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Ecophysiological importance of phototoxins in plant-insect relationships

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J'allais oublier cette artiste qui a bien voulu cristalliser sur toile quelques-unes des beautés incommensurables de la nature rencontrées au hasard de mes excursions.

Abstract

The ecophysiological importance of phototoxins (PTs), a group of plant light activated secondary compounds toxic to a wide range of organisms, was investigated in the context of plant-insect relationships. The efficiency of PTs in protecting plants against herbivores was investigated in the field by measuring the herbivory damage on a series of ten plant species having different levels of phototoxic activity. Regression analyses performed between the herbivory level and the phototoxic activity of plants indicated a lower number of herbivore attacks per leaf and a higher leaf area removed per attack on the most phototoxic plants. There was, however, no effect of the phototoxic activity of plant tissues on the total area per leaf removed by herbivores or the percent of leaf area consumed. Field observations also revealed that specialist insects typically occurred on highly phototoxic plants whereas generalist insects were predominantly restricted to the least phototoxic plants. Thus PTs do not reduce the total herbivory pressure experienced by plants, although they restrict the range of herbivores attacking plants to specialist herbivores, that is those that are likely to have evolved some adaptation.

The purported adaptations of insects to PTs present in their host plants were investigated in a group of specialist and generalist herbivorous insects feeding on the phototoxic foliage of either *Hypericum perforatum* (Guttiferae) or *Viguiera annua* (Asteraceae). This comparative approach identified distinct behavioral and biochemical adaptations that are used by generalist and specialist phytophagous insects to circumvent phototoxicity. Generalist insects, three long-horned grasshoppers collected on *H. perforatum* in the southwest of France, were observed

to selectively feed between the glands located on leaves of *Hypericum perforatum* that contain the PT hypericin. On the other hand, specialist insects, three leaf beetles and one noctuid, all relied on light-avoidance behavioral strategies to prevent photosensitization. Glands containing the PT hypericin were indeed acting as a feeding stimulant to larvae of *Cloantha perspicillaris*, a specialist noctuid on St. John's-wort. Some differences between specialist and generalist insects were also observed in the activities of antioxidant enzymes which are a major biochemical adaptation for insects to tolerate the oxidative stress induced by PTs. Generalist insects relied on high constitutive activities of antioxidant enzymes (glutathione reductase and glutathione S-transferase) whereas insects specialized on phototoxic plants had lower, but PT-inducible activities of antioxidant enzymes.

Some important ecophysiological constraints for plants relying on phototoxic defenses were also identified. For example, the production of PTs and other related defensive traits like the foliar glands into which PTs are sequestered, were shown to be highly dependent on the environmental availability of light and nutrients. For example, the volume per leaf area unit of foliar glands containing PTs and monoterpenes was increased by up to 150-fold in leaves of *Porophyllum ruderale* grown under an optimal compared to a shaded light regime. It was furthermore confirmed that these environmentally-mediated variations in the production of defensive traits in plants affected the performance of herbivorous insects under both laboratory and field conditions.

Different mechanisms of complementary or additive insecticidal defenses between PTs and other defensive traits of plants were also identified. PTs did not confer a complete resistance to safflower, *Carthamus tinctorius* (Asteraceae), and other physico- (water content) chemical (soluble phenolics) traits are efficient complementary lines of defense against generalist herbivorous insects. Volatile monoterpenes and sesquiterpene lactones were also shown to synergize the insecticidal effects of α-terthienyl against European corn borer larvae.

Inconsistencies between the results that were obtained in the present study and predictions based on the classical theory of coevolutionary interactions between host plants and their associated herbivorous insects, led me to propose a modified evolutionary scheme referred to as plant integrated chemical defenses (PICDs). It is suggested that PICDs, for example a diversification in modes of toxicity of different phototoxic derivatives or synergist interactions between PTs and other classes of secondary compounds, may have evolved in phototoxic plants to specifically counter the ecophysiological drawbacks associated with phototoxic chemical defenses.

Résumé

L'importance écophysiologique des phototoxines (PTs), des composés secondaires toxiques envers une gamme diversifiée d'organismes incluant les insectes, a été investiguée dans le cadre des relations plante-insecte. L'efficacité des PTs à protéger les plantes envers les aggressions des organismes herbivores a été étudiée en champs en déterminant les niveaux d'herbivorie chez dix espèces végétales possédant différents niveaux d'activité phototoxique. Une analyse de régression réalisée entre les niveaux observés d'herbivorie et l'intensité phototoxique des différentes espèces a indiqué qu'un nombre moindre de dommages est infligé par feuille, mais qu'une plus grande surface foliaire est consommée par attaque chez les plantes possédant une activité phototoxique plus élevée. Par contre, la phototoxicité des tissus végétaux n'affectait pas la surface totale consommée par feuille ni le pourcentage de surface foliaire consommée. Les études menées en champs ont aussi révélé une présence dominante d'insectes phytophages spécialistes au niveau des plantes fortement phototoxiques alors que des insectes généralistes furent surtout observés sur les plantes possédant une faible activité phototoxique. La présence de PTs ne confère donc pas une meilleure protection aux plantes bien qu'elle restreigne la présence des insectes herbivores généralistes. Il est donc suggéré que certaines adaptations sont requises de la part des insectes phytophages afin de tolérer la présence des PTs dans les tissus végétaux.

Les présumées adaptations des insectes permettant de prévenir ou d'atténuer les effets nocifs de la phototoxicité furent étudiées chez différents insectes spécialistes et généralistes recensés au niveau des feuilles phototoxiques

d'*Hypericum perforatum* (Guttiferae) ou de *Viguiera annua* (Asteraceae). Cette approche comparative a permis d'identifier les adaptations distinctes des insectes généralistes et spécialistes qui leur permettent respectivement de contrer la phototoxicité. Les insectes généralistes, trois sauterelles s'alimentant occasionnellement sur le feuillage du millepertuis dans le sud-ouest de la France, ingéraient spécifiquement les tissus non-phototoxiques d'*Hypericum perforatum* localisés entre les glandes présentes dans les feuilles de cette plante et qui contiennent une PT, l'hypéricine. Par contre, les insectes spécialistes, trois chrysomèles et une noctuelle, avaient tous recours à une réponse comportementale leur permettant d'éviter une exposition directe à la lumière afin de prévenir la phototoxicité. Il fut aussi observé que les glandes foliaires contenant l'hypéricine exercent un effet phagostimulant envers les jeunes stades larvaires de *Cloantha perspicillaris*, une noctuelle spécialiste du millepertuis. Des différences furent aussi observées quant aux activités d'enzymes antioxydantes qui constituent une adaptation biochimique majeure chez les insectes afin d'atténuer le stress oxydatif induit par les PTs. Les insectes généralistes avaient des activités constitutives élevées des enzymes antioxydantes (la péroxydase du glutathion et la S-transférase du glutathion) tandis que les insectes spécialistes possédaient des activités constitutives plus faibles mais qui pouvaient être induites jusqu'à près du double en présence de PTs.

Des contraintes écophysiologiques importantes pour les plantes utilisant une défense phototoxique furent aussi identifiées. Par exemple, les productions des PTs ainsi que d'autres caractéristiques défensives telles que les glandes foliaires dans lesquelles les PTs peuvent être séquestrées, sont contraintes par les niveaux d'éclairage et de minéraux accessibles aux plantes. Par exemple, le volume par unité de surface foliaire des glandes contenant des PTs et des monoterpènes fut augmenté jusqu'au-delà de 150 fois chez des plantes de *Porophyllum ruderale* (Asteraceae) ayant poussé sous un niveau optimal d'éclairage comparativement au traitement où les plantes furent maintenues dans l'ombre. Il fut de plus confirmé que de telles diminutions dans la production des défenses des plantes résultant d'une disponibilité réduite en ressources résultaient en une plus grande vulnérabilité des plantes envers les insectes phytophages.

Différents mécanismes de défenses complémentaires ou additives impliquant les PTs et d'autres caractéristiques défensives des plantes furent aussi identifiés. Il fut démontré que les PTs conféraient une résistance partielle à des plants de *Carthamus tinctorius* (Asteraceae) et que d'autres caractéristiques physico- (contenu en eau) chimique (substances phénoliques solubles) étaient efficaces afin de complémenter les défenses phototoxiques envers des insectes phytophages généralistes. Certains monoterpènes et sesquiterpènes lactones exerçaient des effets synergiques quant à la mortalité induite chez des larves de la pyrale du maïs par l'α-terthiényl.

Certaines incompatibilités observées entre les résultats obtenus dans la présente étude et les prédictions de la théorie classique de coévolution entre les insectes phytophages et leurs plantes hôtes, m'ont amené à proposer une nouvelle hypothèse évolutive appelée l'intégration des défenses chimiques des plantes (IDCPs). Cette hypothèse de l'IDCPs met l'emphase sur les différentes contraintes écophysiologiques, limitations environnementales des ressources requises à la production des substances de défense et les différentes adaptations utilisées par les insectes phytophages pour contrer les défenses phototoxiques, qui peuvent limiter l'efficacité des PTs à protéger les plantes contre les insectes phytophages. Il fut aussi suggéré que l'IDCPs, par exemple une diversification des mécanismes d'action des différents dérivés phototoxiques ou des interactions synergiques entre les PTs et d'autres classes de composés de défense, peut efficacement contrer certaines des lacunes écophysiologiques reliées à une défense de type phototoxique chez les végétaux.

Table of contents

List of tables

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

Chapter 7

Chapter 8

Chapter 10

List of figures

Chapter 1

Chapter 2

Chapter 4

Chapter 5

Chapter 6

Chapter 7

Chapter 8

Chapter 9

Chapter 10

Chapter 1

General introduction

Diversity and defensive role of secondary chemicals in plants

Recent progress in analytical chemistry, especially in chromatography and spectroscopy, has facilitated phytochemical investigations (see review by Phillipson, 1995). Over 30 000 secondary chemicals (SCs) have been so far identified in plants (Bernays & Chapman, 1994), although it is estimated that plants could contain as many as 500 000 differents SCs (Mendelsohn & Balick, 1995). The questions of why so many SCs do occur in plants and which functions do they assume in nature represent a challenging issue for scientists.

The purported significance of SCs as natural defenses against both herbivores and predators is circumstantially suggested by the abundance of SCs in organisms that are more likely to be consumed, whereas they are mostly absent in parasitic animals and large vertebrates which occupy the top of the food chain (Berenbaum, 1995; Berenbaum & Seigler, 1992). It is likely that plants, which remain rooted to the ground for most of their lives and constitute the bottom level of the food chain, had no other reliable evolutionary alternative to prevent a build-up in herbivore populations than to develop a defensive strategy based either on physical barriers (Wilkens *et al.*, 1996; Gowda, 1996; Myers & Bazely, 1991) or chemical toxins (Fraenkel, 1959; Schultz, 1988).

Recent studies have clearly identified the functional efficiency of some SCs to defend plants against pest organisms under natural conditions. Hartmann (1996) cites the case of a selective feeding by rabbits on sweet lupines lacking the quinolizidine alkaloids. This selective feeding pattern results in a lower survival for the sweet compared to the bitter lupines, which produce alkaloids to chemically defend themselves. Dirzo and Harper (1982) also described a similar behaviour in slugs that preferentially feed on the acyanogenic rather than cyanogenic morphs of *Trifolium repens*. Newman *et al*. (1996) demonstrated the key functional role of glucosinolates against four herbivores naturally co-occurring with watercress in fresh water environments. In this study, the consumption rate of herbivores was five- to 25-fold higher on yellow than on green leaves which contained higher levels of glucosinolates. But when the toxic properties of the glucosinolates were neutralized, all herbivores showed some reversal of preference: the green tissues which contained nearly twice the amount of nitrogen compared to yellow tissues, *i.e.*, 6.9% vs 3.8%, were selectively consumed.

Stronger evidence that confirms the efficacy of chemical defenses come from investigations performed with transgenic plant materials. Maize plants genetically engineered with a *Bacillus thurigiensis* toxin suffered only a small fraction of the damage compared to control plants in the field (Koziel *et al*., 1993). Transgenic tobacco plants expressing the cowpea protease inhibitor (CpTI) gene were also clearly more resistant against *Helicoverpa zea* under field conditions (Hoffman *et al*., 1992). Transgenic plants are indeed so efficient in controlling pest insects that private companies have developed and commercialized transgenic varieties for different agricultural crops including cotton, potato and maize (Hughes, 1996).

In experiments performed with transgenic plants, the fact that investigated genomes only differ by the introduced gene of interest also greatly simplifies the interpretation of results regarding the efficiency of chemical defenses to confer insect resistance. This situation represents a tremendous advantage compared to natural systems where background variations in traits of resistance, which are due to both ecological phenotypic plasticity (see review by Via *et al.*, 1995) and quantitative genetic variations among genotypes (see review by Simms & Rausher, 1992), make it very difficult to draw simple conclusions.

Evolutionary implications of secondary chemicals in plant/insect relationships

Phytophagous insects by their outstanding diversity, about 350 000 out of 800 000 identified species (Bernays and Chapman, 1994), and their propensity to reproduce quickly have undoubtedly exerted a strong evolutionary impact on plants. As for plants that are hosting the phytophagous insects, they also represent a numerous group with over 250 000 species have been described in the terrestrial world (Wilson, 1992).

The negative impact exerted by herbivores on plants (Marquis, 1992; Harper, 1977; Coley *et al.*, 1985), which may however be modulated by environmental conditions (Ruohomäki *et al.*, 1996; Whitham *et al.*, 1991; Whitham & Mopper, 1985), suggest the occurrence of conflicting interests between hostplants and phytophagous insects. It is generally estimated that about 10% of annual plant biomass is directly consumed by phytophagous insects (Pimentel *et al.*, 1991), although higher levels may be reached especially for highly nutritive tissues such as seeds (Mattson, 1978; Cerezke & Holmes, 1986). The impact of insect herbivory on plants may still be higher when their ability to transmit viruses and other pathogens to hostplants is considered (Richard & Boivin, 1994).

Ehrlich and Raven (1964) first proposed an evolutionary scheme that not only

only integrates both the concept of variation of SCs among plants and their effects on herbivorous insects, but also provides a theoretical evolutionary framework explaining the diversity of both insects and plants in nature. Their hypothesis, also referred as the escape-and-radiation coevolution, is based on the five following sequential steps:

1. Plants produce novel SCs through mutation or recombination.
2. The new SCs reduce the palatability of these plants to phytophagous insects.
3. Plants with these novel SCs undergo evolutionary radiation in species into a new adaptive zone in which they are free of their former herbivores.
4. A new mutant or recombinant appears in an insect population that is able to overcome the novel plant compounds.
5. These insects enter a new adaptive zone and radiate in species onto the plants containing the novel compounds.

To this day, Ehrlich and Raven's hypothesis still remains the basic reference that sustains the discussion of plant-insect relationships. Some alterations of this model have however been proposed based on either the relative importance of the different steps originally proposed (see review in Menken, 1996) or the influence of biotic and abiotic factors other than SCs (Thompson, 1994).

Experimental data in favour of Ehrlich and Raven's hypothesis

Berenbaum (1983) was the first to provide a case study supporting the escape-and-radiation evolution as predicted by Ehrlich and Raven's hypothesis.

This author essentially proposed that umbellifers had been submitted to two major chemical-mediated evolutionary radiations that were characterized by some alterations of the coumarin biosynthetic pathway, and that resulted in the production of linear furanocoumarins and angular furanocoumarins (Figure 1). A higher number of species per genus occurs in plants in which both linear and angular furanocoumarins are present than for plants producing only linear coumarins or those without furanocoumarins. These observations were used as an argument to support the hypothesis that evolutionary radiation has increased the diversity of plants less suitable to herbivores. Alternatively, Berenbaum (1983) also provided data showing that, for both the Depressariini (Oecophoridae) of North America and the *Papilio* swallowtails, the number of species per insect genus is higher for those feeding on hostplants producing linear and angular furanocoumarins than for those colonizing plants without furanocoumarins. These data provided a substantial argument for the steps 3 and 5 cited above.

Another possible outcome of Ehrlich and Raven's hypothesis is that specialist insects would be confined to feeding on a limited number of hostplants sharing the same type of chemical defenses due to their high level of specialization (Spencer, 1990). The estimate that 50% of all insect species feed on hostplants that belong to a specific genus[1] (Bernays & Chapman, 1994; Fiedler, 1996) supports the specialization process as predicted by the stepwise coevolutionary model. An extensive study made in a Costa Rican deciduous forest from 1977 to 1987 by

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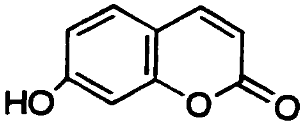
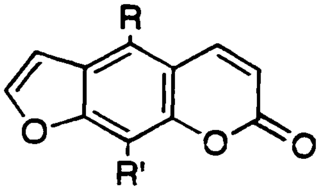
[1] Specialist insects are divided into monophagous and oligophagous insects based on the phylogenetic association of their respective hostplants. Monophagous may colonize a range of plants belonging to a specific genus while oligophagous occur on those from a determined family. As for generalist insects, they may feed on hostplants from different families (Bernays & Chapman, 1994).

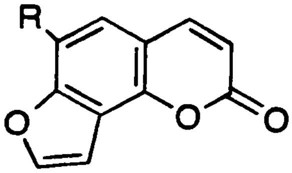
Figure 1. The three major classes of biosynthetically related coumarin derivatives: (A) coumarin, (B) linear furanocoumarin, and (C) angular furanocoumarin.



Coumarins (umbelliferone)



Linear furanocoumarins



Angular furnaocoumarins

Janzen (1988) has shown that more than half of the 3142 species of moth and butterfly caterpillars collected were feeding on only one plant species. Fox and Morrow (1981) even suggested that specialization may occur in different populations of a generalist insect. For example, the Florida population of the eastern tiger swallowtail, *Papilio glaucus*, which has a recorded host list of 530 species from 17 plant families (Scriber, 1984, 1988), exhibits an oviposition preference hierarchy for *Magnolia*, a major host in Florida, while populations from southern Ohio and north central Georgia preferred two characteristic hosts of these regions, *Liriodendron tulipifera* and *Prunus serotina* (Brossart & Scriber, 1995).

Recent investigations, having shown a congruence between the cladograms[2] of both insects and their respective hostplants, also confirm that plant-insect relationships are highly determined by conserved phylogenetic traits (Mitter *et al.*, 1991; Farrell *et al.*, 1992; Farrell & Mitter, 1990) and are potentially due to reciprocal adaptations during the coevolutionary processes. New biological and chemical sensitive techniques (Brown, 1996) have furthermore allowed the characterization of the specificity of some molecular mechanisms involved in different tight associations of specialist insects and their respective hostplants. Cohen *et al.* (1992) showed the xanthotoxin-induced expression of a RNA coding for a specific metabolizing enzyme acting on xanthotoxin in three species of Lepidoptera tolerant to this particular SC.

All those examples are in agreement with the scheme proposed by Ehrlich and Raven, although it is unknown to what extent these coevolutionary processes are specifically mediated by SCs.

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[2] A cladogram is an estimated phylogeny inferring the order of lineage branching based on the assumption that traits evolve in the most parsimonious way (Armbruster, 1992).

Experimental data against Ehrlich and Raven's hypothesis

Two thoroughly critical reviews on coevolutionary processes (Bell, 1997; Thompson, 1994) indicated that the essential prediction of Ehrlich and Raven's hypothesis is that clusters of plant and insect species should show this sequence of plant escape and radiation of species followed by insect colonization and radiation of species. Both authors maintain that none of the few attempts to corroborate this "coupled genetic oscillation" pattern between phytophagous insects and their hostplants in nature has convincingly succeeded. Even the studies for which a parallel cladogenesis between tightly associated insects and their respective hostplants exist (see references above) do not necessarily validate the escape-and-radiation coevolution (Menken, 1996). It is possible that parallel cladogenesis may occur without any reciprocal evolution. It is likely that commensals[3] may speciate in parallel with their hosts without any effect on speciation of their hosts.

Many reasons may explain why a full sequence of the stepwise process involving reciprocal evolutionary change in interacting species is so rarely observed in nature. First, scientists are "locked into the present" (Jarvis & Miller, 1996) so that they may only guess as to the nature of biochemical evolution which is not a simple matter due to the intricacy of the redundancy of SCs occurring in plants as well as the way SCs interact between each other (Jarvis & Miller, 1996; Berenbaum & Zangerl, 1992). The fact that any given population interacts with others, including partners and antagonists, and is also constrained by abiotic environmental conditions, may strongly affect the selective impact of SCs on the output of

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[3] A commensal organism depends on its host to survive although the host is not significantly affected by such a presence.

interactions between herbivores and their hostplants (Bell, 1997).

A few recent studies clearly illustrated how environmental conditions may intervene with the evolutionary path proposed by Ehrlich and Raven. Dobler *et al.* (1996) performed an estimate of the evolutionary history of *Oreina* spp., a genus of specialized leaf beetles, based on a cladistic analysis of 18 allozyme loci. They concluded that hostplant switches in this genus are ecologically dictated by the availability of hostplants in high-altitude habitats rather than by hostplant chemical similarity. Similar results were also obtained for two leaf beetles, *Phratora tibialis* and *Phratora polaris*, that feed on different hosts harboring distinct secondary chemical patterns in different parts of Europe (Köpf *et al.*, 1996). Scriber (1996) also suggested that the local climate regime in a "cold pocket" area in the state of Michigan (USA) may have driven the host shifts in *Papilio canadensis* from its usual hostplants, *Populus* spp., *Prunus* spp., and *Betula* spp., to white ash, *Fraxinus americana*. This localized host shift was speculated by the author to result from a differential phenological response of the different trees to spring warming. Budbreak and leaf expansion were delayed in *F. americana* growing in this "cold " area, foliage of alternative hostplants developed earlier, and thus provided the best nutritional requirements for *P. canadensis* larvae at the given time of their active growth and development.

This recent information that emphasizes the importance of ecological determinants on the pattern of hostplant used by phytophagous insects has led to a reconsideration of the evolutionary role of SCs in plant/insect relationships. For example, the "geographic mosaic theory of coevolution" proposed by Thompson (1994) illustrates the importance attached to geographic variations, which integrate both ecological and phylogenetic constraints. A similar conclusion was also reached by Bennett (1997) who incorporated both ecological and evolutionary considerations to propose a "global synthesis of the organization of life".

Rationale of the present study

Ehrlich and Raven provided an explanatory basis to explain the origin of SCs and their evolutionary importance in plant-insect relationships. Unfortunately, experimental evidence supporting their coevolutionary scheme is lacking. The above discussion has emphasized that some environmental factors may attenuate or prevent the evolutionary impact of SCs between phytophagous insects and their respective hostplants. More studies are, however, required to provide an explanatory basis to why the escape-and-radiation coevolutionary scheme is so difficult to observe and detect in nature. In other words, are there some particular ecophysiological constraints acting on both plants and herbivorous insects that may "put the brakes on" coevolution taking place under natural conditions? It is with this particular question in mind that I investigated the ecophysiological importance of phototoxins in plant-insect relationships.

Phototoxins as plant chemical defenses

Phototoxins (PTs) are light-activated SCs that are ubiquitous in Asteraceae and Umbelliferae (Berenbaum, 1990; Bohlmann *et al.*, 1973) but that also occur at a lower frequency in over 30 different families (Downum *et al.*, 1991). PTs are divided in two types based on their mechanisms of photosensitization. The excitation quantum of energy associated with the photosensitized PT is further dissipated in the cellular environment when the molecule returns to its ground state. In type I process, the dissipation occurs via an electron transfer involving formation of free radicals and often covalent photoadducts between the PT and a nucleic acid. In the type II, the dissipation process relies on an energy transfer to an oxygen molecule resulting in the formation of the reactive singlet oxygen ($^1O_2$) that may induce deleterious photooxidative processes (Foote, 1987). In the present study, the discussion will be focused on plants possessing PTs of type II as they roughly correspond to PTs occurring in the Asteraceae and the Guttiferae families that were investigated.

Photooxidative processes involve chain reactions (Girotti, 1990) that strengthen the toxicity of PTs as evidenced by the broad range of susceptible organisms to PTs from type II. Viruses (Hudson *et al.*, 1993; Lopez-Bazzocchi *et al.*, 1991), microorganisms (McCloud *et al.* 1992; Larson *et al.*, 1988), fungi (Asthana & Tuveson, 1992), nematodes (Gommers & Bakker, 1988), insects (Berenbaum, 1995c; Aucoin *et al.*, 1995), other vertebrates (Philogène *et al.*, 1986), plants (Towers & Arnason, 1988) and, to a lesser extent, rats (Marles *et al.*, 1995), are all sensitive to PTs. Based on these noxious properties, PTs have been considered as SCs involved in the chemical defense of plants against herbivores and pathogens (Arnason *et al.*, 1992; Downum & Nemec, 1987).

PTs are ubiquitously distributed in the Asteraceae: approximately 75% of the investigated species from the Heliantheae tribe produce polyacetylene derivatives susceptible to generating phototoxicity (Christensen & Lam, 1991). Since Asteraceae represents the largest family in terms of species in North America (Bennett, 1996), it is suggested that PTs may represent a common model of SCs in nature.

General organization of the present study

The present study is divided in four sections that respectively examine:

1. The adaptive role of PTs in conferring protection to plants against insect herbivores was investigated in this first section. Results from a field investigation in which herbivory damages were determined on phototoxic and non-phototoxic hostplants are presented in chapter 2. The objective of this field approach was to verify if hostplants harboring PTs obtain a benefit in terms of a reduction in herbivore pressure.
2. The second section deals with both behavioral and biochemical adaptations used by specialist and generalist insects to tolerate PTs occurring in their hostplants. Chapter 3 presents a comparative description of both behavioral responses and antioxidant enzyme activities in a suite of specialist and generalist insects that all feed on the phototoxic leaves of *Hypericum perforatum* in the South of France. Chapter 4 then presents the complementary roles of behavior and antioxidant enzymes used by a leaf beetle feeding on the extremely phototoxic leaves of *Viguiera annua* in a semi-desert area near Tucson, Arizona.
3. The response of insect herbivores to environmentally-mediated variations in the production of SCs was investigated in two model systems. The way nutrient and light availability modulates different physical and chemical traits of *Carthamus tinctorius* seedlings as well as the correlation in the variation in these traits with the performance of three phytophagous insects were investigated in chapter 5. The following chapter (chapter 6) combines field and laboratory observations describing how the presence of secretory cavities modulate the feeding pattern of herbivorous grasshoppers.
4. The last section focusses on why diverse SCs concurrently occur in a given

plant. In chapter 7, it is demonstrated that three different classes of polyacetylenes occurring in the tissues of *Rudbeckia hirta* indeed possess diversified and complementary insecticidal modes of action that, combined together, are likely to confer a more efficient chemical defense against herbivorous insects. The synergist insecticidal interactions between PTs and both volatile monoterpenes (chapter 8) and some sesquiterpene lactones (chapter 9) are presented.

The general discussion (chapter 10) recapitulates the experimental results in a more general evolutionary context to sort out some explanations to the dilemma that SC-mediated coevolutionary interactions are rarely observed in nature. It is suggested that under field conditions the coevolutionary processes in phototoxic plant-insect relationships *sensu* Ehrlich and Raven (1964) are constrained by the ecological plasticity observed in both the production of chemical defenses and in the behavioral responses of phytophagous insects, and by the synergist interactions between different chemical defenses occurring in a single plant.

Chapter 2

Herbivore pressure vs hostplant phototoxicity under natural conditions

Introduction

Many species of Asteraceae produce polyacetylenic secondary chemicals (SCs) (Christensen & Lam, 1991a,b; Bohlmann *et al.*, 1973) many of which are phototoxins (PTs) due to their light-activated activity as reported by many studies (Heitz & Downum, 1995; Guillet, 1994; Arnason *et al.* 1983, 1989, 1992). Based on the remarkable toxicological properties of PTs observed under laboratory conditions, researchers have proposed that PTs may confer an efficient protection to hostplants against pest organisms in nature. Champagne *et al.* (1986), who investigated the toxicological properties of seven polyacetylenes fed in meridic diets to three herbivorous insects, suggested that "... it is probable that photosensitization has real ecological significance in plant-pest interactions under natural conditions". The occurrence of PTs in plants at concentrations that are comparable or higher (mean = 0.94 µmol/g f.w., Table 1) to the concentrations required for acute insecticidal activity (PC_{50}s[1] range from 0.08 to 0.45 µmole/g f.w.; LD_{50}s vary from 0.04 to 2.81 µmole/g f.w. and the mean LC_{50} is 0.19 µmole/g f.w., Table 1) also

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[1] The protective concentration, PC_{50}, is an index of the required concentration of an active ingredient to reduce the feeding activity by 50%.

corroborates the purported defensive function of PTs in nature.

The conjecture of the ecological ability of PTs to provide a protection against herbivores in the field is, however, largely uninvestigated. Such experiment would be all the more justified given the intricacy in the way that plant defenses affect herbivores (see discussion in Firn & Jones, 1996; Berenbaum & Zangerl, 1996). Several other factors that play prominent roles in structuring plant-insect herbivores relationships, such as natural enemies (Bernays and Graham, 1988; Turlings *et al.*, 1995) and hostplant phenology (Mopper and Simberloff, 1995), may indeed "dilute" the functional efficiency of chemical defenses like PTs in the field. The behavioral ability of insects to selectively ingest the least phototoxic tissues (Guillet *et al.*, 1995) was ignored in most bioassays performed under laboratory conditions and may also represent a severe limitation to the efficiency of PTs to defend hostplants against insect herbivores under field conditions.

In the present study, the efficiency of PTs to reduce herbivory was investigated under field conditions by determining the total herbivory on ten plant species possessing different levels of phototoxic activities in their tissues. Given the remarkable insecticidal properties of PTs in laboratory, I hypothesized a negative relationship between total herbivory and phototoxic activity of plant tissues.

Materials and methods

Sites and species investigated

Previous reports on the phototoxic properties of different plants (Towers *et al.*, 1977; Camm *et al.*, 1975; Champagne & Arnason, unpublished results) were used as a guideline for the selection of the species investigated in the present study.

Table 1. Concentration of phototoxins in some Asteraceae and Hypericaceae.

Species	**Phototoxin(s)**	**Concentration of phototoxin(s)**
Eclipta erecta[1]	total major thiophenes	roots: 0.96 µmole/g (d.w.) leaves: 0.03 µmole/g (d.w.)
Tagetes patula[2] (0-150 days old plants)	total major thiophenes	roots: 12-52 µmole/g (d.w.) leaves: 4-10 µmole/g (d.w.) stems: 10-25 µmole/g (d.w.) inflorescences: 4-16 µmole/g (d.w.)
Tagetes minuta[3] (13 and 35 weeks old plants)	total major thiophenes	roots: 0.051-0.577 µmole/g (f.w.)
Tagetes erecta[3] (13 and 35 weeks old plants)	total major thiophenes	roots: 0.157-0.171 µmole/g (f.w.)
Adenophyllum porophylloides[4]	total major thiophenes	total plant: 2.27 µmole/g (d.w.)
Chrysactinia mexicana[4]	total major thiophenes	total plant: 5.46 µmole/g (d.w.)
Dyssodia anthemidifolia[4]	total major thiopenes	total plant: 2.85 µmole/g (d.w.)
Hymenatherum acerosa[4]	total major thiophenes	total plant: 3.96 µmole/g (d.w.)
Hymenatherum pentachaeta[4]	total major thiophenes	total plant: 0.37 µmole/g (d.w.)
Hymenatherum tenuiloba[4]	total major thiophenes	total plant: 2.02 µmole/g (d.w.)
Nicolletia trifida[4]	total major thiophenes	total plant: 1.49 µmole/g (d.w.)
Porophyllum gracile[4]	total major thiophenes	total plant: 3.01 µmole/g (d.w.)
Porophyllum ruderale[4]	total major thiophenes	total plant: 0.53 µmole/g (d.w.)
Porophyllum scoparium[4]	total major thiophenes	total plant: 0.42 µmole/g (f.w.)
Carthamus tinctorius[5]	total major polyacetylenes	cotyledons: 33-85 µmole/g (d.w.) leaves: 30-70 µmole/g (d.w.)
Rudbeckia hirta[5]	pentaynene	inflorescences: 0.03-0.34 µmole/g (f.w.)
Hypericum perforatum[6]	hypericine	leaves: 2 µmole/g (d.w.)

[1]Singh, 1988; [2]Tosi *et al.*, 1988; [3]Croes *et al.*, 1988; [4]Downum *et al.*, 1985; [5]Guillet, unpublished results; [6]Knox *et al.*, 1987.

Table 2. Concentrations of phototoxins required to generate insecticidal effects against phytophagous insects.

