BIOCHEMICAL MODE OF RESISTANCE TO MULTIPLE INSECT PESTS IN A ROMAINE LETTUCE CULTIVAR

By

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BIOCHEMICAL MODE OF RESISTANCE TO MULTIPLE INSECT PESTS IN A ROMAINE LETTUCE CULTIVAR

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Lettuce (*Lactuca sativa* L.) quality and yield can be reduced by feeding of several insect pests. Host plant resistance to these insects is an environmentally sound adjunct to conventional chemical control. In this study I compared the survival, development and feeding behavior of cabbage looper, *Trichoplusia ni* (Hübner) and beet armyworm *Spodoptera exigua* (Hübner) on two romaine lettuce cultivars, resistant 'Valmaine' and susceptible 'Tall Guzmaine'. The survival and development of both species was significantly less on resistant Valmaine than on susceptible Tall Guzmaine. The two insect species showed different feeding preference for leaves of different age groups on Valmaine and Tall Guzmaine.

Latex from Valmaine strongly inhibited feeding of banded cucumber beetle, *Diabrotica balteata* LeConte compared to Tall Guzmaine when applied to the surface of artificial diet in both choice and no-choice tests. In a choice test involving diet disks treated with Valmaine latex from young leaves versus mature leaves, the beetles consumed significantly less diet treated with latex from young than mature leaves. No significance difference in feeding was found between diet disks treated with latex from young and mature Tall Guzmaine leaves in choice tests. Three solvents of differing polarity (water, methanol and methylene chloride) were tested to extract deterrent compounds from latex; Valmaine latex extracted with water:methanol (20:80) strongly

inhibited beetle feeding when applied to the surface of artificial diet. These studies suggest that moderately polar chemicals within latex may account for resistance in Valmaine to *D. balteata.*

Further fractionation of methanolic crude extract of Valmaine latex was done using reverse phase and cation exchange solid-phase extraction to isolate the deterrent compounds. Retention of deterrent compounds on cation exchange resin suggests the presence of compounds with amine group in Valmaine latex. Further bioassay directed fractionation of cation exchange extract using LC/MS indicates the presence of ten compounds in the active fraction between 3 and 4 min. The successful isolation of potent feeding deterrents against *D. balteata* adults provides convincing evidence of a chemical basis for host plant resistance mediated through latex in this cultivar.

Latex from damaged plants of Valmaine was much more deterrent to *D. balteata* adults than latex from undamaged plants when applied on the artificial diet under choice conditions and no such difference was found in Tall Guzmaine choice tests. The activities of three enzymes (phenylalanine ammonia lyase, polyphenol oxidase and peroxidase) significantly increased in Valmaine latex from damaged plants over time (i.e., 1, 3 and 6 d) after feeding initiation, but they remained the same in Tall Guzmaine latex. The constitutive level of phenylalanine ammonia lyase and polyphenol oxidase was also significantly higher in the Valmaine latex than in Tall Guzmaine latex. These studies suggest that latex chemistry may change after damage due to increased activity of inducible enzymes and that inducible resistance appears to act synergistically with constitutive resistance in Valmaine latex.

CHAPTER 1
REVIEW OF LITERATURE

Introduction

Lettuce, *Lactuca sativa* L., a member of the Compositae (Asteraceae), is a rosette plant that is grown commercially for its leaves. The family Compositae includes a wide range of herbaceous plants and accounts for one tenth of known angiosperm species. Lettuce is one of the most important vegetable crops grown in the United States, in terms of quality and quantity as well as its acreage (Ryder 1998). Demand for lettuce grows yearly, probably due to its use as a healthy, low caloric, salad component of meals. It requires minimal processing, and its long storage life, good quality and reputation as healthy food contribute to its increase in salad bars and fast foods (Ferreres et al. 1997).

During 2006, the United States produced 2,935 thousand metric tons of head lettuce, 857.8 thousand metric tons of leaf lettuce, and 990.3 thousand metric tons of romaine lettuce harvested over areas of 71,508, 29,056, and 24,929 hectares, respectively (Agricultural Statistics 2007). Crisphead (iceberg) varieties predominate in the Unites States markets, particularly for extended transport. However, romaine (Cos), butterhead, and leaf type lettuces are also produced in considerable amounts. A number of other varieties that show variation in color from light green and yellow to deep green are also becoming more accepted. Romaine lettuce is the most common leaf lettuce grown throughout the United States.

California is the major producer of lettuce (77% of total production) in the United States (Lauritzen 1999), followed by Arizona, Florida and New Jersey (Kerns et al. 1999, USDA 2002). Lettuce production from the Everglades Agricultural areas in southern Florida contributes 90% of the total state production (Hochmuth et al. 1994).

Origin and History of Lettuce

Lettuce originated in the Mediterranean region and its cultivation may have started in Egypt as early as 4500 years BC (Lindquist 1960). Lettuces were supposedly grown by Persians 500 years BC, and were introduced into China between the years 600 and 900 AD. Lettuces were mentioned in England in the fourteenth century and reached America with Columbus (Davis et al. 1997). In 1494, Columbus introduced a non-heading type of lettuce to the New World. This type quickly formed a seed stalk and in fact did not become a stable food crop. Head lettuce in the United States was first reported in 1543 (Helm 1954). Salad lettuce was popular with the ancient Greeks and Romans and it arrived in the United States during colonial days (Davis et al. 1997). Sturtevant (1886) studied the history of lettuce and observed that 83 distinct varieties of lettuce were grown under nearly 200 names at the New York Agricultural Experiment Station. These varieties were present in three distinct form-species, the lanceolate-leaved, the Cos and the cabbage.

The lanceolate-leaved form was represented by one variety, 'the deer's tongue', and had a chicory-like appearance in some stages of its growth, as mentioned and illustrated by Bauhin (1671). This type of lettuce was submitted under the names *Romaine asperge*, *Lactuca angustana* Hort., and *L. cracoviensis* Hort by Vilmorin (1883).

The Cos lettuce had upright growth of elongated, spatulate leaves. The Cos form was less commonly grown in northern Europe as compared to the south and was seldom cultivated in France and Germany in the sixteenth century.

Cabbage lettuce was characterized by rounded and spatulate leaves, growing less upright than the Cos lettuce. The commentators of the sixteenth and seventeenth centuries deemed this form-species to have been known to ancient Greeks and Romans. Pliny (23-79 AD) and Columella (42 AD) referred to it as a variety, 'Laconicon', and 'Tartesian' or 'Bætica',

respectively. The cabbage lettuce was more wrinkled or blistered than the Cos (Sturtevant 1886). Pinaeus (1561) identified a heading lettuce that closely resembled "the stone tennis ball" variety of lettuce. Botanists were agreed in considering the cultivated lettuce as a modification of the wild species *L. scariola* (de Candolle 1885). In conclusion, these three form-species had different origins from different wild forms that had been cultivated in different regions of the world (Sturtevant 1886).

Types of Lettuce

There are five modern types of lettuce based on morphological features: crisp-head, leaf, butterhead, cos or romaine, and stem (Davis et al. 1997, Ryder 1998). The crisp-head varieties with dense, firm heads and crisp leaves are the most significant commercial types and take about 75 – 130 d from planting to mature. Leaf lettuce varieties have frilled, glossy red or bright green leaves and mature in 45 d from planting. Leaf lettuce is a good type of lettuce for home gardens, as it matures quickly and is easy to grow. Butterhead lettuce generates an unfastened and soft head, and inner leaves have an oily or buttery feel. Butterhead varieties produce high quality lettuce for commercial purposes. They mature slightly earlier than crisp-head varieties. The cos or romaine type of lettuce develops an elongated head of stiff, upright leaves about 80 d from planting. Cos lettuce is an important lettuce type in Europe and is also gaining popularity in the United States. Stem lettuce often is listed in catalogs under the name of Celtuce (CELery - letTUCE). It is grown for its fleshy, elongated stem rather than its leaves.

Insect Pests and Lettuce

Lettuce is vulnerable to attack by several insect pests from seedling to reproductive stages. The estimated average yield loss is 17 and 13% for fall and spring lettuce, respectively, due to attack of various insect pests (Anonymous 2003). Seedling pests are bulb mites (*Rhizoglyphus* spp., *Tyrophagus* spp.), black cutworm (*Agrotis ipsilon* Hufnagel), variegated cutworm

(*Peridroma saucia* (Hübner)), granulate cutworm (*Feltia subterranean* (Fabricius)), darkling beetles (tenebrionids), field cricket (*Gryllus* spp.), garden symphylans (*Scutigerella immaculate* (Newport)), pea leafminer (*Liriomyza huidobrensis* (Blanchard)), serpentine leafminer (*L. trifolii* (Burgess)), vegetable leafminer (*L. sativae* Blanchard), and springtails.

Lepidopterous pests are responsible for major economic yield losses in lettuce, with losses reaching 100% if control measures are not followed (Inglis and Vestey 2001). Important lepidopterous pests include: armyworm (*Pseudaletia unipuncta* Haworth), beet armyworm (*Spodoptera exigua* (Hübner)), corn earworm (*Helicoverpa zea* Boddie), tobacco budworm (*Heliothis virescens* (Fabricius)), cabbage looper (*Trichoplusia ni* (Hübner)), alfalfa looper (*Autographa californica* Speyer), and saltmarsh caterpillar (*Estigmene acrea* [Drury]) (Parenzan 1984, Toscano et al. 1990, McDougall et al. 2002, Anonymous 2003). In Florida, beet armyworm (*S. exigua*), southern armyworm (*S. eridania*), cabbage looper (*T. ni*), corn earworm (*H. zea*), black cutworm (*A. ipsilon*), variegated cutworm (*P. saucia*), and granulate cutworm (*F. subterranea*) are the major lepidopterous pests (Nuessly and Webb 2003).

The coleopterous pests of lettuce include western spotted cucumber beetle, *Diabrotica undecimpunctata howardi* Barber and banded cucumber beetle, *D. balteata* LeConte (Nuessly and Webb 2003). In Florida, cucumber beetles are found throughout the state. The banded species is more common in central and southern Florida whereas, the spotted species is more prevalent in northern Florida. Beetles may cause potential losses of 100%, if not managed. Yield loss with proper management strategies is generally less than 2.5%. Cucumber beetles became a problem on lettuce in Washington, when peas and cucumbers were grown in lettuce growing areas (Inglis and Vestey 2001).

The homopterous pests are foxglove aphid (*Aulacorthum solani* (Kaltenbach)), green peach aphid (*Myzus persicae* (Sulzer)), potato aphid (*Macrosiphum euphorbiae* (Thomas)), lettuce aphid (*Nasonovia ribisnigri* (Mosley)), lettuce root aphid (*Pemphigus bursarius* (L.)), and silverleaf whitefly (*Bemisia argentifolii* Bellows & Perring). In Florida, *Uroleucon pseudoambrosiae* (Olive) is important (Mossler and Dunn 2005). Aphids appear annually in lettuce production fields and cause yield losses generally less than 2% under normal management with insecticides. Losses in Washington can range from 75 to 100% without the timely use of chemical control measures (Inglis and Vestey 2001). Tarnished bug (*Lygus lineolaris* (Palisot) and *L. hesperus* Knight) is also a pest of lettuce. It causes qualitative damage due to discharge of a toxin during feeding that can be sufficiently severe to make the heads unmarketable. This pest arises irregularly every few years, often later in the spring and early summer. Potentially 100% of the acreage can be affected without appropriate management (Kurtz 2001, McDougall et al. 2002, Anonymous 2003).

In the United States, about 93% of the lettuce area is highly dependent upon chemical control for management of economic pests (Agricultural Statistics 2001). Florida, in particular, ranks first among lettuce growing states in the usage of insecticides to manage insect pests. Florida growers applied insecticides on 98 to 100% of the states lettuce acreage with a total annual usage ranging from 1,900 to 4,900 pounds of active ingredient (Mossler and Dunn 2005). High dependence on chemicals poses a potential threat to farmers, the environment, and natural enemies of these insect pests. Dependence on chemicals is also costly. Therefore, there is a need to look for alterative strategies for management of economic insect pests of lettuce. Host plant resistance should be one of the major components of an integrated pest management (IPM) program and can sustain or improve production efficiency in ways that will maintain or enhance

natural resources and the environment (Sharma and Ortiz 2002, Sadasivan and Thayumanavan 2003). Despite noticeable benefits of host plant resistance mediated through chemicals, it may reduce the competitive ability of plants, leading to a trade-off between growth and resistance (Herms and Mattson 1992). The production and maintenance of these chemicals require resources that are then not available for the growth and reproduction of plants. Therefore, metabolic costs are thought to be involved in resistance (Agrawal 1999) and resistance is always affected by metabolic turnover of compounds (Fagerström 1989, Skogsmyr and Fagerström 1992, Gershenzon 1994).

Management of insects based on host plant resistance is more advantageous economically, ecologically and environmentally than management based on chemical measures (Sharma and Ortiz 2002, Sadasivan and Thayumanavan 2003). It is a very targeted and long-lasting approach to manage economic insect pests. Dependence on fewer chemical sprays and increased yields could provide economic benefits. Plant resistance increases ecosystem stability due to conserving species diversity and maintains natural food webs by not disturbing natural enemies of insect pests.

Host Plant Resistance

Plants live in a world that is inhabited by numerous adversaries (biotic and abiotic), the major proportion of which belongs to plant-eating animals, including insects, called herbivores. In spite of the great variety of herbivores, only parts of plants are defoliated, and the majority of plant foliage and reproductive structures survives due to an innate capacity to tolerate herbivory by compensating for resource losses (Constabel 1999, Strauss and Agrawal 1999), or to defend themselves and thus to reduce the amount of damage (Constabel 1999). The ability of the plant to defend itself against herbivores using different strategies is known as host plant resistance. Host plant resistance is considered to be one of the most effective components of an integrated

pest management program and has been exploited to reduce the dependence on chemical insecticides (Panda and Kush 1995). Host plant resistance is usually compatible with other control measures like biological control and cultural controls, and maintains the food web by conserving the natural enemies. Plants possess a natural defensive system incorporating mechanical and chemical factors produced via transcriptional activation of corresponding genes. These defenses operate either constitutively or after damage due to enemy attack (induced resistance) (Vet 1999).

Many studies have investigated host plant preferences of herbivorous insects. Morphological structures like hair and waxes (Lucas et al. 2000), hooks, spikes and trichomes (Gilbert 1971), leaf hardness (Patanakamjorn and Pathak 1967), and physical factors, such as water content (Scriber 1977), and nutrient content (Morrow and Fox 1980) are identified as important factors leading to rejection of or preference for certain plant tissues by an insect. Low nutritional quality of the plants may impede the development of insect herbivores (Scriber and Slansky 1981). Plants are also known to be full of an array of secondary compounds, which may be toxic, lower the nutritional quality of the foliage, or act as antifeedants (Fraenkel 1959, Bernays and Chapman 1977, Rhoades 1979, Scriber and Slansky 1981, Constabel 1999).

Secondary chemicals are not evenly distributed in plant tissues. They are usually concentrated in specialized structures, like vacuoles, idioblasts, glandular trichomes, cavities and canals (Esau 1965, Fahn 1979). Plants sequester secretions within a diversity of canal systems that include laticifers, resin ducts and phloem (Fahn 1979, Metcalfe and Chalk 1983). The canals usually form a complex network and are effectively distributed throughout the plant. Secretions in these canals are characteristically stored under pressure. Damage by insects causes an immediate release of fluids down a force gradient to the place of injury (Buttery and Boatman

1976). Insects may get entrapped due to adhesiveness of some exudates (Farrell et al. 1991). The squirt gun defense mechanism in the forest plant, *Bursera trimera* Bullock is a good example; a fine spray of resins that is released just after attack by the chrysomelids *Blepharida* spp. causes larval mortality (Becerra et al. 2001).

It has been shown that some insects on canal-bearing plants defuse the canalicular reaction before feeding. The cabbage looper, *T. ni*, ruptures *Lactuca* laticifers by making a superficial trench before actual feeding. The trench drains the latex from the distal tip and isolates that particular section from the main canal system (Dussourd and Denno 1991, 1994). Dussourd (1993) compared the survivorship of each instar of *T. ni* and yellow-striped armyworm, *Spodoptera ornithogalli* (Guenee), an insect that does not trench on canal-bearing plants, to the following instar on intact vs. detached leaves of *L. serriola.* The survivorship was high for each instar of *T. ni* on both leaf categories. In contrast, *S. ornithogalli* larvae survived only on detached leaves. Larvae of *S. ornithogalli* in the first and second instar often died with their mandibles glued together with latex. Older larvae tried to feed over and over again but invariably starved to death. Detaching leaves, particularly of plant species with exudates, often modifies their palatability (Bernays and Lewis 1986, Huang et al. 2003c).

Constitutive defense is common to all healthy plants and provides general protection against invasion by herbivores. Constitutive defense has also been referred to as natural or innate defense. On the other hand, induced defense is the mechanism that must be induced or turned on by plant exposure to an herbivore. Unlike constitutive defense, it is not immediately ready to come into play until after the plant is appropriately exposed to herbivore. Constitutive defense is not specific, and is directed toward general strategic defense. Phenolic compounds were previously regarded as quantitative defenses that are always present at high levels in plant tissues

(Feeny 1976). Recently, it has been shown that certain phenolics may increase after insect attack or mechanical wounding (Pullin 1987, Clausen et al. 1989, Ke and Saltveit 1989, Hartley and Lawton 1991, Brignolas et al. 1995, Constabel 1999). Thus, it seems that induced defense may be more cost-effective for plants than constitutive defense under certain conditions. In induced defense, secondary compounds are manufactured as reactions to insect attack or wounding, and there is no need to maintain the compounds at a steady and effective concentration as in the case of constitutive defense (Herms and Mattson 1992, Baldwin 1994, Gershenzon 1994). Induced defense contributes to plant resistance by enhancing the action of natural enemies of insects (Thaler 1999).

Biochemical Basis of Host Plant Resistance

Both proteins and secondary plant compounds contribute to defense in plants. Secondary plant compounds are organic molecules that are not required for normal physiological processes in growth and development. These biochemicals are also called allelochemicals, because they influence the behavior and/or physiology of species other than their own. Generally, secondary plant compounds have been more extensively studied than proteins, possibly due to their interesting structural variety and advanced biological activities (Duffey and Stout 1996).

Host Plant Resistance Due To Proteins

Molecular biology has proven to be a useful tool in host plant resistance research because plant defense responses can be studied at the level of gene expression rather than simply with assays of the encoded proteins. Each protein in a plant is encoded by a single gene, which can be isolated and employed for developing genetically engineered crops with improved pest resistance. Regulation of gene expression is a principal way that defense proteins are generated in plants and has been confirmed by the induction of mRNA after herbivory (Constabel et al. 2000).

Protease inhibitors

Protease inhibitors (PIs) are proteins that strongly bind proteolytic enzymes and thereby hinder their activity (Ryan 1990, Richardson 1991). PIs are classified as inhibitors of serine, cysteine, aspartic, or metallo-proteases (Ryan 1990). These inhibitors effectively block the active site of proteases by binding to it and forming a complex with a low dissociation constant (Terra et al. 1996, Walker et al. 1998). PIs accumulate in tomato leaves in response to insect attack within hours of damage (Green and Ryan 1972). Low molecular weight protease inhibitors, such as leupeptin, calpin inhibitor I, and calpeptin are strong antifeedants for adult western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Kim and Mullin 2003). All PIs possess a di- or tripeptidyl aldehyde moiety, which binds covalently with sulfhydral (SH) group on the taste chemoreceptors of insects (Kim and Mullin 2003). PIs cause hyper-production of insect digestive enzymes, which triggers the loss of sulfur amino acids, and also reduces the quantity of proteins. As a result, insects become weak, exhibit stunted growth and ultimately die (Shulke and Murdock 1983).

Cysteine protease

Cysteine protease is a 33-kDa defense protein, which accumulates in resistant lines of maize (*Zea mays* L.) in response to larval feeding of fall armyworm, *S. frugiperda* (Pechan et al. 2000). It accumulates in the mid whorl of the maize plant within 1 h of infestation and continues to build up for as long as 7 d. This protein hinders larval growth and is responsible for 60 to 80% weight loss (compared to control insects feeding on susceptible lines of maize) (Pechan et al. 2000), which is due to destruction of the peritrophic matrix of the gut and subsequent disruption of the normal digestive mechanism (Pechan et al. 2002).

Oxidative enzymes

Oxidative enzymes include phenol oxidases, peroxidases, and lipoxygenases. These are stress-associated enzymes synthesized in plants (Butt 1980). The oxidative enzymes are involved in anti-nutritive defense in plants against various insect pests (Felton et al. 1989, Duffey and Felton 1991, Duffey and Stout 1996). Systemin, jasmonates, and the octadecanoid defense signaling pathway induce polyphenol oxidase and lipoxygenase in tomato and cotton, and thus support the role of oxidative enzymes in plant defense (Constabel et al. 1995, Thaler et al. 1996, Bi et al. 1997a, Heitz et al. 1997). Bestwick et al. (2001) characterized pro- and antioxidant enzyme activities during the hypersensitive reaction (HR) in lettuce, and reported a prolonged oxidative stress in lettuce cells experiencing HR. This stress is chiefly through a boost in pro-oxidant activities primarily taking place in the absence of enhanced antioxidants.

Polyphenol oxidase. Polyphenol oxidase (PPO) uses molecular oxygen to catalyze the oxidation of monophenolic and *ortho*-diphenolic compounds, and is a key factor for darkening of many fruits and vegetables (Sherman et al. 1991, Steffens et al. 1994, Constabel and Ryan 1998). The expression of PPO is generally high in diseased, insect-damaged and wounded tissues (Mayer and Harel 1979, Stout et al. 1994, Constabel et al. 1995, Thaler et al. 1996). In crops, such as potato, tomato, apple and hybrid poplar, wound-induction at the level of PPO mRNA has been confirmed due to accessibility of PPO cDNA probes (Constabel et al. 1996). PPO contacts its chemical substrates during insect feeding. PPO produces reactive *ortho*-quinones which readily form alkylated amino acids, which ultimately results in protein modification, cross-linking, and precipitation. This protein modification significantly impacts insect pests by preventing efficient digestion and assimilation of nitrogen (Felton et al. 1992, Duffey and Stout 1996).

Wounding induces expression of PPO genes in damaged as well as undamaged (systemically wounded) leaves (Robison and Raffa 1997, Havill and Raffa 1999). Constabel et al. (2000) observed through southern blot analysis that hybrid poplar presumably possesses two PPO genes, with polymorphic alleles at each locus. Similarly, tomato and potato also have seven and six member PPO genes families, respectively (Hunt et al. 1993, Thygesen et al. 1995). Out of seven PPO genes in tomato, only one gene is wound inducible, while the others are regulated by development (Thipyapong and Steffens 1997, Thipyapong et al. 1997). Therefore, the wound-induced increase in PPO activity is through transcriptional activation of PPO genes and *de novo* enzyme synthesis, rather than enzyme activation (Bergey et al. 1996, Constabel et al. 2000). Various plant PPOs require chemical activation to become active, as they are present in latent form in the plant (Jimenez and Garcia-Carmona 1996). The younger leaves show higher PPO activity than older leaves due to buildup of higher levels of PPO mRNA in response to restricted damage of old leaves (Constabel et al. 2000).

Peroxidase. Peroxidase is a heme-containing enzyme that oxidizes a wide range of biological compounds, such as phenolics, indole acetic acid, and ascorbate by utilizing hydrogen peroxide (Butt 1980). Peroxidase plays a key role in lignification of plant tissue. The cell wall peroxidases produce phenoxy radicals from hydroxycinnamyl alcohols that ultimately form lignin by non-enzymatic polymerization (Douglas 1996). These enzymes also perform an important role in suberization of tissues (Kolattukudy 1981). In addition, they are also involved in the construction of cross-links between carbohydrates and proteins in cell walls (Fry 1986, Cassab and Varner 1988). In various plants, like tomato, rice, peanut and bean, peroxidase level is increased after wounding of tissues (Breda et al. 1993, Felton et al. 1994a, Ito et al. 1994, Smith et al. 1994). Peroxidase is also involved in defense by means of cell wall reinforcement

due to its role in lignification and cross-linking of other cell wall components. Ultimately, amplified peroxidase level affects insect performance due to increased leaf toughness (Coley 1983). Bi et al. (1997a) observed induced resistance in cotton to *H. zea* due to increased peroxidase activity in previously damaged cotton foliage or squares. Similarly, Dowd and Lagrimini (1997) found that peroxidase-overproducing transgenic tobaccos, *Nicotiana sylvestris* (Spegazzini and Comes) and *N. tabacum* L., experienced significantly less damage by *H. zea* than did wild plants, suggesting the contribution of peroxidase activity in leaf resistance to chewing insects. Aphid infestation in barley results in ethylene production and subsequent increase in hydrogen peroxide and total peroxidase activity. This highlights the role of ethylene in the oxidative response of infested barley plants (Argandona et al. 2001).

Lipoxygenase. Lipoxygenase employs molecular oxygen to oxygenate unsaturated fatty acids, like linoleic and linolenic acid, and produces fatty acid hydroperoxides (Galliard and Chan 1980, Siedow 1991). Lipoxygenase has a number of important roles in plant defense against insect pests. Lipoxygenase produces a direct antinutritive effect on insects. This adverse effect is due to destruction of polyunsaturated fatty acids, which are key nutrients for most insects (Duffey and Stout 1996). Fatty acid hydroperoxides (plus extra free radicals) generated by lipoxygenase react with essential amino acids and modify proteins. Therefore, lipoxygenase plays an antinutritive role in plant defense similar to PPO and peroxidase (Duffey and Felton 1991).

Proteins of the cell wall

Stresses, including insect pest and pathogen attack, modify cell wall contents of plants, such as carbohydrates, proteins, and phenolics (Bowles 1990, Carpita and Gibeaut 1993). Cell wall proteins, such as proline-rich proteins (PRPs), hydroxyproline-rich glycoproteins (HRGPs),

arabinogalactan proteins, and glycine-rich proteins (GRPs), are induced during wounding of leaves or stems (Showalter 1993).

Secondary metabolism pathways

Phenolics and phenylpropanoids are major classes of phytochemicals responsible for defense reactions in plants. These chemicals are synthesized and accumulated upon insect and pathogen attack, and mechanical wounding**.** The phenylpropanoids are mainly derivatives of phenylalanine (an aromatic amino acid). Plants possess hundreds of phenylpropanoids, with flavonoids and their derivatives constituting the major group (Heller and Forkman 1993). Phenylpropanoid synthesis is always initiated through a common phenylpropanoid pathway. Phenylalanine is converted through a number of steps to hydroxycinnamoyl coenzyme A (CoA) esters, which is a branching point in phenylpropanoid biosynthesis. Lignin precursors, cell wall-bound hydroxycinnamoyl esters, and soluble glucosides are possible end products of different branches and ultimately form lignin and various flavonoids.

Enzymes involved in secondary metabolism

Herbivory and wounding induces various phenylpropanoid enzymes. Phenylalanine ammonia lyase (PAL) is the first enzyme of the phenylpropanoid pathway and catalyzes deamination of phenylalanine to cinnamic acid (Hahlbrock and Scheel 1989). PAL is inducible by insect and pathogen attack, mechanical wounding, exposure to ethylene and abiotic stresses, such as UV light (Hyodo et al. 1978, Jones 1984, Hahlbrock and Scheel 1989, Ke and Saltveit 1989). PAL induction is well documented in certain plant parts, like lettuce leaves (Ke and Saltveit 1986), bean hypocotyls (Cramer et al. 1989), alfalfa (Dixon and Harrison 1990), tobacco leaves (Pellegrini et al. 1994, Fukasawa-Akada et al. 1996), potato tubers and leaves (Rumeau et al. 1990, Joos and Hahlbrock 1992), and parsley (Lois and Hahlbrock 1992). Induction of PAL takes place through transcription of a single or many genes. Endogenous phenylpropanoid

pathway intermediates regulate the level of PAL transcripts by way of a feedback mechanism (Braun and Tevini 1993, Orr et al. 1993). Various other important phenylpropanoid enzymes, in addition to PAL, such as 4-cinnamic acid hydroxylase (4-CH) in *Arabidopsis thaliana* (L.) and pea (*Pisum sutivum* L.) (Frank et al. 1996, Mizutani et al. 1997) and caffeic acid O-methyltransferase in corn (Capellades et al. 1996), are also induced by insect and pathogen attack and/or wounding. Similarly, 4-coumarate CoA ligase in tobacco, *Arabidopsis*, and bean (*Phaseolus vulgaris* L.) is also wound inducible (Smith et al. 1994a, Ellard-Ivey and Douglas 1996, Lee and Douglas 1996). In addition, numerous shikimic acid pathway enzymes responsible for phenylalanine biosynthesis, like 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase in potato tubers (*Solanum tuberosum* L.) (Dyer et al. 1989) and shikimate dehydrogenase from bell peppers (Diaz and Merino 1998) are induced by wounding. Peiser et al. (1998) reported that PAL inhibitors control browning of cut lettuce.

Host Plant Resistance Due To Secondary Plant Compounds

There are wide range of secondary plant compounds found in the plant kingdom (Luckner 1990, Dey and Harborne 1997). This wide diversity of compounds is hypothesized to be the outcome of co-evolution of plants with insects and pathogens (Harborne 1993). Secondary plant compounds are traditionally categorized into three major groups: carbon-based phenolics and terpenes, and nitrogen-containing compounds such as alkaloids (Taiz and Zeiger 1991). In general, carbon-based compounds have been considered cheaper defense tools than nitrogen-containing compounds, as nitrogen is vital and frequently in limited supply for the growth of plants (Bryant et al. 1983). Gonzalez (1977) reported the presence of terpenes, sterols, flavonoids and other phenolics, and alkaloids in lettuce.

Phenolics

Phenolics constitute a diverse group of chemicals, ranging from small phenolic acids to complex polymers such as tannins and lignin (Dey and Harborne 1997). Most phenolic compounds are derivatives of the shikimic acid and phenylpropanoid pathways and bear aromatic rings having one or more hydroxyl groups. Phenolics can be divided into simple phenols and polyphenols based on the number of hydroxyl group attached. Simple phenols include the hydrobenzoic acids (e.g., vanillic acid), the hydroxycinnamic acids (e.g., caffeic acid) and the coumarins (e.g., umbelliferone). Polyphenols are a diverse group of plant phenolics, such as flavonoids (e.g., quercetin) and tannins (e.g., esters of gallic acid) (Schoonhoven et al. 2005). Additional functional groups such as ester, methyl, acetyl or sugar moieties are also found in some complicated phenolics. Stresses such as excessive light, UV, cold, nutrient deficiencies, and attacks by insects and pathogens are most commonly responsible for the induction of phenolics in plants (Dixon and Paiva 1995, Somssich et al. 1996). In intact plants, phenolics are stored in vacuoles in their less toxic glycoside forms as water-soluble compounds (Hösel 1981). The wounding of cells (e.g., by insect attack) causes release of the glycosides from their storage site (Hösel 1981) and ultimately formation of compounds with toxic, deterrent or nucleophilic, and nutritive-value-lowering properties after coming in contact with specific degradative enzymes. For example, toxic hydrogen cyanide is released due to hydrolysis of harmless cyanogenic glycosides by β-glucosidase activity (Wink 1997).

o-Substituted phenolic compounds (e.g., chlorogenic acid) produce *o*-quinones with the help of oxidative enzymes which alkylate amino acids by binding to their nucleophilic groups (Felton et al. 1989, Constabel 1999). This binding hinders the assimilation of essential amino acids and lowers the quality of plant foliage for insects (Felton et al. 1989). As Bi et al. (1997a) observed, wounding of cotton (*Gossypium hirsutum* L.) foliage induced increased activity of

oxidative enzymes that was associated with decreased levels of the nutritional antioxidant ascorbate and increased levels of phenolic prooxidants (i.e., chlorogenic acid) and lipid peroxides. This significant decline in host nutritional quality (due to accumulation of secondary compounds) is responsible for induced resistance in cotton foliage and squares to herbivory by *H. zea,* indicated by a decrease in larval growth when larvae fed on previously damaged foliage or squares compared to the controls. Chemicals like phenolics also play an important role in the inhibition of oviposition on the host plant in addition to reducing larval growth and survival (Dethier 1970, Todd et al. 1971, Chapman 1974, Elliger et al. 1980, Corcuera 1993, Stotz et al. 1999).

Simple phenols such as ferulic, caffeic and *p*-coumaric acids are precursors of lignin. Upon wounding of plant tissue, a subsequent host response occurs involving an intensive accumulation of lignin-like polyphenolics in wounded or ruptured cells. This is followed by a rapid hypersensitive death of the cell, giving rise to a single cell brownish necrosis (Moerschbacher et al. 1990, Nicholson and Hammerschmidt 1992, Wei et al. 1994, Zeyen et al. 1995). Wounding of potato tubers (Hahlbrock and Scheel 1989) and lettuce (Loaiza-Velarde et al. 1997, Loaiza-Velarde and Saltveit 2001) induces an accumulation of phenolic conjugates, such as chlorogenic acid (a caffeic acid conjugate). Hydrogen peroxide is required for the polymerization step in the formation of the poly (phenolic) domain of suberized potato tubers (Razem and Bernards 2002). Ke and Saltveit (1989) also observed an increase in phenolic compounds (e.g., chlorogenic and isochlorogenic acids) and brown stain in lettuce tissue affected by russet spotting.

Phenolics are known to play important role in the host plant resistance (Dethier 1970, Todd et al. 1971, Chapman 1974, Elliger et al. 1980, Corcuera 1993, Stotz et al. 1999). Ikonen et al. (2001) reported feeding deterrence in the willow (*Salix pentandra* L.), to the leaf beetle

(*Lochmaea capreae* L.) due to high levels of chlorogenic acid in the leaves. Cole (1984) correlated resistance to lettuce root aphid with the presence of high amounts of isochlorogenic acid and the enzyme PAL in resistant lettuce cultivars. However, the increased concentration of phenolics in transgenic tobacco showing differential expression of PAL does not substantiate their role in plant resistance against the generalist tobacco hornworm (*Manduca sexta* L.), and the specialist tobacco budworm (*H. virescens* (Fabricius)) (Bi et al. 1997b). Similarly, Eichenseer et al. (1998) also did not find any preference in larvae of *M. sexta* fed transgenic tobacco plants that either under- or over-expressed PAL and consequently with either lower or higher levels of phenolics than normal.

Tannins are complex polyphenols and are more prevalent in woody perennials than in herbaceous plants (Swain 1979). They are often considered as general feeding deterrents in plant-insect interactions, and therefore, play an important role in chemical ecology and defense against insects (Swain 1979, Hagerman and Butler 1991). Based on their structure, they are categorized as condensed tannins, or proanthocyanidins, and hydrolysable tannins, which are gallic acid or ellagic acid esters of various sugars.

Caffeic acid derivatives (Ke and Saltveit 1988) and flavonoids (Hermann 1976) are the two main classes of simple phenols and complex polyphenols, respectively, which have been identified in lettuce. In particular, simple phenolics like monocaeffeoyl tartaric acid, chicoric acid, 5-caffeolyquinic acid and 3,5-di-*O*-caffeoylquinic acid are present in lettuce (Winter and Hermann 1996, Ferreres et al. 1997).

Flavonoids

Plants flavonoids are a large group of phenolic compounds produced by the shikimic acid pathway. Flavonoids are grouped under major classes, such as the flavanones, flavones, flavonols, and isoflavonoids (Harborne 1994). In the biosynthesis of flavonoids, chalcone

synthase (CHS) is the first committed enzyme that catalyzes the formation of chalcone intermediate by condensing three malonyl-CoA and one hydroxycinnamoyl-CoA molecules. CHS is known to be involved in the response to many forms of stress in many plants, including to insects and pathogens (Dangl et al. 1989). Chalcone is then catalyzed to flavanone with the help of the enzyme chalcone isomerase. In the next step, flavonoid biosynthesis splits into different branches. In the first branch, flavones are formed from flavanones due to the action of flavone synthase (Britsch 1990). Secondly, dihydroflavonols, which are precursors of flavonols and anthocyanins, can be synthesized from flavanones by the enzyme flavanone-3-hydroxylase. In the third branch, flavanones can be converted into isoflavanones in a reaction catalyzed by isoflavanone synthase (Dixon et al. 1995).

Flavonoids are found in high concentrations in many plant species under normal conditions as sugar conjugates (Fröst et al. 1977, Feng and McDonald 1989, Jähne et al. 1993, Stapleton and Walbot 1994), and over 50 different glycosides have been identified among the more common-occurring flavonoids (Hermann 1976, 1988). Flavonoid accumulation in leaves is very much increased in response to illumination with the UV-B spectrum of visible light (Koes et al. 1994, Strid et al. 1994). Flavonoids play a role in the protection of plants from the damaging effects of UV-light, as they have good light absorbing properties in the UV spectrum (Markham 1989). Red-pigmented lettuce, such as ‘Lollo Rosso’, contains high concentrations of anthocyanin with antioxidant and free-radical scavenging properties (Gil et al. 1998). Flavonols, such as kaempferol, quercetin and myricetin, and the analogous flavones, apigenin and luteolin are found in vegetables, fruits, and beverages (Hertog et al. 1992, 1993), and are also known to possess antioxidant and free radical scavenging activity in foods (Shahidi and Wanasundara 1992). Flavonols, such as quercetin 3-O-glucuronide, quercetin 3-O-glucoside and quercetin 3-

O-(6-O-malonylglucoside) are also present in lettuce (Winter and Hermann 1996, Ferreres et al. 1997). The lettuce varieties 'Lollo Rosso' and 'Round' contain high amounts of quercetin, varying from 11-911 μg g^{-1} of fresh weight in the outer leaves to 450 mg g^{-1} in the inner leaves (Crozier et al. 1997). The polyphenol compounds (caffeic acid derivatives, quercetin and kaempferol glycosides) are present in higher amounts in lettuce grown in the field than in a greenhouse (Romani et al. 2002).

The behavior, development, and growth of insects are influenced by plant flavonoids (Hedin and Waage 1986). Plant flavonoids act as feeding stimulants for the boll weevil (*Anthonomus grandis* Boheman) in cotton (Hedin et al. 1988), or oviposition stimulants to the citrus-feeding swallowtail butterfly, *Papilio xuthus* L. (Nishida et al. 1987). Flavonoids may be antibiotic substances effective against phytophagous insects (Todd et al. 1971, Chan and Waiss 1978, Chan et al. 1978, Joerdens-Roettger 1979, Elliger et al. 1980, Hanny 1980, Hedin et al. 1983, Peng and Miles 1988, Ridsdill-Smith et al. 1995). Rutherford (1998) observed the involvement of chlorogenates and flavonoids in the resistance of sugarcane to the stalk borer (*Eldana saccharina* Walker). Two extreme types of flavonoid profile were found using near-infrared spectroscopy (NIR), one coupled with susceptibility and other with resistance. Stalk borer larvae could be induced to feed by inclusion of the susceptible-type flavonoid profile into a defined synthetic diet. Subsequent survival of first instar larvae was greater on this diet than on diets containing the resistant-type flavonoid profile.

Terpenoids

Terpenoids are a diverse group of chemicals which all originate from iospentenoid precursors. Based on the number of isoprene (five-carbon) units, terpenoids are classified as mono-, sesqui-, di, tri- or tetra-terpenoids. They are known to have various secondary functions,

like defense against pathogens and insects as well as primary functions, such as membrane components, pigmentation, free radical scavenging, and growth regulators (Harborne 1993). Antibiotic, cytotoxic, and allergenic properties are also associated with terpenoids (Burnett et al. 1978).

