allelochemicals against insect pests may be reduced by numerous biotic and abiotic factors (Panda & Khush, 1995) and in order to offer a reliable potential species to be considered for breeding purposes. In this regard, the study presented here clarifies and substantiates the likely role of the wild potato species *S. immite* for breeding commercial potato cultivars with resistance to CPB.

APPENDICES

Appendix S.1 Supplementary data 1

Liquid Chromatography-Mass Spectrometry (LC-MS) details for glycoalkaloid solanine analysis

Instrument: 1. Quattro micro (mass spectrometer)

2. Shimadzu HPLC system

Polarity ES+

Capillary (kV) 3.2

Source Temperature (°C) 120

Desolvation Temperature (°C) 350

Function type: MRM

Chan Reaction	Dwell (secs)	Cone Voltage (V)	Collision Energy (V)
868.45 > 398.35	0.250	60.0	80.0

Column: Ascentis Express C18, 100x2.1mm, 2.7uM

Mobile phase A: Water + 0.1% formic acid in water B: Acetonitrile

Gradient Table:

Time (min)	Flow Rate	% A	% B
1. 0.00	0.300	90.0	10.0
2. 2.00	0.300	5.0	95.0
3. 3.00	0.300	5.0	95.0
4. 3.01	0.300	90.0	10.0
5. 5.00	0.300	90.0	10.0

Flow rate: 0.3mL/min

Column temperature: 30 °C

Appendix S.2 Supplementary data 2

Liquid Chromatography-Mass Spectrometry (LC-MS) details for glycoalkaloid analysis

(solanine, chaconine and tomatine)

Instrument: 1. XEVO G2-XS QTOF (mass spectrometer)

2. Acquity UPLC system

3. CTC PAL autosampler

Polarity ES-

Capillary (kV) 2

Sampling Cone (V) 40

Source Temperature (°C) 100

Desolvation Temperature (°C) 350

Mass range: 50 to 1500

Column: Ascentis Express C18, 100 x 2.1mm, 2.7 µM

Mobile phase A: Water + 0.1% formic acid in water B: Acetonitrile

Gradient Table:

Time (min)	Flow Rate	%A	%B
1. Initial	0.300	95.0	5.0
2. 1.00	0.300	40.0	60.0
3. 5.00	0.300	0.0	100.0
4. 6.00	0.300	0.0	100.0
5. 6.01	0.300	95.0	5.0
6. 7.00	0.300	95.0	5.0

Flow rate: 0.3mL/min

Column temperature: 40 C

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