Insect	Phototoxin	Treatment and active concentration
Manduca sexta[1]	α-terthienyl	topical application onto late instar LD_{50} = 0.04 μmole/g
Pieris rapae[1]	α-terthienyl	topical application onto late instar LD_{50} = 0.06 μmole/g
Heliothis virescens[1]	α-terthienyl	topical application onto late instar LD_{50} = 1.91 μmole/g
Ostrinia nubilalis[1]	α-terthienyl	topical application onto late instar LD_{50} = 2.81 μmole/g
Manduca sexta[2]	a monothiophene[4]	larvae fed with treated meridic diet LC_{50} = 0.19 μmole/g
Manduca sexta[2]	a monothiophene[4]	neonate larvae offered with treated tobacco leaves PC_{50} = 0.45 μmole/g
	a thiarubrine[5]	neonate larvae offered with treated tobacco leaves PC_{50} = 0.29 μmole/g
Ostrinia nubilalis[2]	a monothiophene[4]	third instar larvae offered with treated corn leaves PC_{50} = 0.21 μmole/g
	a thiarubrine[5]	third instar larvae offered with treated corn leaves PC_{50} = 0.08 μmole/g
Manduca sexta[3]	hypericine	third instar larvae fed with treated meridic diet LD_{50} = 0.03 μmole/g

[1]Arnason *et al.*, 1989; [2]Guillet, 1994; [3]Knox *et al.*, 1987; [4]compound **2** in chapter 7; [5]compound **3** in chapter 7.

Table 3. Phototoxic properties and herbivorous insects ubiquitously occurring on the ten plant species investigated.

Species of plants		Phototoxicity[¶] (mm)		Major herbivorous insects observed
Scientific name[†]	Common name	Leaves[§]	Immature seeds[§]	
*Taraxacum officinale** Weber	Dandelion	0.0 (0.0)	0.0 (0.0)	Unidentified larvae feeding on immature seeds
*Chrysanthemum leucanthemum** L.	Ox-eye daisy	0.0 (0.0)	3.3 (1.9)	*Sparganothis sulfureana* and *Argyrotaenia velutinana*: concealed larvae feeding on the flower heads
*Tragopogon pratensis** L.	Salsify	0.0 (0.0)	0.0 (0.0)	
*Hieracium aurantiacum** L.	Orange hawkweed	0.0 (0.0)	0.0 (0.0)	
*Hypericum perforatum** L.	Common St. John's-wort	4.1 (2.1)	2.3 (1.4)	*Chrysolina quadrigemina*, *C. gemini* – adults and larvae – and *Anaitis plagiata* larvae feeding on leaves and inflorescences
*Sonchus arvensis** L.	Corn sow-thistle	0.0 (0.0)	0.0 (0.0)	
*Erigeron annuus** (L.) Pers.	Annual fleabane	0.0 (0.0)	2.4 (1.8)	*Sparganothis sulfureana* and *Argyrotaenia velutinana*: concealed larvae feeding on the flower heads and leaves
Bidens cernua L.	Nodding beggar-ticks	1.7 (0.5)	6.5 (3.0)	Unidentified beetle: concealed larvae feeding on the flower heads
Bidens frondosa L.	Large-leaved beggar-ticks	0.8 (0.4)	4.9 (1.4)	Unidentified beetle: concealed larvae feeding on the flower heads
*Rudbeckia hirta** L.	Black-eyed Suzan	0.2 (0.1)	10.6 (4.7)	*Sparganothis sulfureana* and *Argyrotaenia velutinana*: concealed larvae feeding on the flower heads; *Chlorochlamys chloroleucaria* larvae feeding on flower heads

[†] Names of plant species followed by an asterisk have been naturalized in the Ottawa/Hull area.

[§] Values provided in brackets represent the standard deviation of the 15 measurements performed on five different plants with three replicates per plant.

[¶] The phototoxicity is quantified by the difference in mm of the inhibition zones of growth of *Saccharomyces cerevisiae* between a photosensitizing and a non photosensitizing light regimes.

The objective was to get a reasonable set of plants harboring different levels of phototoxicity and to test for a correlation between the observed herbivory and the degree of phototoxicity in plants.

Observations were made in the area of Ottawa/Hull from June 10 to August 23, 1996 (specific sites are indicated on the map provided in appendix 1). All species investigated belong to the Asteraceae family except *Hypericum perforatum* which is a member of the Guttiferae family (Table 3). This species, *H. perforatum*, was chosen given the presence in its tissues of hypericin that has a similar mode of photodynamic oxidative action (Hadjur & Jardon, 1995) as the polyacetylenic PTs occurring in the Asteraceae family (see review in Aucoin *et al.*, 1995).

For each species investigated (Table 3), the herbivory was determined on a total of 50 to 86 haphazardly selected plants collected from five to ten different sites. The number of plants of a given species observed per site varied between five to 26 and each site was at least one km away from the others.

Herbivory measurement

Leaves

For each selected plant, the numbers of leaves and the herbivory attacks were determined. Each non-overlapping herbivory damage was considered as a separate attack. The length and width of each herbivory damage was also determined using a millimetric ruler. Under some circumstances, especially for leaves of *H. perforatum* which were often mostly consumed, approximate leaf length and width were considered to evaluate the size of herbivory damages. It was also assumed that herbivory damages had an elliptical shape so that the area consumed may be determined according to equation 1.

$$A = \pi * (L/2) * (l/2) \qquad (1)$$

In this equation, the area consumed and the length and width of a feeding damage are respectively represented by A, L and l. The total leaf area removed by herbivores (A_H) was then calculated for each plant by summing the area of individual damages on all leaves.

For each species, one averaged size plant was collected from five different sites, put in an icebox and brought to the laboratory where leaves were removed then photocopied. The photocopies were then scanned and a non-commercial software provided by Dr. Hans Damman (University of Carleton, Ottawa, CND) was used to convert the digitalized images into quantitative leaf area measurements.

According to the information collected, it was possible to determine for each plant sampled different units of herbivory: (1) mean number of herbivore attacks per leaf, (2) mean leaf area consumed per herbivore attack, (3) mean leaf area fed per leaf, and (4) mean percent of leaf area fed by herbivores that was according to equation 2 where RH, A_H and A_T, respectively, represent the relative herbivory in percentage of leaf area fed, the total leaf area consumed per plant, and the total leaf area of a plant.

$$RH = A_H / A_T * 100 \qquad (2)$$

Inflorescences

The herbivory was determined differently for inflorescences since it was impossible to accurately evaluate the area consumed by herbivores. A qualitative index involving the determination of the presence or absence of herbivory damage on a flower head was therefore used. For plants having more than one flower heads, the number of both total and damaged inflorescences were noticed. The on inflorescences was determined on the same plants as for the leaves.

Phototoxic activity

A yeast inhibition bioassay (Camm *et al.*, 1975; Arnason *et al.*, 1991) was used to verify the phototoxic activity of plant tissues. Sterile petri dishes containing agar and Sabouraud dextrose broth were inoculated with *Saccharomyces cerevisiae*. Small pieces of young newly expanded leaves, about 5 mm X 5 mm, and immature seeds were gently squeezed with a metallic rod and immediately placed on the inoculated agar. To detect phototoxicity, one replicate was irradiated for 4 h under Westinghouse F20T12/bulbs, 30μmole photons/m^2/s in the long-UV range (300-400 nm) as measured with a UVP Model J221 radiometer, while a Kodak CP2B filter (UV cutoff at 400 nm) was maintained over the other replicate serving as a control (no generation of phototoxicity). For the tissues of *H. perforatum* containing hypericin, which requires visible light around 590 nm to be photosensitized, the inoculated dishes were exposer under cool white fluorescents, 150 μmol photon/m^2/s, or maintained in darkness for the non-photosensitizing treatment. Immediately after irradiation, dishes were incubated for another 20 h in darkness at room temperature and the mean zone of the yeast growth inhibition was measured. Phototoxicity was defined by the difference between the zone of growth inhibition for the photosensitization light regime and the zone of growth inhibition for the non-photosensitization light regime. For each species, the phototoxic activity was measured on five plants sampled from five different sites and three replicates were performed on each plant for both leaves and immature seeds. The tissues of plants were kept in an icebox up to 4 h until the bioassay was performed in the laboratory.

Statistical analysis

Regression analyses were performed between the different herbivory indices and the mean phototoxic activity measured using Systat® for Windows™ version 5.

Results

Phototoxic activity

Six out of the ten species investigated did not show any phototoxic activity in leaves and four for the immature seeds (Table 3). For the species harboring a phototoxic activity in at least one of the two parts analyzed, the activity was always greater in immature seeds, 4.43 ± 1.24 mm (mean ± 1 sem), than in leaves, 1.36 ± 0.63 mm, except for *H. perforatum* for which phototoxicity was 78% higher in leaves. An analysis of variance performed to verify if the variable site had a significant effect on the phototoxic activity measured was not significant for each of the ten plant species investigated ($P>0.05$).

Herbivory vs phototoxic activity

Regression analysis revealed that phototoxic properties of plant tissues significantly affect only two out of the five herbivory indices measured. A higher phototoxicity in leaves was correlated with a lower number of attacks per leaf (Fig. 1), although the mean area consumed per attack increased with the phototoxic activity in leaves (Fig. 2). According to the equations obtained by regression analysis, the leaves of plant species having no phototoxicity are exposed to nearly one attack per leaf, although a species harboring a phototoxic activity in its leaves of 4.1 mm (St. John's-wort) was subjected to only 0.32 attack per leaf, *i.e.*, about three times less. The results also indicated that leaf area consumed per attack was more than twice on *H. perforatum* (11.8 mm^2) than on plants showing no phototoxicity (5.3 mm^2).

Phototoxic properties of leaves did not affect the total area fed per leaf (Fig. 3) nor the percentage of leaf area consumed (Fig. 4). The proportion of flower heads that had been attacked by herbivores was also not affected by their phototoxic activities (Figure 5). An analysis of variance performed between each of the herbivory units determined and the variable site was significant ($P<0.05$) for each plant species.

Discussion

Most valuable plant organs are highly protected

It is commonly assumed that plants produce higher levels of chemical defenses in more valuable tissues (Zangerl & Bazzaz, 1992), although the absolute value of a tissue remains an elusive concept (see review by Lerdau, 1996). The concentration of pyrrolizidine alkaloids, which are efficient to repel polyphagous insects, is 50-190 times higher in young leaves of *Cynoglossum officinale* which have a higher photosynthetic rate and a higher nitrogen content compared to older leaves (Van Dam *et al.*, 1996). Young seedlings that are more vulnerable to herbivores and pathogens are also generally better protected than older plants (Maffei *et al.*, 1989; Duriyaprapan & Britten, 1982). The reproductive organs, which are a basic component to a long-term plant fitness, also frequently contain high levels of chemical defenses (Zangerl & Bazzaz, 1992).

The results of the present study confirmed that phototoxicity more often

Figure 1. Effects of the phototoxic activity on the number of herbivore attacks per leaf for ten different plant species in the Ottawa/Hull area. The equation for the linear regression and the squared multiple regression coefficient (r^2) are indicated in the bottom part of the graph. Values for the 6 species of plant with no phototoxic activity in their leaves were pooled. Error bars represent the standard error.

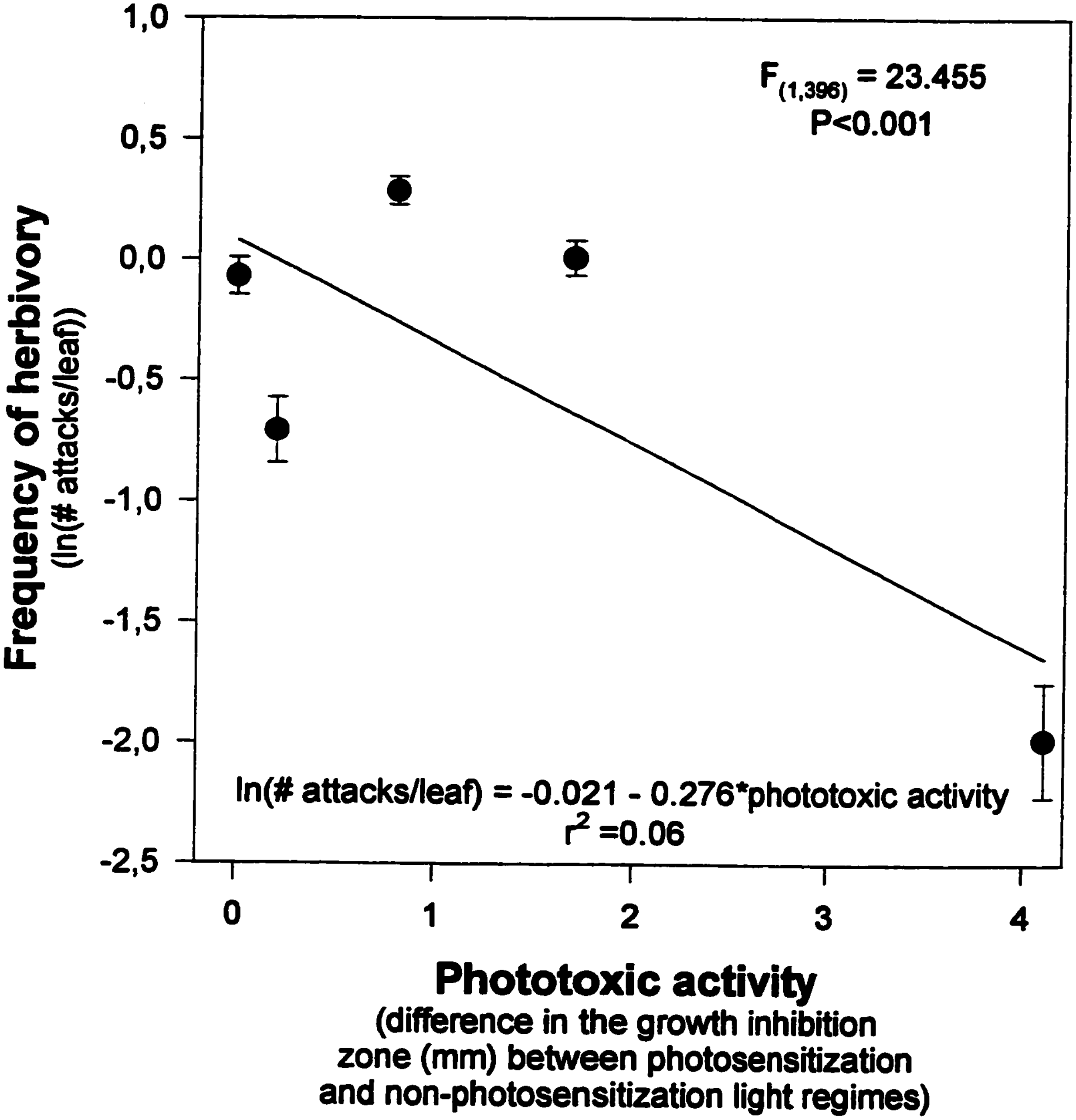

1,0
0,5
0,0
-0,5
-1,0
-1,5
-2,0
-2,5
Frequency of herbivory
(ln(# attacks/leaf))
F(1,396) = 23.455
P<0.001
ln(# attacks/leaf) = -0.021 - 0.276*phototoxic activity
r2 =0.06
0
1
2
3
4
Phototoxic activity
(difference in the growth inhibition zone (mm) between photosensitization and non-photosensitization light regimes)

Figure 2. Effects of the phototoxic activity on the leaf area consumed per herbivore attack for ten different plant species in the Ottawa/Hull area. The equation for the linear regression and the squared multiple regression coefficient (r^2) are indicated in the bottom part of the graph. Values for the 6 species of plant with no phototoxic activity in their leaves were pooled. Error bars represent the standard error.

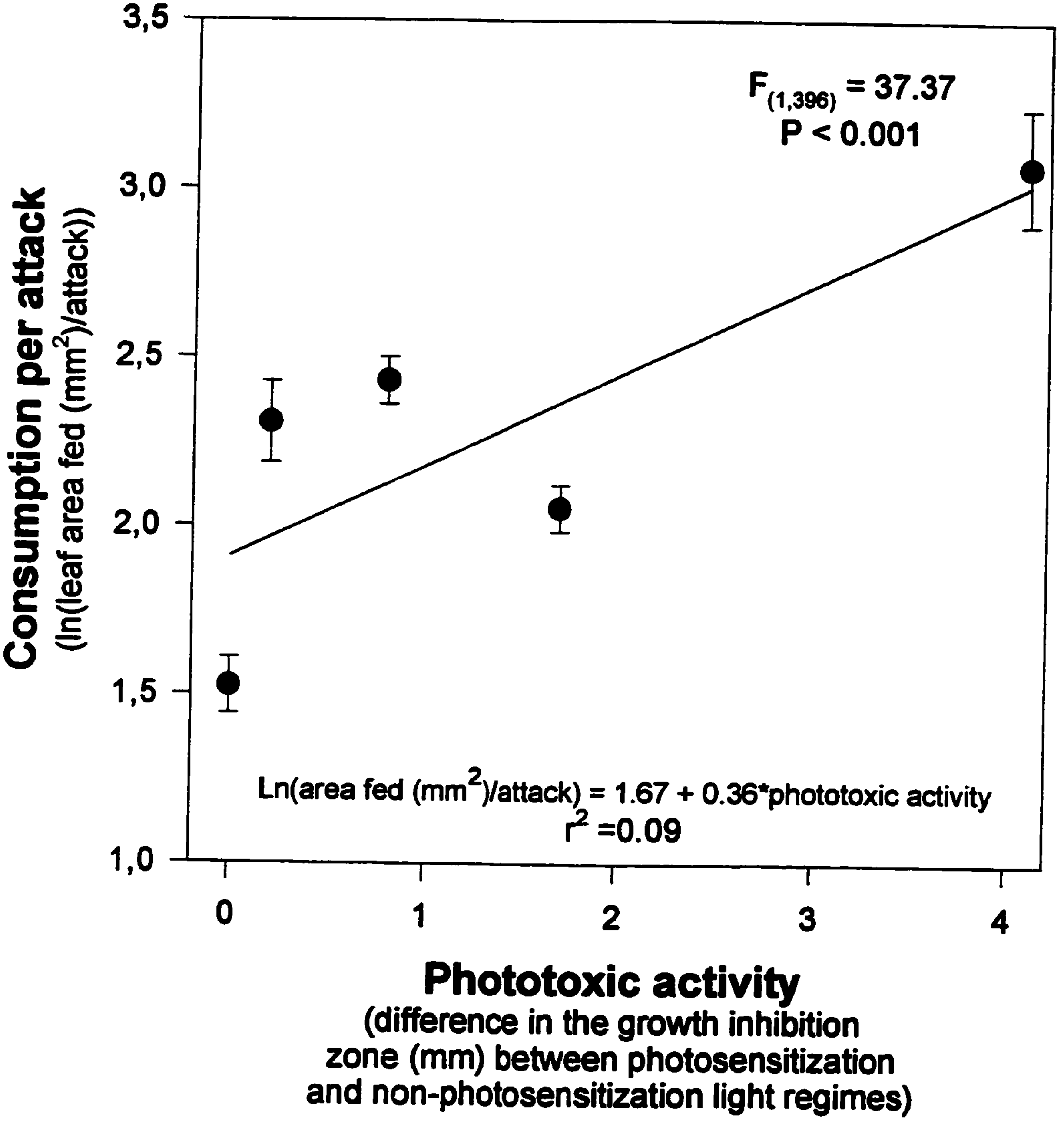
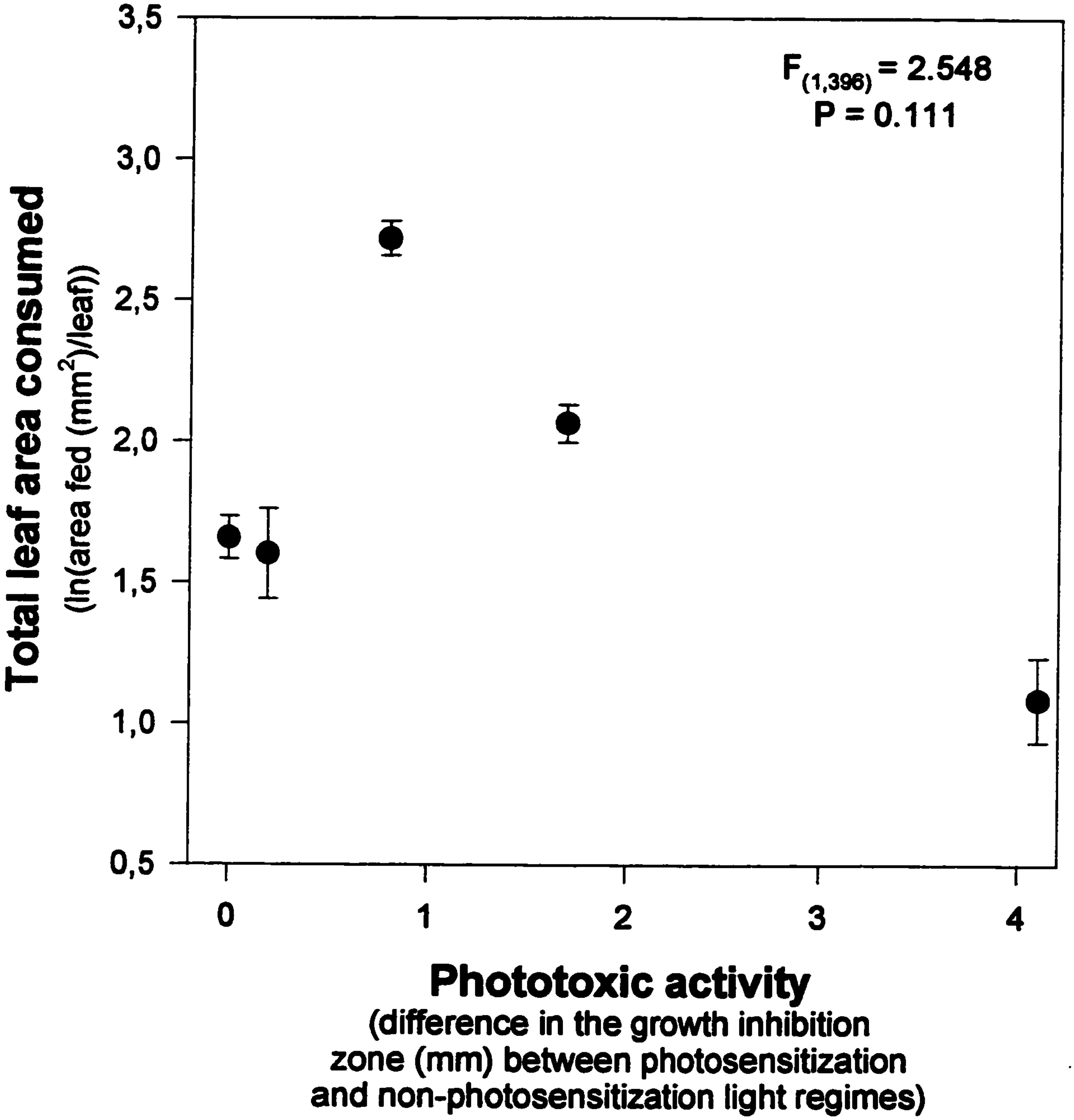
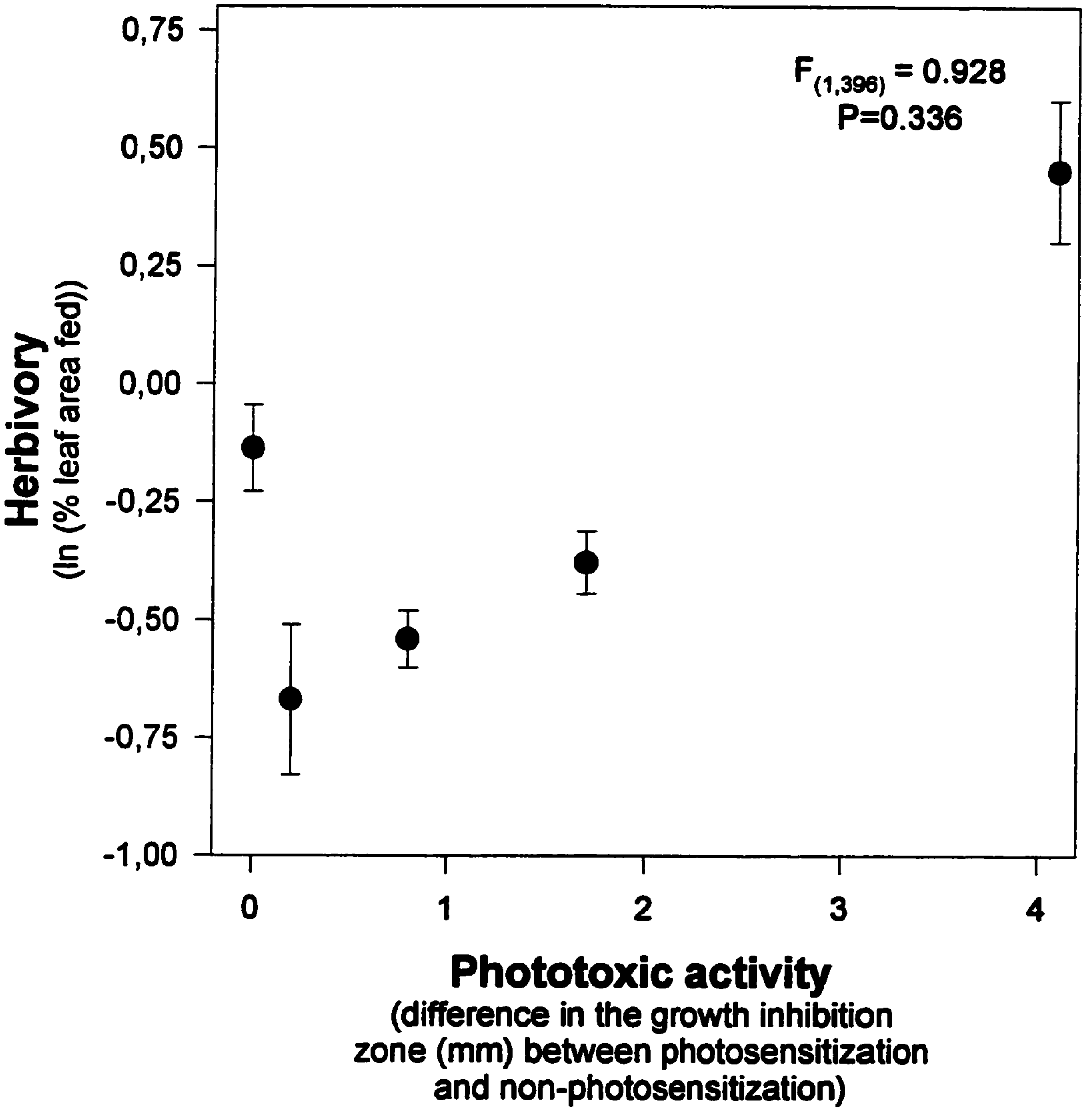

3,5
3,0
2,5
2,0
1,5
1,0
0
1
2
3
4
$F_{(1,396)} = 37.37$
$P < 0.001$
Ln(area fed (mm^2)/attack) = 1.67 + 0.36*phototoxic activity
$r^2 = 0.09$
Consumption per attack
(ln(leaf area fed (mm^2)/attack))
Phototoxic activity
(difference in the growth inhibition
zone (mm) between photosensitization
and non-photosensitization light regimes)

Figure 3. Effects of the phototoxic activity on the total area per leaf fed by herbivores for ten different plant species in the Ottawa/Hull area. Values for the 6 species of plant with no phototoxic activity in their leaves were pooled. Error bars represent the standard error.



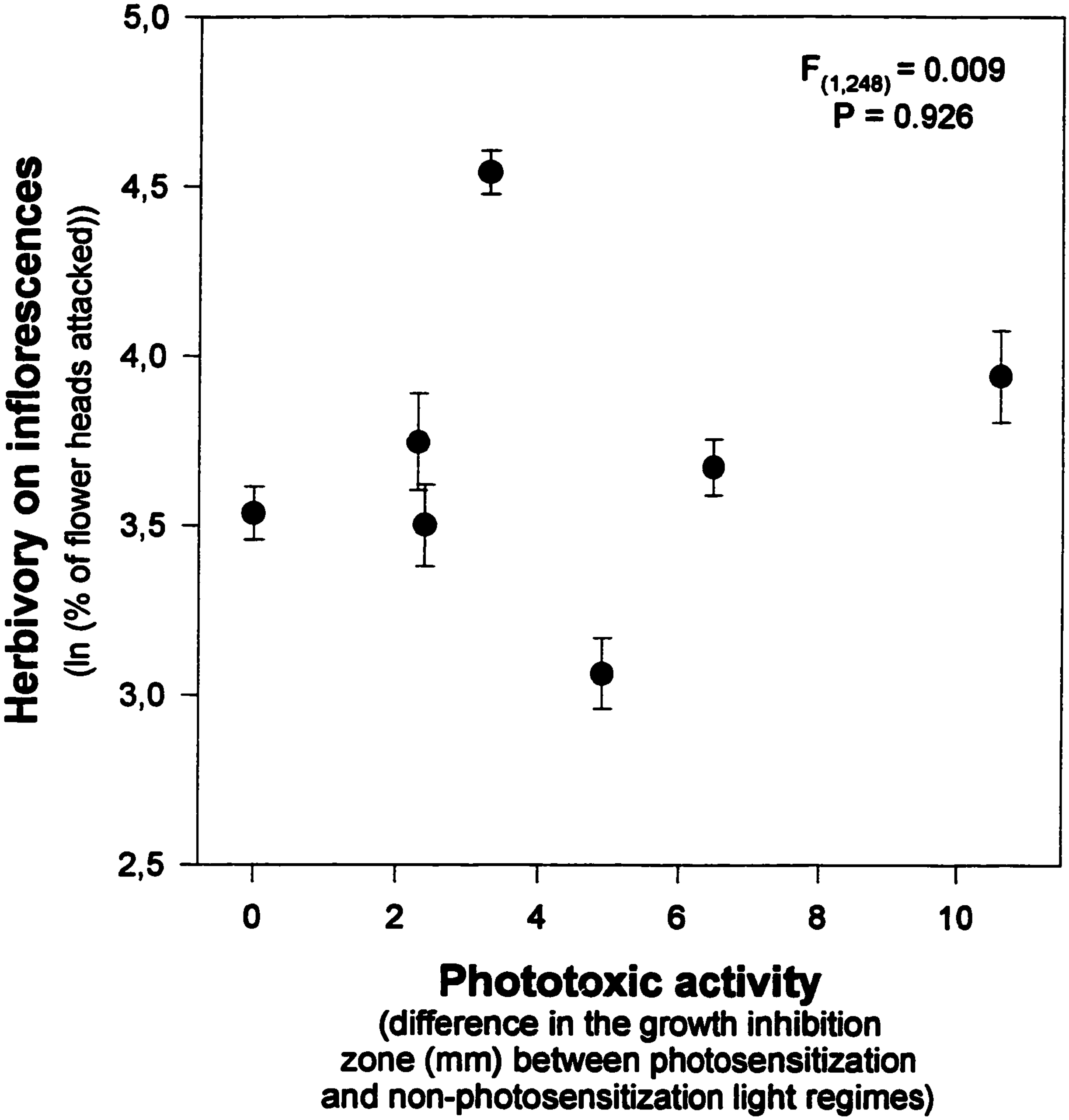
$F_{(1,396)} = 2.548$
P = 0.111
Total leaf area consumed
(ln(area fed (mm^2)/leaf))
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0
1
2
3
4
Phototoxic activity
(difference in the growth inhibition zone (mm) between photosensitization and non-photosensitization light regimes)

Figure 4. Effects of the phototoxic activity on the percentage of leaf area fed by herbivores for ten different plant species in the Ottawa/Hull area. Values for the 6 species of plant with no phototoxic activity in their leaves were pooled. Error bars represent the standard error.



$F_{(1,396)} = 0.928$
P=0.336
0,75
0,50
0,25
0,00
-0,25
-0,50
-0,75
-1,00
Herbivory
(ln (% leaf area fed))
0
1
2
3
4
Phototoxic activity
(difference in the growth inhibition zone (mm) between photosensitization and non-photosensitization)

Figure 5. Effects of the phototoxic activity of immature seeds on the percentage of flower heads attacked by herbivores for ten different plant species in the Ottawa/Hull area. Values for the 4 species of plant with no phototoxic activity in their immature seeds were pooled. Error bars represent the standard error.


$F_{(1,248)} = 0.009$
P = 0.926
Herbivory on inflorescences
(ln (% of flower heads attacked))
5,0
4,5
4,0
3,5
3,0
2,5
0
2
4
6
8
10
Phototoxic activity
(difference in the growth inhibition
zone (mm) between photosensitization
and non-photosensitization light regimes)

occurs in immature seeds than in leaves (Table 3). Furthermore, phototoxicity tended to be higher in immature seeds, 3.0 ± 2.1 (mean ± 1 sd), than in leaves, 1.0 ± 1.5 (T-test, P =0.059). Towers *et al.* (1977) and Camm *et al.* (1975), who used a yeast inhibition growth bioassay similar to the one used in the present study, also reported more ubiquitous phototoxic activities in reproductive organs than in leaves or stems of different Asteraceae species.