Many sesquiterpene lactones accumulate in canals (laticifers) closely associated with the vascular tissues of composit plants (Esau 1965). Damaged laticifers release latex containing sesquiterpene lactones which may have analgesic, antitussive and sedative properties (Gromek et al. 1992). Sesquiterpene lactones are extremely varied in their structure, properties and functions (Rees and Harborne 1985). The main bitter constitutive principles of *Lactuca* species are lactucin, lactucopicrin, 8-deoxylactucin and their derivatives, such as 11,13-dihydro-analogues (Barton and Narayanan 1958, van Beek et al. 1990). Two triterpenes, the quaianolides lactucin, and lactucopicrin have been isolated from dry latex of *L. virosa.* The presence of lactucin, 8-deoxylactucin, and lactucopicrin in lettuce and chicory make them intensely bitter (Price et al. 1990). Wounding of leaves or stems of *Lactuca* species releases a milky latex consisting of 15-oxalyl and 8-sulphate conjugates of lactucopicrin, which ultimately revert to the parent lactone due to hydrolysis of unstable oxalates. The induced quaianolide sesquiterpene lactone phytoalexin, lettucenin A, is also present in *Lactuca* species, but not in chicory (Sessa et al. 2000). Lettucenin A was initially characterized by Takasugi et al. (1985). It is one of the most toxic phytoalexins ever discovered and provides resistance to lettuce downy mildew in certain lettuce cultivars due to its strong antimicrobial properties (Bennett et al. 1994). Bestwick et al. (1995) isolated lettucenin A from lettuce seedlings with the red spot physiological disorder. A 15-glycososyl conjugate of 11,13-dihyrolactucopicrin is found in *L. tartarica* roots (Kisiel et al. 1997). Likewise, the related quaianolide sesquiterpene lactone glycosides, such as picriside A

(lactucin-15-glycoside) and crepiaside A (8-deoxylactucin-15-glycoside), are found in other members of the Lactuceae tribe (Seto et al. 1988).

Host Plant Resistance in Lettuce to Insect Pests

Aphids

Many species of aphids are known to colonize lettuce, but few are responsible for transmission of viruses (Kennedy et al. 1962). Aphids are the most serious pests of lettuce in North America (Alleyne and Morrison 1977, Forbes and Mackenzie 1982, Toscano et al. 1990), Spain (Nebreda et al. 2004), and other areas of Europe (Ester et al. 1993, Ellis et al. 1996, Martin et al. 1996, Monnet and Ricateau 1997, Parker et al. 2002). Reduction in yield of lettuce is due to direct damage caused by aphid feeding and indirect damage by aphid-transmitted virus infections. In addition, marketability of harvested heads is greatly reduced by the physical presence of aphids (Dunn 1959, Rufingier et al. 1997).

The lettuce root aphid, *P. bursarius*, is one of the most important pests of lettuce in the United States (Swift and Lange 1980, Blackman and Eastop 2000), Western Europe, and Canada (Ellis 1991, Reinink and Dieleman 1993). It feeds on the youngest leaves and rapidly colonizes the 'heart' of the lettuce (Forbes and Mackenzie 1982). The lettuce aphid, *N. ribisnigri*, is a major pest in the United States, Czechoslovakia, UK, France, Germany, Netherlands and Switzerland (Reinink and Dieleman 1993, Mosler and Dunn 2005). *Uroleucon ambrosiae* is a pest of hydroponically-grown lettuce in Brazil (Auad and Moraes 2003, Miller et al. 2003) and Turkey (Zeren 1985). Green peach aphid, *M. persicae* (Capinera 2004), and potato aphid, *M. euphorbiae* (Reinink and Dieleman 1989), are active vectors of lettuce yellow virus.

A variety of chemicals are sprayed to control aphids in lettuce. Therefore, to reduce lettuce growers' dependence on insecticides for aphid control, a number of alternative measures must be used as a part of IPM program together with the use of varieties resistant to aphids (Tatchell et

al. 1998). Plant resistance as one of the components of IPM has been extensively studied to manage aphids on lettuce. Successful transfer of resistance from wild to cultivated lettuce has proven useful in controlling *N. ribisnigri* (Eenink et al. 1982). However these varieties afford only slight to no defense against *M. persicae* and *M. euphorbiae* (Reinink and Dieleman 1989, van Helden et al. 1993). Modern varieties of lettuce resistant to *P. bursarius*, such as 'Avoncrisp' and 'Lakeland', possess the dominant *Lra* gene (Dunn 1974, Ellis et al. 1994). The *Lra* gene is also linked to the downy mildew (*Bremia lactucae*) resistance gene, *Dm6* (Harrewijn and Dieleman 1984, Ellis et al. 1994, Ellis et al. 2002). However, the lettuce variety 'Grand Rapid', reported to be resistant to *P. bursarius* (Dunn and Kempton 1980), does not possess *Dm6* (Crute and Dunn 1980). In addition, several factors whose genetic basis have not been identified, such as deficient nutritive value of the phloem sap, phytochemicals (toxic or deterrents), and unacceptability of the plant surface for feeding, provide resistance to aphids (Harrewijn and Dieleman 1984).

Wild lettuce species *L. virosa* L., *L. saligna* L., and *L. perennis* L. are found to be resistant to *M. persicae*, causing aphid mortality and lower nymph production (Eenink and Dieleman 1982). This resistance (governed by additive genes) was transferred to cultivated lettuce by making a series of inter-specific crosses (Eenink et al. 1982). Clones of *M. persicae* exhibit different intensities of aggressiveness on lettuce. The lettuce genotypes selected for partial resistance to the aggressive clone *WMp1* possess complete or almost complete resistance to less aggressive clones (Reinink et al. 1989). *Lactuca virosa* is almost completely resistant to *N. ribisnigri*, causing low feeding rate, adult and nymphal mortality, and reduced reproduction (Eenink and Dieleman 1982). Complete resistance to *N. ribisnigri* is governed by the presence of

the *Nr* gene in the plant, whereas the *Nr* gene provides only partial resistance to *M. persicae*, and no resistance to *M. euphorbiae* (Reinink and Dieleman 1989).

Iceberg lettuce shows resistance towards three main aphids, *N. ribisnigri*, *M. euphorbiae* and *P. bursarius* (Dunn and Kempton 1980). Ester (1998) observed 100% resistance against *N. ribisnigri* and *M. euphorbiae* in aphid-resistant butterhead lettuce cultivars. In Europe, the lettuce butterhead cultivar 'Dynamite' shows high resistance against *N. ribisnigri* and *P. bursarius*, some resistance to *M. euphorbiae* and *U. sonchi*, but no resistance to the glasshouse-potato aphid, *Aulacorthum solani* (Kaltenbach) (van der Arend et al. 1999, van Melckebeke et al. 1999). Butterhead cultivars are moderately to highly resistant to *M. euphorbiae* and *U. sonchi*, whereas crisphead cultivars possess little or no resistance to either aphid species (Reinink and Dieleman 1989). The lettuce cultivar 'Charan' shows partial resistance to *M. euphorbiae* and *U. sonchi* (Reinink et al. 1995). Montllor and Tjallingii (1989) electronically monitored the probing behavior of *M. persicae* and *N. ribisnigri* on susceptible and resistant lettuce lines using a DC amplifier. They proposed the possible involvement of both mesophyll and phloem factors in conferring resistance. van Helden and Tjallingii (1993) also discussed the role of phloem vessels in resistant lettuce. van Helden et al. (1995) compared the phloem sap of both resistant and susceptible cultivars and found no relationship between phloem sap composition and resistance to *N. ribisnigri*. However, later work by van Helden and van der Wal (1996) suggests the presence of a resistance factor against *N. ribisnigri* in lettuce phloem sap. The roots of lettuce cultivars showing resistance to *P. bursarius* have greater concentrations of isochlorogenic acid and PAL as compared to susceptible cultivars (Cole 1984).

Cabbage Looper

Cabbage looper, *T. ni*, is a serious problem in all lettuce growing areas in the United States (Kerns and Palumbo 1996, Kerns et al. 1999, Agnew 2000, Kurtz 2001). It is the predominant

pest of lettuce during autumn in California (Kishaba et al. 1976, Vail et al. 1989). The larvae of *T. ni* often transect leaves with a narrow trench before eating to reduce exposure to exudates, such as latex, during feeding (Dussourd 2003). Cabbage looper larvae develop faster on excised than on attached leaves of prickly lettuce, *L. serriola*, signifying the suitability of these plants when canals are inactivated (Tune and Dussourd 2000). Lactucin from lettuce latex seems to act as a trenching stimulant, but other chemicals, such as phenylpropanoids, monoterpenes, and furanocoumarins, show slight or no activity for inducing trenching (Dussourd 2003). The F_2 plants derived from a cross between *L. sativa* lines and resistant lines of *L. saligna* were resistant (Whitaker et al. 1974) and showed antixenosis toward *T. ni* (Kishaba et al. 1980).

Banded Cucumber Beetle

The banded cucumber beetle, *D. balteata*, is a generalist feeder that feeds upon many plant species. In the early 1900s, this pest was mostly found in Central and South America and Mexico (Saba 1970, Krysan 1986, Bellows and Diver 2002). Later on, it spread into the United States and is now established in Alabama, Arizona, Arkansas, California, Florida, Georgia, Louisiana, Mississippi, New Mexico, North Carolina, South Carolina, and Texas (CABI 2006). It is also found throughout Florida but most commonly in the Lake Okeechobee area (Capinera 1999). It is an economic concern for lettuce cultivation in southern Florida (Nuessly and Nagata 1993). *Diabrotica balteata* has a high reproductive capacity (Pitre and Kantack 1962), and many generations occur throughout the year (Schalk 1986).

Romaine lettuce cultivars ‘Valmaine’ and ‘Tall Guzmaine’ were analyzed to assess the level of resistance to *D. balteata* (Huang et al. 2002). Valmaine was highly resistant whereas Tall Guzmaine was susceptible to *D. balteata*. The mechanism of resistance was determined to be antixenosis and such little feeding occurred on Valmaine that the reproductive structures were not fully developed in adult females (Huang et al. 2002). However, latex from both Valmaine

and Tall Guzmaine showed antifeedant activities when applied to the surface of a preferred food, such as lima bean (*Phaseolus vulgaris* L.) leaves. Valmaine plants that had been previously fed upon showed higher resistance to *D. balteata* than did Tall Guzmaine after previous feeding, suggesting involvement of physical factors and an induced mechanism of resistance in Valmaine against the beetle (Huang et al. 2003b).

Leafminer

Plants in over 47 genera belonging to 10 families have been recorded as hosts of leafminers. The principal leafminer species affecting lettuce include *L. sativae*, *L. trifolii*, *L. huidobrensis* and *L. langei* Frick. Both *L. trifolii* and *L. sativae* are native to America (Waterhouse and Norris 1987). In the United States, these two species are found commonly in the southern United States from Florida to California and Hawaii (Capinera 1999). In Arizona, *L. sativae* is predominant during the period of August to January, whereas during February *L. trifolii* prevails (Kerns and Palumbo 1996, Kerns et al. 1999, Agnew 2000). In recent years, populations of leafminers have increased in coastal areas in California (Kurtz 2001). In central Florida, populations of leafminer are high between May and October, when minimum average temperatures are 25^{o}C, whereas higher temperature in southern Florida favors leafminer populations throughout the year (Anonymous 1999). Leafminer larvae cause damage by mining the leaves, which may result in reduced photosynthetic activity. Younger plants are more vulnerable to leafminer attack and severe damage can kill the plants (Nuessly and Webb 2003).

The romaine lettuce cultivar Valmaine was the most resistant to *L. trifolii* in tests involving three additional lettuce cultivars, ‘Floricos 83’, ‘Parris Island Cos’, and Tall Guzmaine. Adults on Valmaine had significantly reduced levels of feeding, longevity, and fecundity (Nuessly and Nagata 1994). *Liriomyza trifolii* preferred to feed on the middle leaves of Valmaine plants in contrast to Tall Guzmaine where they preferred to feed on the older and younger leaves. When

honey was supplied as a supplement to the diet of Valmaine, female survivorship and reproductive rates increased to levels more similar to Tall Guzmaine suggesting a deficiency in a critical diet component in Valmaine (Nagata et al. 1998).

Mou and Ryder (2003) screened 48 varieties of cultivated lettuce, *L. sativa* and the wild species, *L. serriola*, *L. saligna*, and *L. virosa* for resistance to *L. langei*. Wild species had fewer leafminer stipples per unit area than cultivated lettuce. Iceberg experienced the most stippling damage among the genotypes tested. The progenies of crosses between the resistant genotypes were selected to raise the level of resistance (Mou et al. 2004, Mou and Ryder 2003).

***Helicoverpa* species**

Heliothinae are very destructive pests of many crops and frequently shift to lettuce from surrounding crops, like cotton and corn (Kerns and Palumbo 1996, Kerns et al. 1999, Agnew 2000, Kurtz 2001). Corn earworm, *H. zea,* is found throughout the United States (Capinera 1999). It is found on all Florida vegetable crops (Martin et al. 1976). In Australia, *H. armigera* (Hübner) and *H. punctigera* Wallengren are serious pests of lettuce and can cause extensive damage (Ridland et al. 2002, Dimsey and Vujovic 2003). In India, *H. armigera* is found throughout the year in lettuce fields, but is most active during March and April (Parihar and Singh 1992).

***Spodoptera* species**

Beet armyworm, *S. exigua*, is a polyphagous and widely distributed insect (CABI 1972). It is the key pest of lettuce in the western United States (Metcalf and Flint 1962, Kerns and Palumbo 1996, Kerns et al. 1999, Agnew 2000, Kurtz 2001). In the early stage of crop development (between thinning and cupping stage), it does not cause any economic damage, but feeding during the heading stage makes the lettuce unmarketable (Kerns and Palumbo 1996, Kerns et al. 1999, Agnew 2000). Ghaffar et al. (2002) found the pupal period of *S. exigua* to be

the shortest (5.8 d) on lettuce as compared to eggplant (*Solanum melongena* L.) and field bindweed (*Convolvulus arvensis* L.) (7.6 d).

Bemisia species or strains

The B strain of the cotton whitefly, *Bemisia tabaci* (or *B. argentifolii*, the silverleaf whitefly), is one of the primary pests of fall lettuce in California and Arizona. It causes complete destruction of early fall planted lettuce due to the extraction of large amounts of phloem sap from seedlings (Kerns and Palumbo 1996, Kerns et al. 1999, Agnew 2000). It also causes yellowing and distortion of the leaves and can reduce dry mass accumulation by up to 41%, depending upon population level (Costa et al. 1993). In lettuce, whitefly stylets penetrate epidermal cells and intercellular junctions while feeding. Arrangement of vascular bundles in lettuce affects the feeding behavior of whitefly. The length of the vascular bundle (2.8 mm per 1.0 mm^2 leaf area) is tolerably acceptable to whitefly (Cohen et al. 1996). However, fewer minor veins (fewer vascular bundles) accounts for low success of whitefly on lettuce compared to preferred crops, such as cantaloupe and other cucurbits (Cohen et al. 1998).

Thrips

Western flower thrips, *Frankliniella occidentalis* (Pergande), and onion thrips, *Thrips tabaci* (Lindeman), are prevalent pests of lettuce in Arizona (Kerns and Palumbo 1996, Kerns et al. 1999, Agnew 2000, Kurtz 2001). Western flower thrips is a native of North America, and has a broad host range of more than 500 species representing 50 plant families (Beshear 1983, Yudin et al. 1986). It is most commonly found in California (Bryan and Smith 1956, Rob 1989) and Arizona (Bibby 1958) on lettuce. Thrips adults and larvae puncture and feed from epidermal cells (Nuessly and Webb 2003), and affect quality of lettuce, as they cause leaf stippling and rib discoloration (Kurtz 2001). Romaine lettuce is especially susceptible to thrips in Arizona (Kerns and Palumbo 1996, Kerns et al. 1999, Agnew 2000). In Florida, *Frankliniella* spp. are important

carriers of tomato spotted wilt and escarole necrosis viruses (Nuessly and Webb 2003). Mollema and Cole (1996) found a positive correlation between amino acid concentration in lettuce leaves and western flower thrips damage suggesting that higher concentrations are important for successful thrips development.

Research Goals

Lettuce is an important leafy vegetable grown all over the world. In the United States, romaine lettuce is the most commonly grown leaf lettuce. It is vulnerable to attack by several insect pests during field production. Chemical control measures are the main tools for management of insect pests on lettuce and about 93% of lettuce grown in the United States is under chemical management of noxious insects (Agricultural Statistics 2001). In southern Florida, vegetable farming involves high intensity pesticide usage (>20 pounds of active ingredient pesticide per acre/crop), and often there is more than one crop per year, which further increases the amount of pesticides used (Agricultural Statistics Board 2001). In Florida, lettuce production is more concentrated in the southern part of the state, which is an ecologically sensitive area due to its proximity to the Everglades National Park and heavy precipitation and run-off (Miles and Pfeuffer 1997). High dependence on chemicals can pose a threat to growers and natural enemies of insect pests as well as involves a heavy cost (Sharma and Ortiz 2002, Sadasivan and Thayumanavan 2003). Hence, there is a need to look for alterative tactics for management of economic insect pests.

Host plant resistance is an important component of integrated pest management. Management of insects based on host plant resistance can reduce the sole dependence on chemical usage (Sharma and Ortiz 2002, Sadasivan and Thayumanavan 2003). Thus, it is essential to develop resistant varieties in lettuce to reduce these economic and environmental problems. The romaine lettuce cultivar Valmaine is known to possess a high level of resistance

against *D. balteata* (Huang et al. 2002) and *L. trifolii* (Nuessly and Nagata 1994) as compared to three other cultivars, 'Parris White', 'Short Guzmaine', and Tall Guzmaine. Resistance was highest in Valmaine and lowest in Parris White in confirmation with pedigree analysis (Guzman 1986). Short Guzmaine is the product of Valmaine and 'FL 1142', whereas Tall Guzmaine was selected from progeny of a cross between Short Guzmaine and Parris White. Guzman designed Tall Guzmaine for improvement of certain horticultural characters over Valmaine, such as thermodormancy, premature bolting, and resistance to lettuce mosaic virus and corky root rot. Breeders did not evaluate insect resistance when developing Tall Guzmaine. Further, previously wounded Valmaine plants showed higher resistance to *D. balteata* as compared to Tall Guzmaine suggesting the involvement of an induced mechanism of resistance in Valmaine (Huang et al. 2003b). Thus, it would be helpful to know the biochemical mechanism of resistance in Valmaine to different insects to aid plant-breeding programs in development of new lettuce cultivars with both desirable horticultural characters and insect resistance.

The objectives of this study were the following:

1. To compare survival, development and feeding behavior of cabbage looper and beet armyworm on Valmaine and Tall Guzmaine
2. To determine the potential of latex produced by Valmaine as a defense mechanism against banded cucumber beetle using choice and no-choice tests and isolation of deterrent compounds from the latex using solvent extraction
3. To further isolate deterrent compounds from Valmaine latex using bioassay-directed fractionation of Valmaine latex crude extract
4. To investigate enzyme induction as a possible reason for latex-mediated insect resistance in Valmaine

CHAPTER 2
HOST PLANT RESISTANCE IN ROMAINE LETTUCE AFFECTS LARVAL FEEDING BEHAVIOR AND BIOLOGY OF TRICHOPLUSIA NI AND SPODOPTERA EXIGUA (LEPIDOPTERA: NOCTUIDAE)

Introduction

Over the past 15 yr, romaine lettuce, *Lactuca sativa* L., has been the fastest growing vegetable in terms of production, consumption, and exports in the United States. During the period 2002 to 2004, romaine lettuce accounted for 22% of all lettuce produced in the United States and per capita use of romaine lettuce has tripled (3.7 kg) since 1992-94 (USDA 2005a). Lettuce is vulnerable to attack by several insects including lepidopterans that can be responsible for yield losses of 100% if populations are not managed (Inglis and Vestey 2001). In Florida, the cabbage looper, *Trichoplusia ni* (Hübner), and the beet armyworm, S*podoptera exigua* (Hübner) (Lepidoptera: Noctuidae), are serious pests of lettuce (Nuessly and Webb 2003).

Economic pests are managed chemically on about 89% and 85% of head and other lettuce acreage, respectively, in the United States (USDA 2005b). Florida ranks first among lettuce growing states in the usage of insecticides and growers apply insecticides on 98 to 100% of the state's lettuce acreage to manage these insect pests (Mossler and Dunn 2005). For instance, restricted insecticides such as lambda-cyhalothrin (34% and 32% of head and other lettuce acreage, respectively) and methomyl (32% and 30% of head and other lettuce acreage, respectively) are extensively applied on lettuce (USDA 2005c). Rapid development of insecticide resistance has been reported for *Liriomyza* spp. (Diptera: Agromyzidae) against chlorinated hydrocarbons, organophosphates and the pyrethroid permethrin (Genung 1957, Leibee 1981, Parrella and Keil 1984). The high dependence on chemicals poses a threat to agricultural workers and natural enemies of

these insect pests and increases production cost. Therefore, the implementation of alternative strategies, such as host plant resistance, for the management of economic insect pests should be explored.

'Valmaine' romaine lettuce (Leeper et al. 1963) was the major cultivar grown in Florida before the adoption of 'Tall Guzmaine'. Tall Guzmaine was selected from a cross between 'Short Guzmaine' and 'Parris White' (Guzman 1986). Short Guzmaine was a selection from a cross between Valmaine and 'Florida 1142'. Tall Guzmaine was selected for resistance to thermodormancy, premature bolting, lettuce mosaic virus and corky root rot; however, Guzman did not include insect resistance in his selection criteria (Guzman 1986). Tall Guzmaine was found to be susceptible to the leafminer, *Liriomyza trifolii* (Burgess) (Nuessly and Nagata 1994) and the banded cucumber beetle, *Diabrotica balteata* LeConte (Coleoptera: Chrysomelidae) (Huang et al. 2002) compared to Valmaine. Therefore, I selected the same two cultivars to determine whether resistance in Valmaine extends to a third order containing economically important insect pests of lettuce, the Lepidoptera.

In this study, I tested the performance of two noctuid defoliators important to Florida lettuce production, cabbage looper and beet armyworm on Valmaine and Tall Guzmaine. I chose these two insect species because I was interested in how ecologically similar but behaviorally different defoliators of lettuce would respond to the selected lettuce cultivars. Cabbage loopers trench leaves of latex-bearing plants (Dussourd and Denno 1994), whereas beet armyworms do not. In particular, cabbage looper has been shown to deactivate the canalicular defenses in wild lettuce, *Lactuca serriola* L., by making shallow trenches before actual feeding (Dussourd 1997). The objectives of the

study were to compare the survival, development and feeding behavior of cabbage looper and beet armyworm on resistant Valmaine and susceptible Tall Guzmaine lettuce.

Materials and Methods

Plants

Seeds of two romaine lettuce cultivars, Valmaine and Tall Guzmaine, were provided by R. T. Nagata (Everglades Research and Education Center, University of Florida, FL). Seeds were germinated by placing them overnight in a Petri dish lined with wet filter paper in the laboratory. Germinated seeds were planted in a transplant tray filled with commercial soil mix (MetroMix 220, Grace Sierra, Milpitas, CA) in a greenhouse with natural light at a mean temperature of 27°C (32 to 24°C) and 68% mean RH (44 to 94%). After 2 wk, seedlings were transplanted to plastic pots (15 cm diameter) filled with MetroMix 220. Plants were irrigated daily and fertilized once per week with 10 ml of a 10 g/L solution of soluble fertilizer (Peters 20-20-20, N-P-K, W.R. Grace, Fogelsville, PA) from transplanting of seedlings to the end of the experiment. Four-week-old plants with six to seven true leaves were used in all experiments that were conducted in the greenhouse under ambient light.

Insects

Cabbage looper eggs were supplied by G. L. Leibee (Mid-Florida Research and Education Centre, University of Florida, FL) from a 1-yr-old colony, which was raised on mustard leaves. Eggs were sterilized with 500 ml 0.008 % sodium hypochlorite solution (Clorox, Oakland, CA) for 1 min in a cylindrical container (18 cm diameter by 7.5 cm high). Sterilized eggs were rinsed twice with distilled water and were drained into a nylon strainer. Eggs were inverted into a 177-ml cup under running water until the cup was half filled with water. The remaining half cup was filled with neutralizer (10% sodium

thiosulphate solution) and eggs were soaked in the cup for 2 min. Neutralizer and eggs were drained into a nylon strainer and eggs were rinsed twice with distilled water. After rinsing, eggs were placed on a paper towel in a cylindrical container with plastic screen lid and placed in an incubator at 27 ± 2°C, 70 ± 10% RH, and a photoperiod of 14:10 (L:D) h. Neonates were used for bioassays.

Egg masses of beet armyworm were collected from pepper plants in Citra, FL and the subsequent generations (F3 through F8) were used for bioassays. Eggs were sterilized in the same way as for cabbage looper. Newly emerged larvae were transferred onto pinto bean diet (Guy et al. 1985) in a rectangular container (25 × 25 × 11 cm) with plastic screen lid in an incubator at the same conditions as for cabbage looper. Pupae were placed into paper cups and placed in the incubator. Beet armyworm adults were held in a screen cage (30.5 × 30.5 × 30.5 cm) in the incubator. Two cotton plants with three to four true leaves were used for oviposition and were replaced with fresh ones every other day. Adults were fed a 20% sucrose solution dispensed on a cotton wick. Neonates were used for bioassays.

Neonate Survival and Development to Third Instar

Thirty replicates of each cultivar were set up along greenhouse benches in a randomized complete block design. Experiments on beet armyworm and cabbage looper were done separately under similar greenhouse conditions. Ten neonates were placed in the central whorl of each plant and the plant was covered with a cylindrical screen cage (18.5 cm diameter × 61.0 cm height) to confine the insects for feeding (Fig. 2-1). Plants were dissected 1 wk after infestation to locate the surviving larvae. Larval mortality, weight, instar and feeding behavior were observed and recorded. Instars were determined by measuring head capsule widths (Capinera 2005, 2006). Observations were also made

on the preferred site of feeding on a leaf and within a plant. Larval mortality and weight for each species were analyzed using PROC GLM with cultivar as a fixed effect and replications as random effect (SAS Institute 1999). Tukey's honestly significant difference (HSD) test with a significance level of $\alpha = 0.05$ (SAS Institute 1999) was used for posthoc means separation. Log-likelihood ratio (G^2-test) (Zar 1984) was used to analyze the frequency of surviving instars using JMP release 5.1.2 (JMP Software, SAS Institute Inc., Cary, NC). Differences in the preferred site of feeding within a plant were analyzed by χ^2 goodness of fit tests (Freund and Wilson 1997).

Survival and Development from Neonate to Adult Emergence

Time of development from neonates to adults was investigated on both lettuce cultivars. Thirty replicates of each cultivar were set up along greenhouse benches in a randomized complete block design. Experiments on beet armyworm and cabbage looper were done separately under similar greenhouse conditions. Ten neonates were placed in the central whorl of each plant and the plant was covered with a cylindrical screen cage (Fig. 2-1). Days required to develop from neonate to pupa and from pupa to adult emergence were recorded. Beet armyworm larvae were provided MetroMix 220 in a 5-cm-diameter Petri dish at the base of the plant as a pupation site. Cabbage looper pupated on the plant and on the walls of the container so were not supplied with MetroMix. Pupae were removed from the greenhouse, weighed and put in individual cups in the incubator at 27 ± 2°C, 70 ± 10% RH, and a photoperiod of 14:10 (L:D) h. Emerged adults were sexed, killed and then dried in an oven at 50 ± 5°C, for 3 d. Larval period, pupal fresh weight, pupal period, and dry weight of emerged adults of each insect species were analyzed using PROC GLM with cultivar as a fixed effect and replications as random effect (SAS Institute1999). Tukey's honestly significant difference (HSD) test with a

significance level of α = 0.05 (SAS Institute 1999) was used for posthoc means separation. Percent successful pupation and adult emergence were analyzed by two sample *t*-test using PROC TTEST (SAS Institute1999). A binominal test (using the normal approximation with test statistic *Z*) was used to determine whether the numbers of males versus females deviated from a 1:1 ratio on each cultivar. A Fisher's Exact test of independence was used to test whether the adult sex-ratio differed between the cultivars (Sokal and Rohlf 1995) using JMP release 5.1.2.

Fecundity and Longevity of Subsequent Generation

Fecundity and longevity were measured for nine pairs of newly emerged adults of each species that had been reared on either Tall Guzmaine or Valmaine as larvae. Each pair of adults was confined on a Tall Guzmaine plant using a cylindrical screen cage (18.5 × 61.0 cm) in the greenhouse. Adults were supplied with 20% sucrose solution. Every other day, the lettuce plant was replaced with a fresh plant. Eggs were counted on each plant and totaled over the life of each female. Fecundity and adult longevity of each insect species were analyzed using PROC GLM with cultivar as a main effect (SAS Institute 1999). A simple linear regression analysis was done to study the relationship between adult weight and fecundity using PROC REG (SAS Institute 1999).

Results

Neonate Survival and Development to Third Instar

Larval mortality of cabbage looper and beet armyworm after 1 wk of feeding was significantly higher on Valmaine than on Tall Guzmaine (Fig. 2-2). Cabbage looper mortality was 24 times higher on Valmaine than on Tall Guzmaine ($F = 242.82$; df = 1, 29; $P = 0.0001$) whereas beet armyworm mortality was four times higher on Valmaine than on Tall Guzmaine ($F = 187.54$; df = 1, 29; $P = 0.0001$). Average weight of cabbage

looper feeding for 1 wk on Valmaine (75.4 ± 3.7 mg, mean ± SEM) was significantly lower than that of larvae feeding on Tall Guzmaine (151.2 ± 3.3 mg) ($F = 249.27$; df = 1, 29; $P = 0.0001$). Beet armyworm weight was also significantly lower (1.5 ± 0.1 mg) on Valmaine than on Tall Guzmaine (8.3 ± 0.8 mg) ($F = 68.71$; df= 1, 29; $P = 0.0001$).

The instar of the larvae surviving to plant dissection (1 wk after inoculation as neonates) differed significantly on the two lettuce cultivars for both species (Fig. 2-3). Cabbage looper and beet armyworm developed more slowly on Valmaine than on Tall Guzmaine. More of the surviving neonates of both insect species were in the early instars on Valmaine than on Tall Guzmaine. About 80% of cabbage looper surviving on Valmaine were in either the first or second instar, whereas on Tall Guzmaine about 80% of surviving larvae were in either the third or fourth instar (Fig. 2-3). Of the beet armyworm surviving for 1 wk on Valmaine, 57.7% were in the first instar, whereas 78.8% were in the third instar on Tall Guzmaine (Fig. 2-3).

Larval Feeding Behavior

The insect species behaved differently on the lettuce cultivars. Cabbage looper cut narrow trenches across veins on the leaves and then fed on the area distal to the trench (Fig. 2-4A). This behavior released exudate from the laticifers of the leaves. Beet armyworm did not trench; neonates made shallow scratches between the veins by feeding on parenchymatous tissue and second instars made holes through the leaf (Fig. 2-4B). The preferred site of feeding of cabbage looper ($\chi^2 = 55.42$, df = 2; $P = 0.0001$) and beet armyworm ($\chi^2 = 35.13$, df = 2; $P = 0.0001$) differed between the two cultivars (Fig. 2-5). Cabbage looper preferred to feed on the lowermost fully mature leaves of Valmaine plants and on young and middle-aged leaves of Tall Guzmaine plants (rarely feeding on fully-matured leaves) (Fig. 2-6). Beet armyworm preferred to feed on the lowermost fully

mature leaves of Valmaine plants and on middle-aged leaves of Tall Guzmaine plants. Both insect species preferred to feed on the distal end of leaves. Early instars of cabbage looper preferred to feed on the underside of the leaves, whereas early instars of beet armyworm fed on the upper side of the leaves.

Survival and Development from Neonate to Adult Emergence

Both cabbage looper (F = 82.55; df = 1, 29; P = 0.0001) and beet armyworm (F = 581.58; df = 1, 29; P = 0.0001) took significantly longer time to develop from neonate to pupation on Valmaine than on Tall Guzmaine (Table 2-1). Larval period of cabbage looper and beet armyworm was increased by 2.6 d and 5.9 d, respectively on Valmaine. Feeding on Valmaine resulted in a significant reduction in successful pupation of cabbage looper (t = 9.75; df = 58; P <0.0001) and beet armyworm (t = 13.46; df = 58; P <0.0001) (Table 2-1). Pupae of cabbage looper (F = 41.53; df = 1, 29; P = 0.0001) and beet armyworm (F = 63.84; df = 1, 29; P = 0.0001) weighed significantly less when reared on Valmaine compared to Tall Guzmaine (Table 2-1). The duration of the pupal period of cabbage looper (F = 44.53; df = 1, 29; P = 0.0001) and beet armyworm (F = 30.79; df = 1, 29; P = 0.0001) was significantly increased on Valmaine (Table 2-1), thus delaying adult emergence. Successful emergence of adults from pupae surviving on Valmaine was significantly reduced for cabbage looper (t = 2.40; df = 58; P = 0.0196) but not for beet armyworm (t = 1.40; df = 58; P = 0.1649) (Table 2-1). Adults of cabbage looper (F = 83.02; df = 1, 29; P = 0.0001) and beet armyworm (F = 196.34; df = 1, 29; P = 0.0001) surviving on Valmaine weighed significantly less than those surviving on Tall Guzmaine (Table 2-1). The mean adult sex-ratio of cabbage looper (Z = 0.91, P = 0.3652) and beet armyworm (Z = 0.59, P = 0.5529) did not deviate from a 1:1 ratio on Valmaine. The mean adult sex-ratio of cabbage looper (Z = 1.30, P = 0.1950) and beet armyworm (Z =

1.33, $P = 0.1845$) also did not deviate from a 1:1 ratio on Tall Guzmaine. In addition, the sex-ratios of adult cabbage looper (Fisher's Exact test of independence, $P = 0.1417$) and beet armyworm (Fisher's Exact test of independence, $P = 0.2077$) on the two cultivars did not differ statistically (Table 2-1).

Fecundity and Longevity of Subsequent Generation

Fecundity of cabbage looper ($F = 109.36$; df = 1, 8; $P = 0.0001$) and beet armyworm ($F = 149.14$; df = 1, 8; $P = 0.0001$) on Valmaine was reduced by 62.8 and 67.9%, repectively, compared to that on Tall Guzmaine (Table 2-2). Significant positive linear relationships were found between adult weight and fecundity of both insect species on the two lettuce cultivars (Fig. 2-7). However, neither male nor female longevity of cabbage looper (male: $F = 0.47$; df = 1, 8; $P = 0.5121$; female: $F = 0.47$; df = 1, 8; $P = 0.5121$) nor beet armyworm (male: $F = 0.31$; df = 1, 8; $P = 0.5943$; female: $F = 1.33$; df = 1, 8; $P = 0.2815$) differed on Valmaine or Tall Guzmaine (Table 2-2).

Discussion

Performance of cabbage looper and beet armyworm was greatly reduced on resistant Valmaine compared to Tall Guzmaine. Insects surviving on poor quality hosts are expected to have reduced survival to adult emergence and reduced fecundity (Zalucki et al. 2001), as was shown in my study. Larval survival and development can be reduced on poor quality hosts due to nutritional composition and/or secondary plant metabolites (Scriber and Slansky 1981, Herms and Mattson 1992, Slansky 1992).

Nutritional composition and secondary plant metabolites vary among plants, plant parts and developmental stages (Nelson et al. 1981, Brower et al. 1982). Cabbage looper and beet armyworm larvae preferred to feed on mature leaves of Valmaine (Fig. 2-5). In lettuce, mature leaves are less nutritious than young and middle-aged leaves. Young and

middle-aged lettuce leaves are more metabolically active than mature leaves, and therefore, contain higher amounts of dry matter, ascorbic acid and soluble solids, such as fructose, sucrose, glucose, fructans and other saccharides (McCabe et al. 2001, Siomos et al. 2002). Moreover, mature lettuce leaves always have higher amounts of anti-nutritional constituents, such as nitrates (Siomos et al. 2002). Leaf maturation is accompanied by a decline in the concentrations of proteins and other nutrients (Bernays and Chapman 1994). Therefore, feeding on less nutritious mature leaves of Valmaine may have affected the fitness of cabbage looper and beet armyworm.

Larval avoidance of young and middle-aged leaves of Valmaine may have been due to the presence of high amounts of latex and/or the chemical constituents of latex. Latex from young and middle-aged leaves was pure white and viscous whereas latex from mature leaves was a watery translucent fluid (A. Sethi, pers. obs.). Young leaves of the poinsettia, *Euphorbia pulcherrima* Wilenow contained higher amounts of latex and laticifer starch than mature leaves (Spilatro and Mahlberg 1986). The proportionally higher latex amount may have a specific purpose related to plant defense. The defensive role of latex has been attributed to its sticky nature, which would enable the plant to capture small insects and immobilize the mouthparts of larger insects (Farrell et al. 1991, Dussourd 1993, Dussourd and Denno 1991, 1994). Antiherbivore function of latex has been suggested in many plant systems (Shukla and Krishna-Murti 1971, Fahn 1979, Konno et al. 2004, 2006). The presence of high amounts of latex with its chemical components in young leaves (Kinghorn and Evans 1975, Swain 1977, Rees and Harborne 1985) may provide for their defense compared to mature leaves. In the chicory plant, *Cichorium intybus* L., sesquiterpene lactones were present in the highest amounts in the

most actively growing regions of the plant and possessed antifeedant properties against *Schistocerca gregaria* (Orthoptera: Acrididae) (Rees and Harborne 1985). Various organic compounds, like phenolics and terpenoids have been reported in latex of *Lactuca* spp. (Crosby 1963, Gonzales 1977, Cole 1984, Sessa et al. 2000) and their defensive role as phytoalexins has been reported against plant diseases (Bennett et al. 1994, Bestwick et al. 1995). In lettuce, the density of latex is successively decreased from the base to the apex of the leaf (Small 1916). This may account for the preference of neonate caterpillars in my study to feed on the distal end (apex) of leaves.

Certain plant enzymes, such as phenylalanine ammonia lyase, polyphenol oxidase and peroxidase are known for their defensive role against insects. In lettuce, Campos-Vergas and Saltveit (2002) reported enhanced activity of phenylalanine ammonia lyase upon mechanical wounding in young leaves compared to mature leaves. Phenylalanine ammonia lyase is also more active in aphid-resistant cultivars of *L. sativa* than in susceptible cultivars (Cole 1984).

Outer (i.e., older) leaves of head lettuce exhibit high concentrations of flavonoids, such as quercetin (Hohl et al. 2001). Quercetin and its derivatives are known to act as phagostimulants to many lepidopterans (Simmonds 2003). Therefore, the feeding location of beet armyworm and cabbage looper may have been the end result of both antifeedant properties (either physical or chemical or both) of young and middle-aged leaves and phagostimulant properties of mature leaves.

Cabbage looper exhibited greater fitness than beet armyworm on Valmaine. Larval mortality of cabbage looper was less compared to beet armyworm on Valmaine and other parameters, such as larval weight, pupal weight, percent pupation and adult weight of

cabbage looper were less affected compared to beet armyworm on Valmaine (Table 2-1). Moreover, larval development of cabbage looper was faster than beet armyworm, as cabbage looper larvae entered the fourth larval after 1 wk on Valmaine while beet armyworm larvae were still in the third instar stage (Fig. 2-2). Survival and development of yellow-striped armyworm was also affected greatly on *L. serriola* compared to cabbage looper (Dussourd 1993). This superior performance of cabbage looper may be attributed to their feeding behavior (i.e., trenching on laticiferous plants). Trenching blocks latex flow to intended feeding sites and may act as a counter-adaptation to the plant's defensive secretions (Dussourd and Denno 1994). In spite of their behavioral counter-adaptation, cabbage looper performance was worse on Valmaine than on Tall Guzmaine.