Herbivory patterns as a function of the phototoxic activity of hostplant tissues

During the field investigation of the present study, it was noticed that some plant species were frequently colonized by a limited number of insects, although a more diversified range of mobile insects was observed on other plants. For example, immature seeds of dandelion flower heads were commonly attacked by larvae of an unidentified lepidopteran insect. In one study site located along highway 50 in Gatineau, 87% of the inflorescences were attacked. Apart from the observation on the dandelion inflorescences that were not phototoxic, all other commonly observed herbivorous insects were restricted to phototoxic plants (Table 3). There was thus a trend for hostplants producing PTs to be preferentially colonized by a narrowed suite of herbivorous specialist insects. The significant effects that sampling site had on the different herbivory units for each plant species also indicated a spatial heterogeneous distribution of herbivorous insects.

Insects feeding on phototoxic tissues may possess some specific behavioral or biochemical adaptations to counteract phototoxicity (see review in Aucoin *et al.*, 1995). Some adaptations to phototoxicity have indeed been reported in some of the phytophagous insects listed in Table 3. For example, *Argyrotaenia velutinana* larvae feeding on the phototoxic inflorescences of the common daisy, *Chrysanthemum leucanthemum,* prevent light-induced mortality by tying together the rays of the flower head to build an opaque shelter in which they hide themselves (Guillet *et al.*, 1995). Negative phototaxis involving feeding at dawn and burrowing in the soil in the daytime have also been reported for *Chrysolina quadrigemina* larvae feeding on the phototoxic leaves of *H. perforatum* (Fields *et al.*, 1989). Biochemical adaptations for fecal elimination of hypericin are also likely to be involved in *Chrysolina* spp. Duffey and Pasteels (1993) showed that the uptake of hypericin in the tissues of four *Chrysolina* spp. was kept very low, under 0.96 mμg/beetle, and that 80% of this amount remained in the gut lumen. The same study also showed that 85% of the ingested hypericin dose was eliminated through feces by *Chrysolina brunsvicensis* larvae.

Behavioral and biochemical adaptations conferring tolerance to specialist insects feeding on hostplants producing PTs may provide an explanation as to why phototoxic plants were not better protected than non-phototoxic plants in terms of both absolute and relative leaf area consumed by insect herbivores (Fig. 3 and 4). The phagostimulant effect that PTs exert on adapted specialist insects (Chapter 3) is likely to explain both the preferential occurrence of specialist insects on phototoxic plants as well as a higher leaf area consumed per attack.

Grasshoppers that represent 63% of the total insect biomass occurring in old fields in the Hull/Ottawa area (personal observations) are known to frequently shift to different host plants (Bernays *et al.*, 1992). This behavioral strategy may be efficient for grasshoppers to optimize their blend of nutrient ingested in order to fulfill all their nutritional requirements (Simpson & Raubenheimer, 1996; Raubenheimer & Simpson, 1996). Another distinct feature of generalist insects is that they are usually deterred by "qualitative" toxic chemical defenses (Feeny, 1976;

see review in Bernays & Chapman, 1994) including PTs (Champagne *et al.*, 1986; personal observations). These feeding behaviors of generalist insects are consistent with the experimental results obtained in the present study. Generalist insects were in a minority on highly phototoxic plants likely because of the deterrent properties of these "qualitative" defences. It is also expected that the frequent host shift behavioral strategy practiced by generalist insects may explain the smaller size of feeding damages inflicted to plants with low phototoxicity.

Surprisingly, phototoxicity did not affect the rate of herbivory on immature seeds (Fig. 5) even if these organs exhibited a higher activity than leaves. It is likely that the hiding behavior observed for all insects – most of them were tortricid larvae – feeding on phototoxic immature seeds was efficient enough to prevent the occurrence of phototoxicity (Sandberg & Berenbaum, 1989). The efficiency of concealment in preventing larval mortality due to phototoxicity has been clearly illustrated with *Argyrotaenia velutinana*. When larvae of this insect are removed from the phototoxic inflorescences of *C. leucanthemum* and exposed for 9 hours to sunlight, 40% of the larvae died compared to only 5% for larvae kept in the shade (Guillet *et al.*, 1995).

Conclusion

The present study rejects the hypothesis that plants with a phototoxic activity are less subject to herbivory (Fig. 3-5). The lower number of attacks observed per leaf harboring phototoxicity (Fig. 1) was compensated by greater feeding damages (Fig. 2) so that all things considered the absolute and relative leaf areas conceded to herbivory did not significantly differ between phototoxic and non-phototoxic plants (Figures 3 and 4). It was also circumstantially shown that the differences in the herbivory pattern observed between phototoxic and non-phototoxic plants were related to the contrasting responses of generalist and specialist herbivorous insects to the presence of PTs in plants. All results observed corroborate the van der Meijden's model (1996) predicting that generalist herbivores will perform better on plants lacking or having low concentrations of chemical defences, although specialist insects will have a higher fitness on plants with higher amounts of chemical defences because (1) "they have broken the defence system of ... plants" and (2) they "... use plant chemicals as a cue to find or identify their food plants".

Acknowledgments

Maxime Guillet provided technical assistance for field work.

Chapter 3

Behavioral and biochemical adaptations of generalist vs specialist herbivorous insects feeding on *Hypericum perforatum* (Guttiferae)

Introduction

In his attempt to describe how plant apparency, *i.e.*, the likelihood to be discovered by their enemies in ecological time, may have affected the evolutionary pattern of secondary chemical (SC) production, Feeny (1975) also indirectly classified herbivorous insects as either generalists, those feeding on hostplants harboring "quantitative" defenses, or specialists, which are able to tolerate "qualitative" defenses. By "quantitative" defenses, Feeny (1975) referred to those SCs produced at high concentrations in plants and which exert a dosage-dependent inhibition of food digestibility in both generalist and specialist enemies. As for "qualitative" defenses, they were defined as those SCs occurring at lower concentrations in plants, usually less than 1% d.w., and whose specific mechanisms of toxicity are susceptible to be counteract by specialist enemies.

The specific biochemical adaptations to tolerate "qualitative defenses", as implied in Feeny's model, have been elucidated for few herbivorous insects. For example, the tolerance of the tobacco hornworm, *Manduca sexta*, larvae to nicotine present in tobacco is related to several physiological adaptations, two of which include metabolism which is mediated by a series of cytochrome P-450 (Snyder *et al.*, 1993) as well as nicotine removal mediated by an ATP-dependent toxin pump (a P-glycoprotein analog) located in both the Malpighian tubules and the blood-brain barrier (Murray *et al.*, 1994; Murray, 1996). *Pieris rapae* is a crucifer specialist that resists a cabbage proteinase inhibitor, which normally reduces the efficiency of digestibility by reducing the proteolytic enzyme activity in midgut, through secretion of "inhibitor-resistant" trypsin(s) (Broadway, 1996). Another well-known example is the physiological adaptation of the monarch butterfly, *Danaus plexippus*, which confers the ability to tolerate and sequester the cardenolides present in the common milkweed, *Asclepias syriaca* (see review in Malcolm, 1991).

However, one key premise of Feeny's model that has not receive much attention is the purported higher susceptibility of generalist insects to plants producing "qualitative defenses". This assumption does however not necessarily fit with some recent results suggesting that generalist insects exhibit a tolerance to diverse SCs when offered at concentrations which are representative of those occurring in plants. The migratory grasshopper, *Melanoplus sanguinipes*, and the variegated cutworm, *Peridroma saucia*, release feces in which almost all orally ingested xanthotoxin and digitoxin are metabolized (Smirle & Isman, 1992). Xanthotoxin and digitoxin have been reported as highly toxic to phytophagous insects due to their respective light-activated cross-linking between DNA strands (Berenbaum, 1991) and inhibition of the Na^+-K^+ pumps (Stryer, 1988). A series of studies made by Bernays' group also showed that generally polyphagous and oligophagous grasshoppers do not exhibit any particular disorder following ingestion of different SCs including nicotine, salicin, umbelliferone, allylisothyocyanate and others (Cottee *et al.*, 1988; Bernays, 1990,1991).

The objective of the present study was to compare the behavioral and biochemical adaptive features of generalist and specialist insects feeding naturally on leaves of St. John's-wort, *Hypericum perforatum* L. (Guttiferae). Southwest of France was chosen for the location of the study because the plant is native to Europe and has presumably a long association with its insect fauna while the North American *H. perforatum* is introduced and its fauna recently adapted.

Hypericum perforatum produces in its leaves and inflorescences a phototoxin (PT) called hypericin that is photosensitized by visible wavelengths around 590 nm (Andreoni *et al.*, 1994; Hadjur & Jardon, 1995). The highly phototoxic properties of hypericin (Hudson *et al.*, 1994; Andreoni *et al.*, 1994; Knox *et al.*, 1987; see also reviews by Mitich, 1994 and Bombardelli & Morazzoni, 1995) and its occurrence in plants at relatively low concentrations, approximately 200 ng/g dry wt (Jensen *et al.*, 1995), fit the criteria of a "qualitative" defense *sensu* Feeny. Behavioral strategies (negative phototaxis and selective feeding on least phototoxic parts) and constitutive and hypericin-inducible activities of antioxidant enzymes were examined since these adaptations have been previously reported in phytophagous insects colonizing phototoxic hostplants (Guillet *et al.*, 1995, 1997a, see review in Aucoin *et al.*, 1995). Our working hypotheses were, as predicted by Feeny's model, that (1) specialist insects generally rely on biochemical adaptations, that is, higher constitutive and hypericin-inducible activities of antioxidant enzymes, whereas (2) generalist insects use a more flexible behavioral strategy to prevent toxicity related to hypericin photosensitization.

Materials and methods

Collection of insects in the field

Insects commonly observed to feed on *H. perforatum* in the southwest of France were sampled. Three generalist grasshoppers, *Tettigonia viridissima* Linné (Orthoptera: Tettigoniidae), *Ruspolia nitidula* (Scopoli) (Orthoptera: Tettigoniidae) and *Conocephalus discolor* Thunberg (Orthoptera: Tettigoniidae), were occasionally observed to feed on leaves of *H. perforatum*. These three insects were observed throughout the sites investigated near Bordeaux, in the "Montagne Noire" located at 50 Km north of Carcassonne and at the different altitudes where *H. perforatum* occurs in the French side of the Pyrenees, that is up to 1000 m (Saule, 1991). *Conocephalus discolor* is a widespread insect throughout Western Europe and occurs especially in marshy meadows, riverside vegetation and occasionally abandoned fields (Luquet & Bellmann, 1995). This insect preferentially feeds on grasses but it also consumes other plants and small insects (Luquet & Bellman, 1995). The large conehead cricket, *R. nitidula*, is a polyphagous insect feeding on different grasses throughout southern Europe (Bellman & Luquet, 1991 (reference in Luquet & Bellman, 1995)). The great green bush-cricket, *T. viridissima*, is 4 cm long and represents one of the largest crickets in Europe. It occurs almost everywhere except at high altitude and has become rare in urban zones (Luquet & Bellman, 1995). The great green bush-cricket is mostly carnivorous but has been reported as a pest insect to fruit trees and to garden peas (Chopard, 1951).

Three other insects were observed to feed almost exclusively on *Hypericum* spp. and will thus be referred as specialists for the scope of the present study. Early instar larvae of *Cloantha perspicillaris* Boisd. (Lepidoptera: Noctuidae) were widely found on *H. perforatum* in the outskirts of Paris and Bordeaux, although it was not observed in the Pyrenees or in the area of Carcassonne. *Cloantha perspicillaris* has been reported to specifically colonize *Hypericum* spp. (Dubois &

Dubois Fils, 1880). During the day, young larvae of *C. perspicillaris* were observed on the ground, usually under the hostplants, and had rolled themselves up in a spiral. *Galeruca tanaceti* L. (Coleoptera: Chrysomelidae) was also observed consuming the inflorescences of the phototoxic plant *Chrysanthemum leucanthemum* L. (Asteraceae). *Chrysolina geminata* (Payrtull) (Coleoptera: Chrysomelidae) is another specialist insect whose larvae and adults were frequently observed feeding on *H. perforatum* in the Pyrenees and in the area of Carcassonne, especially in open fields.

. All insects were collected in the field between May and August 1995 and fed with aboveground tissues of *H. perforatum* in plastic containers equipped with a screen cover for holding for 24 to 48 hours until the experiments were performed in the laboratory at the Université de Pau et des Pays de l'Adour, France. Dr. Robert de Gregorio from this university identified the three grasshoppers while Georges Nehr from the Entomology Club of Pau identified the chrysomelid beetles and larvae and adults of *C. perspicillaris*.

Behavioral studies

Selective feeding

Adult grasshoppers, chrysomelid beetles and *C. perspicillaris* penultimate instar larvae were deprived of food overnight (14-16 h) before being offered a leaf of *H. perforatum* with 30 to 35 dark glands in which hypericin is sequestered (Knox & Dodge, 1985; Kingsbury, 1964). The phenological stage of the leaves used in the experiment was standardized by selectively removing newly expanded leaves occurring in the top part of plants that had just begun to produce flowers. The mean length and width of leaves used were 11.4 ± 2.3 mm (mean ± 1 s.d) and 4.6 ± 1.1

mm, respectively. The exact number of dark glands of the selected leaves was recorded. The outline of leaves were also sketched on 1 mm graph paper to determine their areas by visually counting the 1 x 1 mm squares. The leaves were then individually offered to insects. Grasshoppers were individually put in transparent plastic boxes (10 cm x 10 cm x 4 cm) and chrysomelid and *C. perspicillaris* penultimate instar larvae were set in small petri dishes (5 cm in diameter). The leaves were set on a wet squared filter paper (2 cm x 2 cm) to avoid foliar dessication and to maintain humidity inside the experimental containers in which insects were put. The experiment lasted for a period of 1 to 8 h according to the insect species—the experiment was carried on until insects had approximately fed on 50% of the leaf surface area. Insects were then removed and the area fed was determined by visually counting the 1 x 1 mm squares consumed by comparing the leaf area left to the one initially drawn on a 1 mm graph paper. The number of intact glands still present on the leaves and harboring no evidence of physical disturbance was also recorded. A gland was determined as physically disturbed when a dark coloration in the surrounding foliar tissues, which results from a physical breaking of the epidermal layer surrounding the glands, was observed. Twenty-five to 31 insects were used for each insect species but only those for which noticeable leaf consumption was detected were kept in the results, that is 25 to 28 insects per species. The experiment was performed at 25 °C under a dim light regime of 15 µmol PAR m^{-2} s^{-1}. This low light intensity was selected since insects, especially the three specialists, refused to feed under a high light regime.

An index of selective ingestion (*SI*) of non-phototoxic tissues was determined as the ratio of proportions of total leaf area fed and glands fed or physically altered (1).

$$SI = (LA_f / LA_t) / (G_f / G_t) \qquad (1)$$

In this equation, LA_f and LA_t, respectively, represent the leaf area that was eaten by insects and the total area of the leaves that was initially offered to insects. Similarly, G_f and G_t, respectively, refer to the number of glands that were fed or damaged by insects and the total number of glands that was initially recorded on the leaves offered to insects. According to this definition, a value of the *SI* higher than 1 means that insects are selectively feeding on the non-phototoxic foliar tissues located between the dark glands that contain the PT hypericin.

Variation in the number of hypericin-containing glands per leaf of Hypericum *spp. and larval feeding behavior of* Cloantha perspicillaris

Penultimate instar larvae of *C. perspicillaris* were collected on *H. perforatum* in the field in the area of Bergerac 80 km East of Bordeaux. Larvae were deprived of food overnight and they were individually placed into petri dishes as above except that three leaves with different numbers of dark glands were simultaneously offered. One leaf of *Hypericum androsaemum* L. that is lacking the dark glands containing hypericin was used as a control while two other leaves of *H. perforatum* having respectively 5-10 and 35-42 glands were offered. Leaves of similar size and phenological stage were selected (see above) and 31 replicates were performed. Insects were exposed to these three leaves for 2 hours under a dim light, 15 µmol PAR m^{-2} s^{-1} (see note above). Every 15 sec larval behavior was determined regarding their position, head in contact with a leaf or not, and their activity, active motion and feeding. A larva was considered in contact with a leaf when its head was less than approximately 1 mm away from a leaf. For the different calculations performed (Table 2), it was assumed that the behavioral response remained the same over the 15 sec interval between each observation. At the end of the experiment, the leaf area consumed on each leaf was determined as above.

Biochemical studies: antioxidant enzymes

Constitutive and hypericin-induced activities of antioxidant enzymes were determined as described in chapter 4 except that a visible light source provided by cool-white fluorescents,175 µmol PAR m^{-2} s^{-1}, was used to photosensitize hypericin. Hypericin dissolved in 10 µl of ethanol was applied to insects to reach a final approximate concentration of 25 µg/g of insect. Ethanol was dorsally applied on chrysomelid ultimate larval instar, but adult grasshoppers were subcutaneously injected since their sclerified cuticles could prevent the diffusion of hypericin. The injection was performed between the third and fourth abdominal sclerite using a 10 µl Hamilton syringe. Insects were then kept in darkness for 8 h to let hypericin diffuse through larval tissues and were subsequently exposed for 4 h under visible light as above to induce photosensitization. Insects were then dissected on ice to remove midgut and fat body, rinsed in H_2O containing NaCl (0.9%), and homogenized in PBS (0.1M, pH 7.4) containing 1 mM EDTA and centrifuged at 5000g for 5 min at 5°C. For each analysis, tissues from five chrysomelid larvae or three grasshopper adults were pooled and 3-4 replicates were performed per treatment.

Antioxidant enzymes, catalase (CAT, EC 1.11.1.6), glutathione reductase (GR, EC 1.6.4.2) and glutathione transferase (GST, EC 2.5.1.18), were measured as in chapter 4.

Results

Behavioral distinctions between generalist and specialist herbivorous insects feeding on *Hypericum perforatum*

Insect species ($F_{5,158} = 27.1$, $P<0.001$) and insect lifestyle ($F_{1,162} = 27.0$, $P<0.001$), that is specialists vs generalists, significantly affected the index of SI (Table 1). The index of SI for generalist insects was over two-fold higher than for specialist insects, 3.6 ± 0.4 (mean ± 1 s.e.) vs 1.5 ± 0.2 (mean ± 1 s.e.) (data not shown). The large variation observed for the index of SI resulted in overlapping values for five out of the six investigated insects (Table 2). The SI for *R. nitidula* was indeed about five-fold higher, 7.8 ± 1.1 (mean ± 1 s.e.), than the pooled value of all other insects, 1.6 ± 0.1 (mean ± 1 s.e.) (data not shown).

Dark glands located on leaves of *H. perforatum* also affected many characteristics of the feeding pattern of *C. perspicillaris* larvae (Table 3). The acceptability of a leaf as a feeding substrate by *C. perspicillaris* larvae was higher on leaves with dark glands than on the leaf bearing no gland (Table 3). Furthermore, the total duration that *C. perspicillaris* larvae spent in feeding, the total leaf area fed per contact and the rate of tissue ingestion were all higher on leaves harboring the highest number of glands (Table 3). The number of contacts, followed or not by ingestion, that *C. perspicillaris* larvae had with a leaf was, however, not affected by the number of glands present on these leaves (Table 3).

Table 1. Anova values for the index of selective herbivory.

Source	F-ratio	P
Insect species	27.099	<0.001
Lifestyle of insect[§]	27.048	<0.001

[§] The lifestyle refers to the classification of insects in generalist (*Ruspolia nitidula*, *Conocephalus discolor* and *Tettigonia viridissima*) or specialist (*Galeruca tanacetti*, *Chrysomela geminata* and *Cloantha perspicillaris*) based on the specificity of their occurrence on *Hypericum perforatum* in the field.

Table 2. Feeding patterns on leaves of *Hypericum perforatum* for different generalist and specialist herbivorous insects naturally occurring on this hostplant in nature.

Insect species	**Index of selective ingestion**[¶][§]
Generalist insects	
Ruspolia nitidula	7.84^{d} (1.12)
Conocephalus discolor	1.99^{bc} (0.19)
Tettigonia viridissima	0.95^{abc} (0.17)
Specialist insects	
Galeruca tanaceti	1.24^{abc} (0.40)
Chrysomela geminata	1.08^{abc} (0.15)
Cloantha perspicillaris	0.67^{ab} (0.17)

[¶] The selective ingestion was defined as the ratio between the proportion of leaf area fed and the proportion of glands fed or physically disturbed.

[§] Values provided in brackets represent the standard error of the mean ($n = 25\text{-}28$). Values followed by distinct letters differed significantly according to the Tukey's multiple comparison test ($P<0.05$) that was performed after a $\log_e$ transformation of the data.

Table 3. Behavioral responses of penultimate larval instars of *Cloantha perspicillaris* when simultaneously offered three leaves of *Hypericum* spp. harboring different number of glands containing the phototoxin hypericin.

Behavioral trait[¶]	**Number of dark glands containing hypericin per leaf**[§]		
	0	5-10	35-42
Number of contacts	1.4^a (0.4)	1.4^a (0.2)	1.7^a (0.2)
Rate of substrate acceptability (# contacts followed by ingestion over the total # of contacts)	0.174^a (0.055)	0.399^b (0.070)	0.536^b (0.075)
Total duration of active feeding (sec.)	558^a (152)	780^b (122)	1232^c (211)
Total leaf area fed (mm^2)	54.6^a (22.3)	348.2^b (87.2)	651.0^c (83.2)
Rate of ingestion (mm^2/s)	0.087^a (0.021)	0.418^b (0.102)	0.559^b (0.091)

[¶] Observations lasted over a period of 2 hours and values provided in brackets represent the standard error of the mean. Values on the same row and followed by distinct letters differed significantly based on the Dunn's procedure statistical analysis ($P<0.05$).

[§] Leaves of *Hypericum androsaemum* with no dark gland were used for the 0 treatment while leaves of *Hypericum perforatum* were used for the two other treatments. It was possible to obtain leaves of *H. perforatum* having 5-10 and 35-42 glands respectively owing to the natural variation observed for this trait in nature.

Antioxidant enzyme activities in tissues of generalist and specialist herbivorous insects feeding on *Hypericum perforatum*

All factors investigated apart from hypericin, that is insect species, insect lifestyle and insect tissue, significantly affected the activities of the three antioxidant enzymes (Table 4). The only exception was for the effect of insect tissue on the GR activity, although a borderline significance was obtained for this trait (P=0.055).

Pre-treatment of insects with hypericin only affected the activity of GST, which was increased by 47% (Tables 4 and 5). This particular sensitivity of the activity of GST to hypericin was essentially due to the two-fold increase observed for specialist insects, 149 ± 15 vs 315 ± 38 units for treatments without and with hypericin, respectively. No significant effect of hypericin was, however, noticed for generalist insects, 302 ± 34 vs 348 ± 28 units for treatments without and with hypericin, respectively (Table 5). These differences resulted in a significant interaction between insect lifestyle and hypericin on the GST activity (P=0.050) while no such interaction was obtained either for CAT (P=0.103) or for GR (P=0.131) (data not shown).

The lifestyle of herbivorous insects affected the activity of all three enzymes investigated, although the specific trends differed for the different enzymes. Specialist insects had a 2.3-fold higher activity of catalase than generalists, although the opposite was observed for GR and GST which were, respectively, 6.7 and 1.4 higher in generalist insects (Table 5).

The detailed results of all treatments for which antioxidant enzymes were determined are provided in Tables 6 and 7.

Discussion

The specific relevance of insect behavioral strategies to subdue the phototoxic defenses in *Hypericum perforatum*

Negative phototropism

Photosensitization of hypericin increases its biological activity by almost two-order of magnitude (Andreoni *et al.*, 1994; Hudson *et al.*, 1991; Carpenter & Kraus, 1991), although this phototoxic effect may be modulated by reaction parameters (Hudson *et al.*, 1994). It is thus not surprising that herbivorous animals occasionally exposed to hypericin in nature have evolved some adaptations to prevent or attenuate the light-activated toxicity of hypericin.

Herbivorous insects feeding on plants containing PTs may rely on a negative phototropism to circumvent phototoxicity. For example, concealment in the soil to avoid exposure to the daylight is efficient to reduce mortality of *Chrysolina quadrigemina* larvae that feed on phototoxic leaves of *H. perforatum* (Fields *et al.*, 1990). Although the present study was not designed to investigate the light-avoidance response of insects, observations made under field and laboratory conditions revealed the importance of this strategy for insects feeding on the phototoxic leaves of St. John's-wort. In the field, all *C. perspicillaris* older larval instars were hiding under the leaves on which they were feeding whereas, in presence of the daylight, most first and second instars were found buried in the one cm uppermost soil layer underneath *H. perforatum* plants. It is likely that the feeding stimulant effect that hypericin exerts on *C. perspicillaris* larvae (Table 3) optimizes the functional efficacy of this light avoidance response that involves at least daily withdrawal of larvae from their hostplant. Hypericin, which represents a distinct chemical "fingerprints" of *H. perforatum*, and a few other *Hypericum* spp.,

Table 4. Anova values for the activities of antioxidant enzymes.

Enzyme	Source	F-ratio	P
Catalase	Lifestyle of insect	5.484	0.022
	Insect species	10.659	<0.001
	Insect tissue	6.761	0.012
	Hypericin	1.698	0.197
Glutathione reductase	Lifestyle of insect	64.022	<0.001
	Insect species	39.610	<0.001
	Insect tissue	3.812	0.055
	Hypericin	1.624	0.207
Glutathione S-transferase	Lifestyle of insect	7.807	0.007
	Insect species	7.606	<0.001
	Insect tissue	8.240	0.006
	Hypericin	10.586	0.002

[§] The lifestyle refers to the classification of insects in generalist (*Conocephalus discolor* and *Tettigonia viridissima*) or specialist (*Galeruca tanacetti* and *Chrysomela geminata*) based on the specificity of their occurrence on *Hypericum perforatum* in the field.

Table 5. Mean values for the effects of insect lifestyle and hypericin on the activity of catalase, glutathionse S-transferase and glutathione reductase.

Source	Enzymatic activity		
	Catalase[§]	Glutathione reductase[§]	Glutathione S-transferase[§]
Hypericin			
-hypericin	798^{a} (43)	22.4^{a} (6.3)	225^{a} (23)
+hypericin	1515^{a} (91)	31.8^{a} (7.2)	331^{b} (23)
Lifestyle of insects			
generalist	529^{a} (49)	48.0^{b} (5.4)	325^{b} (22)
specialist	1783^{b} (82)	6.2^{a} (1.2)	232^{a} (25)
Hypericin * lifestyle of insects			
-hypericin, generalist	605^{a} (34)	39.4^{b} (2.4)	302^{b} (34)
-hypericin, specialist	990^{ab} (15)	5.4^{a} (0.6)	149^{a} (15)
+hypericin, generalist	455^{a} (28)	56.6^{b} (2.8)	348^{b} (28)
+hypericin, specialist	2575^{b} (38)	7.0^{a} (0.9)	315^{b} (38)

[§] Values provided in brackets represent the standard error of the mean (n=3-4). For each enzyme, values from a given source that are followed by distinct letters differed significantly based on Tukey's multiple comparison tests ($P<0.05$). Units of activities for catalase, glutathione reductase and glutathione S-transferase are µmole H_2O_2 decomposed/mg protein/min, units/mg protein/min and nmole 1-chloro-2,4-dinitrobenzene conjugate/mg protein/min respectively.

Table 6. Activities of antioxidant enzymes in generalist grasshoppers feeding occasionally on phototoxic leaves of *Hypericum perforatum* in absence or presence of hypericin.

Insect species	Tissue analyzed	Hypericin†	Enzyme		
			Catalase§	Glutathione reductase§	Glutathione S-transferase§
Tettigonia viridissima	Midgut	-	885 (94)	53.0 (5.2)	508 (24)
		+	547* (65)	57.3 (3.4)	431 (28)
	Fat body	-	168 (16)	9.2 (2.1)	201 (25)
		+	97* (12)	6.9 (0.4)	304* (15)
Conocephalus discolor	Midgut	-	640 (43)	40.0 (4.5)	307 (17)
		+	482 (50)	99.0* (5.9)	446* (32)
	Fat body	-	730 (32)	55.5 (2.9)	191 (14)
		+	694 (34)	63.0 (7.1)	211 (25)

§Units of activities for catalase, glutathione reductase and glutathione S-transferase are µmole H_2O_2 decomposed/mg protein/min, units/mg protein/min and nmole 1-chloro-2,4-dinitrobenzene conjugate/mg protein/min respectively. Values provided in brackets represent the standard deviation. Values from the treatments with hypericin (+) that are followed by an asterisk are significantly different than the corresponding treatments without hypericin (LSD, $P<0.05$).

†Hypericin was topically applied at an approximate concentration of 25 µg/g of insect. Symbols - and + refer to without and with hypericin respectively.

Table 7. Activities of antioxidant enzymes in specialist chrysomelid beetle larvae naturally feeding on phototoxic leaves of *Hypericum perforatum* in absence or in presence of hypericin.

Insect species	**Tissue analyzed**	**Hypericin**	**Enzyme**		
			Catalase[§]	**Glutathione reductase[§]**	**Glutathione S-transferase[§]**
Geleruca tanaceti	Midgut	0	208 (31)	4.1 (0.2)	217 (16)
		+	783* (46)	6.4* (0.4)	494* (27)
	Fat body	-	3569 (252)	10.3 (0.4)	184 (12)
		+	8971* (710)	4.8* (0.6)	316* (29)
Chrysomela geminata	Midgut	-	94 (11)	4.2 (0.4)	102 (15)
		+	103 (17)	9.6* (1.9)	103 (17)
	Fat body	-	89 (7)	5.1 (0.4)	94 (20)
		+	444* (28)	8.2* (0.3)	346* (23)

[§] Units of activities for catalase, glutathione reductase and glutathione S-transferase are µmole H_2O_2 decomposed/mg protein/min, units/mg protein/min and nmole 1-chloro-2,4-dinitrobenzene conjugate/mg protein/min respectively. Values provided in brackets represent the standard deviation.

[¶] Values from the treatments with hypericin (+) that are followed by an asterisk are significantly different than the corresponding treatments without hypericin (LSD, P<0.05).

[†] Hypericin was topically applied at an approximate concentration of 10 mg/g of insect. Symbols - and + refer to without and with hypericin respectively.

may guide *C. perspicillaris* larvae to reach an appropriate hostplant when they are leaving the soil at sunset.

The second observation suggesting that herbivorous insects feeding on *H. perforatum* rely on a negative phototaxis strategy, is supported by the fact that all chrysomelid and *C. perspicillaris* larvae refuse to feed on leaves when they were irradiated with a light regime representative of open sites in nature, that is 150 ± 25 µmole PAR m^{-2} s^{-1} (Downum *et al.*, 1991; Fitter & Hay, 1991). Insects were, however, observed to feed when put under a dim light (15 µmol PAR m^{-2} s^{-1}, see comment in materials and methods).

Selective feeding on the least phototoxic parts

The distribution of the SCs in spatially discrete plant tissues is likely to result in a heterogeneous feeding pattern involving more damage on parts lacking or harboring the lowest amount of chemical defenses (see references in chapter). For example, *Chlorochlamys chloroleucaria* larvae feed preferentially on the non-phototoxic pollen compared to other phototoxic parts of the inflorescence of *Rudbeckia hirta* (Guillet *et al.*, 1995). St. John's-wort may be particularly vulnerable to such a selective feeding strategy since the glands containing hypericin occur restrictively on a narrow stripe of tissues located on the leaf edge (see figure in Fields *et al.*, 1991). This spatial arrangement of the SCs in *H. perforatum* lets the central part of leaf blades unprotected. The present results indeed indicated that generalist grasshoppers selectively feed on the non-phototoxic parts since the proportion of leaf area on which they fed was 3.6-fold higher than the proportion of glands ingested or physically disturbed, that is a mean *SI* of 3.6 ± 0.5 (mean ± 1 s.e., data derived from Table 2). The feeding pattern of specialist insects was, however, not affected by the presence of the glands in leaves of *H. perforatum* since their mean *SI*, 1.5 ± 0.19 (mean ± 1 s.e., data derived from Table 2), did not significantly differ from the neutral value of 1 (the 95% CI for the $\ln_e[SI]$ was [-0.119, 0.225] and thus overlapped the $\ln_e[1]$ or 0) that refers to equivalent proportions of leaf area ingested and glands fed or damaged. In other words, the number of glands containing hypericin fed on or physically disturbed by specialist insects was proportional to the leaf area ingested. This absence of behavioural avoidance to ingest phototoxic glands in specialist insects is consistent with the phagostimulant effect that these glands exerted on *C. perspicillaris* larvae (Table 3). Tarsal chemoreceptors with a cell responding specifically to hypericin have already been identified in *Chrysolina brunsvicensis*, a specialist insect feeding on *H. perforatum*, which indeed exploits hypericin as a feeding stimulant (Rees, 1969).

The role of antioxidant enzymes in herbivorous insects to counteract the phototoxic defenses in *Hypericum perforatum*

It is generally accepted that antioxidant compounds and antioxidant enzymes provide protection to prevent or reduce the oxidative damages caused by singlet oxygen to herbivorous insects feeding on hostplants containing PTs of type II (Aucoin *et al.*, 1990, 1995; Guillet *et al.*, 1997b). Comparative studies regarding the activities of antioxidant enzymes between insects from different orders and lifestyles have, however, rarely been performed. A few investigations have compared the activities of antioxidant enzymes between different lepidopterous insects including some susceptible and some that are more tolerant to PTs (Aucoin *et al.*, 1991; Pardini *et al.*, 1989; Pritsos *et al.* 1991). These studies suggested that higher constitutive levels of antioxidant enzymes occur in insects that are more likely to be exposed to PTs in nature.

The present study provided unexpected results since generalist insects had a higher constitutive level than specialist insects for two antioxidant enzymes, GR and GST (Table 5). These two enzymes have been previously identified as important biochemical features conferring to insects a tolerance to the PT-mediated oxidative stress (Ahmad *et al.*, 1992, 1994). Even by considering the two-fold inducible activity of the GST in specialist insects (Table 5), the final level reached is only comparable to the constitutive activity observed in generalist insects. These results cast doubt on the widely accepted concept that generalist insects have less efficient biochemical adaptive features to tolerate "qualitative" defenses than specialists insects (Feeny, 1976). One plausible explanation for the present results is the non-specific roles assumed by antioxidant enzymes that are triggered by different exogenous and endogenous factors in all aerobic organisms (see review in Eisenstark *et al.*, 1995). Multiple genes for GST are found in vertebrates where each locus has its own promoter and some inducers affect only specific loci in a GST gene family (Pickett *et al.*, 1984). Multiple forms of GST occur in *Drosophila melanogaster* (Ottea *et al.*, 1987; Jansen *et al.*, 1984) and it is likely that many dietary constituents other than PTs affect the expression of the different forms (Harshman *et al.*, 1991).

Conclusion

The causal factors that allow herbivorous insects to colonize a limited number of hostplants represent a fundamental question that must be answered to better understand the dynamics of plant-insect relationships on both ecological and evolutionary time scales. The scientific literature has historically focused on interspecific variations of SCs as the major constraint limiting the range of hostplants accessible to insects (Dethier, 1954; Fraenkel, 1959; Ehrlich & Raven, 1964; Berenbaum, 1983; Schultz, 1988; Firn & Jones, 1996). The present study adds to that literature by indicating that adaptations to phototoxic defenses are achieved largely by behavioral feeding strategies used by both specialist and generalist insects, rather than biochemical adaptations.

Acknowledgments

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Chapter 4

Phototoxic polyacetylenes from *Viguiera annua* (Asteraceae) and adaptations of a chrysomelid beetle, *Zygogramma continua*, feeding on this plant

Introduction

More than 1000 species of Asteraceae are now reported to produce polyacetylene derivatives (Bohlmann *et al.*, 1973; Christensen & Lam, 1990, 1991) many of which exert potent insecticidal effects when sensitized by near-UV light (Champagne *et al.*, 1986). This phototoxicity process which is due to the production of activated oxygen species or other radicals damage the lipid membranes (Aucoin *et al.*, 1995). However, some insects that are often exposed to phototoxic polyacetylenic derivatives in their host plants possess either some light avoidance behavior (Guillet *et al.*, 1995) or enzymatic and non-enzymatic antioxidant adaptations that may protect them against phototoxicity (Aucoin *et al.*, 1995; Berenbaum, 1994).

A phototoxic plant, *Viguiera annua* (M.E. Jones) Blake (Asteraceae), as well as a chrysomelid beetle, *Zygogramma continua* Le Conte (Coleoptera: Chrysomelidae), feeding preferentially on this plant were investigated in the present study. *Viguiera* is an important genus of the subtribe Helianthinae and is mostly found in Central America, South America and in the Southwestern United States (Robinson, 1981, Franco-Vizcaino *et al.*, 1993). The genus *Viguiera* appears to be represented by opportunistic species that grow and develop quickly after the first rains of the wet season (Genin & Pijoan, 1993; Gray, 1982) as was also observed for the annual goldeneye, *V. annua*, in our field survey near Tucson, Arizona (USA). The annual goldeneye has been reported as potentially poisonous to livestock due to its ability to sequester potassium nitrate in its tissues at up to 4.7% d.w. (Williams, 1989). In rangelands of New Mexico, *V. annua* occupies up to 50% of the total foliar cover (Williams, 1990).

The insect genus *Zygogramma* has been reported to be distributed in Mexico, Costa Rica, Guatemala and Nicaragua (Wilcox, 1983). *Zygogramma* contains several species that are known to feed restrictively on a particular hostplant. For example, *Z. bicolorata* and *Z. suturalis* have sufficiently a narrow host range that they have been introduced into Europe as biological control agents for their respective hostplant, *Parthenium hysterophorus* and *Ambrosia artemisifolia* (Jayanth & Visalakshy, 1996; Igrc *et al.*, 1995).

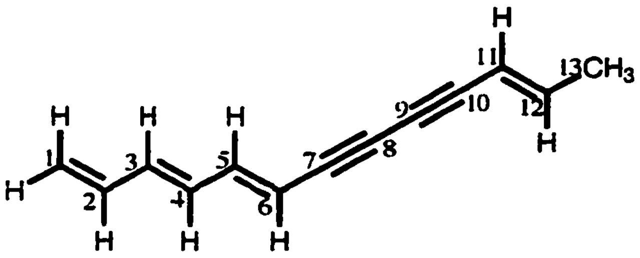
In the present study, we elucidated the structures of two phototoxic polyacetylenes, *trans*-1,3,5,11-tridecatetraen-7,9-diyne (**1**) and 1,*cis*-3,*trans*-5,*trans*-11-tridecatetraen-7,9-diyne (**2**) (Fig. 1), which occur in the leaves of *V. annua*. The behavioral and biochemical investigations of *Z. continua* larvae that were observed to selectively feed on the phototoxic leaves of *V. annua* suggested that both light-avoidance behavior and inducibility of some antioxidant enzymes are useful to circumvent plant phototoxic defences.

Materials and methods

Extraction and isolation of polyacetylenes

Plants of *V. annua* as well as the chrysomelid larvae were collected in the Buenos Aires National Wildlife Refuge about 100 km South of Tucson, Arizona

Figure 1. Phototoxic polyacetylenes isolated in leaves of *Viguiera annua* (Asteraceae).



H
H
H
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1
2
3
4
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6
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8
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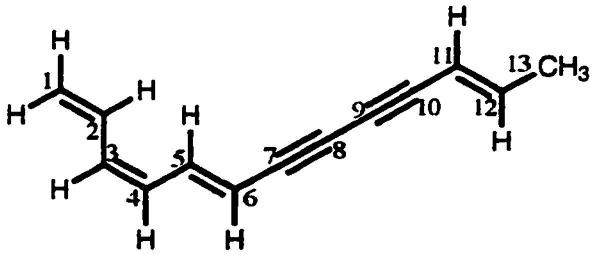
10
11
12
13
CH_3
H
H
H
H

1



H
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CH_3
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2

(USA). The plants were identified at the herbarium of the University of Arizona in Tucson. For the phytochemical isolations, seeds collected were sown in vermiculite and grown to maturity in a greenhouse. Foliar tissues, 650 g, were homogenized in 2.0 l EtOH (95%), filtered, and then combined with 2 l H_2O before being partitioned twice with a total volume of 1.5 l hexane. The hexane fraction was reduced to a volume of 2 ml by rotary evaporation, and the polyacetylenes were then separated by preparative HPLC (model LC-908, Japan Analytical Industry Co. Ltd) using a reverse phase C-18 column with acetonitrile:water (7:3) at a flow rate of 3 ml/min. The two major peaks determined by UV absorption at 254 nm and by refractive index, were separately collected, evaporated to dryness, and then submitted for GC-MS and ^1H and ^{13}C NMR analysis.

Compound 1: ^1H NMR (500 MHz, C_6D_6) δ 6.64 (1 H, dd, J = 15.5 Hz, 10.2 Hz, H-5), 6.24-6.14 (3 H, m, H-2,3,4), 6.11 (1 H, dd, J = 15.8 Hz, 6.9 Hz, H-12), 5.68 (1 H, d, J = 15.5 Hz, H-6), 5.49 (1 H, dd, J = 15.8 Hz, 1.4 Hz, H-11), 5.14 (1 H, d, J = 14.0 Hz, H-1_{trans}), 5.02 (1 H, d, J = 8.8 Hz, H-1_{cis}), 1.48 (3 H, dd, J = 6.9 Hz, 1.4 Hz, H_3-13); ^{13}C NMR (125 MHz, C_6D_6) δ 145.12 (C-5), 144.65 (C-12), 137.31 (C-3*), 136.84 (C-4*), 132.34 (C-2*), 120.51 (C-1), 110.27 (C-6), 109.89 (C-11), 82.91 (C-7*), 81.36 (C-8*), 78.00 (C-9*), 72.90 (C-10*), 18.77 (C-13); MS *m/z* 168 (M, 78), 167 (28), 166 (21), 165 (57), 153 (40), 152 (100), 151 (16), 141 (16), 139 (19), 128 (17), 115 (35), 77 (15), 63 (18), 39 (16). * assignment ambiguous.

Compound 2: ^1H NMR (500 MHz, C_6D_6) δ 7.00 (1 H, ddd, J = 15.4 Hz, 11.4 Hz, 1.0 Hz, H-5), 6.39 (1 H, ddt, J = 16.7 Hz, 11.4 Hz, 1.0 Hz, H-2), 6.00 (1 H, dd, J = 15.8 Hz, 6.9 Hz, H-12), 5.76 (1 H, dt, J = 11.4 Hz, 0.9 Hz, H-3), 5.61 (1 H, t, J = 11.4 Hz, H-4), 5.38 (1 H, d, J = 15.4 Hz, H-6), 5.31 (1 H, ddd, J = 15.8

Hz, 1.8 Hz, 1.0 Hz, H-11), 5.00 (1 H, dt, J = 16.7 Hz, 0.9 Hz, H-1_{trans}), 4.92 (1 H, d, J = 11.4 Hz, H-1_{cis}), 1.24 (3 H, dd, J = 6.9 Hz, 1.8 Hz, H_3-13); ^{13}C NMR (125 MHz, C_6D_6) δ 143.60 (C-12), 139.58 (C-5), 133.23 (C-3), 132.09 (C-2), 128.77 (C-4), 119.87 (C-1), 111.37 (C-6), 110.35 (C-11), 82.89 (C-7*), 81.22 (C-8*), 78.85 (C-9*), 73.72 (C-10*), 18.48 (C-13); MS *m/z* 168 (M, 77), 167 (31), 166 (29), 165 (66), 153 (42), 152 (100), 151 (15), 141 (17), 139 (20), 128 (17), 115 (35), 90 (15), 89 (21), 78 (15), 77 (15), 63 (20), 39 (17). * assignment ambiguous.

Light avoidance behavior of *Zygogramma continua* larvae

The coleopteran larvae collected on the leaves of *V. annua* were identified by Dr. Carl Olson from the Department of Entomology at the University of Arizona as *Zygogramma continua* Le Conte (Coleoptera: Chrysomelidae). Intense phototoxicity of the *V. annua* leaves was detected using a standard yeast inhibition assay (Camm *et al.*, 1975). For the behavioral experiment, oblong-elongated leaves of *V. annua*, *ca* 12 cm long and 0.8 cm wide, were cut in squares (0.9 cm x 0.9 cm) and individually placed in a compartment of a Falcon tray (1.6 cm in diameter). One third instar larva was deposited onto each leaf section and the trays were recovered with a plastic wrap transparent to visible and near-UV wavelengths. Trays containing larvae and leaves were then placed under visible light (cool-white, 80W/m^2), near-UV light (300-400 nm) (Westinghouse F20T12/BLB bulbs, 1.0 mW/cm^2) or kept in darkness at room temperature. Observations were made every 15 min over a 3 h period on the relative position of the larvae compared to the leaf squares. Larvae without any contact with the leaf tissues were classified as "escaped" while those still in contact with the leaf were divided in two groups depending on whether their heads were located above or under the leaves. At the end of the 180 min observation period, the feeding activity of each larva was determined by placing the leaf parts over a 1 mm graph paper and then evaluating the area removed using a dissecting microscope. Due to a limitation in the availability of third instar larvae, the experiment was repeated twice using each time 20 larvae per light treatment so that 120 larvae were used in total (3 treatments * 20 larvae/treatment * 2 experiments). For statistical analysis, larvae having either escaped or moved under the leaf were considered as responding while those remaining above the leaf were counted as non-responding. The Kruskall-Wallis test for nonparametric data was used and then the Dunn procedure was performed to compare the insect response between the different light regimes (Rosner, 1995).

Biochemical adaptations of *Zygogramma continua* larvae: Inducibility of antioxidant enzymes in presence of α-terthienyl

The hypothesis that inducible antioxidant enzymes may buffer the oxidative stress generated by polyacetylenic derivatives in insects was tested using third instar *Z. continua* larvae. Because the polyacetylenes contained in *V. annua* had not yet been purified at the time of our field survey, we used α-terthienyl as an alternate photo-oxidative polyacetylenic derivative (Scaiano *et al.*, 1989). Because oral administration with a feeding syringe would have been impractical due to the small size of *Z. continua* larvae, so topical applications were performed. Five μl of EtOH containing 10 μg of α-terthienyl (control insects received only EtOH) were dorsally applied on each larva. Larvae were then kept in darkness for 8 h to let the α-terthienyl diffuse through larval tissues. Our previous work has shown that over 50% of the topically applied α-terthienyl diffused in the internal tissues of lepidopteran insect larvae in this time frame (Iyengar *et al.*, 1987). The fact that the toxicokinetic parameters of α-terthienyl are comparable in both topical application or oral ingestion (Iyengar *et al.*, 1987) was used to justify our experimental approach. *Zygogramma continua* larvae were subsequently exposed for 4 h under near-UV irradiation as above to stimulate the α-terthienyl photosensitization. Insects were then dissected on ice, rinsed in H_2O containing NaCl (0.9%), homogenized in phosphate buffer (0.1M, pH 7.4) containing 1mM EDTA and centrifuged at 5 000*g* for 5 min. Tissues of 10 insects were pooled for each analysis and 3-5 replicates were performed for each treatment.

Activities of superoxide dismutase (SOD, EC 1.15.1.1) and catalase (CAT, EC 1.11.1.6) were determined as described in Aucoin *et al.* (1991) and glutathione reductase (GR, EC 1.6.4.2) and glutathione S-transferase (GST, EC 2.5.1.18) were performed as in Lee & Berenbaum (1989) and Lee (1991), respectively. Protein content was determined as in Bradford (1976) using bovine serum albumine as a standard.

CAT was determined by monitoring the breakdown of H_2O_2 at 240 nm. One ml of H_2O_2 (50 mM) solution was added in a cuvette and mixed with 2.5-5 µl of insect sample before to read the absorbance at 240 nm for 1 min. In this assay, 1 unit of CAT is defined as 1 µmol H_2O_2 decomposed/mg protein/min at 25 °C.

SOD was determined using a cytochrome c reduction assay which was monitored at 550 nm. A xanthine-xanthine oxidase system was used as the source of superoxide (O_2^-). The inhibition of the reduction of cytochrome c by O_2^- was measured by adding insect sample. The initial rate of cytochrome c reduction was determined by adding 20 µl of xanthine oxidase (0.2 U/ml) in a 1 ml cuvette containing 950 µl of potassium phosphate buffer (0.1 M, pH 7.6) containing 2 µM cytochrome c, 5 µM xanthine, and 0.1 mM EDTA, and measuring the absorbance at

550 nm for 2 min. For each sample, two other measurements were made under the same conditions but with 10 and 25 µl of insect sample added. Mean slopes over the two min. reading at 550 nm were determined for each measure and the volume required to inhibit by 50% the initial rate of reduction of cytochrome c was determined using a linear regression. One unit of SOD activity was defined as the amount required to inhibit by 50% the rate of reduction of cytochrome c at 25 °C (pH 7.6) and was expressed in unit/mg protein.

GR assay was determined based on NADPH oxidation in the presence of exogenous oxidized glutathione (GSSG) (Racker, 1955). The 1 ml reaction mixture contained 0.6 ml PBS (0.1 M, pH 7.6), 100 µl NADPH (1 mM), 100 µl of bovine serum albumine (1% w/v solution in PBS), 100 µl GSSG (2% solution in PBS), and 100 µl of insect sample. Endogenous oxidation of NADPH was measured in the absence of insect sample and used a correction factor. One unit of GR was defined as as the oxidation of 1 nmol of NADPH oxidized/min/mg protein.

GST assay was determined based on the change in absorbance at 340 nm related to the production of CDNB-conjugate (Lee & Berenbaum, 1992). The 1 ml reaction mixture contained PBS (0.1 M, pH 7.6, 1 mM EDTA), 1 mM GSH and 2-5 µl of enzyme source. The reaction mixture was equilibrated for 1 min before adding 20 µl of CDNB (50 mM) and the absorbance was monitored at 340 nm for 2 min. One unit of GST activity was determined as nmol CDNB conjugate/mg protein/min using an extinction coefficient of 9.6 $mM^{-1}*cm^{-1}$ for S-(2,4-dinitrophenyl) glutathione.

Results and discussion

Structure elucidation of polyacetylenes

The U.V. spectra of **1** and **2**, displaying λ^{hexane} at 360, 334, 315, 280, 265 and 255 nm, suggested the presence of triene-diyne-ene chromophores (Bohlmann *et al.*, 1973). Mass spectral analysis provided a M_r of 168 for each of the 2 compounds. They were determined to be closely related structural isomers of one another, with the molecular formula $C_{13}H_{12}$, as verified via ^{13}C NMR spectroscopy, by the presence of 13 carbon signals. ^1H-^1H coupling patterns, along with data from 2D COSY and HMQC NMR experiments, confirmed the arrangement of double and triple bonds. Compound **1**, the all-*trans* geometrical isomer, displayed typical ^1H-^1H coupling constants (J = 12-18 Hz) across each of its *trans* double bonds: 14.0 Hz (H-1_{trans},H-2), 15.5 Hz (H-5,H-6), and 15.8 Hz (H-11,H-12) (H-3 and H-4 resonated at almost identical frequencies, thereby making for intractable second order coupling patterns). Compound **2** had a ^1H NMR spectrum very similar to that of compound **1**, only in this case, the C3-C4 double bond clearly showed *cis* geometry (J = 6-12 Hz). The *trans* and *cis* ^1H-^1H coupling constants observed for compound **2** were 16.7 Hz (H-1_{trans},H-2), 11.4 Hz (H-3, H-4), 15.4 Hz, (H-5,H-6), and 15.8 Hz (H-11,H-12). Compounds **1** and **2** thus differ only in their C3-C4 double bond. The concentrations of compounds **1** and **2** in the leaves of *V. annua* were *ca* 5 mg/g dry wt and 3.5 mg/g dry wt respectively. The 1,3,5,11-tridecatetraen-7,9-diyne has been reported to occur in 27 species of the Heliantheae tribe (Christensen & Lam, 1991).

Insect tolerance to phototoxins: behavioral and biochemical adaptations

The compounds **1** and **2** when tested in our standard yeast bioassay (Arnason *et al.*, 1991) were found to be highly phototoxic (with UV treatment, 22 mm of inhibition for 10 µg applied to a paper disk of 7 mm diameter, but no inhibition in dark). In previous experiments, we also showed that polyacetylenes with similar chromophores to compounds **1** and **2** were highly phototoxic to mosquito larvae (Arnason *et al.*, 1981).

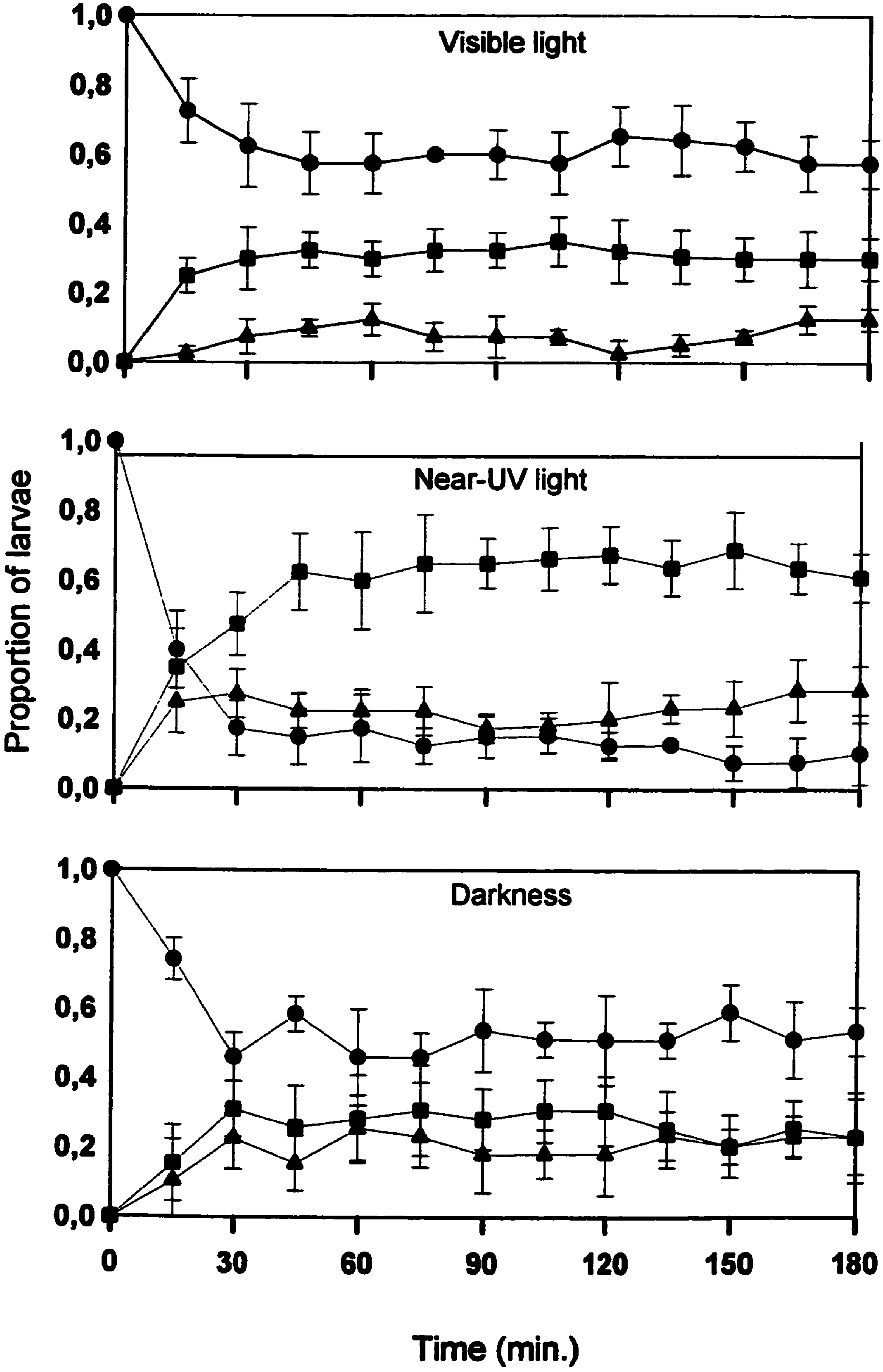
In a laboratory behavioral experiment (Fig. 2) beetle larvae assumed positions on the phototoxic leaves of *V. annua,* that were influenced by the light regime (Kruskall-Walis test, $P<0.001$). From 45 min until the end of the observation period, the number of larvae that had either escaped, that were no longer touching the leaves, or that had concealed their head under the leaf, was significantly higher for the near-UV treatment than in the visible light or dark treatments (Dunn's procedure, $P<0.05$). For example, about 55% of the larvae remained above the leaves after 45 min in both visible light and darkness, while only 16% remained exposed to the presence of photosensitizing near-UV light (Fig. 2).

Under field conditions, the first *Z. continua* larval instars were often observed hiding at the basal part of a leaf with their heads located between a node and the base of a petiole on *V. annua* plants. Such larval concealment in fields may constitute an analogous hiding behavior to that detected in our lab experiment.

In the laboratory experiment, the feeding activity was also affected by the light regime since the area of tissues ingested was nearly two-fold higher in darkness than either under near-UV or visible light (Table 1).

We had previously reported the efficiency of behavioral adaptations alone to reduce the mortality of insects feeding on phototoxic plants in nature (Guillet *et al.* 1995; Fields *et al.*, 1989). However, we hypothesized that antioxidant capacity may also be induced in these insects after exposure to phototoxins, in order to enhance the protection of insects. Table 2 shows induction by the related phototoxin

Figure 2. Effects of light regimes on the behavioral response of third instar *Zygogramma continua* larvae feeding on phototoxic leaves of *Viguiera annua*. The symbols ●, ■, and ▲ respectively represent the proportions of larvae above the leaves, under the leaves and those without any contact with the leaves. Each point represents the average of duplicate trials and vertical bars represent the standard deviation.



Visible light
Near-UV light
Darkness
Proportion of larvae
1,0
0,8
0,6
0,4
0,2
0,0
0
30
60
90
120
150
180
Time (min.)

Table 1. Feeding activity of *Zygogramma continua* third instar larvae on phototoxic leaves of *Viguiera annua* under different light regimes.

Treatment	Ingestion[†] (mm^2/larva/3 hr)
Near-UV light	1.77[a] (0.22)
Visible light	1.61[a] (0.26)
Darkness	3.27[b] (0.19)

[†] Values followed by the same letter are not significantly different (LSD, $P<0.05$). Values provided in brackets represent the standard error of the mean.

Table 2. Effects of α-terthienyl topical applications on the activities of antioxidant enzymes in third instar *Zygogramma continua* larvae[†].

	CAT[‡]			SOD[‡]			GST[‡]			GR[‡]		
tissue	-αT	+αT	ind (%)	-αT	+αT	ind (%)	-αT	+αT	ind (%)	-αT	+αT	ind (%)
midgut	222 (57)	189 (42)	-17	4.9 (0.4)	3.2* (0.5)	-35	1420 (85)	2005* (90)	+44	1.9 (0.3)	2.8* (0.3)	+45
fat body	1174 (239)	1372 (150)	+17	2.9 (0.3)	2.3 (0.3)	-21	1270 (70)	1840* (50)	+45	1.6 (0.4)	2.5* (0.2)	+50

[†] Activity units for CAT, SOD, GST and GR are µmole H_2O_2 decomposed/mg protein/min, units/mg protein/min, nmole 1-chloro-2,4-dinitrobenzene conjugate/mg protein/min and nmole NADPH oxidation/mg protein/min respectively.

[‡] Values in brackets represent the standard deviation. Treatments with and without α-terthienyl are referred to as +αT and -αT. Values from the +α-T treatments that are followed by an asterisk are significantly different than the corresponding -α-T treatments (LSD, $P<0.05$). Ind represents the relative inducibility of enzymatic activity between treatments +α-T and -α-T.

α-terthienyl of glutathione-S-transferase (GST) activity and glutathione reductase (GR) activity in both midgut and fat body ($P<0.05$). Conversely, superoxide dismutase (SOD) activity was inhibited in the midgut and catalase (CAT) activity appeared unaffected when *Z. continua* larvae were topically applied with α-terthienyl. These results suggest a predominant role for GST and GR in the tissues of *Z. continua* to buffer the α-terthienyl-induced oxidative stress that leads to the peroxidation of lipids in sensitive insects (Aucoin *et al.*, 1995) and ultimately to larval mortality. The previous demonstration of the importance of a high GSH:GSSG ratio in tobacco hornworm larvae to prevent the α-terthienyl-induced peroxidation of lipids (Aucoin *et al.*, 1995) and our current results with the glutathione-dependent enzymes, GST and GR, indicate that glutathione is an important defense against photoxidants.

A plausible explanation for the fact that SOD and CAT activities were not induced by α-terthienyl may be that it is primarily a generator of singlet oxygen that can lead to lipid peroxidation without the involvement of either superoxide or hydrogen peroxide. Nivsarkar *et al.* (1991) demonstrated that SOD was inhibited by α-terthienyl in the anal gills of mosquito larvae.

The activities of antioxidant enzymes in *Z. continua* larvae were also affected by the nature of the tissues (Table 2). For example, CAT was five-fold more active in the fat body than in the midgut while GST and GR had similar activity in both tissues (less than 22% variation). SOD showed a slight trend to higher activity in the midgut than in fat body either for α-terthienyl treated larvae (39% higher) or control larvae (69% higher).

While it is unknown if the integrated behavioral and biochemical strategies reported in *Z. continua* larvae have directly arisen due to the presence of phototoxins in its hostplant, these adaptive traits represent major features that make this insect tolerant to the phototoxic polyacetylenes present in *V. annua*. In terms of an optimal strategy for the chrysomelid, it is hypothesized that the behavioral response of *Z. continua* larvae is the primary defence. The substitution of biochemical for behavioral adaptation when light avoidance is not feasible, is the best strategy to minimize the cost required to prevent phototoxicity.

Acknowledgements

Part of the work was performed in E.A. Bernays's laboratory at the University of Arizona in Tucson. M. Singer and D. Champagne provided helpful indications for locating field sites in Tucson. D. Chaurest performed the NMR analysis and helped with the structure elucidation of both polyacetylenes.

Chapter 5

How do light and nutrients affect the production of chemical defenses and the palatability of *Carthamus tinctorius* plants to generalist herbivorous insects?

Introduction

The toxicological properties of some polyacetylenic derivatives, also referred to as phototoxins (PTs), are increased by more than one order of magnitude in the presence of near-UV light (Arnason *et al.*, 1981a; Champagne *et al.*, 1984, 1986; Marles *et al.*, 1992; Lozoya & Gaspar, 1993). The tight relationship between the absorption spectra of different polyacetylenes and their action spectra on mosquito larvae (Arnason *et al.*, 1981b) corroborates the photosensitization process involved.

One expectation for plants producing PTs is that a variation in the light intensity will affect the efficacy of the photosensitization process and thus the protection against pest organisms including herbivorous insects. In agreement with this prediction, the proportion of phototoxic inflorescences of *Chrysanthemum leucanthemum* (Asteraceae) damaged by larvae of the red-banded leaf roller, *Argyrotaenia velutinana* (Lepidoptera: Tortricidae), was an order of magnitude higher in the shade than in open sites (Guillet *et al.*, 1995). Such differential herbivory between phototoxic plants exposed to different light regimes may result in a fitness gradient with hostplants in open habitats performing the best. Over evolutionary time, this spatially heterogeneous herbivore pressure may result in a restricted distribution of phototoxic plants to sunny habitats as observed with the phototoxic species of the Rutaceae family (Berenbaum, 1981).

The interactions between abiotic environmental traits, especially light intensity and nutrient availability (Coley, 1983; Bryant *et al.*, 1983), and production of SCs represent an alternative explanation for the restrictive distribution of phototoxic plants in open habitats. A higher light intensity generally increases the phenolic content and the leaf toughness that are both negatively correlated with the herbivory experienced by hostplants (Shure & Wilson, 1993). A similar increase in response to a higher light regime is also expected for all carbon-based defenses (Bryant *et al.*, 1983) including polyacetylenic PTs produced in some Asteraceae species. According to this scheme, it is predicted that phototoxic plants would occur more frequently in open habitats since individuals growing in the shade would have less chemical defenses, and thus be exposed to a higher rate of herbivory.

The present study tested the hypothesis that shaded phototoxic plants suffer greater herbivory than phototoxic plants grown in sunny habitats because of a lower production of chemical defenses. More precisely, the effects of nutrients and light on the production of SCs were both considered since nutrients not only affect the production of SCs, but they also influence the effects of light on the production of SCs in plants (see review in Herms & Mattson, 1992). As a first step, the effects of realistic variation of light and nutrients on the levels of chemical defenses of the phototoxic plant *Carthamus tinctorius* (Asteraceae) were determined. I then measured the performance of three herbivorous insects offered *C. tinctorius* foliage grown under different light and nutrient combinations. A multiple regression analysis was used to identify which defensive traits affected by light and nutrient levels are the most strongly correlated with insect fitness.