Lettuce possesses little tolerance for cosmetic damage and foliar feeding by lepidopterous pests greatly affects its marketable production. The consumer, especially in developed countries, will not accept produce unless it is free of all insects and blemishes at harvest. Further, lettuce is a short-season crop and insufficient time may be present between treatment of chemical and harvest for pesticide residues to decline to acceptable levels (Norris et al. 2003). This limits the use of chemicals in lettuce production that do not break down rapidly. Therefore, host plant resistance is an economically, ecologically and environmentally advantageous method of insect management. The results of my study have confirmed that Valmaine expresses considerable resistance to lepidopterous pests in spite of their counter-strategies against plant resistance. In general, multiple-insect resistance is thought to be more desirable than single-insect resistance (Smith 1989). Feeding on Valmaine resulted in reduced vigor of both insect species, which

ultimately could make them more susceptible to other biotic and abiotic factors. However, additional research is required to determine the biochemical basis of multiple-insect resistance in lettuce. Understanding the mechanism of resistance will certainly aid in the development of lettuce cultivars with improved pest resistance and may result in reduced pesticide usage.

Table 2-1. Performance of cabbage looper and beet armyworm released as neonates onto Valmaine and Tall Guzmaine lettuce.

Species	Cultivar	Larval period (days)	% Pupation	Pupal weight (mg)	Pupal period (days)	% Adult emergence	Adult weight (mg)	Sex-ratio (male: female)
Cabbage looper	Valmaine	11.7 ± 0.2a	49.3 ± 2.0b	172.2 ± 4.5b	8.7 ± 0.2a	82.4 ± 2.5a	23.4 ± 0.6b	1.18 : 1a
	Tall Guzmaine	9.1 ± 0.3b	79.0 ± 2.3a	206.5 ± 2.8a	7.8 ± 0.1b	90.7 ± 1.3a	29.7 ± 0.6a	1 : 1.20a
Beet armyworm	Valmaine	19.3 ± 0.3a	27.3 ± 2.6b	51.4 ± 1.4b	7.5 ± 0.1a	86.6 ± 2.7a	9.1 ± 0.3 b	1.20 : 1a
	Tall Guzmaine	13.4 ± 0.1b	65.3 ± 3.8a	68.9 ± 1.3a	6.7 ± 0.1b	93.9 ± 2.0a	14.9 ± 0.3a	1 : 1.22a

Means ± SEM followed by different letters for each parameter within insect species differed significantly ($P \leq 0.05$) using ANOVA and Tukey's HSD test for larval period, pupal weight, pupal period and adult weight, two sample t-test for % pupation and % adult emergence, and Fisher's Exact test of independence for sex-ratio

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Table 2-2. Fecundity and longevity of subsequent generation of cabbage looper and beet armyworm reared on Valmaine and Tall Guzmaine lettuce.

Species	Cultivar	Fecundity	Male longevity (d)	Female longevity (d)
Cabbage looper	Valmaine	146.4 ± 8.4a	11.8 ± 0.2a	10.1 ± 0.2a
	Tall Guzmaine	393.3 ± 18.1b	12.0 ± 0.3a	10.3 ± 0.3a
Beet armyworm	Valmaine	123.2 ± 10.3a	6.4 ± 0.2a	8.1 ± 0.2a
	Tall Guzmaine	383.6 ± 17.7b	6.3 ± 0.2a	8.4 ± 0.2a

Means ± SEM followed by different letters for each parameter within insect species differed significantly ($P \leq 0.05$) using ANOVA and Tukey's HSD test.

Figure 2-1. Experimental setup to study cabbage looper and beet armyworm neonate survival and development to third instar.

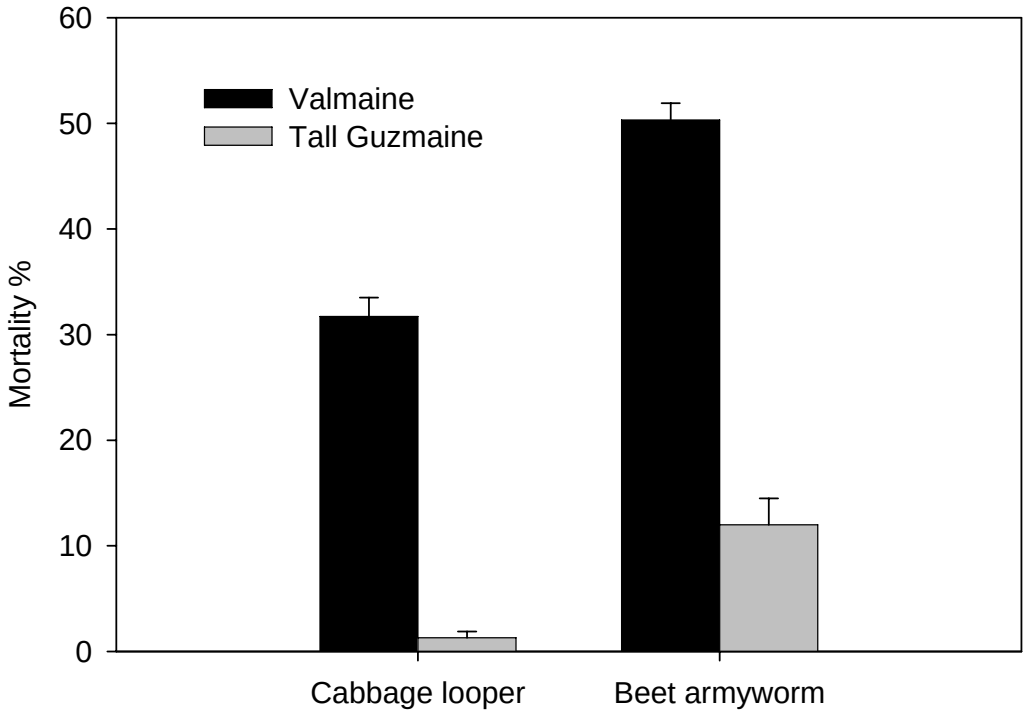


Figure 2-2. Larval mortality of cabbage looper and beet armyworm after 1 wk of feeding on resistant Valmaine and susceptible Tall Guzmaine lettuce. Error bars indicate 1 SEM

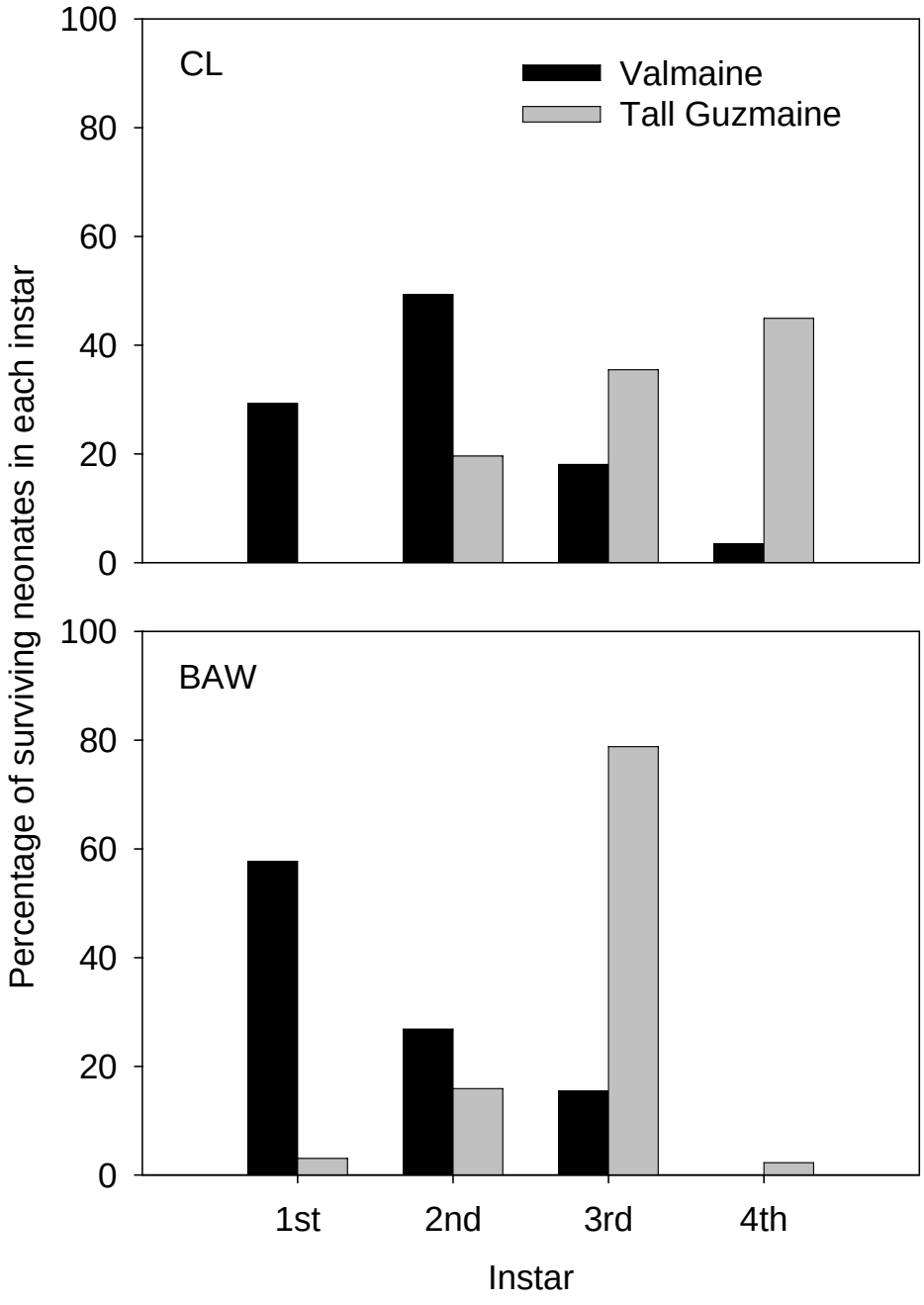


Figure 2-3. Instars of cabbage looper (CL) and beet armyworm (BAW) surviving for 1 wk on resistant Valmaine and susceptible Tall Guzmaine lettuce. G^2 tests indicated that the instar distribution on Valmaine differed significantly from that on Tall Guzmaine ($P < 0.05$).

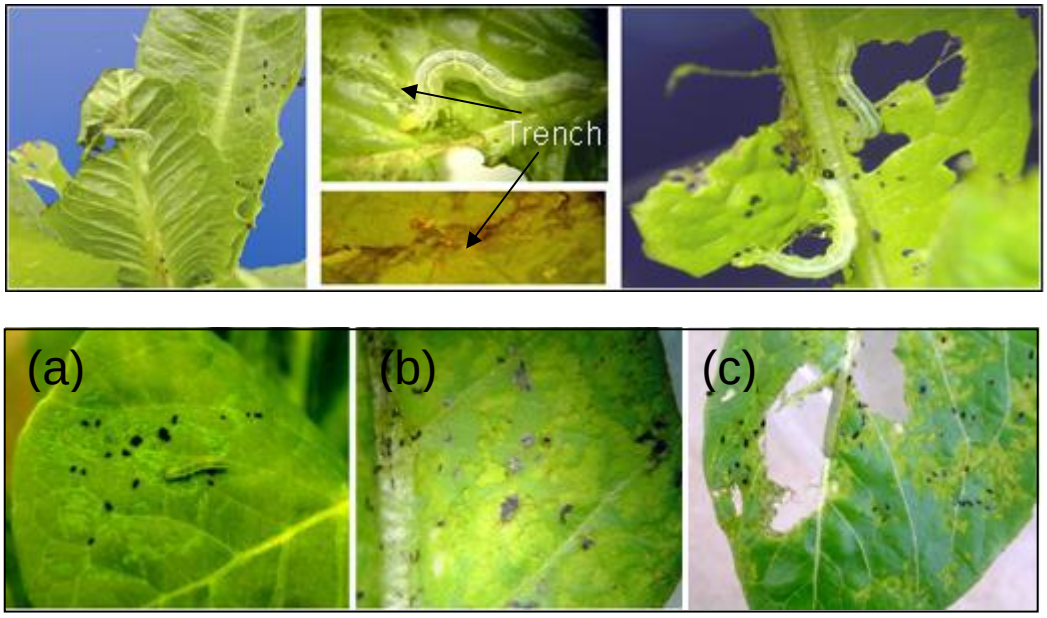


Figure 2-4. Feeding of two lepidopterans on lettuce. A) Cabbage looper cutting narrow trenches on romaine lettuce, B) Beet armyworm damage on romaine lettuce: (a) & (b) shallow scratches, (c) holes.

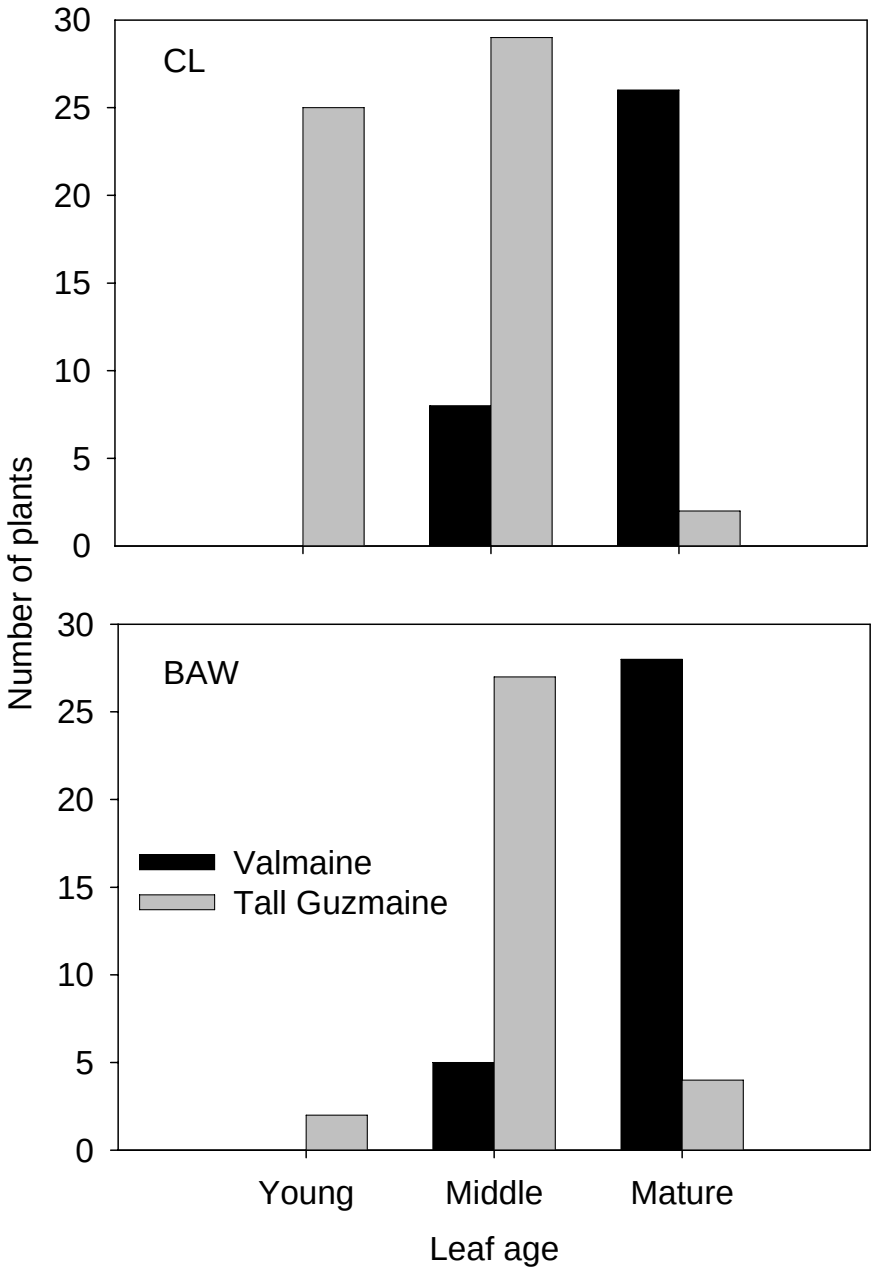


Figure 2-5. Feeding preference of cabbage looper (CL) and beet armyworm (BAW) larvae among lettuce leaves of different ages on resistant Valmaine and susceptible Tall Guzmaine. The y-axis depicts the total number of plants (out of 30) on which at least some feeding occurred on leaves of the specified age group.

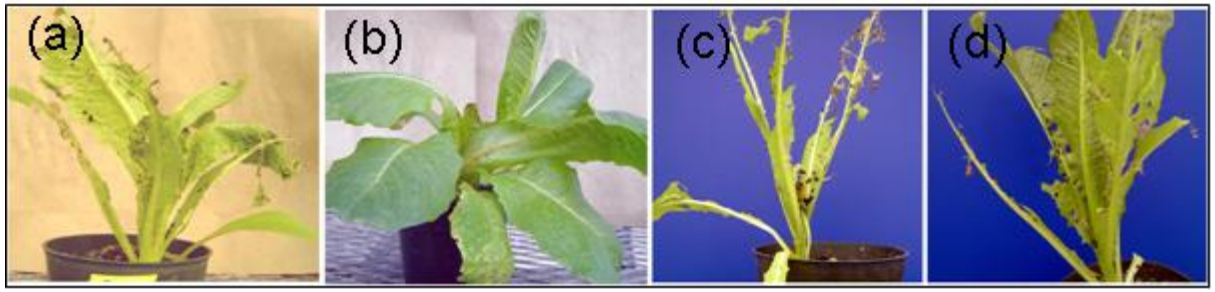


Figure 2-6. Feeding behavior of beet armyworm on (a) Tall Guzmaine and (b) Valmaine, and of cabbage looper on (c) Tall Guzmaine and (d) Valmaine.

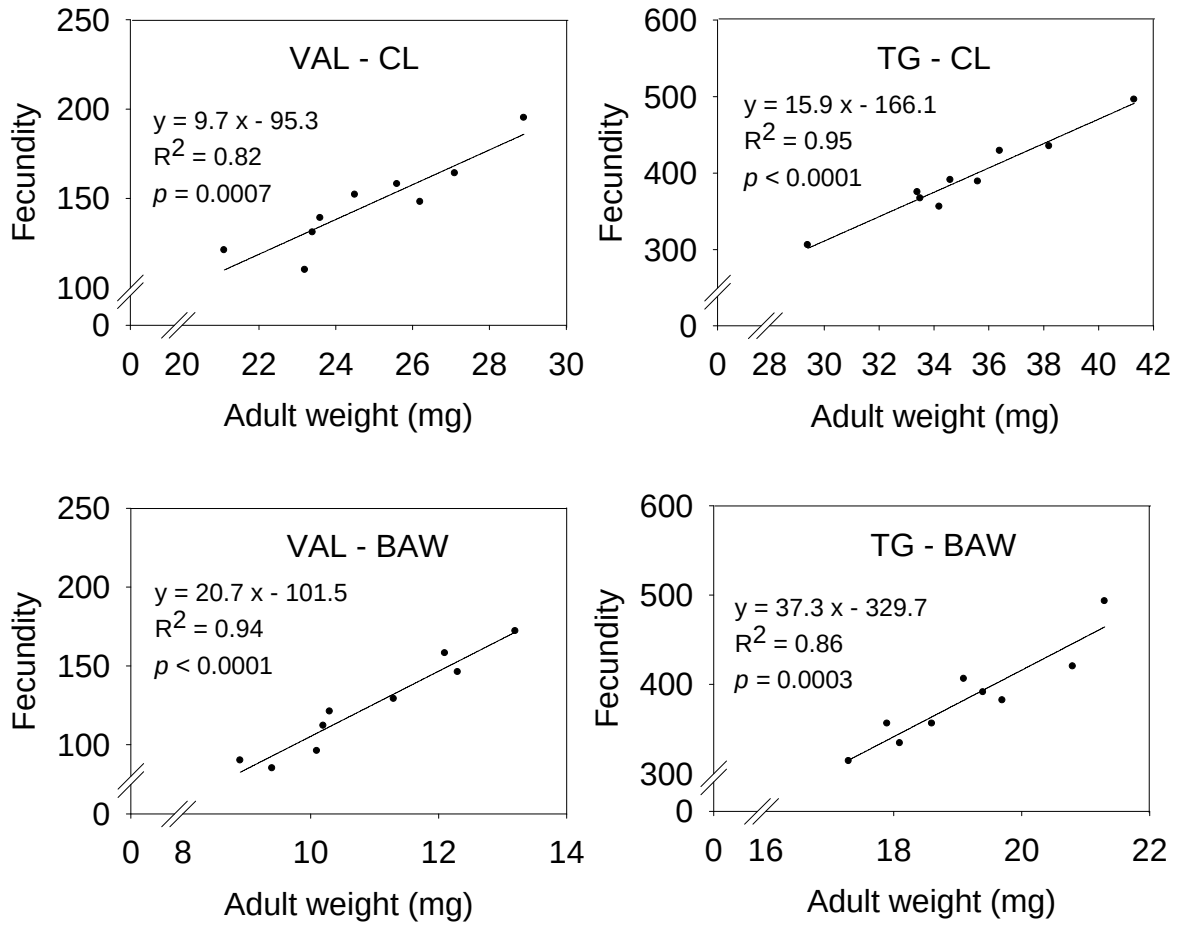


Figure 2-7. Relationships between adult weight and fecundity of cabbage looper (CL) and beet armyworm (BAW) that developed from larvae reared on resistant Valmaine (VAL) or susceptible Tall Guzmaine (TG) lettuce.

CHAPTER 3
ROMAINE LETTUCE LATEX DETERS FEEDING OF BANDED CUCUMBER BEETLE (COLEOPTERA: CHRYSOMELIDAE)

Introduction

Latex is the common term used to describe a frequently milky plant exudate which is typically stored under positive pressure within specialized vessels called laticifers (Fig. 3-1). These laticifers accompany the vascular bundles and ramify into the mesophyll to reach the epidermis (Hayward 1938, Esau 1965, Metcalfe 1967, Olson et al. 1969, Metcalfe and Chalk 1983, Fahn 1990, Kekwick 2001). About 12,500 to 20,000 plant species, belonging to >900 genera from about 40 families, most of which are dicotyledons, are known to exude latex (Esau 1965, Metcalf 1967, Lewinsohn 1991, Kekwick 2001, Evert 2006). Latex contributes to plant defense in two different ways; physical properties (stickiness) and chemical properties (toxic constituents). Stickiness can result in the entrapment or gumming up of the mouthparts of herbivorous insects (Dillon et al. 1983, Dussourd 1993, 1995, Zalucki and Malcolm 1999). Latex contains toxic constituents including alkaloids (Roberts 1987, Valle et al. 1987, Konno et al. 2006), cardiac glycosides (Zalucki and Brower 1992, Zalucki and Malcolm 1999), and terpenoids (Evans and Schmidt 1976, Rees and Harborne 1985, Spilatro and Mahlberg 1986). Some insects circumvent the mechanical stickiness and toxic effects of latex by severing latex-bearing veins or by cutting trenches prior to consuming the distal tissue (Dussourd 1993, Zalucki and Malcolm 1999, Sethi et al. 2006).

Lettuce, *Lactuca sativa* L., is one of the most important vegetable crops grown throughout the world and its production grows annually (USDA 2005a). As a cultivated crop, lettuce is vulnerable to attack by various insect pests including the banded cucumber beetle, *Diabrotica balteata* LeConte (Nuessly and Nagata 1993). This insect

has a host range of >50 plant species in 23 families (Saba 1970) and a high reproductive potential of >800 eggs per female with a 2 to 8 wk oviposition period (Pitre and Kantack 1962). It can be found throughout the year in the southern United States (Schalk 1986). In southern Florida, foliar feeding by *D. balteata* adults leads to economic damage in lettuce due to reduction in stand and marketability, decreased photosynthetic area, frass contamination of the heads, and increased vulnerability to diseases. Chemical control of soil-borne eggs, larvae and pupae of this insect has been ineffective (Schalk et al. 1986) and control of the adult is the sole promising option (Schalk et al. 1990). As a result, growers currently are dependent on pesticides (Nuessly and Nagata 1993) which can pose a threat to the environment, farm workers and natural enemies of insect pests, and increase production costs.

Host plant resistance was explored as an alternative strategy for the management of this economic insect pest in a cos or romaine lettuce cultivar, 'Valmaine' (Nuessly and Nagata 1994, Huang et al. 2002, Sethi et al. 2006). A high level of resistance was reported in Valmaine, compared to the closely related susceptible cultivar 'Tall Guzmaine' against serpentine leafminer, *Liriomyza trifolii* (Burgess) (Nuessly and Nagata 1994), banded cucumber beetle (Huang et al. 2002) and two lepidopterans, *Trichoplusia ni* (Hübner) and *Spodoptera exigua* (Hübner) (Sethi et al. 2006). These studies suggested that Valmaine lacks feeding stimulants or contains feeding deterrents, either in the leaf cuticle or the leaf interior. Huang et al. (2003a) reported that leaf surface chemicals were not responsible for resistance in Valmaine and suggested chemicals inside the leaf may play a role. However, incorporation of freeze-dried leaves of Valmaine into an artificial diet did not deter feeding by *D. balteata* adults and neither did

application of Valmaine latex on the leaf surface of a favorite food, lima bean (Huang et al. 2003b). It is possible that the activity of physical and/or chemical defenses in latex or leaf tissue may have been reduced or eliminated when whole leaves were dried and powdered. Furthermore, the physical and chemical properties of latex may have changed when applied on lima bean leaves due to drying of the latex and/or oxidation of chemical constituents.

In free-choice situations *L. trifolii* (Nuessly and Nagata 1994), *D. balteata* (Huang et al. 2002), *T. ni* and *S. exigua* (Sethi et al. 2006) preferred to feed on mature leaves of Valmaine over young or middle-aged leaves. The avoidance of young and middle-aged leaves of Valmaine may have been due to the presence of high amounts of latex and/or the chemical constituents of latex. The latex from young and middle-aged leaves is pure white and viscous, whereas latex from mature leaves is watery and translucent (Sethi et al. 2006).

In this study, I report on the possible deterrent role of latex against beetle feeding on artificial diet treated with freshly extracted latex from either Valmaine or Tall Guzmaine in choice and no-choice conditions. Additional tests were conducted using latex extracted from young versus mature leaves of these two cultivars to study the role of leaf age in the expression of latex deterrence. Lastly, samples of supernatant material collected following dissolution of latex from both cultivars in water/methanol combinations or methylene chloride and centrifugation were applied to diet disks under no-choice situations to determine whether differences in latex chemistry between Valmaine and Tall Guzmaine contribute to the multiple insect resistance observed on Valmaine.

Materials and Methods

Plants and Insects

Valmaine and Tall Guzmaine seeds were germinated by placing them overnight in a Petri dish lined with a wet filter paper in the laboratory. Germinated seeds were planted in a transplant tray filled with commercial soil mix (MetroMix 220, Grace Sierra, Milpitas, CA) in a greenhouse with natural light at a mean temperature of 27ºC (32 to 24 ºC) and 68% mean R.H. (44 to 94%). After 2 wk, seedlings were transplanted into 15-cm-diameter plastic pots filled with MetroMix 220. Plants were irrigated daily and fertilized once a week with 10 ml of a 10 g/l solution of soluble fertilizer (Peters 20-20-20, N-P-K, W.R. Grace, Fogelsville, PA).

Bush lima bean (*Phaseolus lunatus* L.) cultivar Fordhook 242 (Illinois Foundation Seeds, Champagne, IL) was grown as an adult food source for the colony. Seeds were planted in a transplant tray filled with MetroMix 200. Lima bean plants were irrigated daily and fertilized once a week after the first true-leaf stage with the same solution used for lettuce plants.

Adults of *D. balteata* were used because previously the same insect species was used by Huang et al. (2003b) as explained above. In addition, R. T. Nagata (Everglades Research and Education Center, University of Florida, FL) used the same insect species to track resistance in lettuce breeding lines. Further, adults of *D. balteata* were easy to rear and handle during bioassays. A colony of *D. balteata* was established in 2003 from a wild population of adults collected from spiny amaranth, *Amaranthus spinosus* L. and primrose willow, *Ludwigia peruviana* L. in Belle Glade, FL. The colony was supplemented with wild individuals to increase genetic diversity in 2005 and 2006. Adults of *D. balteata* were fed on lima bean leaves and sweet potato tubers, and larvae

were reared on corn seedling roots (H93 × FB37, Illinois Foundation Seeds Inc., IL) as per Huang et al. (2002) (Fig. 3-2).

Adults of the *D. balteata* colony were confined in a ventilated plexiglas cage (30.5 × 30.5 × 30.5 cm) in an incubator at 27 ± 2 ºC and R.H. 70 ± 10% with a photoperiod of 14:10 (L:D) h (Fig. 3-2A). Oviposition was facilitated by providing two domed-shaped plastic containers (8.4 cm diameter × 7 cm high) with mesh-covered lids (0.1 × 0.2 cm). These plastic containers were covered with inverted strawberry baskets upon which lima bean leaves were placed (Fig. 3-2B). The plastic containers were filled with upright small glass vials (20 ml) to hold tightly one moist layer of cotton balls. Two circular pads, each containing four layers of premium paper towels (Kimberly-Clark Co., Roswell, GA), and four layers of cheesecloth were placed between the layer of cotton balls and the meshed lid.

The cheescloth and paper towel pads with eggs were collected every 2 d and kept in a Petri dish with a screened lid in the same incubator. Three-day-old eggs were dipped in sodium hypochloride solution (15 ml Clorox in 485 ml water, The Clorox Co., CA) for 1 min and then rinsed thrice with deionized water to affect surface sterilization. The sterilized eggs were replaced in the incubator and covered with a wet paper towel within a cylindrical container (18 cm diameter × 7.5 cm high) with a screened lid (Fig. 3-2C). On the following day, 9 to 10 germinated corn seeds were put in the container as food for the emerging larvae.

Larval *D. balteata* were raised on germinated corn seeds in containers designed to maintain sufficient moisture for seed growth without drowing the larvae or covering them with soil. A preassembled germination paper with a wick stapled on each end was placed

at the botton of a rectangular plastic container (32.5 × 17.2 × 10 cm) and covered with a single layer of pregerminated corn seeds. The seeds were covered with wet paper towel and the container was placed on the top of a water-filled tray in such a way that the two wicks were suspended in the water. Pregerminated corn seeds were prepared by soaking dried seeds overnight in a Clorox soloution (16 ml/L of water). They were rinsed with deionized water the following morning and stored in a refrigerator until needed. These larval rearing containers were covered with a screened lid. One-day-old emerged larvae on germinated corn seeds were next transferred to the rectangular rearing containers stocked with 3-d-old germinated corn seedlings and kept at 27 ± 2 ºC with a photoperiod of 14:10 (L:D) h in a rearing room (Fig. 3-2D, E). After 1 wk of larval rearing in these containers, the larvae were transferred to a second container with germinated corn to complete their larval development (Fig. 3-2F, G, H).

Two days after putting larvae in the second container, third instar larvae were collected into a container (18 cm × 7.5 cm high) filled with moistened and autoclaved MetroMix 220 (Fig. 3-2I) to allow for pupation and adult emergence. The container was covered with a dampened towel to retain moisture. After 10 d, the emerged adults were transferred into the screen cage mentioned above (Fig. 3-2J).

Artificial Diet Preparation

Dry mix for artificial diet is commercially available and has been shown to support the adult stage of *D. balteata* (Creighton and Cuthbert 1968). All materials required for preparing and dispensing the diet were thoroughly sanitized with sodium hypochlorite solution (Clorox, Oakland, CA) diluted 1:5 with deionized water. A 100-ml quantity of southern corn rootworm artificial diet (Bio-Serv, Frenchtown, NJ) (Creighton and Cuthbert 1968) was prepared as follows. Sterile deionized water (100 ml) and agar (1.74

g) were heated on a hot plate to boiling. Once the agar had cooled to approximately 40 ºC, KOH solution (1 ml) and diet dry mix (14.91 g) were added to it and thoroughly mixed to avoid the formation of lumps. The liquid diet was dispensed into two glass Petri dishes (9 cm diameter). The diet was allowed to cool before the Petri dishes were covered with glass lids. The Petri dishes were wrapped completely in plastic wrap and aluminum foil, and stored in a refrigerator (4-6 ºC) for up to 3 h.

Latex Collection and Solvent Extraction

Latex (70 µl) was collected from the bases (where leaf lamina joins the stem) of young and middle-aged leaves of individual plants, sites where there was a rapid exudation of latex upon cutting (Fig. 3-3). The cuts were made using a disposable scalpel blade (Feather, Osaka, Japan). The latex was collected using a silanized 100-µl glass capillary tube inserted into a microdispenser (Drummond Scientific Company, Broomall, PA) 60 s after the leaf base was cut.

In a pilot study, I incorporated fresh latex into the artificial diet for *D. balteata* adults at two concentrations (0.1 and 0.2%) and recorded diet consumption by *D. balteata* adults to investigate the potential of Valmaine latex as a mechanism of multiple insect resistance. Latex did not deter feeding of *D. balteata* adults when presented in this manner. Therefore, in this study, I applied freshly extracted latex from either Valmaine or Tall Guzmaine to artificial diet and confined *D. balteata* adults under choice and no-choice conditions to investigate the possible deterrent role of latex against beetle feeding. A 1.5-cm-diameter cork borer was used to punch out disks (1 cm thick) from cooled artificial diet. Latex (70 µl) from an individual plant was applied, immediately after collection, onto the top surface and sides of a diet disk using a microdispenser.

I chose artificial diet as a substrate for application of the latex because it kept latex moist for a longer time by providing more moisture compared to lima bean leaves. In addition, latex treated diet disks facilitated the direct exposure of *D. balteata* adults to latex. As the diet disks were totally covered with latex on all sides, it somewhat simulated the natural situation where an insect gnawing a lettuce plant is directly exposed to latex.

Four different solvent combinations, i.e., water, water:methanol (20:80), water:methanol (50:50), and methylene chloride were used to extract chemical constituents of latex (Fig. 3-5A). Latex (70 µl) was collected from an individual plant in the same way as explained above and immediately dissolved in 10 times volume of the solvent (Fig. 3-4). After dissolution, samples were centrifuged at 1200 ×g for 20 min and supernatant was collected (Fig. 3-5B, C). The supernatant was reduced down to 1/10 volume by evaporating with nitrogen gas. An amount of extract, equivalent to 70 µl latex, was applied to each diet disk for use in the following bioassays (Fig. 3-4).

Bioassay Conditions

For all experiments described below, an experimental unit consisted of two diet disks and three pairs of unfed *D. balteata* adults within a plastic ventilated container (10 × 10 × 8 cm). Unfed adults that had emerged within 48 h of the start of the experiment were used in all tests. The diet disks were placed on the bottom of the container and beetles were allowed to feed on the diet for 16 h. Each experimental unit was replicated 15 times. The experiments were carried out at 25 ± 1ºC in a laboratory under a photoperiod of 14:10 (L:D) h. In all tests, the number of adults feeding on each diet disk was recorded 15, 30, 60 and 90 min after their release into the bioassay chambers.

Choice Tests and No-choice Tests with Fresh Latex

Choice tests were conducted to determine whether *D. balteata* adults showed a preference between diet disks treated with latex from Valmaine or Tall Guzmaine. Three treatment combinations were studied: latex from Valmaine versus latex from Tall Guzmaine, latex from Valmaine versus control (untreated diet without latex), and latex from Tall Guzmaine versus control (Fig. 3-6A). Three treatments (latex from Valmaine, latex from Tall Guzmaine, and control) also were studied in a no-choice situation, with each experimental unit containing two disks of the same treatment (Fig. 3-6B).

Dry weight of diet consumed in a 16-h period was calculated for comparison among the treatments. To determine dry weight, an additional 10 diet disks from each treatment (Valmaine latex-treated, Tall Guzmaine latex-treated and control) were weighed individually (disk fresh weight) before they were put into an oven at 50 ± 5ºC. After 3 d, these diet disks were reweighed individually (disk dry weight). A dry/fresh weight ratio was calculated for each diet disk and averaged over the 10 disks. The diet fresh weight was determined for each disk for each treatment prior to the start of each experiment. After 16 h of exposure to beetle feeding, the diet disk was dried in the oven for 3 d as above, reweighed and then multiplied by the corresponding average dry/fresh weight ratio. The dry weight of diet consumed was calculated as the difference between initial and final dry weights.

Choice Tests Using Latex from Young and Mature Leaves

Choice tests were conducted to determine whether *D. balteata* adults exhibit any preference between diet disks treated with latex from young or mature leaves of either Valmaine and Tall Guzmaine. Two treatment combinations were studied: latex from young leaves versus latex from mature leaves of Valmaine and latex from young leaves

versus latex from mature leaves of Tall Guzmaine. The dry weight consumption of young and mature latex-treated diets of each cultivar in 16 h was recorded as described above. Total diet consumed per three pairs of adults for 16 h was calculated by adding consumption of the two diet disks in each container in each treatment.

No-Choice Tests Using Latex Extracts

Fifteen treatments were studied: five for Valmaine - latex dissolved in water, water:methanol (20:80, % v/v), water:methanol (50:50), methylene chloride, and fresh latex without solvent; five for Tall Guzmaine - latex dissolved in water, water:methanol (20:80), water:methanol (50:50), methylene chloride, and fresh latex without solvent; and five for control - the four solvent combinations without latex and untreated diet. Each experimental unit contained two disks of the same treatment. The dry weights of Valmaine- and Tall Guzmaine-extract treated and control diets disks consumed in 16 h were calculated as above.

Beetle Behavior in Response to Contacting Latex

Observations were made on beetle behavior in response to contacting latex and latex extracts on diet disks in the choice and no-choice tests described above. In addition, freshly collected latex (1 µl) from both Valmaine and Tall Guzmaine plants was applied to mouthparts of beetles (10 for each) using a microdispenser. Using a microscope, salivation by treated beetles was observed immediately after latex application and mobility of mouthparts was observed after 24 h to distinguish if toxic constituents or stickiness contributed to the feeding deterrence of Valmaine lettuce. Individual beetles also were confined on young leaves of either Valmaine or Tall Guzmaine (10 plants for each cultivar) and observed for 90 min using a microscope to closely observe their feeding behavior in response to contacting latex during test bites.

Statistical Analysis

For all choice and no-choice tests using latex, the number of adults feeding on diet 15, 30, 60 and 90 min after release was analyzed as a repeated measures design using Proc GLIMMIX (SAS Institute 2003). In each choice test (Valmaine versus Tall Guzmaine, Valmaine versus control, and Tall Guzmaine versus control), data on number of adults feeding were analyzed as a 2 × 4 factorial design separately, in which latex was treated as one factor with two levels, and time interval after beetle release was treated as the other factor with four levels (15, 30, 60 and 90 min). In no-choice tests, data on number of adults feeding were analyzed as a 3 × 4 factorial design, in which latex was treated as on factor with three levels (Valmaine, Tall Guzmaine and control), and time interval after beetle release was treated as the other factor with four levels. Both variables (latex and time interval) were fixed. Fifteen groups of six beetles (i.e., replications) were randomly assigned to each level of latex, meaning that beetles were nested within latex levels. Each group of six beetles was tested four times (levels of time interval). The model was number of beetles feeding = [latex] [replications(latex)] [time interval] [latex*time interval]. The error degree of freedom for latex effect was calculated as levels of latex(replications – 1). The error degree of freedom for time interval effect and interaction was calculated as levels of latex(levels of time interval – 1) (replications – 1).

In no-choice tests using latex extracts, the data on number of beetles feeding was analyzed using Proc GLM (SAS Institute 2003) separately at each time interval with latex extract as a fixed effect and replications as a random effect. The error degree of freedom for latex extract effect was calculated as (levels of latex extract -1)(replications -1).

The dry weights of Valmaine, Tall Guzmaine and control diets consumed in 16 h were analyzed by paired *t*-tests using Proc MEANS (SAS Institute 2003) for all choice

tests and by ANOVA using Proc GLM with latex as a fixed effect and replications as a random effect (SAS Institute 2003) for all no-choice tests. The total dry weight consumed by adding consumption of the two diet disks in each chocie test using latex from young and mature leaves including control disks was also analyszed by ANOVA using Proc GLM with latex as a fixed effect and replications as a random effect (SAS Institute 2003). The error degree of freedom for latex/latex extract effect was calculated as (levels of latex/ latex extract -1)(replications -1). Tukey's honestly significant difference (HSD) test with a significance level of $\alpha = 0.05$ (SAS Institute 2003) was used for post hoc means separation.

Deterrence coefficients (relative and absolute) were calculated (Nawrot et al. 1986) based on the amount of diet consumed. All the data from both choice and no-choice tests were pooled and used to determine coefficients using the following equations:

$$\text{Relative (R)} = [(C - T) / (C + T)] \times 100 \qquad \text{(Choice Test)}$$

$$\text{Absolute (A)} = [(CC - TT) / (CC + TT)] \times 100 \qquad \text{(No-choice Test)}$$

where C and CC are the consumption of control diet (without latex) in choice and no-choice tests, respectively; and T and TT are the consumption of latex-treated diet (Valmaine or Tall Guzmaine) in choice and no-choice tests, respectively. The deterrent activity of the latex-treated diets was expressed by the total coefficient of deterrence (D = A + R). The deterrence coefficients were analyzed by two-sample *t*-tests using PROC TTEST (SAS Institute 2003).

Results

Latex Choice and No-Choice Tests

Treatment of latex had significant effect on the number of insects feeding in all three choice tests, Valmaine (Val) versus Tall Guzmaine (TG) ($F = 64.83$; df = 1, 28; $P = 0.0001$), Valmaine versus control ($F = 99.27$; df = 1, 28; $P = 0.0001$), and Tall Guzmaine versus control ($F = 5.68$; df = 1, 28; $P = 0.0241$). Beetles avoided feeding on diet treated with Valmaine latex (Fig. 3-6A; 3-7A, B). The number of insects feeding on diet treated with Valmaine latex was negligible compared to the number feeding on diet treated with Tall Guzmaine latex (Fig. 3-7A) and control diet (Fig. 3-7B). The number of insects feeding increased over time (i.e., 15, 30, 60 and 90 min) in all choice tests (Val vs. TG: $F = 7.28$; df = 3, 84: $P = 0.0002$, TG vs. control $F = 9.83$; df = 3, 84: $P = 0.0001$, Val vs.. control: $F = 24.87$; df = 3, 84: $P = 0.0002$) (Fig. 3-7A, C). Significant interactions were found between latex treatment and time interval in the choice tests involving Valmaine and Tall Guzmaine ($F = 8.56$; df = 3, 84; $P = 0.0001$) and Valmaine and the control diet ($F = 28.47$; df = 3, 84; $P = 0.0001$). In contrast, there was no significant interaction found in the choice test between Tall Guzmaine latex-treated diet disks and control disks ($F = 1.44$; df = 3, 84; $P = 0.2374$) (Fig. 3-7C). Beetles consumed significantly less diet treated with Valmaine latex (Table 3-1). Beetles ate 2.9 times more on Tall Guzmaine latex treated diet than diet treated with Valmaine latex in a choice between Valmaine and Tall Guzmaine. Beetles also consumed 4.5 times more control diet than diet treated with Valmaine latex in a choice between Valmaine and control. Beetles also consumed 1.5 times less diet treated with Tall Guzmaine than control diet.

In no-choice tests, latex also had significant on the number of insects feeding on diets ($F = 109.46$; df = 2, 42; $P = 0.0001$). Significantly fewer insects fed on diet treated

with Valmaine latex than on Tall Guzmaine latex-treated and control diets (Figs. 3-6B, 3-8). No significant interaction was found between latex treatment and time interval (F = 1.74; df = 3, 126; P = 0.1179). Beetles consumed 4.7 and 6.3 times more Tall Guzmaine latex-treated and control diets, respectively, than Valmaine latex-treated disks (F = 168.31; df = 2, 42; P = 0.0001) (Table 3-1).

Valmaine latex exhibited strong deterrence against beetles in both choice and no-choice bioassays (Table 3-2). Both relative and absolute coefficients of deterrence for Valmaine latex-treated diets were significantly higher than those for Tall Guzmaine latex-treated diets. The total coefficient of deterrence of Valmaine latex was 3.9 times higher than that of Tall Guzmaine latex.

Choice Tests Using Latex from Young and Mature Leaves

In Valmaine choice test, latex significantly affected the number of insects feeding on diet (F = 61.87; df = 1, 28; P = 0.0001), but it was not significantly affected by latex treatment in Tall Guzmaine choice tests (F = 1.84; df = 1, 28; P = 0.812). Significantly fewer insects fed on diet treated with latex from young leaves than on diet treated with latex from mature leaves of Valmaine (Figs. 3-9, 3-10A). Adult preference for diet treated with latex from mature leaves of Valmaine increased significantly with time (F = 30.95; df = 3, 84; P = 0.0001) (Fig. 3-10A). In the Tall Guzmaine latex choice test, the number of beetles feeding on diet disks treated with latex from young leaves did not differ significantly from that on disks treated with latex from mature leaves (Figs. 3-9, 3-10B). The number of beetles feeding on both Tall Guzmaine diets increased significantly with time (F = 39.44; df = 3, 84; P = 0.0001) (Fig. 3-10B).

Beetles consumed 7.2 times more diet treated with latex from mature Valmaine leaves than treated with latex from young Valmaine leaves (Table 3-3). Diet consumption

did not differ significantly between diet disks treated with latex from young and mature leaves of Tall Guzmaine. The total diet consumed in the Valmaine latex choice test (sum of the consumption on the two disks) did not differ significantly from the amount eaten in the Tall Guzmaine latex choice test but was significantly less than the amount consumed in the control diet test.

No-Choice Tests Using Latex Extracts

Water extracts of both Valmaine and Tall Guzmaine were yellow in color, but the color of the Valmaine extract was more intense than that of the Tall Guzmaine extract (Fig. 3-5C). Water:methanol (20:80) extracts of both cultivars were colorless. The water:methanol (50:50) extract of Tall Guzmaine was colorless, but it was yellow in the case of Valmaine. Methylene chloride extracts of both cultivars were white in color and sticky.

Treatment of latex extracts had significant on the number of insect feeding on diet after 15 min ($F = 11.97$; df = 14, 196; $P = 0.0001$); 30 min ($F = 12.60$; df = 14, 196; $P = 0.0001$); 60 min ($F = 24.42$; df = 14, 196; $P = 0.0001$); and 90 min of release ($F = 31.93$; df = 14, 196; $P = 0.0001$). Significantly fewer insects fed on diet disks treated with a water:methanol (20:80) extract of Valmaine latex than on diets treated with all other Valmaine and Tall Guzmaine latex extracts, as well as all the control diets (Figs. 3-11, 3-12). In addition, diet consumption was also significantly affected by the latex extract treatment ($F = 95.01$; df = 14, 196; $P = 0.0001$). Beetles consumed significantly less diet treated with water:methanol (20:80) extract of Valmaine latex than diet treated with any other latex extract or control diet (Fig. 3-13). The number of insects feeding on disks (Fig. 3-12) and amount consumed (Fig. 3-13) on diet disks treated with the

water:methanol (20:80) extract of Valmaine latex were equivalent to those values for diet treated with fresh Valmaine latex.

Beetle Behavior in Response to Contacting Latex

In latex choice tests, beetles flew immediately to the roof and walls of the container whenever they approached the Valmaine latex-treated diet disk, whereas the beetles started feeding on the Tall Guzmaine latex-treated diet disk whenever they approached it. In latex no-choice tests, beetles generally returned to the roof and walls of the container after approaching several times the Valmaine latex-treated diet disks. The behavior of the beetles on diet treated with water:methanol (20:80) extracts of Valmaine latex was similar to that for diet treated with pure Valmaine latex. Before biting a latex-treated diet disk, beetles inspected it at a close range, antennating and palpating it. In cases where the beetles landed directly on a disk, they appeared to sense the deterrent with their tarsi, even before antennating and palpating the disk, and flew back to the container walls immediately. Beetles performed frequent and more vigorous grooming of antennae and tarsi by passing them through mouthparts after contact with Valmaine latex compared to Tall Guzmaine latex. Further tarsal grooming was also done by scraping the legs on the elytra.

Beetles salivated more when Valmaine latex was applied to their mouthparts with a microdispenser compared to Tall Guzmaine latex. Mandibles and maxillae were not gummed up and were moving freely 24 h after application of either Valmaine or Tall Guzmaine latex, but there were some traces of dried latex on the labium and tarsi. During test bites on a lettuce leaf and contact with the exuded latex, the beetles moved away from the feeding site very quickly and started test bites somewhere else. The response was very vigorous on Valmaine. On Tall Guzmaine, beetles resumed test bites in close

proximity to the previous bites, but on Valmaine tests bites were much farther away from the previous bites.

Discussion

Evidence presented here indicates that resistance found in Valmaine romaine lettuce against *D. balteata* is due to latex. Adult *D. balteata* were found more frequently on diets treated with latex from Tall Guzmaine than on diets treated with Valmaine latex in both choice and no-choice tests. The alighting behavior of the beetles observed in my choice and no-choice tests suggests that contact chemosensilla are present on their antennae, palps and tarsi (Chapman 2003). These types of chemosensilla have been reported in other chrysomelids, such as on the antennae of the cabbage stem flea beetle, *Psylliodes chrysocephala* L. (Isidoro et al. 1998), maxillary appendages of the western corn rootworm, *D. virgifera virgifera* LeConte (Chyb et al. 1995, Eichenseer and Mullin 1996), and tarsomeres of the Klamath beetle, *Chrysolina brunsvicensis* Gravenhorst (Rees 1969). Such chemsensilla have been found to discriminate between phagostimulants and phagodeterrents. Antennal and tarsal grooming, similar to that observed by us with *D. balteata*, has been reported in the crucifer flea beetle, *Phyllotreta cruciferae* Goeze as an important part of the prefeeding behavior for recognizing host and non-host crucifers (Henderson et al. 2004).

Adult *D. balteata* consumed significantly less Valmaine latex-treated diet compared to Tall Guzmaine latex-treated diet in both choice and no-choice tests. Huang et al. (2003b) reported that latex from both Valmaine and Tall Guzmaine was very deterrent to beetle feeding when applied on lima bean leaves. I believe that Tall Guzmaine latex in the studies of Huang et al. (2003b) showed very high deterrence due to changes in its chemical properties (possibly oxidation) after drying on the lima bean leaf

surface. In my studies, the precisely defined quantities of latex (70 µl) applied to diet disks did not dry significantly within the 16-h exposure period to beetles due to moisture from the artificial diet, which perhaps prevented changes in the chemical properties of the latex. Both cultivars produce latex upon wounding but the much higher coefficient of deterrence for Valmaine latex compared to Tall Guzmaine latex observed in my study argues that Valmaine latex is more deterrent than Tall Guzmaine due to its physical or/and chemical properties. These properties may be due to the original chemicals produced by the plants or new chemicals produced by the action of certain plant enzymes, such as phenylalanine ammonia lyase, polyphenol oxidase and peroxidase. Valmaine also partially or totally lost its resistance in feeding bioassays using detached leaves or leaf disks, where latex exudation was greatly reduced (Huang et al. 2003c). This further provided evidence about the defensive role of latex in resistant Valmaine.

The strong deterrent activity of Valmaine latex extracted with water:methanol (20:80) provides compelling evidence for the chemical basis of resistance in Valmaine against *D. balteata*. The ability of water:methanol (20:80) to extract deterrent constituents from Valmaine latex suggests that moderately polar compounds in Valmaine latex account for its feeding deterrence. The defensive role of latex due to it chemical constituents against insects has been reported in many plant systems, such as milkweed (Dussourd and Hoyle 2000), mulberry (Konno et al. 2006), papaya (Konno et al. 2004), and chicory (Rees and Harborne 1985). Various organic compounds, such as phenolics and terpenoids have been reported in latex of *Lactuca* spp. (Crosby 1963, Gonzalez 1977, Cole 1984, Sessa et al. 2000), and their defensive role as phytoalexins has been reported against plant diseases (Bennett et al. 1994, Bestwick et al. 1995).

Latex from young leaves of Valmaine strongly deterred the feeding of *D. balteata* adults in a choice between diets treated with latex from young and mature leaves. Sethi et al. (2006) found that latex from young and mature leaves differed in terms of milkiness and viscosity. Such differences in milkiness arise due to differences in the refractive indices of the dispersing particles (mainly terpenoids) and the dispersing medium (Esau 1965, Fahn 1990). Thus, latex from young leaves may be richer in dispersing particles, and the relatively higher amount of dispersing particles may have a specific purpose related to plant defense. Young leaves are typically better defended than mature leaves due to the presence of higher quantities of latex and its associated chemical components (Swain 1977, Spilatro and Mahlberg 1986). In the chicory plant, *Cichorium intybus* L., sesquiterpene lactones were present in the highest amounts in the most actively growing regions of the plant and possessed antifeedant properties against *Schistocerca gregaria* (Orthoptera: Acrididae) (Rees and Harborne 1985). Young vines of sweetpotato, *Ipomoea batatas* (L.) Lam., possessed more latex and exhibited less damage due to the sweetpotato weevil, *Cylas formicarius* (F.) (Coleoptera: Curculionidae) than mature vines (Data et al. 1996). Latex exudation is higher in growing regions than in mature regions of great bindweed, *Calystegia silvatica* (Kit.) Griesb (Condon and Fineran 1989).

Anatomy of laticifers changes during the course of their ontogeny (Olson et al. 1969). The number of laticifers and their contents decrease with increasing proximity to roots (Condon and Fineran 1989, Monacelli et al. 2005). In mature leaves, the protoplast of laticifers degenerates near senescence indicating a low level of metabolism (Fineran 1982, 1983). Fusion of latex particles has also been seen in mature leaves when much of the latex has already vanished (Dickenson 1963, Heinrich 1967, Fineran 1982). Plug-like

masses of callose have been reported at the bases of mature leaves and no or much reduced amounts of latex exude when such leaves are severed, completely or partially, from the plant (Spencer 1939). Young leaves have discrete files of laticifers separated by end walls, while laticifers differentiate by breakdown of end walls in mature leaves (Condon and Fineran 1989). Thus, laticifers of young leaves may have more turgor pressure resulting in profuse latex exudation compared to mature leaves, making it more likely that insect mandibles will be exposed to latex during test bites on intact leaves.

My data support a hypothesis that latex has a definite role in the expression of resistance in Valmaine lettuce to *D. balteata*, and differences in latex chemistry between the two cultivars may account for this. Future research on the isolation of latex constituents and their biological activity is required to better understand the mechanism of resistance in Valmaine lettuce. This knowledge may contribute to the development of new cultivars expressing insect resistance along with superior horticultural traits through conventional and transgenic approaches.

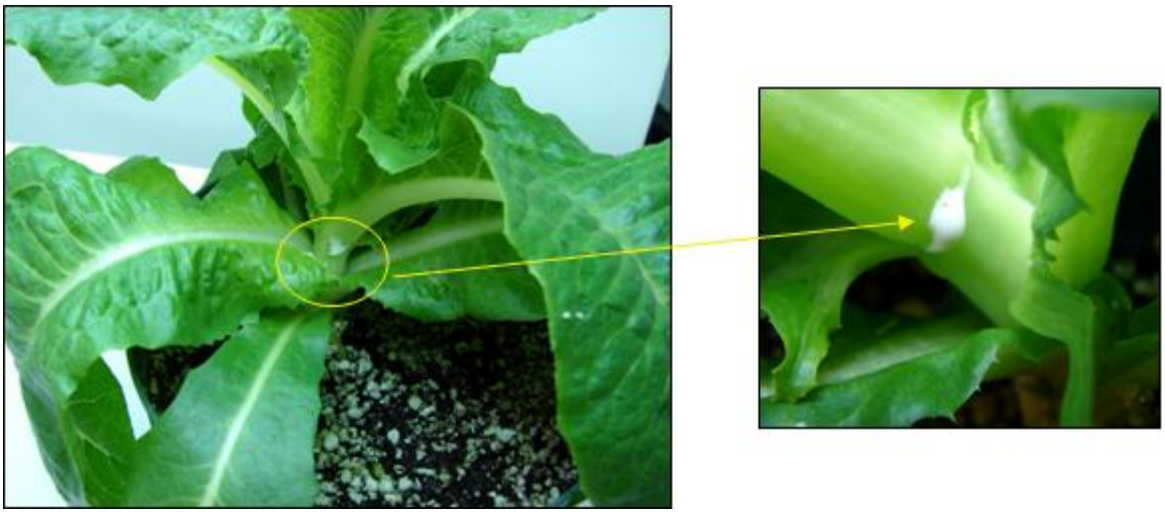


Figure 3-1. Wounding of lettuce releases a milky fluid called latex.

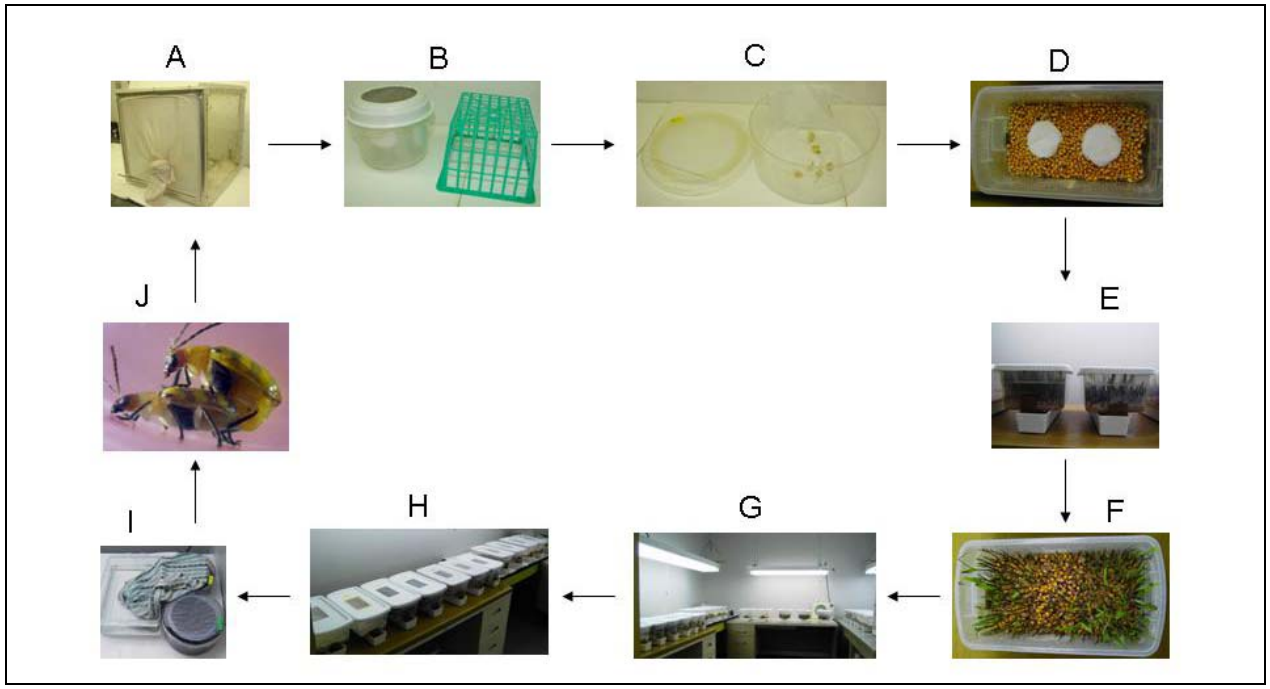


Figure 3-2. Colony rearing of *D. balteata*. See text for description of each stage of colony maintenance.

Figure 3-3. Collection of latex from romaine lettuce, application on artificial diet disk and bioassay setup.

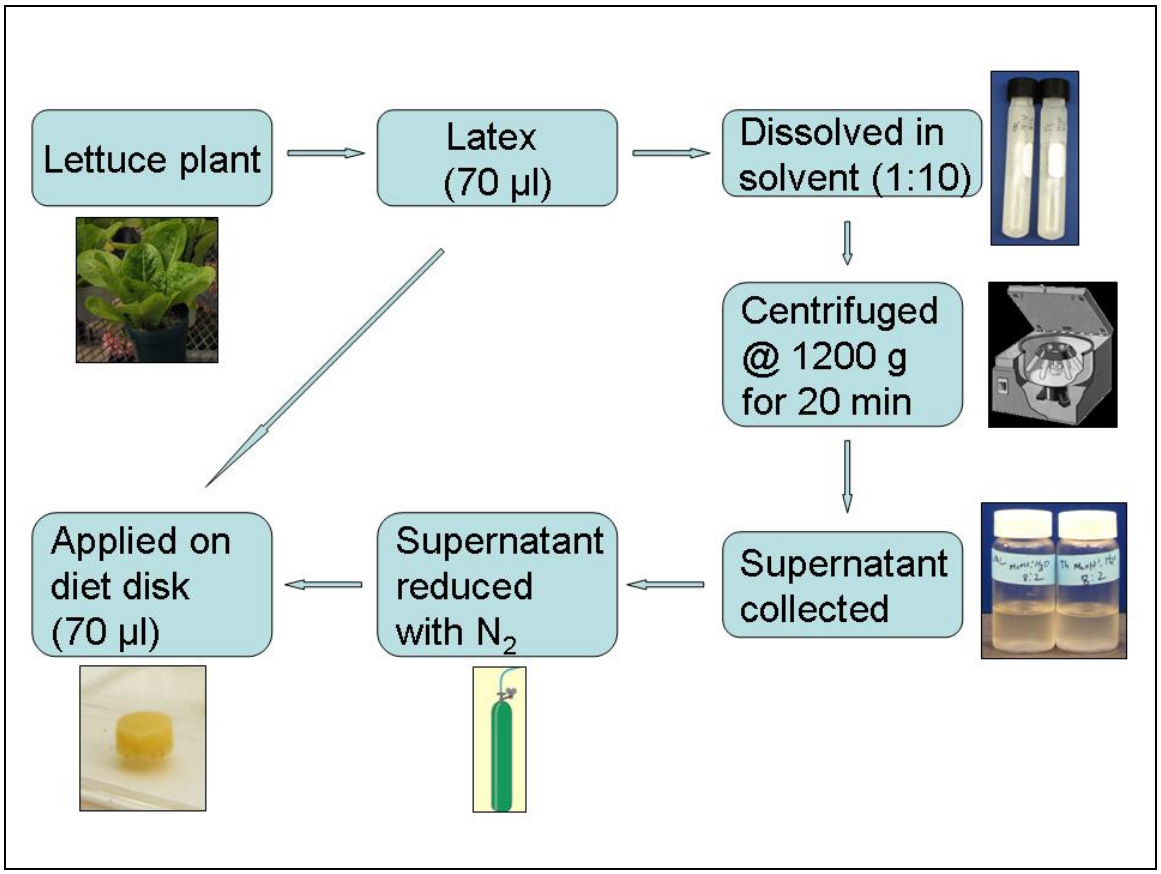
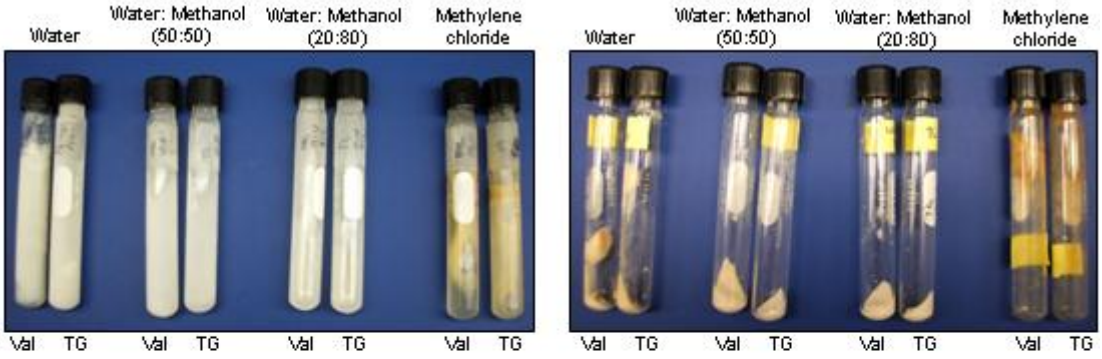
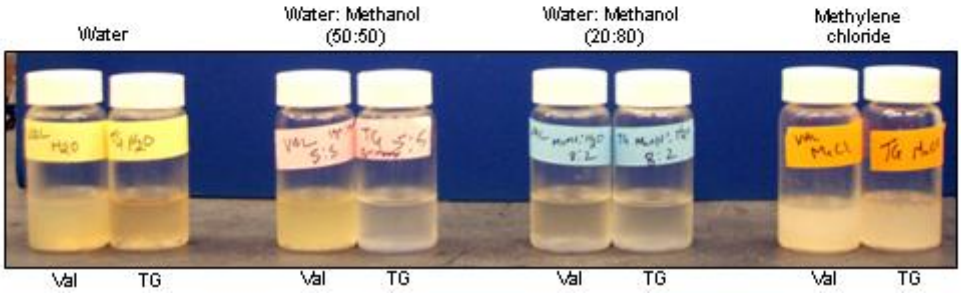


Figure 3-4. Scheme of latex solvent extraction.



A B

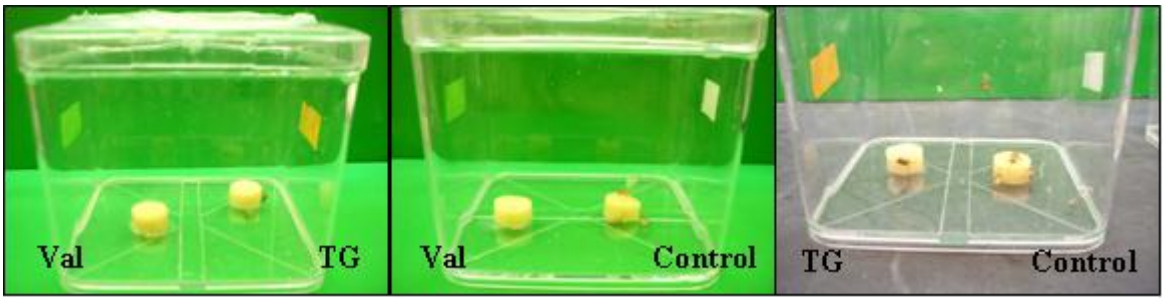


C

Figure 3-5. Latex dissolution in different solvents. A) Latex dissolved in different solvents, B) pellet settled down after centrifugation, and C) supernatant collected after centrifugation.

A

Choice tests



B

No-choice test

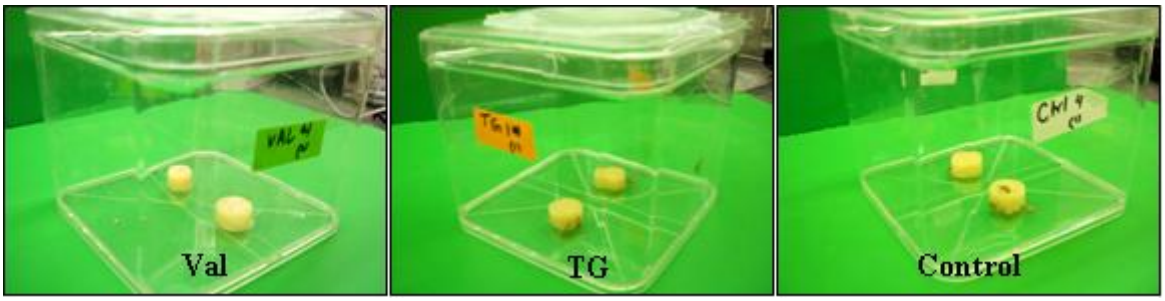


Figure 3-6. Feeding bioassays using fresh latex. A) Choice tests: Valmaine (Val) versus Tall Guzmaine (TG), Valmaine versus control, Tall Guzmaine versus control. B) No-choice tests: Valmaine (Val), Tall Guzmaine (TG), control.

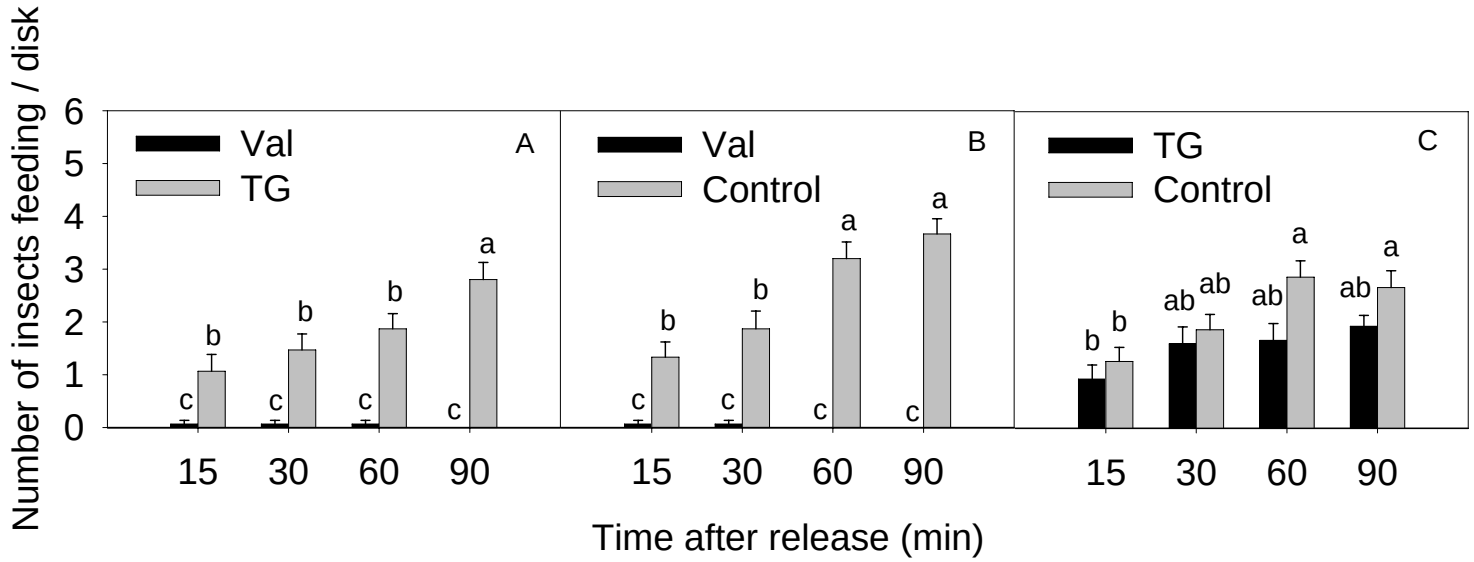


Figure 3-7. Mean number of *D. balteata* adults feeding on artificial diet disks treated with latex from resistant Valmaine (Val), disks treated with latex from susceptible Tall Guzmaine (TG), and control diet disks in choice tests. Error bars indicate SEM. Bars topped with different letters within same panel differ significantly at the 0.05 level (Tukey's HSD test).

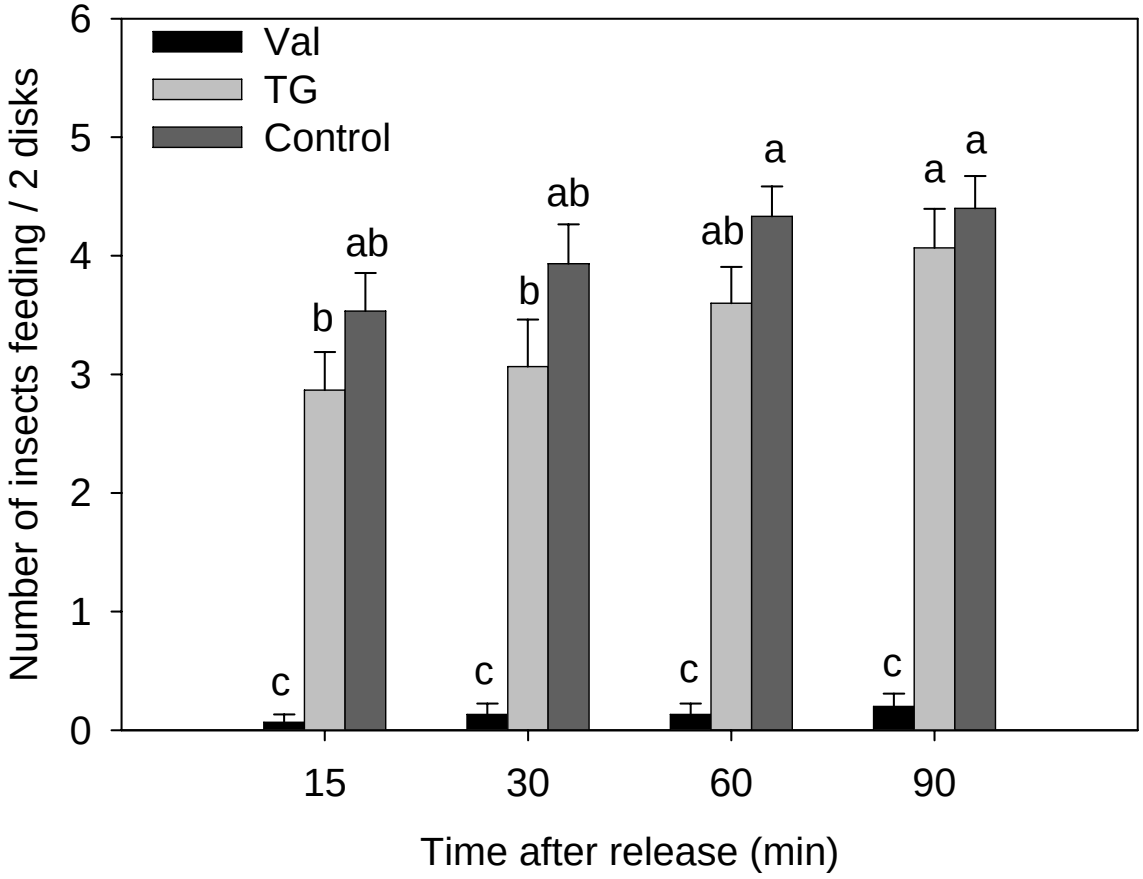


Figure 3-8. Mean number of *D. balteata* adults feeding on two artificial diet disks treated with latex from resistant Valmaine (Val), disks treated with latex from susceptible Tall Guzmaine (TG), and control diet disks in no-choice tests. Error bars indicate SEM. Bars topped with different letters differ significantly at the 0.05 level (Tukey's HSD test).

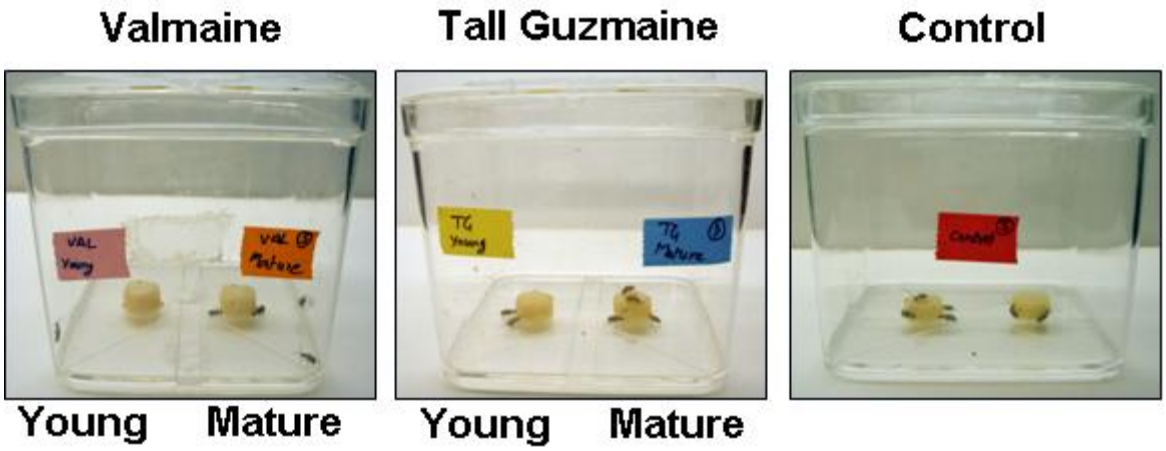


Figure 3-9. Choice tests using *D. balteata* adults on two artificial diet disks treated with latex from young and mature leaves of the same cultivar.

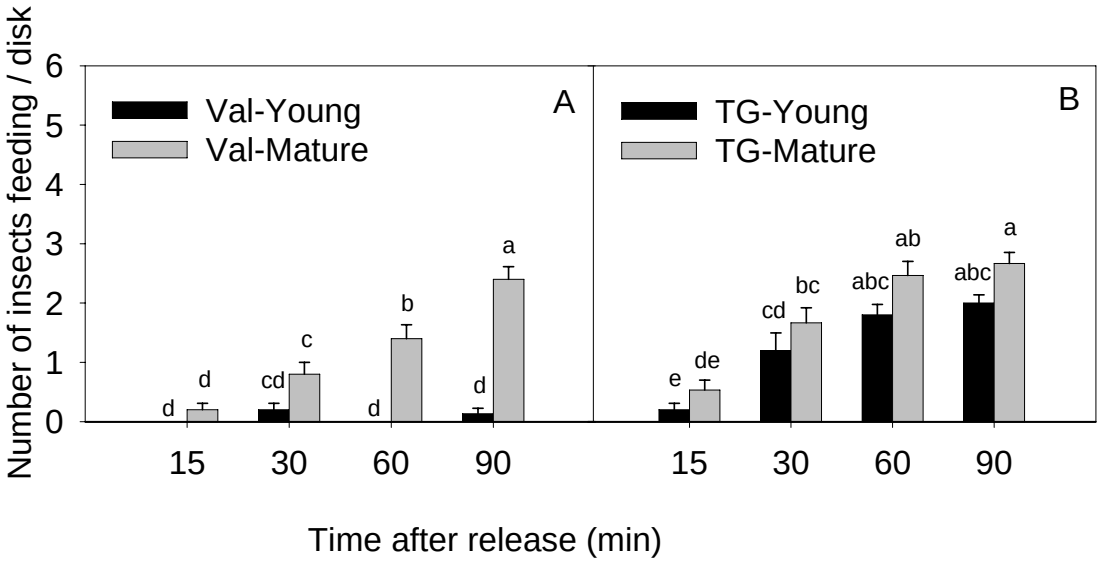


Figure 3-10**.** Number of *D. balteata* adults feeding on artificial diet disks treated with latex from young or mature leaves of resistant Valmaine (Val) (A) and susceptible Tall Guzmaine (TG) (B) in choice tests. Error bars indicate SEM. Bars topped with different letters within same panel differ significantly at the 0.05 level (Tukey's HSD test).