Materials and methods

Experimental design

Safflower was grown under different combinations of light and fertilization to determine how the production of chemical defenses is affected by these two abiotic factors. An incomplete factorial design involving a checkerboard pattern of the combinations between five light and ten fertilization regimes was used for a total of 25 treatments (Table 1). As five replicates per treatment were used and as each replicate had to be duplicated for measurements of both constitutive and inducible amount of PTs, a total of 250 pots were set up.

Light regimes

The light regimes were controlled through open-ended opaque cardboard boxes (70 cm of length, 40 cm of width and 30 cm of height) to which different thicknesses of white nylon fabric were stapled on top. The boxes of 70 cm of length were set down side by side on a table of 65 cm of width bordered with wooden planks of 10 cm of height in such a way that proper ventilation could be maintained inside the boxes through the lateral openings (10 cm x 65 cm). Using a spectrophotometer, the nylon fabric used to cover the boxes was shown to act as a neutral density filter between 300 and 700 nm and to allow a transmission of 38.5 ± 2.4 % (mean ± sd) of the incident light per thickness. By using 0, 1, 2, 4 or 6 thicknesses of nylon fabric, it was possible to grow the seedlings under a gradient of light regimes as indicated in Table 1.

Table 1: Checkerboard experimental design: all cases with hatching were not tested.

Fertilization regimes[§]	Light regime (μmol photons/m²/s)				
	63	92	142	188	278
0	▨	tested	▨	tested	▨
0.025	tested	▨	tested	▨	tested
0.05	▨	tested	▨	tested	▨
0.1	tested	▨	tested	▨	tested
0.2	▨	tested	▨	tested	▨
0.4	tested	▨	tested	▨	tested
0.6	▨	tested	▨	tested	▨
0.8	tested	▨	tested	▨	tested
0.9	▨	tested	▨	tested	▨
1.0	tested	▨	tested	▨	tested

[§] The units for the fertilization regimes represent the proportion of a stock solution prepared with 0.92 g/l of 20-20-20 commercial fertilizer and which was used as watering solution. For example, plants treated accordingly to the fertilization regime 0.1 were watered with a solution containing 1 part of the stock solution and 9 parts of distilled water.

Fertilization regimes

Dissolved fertilizer was provided at various strengths 4-5 times per week to provide water and a range of nutrient levels. The amount of solution applied at each watering ranged from 150-300 mL per pot depending on the evapotranspiration rate. A commercial 20-20-20 fertilizer containing micronutrients (Plant-Prod, Brampton, Ontario, CND) was used at a concentration of 0.92 g/l of distilled water to prepare a stock solution. The concentration of the stock solution was calculated based on the nitrogen equivalent of a recommended watering solution, *i.e.*, 15 mM of NO_3^- (Machlis & Torrey, 1956). Over the course of the three weeks that the experiment lasted, each pot received a total of 2.1 L of watering solution. The unit used to describe the fertilization regimes is the proportion of the stock solution in the corresponding watering solution. For example, the fertilization regime 0.1 was a solution made from 1 part of the stock solution and 9 parts of distilled water.

Mechanically simulated herbivory

A mechanically simulated herbivory treatment was used to determine the inducible accumulation of the PTs in *C. tinctorius*. The protocol described by Dr. Kelsey Downum (personal communication) was used because it optimizes the inducible accumulation of PTs in *C. tinctorius*. According to this procedure, the adaxial epidermis of leaves and cotyledons of *C. tinctorius* plants were pricked with a needle at an approximate rate of one prick per 4 mm^2. Needles were inserted through a Styrofoam block (1.5 cm x 1.5 cm x 1.5 cm) to form a grid pattern of 2 mm of width. The needles were arranged so that the points were sticking out by 1 mm on the opposite surface from which they had been inserted in the Styrofoam block. The mechanically simulated herbivory was then performed by putting a finger under a leaf and gently pressing with the Styrofoam block to prick the upper side. Particular attention was paid to avoid piercing through the entire leaf when the pricking was performed. A delay of 24-30 h between the simulated herbivory treatment and the sampling of plants for the analysis of PTs was allowed for chemicals to accumulate.

Growth conditions and disposition of the plants

The experiment was performed from 23rd February to 15th March 1996 in a greenhouse equipped with a lighting system of cool white fluorescent and incandescent bulbs. The lighting system was daily set on and off at 8:00 and 24:00, respectively, for a total photoperiod of 16 hours. The maximum day temperature oscillated between 29°C and 35°C according to sunshine.

Seeds of *C. tinctorius* were purchased from Ritchie Feed & Seed Ltd (Ottawa, Ontario, CND) and sown in 15 cm plastic pots filled with a mix of vermiculite and perlite (1:1) pre-humidified with distilled water. Approximately ten seeds were planted in each pot but the number of seedlings per pot was further standardized to five about one week after germination had started, *i.e.* 14 days after sowing.

Immediately after sowing, the pots were maintained under the assigned light and fertilization regimes. Ten pots, five fertilization regimes in duplicate for measurements of both constitutive and inducible concentrations of PTs, were grouped together in every cardboard box. A total of 25 boxes were required since there were 5 light regimes, and each treatment was replicated five times. The positions of the 25 boxes were randomized within the greenhouse every week. The same ten pots were maintained in the same box throughout the experiment (nested design), although the position of each pot within the box was weekly randomized.

Sampling and tissue analysis

In each pot, three out of the five seedlings were required for the different analyses performed: two plants were used for the analysis of PTs and one to measure the water, carbon, nitrogen, chlorophyll and soluble phenolic contents. All measurements were performed for the first sampling (t = 20 days), although only the concentrations of PTs were determined following the mechanically simulated herbivory treatment which was sampled the following day (t = 21 days).

Chemical analysis of phototoxins

The plants selected for PT analysis were rinsed in water to remove vermiculite and perlite from the roots, gently pressed between absorbent paper to remove excess water, weighed to determine the fresh weight, then dipped in 100 ml of hexane. Plants were maintained in hexane with constant agitation for 72 hours to extract the PTs. One ml of hexane was then removed and kept in a freezer for HPLC analysis of the PTs. The recovery of compounds **1** and **2** (Fig. 1) using this procedure was over 92%. After extraction of polyacetylenes in hexane, the plants were dried for 48 hours at 70 °C to determine the dry weight. The inducible PT concentration was determined as the difference in concentrations between induced (simulated herbivory treatment) and non-induced plants.

A Beckman Gold System HPLC including a solvent module (model 126), a UV-detector (model 168) set to 340 nm and an autosampler (model 502), was used to analyze the two main polyacetylenic PTs present in *C. tinctorius*. A good separation of the major chemical constituents was obtained with a C-18 reverse phase column (Beckman, 5 ODS, 25 cm x 4.6 mm) using acetonitrile:water (7:3) solvent system at a flow rate of 1.0 ml/min. Under these conditions, the retention times for 1,3,11-tridecatriene-5,7,9-triyne (**1**) and 3,11-tridecadiene-5,7,9-triyne-1,2-diol (**2**) (Fig. 1) were 9.0 and 15.1 min., respectively. Pure standards of both compounds, **1** and **2**, were obtained by preparative HPLC as described in Guillet *et al.* (1997). The integrated area of the appropriate peaks was used to determine the concentrations of **1** and **2** with reference to standard curves made for each compound at concentrations 0, 10, 25, 50, 100, and 250 µg/ml. Molecular structures of **1** and **2** were determined based on UV and MS spectra and by comparing this information to previously reported values (Bohlmann *et al.*, 1966, 1973).

Chemical analysis of nitrogen, carbon, chlorophyll and soluble phenolics

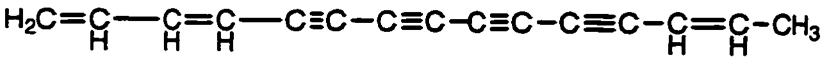
Another haphazardly selected plant from each pot was rinsed in water and weighed after the excess of water had been removed. Leaves were kept for 48 hours in a drying oven at 70°C before determining the dry weight. The percentage of water in the tissues was then calculated according to equation **1** where WC, FW, and DW, respectively, represent the water content in percent, and the fresh and dry weights of tissues.

$$WC = (FW - DW) / FW * 100 \qquad (\mathbf{1})$$

The dried tissues were ground using a motorized mill (Arthur H. Thomas Co., Philadelphia, USA) and this material then served for different chemical analysis.

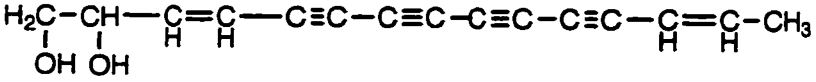
Approximately 10 mg of the ground tissues were sent to the Department of Chemistry at the University of Ottawa for the analysis of carbon and nitrogen

Figure 1. Molecular structures of the two major polyacetylenic phototoxins occurring in *Carthamus tinctorius*.



$H_2C{=}CH{-}CH{=}CH{-}C{\equiv}C{-}C{\equiv}C{-}C{\equiv}C{-}C{\equiv}C{-}CH{=}CH{-}CH_3$

1



$H_2C(OH){-}CH(OH){-}CH{=}CH{-}C{\equiv}C{-}C{\equiv}C{-}C{\equiv}C{-}C{\equiv}C{-}CH{=}CH{-}CH_3$

2

contents using a C-N-H analyzer (Perkin-Elmer, Series 2 CHMS/O Analyzer 2400). Another 10 mg of dried and ground tissues were used for the determination of chlorophyll and soluble phenolic concentrations. Phenolics were extracted as described in Yao *et al.* (1995), except that the procedure was adapted for dry tissues. The 10 mg of leaf powder were added to 1.0 ml of methanol in a 1.5 ml centrifuge tube, incubated for 30 min at 60 °C, then centrifuged 15 min at 16 000*g*. An aliquot of 0.1 ml of the supernatant was added to 0.9 ml of methanol before to measure the absorbance at 645 nm and 663 nm. From these measurements, the total concentration of chlorophyll *a* and *b* was determined as described in Arnon (1949). A second aliquot of 0.2 ml of the supernatant was added to 0.9 ml of the Folin-Ciocalteau reagent prepared as described by the manufacturer (BDH, Toronto, CND). Samples were vortexed then kept at room temperature for 30 minutes before recording the absorbance at 750 nm. The concentration of soluble phenolics in safflower seedlings were expressed in µg of equivalent catechol /g d.w. according to a standard curve made with catechol at 0, 8, 40 and 200 µg/ml.

Herbivory measurements

Safflower plants were grown for three weeks according to a factorial design involving three light (0, 2 and 6 fabric thicknesses) and three fertilization (0, 0.2 and 1.0) regimes as described above then submitted to different herbivorous insects. The objective was to determine how the variations in the phytochemical characteristics (above sections) affect the feeding pattern of polyphagous herbivorous insects. To prevent artefacts in feeding activity due to potential effects of light on insect behavior (Guillet *et al.*, 1997; Carroll *et al.*, 1997; Fields *et al.*, 1990), the assays were performed under standardized conditions, 160 ± 50 µmol photons/m^2/s^1 (mean ± sd), a photoperiod of 16 h and an ambient temperature of 25 °C, although the plants had been grown under different light and fertilization regimes for three weeks before placement in the growth chambers. *Carthamus tinctorius* plants were cut near the ground with a razor blade then transferred into a 7 ml glass vial filled with distilled water. The vials were secured with cotton and sealed around the stems using parafilm then individually offered to an insect. Thirty-two replicates were performed for each treatment and for each insect species tested.

The green peach aphid

The green peach aphid, *Myzus persicae* (Sulzer) (Homoptera: Aphididae), is a phloem-feeding polyphagous insect that may damage several crop cultures especially in greenhouses, but also under field conditions (Richard & Boivin, 1994). Second instar aphids were removed from an infested safflower plant then transferred to safflower plants prepared as above at a rate of one insect per plant. The experimentation lasted seven days and distilled water was added in vials twice during this period by using a syringe to pierce the parafilm. The insect performance was evaluated by the number of aphids observed on each plant. Under these conditions, only offspring from the second generation, those produced from the aphid initially added, were observed.

The European corn borer

The European corn borer, *Ostrinia nubilalis* Hübner (Lepidoptera: Pyralidae), is also a polyphagous insect that infests over 200 herbaceous species including some Asteraceae that have been reported to produce PTs (Hudon *et al.*, 1989). Some investigations performed with the European corn borer showed that a behavioral ability to prevent the ingestion of PTs (Aucoin *et al.*, 1995; Champagne *et al.*, 1986) and a high metabolization rate of PTs (Iyengar *et al.*, 1987, 1990) combined with a high level of antioxidant enzymes (Aucoin *et al.*, 1991) may explain the tolerance of this insect to PTs.

Early third instar *O. nubilalis* larvae obtained from a laboratory colony maintained as described in Guthrie *et al.* (1985) were weighed and individually added on a safflower plant placed in a vial as above. A 500 ml Mason jar was turned upside down over the seedling to prevent the escape of the larvae. The jar was deposited on 1 mm plastic spacers to avoid condensation inside the container. The bioassay lasted 48 h and the larval weight was determined at the end of the experiment. The relative larval weight gain [(final weight - initial weight) / initial weight] was calculated as a performance index.

The red-legged grasshopper

Adult males of the red legged grasshopper, *Melanoplus femurrubrum femurrubrum* (De Green) (Orthoptera: Acrididae), were collected in a hay field located along the Promenades des Fées road in Hull, Québec, Canada. The genus *Melanoplus* is very widespread in North America and large populations commonly occur that damage many herbaceous plant spp. *Melanoplus femurrubrum femurrubrum* is one of the most numerous grasshoppers in the Ottawa area and it was one of the main species involved in the invasions of the city of Montréal in 1948, 1960 and 1971 (Vickery *et al.*, 1974).

The adult grasshoppers were collected during the day, deprived of food overnight, and then individually offered a safflower plant prepared as above for 24 hours. A 1000 ml Mason jar was turned upside down over the seedlings to prevent the grasshoppers from escaping. The jar was deposited on 1 mm plastic spacers to avoid condensation inside the container and at the end of the experiment the feces were dried and weighed as an index of feeding activity.

Statistical analysis

Regression analyses were performed to determine if light and fertilization affect the different physical and chemical traits determined on *C. tinctorius* and the performance of herbivorous insects fed with these plants. The insect performances were compared between the different light and nutrient regimes using the Tukey multiple comparison test. A stepwise multiple regression was also performed to determine which of the physical and chemical characteristics measured on safflower plants significantly affect the performance of the three phytophagous insect investigated. For the experiment with the red-legged grasshopper, the weight of the feces had to be log-transformed to satisfy the statistical assumptions of regression models.

Results

Light- and fertilization-mediated variations in growth and phytochemical aspects of *Carthamus tinctorius*

The nutrient and light regimes significantly affected the growth and most of the physical (water content) and chemical (nitrogen, soluble phenolics and constitutive and inducible PTs) parameters determined in *C. tinctorius* (Table 2). Of all traits measured, only the constitutive concentration of PTs was not affected by light whereas fertilization did not alter either the inducible concentration of PTs

Table 2. P values for the regression analyses between the phytochemical and physical parameters determined on *Carthamus tinctorius* plants and the light and fertilization regimes under which the plants were grown for three weeks.

Dependent variable	Independent variable			
	Light		Fertilization	
	F[§]	P	F[§]	P
Biomass (dry weight)	196.98 (1,228)	<0.0001	78.72 (1,228)	<0.0001
Water content (% of fresh weight)	140.26 (1,228)	<0.0001	29.85 (1.228)	<0.0001
Nitrogen (% of dry weight)	13.09 (1,109)	<0.0001	179.40 (1,109)	<0.0001
Constitutive concentration of the two main phototoxins (dry weight basis)	1.63 (1,110)	0.2043	973.59 (1,110)	<0.0001
Inducible concentration of the two main phototoxins (dry weight basis)	29.05 (1,103)	<0.0001	0.55 (1,103)	0.4616
Total concentration of the two main phototoxins (dry weight basis)	8.39 (1,101)	0.0046	86.53 (1,101)	<0.0001
Concentration of total soluble phenolics (dry weight basis)	5.51 (1,109)	0.0207	0.04 (1,109)	0.8394

[§] The values provided in brackets represent the degrees of freedom for the regression and the residuals respectively.

nor the concentration of total soluble phenolics (Table 2).

The growth of safflower plants improved proportionally to the nutrient and light availability (Fig. 2). Fertilization and light affected in opposite ways nitrogen and water contents. An increase in light intensity resulted in lower nitrogen and water contents, whereas an increase in the nutrient available to safflower plants increased both nitrogen and water contents (Fig. 3, 4).

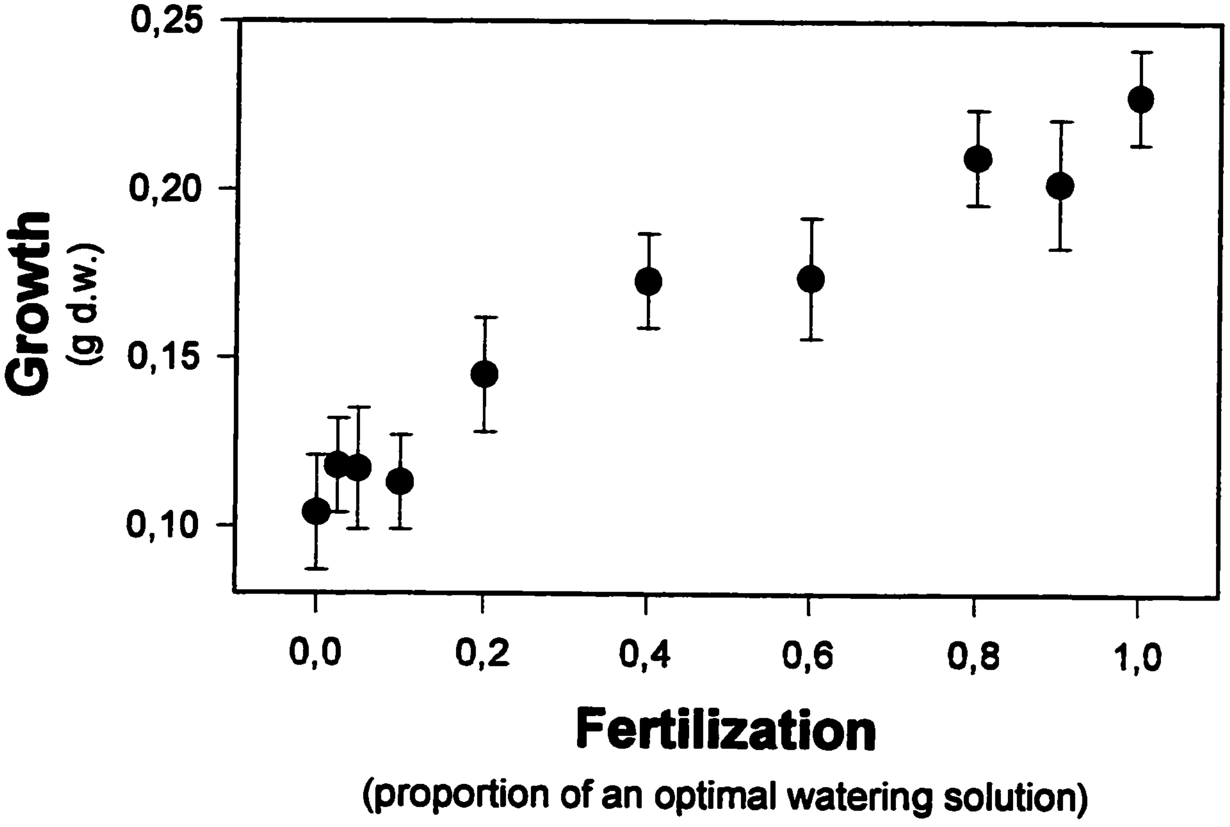
The constitutive concentration of PTs in *C. tinctorius* was related negatively to the nutrient availability, with a variation of up to 2.6-fold observed (Fig. 5). Light had no effect on the constitutive level of PTs (Table 2). The reverse trend was, however, observed with the induced production of PTs in response to mechanically simulated herbivory. Fertilization had no effect on this variable, but light promoted a higher response (Fig. 6). It is worth noting that the total concentration of PTs in induced plants increased with light intensity, although the constitutive amount of PTs was not altered by light (Fig. 5, 7).

The total soluble phenolics were only affected by light (Table 2). Safflower seedlings grown under a high light regime produced less soluble phenolics (Fig. 8).

Performance of herbivorous insects fed with *Carthamus tinctorius* foliage obtained from plants grown under different combinations of light and fertilization

The three herbivorous insects responded differently to the conditions under which the safflower plants offered were grown (Table 3). Light and fertilization regimes under which safflower plants were grown affected larval weight gain of the European corn borer. Only the fertilization altered the reproductive output of the

Figure 2: Growth of *Carthamus tinctorius* plants as a function of (A) fertilization and (B) light regimes. Values provided represent the least squares means obtained from a two-way incomplete factorial design with 10 light and 5 fertilization regimes.


0,25
0,20
0,15
0,10
Growth
(g d.w.)
0,0
0,2
0,4
0,6
0,8
1,0
Fertilization
(proportion of an optimal watering solution)

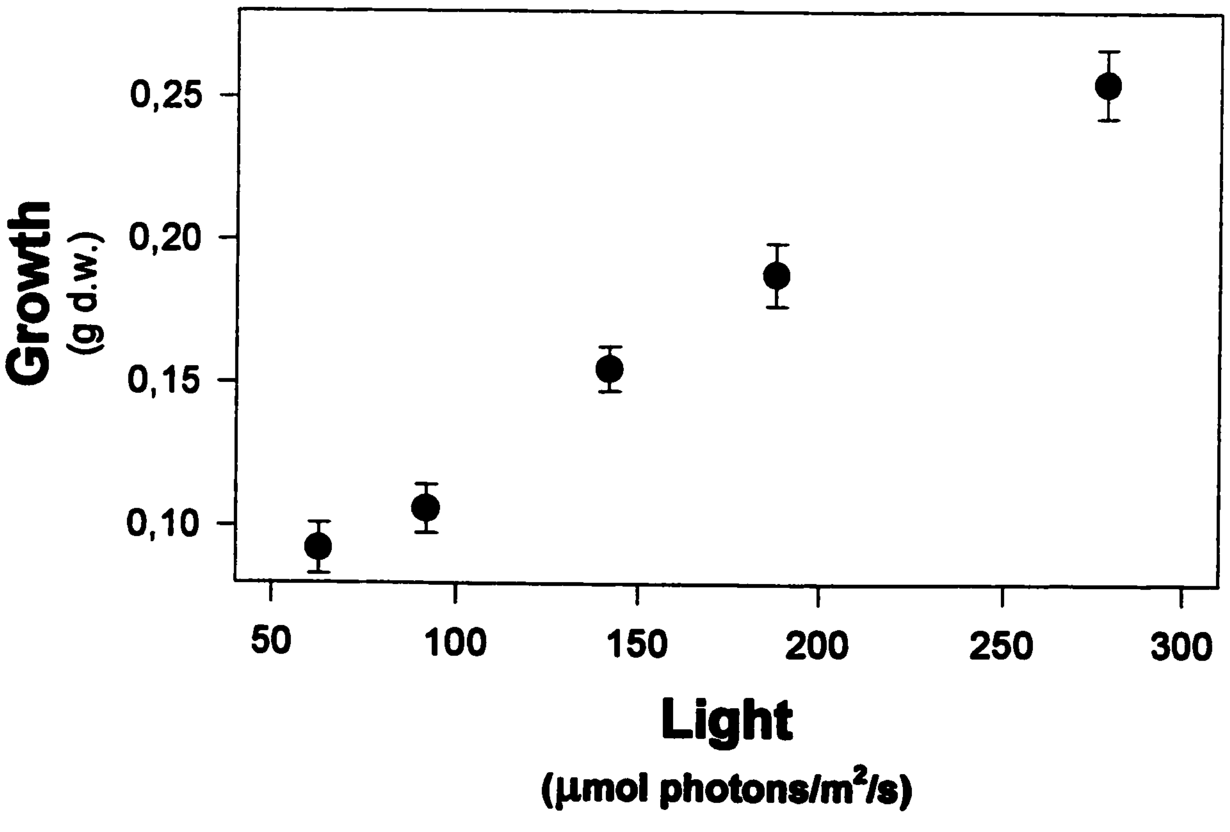
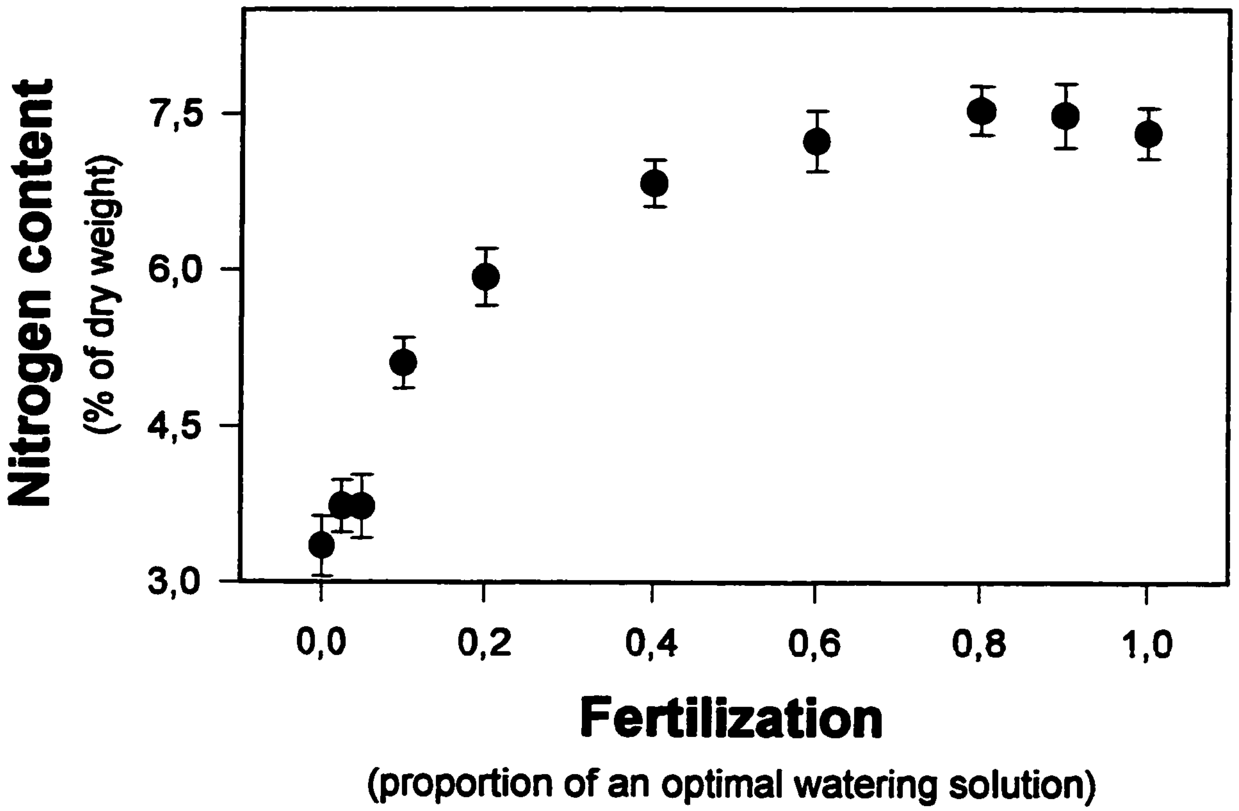

0,25
0,20
0,15
0,10
Growth
(g d.w.)
50
100
150
200
250
300
Light
(µmol photons/m²/s)

Figure 3. Nitrogen content in *Carthamus tinctorius* plants as a function of (A) fertilization and (B) light regimes. Values provided represent the least squares means obtained from a two-way incomplete factorial design with 10 light and 5 fertilization regimes.


Nitrogen content
(% of dry weight)
7,5
6,0
4,5
3,0
0,0
0,2
0,4
0,6
0,8
1,0
Fertilization
(proportion of an optimal watering solution)

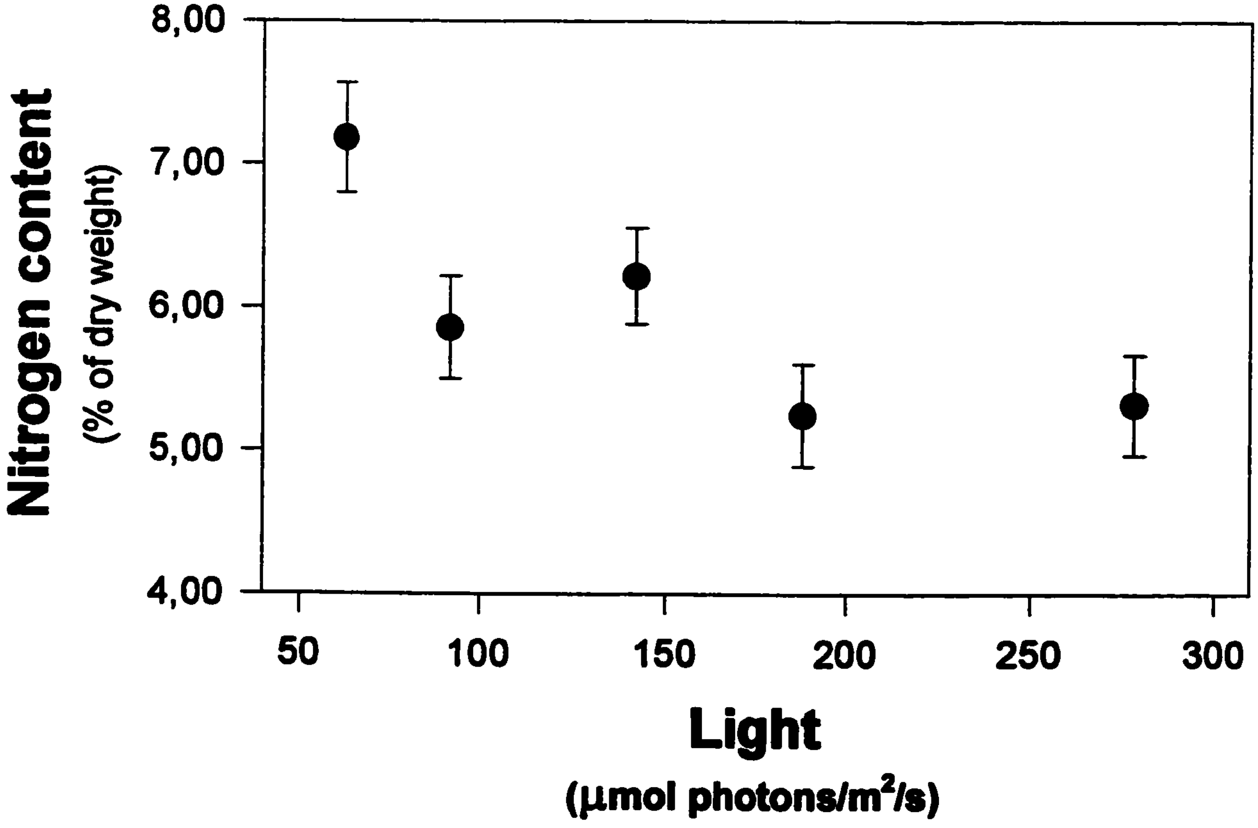
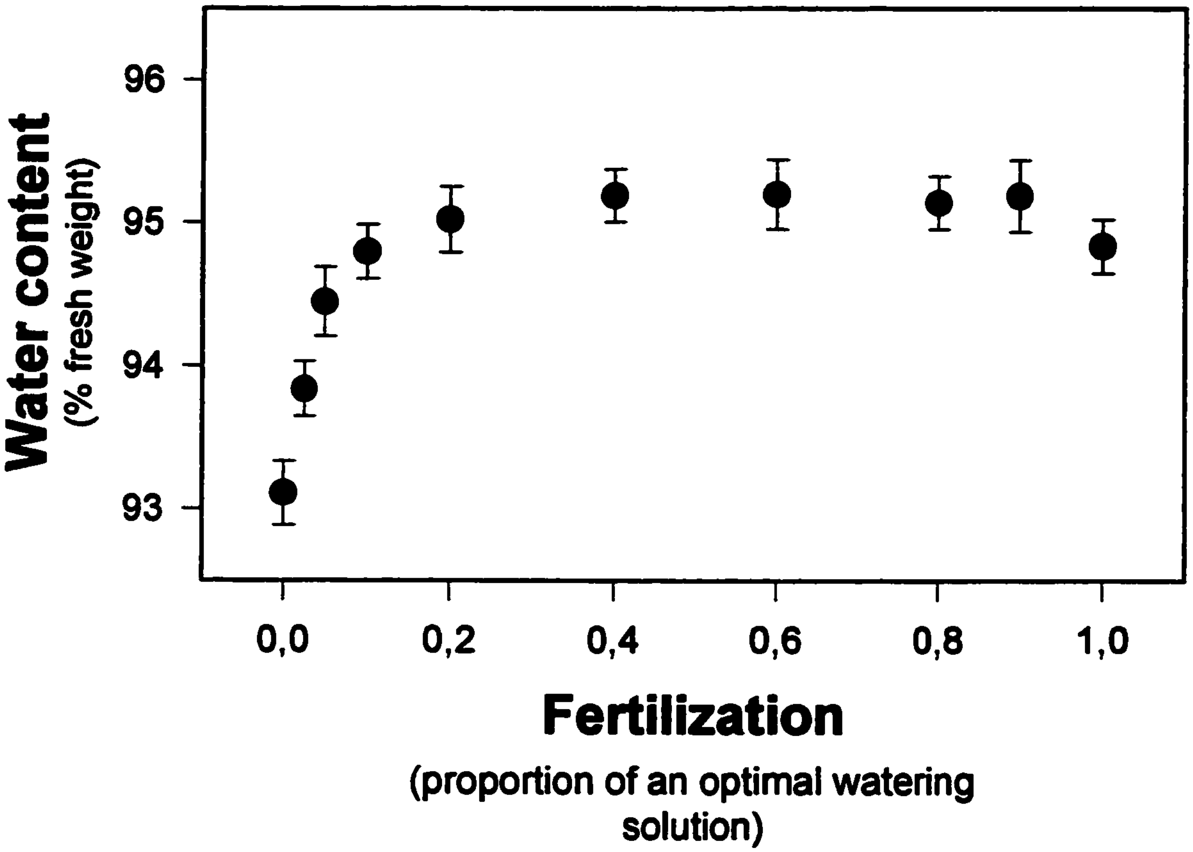

Nitrogen content
(% of dry weight)
8,00
7,00
6,00
5,00
4,00
50
100
150
200
250
300
Light
(µmol photons/m²/s)

Figure 4. Water content in *Carthamus tinctorius* plants as a function of (A) fertilization and (B) light regimes. Values provided represent the least squares means obtained from a two-way incomplete factorial design with 10 light and 5 fertilization regimes.