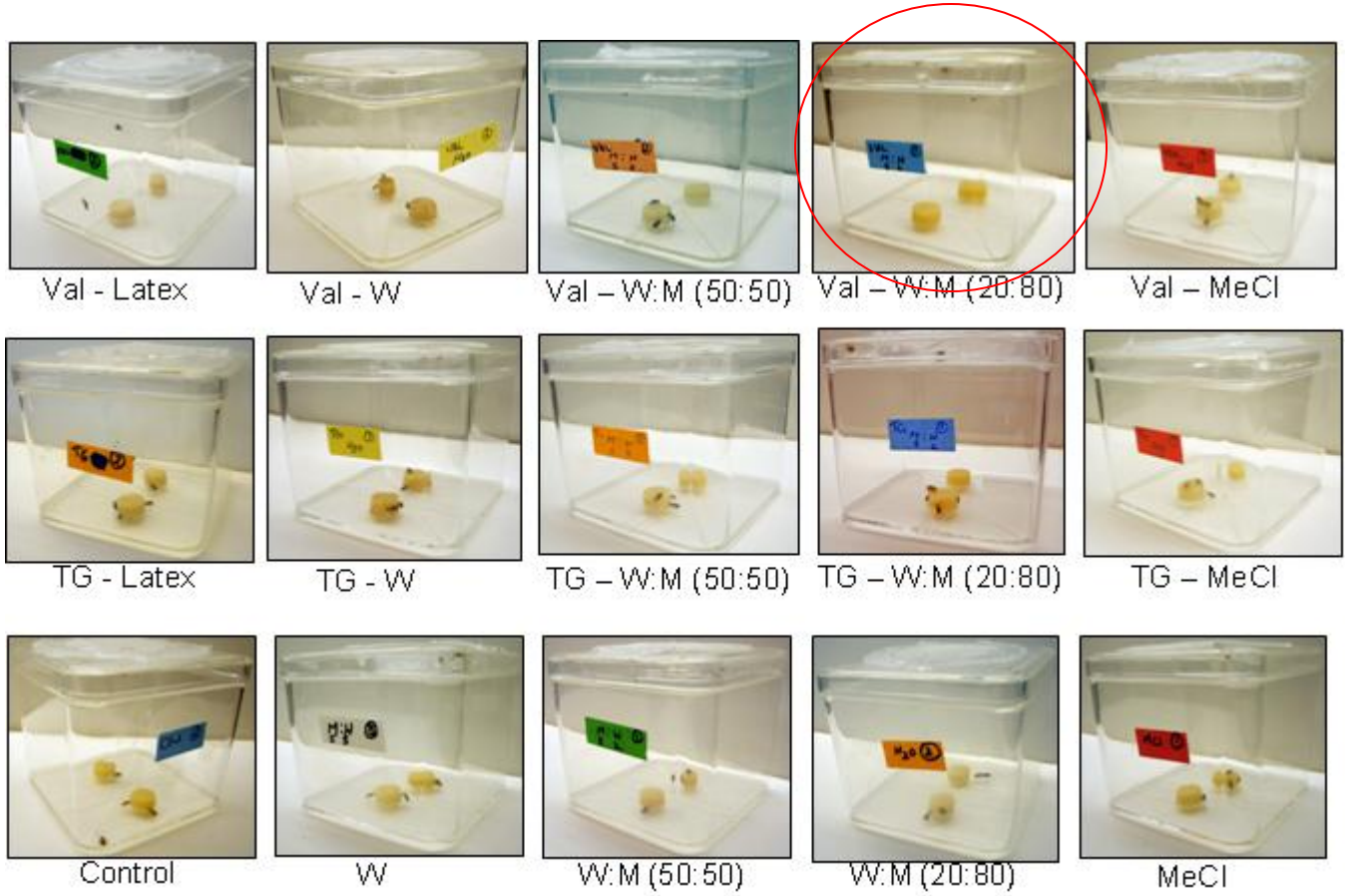


Figure 3-11. No-choice tests using *D. balteata* adults when both the disks were smeared with either Valmaine latex extract or Tall Guzmaine latex extract. W – Water, M – Methanol, MeCl – Methylene chloride. Red circle indicates the most deterrent extract comparable to fresh latex.

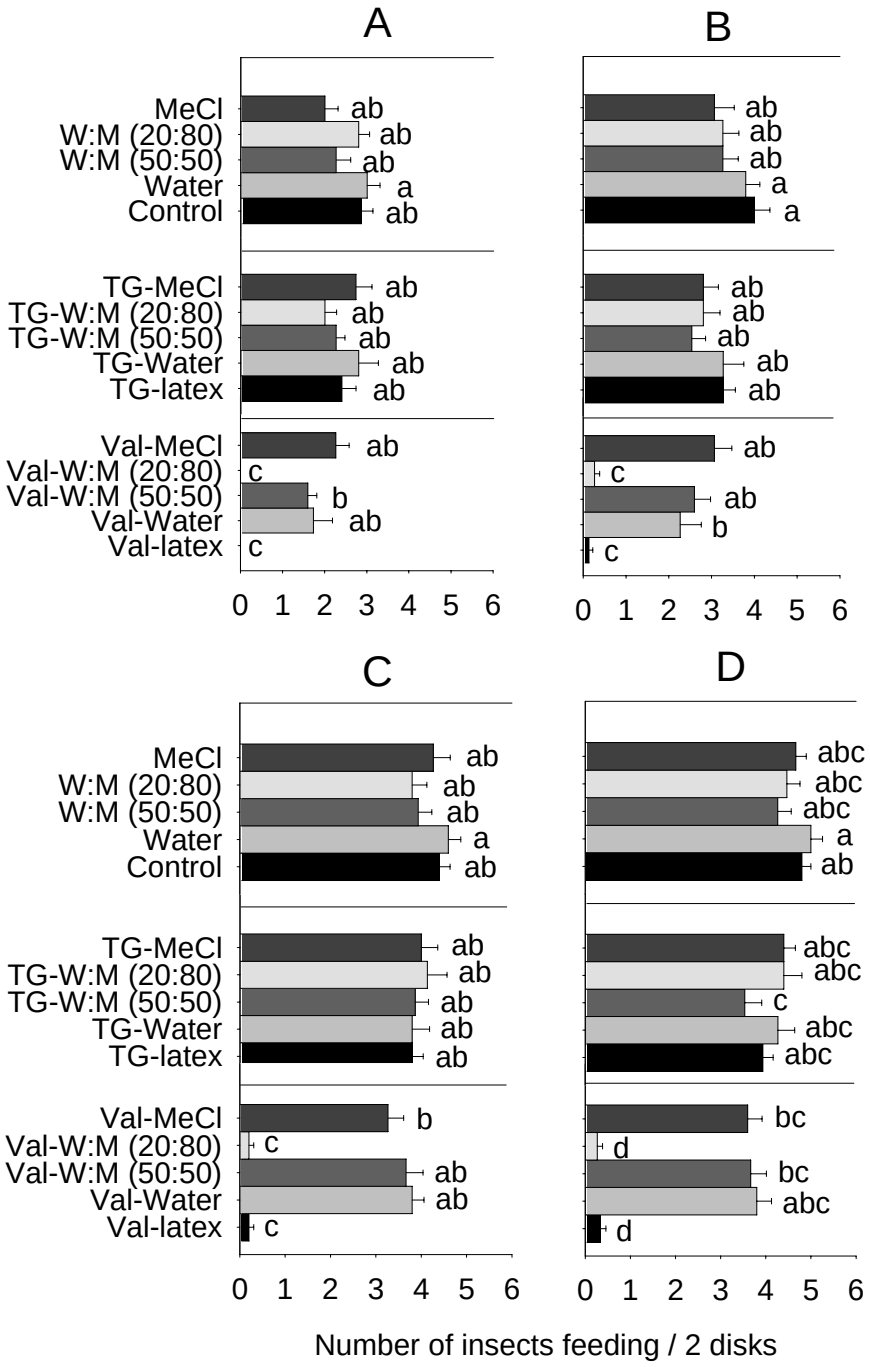


Figure 3-12. Mean number of *D. balteata* adults feeding on two artificial diet disks treated with latex extracts from resistant Valmaine (Val) and susceptible Tall Guzmaine (TG), and controls in no-choice test. Error bars indicate SEM. Bars topped with different letters within same panel (A, B, C, D) differ significantly at the 0.05 level (Tukey's HSD test). A) 15 min, B) 30 min, C) 60 min, and D) 90 min.

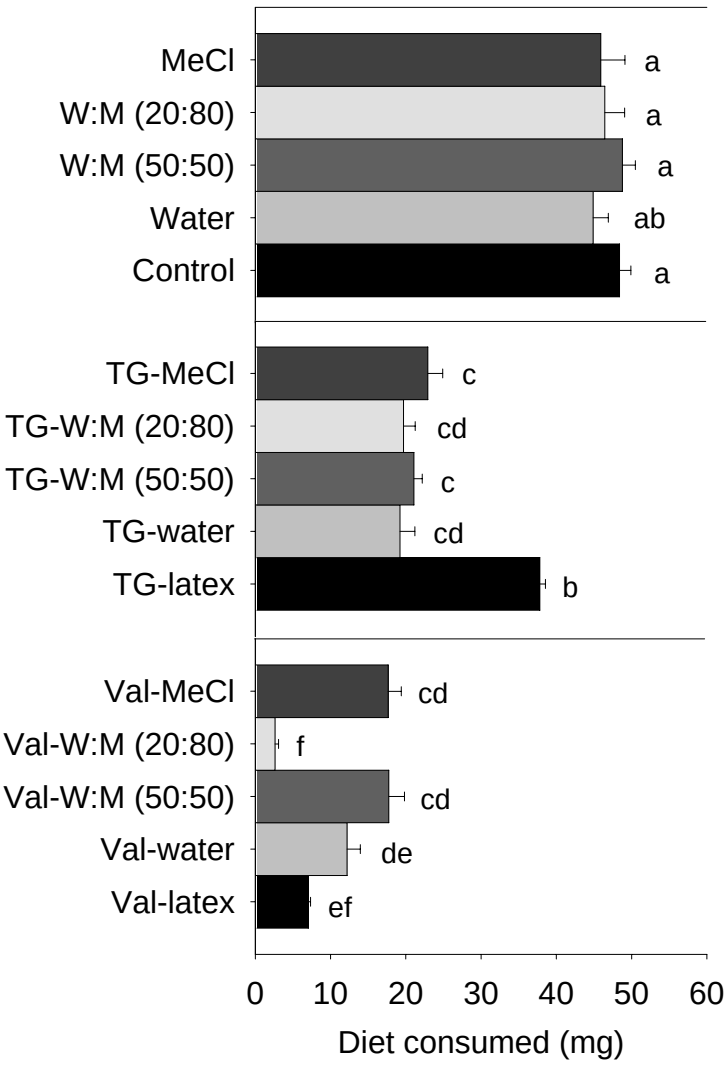


Figure 3-13. Dry weight of diet consumed by six *D. balteata* adults in 16 h when both diet disks were treated with Valmaine (Val) or Tall Guzmaine (TG) latex extracts under no-choice situations. Error bars indicate SEM. Bars topped with different letters differ significantly at the 0.05 level (Tukey's HSD test).

Table 3-1. Dry weight consumption of diet disks treated with Valmaine (Val) or Tall Guzmaine (TG) latex under choice and no-choice tests by six *D. balteata* adults in 16 h.

Tests	Mean diet consumed ± SEM (mg)			*P* value
	Treatment			
	Val latex	TG latex	Control	
Choice*				
Val vs. TG	5.4 ± 0.5	15.5 ± 0.7	-	0.0001
Val vs. Control	5.5 ± 0.5	-	24.7 ± 0.5	0.0001
TG vs. Control	-	14.4 ± 0.5	21.9 ± 0.6	0.0001
No-Choice†	7.3 ± 0.4c	34.6 ± 1.1b	46.2 ± 2.4a	0.0001

* *P* value from paired *t*-test. †Means ± SEM followed by different letters in no-choice test differed significantly ($P \leq 0.05$) using ANOVA ($F = 168.31$; df = 2, 42; $P = 0.0001$) and Tukey's HSD test.

Table 3-2. Feeding deterrent activity of latex against *D. balteata* adults when artificial diet disks were treated with latex from either resistant Valmaine (Val) or susceptible Tall Guzmaine (TG) in choice and no-choice tests.

Latex	Deterrence coefficients		
	Relative	Absolute	Total
Val	63.6	72.7	136.3
TG	20.7	14.4	35.0
P value	0.0001	0.0001	0.0001

P value from two sample *t*-test.

Table 3-3. Dry weight of diet consumed by six *D. balteata* adults in 16 h when given a choice between diet disks treated with latex from either young or mature leaves of resistant Valmaine or susceptible Tall Guzmaine lettuce cultivars.

Cultivar	Choice	Diet consumed (mg)^	Total diet consumed (mg)†
Valmaine	Young latex-treated diet vs.	3.7 ± 0.6b	29.8 ± 2.2 b
	mature latex-treated diet	26.1 ± 1.9a	
Tall Guzmaine	Young latex-treated diet vs.	18.1 ± 2.0a	38.7 ± 3.9ab
	mature latex-treated diet	20.5 ± 2.2a	
Control*	-	-	50.1 ± 5.2a

*Both disks were untreated in control diet. ^ Means ± SEM followed by different letters within cultivar differed significantly using paired *t*-test. †Means ± SEM followed by different letters within column differed significantly ($P \leq 0.05$) using ANOVA ($F = 6.69$; df = 2, 42; $P = 0.0030$) and Tukey's HSD test.

CHAPTER 4
BANDED CUCUMBER BEETLE (COLEOPTERA: CHRYSOMELIDAE) RESISTANCE IN ROMAINE LETTUCE: UNDERSTANDING LATEX CHEMISTRY

Introduction

Host plant resistance is an important potential component of any integrated pest management program for a crop pest. Many plants produce compounds that mediate host plant suitability to insect herbivores (Rosenthal and Berenbaum 1991). These biologically active compounds are frequently present in viscous secretions, such as latex or resin, within specialized canal systems separate from the vascular system (Fahn 1979, Metcalf and Chalk 1983, Farrell et al. 1991). Thus, insect mouthparts get exposed to these compounds during test bites due to copious flow of latex at the damage site (Farrell et al. 1991). The common components of latex are polyisoprene, proteins, amino acids, fatty acids, tetracyclic triterpenoids, glycerides, waxes, starch, flavonoids, alkaloids, water, organic and inorganic salts and many unidentified compounds (Nielson et al. 1977, Spilatro and Mahlberg 1986, Gazeley et al. 1988). Examples of some compounds found in latex with activity against different insect pests include diterpenes (Evans and Schmidt 1976, Noack et al. 1980) and nonprotein amino acids in *Euphorbia* (Haupt 1976), cardenolides in milkweed (Seiber et al. 1982, Nishio et al. 1983), alkaloids in poppy (Roberts 1987, Matile 1976) and mulberry (Konno et al. 2006), sesquiterpene lactones in chicory (Rees and Harborne 1985), and cysteine proteases in papaya and fig (Konno et al. 2004). However, latex of most of the laticiferous species within the Apocynaceae, Compositae, Euphorbiaceae, Musaceae, Papaveraceae, and Urticaceae has not been chemically characterized.

A cultivar of romaine lettuce (*Lactuca sativa* L.), ‘Valmaine’, possesses insect resistance against leafminer, *Liriomyza trifolii* (Burgess) (Nuessly and Nagata 1994),

banded cucumber beetle, *Diabrotica balteata* LeConte (Huang et al. 2002) and two lepidopterans, *Trichoplusia ni* (Hübner) and *Spodoptera exigua* (Hübner) (Chapter 2, Sethi et al. 2006). Latex from Valmaine applied to artificial diet deters feeding of *D. balteata*. Further, a crude extract prepared by dissolving Valmaine latex in a water:methanol mixture (20:80, % v/v) also strongly inhibits beetle feeding when applied to the surface of artificial diet (Chapter 3, Sethi et al. 2007). This suggests that Valmaine latex contains deterrent compounds which are responsible for its resistance against multiple species of mandibulate insects.

Here, I describe the isolation and characterization of deterrent compounds from Valmaine latex against *D. balteata* adults using bioassay-directed fractionation.

Materials and Methods

Plants and Insects

Seeds of the romaine lettuce cultivar, Valmaine were germinated by putting them on moist filter paper in a Petri dish. Germinated seeds were planted in soil-less media (MetroMix 220 potting mixture, Grace Sierra, Milpitas, CA) and healthy seedlings were transplanted into 15-cm-diameter plastic pots after 2 wk in a greenhouse with natural light at a mean temperature of 27 °C (22 to 30°C) and 68% mean R.H. (48 to 93%). Plants were fertilized with 10 ml of a 10 g/l solution of Peters 20-20-20 (N-P-K) (W.R. Grace, Fogelsville, PA) once a week. Bush lima bean (*Phaseolus lunatus* L.) plants of the 'Fordhook 242' cultivar (Illinois Foundation Seeds, Champagne, IL) were grown in transplant trays and fertilized with the same solution used for lettuce plants.

A wild population of D. balteata adults was collected from spiny amaranth, *Amaranthus spinosus* L. and primrose willow, *Ludwigia peruviana* L. in Belle Glade, FL in 2003. A colony was established by raising adults on lima bean leaves and slices of

sweet potato tubers, and larvae were fed on corn seedling roots as per the methods of Huang et al. (2002) (Chapter 3). Wild individuals were added to the colony in 2005 and 2006 to increase genetic diversity. Unfed adults that had emerged within 48 h of the start of the experiment were used in all bioassays.

Assay for Feeding Deterrence

Extracts/fractions from latex obtained as described below were bioassayed on artificial diet for feeding deterrence towards *D. balteata* adults under no-choice conditions. The southern corn rootworm artificial diet (Bio-Serv, Frenchtown, NJ), and disks of diet for use in the assays, were prepared as described in Chapter 3 (Sethi et al. 2007). An experimental setup consisted of two diet disks placed on the bottom of a plastic container (10 × 10 × 8 cm) with screen lid and three male-female pairs of beetles. Both diet disks in each container were treated with the same kind of extract/fraction. The beetles were allowed to feed on the diet for 16 h. The experiments were carried out at 25 ± 1°C in a laboratory under a photoperiod of 14:10 (L:D).

In all bioassays, the number of adults feeding on two diet disks was recorded 90 min after their release into the container. The dry weights of diet consumed in 16 h were also recorded. To compensate for the weight associated with moisture loss during the feeding tests, individual fresh weights of 10 diet disks were recorded before they were dried in an oven at 50 ± 5^{o}C. Individual dry weights of these disks were recorded after 3 d and an average dry/fresh weight ratio was calculated. The diet disks for bioassays were weighed prior to the bioassay setup. At the end of the experiment, the remaining portions of the disks were reweighed after drying for 3 d in the oven. The amount of dry weight of diet consumed was calculated as the difference between initial and final dry weights. Dry

weights of diet disks consumed by the beetles were computed by multiplying fresh weight by the average dry/fresh weight ratio.

Latex Collection and Crude Extract Preparation

Cuts were made near the leaf-bases of young and middle-aged leaves of lettuce plants (9-10 true-leaf stage) using a disposable scalpel blade (Feather, Osaka, Japan). Fresh latex (70 µl) was collected from each plant using a 100-µl silanized glass capillary tube inserted into a microdispenser (Drummond Scientific Company, Broomall, PA) and immediately dissolved in 10× volume of water:methanol (20:80) mixture. After dissolution, samples were centrifuged at 1200 ×g for 20 min and then the supernatant was collected. The supernatant (hereafter termed crude extract) was concentrated to 0.1× volume (the original latex volume) by evaporation under a gentle stream of nitrogen (Chapter 3, Sethi et al. 2007).

Fractionation of Crude Extract Using Reversed-Phase (C-18) Cartridge

Reversed phase separations involve a polar or moderately polar sample matrix (mobile phase) and a nonpolar stationary phase. The analyte of interest is moderately- to non-polar. Alkyl bonded silica (C-18) is the most commonly used stationary phase in solid-phase extraction (SPE) (Hennion 1999). The crude extract was first fractionated using a C-18 cartridge (300 mg, Alltech Associates, Inc., IL) (Fig. 4-1). Prior to application of the crude extract, the C-18 cartridge was pre-conditioned with 10 ml methanol and then with 10 ml water. The crude extract was percolated through the C-18 cartridge at a rate of approximately 1 drop per 1.5 s using positive pressure, and the unbound fraction was collected. After percolation of the crude extract, the cartridge with retained compounds was washed with 10× volumes of a stepwise gradient of water-

methanol mixtures [water, water:methanol (80:20, % v/v), water:methanol (40:60), and water:methanol (5:95)] to elute retained compounds. After percolation of each water-methanol mixture, subsequent fractions were collected and each fraction concentrated back to 0.1× volume under a nitrogen stream.

In reversed-phase SPE procedures using C-18 packing, trapping of the analyte is optimized by adjusting the pH of the conditioning solution or aqueous sample, or by adding a small percentage of organic solvent to the sample before percolation (Hennion 1999, Simpson 2000). Adjustment of the sample pH greatly enhances retention of ionizable compounds under their neutral form on C-18 packing by making them sufficiently hydrophobic (Pichon 2000). The sample pH can also be adjusted for sample clean-up so that unwanted compounds in the sample are retained on the SPE packing (Hennion 1999, León-Gonaźalez and Pérez-Arribas 2000, Simpson 2000). Therefore, the above extractions were repeated separately using crude extract acidified or basified to three different pH, i.e., at original (6.5), acidic (3.0) and alkaline (9.0) pH. Acidification and basification of crude extract was achieved by adding 1 N phosphoric acid and 1 N ammonium hydroxide, respectively.

The crude extract, unbound fraction, four eluted fractions [water, water:methanol (80:20), water:methanol (40:60) and water:methanol (5:95)] and the combination of all four eluted fractions were applied to artificial diet disks for deterrence bioassays under no-choice conditions. An amount of each extract/fraction, equivalent to 70-µl latex, was applied to each diet disk. For controls, five water-methanol mixture combinations without latex extract (including water:methanol (20:80)) and untreated diet disks were used. Each

experimental unit was replicated nine times for extracts at the original pH and six times each for extracts acidified to pH 3.0 or basified to pH 9.0.

Fractionation of Crude Extract Using C-18, SAX and SCX Cartridges Connected in Series

Ion-exchange SPE is also a commonly used method for the extraction of charged compounds. Negatively (anionic) and positively (cationic) charged compounds can be isolated on anion exchange (SAX) and cation exchange (SCX) stationary phases, respectively. Subsequently, these charged compounds can be eluted using a solution of high ionic strength that displaces the absorbed compounds (Hennion 1999). The crude extract at original pH was next fractionated using C-18, SAX (functional group: quaternary ammonium, counter ion: acetate) and SCX (functional group: sulphonic acid, counter ion: hydrogen) cartridges (Alltech Associates, Inc., IL) connected in series (Fig. 4-2). Prior to crude extract application, C-18 cartridges were pre-conditioned with 10 ml methanol and then with 10 ml water; SAX and SCX cartridges were pre-conditioned with 10 ml water. The samples were passed by positive pressure through the cartridges at a flow rate of approximately 1 drop per 1.5 s. The crude extract at original pH (6.5) was percolated through a C-18 cartridge and the unbound fraction was collected. Then, the C-18 unbound fraction was percolated through SAX and SCX cartridges connected in series and the unbound fraction was collected. After percolation of the C-18 unbound fraction, SAX and SCX cartridges with retained compounds were washed separately with 10× volumes of a stepwise gradient of NaCl solutions (0.1, 0.5 and 1 M) to elute retained compounds. After percolation of each NaCl solution, subsequent fractions were collected and concentrated back to 0.1× volume under a nitrogen stream.

An amount of each extract/fraction, equivalent to 70 µl latex, was applied to each diet disk for use in the bioassays. Nine treatments were studied: crude extract; C-18 unbound fraction; SAX and SCX unbound fraction; and 0.1, 0.5, 1M NaCl fractions from each SAX and SCX cartridge. Controls consisted of untreated diet disks, disks treated with water:methanol (20:80) mixture, and disks treated with 0.1, 0.5 or 1 M NaCl solutions. Each experimental unit was replicated nine times. The 0.5 M-NaCl SCX fraction exhibited the strongest deterrent activity and was termed "SCX fraction" in the following LC/MS separations.

LC/MS Separation of SCX Fraction

LC/MS helps in the fractionation of a sample with simultaneous characterization of chemical compounds. Fractionating increases the sample simplicity and ultimately makes the characterization of the compounds much easier. The SCX fraction was further fractionated by LC/MS. A Thermo Finnigan LCQ Deca XP Max was used employing electrospray ionization (ESI) (sheath gas, 25 arbitrary units; sweep gas, 10 arbitrary units; spray voltage, 5.00 kV; capillary temperature 285°C; and capillary voltage, 3.0 V) with the Thermo Separations spectra HPLC system (quaternary pump P4000, autosampler AS 3000, and diode array detector UV6000). Separations were performed on a PLRP-S column (100 Å, 3 µm, 150 × 4.6 mm, Polymer Laboratories. Ltd., UK) with solvent A (water with 10 mM ammonium formate) and solvent B (90 acetonitrile:10 water with 10mM ammonium formate, v:v) as mobile phases for 40 min. Elution was performed using two solvent gradient systems with column temperature maintained at 60°C and a flow rate of 1.0 ml/min. The first gradient elution system employing solvent A at pH 9.0 began with 95:5 percent (A and B) and reached 45:55 at 25 min, followed by gradient to

0:100 in 5 min. The solvent was then kept at the final composition for 5 min.. The second gradient, with solvent A at pH 10, began with 100:0 percent (A and B) and reached 0:100 at 25 min. It was then kept at that composition for 10 min. UV absorption was monitored at 190 - 800 nm, and a low-volume micro needle valve splitter P450 (Upchurch Scientific, Oak Harbor, WA) was used to split the solvent flow between the UV detector and MS electrospray interface up to 90:10, making it possible to collect 90% of the eluted material in one minute fractions for bioassay while simultaneously obtain LC/MS spectra.

In the first gradient elution system at pH 9.0, fractions collected each minute were recombined into six major fractions (Fig. 4-3) and concentrated to a volume equivalent to 70 µl of latex to treat one diet disk. Then, these six fractions (#0-3, #4-7, #8-11, #12-15, #16-20, and #21-40) and the combination of eluted fractions were applied on the surface of artificial diet disks. Each experimental unit was replicated six times and each unit had two diet disks treated with same kind of fraction under no choice conditions. Untreated diet and diets treated with crude extract and SCX fraction were used for the controls.

In the second gradient elution system at pH 10.0, fractions were collected each minute but only eleven fractions were used for bioassays under no-choice conditions (#2, #3, #4-6, #20, #21, #22, #23, #24, #25, #26, and #27). Controls consisted of untreated diet disks and disks treated with crude extract and SCX fraction. Each experimental unit was replicated three times.

Statistical Analysis

In all no-choice tests, number of adults feeding on two diet disks 90 min after beetle release and the dry weights of diets consumed in 16 h were analyzed using Proc GLM (SAS Institute 2003) with latex fraction as a fixed effect and replications as a random effect. The error degree of freedom for latex fraction effect was calculated as

(levels of latex fraction -1)(replications -1). Tukey's honestly significant difference (HSD) test with a significance level of $\alpha = 0.05$ (SAS Institute 2003) was used for post hoc means separation.

Results

Fractionation of Crude Extract Using C-18 Cartridge

Water and water:methanol (40:60) fractions were light yellow and milky white, respectively; the 80:20 and 5:95 water:methanol fractions were colorless (Fig. 4-4).

Fractionation at original pH. Fractionation of the crude extract at its original pH and subsequent bioassays indicated that the unbound fraction had activity equivalent to that of the crude extract (Fig. 4-5). Latex fraction had significant effect on the number of insects feeding on diet disks ($F = 12.05$; df = 12, 96; $P = 0.0001$). Fewer insects were counted 90 min after their release on diet disks treated with the unbound fraction than on disks treated with any other C-18 water-methanol mixture fraction or on control diet disks (Fig. 4-6A). Latex fraction also significantly affected diet consumption by beetles ($F = 39.40$; df = 12, 96; $P = 0.0001$). Beetles consumed significantly less diet treated with the unbound fraction than diet treated with any other water-methanol mixture fraction or control diet (Fig. 4-7A).

Fractionation of crude extract at pH 3.0. Fractionation of the crude extract acidified to pH 3.0 on the C-18 cartridge and subsequent bioassays revealed that some of the deterrent compounds were retained on the C-18 resin. Latex fraction significantly affected the number of beetles feeding ($F = 5.03$; df = 12, 60; $P = 0.0001$). Significantly more beetles were counted 90 min after their release on diets treated with the water-methanol mixture extracts compared to diet treated with the unbound fraction (Fig. 4-6B). Latex fraction also had significant effect on diet consumption ($F = 11.49$; df = 12, 60; $P =$

0.0001). The unbound fraction was still deterrent to beetle feeding as diet consumption was significantly less on it, similar to that on the crude extract. But, in addition, the water fraction also had some deterrent activity (Fig. 4-7B).

Fractionation of crude extract at pH 9.0. Bioassays of fractions obtained by passing the crude extract basified to pH 9.0 over C-18 cartridge identified deterrent activity again in the unbound fraction with some deterrent activity in the water fraction. Latex fraction had significant effect on the number of insects feeding on diet ($F = 4.08$; df = 12, 60; $P = 0.0001$). After 90 min, the number of insects feeding on diet treated with the unbound fraction did not differ significantly from the number feeding on diet treated with the crude extract (Fig. 4-6C). Latex fraction also affected diet consumption by beetles ($F = 4.57$; df = 12, 60; $P = 0.0001$). Beetles consumed similar amounts of diet treated with the unbound fraction and the crude extract (Fig. 4-7C).

Fractionation of Crude Extract Using C-18, SAX and SCX Cartridges Connected in Series

The 0.1M NaCl fraction eluted from the SAX cartridge was colorless, but the other two fractions (0.5 and 1M NaCl) were yellow (Fig. 4-8). All three fractions eluted from the SCX cartridge were colorless.

The deterrent activity of the 0.5 M NaCl fraction obtained from the SCX cartridge was similar to that of the crude extract (Fig. 4-9). Latex fraction had significant effect on the number of insects feeding on diet ($F = 31.75$; df = 13, 104; $P = 0.0001$). Significantly fewer insects were counted on the diet disks treated with the 0.5 M NaCl fraction from either the SAX or SCX cartridge 90 min after their release (Fig. 4-10) compared to all other fractions. Application of latex fraction also significantly affected diet consumption by beetles ($F = 54.67$; df = 13, 104; $P = 0.0001$). Beetles consumed significantly less diet

treated with the 0.5 M NaCl fraction eluted from the SCX cartridge than diet treated with any other fraction from the SAX or SCX cartridges (Fig. 4-11). Beetles also consumed significantly less diet treated with the 0.5 M NaCl fraction from the SAX cartridge but not as little as they did on disks treated with the crude extract.

Fractionation of SCX Fraction Using LC/MS

At pH 9.0 of solvent A. Application of latex fraction significantly affected both the number of beetles counted on diet disks and the amount that they consumed (number of beetles on disks: $F = 18.78$; df = 9, 45; $P = 0.0001$; consumption: $F = 88.34$; df = 9, 45; $P = 0.0001$). Fewer beetles were counted on and consumed less of the diet disks treated with the crude extract, the SCX fraction, LC/MS fractions #0-3, fractions #21-40 as well as the combination of all LC/MS fractions (Figs. 4-12, 4-13). Some weak feeding deterrent activity was also found in the #4-7 fraction.

At pH 10.0 of solvent A. Latex fraction treatment has significant effect on the number of insects feeding on diet ($F = 11.92$; df = 13, 26; $P = 0.0001$). Diets treated with fraction #3 were as deterrent to feeding as were disks treated with either the crude extract or the SCX fraction (Fig. 4-14). Diet consumption by beetles was also significantly affected due the treatment of latex fractions ($F = 26.74$; df = 13, 26; $P = 0.0001$). Consumption was the lowest on the diet disks treated with the crude extract, the SCX fraction and the #3 fraction (Fig. 4-15). This fraction was estimated to contain about 10 peaks based on UV absorption (190 – 450 nm) and M+1 ions produced when analyzed using positive ion electrospray LC/MS (Fig. 4-16).

Discussion

The deterrent activity of the unbound fraction of the reversed-phase extraction at the original pH indicates that the deterrents compounds were not retained on C-18 resin.

Reversed phase extractions using C-18 involve a polar or moderately polar sample matrix (mobile phase) and a nonpolar stationary phase. The analytes of interest retained on the cartridge are moderately- to non-polar. So, this indicates that the deterrent compounds in the crude extract are highly polar. Many biologically active compounds are known to exist in their glycosidic form. By binding to sugars, the toxicity of these compounds is reduced and their solubility is increased which facilitates their storage in large amounts. These compounds become more active after coming in contact with specific degradation enzymes (Harborne 1979, Schoonhoven et al. 2005). In both lettuce and chicory (*Chicorium intybus* L.), most of the sesquiterpenes are found in glycosidic form and the bitterness of the plants is associated with them (Price et al. 1990). Tamaki et al. (1995) also reported that 44, 34 and 56% of sesquiterpene lactones were in their bound form in the wild lettuce species, *L. saligna* and *L. virosa*, and cultivated lettuce, respectively. These sesquiterpenes exhibited low retention on C-18 cartridges (Schenck 1966, Tamaki et al. 1995). Phenolic glycosides found in white grub-infested sugarcane (Nutt et al. 2004) and in white lupin (*Lupinus albus* L.) (Stobiecki et al 1997) also exhibited low retention due to their high polarity and solubility in water.

The crude extract was fractionated at two extreme pH levels with the intention of better retaining deterrent compounds with either acidic or alkaline characteristic. In my study, diet consumption data indicate that some of the deterrent compounds in the crude extract were retained on the C-18 packing both at acidic and basic pH. Some of the compounds with deterrent activity were eluted by water at both pH levels, but also by water:methanol (40:60) at basic pH.

Ion exchange solid-phase extraction is commonly used for the extraction of compounds that are charged when in an aqueous solution. In my study, the deterrent compounds were retained on the SCX packing after percolation of the unbound fraction from the C-18 cartridge, and were eluted with 0.5 M NaCl solution. Retention of deterrent compounds on SCX suggests a basic nature for the compounds.

During the fractionation of the SCX fraction using LC/MS with a mobile phase at pH 9.0, the deterrent activity was found in the very early fractions, between 0 and 3 min, indicating that this pH was not high enough to fully deprotonate a basic column, or that the early elution could be due to additional polar constituents of the molecule, for example sugars. Some deterrent activity was also found in the later fraction eluting between 21 and 40 min which might indicate the aglycon form of an earlier eluting glycosidic compound. When the pH of the mobile phase was raised to 10.0 and the gradient elution system slightly changed to accommodate very polar compounds the deterrent activity was retained on the column and only found in the fraction eluting between 3 and 4 min and not in the later fractions. The change in pH appears to have neutralized very basic compounds, and ultimately resulting in their retention on the column. However, the loss of activity in the later elutin fraction can for the moment not be easily explained.

Based on UV absorption and MS data, there are more than ten compounds present in the fraction between 3 and 4 min, some of these compounds having substituted aromatic group characteristics. Substituted aromatic compounds previously were reported in lettuce, such as sesquiterpene lactones (lactucin, molecular weight 276; and lactucopicrin, molecular weight 410) (Sessa et al. 2000) (Fig. 4-17) and flavonoids

(flavonol glycosides, flavone glycoside and anthocyanidin glycosides) (Dupont et al 2000) (Fig. 4-18). But their biological activity against insects has not been reported in lettuce. However, sesquiterpene lactones provide resistance against lettuce downy mildew and the red spot physiological disorder in certain lettuce cultivars due to its strong antimicrobial properties (Bennett et al. 1994, Bestwick et al. 1995). Sesquiterpene lactones play an antifeedant role in the closely related plant species chicory against *Schistocerca gregaria* (Forsk.) (Rees and Harborne 1985).

The successful isolation of potent feeding deterrents for banded cucumber beetle from a crude extract of romaine lettuce latex provides convincing evidence of a chemical basis for host plant resistance in this variety. Deterrent compounds can be extracted using reversed-phase and cation exchange cartridges (SCX) linked in series, and their retention on cation exchange indicates that they are basic. In addition, LC/MS analysis indicates the presence of substituted aromatic compounds. The chemical composition of the fraction between 3 to 4 min is being investigated. Understanding the defensive role of latex and its deterrent constituents (apart from physical defense due to stickiness) will help to better comprehend the mechanisms of insect-plant interactions. Furthermore, qualitative and quantitative knowledge of these biologically active compounds may help plant breeders select for genotypes with an inherently high level of resistance using these compounds as markers. Insect-susceptible but otherwise horticulturally superior cultivars could also be made more resistant through genetic engineering.

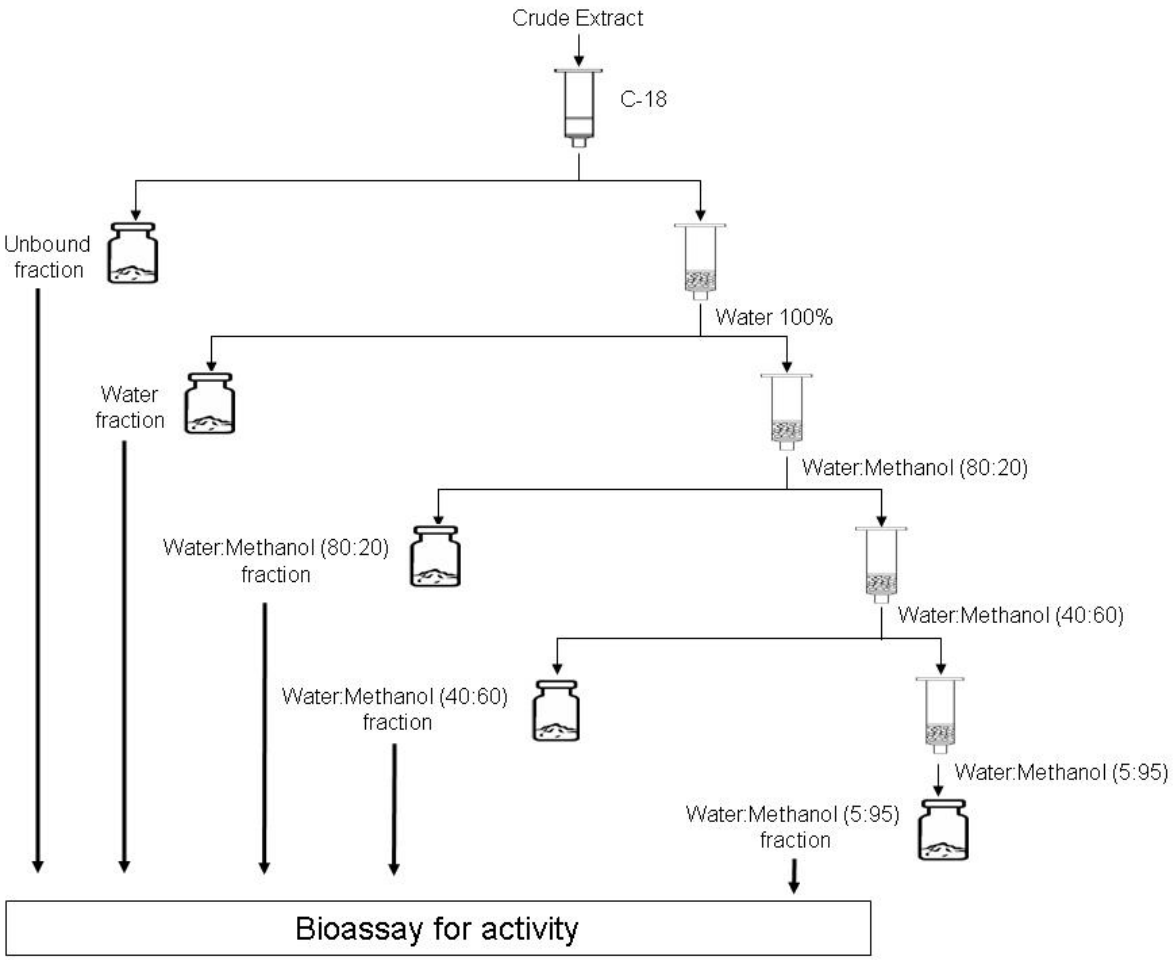


Figure 4-1. Scheme for solid-phase extraction and fractionation of crude extract after passing through reversed-phase (C-18) cartridge.

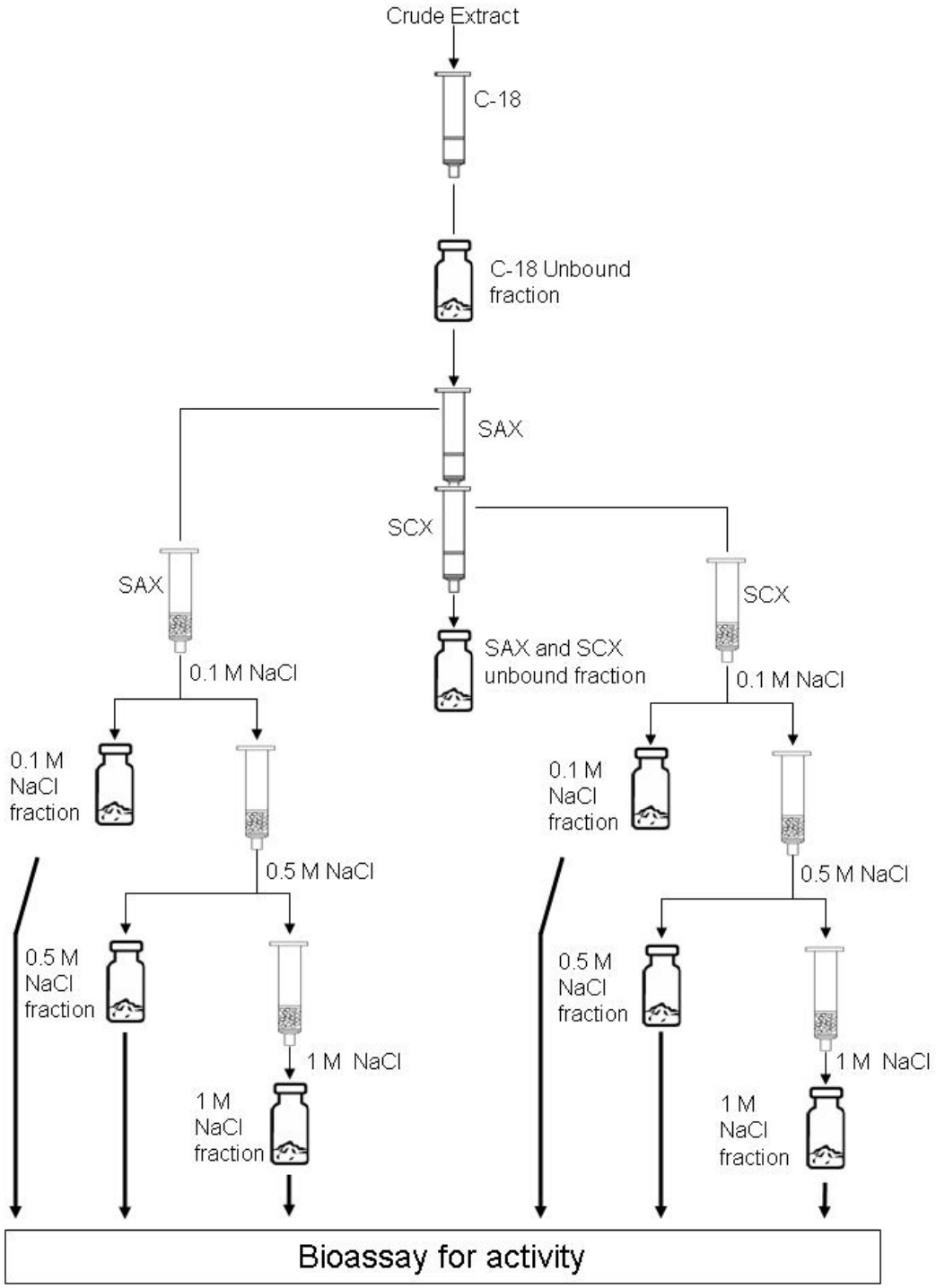


Figure 4-2. Scheme for solid-phase extraction and fractionation of crude extract after passing through reversed-phase (C-18), anion (SAX) and cation (SCX) exchange cartridges connected in series.

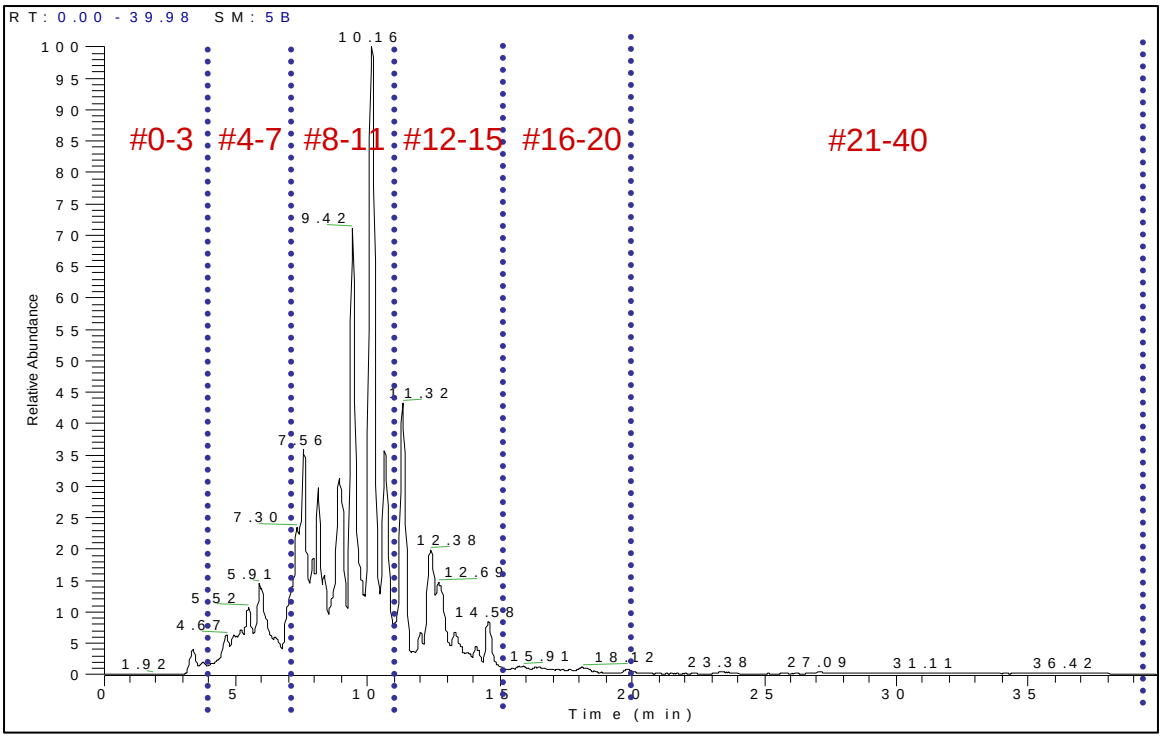


Figure 4-3. Fractions obtained after HPLC analysis of cation exchange (SCX) fraction.

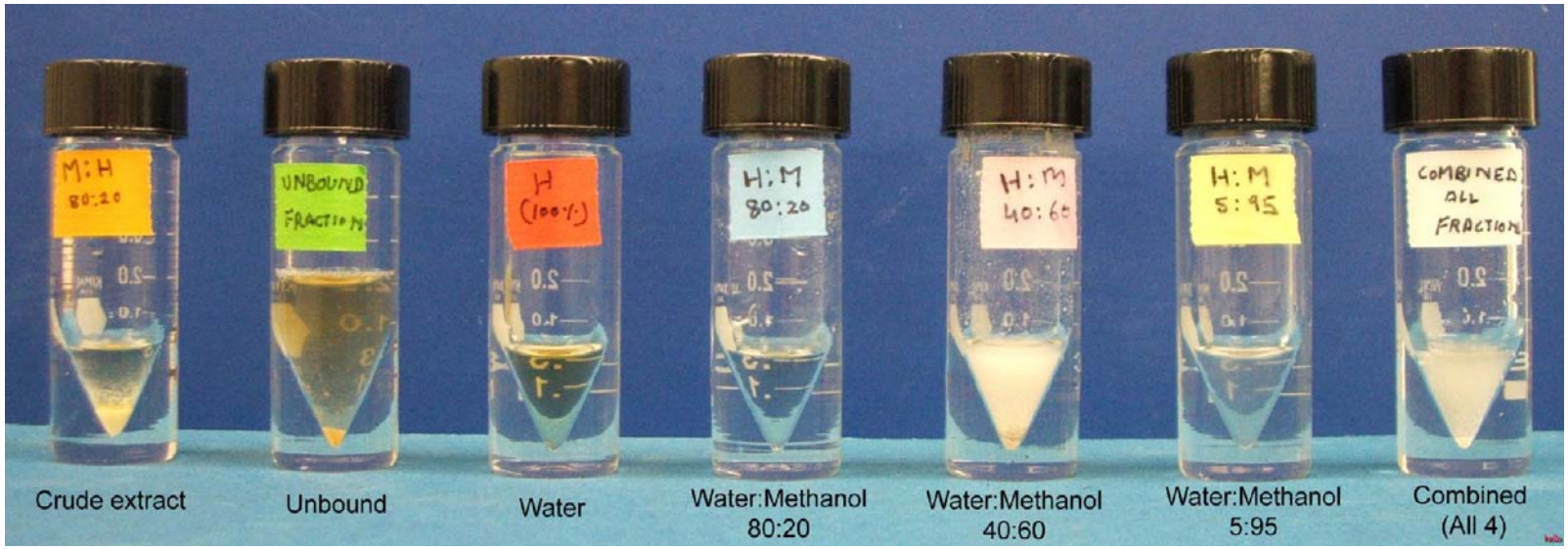


Figure 4-4. Color characteristics of fractions obtained after passing crude extract through reversed phase C-18 cartridge.

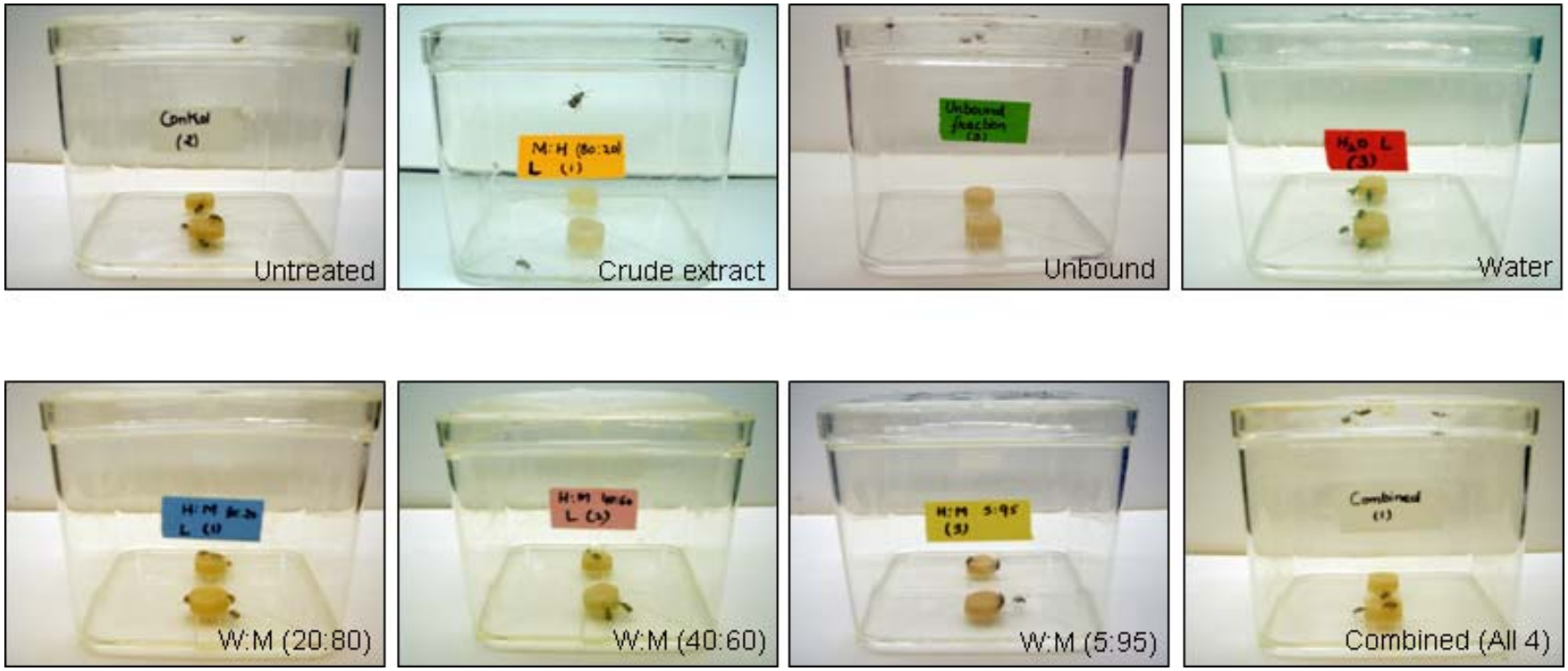


Figure 4-5. Bioassays of C-18 fractions applied on artificial diet disks using *D. balteata* adults under no-choice conditions.

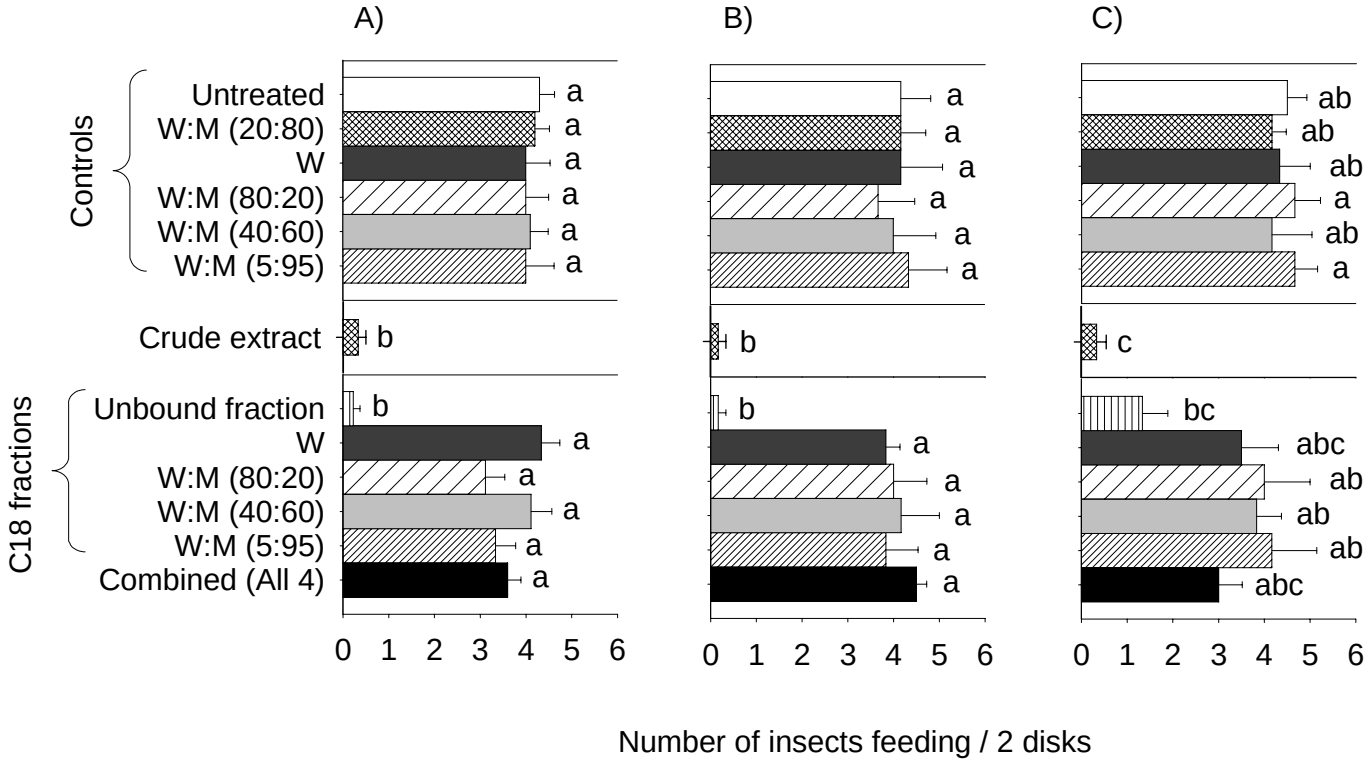


Figure 4-6. Mean number of *D. balteata* adults feeding after 90 min on two artificial diet disks treated with fractions obtained after passing crude extract at three pH levels through C-18 cartridge: A) original (pH 6.5), B) acidic (pH 3.0), and C) basic (pH 9.0). Error bars indicate SEM. Bars topped with different letters within same panel (A, B or C) differ significantly at the 0.05 level (Tukey's HSD test). (W – Water, M – Methanol).

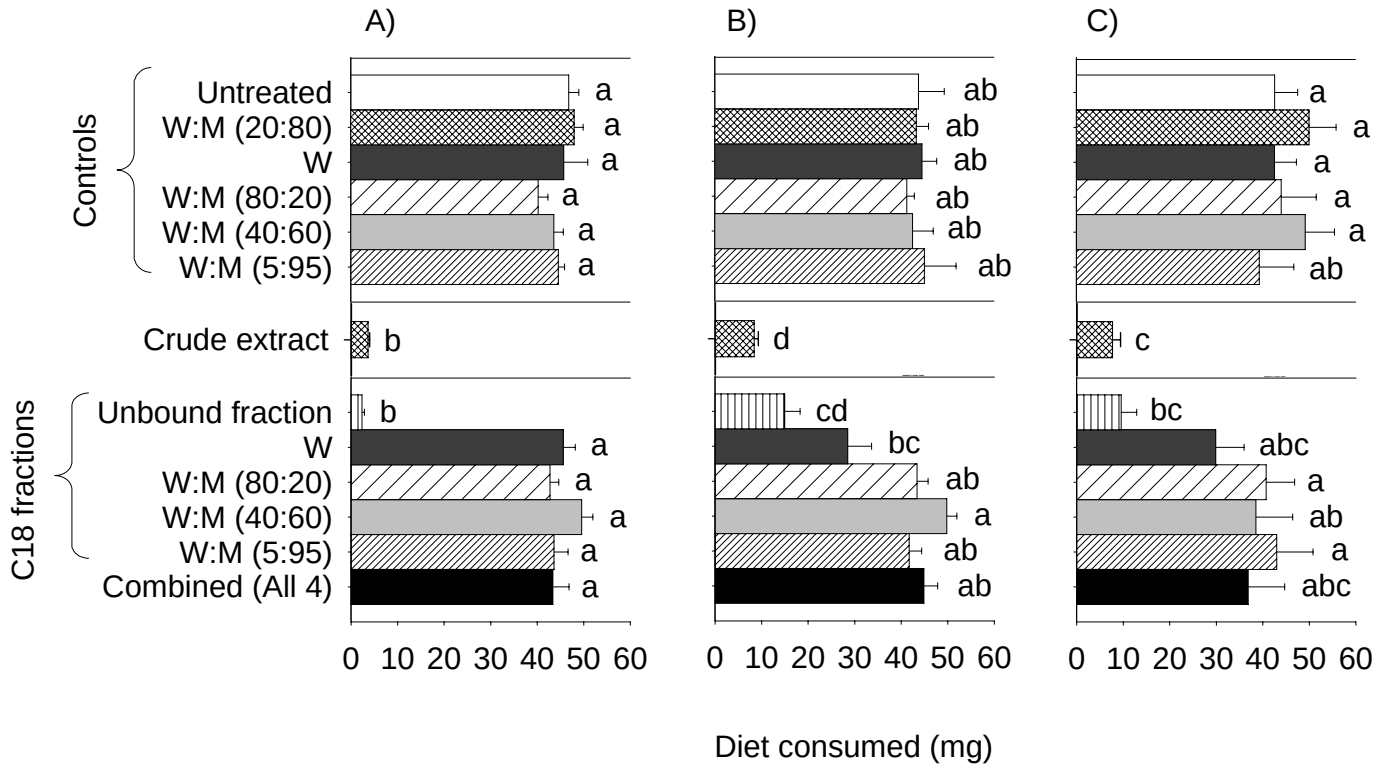


Figure 4-7. Dry weight of diet consumed by *D. balteata* adults when disks were treated with fractions obtained after passing crude extract with different pH levels through C-18 cartridge: A) original (pH 6.5), B) acidic (pH 3.0), and C) basic (pH 9.0). Error bars indicate SEM. Bars topped with different letters within same panel (A, B or C) differ significantly at the 0.05 level (Tukey's HSD test). (W – Water, M – Methanol).

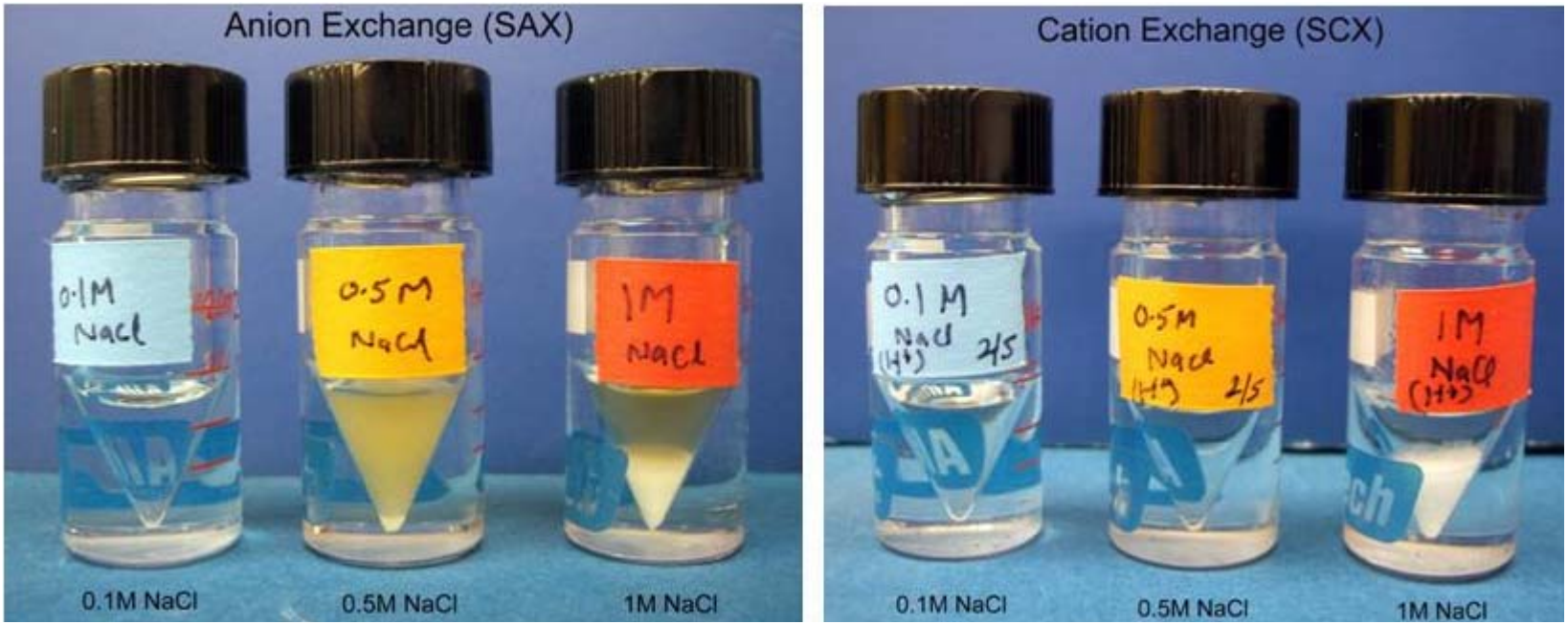


Figure 4-8. Color characteristics of fractions obtained after passing C-18 unbound fraction through anion (SAX) and cation (SCX) exchange cartridges connected in series.

Anion Exchange (SAX) Fractions

Cation Exchange (SCX) Fractions

Figure 4-9. Bioassays of ion-exchange fractions applied on artificial diet disks using *D. balteata* adults under no-choice conditions.

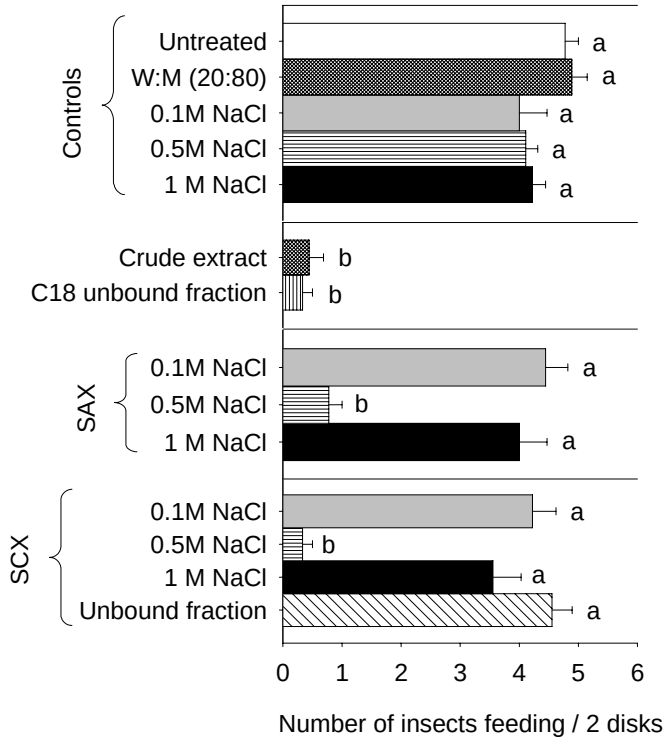


Figure 4-10. Mean number of *D. balteata* adults feeding after 90 min on diet disks treated with ion-exchange fractions obtained by passing C-18 unbound fraction (original pH 6.5) through anion (SAX) and cation (SAX) exchange cartridges connected in series. Error bars indicate SEM. Bars topped with different letters differ significantly at the 0.05 level (Tukey's HSD test).

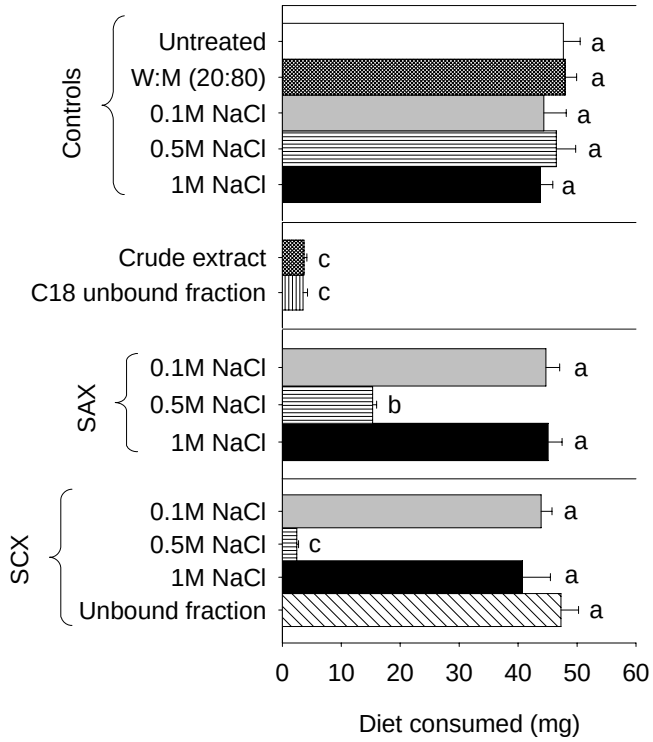


Figure 4-11. Dry weight of diet consumed by *D. balteata* adults when disks were treated with ion-exchange fractions obtained after passing C-18 unbound fraction (original pH 6.5) through anion (SAX) and cation (SAX) exchange cartridges connected in series. Error bars indicate SEM. Bars topped with different letters differ significantly at the 0.05 level (Tukey's HSD test).

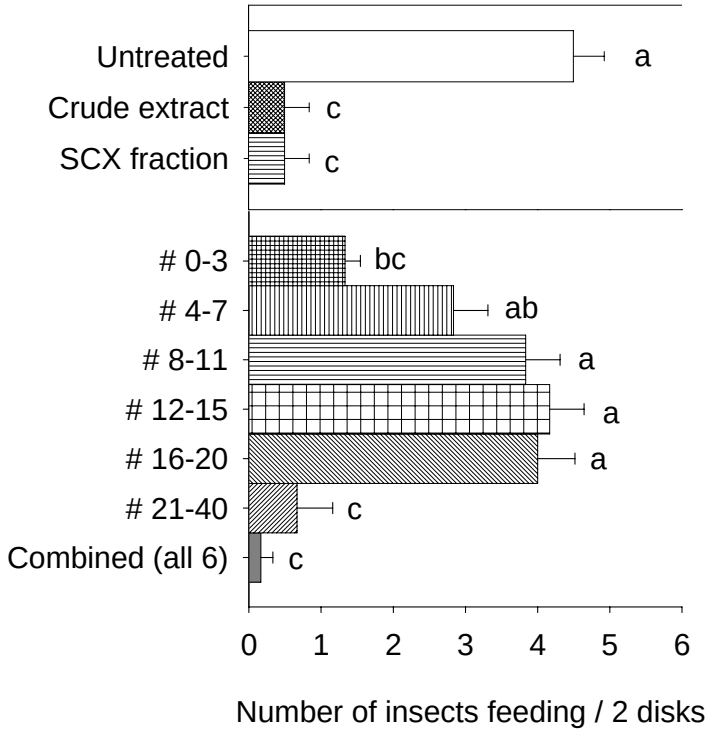


Figure 4-12. Mean number of insects feeding after 90 min on diet disks treated with fractions obtained after LC/MS analysis of cation exchange fraction (SCX) at pH 9.0 of the mobile phase. Error bars indicate SEM. Bars topped with different letters differ significantly at the 0.05 level (Tukey's HSD test).

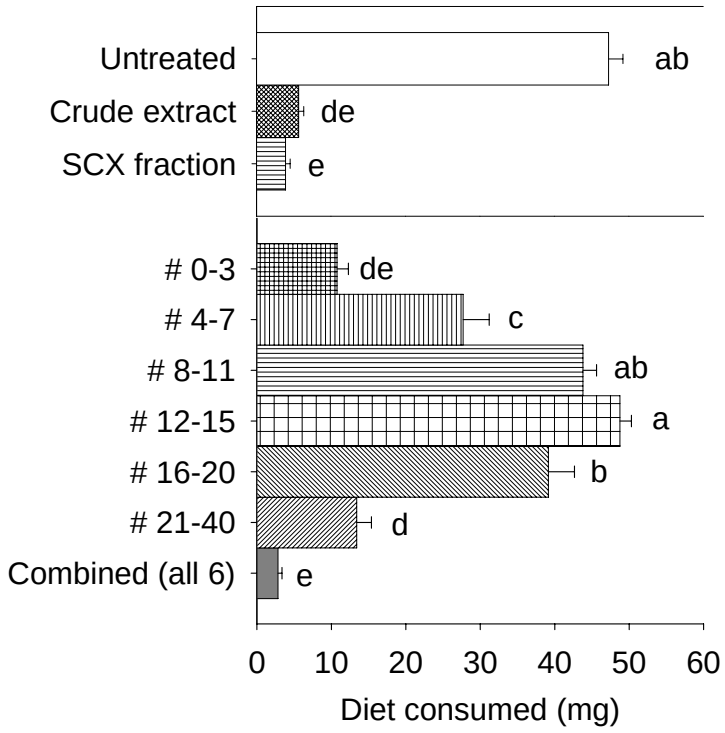


Figure 4-13. Dry weight of diet consumed by *D. balteata* adults when disks were treated with fractions obtained after LC/MS analysis of cation exchange fraction (SCX) at pH 9.0 of the mobile phase. Error bars indicate SEM. Bars topped with different letters differ significantly at the 0.05 level (Tukey's HSD test).

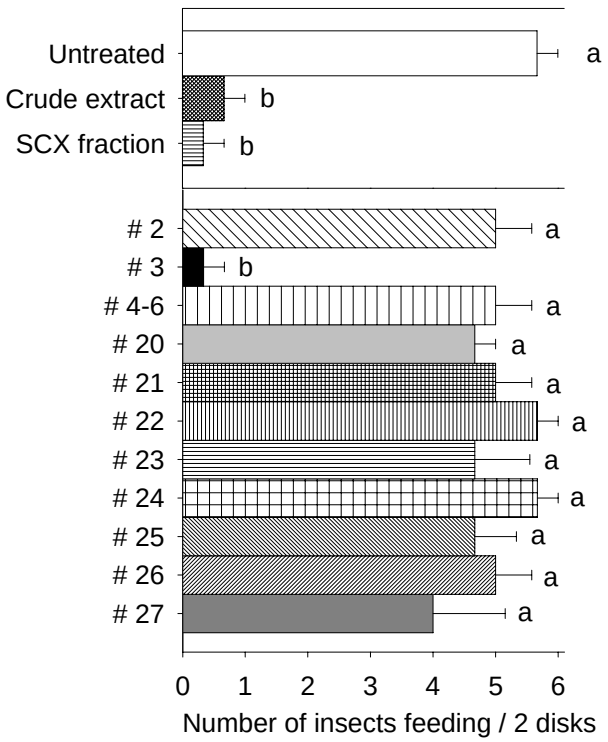


Figure 4-14. Mean number of insects feeding after 90 min on diet disks treated with fractions obtained after LC/MS analysis of cation exchange fraction (SCX) at pH 10.0 of the mobile phase. Error bars indicate SEM. Bars topped with different letters differ significantly at the 0.05 level (Tukey's HSD test).

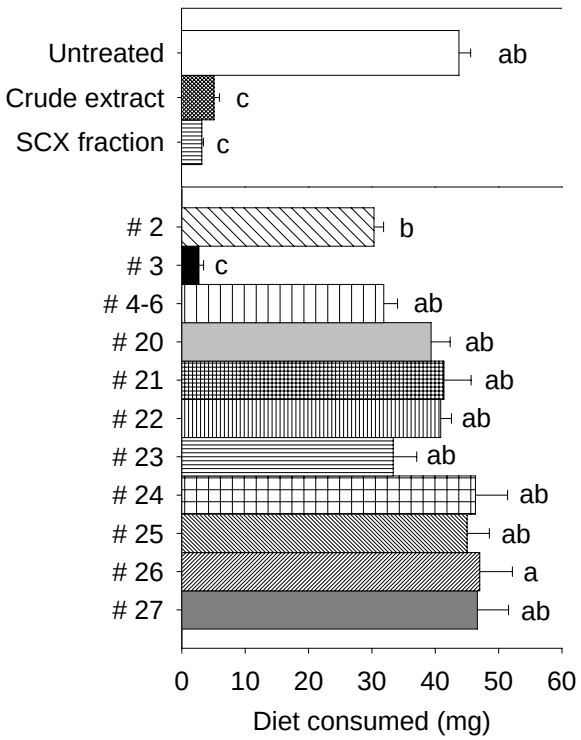


Figure 4-15. Dry weight of diet consumed by *D. balteata* adults when disks were treated with fractions obtained after LC/MS analysis of cation exchange fraction (SCX) at pH 10.0 of the mobile phase. Error bars indicate SEM. Bars topped with different letters differ significantly at the 0.05 level (Tukey's HSD test).

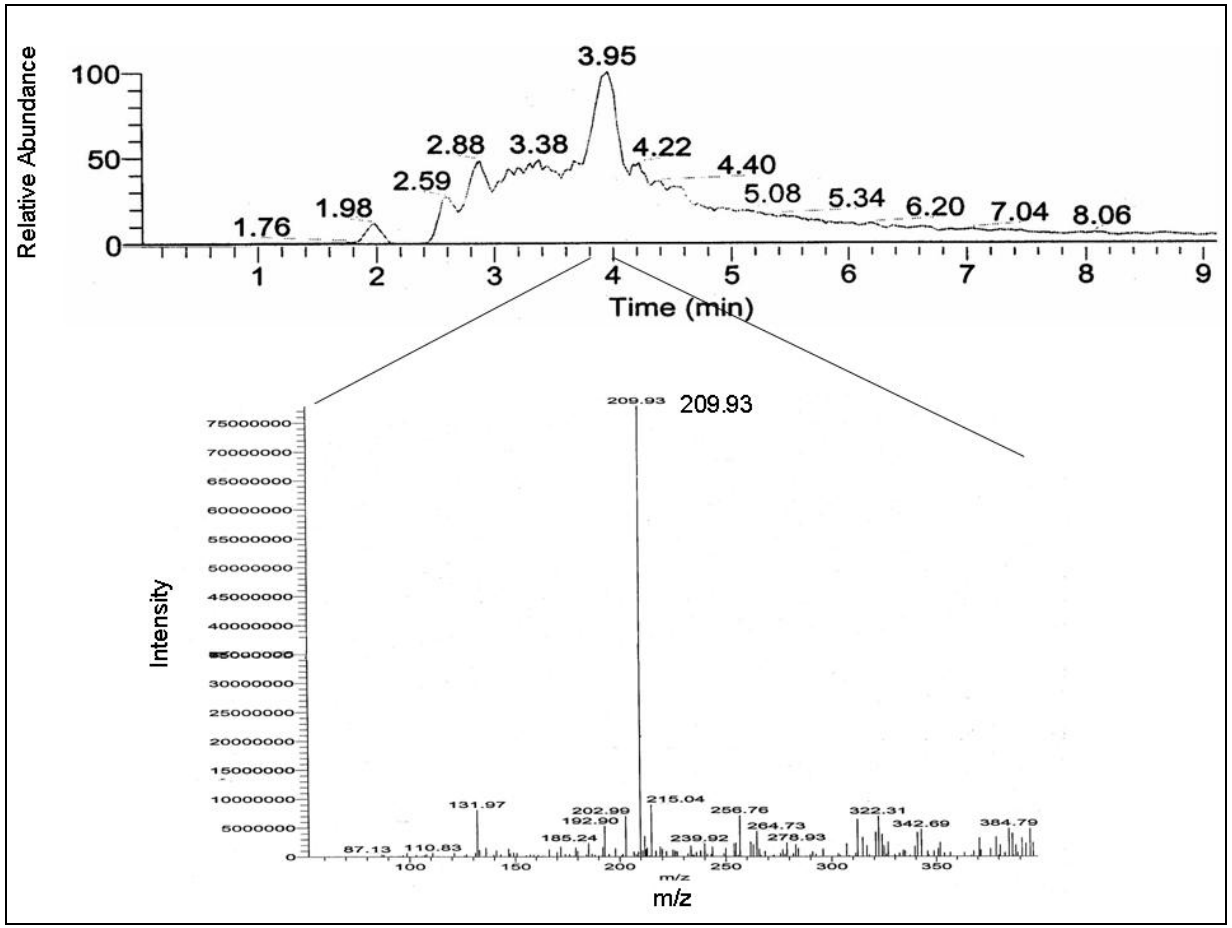
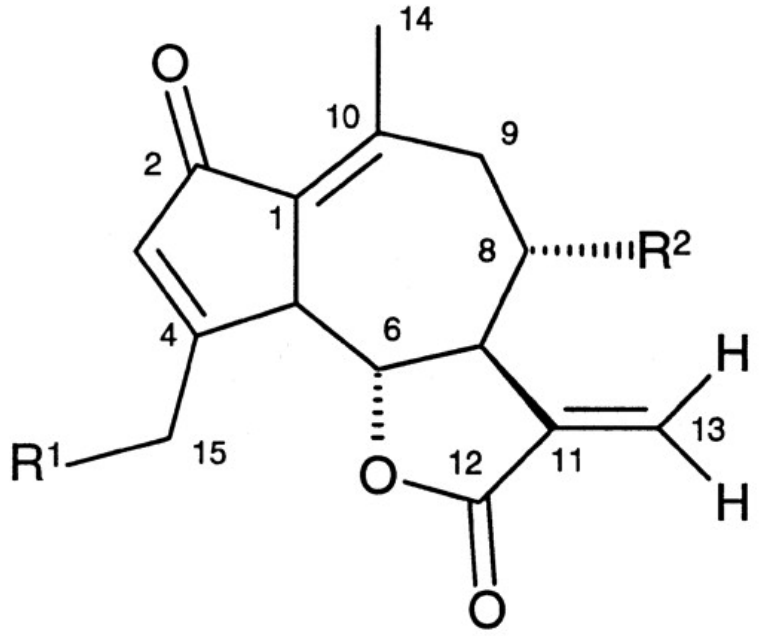


Figure 4-16. Electrospray LC/MS total negative ion trace of active fraction between 3 and 4 min.



lactucin $R^1 = OH; R^2 = OH$

lactucopicrin $R^1 = OH;$

$R^2 = OCOCH_2$  OH

Figure 4-17. Structure of sesquiterpene lactones characterized in lettuce (Sessa et al. 2000).