Water content
(% fresh weight)
93
94
95
96
0,0
0,2
0,4
0,6
0,8
1,0
Fertilization
(proportion of an optimal watering solution)

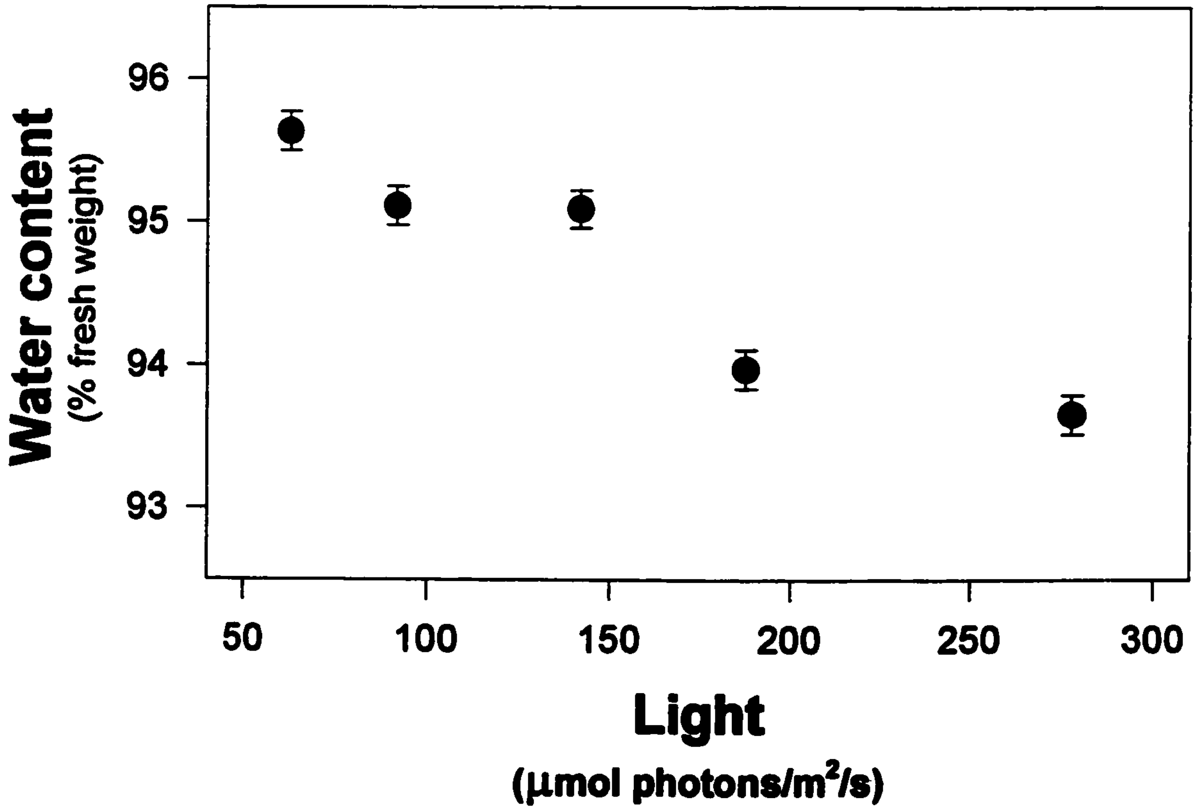
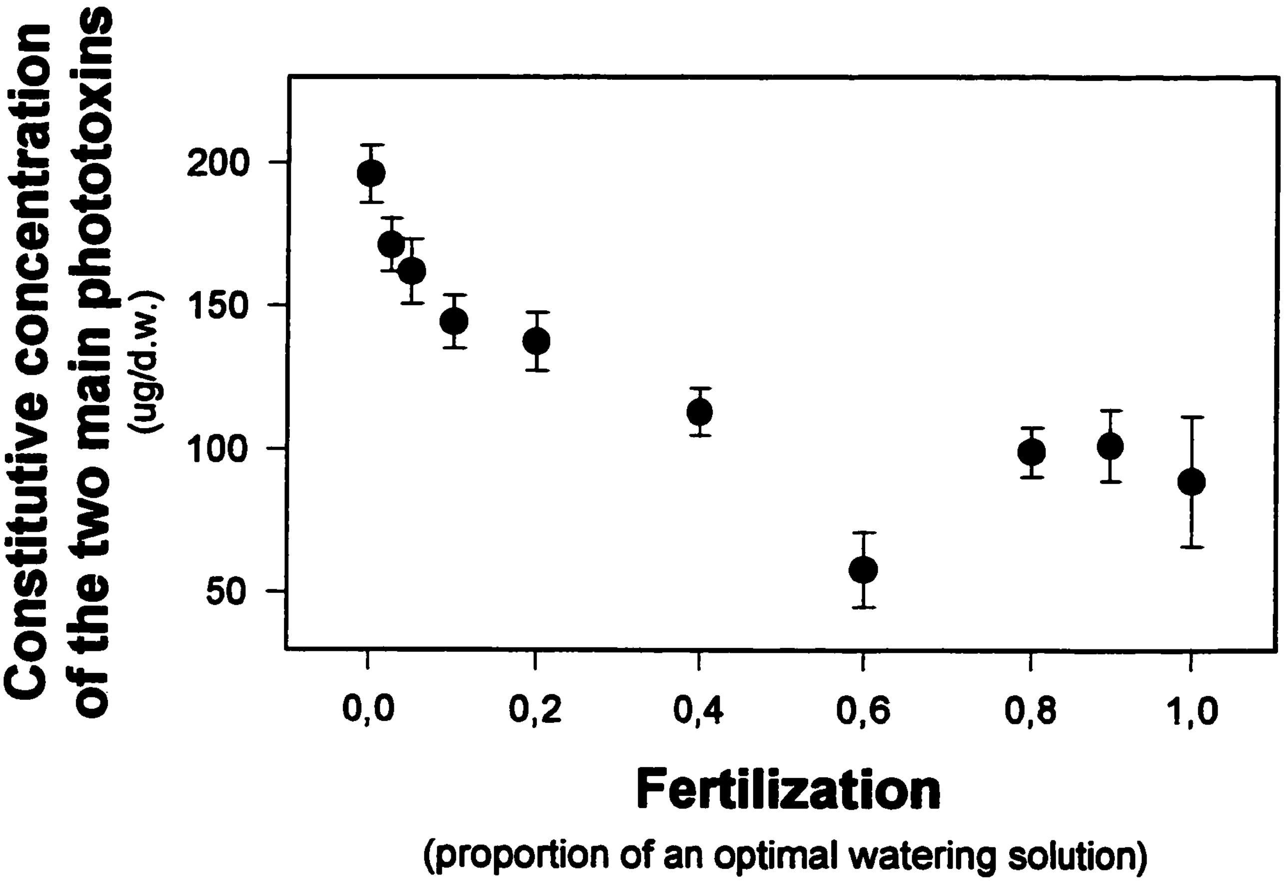

Water content
(% fresh weight)
93
94
95
96
50
100
150
200
250
300
Light
(µmol photons/m²/s)

Figure 5. Constitutive concentration of the two main phototoxins (see Figure 1) in *Carthamus tinctorius* plants as a function of (A) fertilization and (B) light regimes. Values provided represent the least squares means obtained from a two-way incomplete factorial design with 10 light and 5 fertilization regimes.


Constitutive concentration
of the two main phototoxins
(ug/d.w.)
200
150
100
50
0,0
0,2
0,4
0,6
0,8
1,0
Fertilization
(proportion of an optimal watering solution)

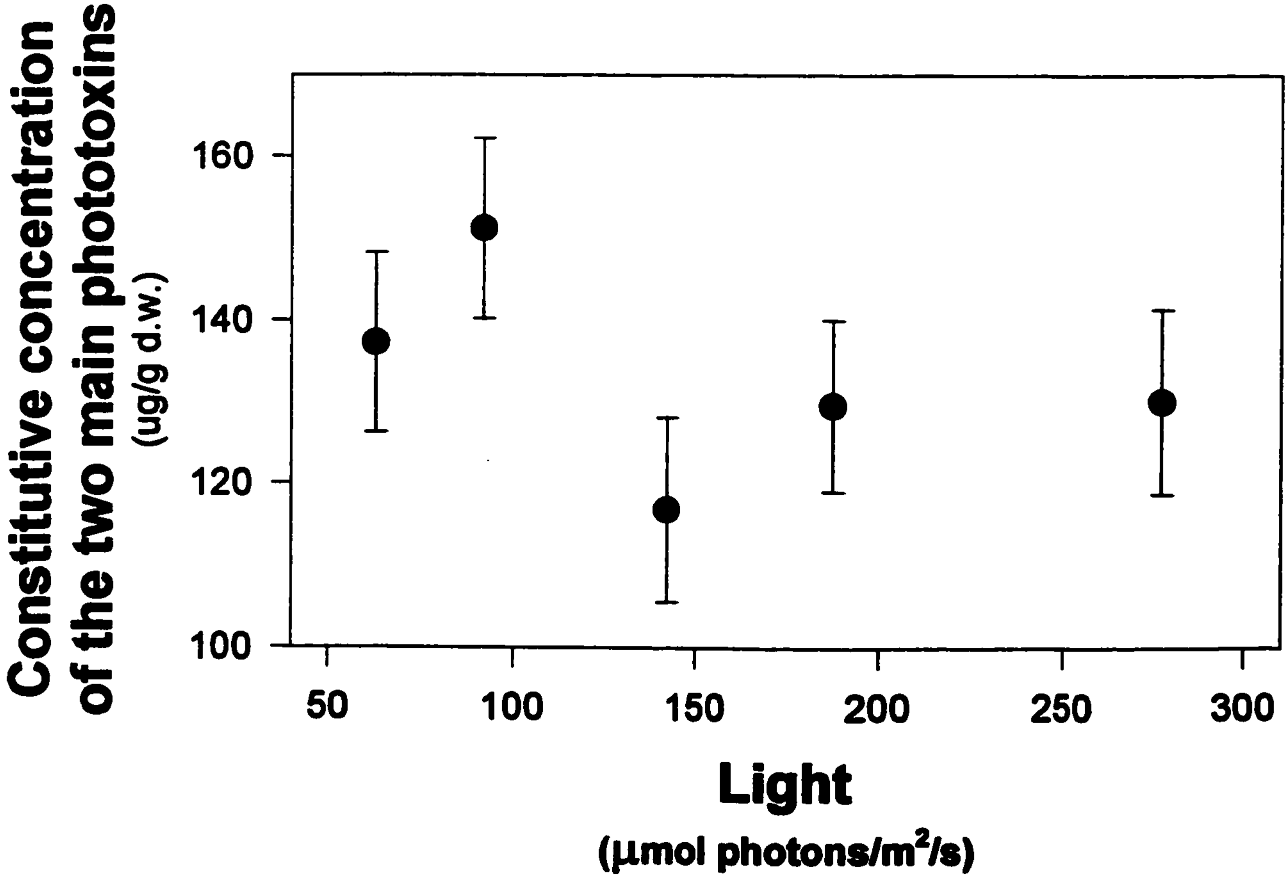
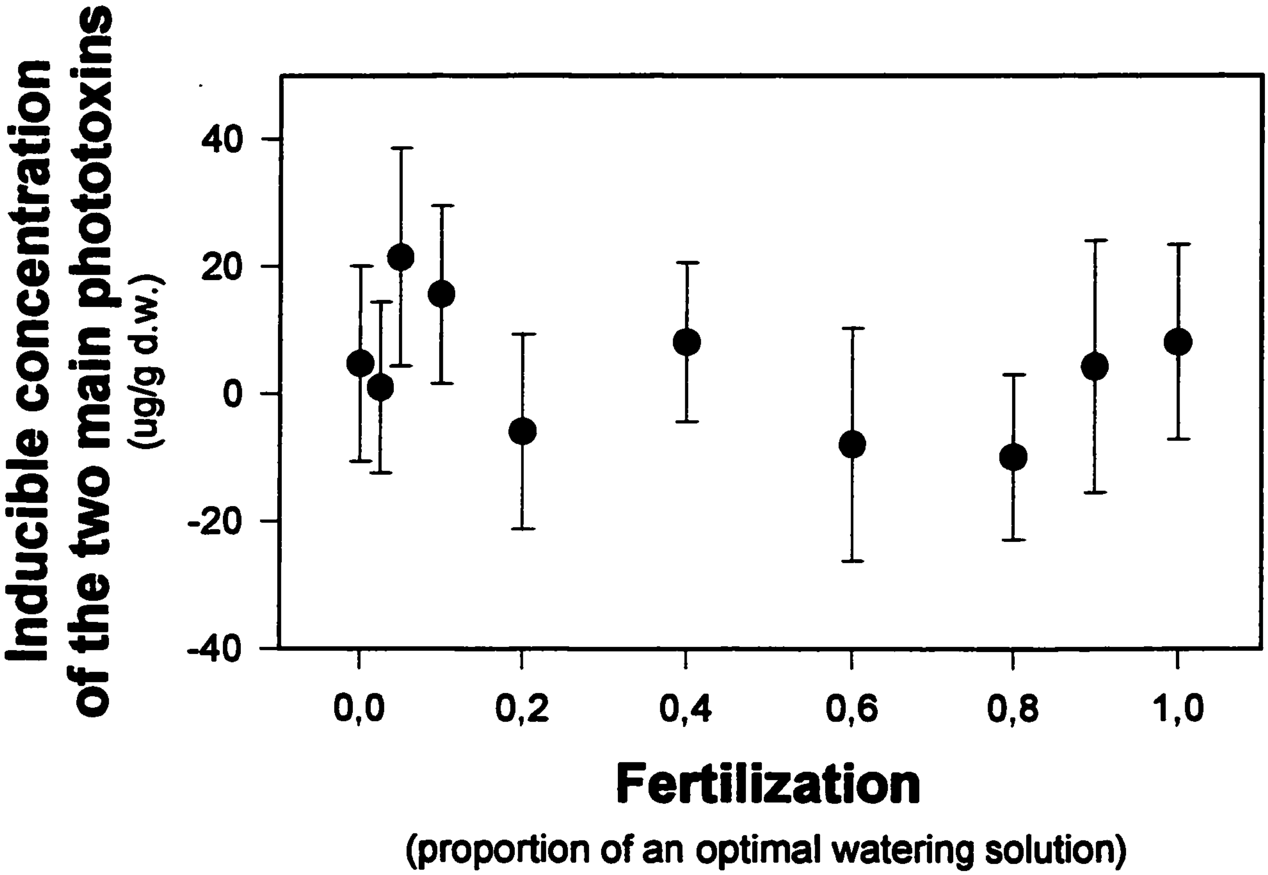

Constitutive concentration
of the two main phototoxins
(ug/g d.w.)
160
140
120
100
50
100
150
200
250
300
Light
(µmol photons/m²/s)

Figure 6. Inducible concentration of the two main phototoxins (see Figure 1) in mechanically-damaged *Carthamus tinctorius* plants as a function of (A) fertilization and (B) light regimes. Values provided represent the least squares means obtained from a two-way incomplete factorial design with 10 light and 5 fertilization regimes.


Inducible concentration of the two main phototoxins
(ug/g d.w.)
40
20
0
-20
-40
0,0
0,2
0,4
0,6
0,8
1,0
Fertilization
(proportion of an optimal watering solution)

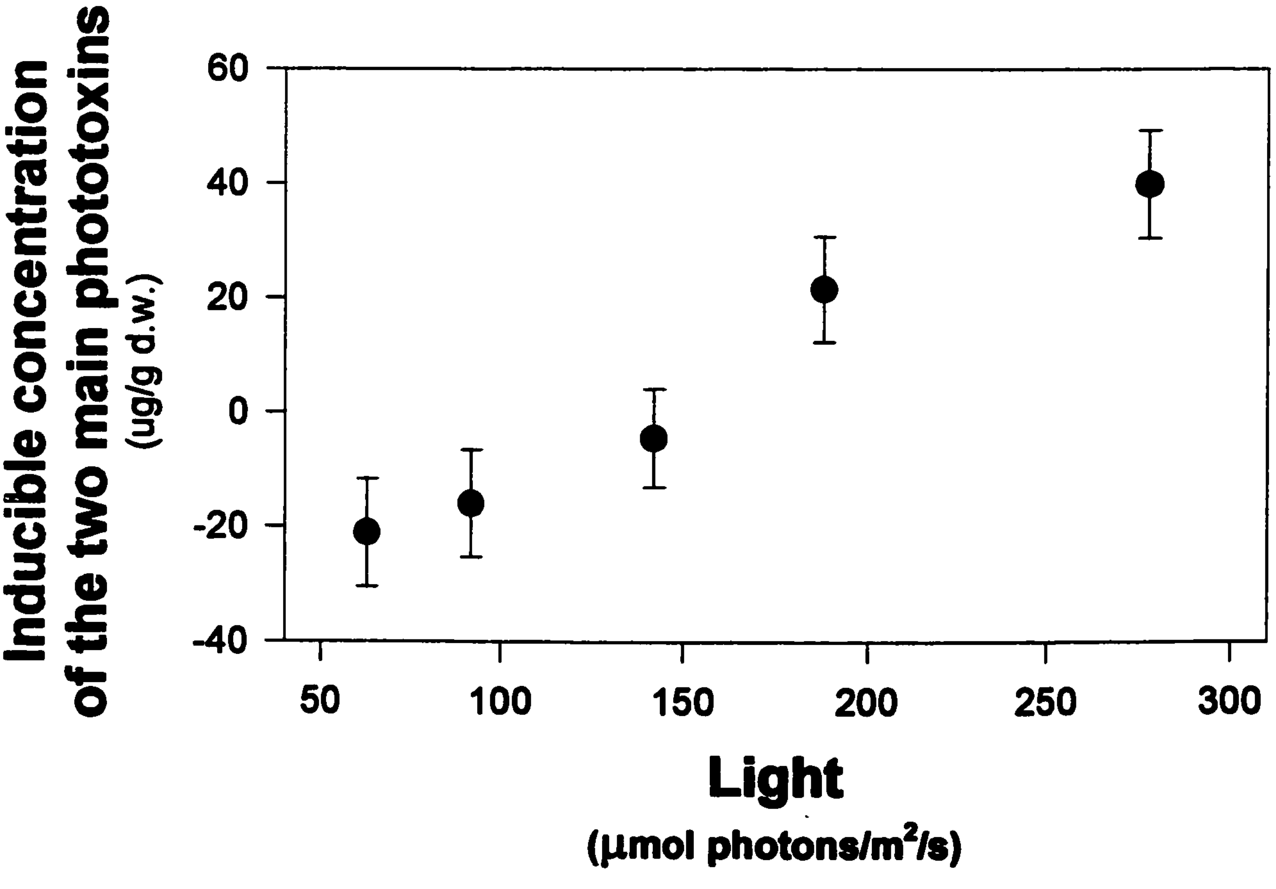
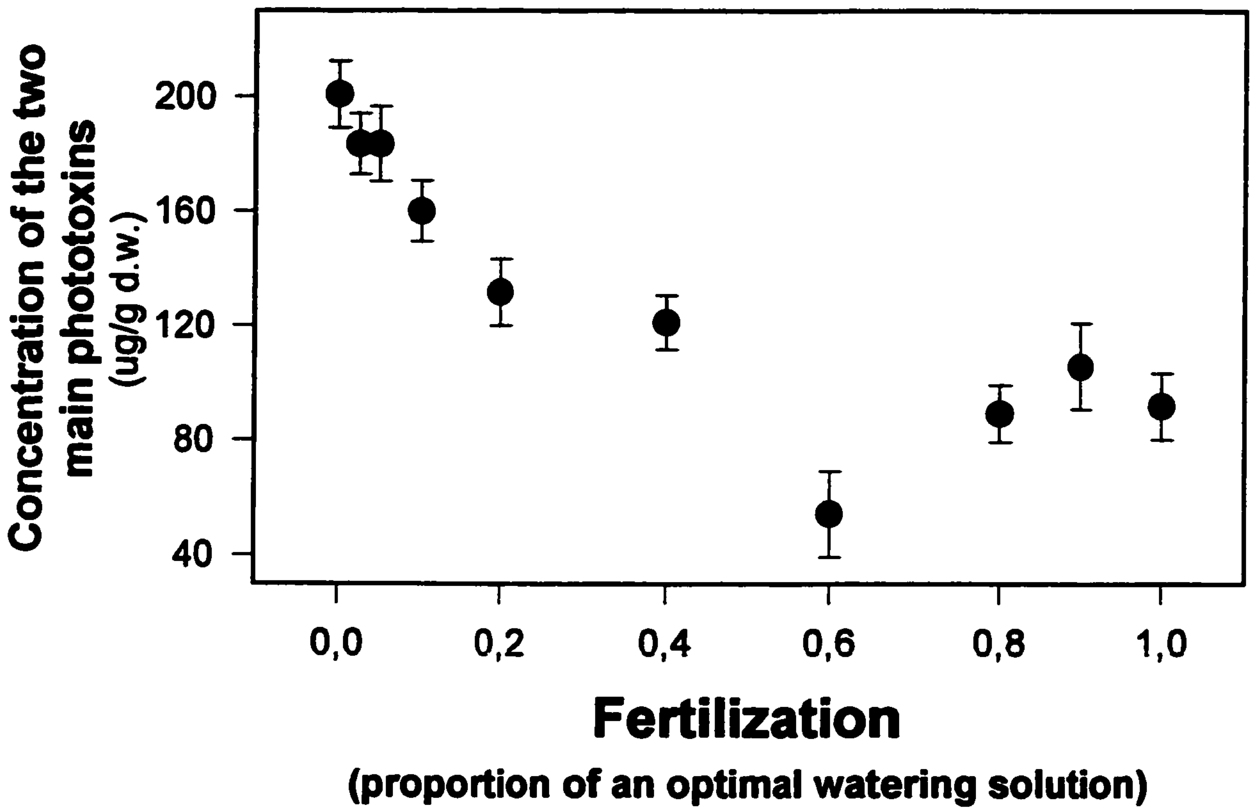

Inducible concentration of the two main phototoxins
(ug/g d.w.)
60
40
20
0
-20
-40
50
100
150
200
250
300
Light
(µmol photons/m²/s)

Figure 7. Concentration of the two main phototoxins (see Figure 1) in mechanically-damaged *Carthamus tinctorius* plants as a function of (A) fertilization and (B) light regimes. Values provided represent the least squares means obtained from a two-way incomplete factorial design with 10 light and 5 fertilization regimes.


Concentration of the two main photoxins
(ug/g d.w.)
200
160
120
80
40
0,0
0,2
0,4
0,6
0,8
1,0
Fertilization
(proportion of an optimal watering solution)

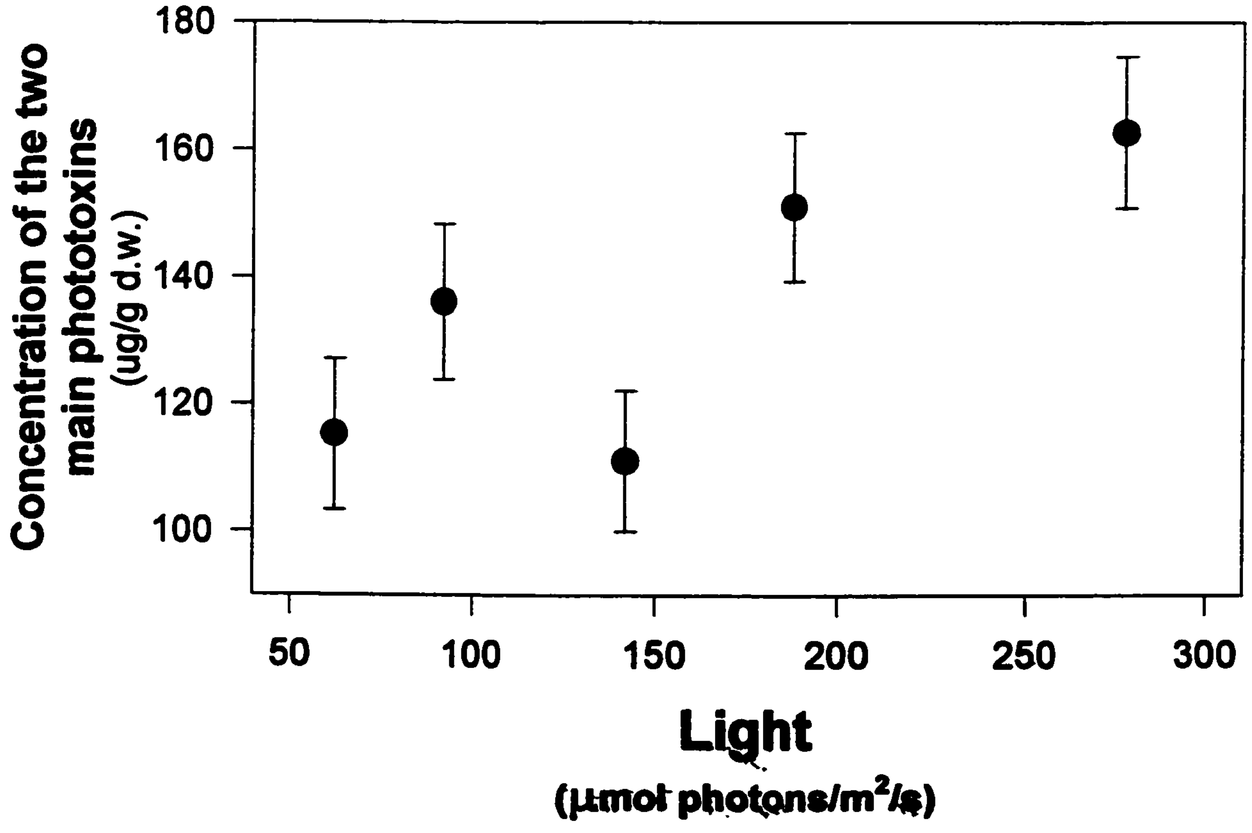
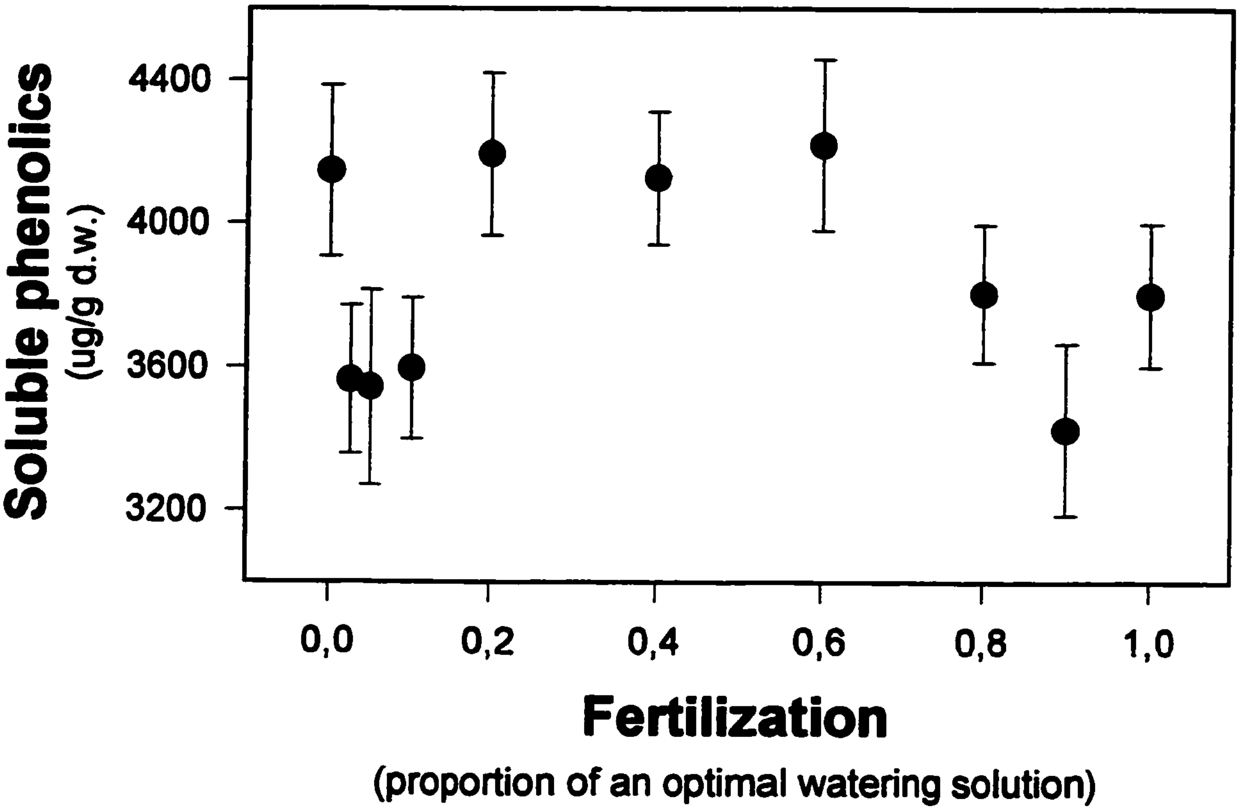

Concentration of the two main phototoxins
(ug/g d.w.)
180
160
140
120
100
50
100
150
200
250
300
Light
(µmol photons/m²/s)

Figure 8. Concentration of total soluble phenolics in *Carthamus tinctorius* plants as a function of (A) fertilization and (B) light regimes. Values provided represent the least squares means obtained from a two-way incomplete factorial design with 10 light and 5 fertilization regimes.


Soluble phenolics
(ug/g d.w.)
4400
4000
3600
3200
0,0
0,2
0,4
0,6
0,8
1,0
Fertilization
(proportion of an optimal watering solution)

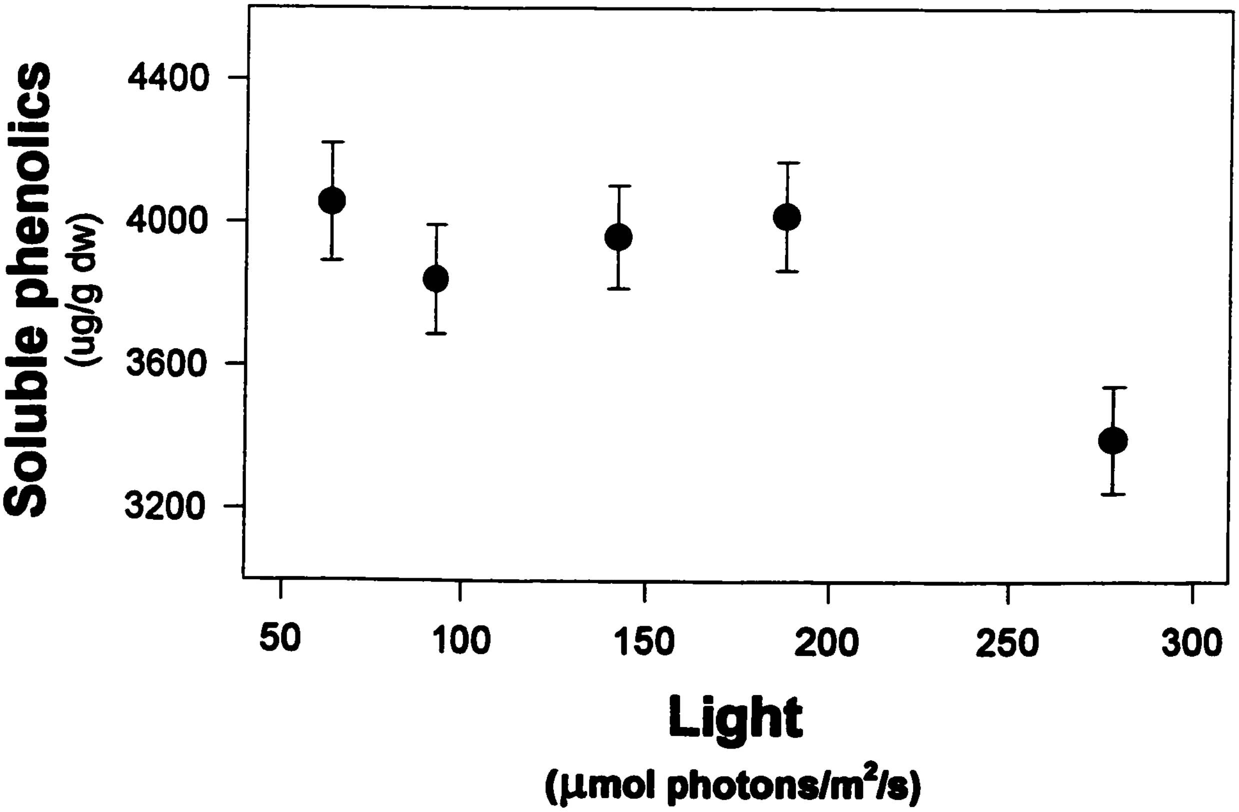

Soluble phenolics
(ug/g dw)
4400
4000
3600
3200
50
100
150
200
250
300
Light
(µmol photons/m²/s)

Table 3: P values for the regression analyses between performance of three herbivorous insects and light and fertilization regimes under which the plants of *Carthamus tinctorius* offered to insects were grown.

Dependent variable	Independent variable			
	Light		Fertilization	
	F[§]	P	F[§]	P
Green peach aphid (# of individuals)	0.13 (1,286)	0.7227	276.02 (1,286)	<0.0001
Weight gain of the European corn borer (g/g/day)	48.96 (1,286)	<0.0001	8.38 (1,286)	0.0041
Feces of the red-legged grasshopper ($\ln_e$(mg d.w.))	5.16 (1,232)	0.0024	2.36 (1,232)	0.1256

[§] The values provided in brackets represent the degrees of freedom for the regression and the residuals respectively.

green peach aphid and only the light intensity significantly modified the amount of feces produced by red-legged grasshopper adults (Tables 4-5). The highest performances for *M. femurrubrum femurrubrum* and *O. nubilalis* were obtained at the lowest light intensity, although the reproductive output of *M. persicae* was not affected by light (Table 4). At the highest fertilization regime, both *O. nubilalis* and *M. persicae* performed the best, although *M. femurrubrum femurrubrum* produced more feces at lower fertilization regimes (Table 5).

Multiple regressions between traits of safflower plants grown under different combinations of light and fertilization regimes and the performances of the three insects tested are presented in Tables 6-8. The reproductive output of the green peach aphid decreased with increasing concentrations of both constitutive PTs and soluble phenolics, whereas a higher nitrogen content in the tissues increased the number of aphids (Table 6). Both the water content and the inducible accumulation of PTs in the tissues did not affect the fitness of *M. persicae*.

The relative weight gain of European corn borer larvae was not affected by any of the phytochemical characteristics measured. Water content best predicted the growth rate of *O. nubilalis* larvae (Table 7). According to the subset model generated, a higher water content in the tissues of *C. tinctorius* improved the relative growth rate of *O. nubilalis* larvae.

Finally, the amount of feces produced by red-legged grasshopper adults correlated positively with the water content and both the constitutive and inducible concentrations of PTs in safflower plants that were offered. Total soluble phenolics in *C. tinctorius* plants were inversely related to the amount of feces produced by *M. femurrubrum femurrubrum* adults whereas nitrogen content of safflower tissues had no effect on this trait (Table 8).

Table 4. Performance of three herbivorous insects offered *Carthamus tinctorius* plants grown under different light regimes[†].

Variable	Light regime (μmol photons/m^{-2}/s)		
	63	142	278
Feces produced by adults of *Melanoplus femurrubrum femurrubrum*[§] (ln(mg d.w.))	0.64^a (0.08)	-0.16^b (0.06)	0.22^c (0.09)
Relative growth rate of third instar *O. nubilalis* larvae[§] (g/g/day)	0.112^a (0.006)	0.072^b (0.007)	0.038^c (0.004)
Reproductive output of *Myzus persicae*[§] (# of individuals)	30.7^a (1.3)	32.2^a (1.5)	30.3^a (1.4)

[†] Values provided represent the least squares means obtained from a two-way experimental factorial design with 3 light and 3 fertilization regimes.

[§] For each row, values followed by a distinct letter differ (Tukey multiple comparison test, P<0.05). Values provided in brackets represent the standard error of the mean.

Table 5: Performance of three herbivorous insects offered *Carthamus tinctorius* plants grown under different fertilization regimes[†].

Variable	**Fertilization regime[¶]** (relative unit)		
	0.0	0.2	1.0
Feces produced by adults of *Melanoplus femurrubrum femurrubrum*[§] (ln(mg d.w.))	0.30^{a} (0.09)	0.26^{a} (0.09)	0.11^{a} (0.10)
Relative growth rate of third instar *O. nubilalis* larvae[§] (g/g/day)	0.046^{a} (0.010)	0.088^{b} (0.009)	0.088^{b} (0.008)
Reproductive output of *Myzus persicae*[§] (# of individuals)	19.8^{a} (1.0)	29.2^{b} (1.1)	44.5^{c} (1.1)

[†] Values provided represent the least squares means obtained from a two-way factorial experimental design with 3 light and 3 fertilization regimes.

[§] For each row, values followed by a distinct letter differ (Tukey multiple comparison test, P<0.05). Values provided in brackets represent the standard error of the mean.

[¶] The units for the fertilization regimes represent the proportion of a stock solution prepared with 0.92 g/l of 20-20-20 commercial fertilizer and which was used as watering solution.

Table 6. Multiple stepwise regression between the number of individuals produced by a second instar green peach aphid, *Myzus persicae* placed individually for one week on *Carthamus tinctorius* plants grown under different combinations of light and fertilization regimes, and the phytochemical and physical characteristics of the plants.

	Regression coefficient	Standardized regression coefficient	Standard error of the standardized regression coefficient	Probability value
Independent variables included in the subset model				
Constant	94.23545			<0.00001
Constitutive concentration of phototoxins	-0.19451	-0.54045	0.06882	<0.00001
Concentration soluble phenolics	-0.01382	-0.33427	0.04596	<0.00001
Concentration of nitrogen	2.44906	0.32579	0.07175	0.00001
Independent variables not included in the subset model				
Water content				0.77710
Inducible concentration of phototoxins				0.33297

Comments: The multiple r^2 of the above subset model was 0.736 and the ANOVA was significant: $F_{(3,284)} = 111.619$ and $P < 0.00001$.

Table 7. Multiple stepwise regression between the relative weight gain (g/g/day) of the European corn borer larvae, *Ostrinia nubilalis* (Lepidoptera: Pyralidae), placed individually for 48 hours on *Carthamus tinctorius* plants grown under different combinations of light and fertilization regimes, and the phytochemical and physical characteristics of the plants.

	Regression coefficient	Standardized regression coefficient	Standard error of the standardized regression coefficient	Probability value
Independent variables included in the subset model				
Constant	-2.26197			<0.00001
Water content	0.02473	0.46172	0.05246	<0.00001
Independent variables not included in the subset model				
Constitutive concentration of phototoxins				0.62665
Concentration soluble phenolics				0.56888
Inducible concentration of phototoxins				0.77528
Concentration of nitrogen				0.55466

Comments: The multiple r^2 of the above subset model was 0.213 and the ANOVA was significant: $F_{(1,286)} = 77.491$ and $P < 0.00001$.

Table 8. Multiple stepwise regression between the feces dry weight ($\ln_e$(dw in mg)) of the red-legged grasshopper adults, *Melanoplus femurrubrum femurrubrum*, individually offered for 24 hours *Carthamus tinctorius* plants grown under different combinations of light and fertilization regimes, and the phytochemical and physical characteristics of the plants.

	Regression coefficient	Standardized regression coefficient	Standard error of the standardized regression coefficient	Probability value
Independent variables included in the subset model				
Constant	-25.09504			0.00056
Water content	0.27929	0.52237	0.14032	0.00026
Constitutive concentration of phototoxins	0.00716	0.35187	0.10025	0.00055
Concentration soluble phenolics	-0.00054	-0.22732	0.07156	0.00147
Inducible concentration of phototoxins	0.00961	0.32085	0.13589	0.01920
Independent variables not included in the subset model				
Concentration of nitrogen				0.24534

Comments: The multiple r^2 of the above subset model was 0.116 and the ANOVA was significant: $F_{(4,229)} = 7.487$ and $P = 0.00001$.

It is important to consider the proportion of variance in the performances of insects explained by the different models. Seventy-four percent, 21% and 12% of the variations observed in the reproductive output of *M. persicae*, the relative larval weight gain of *O. nubilalis* and the feces produced by *M. f. femurrubrum* were respectively explained by the models generated (Tables 6-8).

Discussion

Effects of light and nutrients on phytochemistry of *Carthamus tinctorius*

Herms and Mattson (1992) recently proposed an integrated model to predict how ecological variations in the availability of resources may affect the physical and phytochemical characteristics of plants. Their hypothesis essentially assumes that the plant photosynthetic apparatus is less vulnerable to a resource shortage growth processes. The consequences of this assumption according to Herms and Mattson is that a resource depletion results in an overproduction of carbohydrates that will be allocated to different cellular differentiation processes, including the production of carbon-based chemical defenses like phenolics and PTs. Herms and Mattson's hypothesis shares many features with previous models based on the concept of overflow metabolism (Bryant et al., 1983; Robinson, 1974).

The results obtained in the present study only partially agree with the theoretical predictions of the resource availability models. A lower availability of nutrients to safflower resulted in a lower water content of the tissues, *i.e.* a higher toughness, and a higher constitutive concentration of PTs, although the inducible accumulation of PTs and the soluble phenolics remained unchanged (Fig. 4 ,5, 7, 8). These different patterns of production of phytochemicals in response to a variation in nutrient availability may explain the controversy in the literature regarding the validity of the theoretical models (see review in Lerdau *et al.*, 1994). Muzika (1993) similarly reported that in *Abies grandis*, nitrogen fertilization leads to a reduction of the concentration of phenolic compounds, but the cumulative concentration of 12 different terpenes was not affected.

The level of PTs in *C. tinctorius* was particularly affected by light and nutrient regimes. Variation in nutrient levels caused an over three-fold variation in the concentration of the two main PTs in *C. tinctorius* plants induced by a simulated herbivory treatment. The lowest value was 58 µg/g d.w., for an intermediate level of nutrients (treatment 0.6), and the highest value was 203 µg/g d.w. for the unfertilized plants (treatment 0.0). The effects of light in induced safflower plants was less pronounced with the highest concentration of the two main PTs, 163 µg/g d.w., under the highest light regime and the lowest concentration, 116 µg/g d.w., in plants grown at the lowest light intensity. Given the importance of polyacetylenic PTs in conferring resistance to safflower against a pathogen causing stem rot, *Phytophthora drechsleri* (Allen & Thomas, 1971, 1972), it is suggested that variations in PT contents observed in the present study will alter the chemical resistance of safflower.

Effects of light- and fertilization-mediated variations of physical and phytochemical traits in *Carthamus tinctorius* and performance of herbivorous insects

The physical and phytochemical traits conferring the most efficient protection to *C. tinctorius* plants differed according to the different herbivorous insects tested. The water content of the safflower plants appeared to be an important physical characteristic affecting the performance of herbivorous insects: both the larval growth rate of *O. nubilalis* larvae and the feces produced by the red-legged grasshopper adults increased with the water content of *C. tinctorius* plants. Leaf toughness, which tends to vary inversely with the water content, has been recognized as a resistance factor to different generalists and specialists insects (see reviews by Vicary & Bazely, 1993 and Philogène & Arnason 1995). An increase of leaf toughness in *Zea mays* foliage was negatively correlated with the larval leaf consumption of the European corn borer (Hartley & Ford, 1989; Bergvinson *et al.*, 1994a, 1994b). The results obtained in the present study did not only corroborate this particular vulnerability of *O. nubilalis* larvae to the water content of safflower plants, but also their insensitivity to PTs. The relative growth rate of European corn borer larvae was indeed unaffected by the variations in levels of PTs in *C. tinctorius* plants grown under different light and nutrients regimes (Table 7). The ability of European corn borer larvae to catabolize α-terthienyl (Iyengar *et al.*, 1987, 1990), and possibly other biosynthetically related polyacetylenic PTs like those occurring in safflower, is likely to explain the neutral response of this insect to variations in PTs. Furthermore, the light-avoidance behavioral strategy of *O. nubilalis* larvae – larvae adapted a stem boring habit on safflower – is undoubtedly efficient in attenuating the light-enhanced toxicity of PTs.

The specific pattern observed with the performance of the red-legged grasshopper was surprising given the positive effect of both constitutive and inducible concentrations of PTs on the amount of feces produced. Assuming a constant digestibility between the different treatments, these results would mean that *M. femurrubrum femurrubrum* fed more on safflower seedlings containing higher concentrations of PTs. The absence of a correlation between behavioral deterrence and oral toxicity for different toxic phytochemicals in different grasshoppers (Bernays, 1991; Cottee *et al.*, 1988) suggests that grasshoppers may tolerate a large range of toxins. Such a tolerance of generalist grasshoppers to phytochemicals can be explained by their ability to metabolize and excrete a large range of plant toxins (Smirle & Isman, 1992).

Only the reproductive output of the green peach aphid was observed to be negatively correlated with the variations of PTs levels (Table 6). The presence of PTs on the leaf surface of *C. tinctorius* (personal observation) may make aphids particularly vulnerable to these chemicals given their permanent contact with leaf epidermis. The uptake of PTs in aphids is likely to be made easier given the lipophilic properties of polyacetylenic derivatives and the thin cuticle of aphids in general. The positioning of aphids on surfaces of leaves even in the presence of the daylight combined with their thin, and sometimes even translucent, cuticles are likely to make easier the transmission of photosensitizing wavelengths through tissues. All these characteristics of aphids are likely to explain why they were observed to be relatively more susceptible to variations of PTs levels in safflower compared to both *M. femurrubrum femurrubrum* and *O. nubilalis* (Tables 6-8).

Do environmental-mediated phytochemical variations in plants harboring a phototoxic chemical defense may explain their specific distribution in open microhabitats?

For two of the three investigated insects, performance was significantly higher when safflower seedlings were grown in the shade, although the reproductive output of *M. persicae* was unaffected by this variable (Table 4). The present results thus agree with previous studies which reported higher herbivory damage on phototoxic host plants occurring in the shade (Guillet et al., 1995;

Berenbaum, 1981). However, the improved performance of herbivorous insects fed with safflower plants grown in the shade appear to be unrelated to the predicted lower concentrations of carbon-based chemical defences (see review in Herms & Mattson, 1992). First, the constitutive concentration of the two main PTs was not affected by the light regime (Table 2). Second, the multiple regressions indicated that PTs did not affect the larval relative growth rate of *O. nubilalis* (Table 6) while PTs stimulated the production of feces by *M. femurrubrum femurrubrum* adults and thus, probably, the feeding activity (Table 7). Finally, the concentration of total soluble phenolics was lower at the highest light intensity and lower for shaded regimes (Fig. 8). It was, however, observed that variations in the water content was the best variable explaining the higher performances of both *M. femurrubrum femurrubrum* and *O. nubilalis* on safflower plants grown in the shade. The working hypothesis of the present study, which assumed that higher herbivore pressure on phototoxic hostplants growing in the shade was caused by a lower production of carbon-based chemical defences, is thus rejected.

It is finally suggested that the light-enhanced toxicity of PTs may be more efficient under natural conditions since the near-UV photosensitizing regime encountered in field conditions is approximately 28 µmol photons/m^2/s (Downum *et al.*, 1991), although the measured level in the present greenhouse study was only 2 ± 1 µmol photons/m^2/s. A strengthened phototoxic effects in the field would indeed reinforce the tendency of herbivorous insects to perform better on shaded phototoxic hostplants as observed in the present study.

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Chapter 6

Production of Glands in Leaves of *Porophyllum* spp. (Asteraceae): Ecological and Genetic Determinants, and Implications for Insect Herbivores

Introduction

Allelochemicals, secondary plant metabolites that are often toxic to herbivores and pathogens, are either stored in non-active forms within the cytoplasm or, if active, within specialized structures such as glandular trichomes, secretory canals, resin ducts, or glands. These sequestering structures (SSs) are believed to have evolved to avoid cell autotoxicity (Gershenzon, 1994; McKey, 1979). In support of this interpretation, several studies have shown that the compounds contained in SSs can cause extensive damage to plant tissues (Duke *et al.*, 1988, 1994; Loveys *et al.*, 1992; Shower & Erner, 1989; Knox & Dodge, 1985).

Evidence is now accumulating to show that number, size, or content of SSs may be affected by leaf ontogeny (Bussell *et al.*, 1995; Bourret *et al.*, 1994; Werker *et al.*, 1993), nutrient availability (Mutikainen & Walls, 1995), previous exposure to herbivores (Myers & Bazely, 1991), light quality (Tanaka *et al.*, 1989) and light intensity (Upadhyaya & Furness, 1994; Yamaura *et al.*, 1989). Some studies have also indicated a genetic basis for the production of SSs (Kokkini *et al.*, 1994; Agren & Schemske, 1993; Pollard & Briggs, 1982, 1984). There is evidence that SSs and their contents influence herbivores and that they indeed often improve the resistance of plants to herbivores (Hu & Zhao, 1995; Nihoul, 1993; Myers & Bazely, 1989; Duffey, 1986; Levin, 1975). Due to the important impact that biotic and abiotic parameters exert on the production of SSs, it is expected that environmental conditions will modulate the defensive state of plants as well as the herbivory they experience.

In this study, we examined the genetic and phenotypic variations in production of SSs in two species of *Porophyllum* (Asteraceae), *P. ruderale* (Jacq.) Cass. var. *macrocephalum* (DC.) and *P. gracile* Benth., which develop foliar glands containing secondary metabolites toxic to insect herbivores (Arnason, unpublished data). We also investigated if the pattern of insect herbivory is affected by the variation in the density of SSs present on leaves of *Porophyllum* spp. under both natural and laboratory conditions.

Materials and methods

Plant material

Porophyllum gracile is a perennial with narrow threadlike leaves that occurs on rocky slopes and mesas and in washes from Arizona to southern California in mountains and deserts (Rickett, 1966a). *Porophyllum ruderale* differs from other *Porophyllum* species by having larger, oval leaf-blades of about an inch in length and is found on rocky slopes and in cañons from southern Arizona to South America (Rickett, 1966b). The presence of translucent oil-glands near the leaf margin is a characteristic of the genus *Porophyllum* (Munz & Keck, 1968). These ellipsoidal glands contain pressurized volatile monoterpenes that are ejected when the structures are physically damaged. Seeds of *P. gracile* were collected in the area of Tucson, USA, while seeds of *P. ruderale* were provided by Dr. Lozoya from Xochitepec, Mexico.

Number of glands and herbivory pressure on *P. gracile* in the field

It was initially hypothesized that glands produced in the leaves of *Porophyllum* spp. may affect the response of herbivores under field conditions. A field survey was thus performed to determine if the mean number of glands per leaf in *P. gracile*, a trait which was noticeably variable under field conditions, was related to the herbivory pressure to which the plants were subjected. Observations were made in August 1994 in ten different undisturbed sites located within 25 km of Tucson (Arizona, USA) and which were separated by at least 1 km. In each site, a single randomly selected plant was sampled to get an estimate of the variation in the number of glands per leaf at sites within this area. On each sampled plant, three branches approximately 15-20 cm long and arising from mid-canopy were removed and the number of glands on each intact leaf was counted. Although this sampling procedure does not allow to test for within-site differences, it minimized the probability that the plants sampled were closely related. This was important for the investigation of the inheritance of this trait (see next section). An ANOVA was performed to test for between-plant variation in the number of glands per leaf.

The proportion of damaged leaves relative to the total number of leaves was calculated for each sampled plant. This herbivory index provides an estimate of the pressure exerted by herbivores on *P. gracile*. A Pearson's correlation analysis was then performed on the data to test whether herbivory was correlated with variation in the mean number of glands per leaf.

Genetic basis for the number of glands in *P. gracile*

To determine whether the variation in the production of glands per leaf observed in the field is under genetic control in *P. gracile*, seeds were collected from each of the 10 plants sampled (see above) and the resulting seedlings were grown under identical environmental conditions. The seeds were soaked in distilled water for 48 h until radicles appeared and then transferred individually to 10-cm diameter pots filled with sterile vermiculite. Ten seedlings from each plant from which the seeds had been harvested, were grown to the fourth leaf stage under optimal controlled conditions [25 °C, 75% RH and a photoperiod of 16 h under cool white fluorescent and incandescent lighting (380 μmol photon m^{-2} s^{-1})] and fertilized twice a week with 50 ml of complete growth solution (see below). At harvest, the number of glands per leaf was counted and averaged for the seedlings of each potential genotype. A Pearson's correlation analysis was then performed between the mean number of glands per leaf produced under controlled laboratory conditions and the mean number of glands per leaf observed in the field. A significant positive correlation would indicate that this trait is inherited.

Effects of resource availability on the number of glands in *P. gracile* and *P. ruderale*

We tested whether resource availability affects the number of glands by submitting seedlings of *P. gracile* and *P. ruderale* to nitrogen and light stressed conditions. Because such stresses may also affect leaf size, the total volume of glands relative to leaf area was thought to be more representative of the investment in gland production than gland number as used for genetic studies on *P. gracile* (see above). Seeds for this experiment were initially collected from a single plant of each species to avoid potential artefacts due to genetic differences.

Seeds of *P. gracile* and *P. ruderale* were germinated as described above and seedlings were placed singly in 10-cm pots filled with sterile vermiculite. A two-way factorial design was used with two intensities of light (380 μmol photon m^{-2} s^{-1} vs 50 μmol photon m^{-2} s^{-1}, cool white fluorescents supplemented with incandescent bulbs) and two levels of nitrogen provided twice a week through 50 ml per pot of watering solution with or without NO_3^- as described in Machlis & Torrey (1956). The complete growth solution contained 15 mM of NO_3^- [5 mM of $Ca(NO_3)_2$ and 5 mM of KNO_3], 2 mM $MgSO_4$, 1 mM KH_2PO_4, 0.1 mM of Fe-EDTA and micronutrients. In the solution without NO_3^-, $Ca(NO_3)_2$ and KNO_3 were replaced with $CaCl_2$ and KCl, respectively. Each treatment was replicated 10 times. The size and the number of glands were measured when the seedlings had one and two pairs of true leaves. The length and width of the glands were measured using a binocular microscope fitted with a micrometer. To estimate the relative resource allocation to gland production, leaf length and width were also measured. The ellipsoidal shape of the glands was confirmed by microscopy and volume of glands and leaf area were then approximated using the following formulae:

$$V = 4/3\ \pi L l^2 \qquad (1)$$

$$S = \pi L l \qquad (2)$$

where V represents the volume of a gland, S the one-side leaf area, and L and l the half-length and half-width, respectively, of either glands or leaves. An allocation index (AI) for the production of glands was determined by dividing the total volume of glands in a leaf (V_{tot}) by the one-side surface area of this leaf (S).

$$AI = V_{tot} / S \qquad (3)$$

Repellent properties of volatiles released from glands of *P. gracile* and *P. ruderale*

The potential repellent effect of the volatiles released from damaged glands against a generalist insect herbivore was investigated. Field observations near Tucson, AZ, indicated that grasshoppers were the major herbivores that inflicted damage on *P. gracile*. The genus *Melanoplus* is widespread throughout North America and the large populations that commonly occur are known to damage many plant species. We collected and used adults of *Melanoplus femurrubrum femurrubrum* (De Green) (Orthoptera: Acrididae), a generalist herbivore common in the Ottawa/Hull area, to test the repellent properties of the volatiles released from *P. gracile* and *P. ruderale* foliar glands.

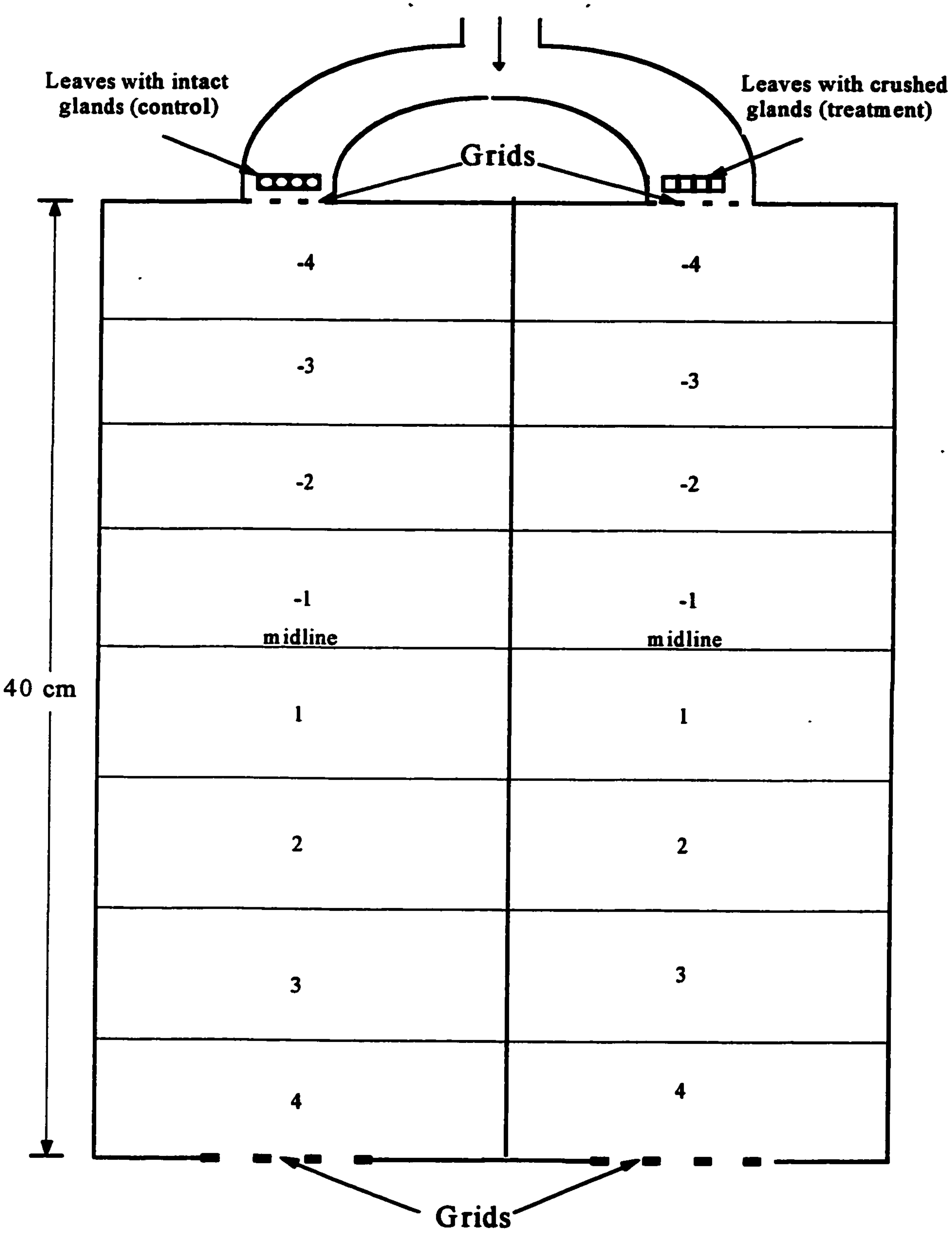
Fresh leaves of either *P. gracile* or *P. ruderale*, each bearing 6-7 glands between 2.0-2.5 mm in length, were removed from plants grown under greenhouse conditions and placed in a modified Y-tube olfactometer (Fig. 1). One side of the apparatus received leaves in which the glands had previously been crushed with the tip of a pencil while the other had leaves with intact glands (control). A plexiglass barrier prevented the air-flows in the two parts of the olfactometer from mixing. A plexiglass sheet also covered the whole olfactometer. Insects were deprived of food for a period of 10 to 15 h prior to each trial, and five adult insects were then placed on the midline of each side of the olfactometer (see Fig. 1). The positions of all insects were noted at 1, 3, 5, 10, 15, 20, 25 and 30 min after the beginning of the trial using equidistant lines marked on the base to create distinct areas. Positive values indicated movement away from the source of volatiles whereas negative values indicated movement toward the source (Fig. 1). The experiment was replicated 10 times for each plant species with the top plexiglass part of the olfactometer removed between each replicate for at least 30 min to let the volatiles disperse and the position of control and treated leaves alternated between replicates. The position of the five insects was averaged for each observation time.

Deterrent properties of the foliar glands of *Porophyllum ruderale*

The pattern of herbivory observed on leaves of *P. gracile* in the field near Tucson, AZ, indicated that insects occasionally feed on these glands. It thus appeared relevant to study the deterrent properties of the compounds present in the glands of *Porophylum* spp. However, we investigated this aspect only with *P. ruderale* since it was difficult to measure area loss due to herbivory on the narrow, threadlike leaves of *P. gracile.*

Adults of *M. femurrubrum femurrubrum* were again used as an insect model. To test for any deterrent effect of the compounds present in the

Figure 1. Apparatus used to determine the repellent properties of the volatiles sequestered in the foliar glandular structures of *Porophyllum gracile* and *P. ruderale*. The apparatus was placed flat on a table and covered with a plexiglass sheet in order to be able to make the observations. The leaves were inserted at the positions indicated just before placing *Melanoplus femurrubrum femurrubrum* adults along the midline (5 individuals per half chamber/replicate).


Leaves with intact glands (control)
Leaves with crushed glands (treatment)
Grids
-4
-4
-3
-3
-2
-2
-1
-1
midline
midline
40 cm
1
1
2
2
3
3
4
4
Grids

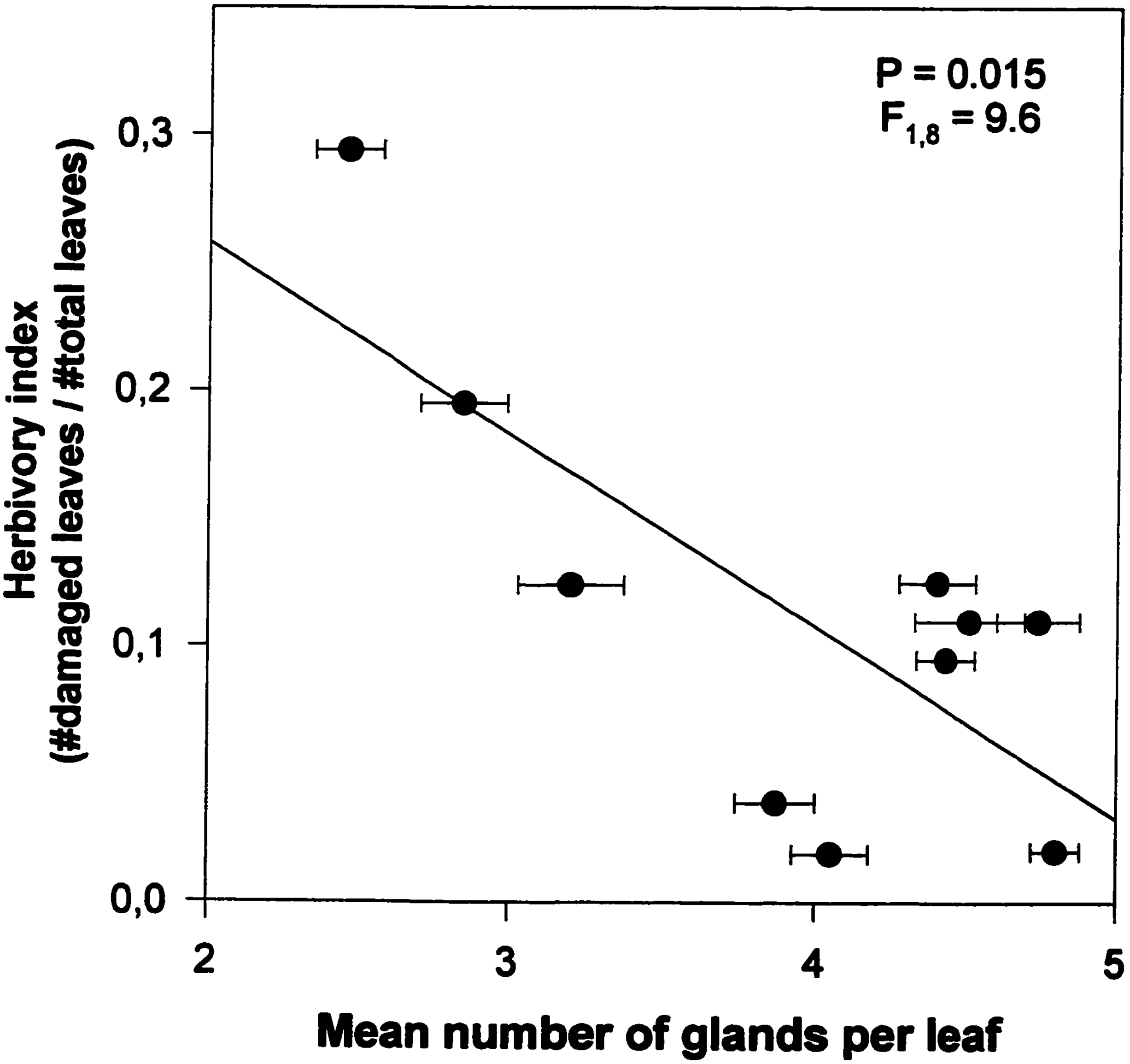
glands, the insects were individually placed in 950-ml Mason jars and were offered a choice of three leaves bearing respectively 1-2, 3-5, or 7-9 intact glands measuring 2.0-2.5 mm in length. All the leaves selected for the bioassay were approximately of the same age (5-7 weeks) and had a similar lamina length (16.4 ± 2.4 mm in length). The petioles of the leaves were inserted in a 2.5 ml vial filled with distilled water and secured with a cotton ball. The trials lasted for eight hours at room temperature (25±2 °C). Twenty-five replicates were performed but only those in which consumption occurred were kept for analysis (n=11). Leaf consumption was determined as area removed using a binocular microscope and 1 mm grid graph paper.