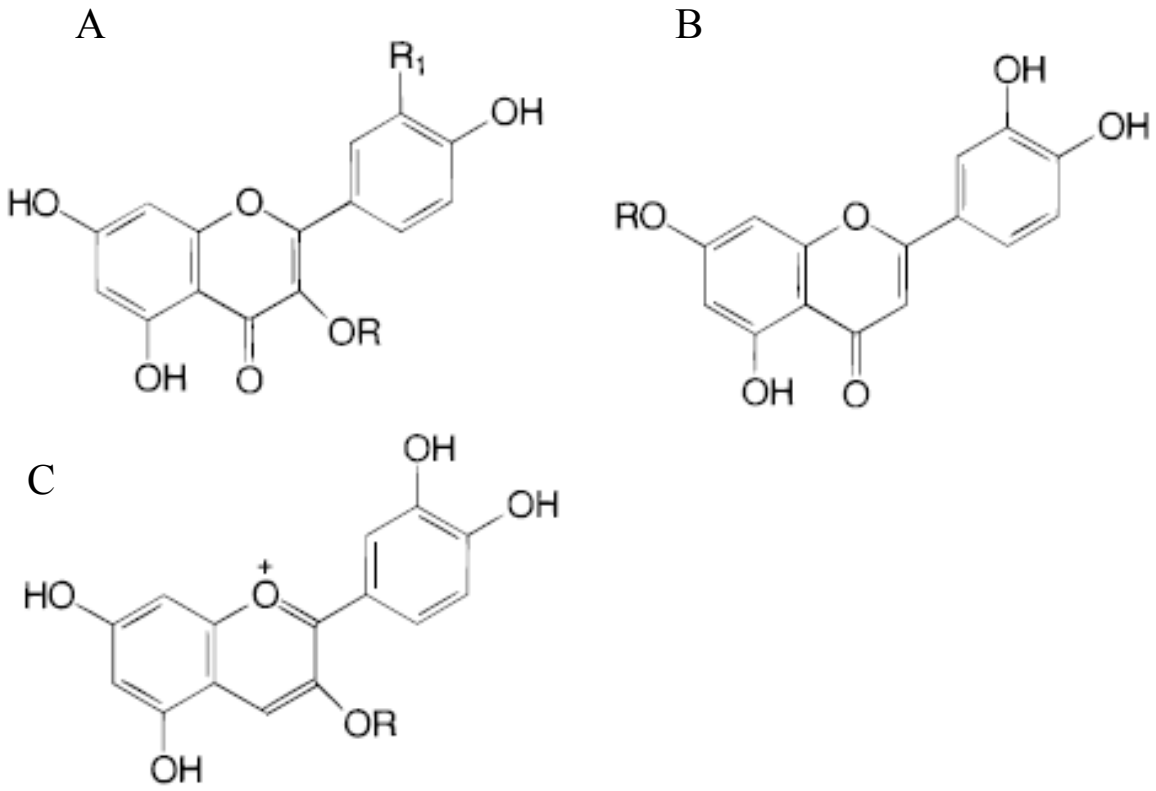


Figure 4-18. Chemical structures of flavonoids found in lettuce A) flavonol glycosides; B) flavone glycoside; and C) anthocyanidin glycosides (kaempferol if R1 = H; quercetin if R1 = OH) (R = glycoside) (Dupont et al. 2000).

CHAPTER 5
INVESTIGATING ENZYME INDUCTION AS A POSSIBLE REASON FOR LATEX-MEDIATED INSECT RESISTANCE IN ROMAINE LETTUCE

Introduction

Lettuce (*Lactuca sativa* L.) is one of the most important vegetable crops grown throughout the world (Ryder 1998). Lettuce growers suffer huge economic losses due to various insect pest infestations because of the very high cosmetic standards demanded by consumers (Palumbo et al. 2006). The romaine lettuce cultivar, 'Valmaine' exhibits a high level of resistance against various insects, including the leafminer, *Liriomyza trifolii* (Burgess) (Nuessly and Nagata 1994), banded cucumber beetle, *Diabrotica balteata* LeConte (Huang et al. 2002) (Fig. 5-1), and two lepidopterans, *Trichoplusia ni* (Hübner) and *Spodoptera exigua* (Hübner) (Chapter 2, Sethi et al. 2006). Valmaine's resistance would be useful in an integrated pest management program however this cultivar is not popular among growers because of its susceptibility to thermodormancy, premature bolting, lettuce mosaic virus and corky root rot (Guzman 1986). Plant breeders have attempted to improve the horticultural characteristics of Valmaine through breeding, but unfortunately the horticulturally improved and currently used cultivar, 'Tall Guzmaine' lost resistance to insects during the process (Chapter 2, Sethi et al. 2006).

My previous research revealed that Valmaine latex placed on artificial diet deterred *D. balteata* feeding, whereas latex from Tall Guzmaine did not (Chapter 3, Sethi et al. 2007). I hypothesize that feeding deterrence due to constitutive levels of compounds in latex may explain the mechanism of multiple insect resistance in Valmaine. Furthermore, previously wounded Valmaine plants showed an increased localized resistance to feeding by *D. balteata* compared to unwounded plants, suggesting the involvement of an

inducible mechanism of resistance (Huang et al. 2003b). Tall Guzmaine showed no such inducible resistance.

Latex is an aqueous suspension or solution of complex mixtures of molecules found in specialized secretory cells of plants known as laticifers (Evert 2006). Laticifers possess high metabolic activity. In addition to synthesizing numerous molecules (lipids, sugars and proteins) required to achieve their basic physiological functions, laticifers are also known to synthesize and store diverse secondary metabolites in appreciable amounts in latex (Moussaoui et al. 2001). Many defensive compounds with demonstrated negative impact on insect fitness are stored in latex (Evans and Schmidt 1976, Haupt 1976, Matile 1976, Noack et al. 1980, Seiber et al. 1982, Nishio et al. 1983, Rees and Harborne 1985, Roberts 1987, Konno et al. 2004, 2006; Ramos et al. 2007). Activity of phenylalanine ammonia lyase, polyphenol oxidase and many other defense-related enzymes is much higher in the laticifers than in the leaves of rubber tree (*Hevea brasiliensis* H.B.K.) (Broekaert et al. 1990, Kush et al. 1990, Martin 1991, Gidrol et al. 1994, Pujade-Renaud et al. 1994, Wititsuwannakul et al. 2002). Wounding of laticifers is also known to induce other defense-related enzymes in latex of papayas (Azarkana et al. 2004, Kydt et al. 2007), fig tree (*Ficus carica* L.) (Kim et al. 2003, Taira et al 2005), rooster tree (*Calotropis procera* Ait.) (Freitas et al. 2007), and Albanian spurge (*Euphorbia characias* L.) (Mura et al. 2005, 2007; Fiorillo et al. 2007). Thus, plant latex acts as a chemical defense due to alteration in its constituents upon insect damage.

The purpose of this study was to investigate the role of inducible enzymes in the latex-mediated multiple insect resistance in Valmaine. I asked the questions of whether enzyme activities changed after insect feeding damage, how quickly this change

occurred, how long the elevated levels lasted, and whether elevated enzyme activity was correlated with increased feeding deterrent activity in latex. Hence, choice experiments were conducted with *D. balteata* adults between diets treated with latex from damaged and undamaged plants within the same cultivar (Valmaine and Tall Guzmaine) to look for changes in latex chemistry after beetle feeding. The induction of defense-related enzymes, in particular phenylalanine ammonia lyase, polyphenol oxidase and peroxidase in latices of resistant Valmaine and susceptible Tall Guzmaine was also compared with and without *D. balteata* feeding damage.

Materials and Methods

Plants

The seeds of romaine lettuce cultivars Valmaine (resistant) and Tall Guzmaine (susceptible) were germinated overnight on moistened filter paper. The germinated seeds were planted in transplant trays filled with Metro Mix 200 (Grace Sierra, Milpitas, CA) and healthy seedlings were transplanted 2 wk later into 15-cm-diameter plastic pots. The plants were watered daily and fertilized with 15 ml of Peters 20-20-20 solution (W.R Grace, Fogelsville, PA) every week. Six-week-old lettuce plants (9-10 true-leaf stage) were used for the experiments. Bush lima bean seeds (*Phaseolus lunatus* L.) of the cultivar Fordhook 242 (Illinois Foundation Seeds, Champagne, IL) were planted in transplant trays filled with Metro Mix 200 and fertilized with the same solution used for lettuce plants.

Insects

The colony of *D. balteata* was started from adults collected from weeds (spiny amaranth, *Amaranthus spinosus* L. and primrose willow, *Ludwigia peruviana* L.) in Belle Glade, Florida in 2003. New adults were added to the established colony in 2005 and

2006 to increase genetic diversity. Larval stages were reared on the roots of corn seedlings and adults were fed on lima bean leaves and sweet potato tubers (Chapter 3, Huang et al. 2002). Unfed adults, within 48 h of emergence, were used for the experiments.

Artificial Diet

Freshly-made southern corn rootworm artificial diet (Bio-Serv, Frenchtown, NJ) was used in all experiments. The diet was prepared according to methods previously described (Chapter 3, Sethi et al. 2007). One-cm-thick disks were punched out from cooled artificial diet using a 1.5-cm-diameter cork borer.

Bioassay Conditions for Feeding Damage

One hundred and eighty plants of each cultivar were placed individually in cylindrical screen cages (18.5 cm diameter × 61.0 cm height) for use in collecting latex from plants after timed, continuous intervals of *D. balteata* feeding. Two male-female beetle pairs were placed into half (90 plants) of the cages of each cultivar, while the other 90 plants of each cultivar were used as undamaged checks. Beetles were allowed to feed on the plants for 6 d. Females were weighed individually before releasing them on the plants, and again at either 1, 3 or 6 d after they were released into the cages, to determine weight change. Latex was collected from plants 1, 3 and 6 d after they were released into the cages. Out of these 180 plants of each cultivar, latex was collected from 60 plants (30 damaged and 30 undamaged checks) at each time interval (1, 3, and 6 d) after initiation of feeding damage. Out of each batch of 30 plants, latex from 15 plants was used for diet disk choice tests and latex from the other 15 was used to assess enzyme activity, as explained below. Each group of 15 plants was further divided into 5 groups (replicates) of 3 plants for the collection of latex. An aloquot of 70 µl of latex was collected from

each group of three plants for use in the assays described below. Latex was collected using a silanized microdispenser (Drummond Scientific Company, Broomall, PA) from the leaf base (site of leaf lamina attachment to the stem, and of rapid latex exudation upon cutting) of young and middle-aged leaves of individual plants 60 s after cutting the tissue with a disposable scalpel blade (Feather, Osaka, Japan). The experiments were carried out at 25 ± 1°C in a laboratory under a photoperiod of 14:10 (L:D) h.

Choice-tests Using Latex from Damaged and Undamaged Plants

Latex (70 µl) collected from plants as described above was applied onto the top and side surfaces of a diet disk, immediately after collection. The experimental unit for the choice-test bioassay consisted of two diet disks, one treated with latex from beetle-damaged plants and the other one with latex from undamaged checks within each cultivar. In the control experimental units, two untreated diet disks were used. The diet disks were placed on the bottom of a plastic ventilated container (10 × 10 × 8 cm) and three male-female pairs of beetles were allowed to feed on the disks for 24 h at 25 ± 1°C in a laboratory. The number of adults feeding on each diet disk was recorded 1, 2, 3 and 4 h after their release into the bioassay units. Dry weight of the diet consumed in 24 h was calculated as previously described in Chapter 3 and in Sethi et al. 2007. Total diet consumed per three pairs of adults in 24 h was calculated by adding the consumption of the two diet disks in each repplicate of each treatment.

Enzyme Activity Assays

Activity of the enzymes phenylalanine ammonia lyase, polyphenol oxidase and peroxidase was assayed in the latices of Valmaine and Tall Guzmaine 1, 3 and 6 d after

initiation of beetle damage. Collected latex was dispensed into a -20°C chilled, 1.5-ml micro-centrifuge tube, on ice and immediately stored at -80°C until analyses.

Frozen latex (70 µl) was dissolved in 5 ml of 50 mM potassium phosphate buffer (pH 6.2) and centrifuged at 48,500 ×g for 45 min at 4°C (Model J2-HS, Beckman Instruments, Fullerton, CA). The supernatant was collected and stored at -80°C until analyses. Total protein and enzyme activities were determined using a spectrophotometer (Model DU 640, Beckman Instruments, Fullerton, CA). Total protein was estimated according to the modified Lowry's method (Peterson 1977) using the Folin-Ciocalteau phenol reagent (Pierce Chemical, Rockford, IL) and bovine serum albumin as a standard.

Phenylalanine ammonia-lyase (PAL). PAL activity in latex was measured as described by Ke and Saltveit (1986) and Campos-Vergas and Saltveit (2002) with slight modifications. The supernatant was analyzed for PAL activity after addition of 200 µl of supernatant to 400 µl of 50 mM L-phenylalanine (dissolved in 20 mM potassium phosphate buffer, pH 8.8) and 400 µl of 50 mM potassium phosphate buffer pH (8.8) and incubated at 40°C for 30 min. The absorbance was measured at 290 nm before and after incubation. PAL activity was expressed as the amount of PAL (µmol mg-1 h-1) that produces 1 µmol of cinnamic acid in 1 h. Cinnamic acid (0 – 400 µmol at an increment of 15 µmol) was used as a reference for quantification of PAL activity.

Polyphenol oxidase (PPO). PPO activity was assayed following the methods of Sirinphanic and Kader (1985) and Loiaza-Velarde et al. (1997) with slight modifications. PPO activity was assessed by incubating 10 µl of supernatant with 500 µl of 1.6% catechol (Sigma, St. Louis, MO), 100 µl of 50 mM potassium phosphate buffer (pH 6.2) and 390 µl distilled water. Absorbance of the mixture was read at 480 nm over a period

of 2 min. One unit of PPO activity was defined as the amount of enzyme that produced an increase in absorbance of 0.1 per min at 480 nm. The linear portion of the curve was used to estimate the reaction rate.

Peroxidase (POX). The activity of POX was determined using the methods of Loiaza-Velarde et al. (1997) with slight modifications. The POX activity was determined by combining 10 µl of H2O2 (30%, v/v) in 50 µl of supernatant, 300 µl of 18 mM guaiacol, 100 µl of 50 mM potassium phosphate buffer (pH 6.2) and 540 µl of distilled water. Absorbance of the resulting mixture was examined at 420 nm over a period of 2 min. The POX activity (µmol mg protein-1 min-1) was determined using guaiacol molar absorptivity (ε = 26.6 M-1 cm-1) at 420 nm. The reaction rate was calculated using the linear portion of the curve.

Statistical Analysis

Data on number of insects feeding on diet disks treated with latex collected from plants with and without prior beetle exposure were analyzed as a repeated measures design using Proc GLIMMIX (SAS Institute 2003). Separate analyses were run for disks from each cultivar at each prior beetle exposure interval (1, 3 and 6 d). The variables latex (from damaged or undamaged plants) and time interval after beetle release (1, 2, 3 and 4 h) were fixed. Fifteen groups of six beetles (i.e., replications) were randomly assigned to each level of latex and tested four times (1, 2, 3 and 4 h). Data on dry weight of diet consumed under choice tests were analyzed using PROC GLM (SAS Institute 2003) with latex and time interval after beetle release as fixed effects. Replications were treated as a random effect for each cultivar.

Data on enzyme activities were analyzed using PROC GLM (SAS Institute 2003) with cultivar, latex treatment (damaged or undamaged), and time interval after feeding

initiation on plants as fixed effects. Replications were again treated as a random effect. Data on beetle fresh weight gain were analyzed using PROC GLM (SAS Institute 2003) with cultivar and time interval after feeding initiation as fixed effects, and replications as a random effect. Tukey's honestly significant difference (HSD) test with a significance level of $\alpha = 0.05$ (SAS Institute 2003) was used for post hoc means separation. Simple regression analysis was done to study the relationship between beetle fresh weight gain and enzymatic activities using PROC REG (SAS Institute 2003).

Results

Oberservations of Latex Characteristics from Damaged and Undamaged Plants

The latex from Valmaine plants damaged for 3 or 6 d browned faster and to a deeper hue than did latex collected after 1 d of feeding damage. However, no such differences were noted in the latex of Tall Guzmaine. The quantity of latex exuded by Tall Guzmaine plants decreased with the duration of feeding damage. Tall Guzmaine latex collected after 3 and 6 d of feeding damage was also less viscous, and more watery and translucent, while latex quality in Valmaine did not differ visually.

Choice-tests Using Latex from Damaged and Undamaged Plants

In case of Valmaine choice tests, type of latex 1 d after feeding initiation did not have significant effect on the number of insects feeding on the diet disks ($F = 2.0851$; df = 1, 8; $P = 0.1585$), but latex after 3 ($F = 18.96$; df = 1, 8; $P = 0.0001$) and 6 d ($F = 14.43$; df = 1, 8; $P = 0.0005$) after feeding initiation had significant effects. The number of *D. balteata* adults feeding on disks treated with latex from Valmaine plants that had been fed on for 1 d was not significantly different from the number feeding on disks treated with latex from undamaged Valmaine plants (Fig. 5-2 and 5-3). However, there were significant differences between disks treated with Valmaine latex from plants with

and without feeding after 3 and 6 d. In Tall Guzmaine choice tests, latex anytime after feeding initiation did not have any significant effect on the number of beetle feeding on diet disks (1 d: $F = 0.0753$; df = 1, 8; $P = 0.7855$; 3 d: $F = 0.800$; df = 1, 8; $P = 0.7791$, 6 d: $F = 0.0468$; df = 1, 8; $P = 0.8301$). The number of beetles feeding on diet disks treated with latex from damaged Tall Guzmaine plants or with latex from undamaged plants did not differ significantly at any time after initiation of feeding damage (Fig. 5-2 and 5-4).

In the Valmaine choice test, latex (damaged or undamaged) had significant effect on diet consumption by the beetles ($F = 72.02$; df = 1, 24; $P = 0.0001$). Adults of *D. balteata* consumed significantly less diet treated with latex from damaged plants than diet treated with latex from undamaged plants (Fig. 5-5). Time interval (1, 3, and 6 d) after feeding initiation on plants did not have significant effect on diet consumption by the beetles ($F = 1.08$; df = 2, 24; $P = 0.3548$). But there was significant interaction between latex and time interval ($F = 3.67$; df = 2, 24; $P = 0.0406$). The amount of diet eaten from disks treated with latex from damaged plants decreased with increasing duration of beetle feeding on plants, whereas the amount of diet eaten from disks treated with latex from undamaged plants was constant across the three time intervals after initiation of feeding (Fig. 5-5). In the Tall Guzmaine choice test, latex did not have any significant effect on diet consumption by beetles ($F = 0.2160$; df = 1, 24; $P = 0.6463$). Diet consumption by *D. balteata* adults on diet treated with latex from damaged plants did not differ significantly than on diet treated with latex from undamaged plants (Fig. 5-5). Neither significant effect of time interval ($F = 0.60$; df = 2, 24; $P = 0.5592$), nor significant interaction between latex and time interval ($F = 2.04$; df = 2, 24; $P = 0.1521$) on diet consumption was found.

Treatment of latex significantly affected the total diet consumption in choice tests (F = 235.08; df = 2, 33; P = 0.0005). Total diet consumed by six *D. balteata* was significantly less on Valmaine latex treated diet compared to Tall Guzmaine latex treated and control diets (Table 5-1). Diet consumption did not change significantly when disks were treated with latex collected from damaged plants at different time intervals (F = 1.11; df = 2, 33; P = 0.3412). No significant interaction was found between type of latex and time interval after feeding initiation (F = 0.6330; df = 4, 33; P = 0.6425).

Total Protein Content

Type of cultivar had significant effect on the total protein content (F = 91.77; df = 1, 47; P = 0.0001). Total protein content was significantly higher (1.3 fold) in Valmaine latex than in Tall Guzmaine latex (Fig. 5-6). No significant effect of treatment (damaged or undamaged) was found on total protein content (F = 1.49; df = 1, 47; P = 0.2281). But significant effect of time interval after feeding damage (1, 3 and 6 d) was found (F = 5.29; df = 2, 47; P = 0.0084). Significant interactions were found between cultivar and treatment (damaged or undamaged) (F = 16.70; df = 1, 47; P = 0.0002), and between cultivar and time interval after feeding damage (F = 7.61; df = 2, 47; P = 0.0013). Total protein content in Valmaine after 6 d of feeding damage was 1.36 fold higher than after 1 d. There was no increase protein content of Tall Guzmaine latex through time.

Phenylalanine Ammonia Lyase

The effect of cultivar was significant on PAL activity (F = 289.82; df = 1, 47; P = 0.0001). The activity of PAL was significantly higher (3.44 fold) in Valmaine latex than in Tall Guzmaine latex (Fig. 5-7). Both treatment (F = 98.45; df = 1, 47; P = 0.0001) and time interval after feeding initiation (F = 7.96; df = 2, 47; P = 0.0010) had significant effect on PAL activity. Significant interactions were found between cultivar and

treatment (F = 20.96; df = 1, 47; P = 0.0001), and between cultivar and time interval after initiation of feeding damage (F = 7.36; df = 2, 47; P = 0.0016). PAL activity in Valmaine latex was significantly increased after 3 d (1.81 fold) and 6 d (1.54 fold) of feeding damage, relative to 1 d after initiation of feeding. No increase was seen in PAL activity in the latex of Tall Guzmaine through time**.**

Polyphenol Oxidase

Type of cultivar had significant effect on PPO activity (F = 358.32; df = 1, 47; P = 0.0001). The activity of PPO was significantly higher (4.37 fold) in Valmaine latex than in Tall Guzmaine latex (Fig. 5-8). Both treatment (F = 80.31; df = 1, 47; P = 0.0001) and time interval after feeding initiation (F = 8.25; df = 2, 47; P = 0.0008) had significant effect on PPO activity. Significant interactions were found between cultivar and treatment (F = 74.86; df = 1, 47; P = 0.0001), and between cultivar and time interval after feeding damage (F = 11.65; df = 2, 47; P = 0.0016). PPO activity was significantly increased 3 d (1.74 fold) and 6 d (1.78 fold) after feeding damage in Valmaine latex, but not in Tall Guzmaine latex.

Peroxidase

The POX activity was significantly affected by the type of cultivar (F = 35.49; df = 1, 47; P = 0.0001). The activity of POX was significantly higher (2.1 fold) in Valmaine latex than in Tall Guzmaine latex (Fig. 5-9). Both treatment (F = 39.29; df = 1, 47; P = 0.0001) and time interval after feeding initiation (F = 4.92; df = 2, 47; P = 0.0113) had significant effect on POX activity. Significant interactions were found between cultivar and treatment (F = 35.45; df = 1, 47; P = 0.0001), and between cultivar and time interval after feeding damage (F = 5.16; df = 2, 47; P = 0.0094). POX activity was significantly

increased 3 d (1.56 fold) and 6 d (2.1 fold) after feeding damage in Valmaine latex but not in Tall Guzmaine latex.

Relationship between Female Weight Gain and Enzyme Activity

Cultivar had significant effect on gain in female fresh weight (F = 1269.92; df = 1, 23; P = 0.0001). Female beetles weighed significantly less when fed on Valmaine than Tall Guzmaine (Fig. 5-10). Both time interval after feeding initiation on plants (F = 30.42; df = 2, 23; P = 0.0001) and interaction between cultivar and time interval (F = 161.35; df = 2, 23; P = 0.0001) had significant effect on female fresh weight gain. Females feeding on Tall Guzmaine weighed 2.2, 12.1, and 50.8 times more than the females on Valmaine after 1, 3 and 6 d of feeding on the plants, respectively. Beetles lost weight over time on Valmaine whereas they gained weight on Tall Guzmaine (Fig. 5-10). Furthermore, a significant negative relationship was found between female fresh weight gain and activities of each enzyme (PAL, PPO and POX) in latex from damaged plants of Valmaine (Fig. 5-11). No significant relationship was found between female fresh weight gain and any of the enzyme activities of latex from Tall Guzmaine.

Discussion

Valmaine latex from damaged plants was more deterrent compared to latex from undamaged plants. This may be due to the change in the concentration of its constituents. Upon wounding, latex turns brown after sometime due to the production of quinones that are catalyzed by PPO. The browning potential of the latex from damaged Valmaine plants was observed to increase with time after feeding damage. The browning is much darker in color in a disease-resistant clone of rubber tree than in a susceptible clone (Wititsuwannakul et al. 2002). Increased intensity of browning may be due to the higher activity of PPO. The intensity of browning was observed to remain the same in Tall

Guzmaine latex after beetle damage. In fact, the intensity of browning was less in latex from undamaged Tall Guzmaine plants than in latex from undamaged Valmaine plants. Tall Guzmaine damaged plants produced less latex which was also less viscous, and more watery and translucent, while the amount of latex production and its viscosity and color (miliky white) remained the same in Valmaine latex even after beetle damage. Such differences in milkiness arise due to differences in the refractive indices of the dispersing particles (mainly terpenoids) and the dispersing medium (Esau 1965, Fahn1990). Thus, the production of these dispersing particles in Tall Guzmaine may have been reduced after feeding damage or the loss of large amounts of latex during beetle feeding may have reduced the concentration of these compounds. The amount of total protein increased in latex from Valmaine after beetle damage while it did not change in Tall Guzmaine. Ni et al. (2001) also found a significant increase in the total protein content in wheat cultivars after damage by the Russian wheat aphid.

The activities of all three enzymes, PAL, PPO and POX were increased significantly in Valmaine latex after 3 d of *D. balteata* feeding damage while they were same in Tall Guzmaine latex. Even the constitutive level of PAL and PPO in undamaged plants was significantly higher in Valmaine latex than in Tall Guzmaine latex. Alteration in the levels of these enzymes due to insect feeding has been observed by many other workers (Green and Ryan 1972, Cole 1984, Hildebrand et al. 1986, Felton 1989, Felton et al. 1994a, b; Miller et al. 1994, Rafi et al. 1996, Jerez 1998, Stout et al. 1999, Constabel et al. 2000, Chaman et al. 2001, Ni et al. 2001, Heng-Moss et al. 2004). The rate of secondary metabolism via the phenylpropanoid pathway, leading to production and accumulation of soluble phenolic compounds, is greatly increased after wounding of

lettuce tissue (Tomás-Barberán et al. 1997, Saltveit et al. 2005). The production of phenylpropanoid compounds plays an important role in plant defense (Hahlbrock and Scheel 1989). Phenylalanine ammonia lyase is the first committed enzyme in the phenylpropanoid pathway (Dixon and Paiva 1995). Its de novo synthesis and increased activity is an initial response to wounding (Lopez-Galvez et al. 1997, Thomás-Barberán et al. 1997, Campos-Vergas and Saltveit 2002) that ultimately results in increased concentrations of phenolic compounds (Loaiza-Velarde et al. 1997). The phenylpropanoid pathway starts with the deamination of phenylalanine to cinnamic acid due to the action of PAL. The enhanced activity of PAL results in an increased production and accumulation of several phenolic compounds that are sequestered in the vacuole. These compounds can be oxidized to strong electrophillic quinones (brown substances) by PPO when membranes become disrupted. In addition, wounding also results in an increased expression of POX and lignin formation (Luh and Phithakpol 1972, Ribereau Gayon 1972, Robinson 1972, Hanson and Havir 1979, Rhodes et al. 1981).

Higher activity of PAL was found in resistant cultivars of lettuce infested with lettuce root aphid, *Pemphigus bursarius* (L.) (Cole 1984) and barley infested with greenbug, *Schizaphis graminum* (Rondani) (Chaman et al. 2003). The activity of PAL was also increased in strawberry leaves as a result of infestation by twospotted spider mite, *Tetranychus urticae* (Inoue et al. 1985).

Insect resistance in many plant species (soybean, tomato, potato, cotton, rubber tree, poplar and barley) has been associated with higher activity of PPO (Gregory and Tingey 1981, Hedin et al. 1983, Felton et al. 1989, Duffey and Felton 1991, Steffens and

Walter 1991, Bi et al. 1993, Felton et al. 1994a, Constabel et al. 1996, Wititsuwannakul et al. 2002, Wang and Constabel 2004, Chaman et al. 2001). Peroxidase activity is also known to increase in tomato and barley after infestation with corn earworm, *Helicoverpa zea* Boddie (Stout et al. 1999) and greenbug (Chaman et al. 2001), respectively.

Earlier tests by Huang et al. 2003 found only localized induced resistance in Valmaine after 2 d of *D. balteata* damage. It is possible the 2 d feeding duration was not long enough to induce increased resistance (Schoonhoven et al. 2006). In our study, significant increases in the levels of all the three enzymes (PAL, POX and PPO) were only found at 3 and 6 d after feeding damage, but not after 1 d of feeding on Valmaine. Female beetles confined for 1 d on Valmaine plants had gained weight, lending support to the hypothesis that increased resistance is only induced after at least 2 d of feeding. Beetles were observed tunneling, and presumably feeding, in the midrib tissue near the proximal end of the leaf. However, after 3 d, beetles did not feed much and lost weight over the remaining 3 d of the experiment. So, beetles may have stopped feeding due to induced resistance. Under these conditions, plants may have reached an equilibrium of defensive compounds concentrations and enzyme activities, and stopped further increment in the activities of these enzymes to save energy for development and growth. I also found a strong relationship between female weight gain and activities of all the three enzymes (PAL, PPO and POX), indicating a possible correlation between increased enzymes activities and decreased beetle fitness.

Based on my results, I hypothesize that increased levels of PAL, PPO and POX in Valmaine after *D. balteata* damage result in increased production of secondary metabolites and other unknown defensive compounds. Consequently, induced resistance

in Valmaine acts synergistically with the constitutive resistance of latex and ultimately enhances its resistance against *D. balteata*. Further research is required to characterize these damage-inducible enzymes at the molecular level to support breeding programs for the development of resistant cultivars with superior horticultural traits using either conventional or transgenic approaches.

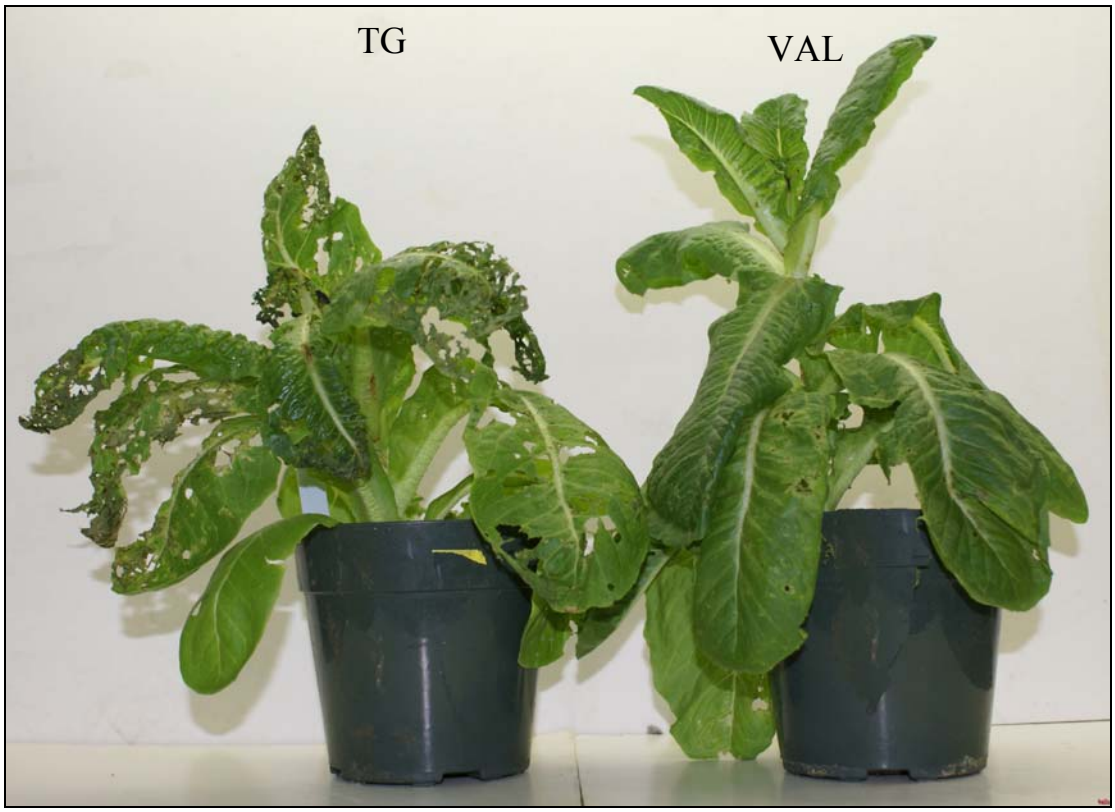


Figure 5-1. Feeding damage caused by *D. balteata* adults on two lettuce cultivars, Valmaine (VAL) and Tall Guzmaine (TG).

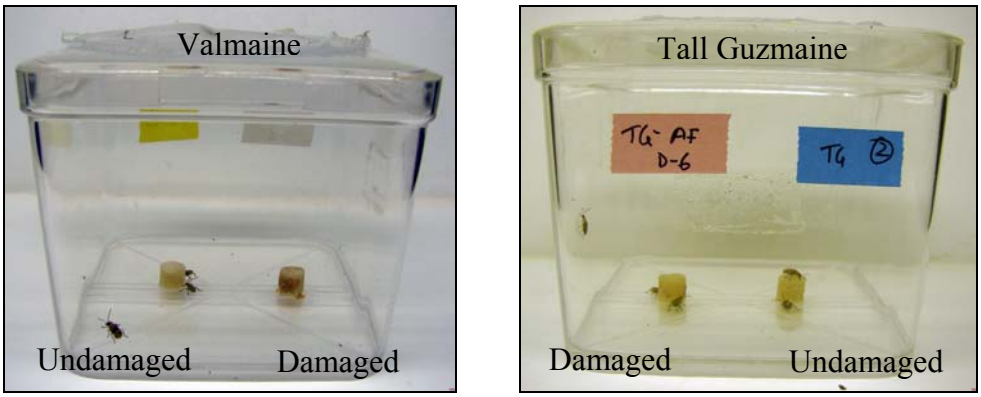


Figure 5-2. Adults of *D. balteata* feeding on diet disks treated with latex from damaged and undamaged plants of two lettuce cultivars, Valmaine and Tall Guzmaine.

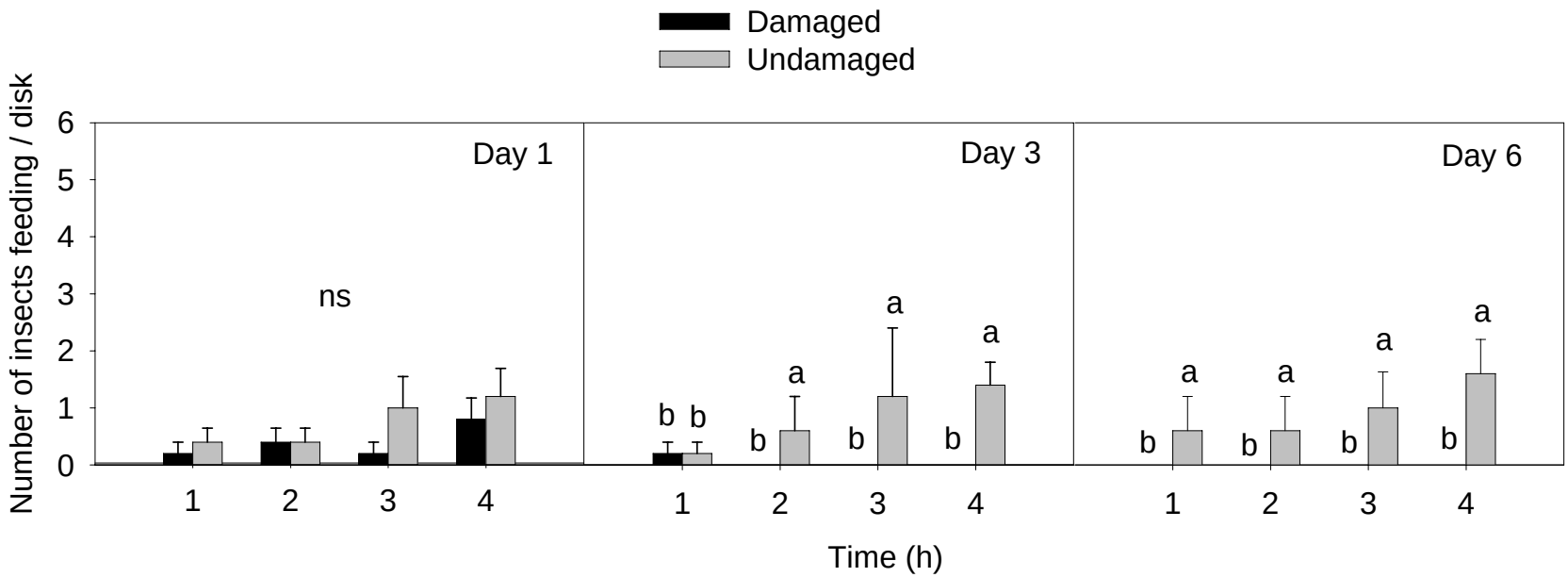


Figure 5-3. Number of *D. balteata* adults feeding on artificial diet disks in a choice between latex from damaged and undamaged plants of Valmaine after 1, 2, 3 and 4 h of their release. Error bars indicate SEM. Bars topped with different letters within panel (day 1, 3 or 6) differ significantly at the 0.05 level (Tukey's HSD test).

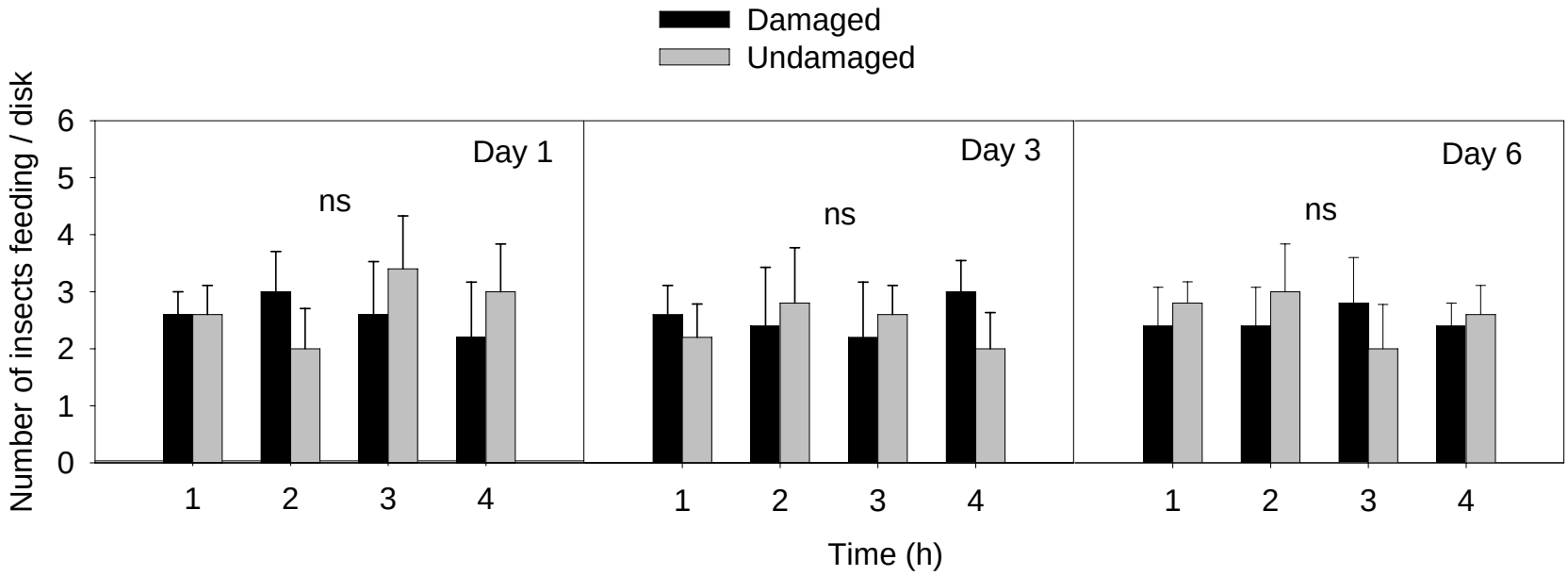


Figure 5-4. Number of *D. balteata* adults feeding in a choice test using two artificial diet disks treated with damaged and undamaged plants of lettuce cultivar, Tall Guzmaine after 1, 2, 3 and 4 h of their release. Error bars indicate SEM. Bars topped with different letters within panel (day 1, 3 or 6) differ significantly at the 0.05 level (Tukey's HSD test).

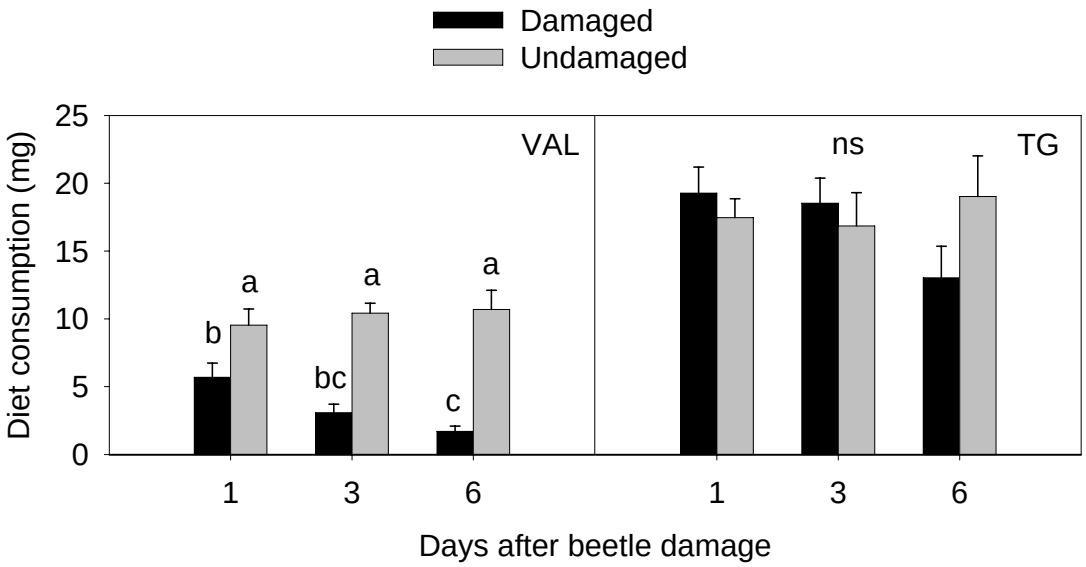


Figure 5-5. Artificial diet consumption after 24 h by *D. balteata* adults in choice test using two diet disks treated with latex from damaged and undamaged plants of two lettuce cultivars, Valmaine (VAL) and Tall Guzmaine (TG). Error bars indicate SEM. Bars topped with different letters with panel (VAL or TG) differ significantly at the 0.05 level (Tukey's HSD test).

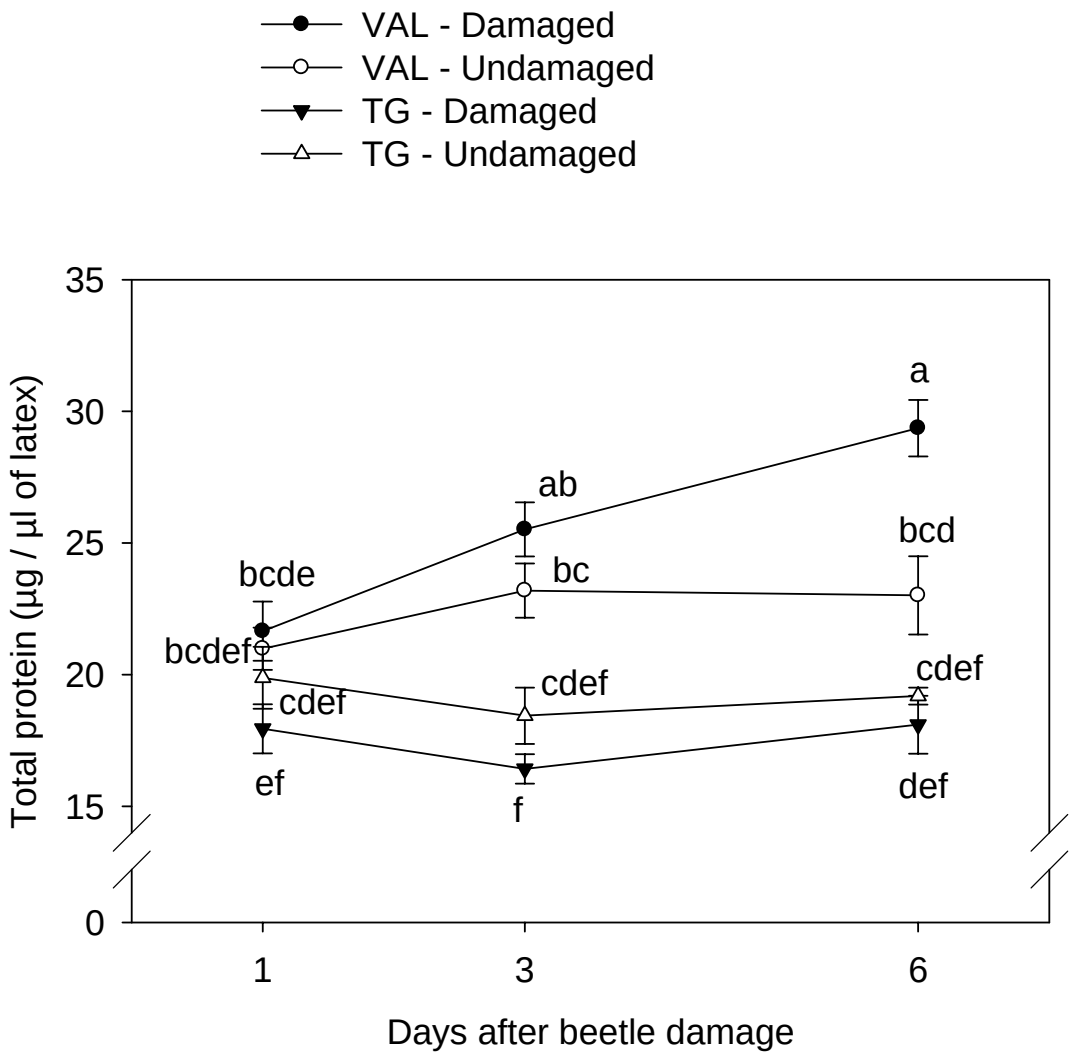


Figure 5-6. Total protein content in two lettuce cultivars, Valmaine (VAL) and Tall Guzmaine at 1, 3 and 6 d after initiation of feeding damage by adults of D. balteata. Error bars indicate SEM. Points topped with different letters differ significantly at the 0.05 level (Tukey's HSD test).

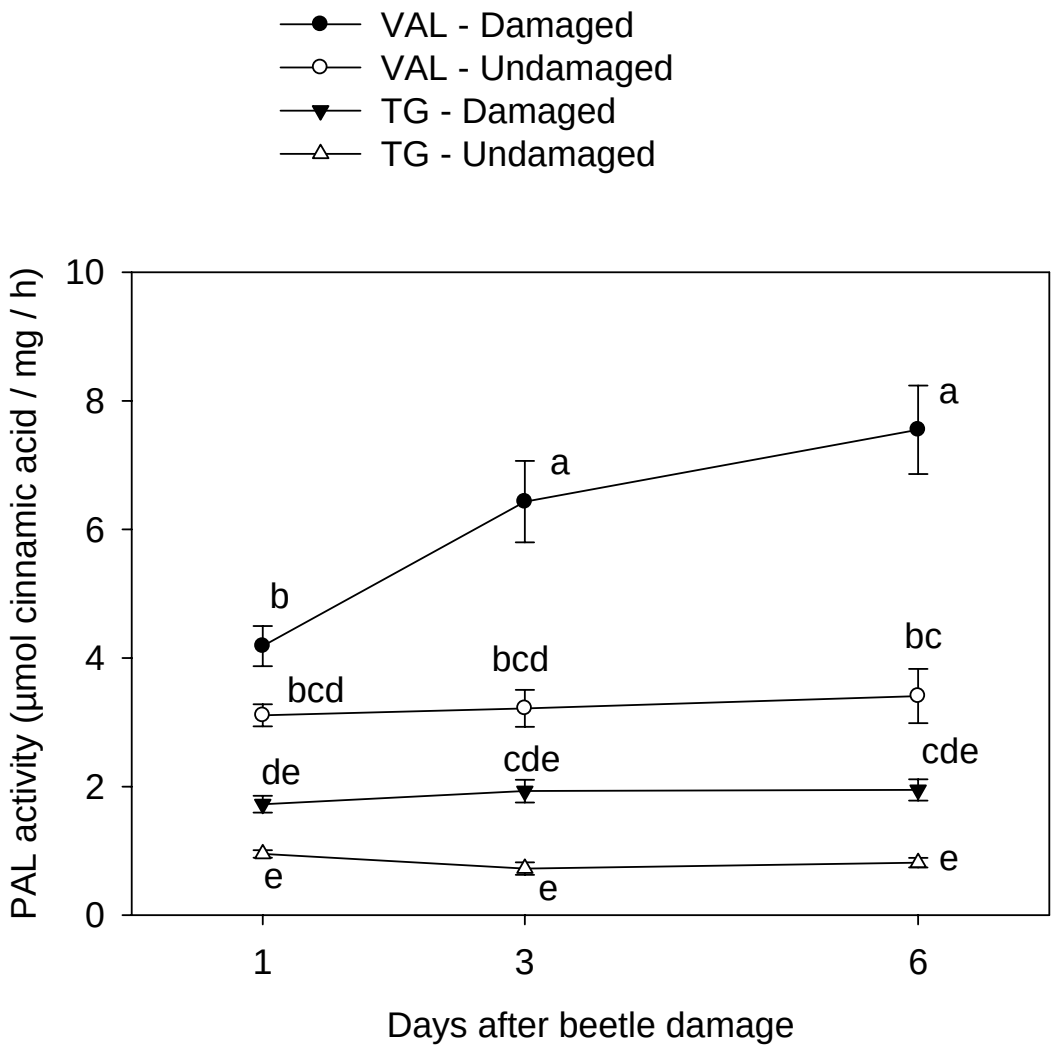


Figure 5-7. Activity of phenylalanine ammonia lyase (PAL) in two lettuce cultivars, Valmaine (VAL) and Tall Guzmaine at 1, 3 and 6 d after initiation of feeding damage by adults of *D. balteata*. Error bars indicate SEM. Points topped with different letters differ significantly at the 0.05 level (Tukey's HSD test).

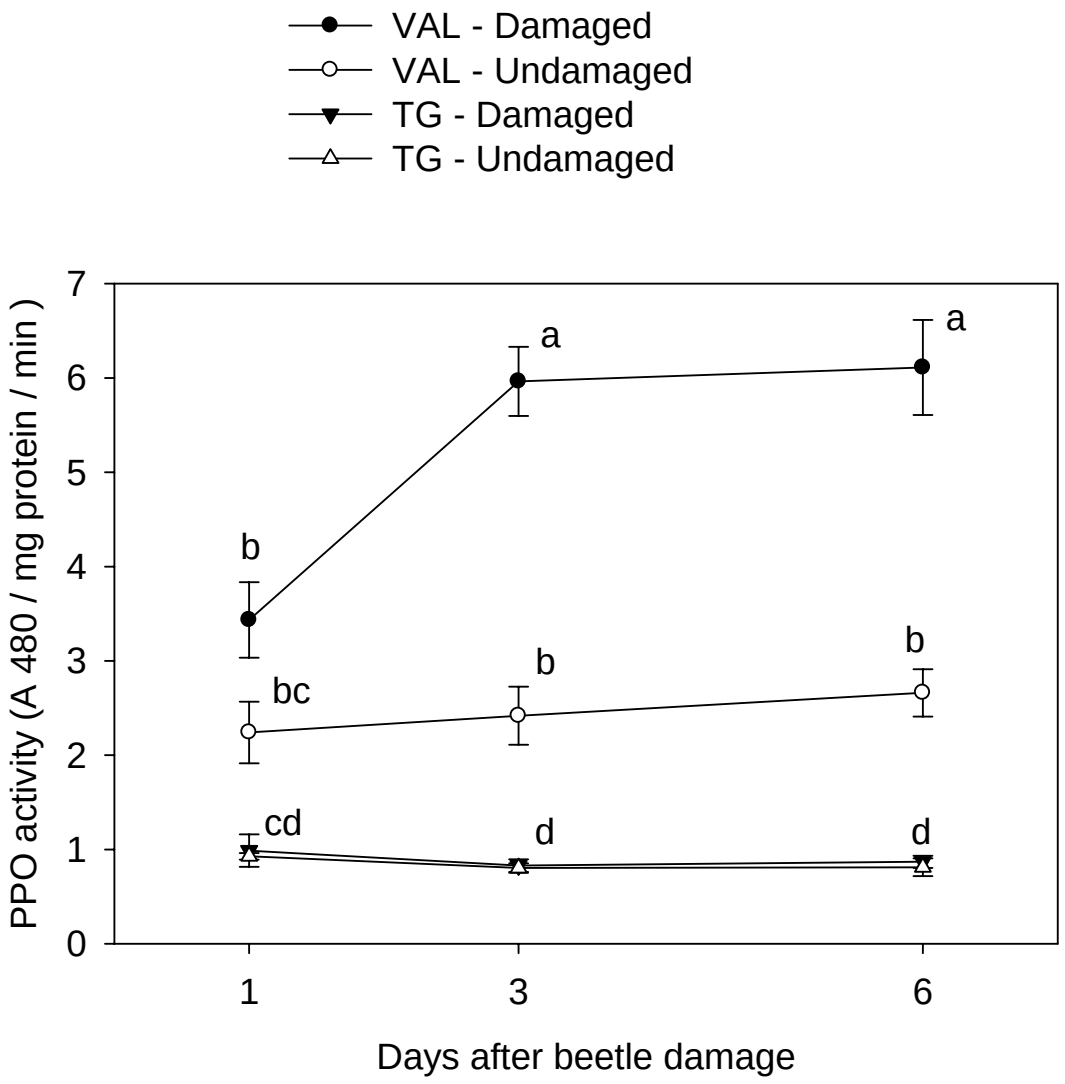


Figure 5-8. Activity of polyphenol oxidase (PPO) in two lettuce cultivars, Valmaine (VAL) and Tall Guzmaine at 1, 3 and 6 d after initiation of feeding damage by adults of *D. balteata*. Error bars indicate SEM. Points topped with different letters differ significantly at the 0.05 level (Tukey's HSD test).

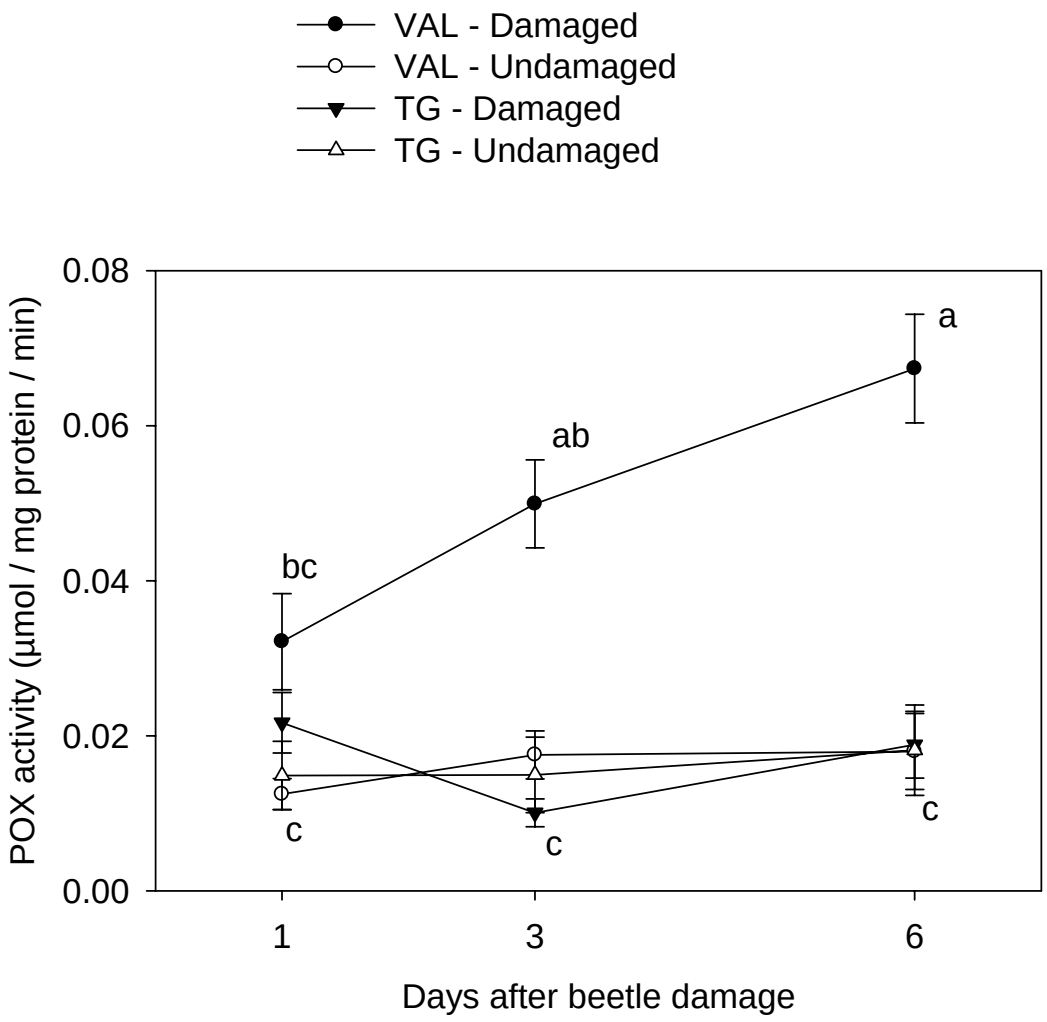


Figure 5-9. Activity of peroxidase (POX) in two lettuce cultivars, Valmaine (VAL) and Tall Guzmaine at 1, 3 and 6 d after initiation of feeding damage by adults of *D. balteata*. Error bars indicate SEM. Points topped with different letters differ significantly at the 0.05 level (Tukey's HSD test).

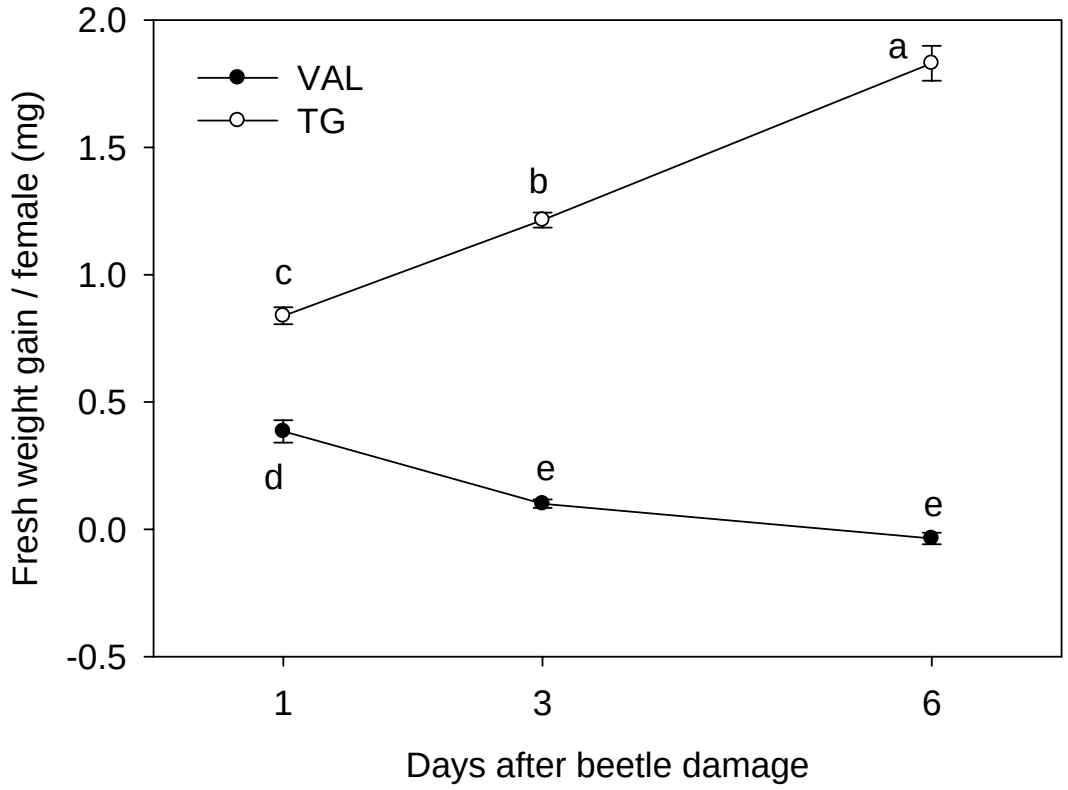


Figure 5-10. Gain in fresh weight of *D. balteata* females over a 6-d period of feeding on two romaine lettuce cultivars, Valmaine (VAL) and Tall Guzmaine (TG). Error bars indicate SEM. Points topped with different letters differ significantly at the 0.05 level (Tukey's HSD test).

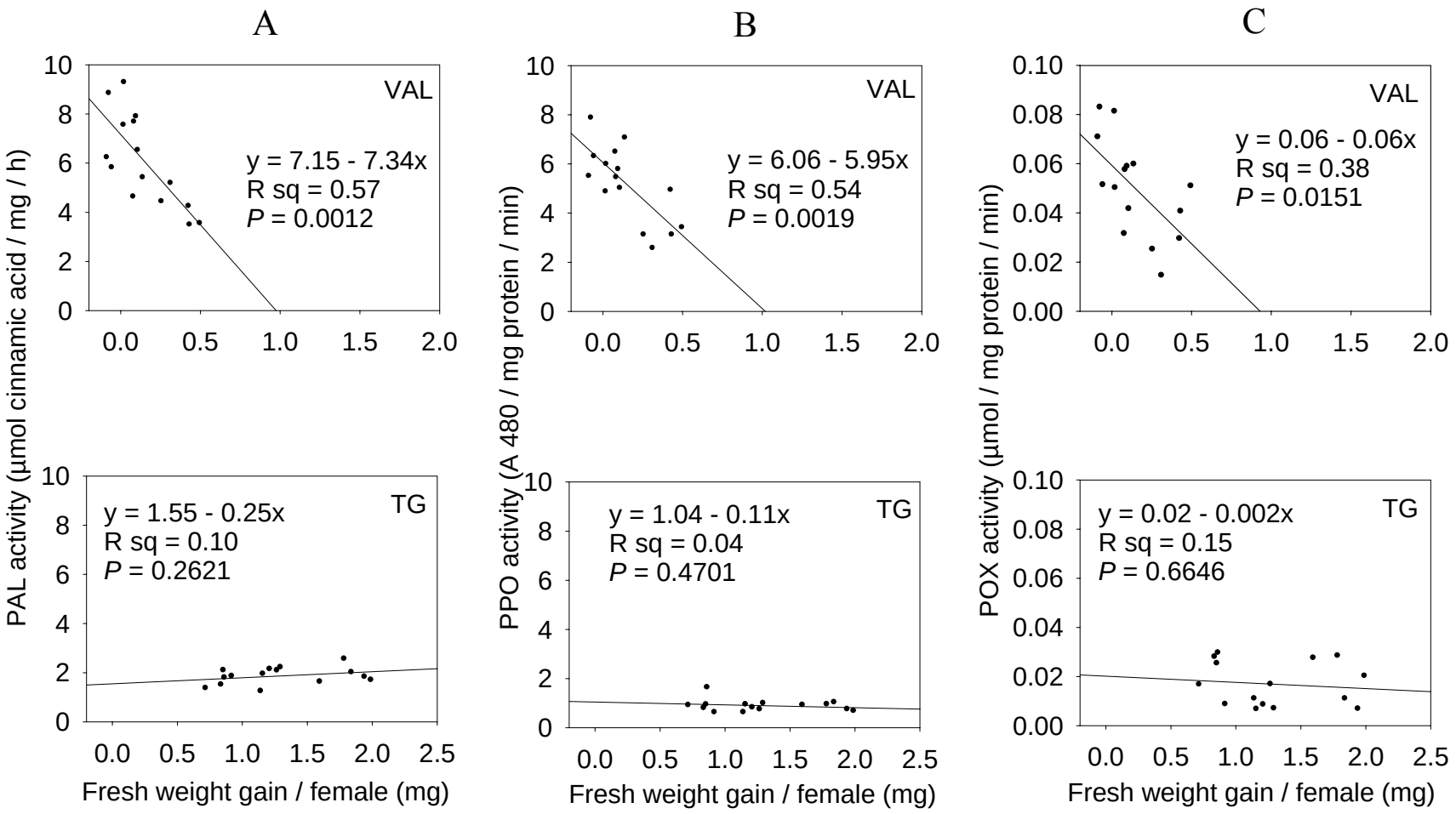


Figure 5-11. Relationship between fresh weight gained by D. balteata females feeding on two lettuce cultivars, Valmaine (VAL) and Tall Guzmaine (TG) and activity of A) PAL, B) PPO and C) POX enzymes after 1, 3 and 6 d of feeding damage.

Table 5-1. Total diet consumption by six *D. balteata* adults on two diet disks treated with latex from same lettuce cultivar, Valmaine or Tall Guzmaine after 24 h of their release.

Cultivar	Days after Damage	Total Diet Consumption (mg)
Valmaine	1	15.2±1.8c
	3	13.5±1.2c
	6	12.4±1.2c
Tall Guzmaine	1	36.7±1.9b
	3	35.4±0.9b
	6	32.1±3.8b
Control	1	45.8±1.2a
	3	47.3±2.6a
	6	46.8±1.5a

Means ± SEM followed by different letters within column differed significantly ($P \leq 0.05$) using ANOVA and Tukey's HSD test.

CHAPTER 6
SUMMARY

Lettuce, *Lactuca sativa* L., is one of the most important vegetable crops grown throughout the world, especially in the United States. California is the major producer of lettuce in the United States (77 % of all lettuce harvested) followed by Arizona, Florida and New Jersey. In Florida, lettuce production from the Everglades Agricultural areas in south Florida contributes 90% of the total state production. Lettuce suffers economic losses due to several insect pests, such as cabbage lopper, *Trichoplusia ni* (Hubner); beet armyworm, *Spodoptera exigua* (Hubner); banded cucumber beetle, *Diabrotica balteata* Leconte; and leafminer, *Liriomyza trifolii* (Burgess). For the management of these pests, growers are dependent on pesticides. Approximately 93% of the lettuce acreage in the United States is treated with the insecticides. Florida ranks first among lettuce growing states in the usage of insecticides to manage these insect pests. Therefore, there is a need to look for alterative strategies for management of economic insect pests. Management of insects with host plant resistance is an important component of integrated pest management strategies.

The romaine lettuce cultivar, 'Valmaine' is known to possess a high level of resistance to *D. balteata* and the leafminer. *Diabrotica balteata* feeding is increased after removal of leaf surface chemicals in Valmaine with methylene chloride, but these surface chemicals did not show any deterrence when applied to leaf surfaces of palatable lima bean at different concentrations. Therefore, it seems that internal factors are involved rather than external chemical factors in imparting resistance against *D. balteata* in Valmaine. Further, previously wounded Valmaine plants showed an increased localized

resistance to *D. balteata* compared to unwounded plants suggesting the involvement of an inducible mechanism of resistance.

The purpose of this research was to investigate the extent of resistance in the lettuce cultivar Valmaine against another order of economically important lettuce pests, the Lepidoptera. The second objective was to identify the mechanism of this multiple insect resistance.

To address the first objective, I compared the survival, development and feeding behavior of cabbage looper and beet armyworm on two romaine lettuce cultivars, resistant Valmaine and the closely-related susceptible 'Tall Guzmaine'. Larval mortality of both species was significantly higher on Valmaine than on susceptible Tall Guzmaine. Significant difference between the cultivars was also observed in development. Larvae weighed six times (beet armyworm) and two times (cabbage looper) more after feeding for 1 wk on Tall Guzmaine than on Valmaine. Larval period was 5.9 (beet armyworm) and 2.6 d (cabbage looper) longer on Valmaine than on Tall Guzmaine. Pupal duration of both insect species was also increased by almost 1 d by feeding on Valmaine compared to Tall Guzmaine. Weights of the pupa and adult of both insect species were reduced on Valmaine compared to Tall Guzmaine. The sex ratio of progeny did not deviate from 1:1 when larvae were reared on resistant Valmaine. The fecundity of cabbage looper and beet armyworm moths that developed from larvae reared on Valmaine was about one third that of moths from Tall Guzmaine, but adult longevity did not significantly differ on the two lettuce cultivars.

Feeding behavior of these moth species was also significantly affected by lettuce cultivar. The two insect species showed different feeding preference for leaves of

different age groups on Valmaine and Tall Guzmaine. Cabbage looper preferred to feed on the lowermost fully mature leaves of Valmaine plants and on young and middle-aged leaves of Tall Guzmaine plants (rarely feeding on fully-matured leaves). Beet armyworm preferred to feed on the lowermost fully mature leaves of Valmaine plants and on middle-aged leaves of Tall Guzmaine plants. Both insect species preferred to feed on the distal end of leaves. Early instars of cabbage looper preferred to feed on the underside of the leaf, whereas early instars of beet armyworm fed on the upper side of the leaf. Cabbage loopers also cut narrow trenches on the leaf before actual feeding to block the flow of latex to the intended site of feeding. In contrast, beet armyworms did not trench; neonates made shallow scratches between the veins by feeding on parenchymatous tissue and second instars made holes through the leaf. The different feeding behavior of the two species on Valmaine may explain the superior performance of cabbage looper compared to beet armyworm.

Lettuce is a laticiferous plant, meaning that it produces a white milky fluid after tissue damage. Latex is stored under pressure in the laticifers. Plant latex is a known defense in certain plants through its physical and chemical properties against several insects. Therefore to address my second objective, i.e. identification of mechanism of resistance in Valmaine romaine lettuce, I hypothesized that latex also plays a defensive role in lettuce. I again used two romaine lettuce cultivars, Valmaine (resistant) and Tall Guzmaine (susceptible) to study the potential of latex as a defense mechanism against *D. balteata*.

Latex from Valmaine strongly inhibited *D. balteata* feeding compared to Tall Guzmaine when applied to the surface of artificial diet. The amount of diet consumed

from Valmaine latex treated disks was significantly less than that consumed from diet treated with Tall Guzmaine latex, in both choice and no-choice tests. The number of adults feeding on diet treated with Valmaine latex was less compared to Tall Guzmaine latex treated diet after 15, 30, 60 and 90 minutes of their release. These studies suggest that latex may account for resistance in Valmaine to *D. balteata.*

All four species that have been tested on Valmaine and Tall Guzmaine (*D. balteata*, leafminer, cabbage looper and beet armyworm) prefer to feed on the lowermost fully matured leaves of resistant cultivar Valmaine. Therefore I decided to test whether this kind of behavior is mediated through any differences in the properties of latex from young and mature leaves. Latex from the young leaves is more viscous and solid white, whereas it is more watery and translucent in the mature leaves. Hence, I conducted choice tests using two artificial diet disks, one treated with latex from young leaves and the second one treated with latex from mature leaves.

There was a significant interaction between leaf age and variety on diet consumption by the beetles. In Valmaine latex treated choice tests, the beetles consumed significantly less diet treated with latex from young leaves than that consumed from diet treated with latex from mature leaves. No significance difference in diet consumption was found between diets treated with latex from young and mature leaves in Tall Guzmaine latex treated choice tests. So, this may explain insect avoidance of young and middle-aged leaves of Valmaine.

After these studies, I was confident that the multiple insect resistance observed in Valmaine was mediated through latex. So in order to further investigate whether this resistance was due to physical or chemical properties of latex, I prepared a crude extract

by dissolving latex in different solvents. Three solvents of differing polarity (water, methanol and methylene chloride) were tested to extract and compare deterrent compounds from Valmaine and Tall Guzmaine latex.

Solvents and the interaction of solvent with lettuce cultivar had significant deterrent affects on beetle feeding. Valmaine latex extracted with water:methanol (20:80) strongly inhibited beetle feeding when applied to the surface of artificial diet. The percentage of beetles feeding on diet treated with Valmaine water:methanol (20:80) extract was less compared to Tall Guzmaine water:methanol (20:80) extract treated diet at intervals of 15, 30, 60 and 90 min after their release. The amount of diet consumed in no-choice tests from disks treated with Valmaine water:methanol (20:80) extract was significantly less than that consumed from diet disks treated with Tall Guzmaine methanol:water (80:20) extract.

To study the role of physical properties of latex in Valmaine resistance, I conducted a small study by applying fresh latex on the mandibles of *D. balteata* adults. Beetles salivated more when Valmaine latex was applied to their mouthparts compared to Tall Guzmaine latex but mandibles and maxillae were not gummed up and were moving freely 24 h after application of either Valmaine or Tall Guzmaine latex (although there were traces of dried latex on the labium and tarsi). These studies strongly indicated a biochemical rather than physical basis of resistance in Valmaine to *D. balteata*. The ability to extract deterrent compounds in water:methanol (20:80) suggested that moderately polar chemicals within latex may account for the observed resistance.

The next series of steps were conducted to isolate deterrent chemicals from the crude Valmaine latex extract (water:methanol, 20:80). The crude extract was first passed

through C-18 cartridges at three different pH levels (natural, acidic and alkaline) to evaluate its relative polarity. No significant deterrent activity was found in the fraction eluted from the cartridge using a step gradient of water:methanol mixtures. The activity was only found in the unbound fraction eluting from the C-18 cartridge, indicating that the deterrent compounds were highly polar. Next, the C-18 unbound fraction was passed through anion exchange and cation exchange cartridges connected in series. The retained compounds on both ion exchange cartridges were tested for feeding deterrence after they were eluted using a NaCl salt gradient. The 0.5M fraction obtained from the cation exchange cartridge possessed the highest deterrent activity. Retention of the deterrent compounds in Valmaine latex on the cation exchange column indicates its basic nature. A fraction eluting between 3 and 4 min exhibited the strongest deterrent activity during further fractionation of cation exchange extract using HPLC-MS. UV absorption and MS data indicated the presence of ten compounds in this active fraction and some of these compounds have substituted aromatic structure. Hence, these results strongly support my hypothesis that unacceptability of Valmaine to *D. balteata* is primarily due to chemical constituents of latex.

Previous research showed that there was a localized induced resistance in Valmaine plants after feeding by *D. balteata*. In general, induced resistance involves changes in plant defensive chemistry due to alteration in the levels of various enzymes, such phenylalanine ammonia lyase (PAL), polyphenol oxidase (PPO) and peroxidase (POX). Therefore, my next steps were to evaluate the potential activity of these three enzymes in Valmaine and Tall Guzmaine lettuce. The questions I tried to answer were if, and how quickly such enzymes could be activated after beetle damage. If such enzymes were

present and inducible by beetle feeding then for how long were levels increased, and did their higher activity correlate with feeding deterrent activity in the latex. To answers these questions, I first tested for inducible enzymatic activity by giving *D. balteata* adults a choice between diets treated with latex from either damaged or undamaged plants. Separate tests were run with extracts from Valmaine and Tall Guzmaine. I investigated the expression of inducible enzymes phenylalanine ammonia lyase, polyphenol oxidase and peroxidase in the latex of both damaged and undamaged plants of Valmaine and Tall Guzmaine. Diet consumption was significantly reduced when disks were treated with latex collected from beetle-damaged Valmaine plants 3 and 6 d after feeding initiation. No significant difference was found in the diet consumption when disks were treated with latex from beetle-damaged Tall Guzmaine plants. Activities of all the three enzymes were significantly enhanced in Valmaine latex after 3 and 6 d of damage, whereas activity remained low in latex from damaged Tall Guzmaine plants. The constitutive levels of PAL and PPO were also significantly higher in latex from undamaged Valmaine compared to Tall Guzmaine plants. So, it seems that Valmaine is better defended in terms of higher expression of these enzymes both at constitutive and induced levels. On Valmaine, beetles gained weight after 1 d of feeding, but then lost weight after being confined on the plants for 3 and 6 d. Fresh weight gain of female *D. balteata* fed Tall Guzmaine plants increased in a linear fashion over the 6 d exposure period. However, a significant negative relationship was found between weight gain and activities of PAL, PPO and POX in Valmaine latex. These studies suggest that latex chemistry may change after beetle feeding damage due to increased activity of inducible

enzymes, and that inducible resistance appears to act synergistically with constitutive resistance in Valmaine latex.

Based on my findings, it appears that Valmaine possesses both constitutive and induced resistance mechanisms, and both are mediated through latex. Solvent extraction studies of the deterrent compounds suggest the presence of biologically active nitrogenous compounds in Valmaine latex, while enzyme induction studies after insect damage indicate an increase in the phenolic compounds. Hence, constitutive and induced defenses in Valmaine may involve different biochemicals. In a situation where there is no constant insect pressure, Valmaine exhibits a constitutive defense and is a non-preferred host. However, in situations where there is prolonged insect pressure, and those insects either have no choice but to feed on Valmaine or are not significantly deterred by the constituitive defenses, inducible enzymatic activity in Valmaine may turn on the second line of defense to protect itself from further damage. Therefore, both types of defenses might be acting synergistically in Valmaine.

Further, Valmaine exhibited resistance only against insects having chewing mouthparts (*D. balteata* adults, leafminer maggots and beet armyworm and cabbage looper caterpillars) and not against insects having sucking mouthparts, such as whitefly (unpublished, Heather McAuslane), aphids (unpublished, Gregg Nuessly) and thrips (unpublished, Amit Sethi). This dichotomy may be an outcome of the mechanism of resistance in Valmaine. Because latex is found in laticifers which run parallel to the vascular system in the plant, chewing insects accidentally rupture the laticifers when attempting to feed on lettuce, resulting in their exposure to latex-borne feeding deterrents. On the other hand, most of the successful sucking insects are known to feed

intercellularly and in this way avoid or reduce the frequency of rupturing laticifers. This may explain why Valmaine only possesses resistance against chewing insects and not against sucking insects.

Based on studies done so far, I propose a biochemical basis for host plant resistance in Valmaine. Further research is required to identify the deterrent compounds both at constitutive and induced levels and also to characterize these inducible enzymes at the molecular level so that both can be used as selection markers during breeding programs to improve lettuce varieties.

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BIOGRAPHICAL SKETCH

Amit Sethi was born August 7, 1977, in Abohar, Punjab, India. He received his bachelor's degree in agriculture with honors in plant protection from the Department of Entomology, Punjab Agricultural University, Ludhiana, India in 1998. He also received the merit fellowship during his bachelor's degree. He obtained his master's degree in entomology from the same institute in 2000, and also received Novartis crop protection fellowship. He worked as a research fellow in the same department for 3 years. While working, he also obtained his M.B.A. in Operational Management from the Indira Gandhi National Open University, New Delhi, India. In 2004, he began his Ph.D. program at the University of Florida to study the biochemical basis of host plant resistance in romaine lettuce under the supervision of Dr. Heather J. McAuslane in the Department of Entomology and Nematology. He received many research and travel grants from the department, university and also from various scientific societies. He won awards (eight) for all of his poster and oral presentations at various state, regional and national scientific meetings. He was an extremely good citizen in the department and the university community. He served as historian for the Graduate Student Organization of the department and he was involved in its many outreach and fundraising (snack-bar coordinator) activities. He was active on the department's Social Committee and he had served as coordinator of the Seminar Committee for several years. This committee was totally responsible for organizing the weekly departmental seminars with local and national speakers. He was Mayor of his married student housing complex and serves in a leadership role on the Mayors' Council, a committee of the University's Student Government. His long term goal is finding a challenging position in molecular and

chemical ecology highlighting insect-plant interactions with both teaching and research responsibilities.