Results

Number of glands and herbivory pressure on *P. gracile* in the field

The population of *P. gracile* growing in the Tucson area showed a significant variation (nearly two-fold overall) in the mean number of glands produced per leaf (Fig. 2; Univariate ANOVA plant effect, $F_{9,1160} = 47.8$, $P<0.0001$). Herbivory significantly decreased as the number of glands per leaf increased (Fig. 2; Pearson's correlation coefficient = -0.733, Bartlett chi-square statistic = 5.8, P=0.0158) consistent with the hypothesis that glands are defensive. Our data indicate that individuals of *P. gracile* bearing 2.5 glands per leaf suffered a five-fold higher herbivory pressure on average than those bearing 4.8 glands per leaf (21.7% vs 4.3% of the leaves were damaged, respectively).

Figure 2. Herbivory as measured by the ratio of the number of damaged leaves to the total number of leaves on *Porophyllum gracile* (Asteraceae) as a function of the number of glands per leaf for ten plants sampled in the field in the area of Tucson (Arizona, USA). For each plant, the mean and the standard error of the number of glands per leaf were determined from 73-209 leaves.


P = 0.015
$F_{1,8} = 9.6$
Herbivory index
(#damaged leaves / #total leaves)
0,3
0,2
0,1
0,0
2
3
4
5
Mean number of glands per leaf

Genetic vs ecological determinants of the number of glands in *Porophyllum* spp.

There was no correlation between the mean number of glands per leaf observed in the *P. ruderale* plants sampled from the different locations around Tucson and the data obtained for the progeny of these same plants when seedlings were grown under controlled conditions (Fig. 3; Pearson's correlation coefficient = -0.032, Bartlett chi-square statistic = 0.008, P=0.93). Thus, the variation in the number of glands per leaf in *P. gracile*, as observed in the field, does not appear to be due to genetic variation.

Effects observed in the nitrogen and light stress experiments suggested that the variation in allocation to gland production, as observed in the field, was more likely due to differences in resource availability. Both light intensity and nitrogen fertilization generally increased the number of glands per leaf in both *Porophyllum* species, with light being more stimulatory than fertilization (Tables 1,2). The magnitude and direction of the effect, however, depended on the developmental stage of the plants (Tables 1,2) and this is confirmed by the significant interactions between light and developmental stage and between nitrogen and developmental stage (Table 2). The pattern of stimulation by both light and nitrogen, was in most cases more marked when allocation to gland production was expressed in terms of either gland size, total volume of glands per leaf, or total volume of glands per leaf area. The results indicate that allocation to gland production for both *P. gracile* and *P. ruderale* peaks when both light and nitrogen are high (Table 1). When quantified in terms of μl of gland per mm^2 of leaf area, the variation observed spanned two orders of magnitude.

Table 1 Light intensity (L) and nitrogen (N) effects on the production of glands in *Porophyllum ruderale* and *P. gracile* (Asteraceae). + and - refer to high and low regimes as described in the materials and methods section. The values are the mean of ten replicates and the standard deviations are indicated in brackets.

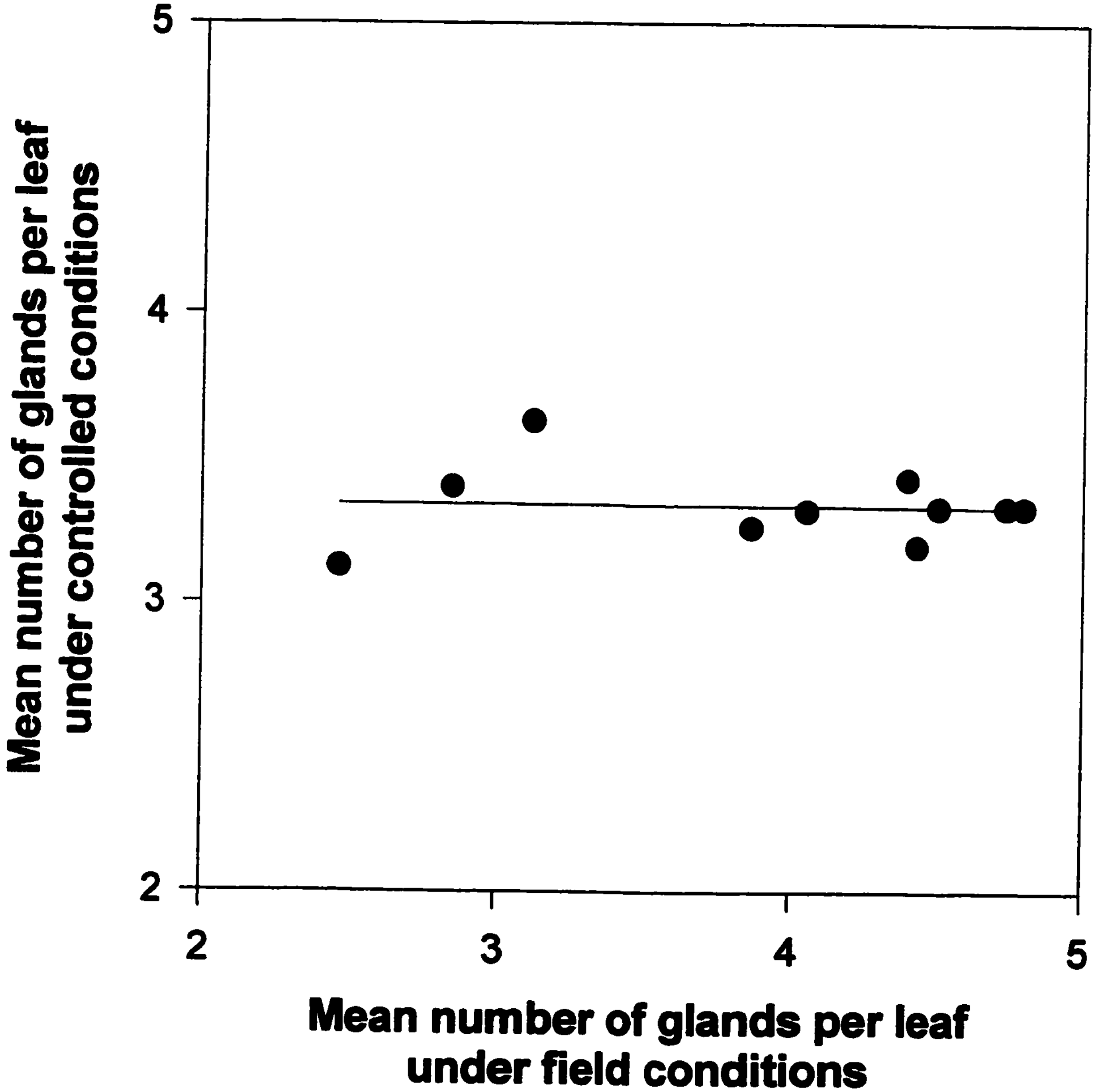
Developmental stage	First pair of true leaves developed				Second pair of true leaves developed							
Tissue sampled	First pair of leaves				First pair of leaves				Second pair of leaves			
Treatment	+L+N	+L-N	-L+N	-L-N	+L+N	+L-N	-L+N	-L-N	+L+N	+L-N	-L+N	-L-N
Porophyllum gracile												
Number of glands per leaf	3.00 (1.10)	2.70 (0.78)	0.60 (0.66)	1.20 (0.78)	3.20 (0.87)	2.90 (0.94)	2.40 (0.49)	1.00 (0.63)	2.60 (0.80)	3.10 (0.30)	3.30 (0.46)	1.20 (0.87)
Mean volume of a gland (10^{-3}µL)	37.9 (18.8)	4.9 (3.0)	0.8 (0.6)	1.0 (0.3)	44.4 (26.2)	13.4 (2.4)	6.6 (2.4)	0.7 (0.2)	57.7 (18.5)	4.1 (3.0)	2.0 (0.9)	2.5 (0.9)
Volume of glands per leaf (10^{-3}µL)	126 (82)	13 (8)	0.5 (0.5)	1.2 (1.4)	133 (78)	39 (14)	16 (16)	0.7 (0.5)	165 (106)	13 (9)	7 (4)	3 (2)
Volume of glands per leaf area (10^{-3}µL/mm^2)	8.40 (4.41)	0.47 (0.27)	0.02 (0.03)	0.05 (0.06)	4.92 (2.94)	1.14 (0.28)	0.47 (0.13)	0.04 (0.03)	2.78 (2.36)	0.56 (0.27)	0.38 (0.14)	0.10 (0.06)
Porophyllum ruderale												
Number of glands per leaf	7.00 (0.45)	6.41 (1.00)	4.40 (0.91)	1.50 (0.50)	6.20 (0.75)	7.00 (0.70)	5.30 (1.10)	5.50 (0.50)	3.00 (0.63)	6.56 (1.42)	0.70 (0.46)	0.20 (0.40)
Mean volume of a gland (10^{-3}µL)	13.0 (6.2)	9.5 (5.2)	0.7 (0.1)	0.7 (0.1)	378.5 (58.2)	95.2 (29.5)	5.6 (1.0)	2.4 (0.6)	139.7 (36.6)	3.2 (2.0)	0.9 (0.6)	0.2 (0.3)
Volume of glands per leaf (10^{-3}µL)	91 (45)	60 (15)	3 (1)	1 (0.4)	2332 (377)	666 (206)	29 (2)	14 (4)	406 (106)	22 (15)	1 (0.9)	0.2 (0.3)
Volume of glands per leaf area (10^{-3}µL/mm^2)	0.44 (0.16)	0.31 (0.15)	0.15 (0.11)	0.09 (0.07)	15.99 (3.92)	2.00 (0.34)	0.28 (0.09)	0.17 (0.08)	10.52 (2.99)	0.04 (0.02)	0.07 (0.07)	0.07 (0.14)

Table 2 P values of ANOVA on effects of developmental stage, light and nitrogen on production of foliar glands in *Porophyllum ruderale* and *P. gracile* (Asteraceae).

	Number of glands per leaf	Mean volume of a gland[§] (µl)	Volume of glands per leaf[§] (µl)	Volume of glands per leaf area[§] (µl/mm²)
Porophyllum ruderale				
Developmental stage	< 0.0001 ($F_{2,108}=170.2$)	< 0.0001 ($F_{2,108}=277.8$)	< 0.0001 ($F_{2,108}=339.6$)	< 0.0001 ($F_{2,108}=88.4$)
Light	< 0.0001 ($F_{1,108}=406.9$)	< 0.0001 ($F_{1,108}=1378.6$)	< 0.0001 ($F_{1,108}=1553.0$)	< 0.0001 ($F_{1,108}=309.9$)
Nitrogen	0.5868 ($F_{1,108}=0.3$)	< 0.0001 ($F_{1,108}=198.4$)	< 0.0001 ($F_{1,108}=149.7$)	< 0.0001 ($F_{1,108}=137.1$)
Dev. stage*Light	<0.0001 ($F_{2,108}=39.0$)	<0.0001 ($F_{2,108}=23.1$)	<0.0001 ($F_{2,108}=25.3$)	<0.0001 ($F_{2,108}=24.7$)
Dev. stage*Nitrogen	<0.0001 ($F_{2,108}=39.5$)	<0.0001 ($F_{2,108}=57.4$)	<0.0001 ($F_{2,108}=21.3$	<0.0001 ($F_{2,108}=28.4$)
Light*Nitrogen	<0.0001 ($F_{1,108}=56.4$)	< 0.0001 ($F_{1,108}=40.5$)	0.0842 ($F_{1,108}= 3.0$)	< 0.0001 ($F_{1,108}=96.9$)
Dev. stage* Light*Nitrogen	0.0001 ($F_{2,108}=10.3$)	<0.0001 ($F_{2,108}=12.9$)	0.0005 ($F_{2,108}=8.2$)	<0.0001 ($F_{2,108}=43.3$)
Porophyllum gracile				
Developmental stage	0.0001 ($F_{2,108}=12.3$)	<0.0001 ($F_{2,108}=18.3$)	<0.0001 ($F_{2,108}=19.9$)	<0.0013 ($F_{2,108}=7.1$)
Light	< 0.0001 ($F_{1,108}=91.3$)	< 0.0001 ($F_{1,108}=406.9$)	< 0.0001 ($F_{1,108}=362.7$)	< 0.0001 ($F_{1,108}=359.0$)
Nitrogen	< 0.0004 ($F_{1,108}=13.5$)	< 0.0001 ($F_{1,108}=116.8$)	< 0.0001 ($F_{1,108}=79.6$)	< 0.0001 ($F_{1,108}=85.7$)
Dev. stage*Light	0.0005 ($F_{2,108}=8.2$)	0.0103 ($F_{2,108}=4.8$)	<0.0001 ($F_{2,108}=11.7$)	0.0001 ($F_{2,108}=19.5$)
Dev. stage*Nitrogen	0.0044 ($F_{2,108}=5.7$)	0.0331 ($F_{2,108}=3.5$)	0.0057 ($F_{2,108}=5.4$)	0.0079 ($F_{2,108}=5.1$)
Light*Nitrogen	0.0009 ($F_{1,108}=11.8$)	<0.0001 ($F_{1,108}=41.3$)	0.0001 ($F_{1,108}=9.4$)	0.0048 ($F_{1,,108}=8.3$)
Dev. stage* Light*Nitrogen	<0.0001 ($F_{2,108}=13.9$)	<0.0001 ($F_{2,108}=38.2$)	<0.0001 ($F_{2,108}=20.3$)	<0.0001 ($F_{2,108}=31.4$)

[§] A logarithmic transformation of the data was performed before the analysis of variance.

Figure 3. Variation in the mean number of glands per leaf in *Porophyllum gracile* (Asteraceae): comparison between the values observed under field conditions (each point is the average of 73-209 leaves collected from a single plant) and those obtained under standardized growth conditions (each point is the average of 40 leaves collected from ten seedlings).


5
4
3
2
2
3
4
5
Mean number of glands per leaf under controlled conditions
Mean number of glands per leaf under field conditions

Repellent and deterrent properties of the glands in *Porophyllum* spp.

The chemical content of the glands located on the leaves of *Porophyllum* spp. exerts both long- (repellent) and short- (deterrent) range effects on a generalist feeder such as the red-legged grasshopper. Insects moved away from volatiles released from crushed glands of both *Porophyllum* species (Fig. 4). Grasshoppers also fed less on leaves of *P. ruderale* bearing more glands (Table 3) as might be expected from field observations on *P. gracile* (Fig.2).

Discussion

Production of glands in *Porophyllum* spp. relates to the availability of both carbon and nitrogen

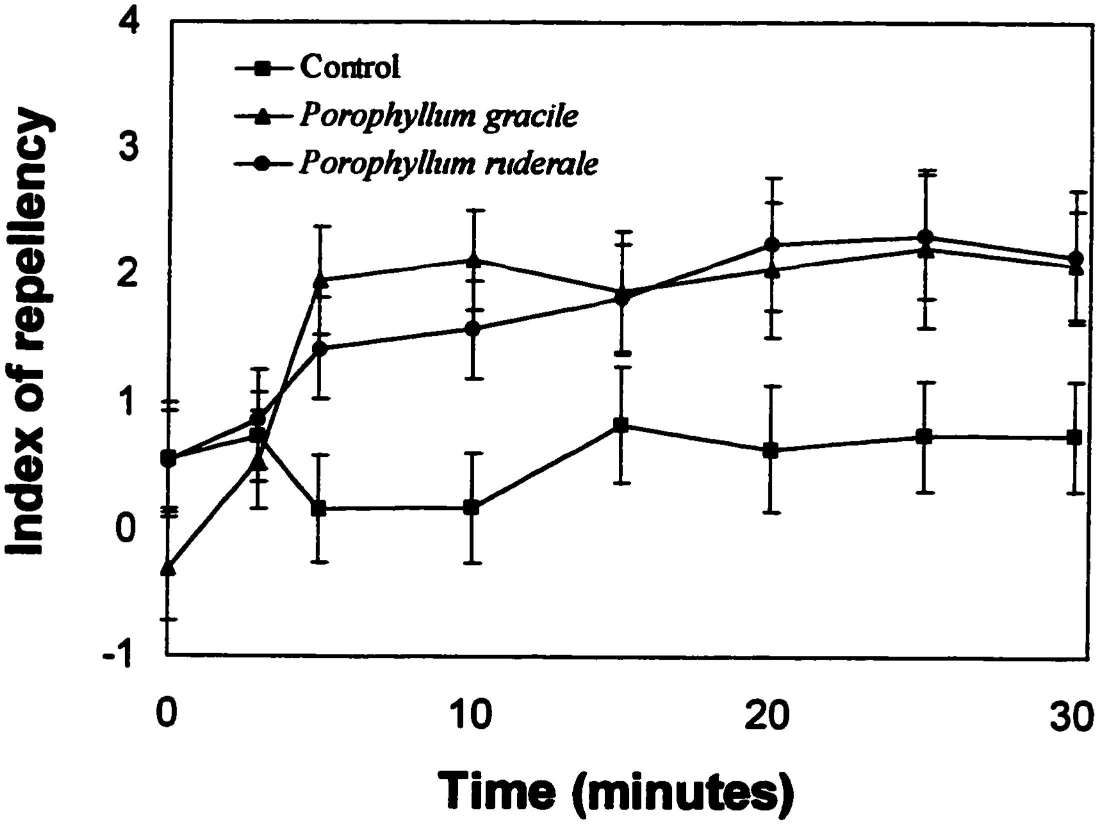
Variation in the mean number of glands per leaf observed on individuals of *P. gracile* in the field disappeared when seedlings obtained from the seeds of the same individuals were grown under controlled conditions (Fig. 2 and 3). This suggests that SSs in *P. gracile* are not under strong genetic control. The variation in the production of glands observed as a result of varying resource availability under laboratory conditions (Table 2) is thus likely to be a better alternative explanation for the variation of this trait in nature.

Carbon-based allelochemicals such as terpenes are sometimes considered to be inexpensive to produce because their biosynthesis may partially depend on an excess of carbohydrates produced in plants (Herms & Mattson, 1992; Bryant *et al.*, 1983). However, such a passive interpretation of the physiological processes involved in the production of chemical

Table 3 Apparent deterrency exerted by the glands on leaves of *Porophyllum ruderale* against *Melanoplus femurrubrum femurrubrum* adults. Standard errors are provided in brackets and values followed by different letters differ significantly ($P<0.05$, Tukey's test).

Choice test: *M. femurrubrum femurrubrum* adults offered leaves bearing different numbers of glands			
	1-2 glands	3-5 glands	7-11 glands
leaf area fed (mm^2)	96.4^{a} (17.3)	71.4^{ab} (16.5)	51.3^{b} (19.5)

Figure 4. Repellent effects associated with the volatiles emitted from the foliar glands of *Porophyllum gracile* and *P. ruderale* against *Melanoplus femurrubrum femurrubrum* adults. A positive value indicates that insects were moving away from the emitting source while a negative value indicates that they were attracted toward the emitting source (mean ± s.d.).


4
3
2
1
0
-1
Index of repellency
0
10
20
30
Time (minutes)
Control
Porophyllum gracile
Porophyllum ruderale

defences in plants appears not to apply for *Porophyllum* spp. In fact, light, nitrogen and their interaction all significantly affected the pattern of resource allocation to gland production (Tables 1 and 2). The importance of light, and hence carbon, to the production of glands seems reasonable for plants growing in high-light environments. The annual total of global radiation in the Sonoran desert where some of our observations were made is about 700 GJ/km^2, which represents one of the highest values recorded anywhere (Larcher, 1995).

The requirement for nitrogen may, however, represent an important ecological constraint to the production of glands in *Porophyllum* spp. since desert soils contain low levels of this nutrient (Bowers, 1982). Knight (1991) reported that arid regions are particularly conducive to ammonia volatilization due to extreme cycles of wetting-drying and high temperatures, and that the low vegetative cover in deserts favours nitrogen losses by wind and water erosion. The high nitrogen requirement for the production of glands in *Porophyllum* may be explained by the different physiological processes involved during the production of SSs (see reviews by Simms, 1992, and Zangerl & Bazzaz, 1992). Additional, indirect costs, such as the prevention of autotoxicity may also drain some nitrogen since they rely on enzymatic processes. For example, thiophene derivatives like those occurring in the glands of *P. gracile* and *P. ruderale* (Arnason, unpublished) may generate an oxidative stress (Hasspieler *et al.*, 1990) that the plant may circumvent by antioxidant enzymes (Larson, 1995), as is the case for insects (Aucoin *et al.*, 1995; Felton & Summers, 1995; Lee & Berenbaum, 1990).

Although the production of glands in *Porophyllum* spp. is likely to be constrained by environmental factors, especially light and nitrogen availability, it provides a potential increase in fitness associated with a decrease in herbivory (Fig. 2 and Table 3).

Environmental constraints on the production of sequestering structures in plants

Accumulation of chemical defences in discrete SSs involves some ecological constraints for plants as some insects may acquire appropriate behaviours for circumventing such spatially discrete protection (Dussourd & Denno, 1994; Dussourd & Eisner, 1987). Avoidance of the content of SSs during feeding is a simple and efficient behavioural strategy to prevent intoxication which seems particulary appropriate for the first larval instars that are generally more sensitive to allelochemicals. First-instar *Heliothis virescens* (F.) larvae, which are sensitive to gossypol, feed all around the glands present on cotton leaves into which gossypol is sequestered (Parrott *et al.*, 1983). Similar results were also obtained with first-instar *Anaitis plagiata* (L.) larvae which selectively avoid feeding on the black glands containing hypericin in *Hypericum perforatum* L. leaves (Fields *et al.*, 1991). In both these insect species, however, higher instars were reported to consume the glands on their respective host plants.

In our study, the preference even of adult *M. femurrubrum femurrubrum* for leaves bearing fewer glands (Fig. 4, Table 3) constitutes an efficient behavioural strategy for reducing the exposure of insects to the allelochemicals found in the glands of *Porophyllum* spp. Although *M. femurrubrum femurrubrum* adults bit into glands, in such cases, they rapidly retreated when the pressurized glandular contents sprayed onto their mouth parts. Such hasty retreats, as well as being in apparent agreement with the deterrent and repellent properties of the glandular content (Fig. 4, Table 3), could also constitute the first step of a more global behavioural strategy involving repetitive assaults to deplete the glands of their volatile defences. Dussourd & Denno (1991) reported a similar strategy used by adults of a katydid, *Scudderia furcata* Brunner von Wattenwyl (Orthoptera: Tettigoniidae), that bit repeatedly into the stem, petiole, and midrib of *Apocynum cannabinum* L. (Apocynaceae) to drain the latex before feeding on the leaves.

Conclusion

We have shown a relationship between the average number of glands per leaf in *P. gracile* and the operational resistance *sensu* Rauscher & Simms (1989) against herbivores both under field and laboratory conditions. However, there are two major ecological trade-offs for *Porophyllum* spp. which rely on the production of glands containing allelochemicals to defend their tissues. The first relates to the conditional availability of both light and nitrogen resources that allow the production of the glands. The second is due to the fact that accumulation of allelochemicals in SSs, despite being a good strategy for plants to reduce risks of autotoxicity, opens the door to insects that have the behavioural ability to feed selectively on tissues harbouring fewer SSs.

Acknowledgement

I am grateful to Donald E. Champagne and Mike Singer for field assistance in Arizona. Part of this work was performed in the laboratory of E.A. Bernays at the University of Arizona in Tucson.

Chapter 7

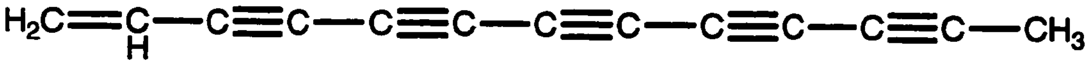
Multiple modes of insecticidal action of three classes of polyacetylene derivatives from *Rudbeckia hirta* (Asteraceae)

Introduction

Over 750 naturally occurring polyacetylene derivatives (PADs) are found in the Asteraceae family (Bohlmann *et al.*, 1973). Straight chain PADs are the most common, while thiophenes and thiarubrines (Fig. 1) are sulphur heterocycles that are not as widely distributed. Among the approximately 500 species of plants in the subtribe Heliantheae that have been investigated, 204 distinct PADs occur in 377 species, 55 thiophenes were found in 114 species and 6 different thiarubrines were detected in 37 species (Christensen & Lam, 1991). From feeding experiments with radioactive tracers, it has been unequivocally established that straight chain PADs are the precursors of thiophenes and thiarubrines (Gomez-Barrios *et al.*, 1991; Jente *et al.*, 1988; Singh, 1988).

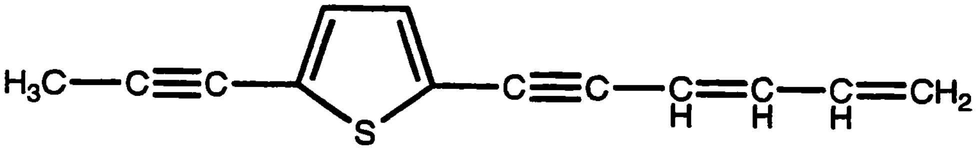
While the light-dependent toxicity (phototoxicity) of thiophenes and some straight chain PADs to insects is well established (Arnason *et al.*, 1981; Marles *et al.*, 1991), there is little information on the insecticidal properties of thiarubrines or the effect of light on these activities. There exist only two references in the

Figure 1. Polyacetylene derivatives occurring in *Rudbeckia hirta* (Asteraceae).

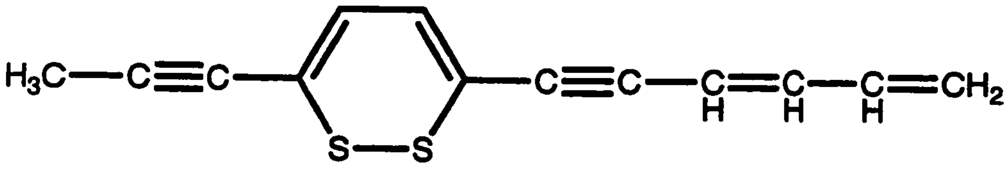


$$H_2C{=}CH{-}C{\equiv}C{-}C{\equiv}C{-}C{\equiv}C{-}C{\equiv}C{-}C{\equiv}C{-}CH_3$$

1



2



3

literature indicating that thiarubrines act as feeding deterrents against the cockroach *Blatta orientalis* and *Ostrinia nubilalis* Hübner (Lepidoptera: Pyralidae) larvae at concentrations of 10 µg/g and 100 µg/g, respectively (Ellis *et al.*, 1995; Aucoin *et al.*, 1995).

Rudbeckia hirta L. (Asteraceae), the black-eyed Susan, is a species containing all three classes of PADs (Fig. 1) and which has been reported as exhibiting phototoxic properties in the stem, root and flower (Camm *et al.*, 1975). An ethanol extract of *R. hirta* was shown to exert repellent effects against the Japanese beetle (Metzger & Grant, 1932). In previous studies, we also showed that tortricid and geometrid larvae that attack the phototoxic inflorescences of *R. hirta* in Eastern North America are sensitive to the phototoxic properties of this plant species (Guillet *et al.*, 1995).

In the present study, we isolated or synthesized and then examined the insecticidal properties of the three related PADs occurring in *R. hirta* (Fig. 1) under different light regimes. The objective was to investigate how different classes of PADs have their toxicity to insects modulated by light. Because of the brief availability of the natural herbivorous larvae, we conducted the present study on the toxic and phototoxic properties of the PADs using laboratory colonies of insects, *Aedes atropalpus* Say (Diptera: Culicidae) and *Manduca Sexta* (L.) (Lepidoptera: Sphingidae), that are known to be sensitive to phototoxins (Arnason *et al.*, 1981; Marles *et al*, 1991; Downum *et al.*, 1984; Champagne *et al.*, 1986).

Materials and methods

Purification of polyacetylene derivatives

The inflorescences and roots of *R. hirta* collected in the Ottawa/Hull area were separately homogenized in ethanol-water (1:1), filtered and then extracted with hexane. The hexane fraction from the inflorescences contained the tridecapentaynene (**1**) which was further purified via repeated TLC (silica gel 60, art. 5721, Merck) using hexane : ethyl acetate (9:1) as the eluent system. The thiarubrine (**3**) was purified from the root hexane fraction as described by Constabel *et al.* (1988) while the thiophene (**2**) was synthesized according to a reported procedure (Atkinson & Curtis, 1965). The identity of each compound was confirmed by comparison of UV and MS data with published literature values (Bohlmann *et al.*, 1973; Constabel *et al.*, 1988) and purity of each compound was checked by HPLC.

Insecticidal activity

Insecticidal activity of the three PADs were evaluated against second instar larvae of *A. atropalpus* as described in Marles *et al.* (1991). Larvae were incubated in 5 ml of distilled water in scintillation vials for 24 h in the presence of different concentrations of the PADs indicated in Fig. 1. Test compounds were added in 25 µl of EtOH and the control received an equal amount of solvent. To test the light-modulated toxicity of PADs, experiments were performed under three light regimes: 1) darkness, 2) visible light (cool white, 350 µmol photons m^{-2} s^{-1}), 3) visible light (as before) supplemented with near-UV provided by blacklight bulbs (Westinghouse F20T12/BLB, 30 µmol photons m^{-2} s^{-1}) having an emission range between 300-400 nm (17). For each treatment, 3 to 5 replicates of 20 to 25 larvae each were performed. The data were submitted to probit analysis to determine the LC_{50} and the 95% fiducial limits (Finney, 1964). Treatments for which the 95% fiducial limits were not overlapping were considered as significantly different.

Light-independent insecticidal properties of PADs were also determined using *M. sexta* as a phytophagous insect model maintained as described by Stewart & Philogène (1983). The compounds dissolved in EtOH were added (5 µl of EtOH/g of diet) and mixed into a warm meridic diet (35 °C) to reach a concentration of 50 µg/g of diet. As a comparison, the concentrations of compounds **2** and **3** in root cultures of *R. hirta* were 70 µg/g dry wt and 440 µg/g dry wt, respectively (Constabel *et al.*, 1988). The control treatment received the same amount of EtOH as the treatments. Neonate larvae (20 per treatment, 3 replicates per treatment) were fed for 4 days in darkness with a diet treated with either compound **2** or **3**. Since HPLC measurements indicated that the half-lives of compounds **2** and **3** in the diet were between 2.8 and 4 days, the remaining diets were replaced by freshly prepared ones every two days during the experiment. Using this protocol, it was impossible to test compound **1** because of its instability in the diet (it was completely degraded after 24 h).

Results and discussion

Only the tridecapentaynene (**1**) was highly toxic to *A. atropalpus* larvae under all the three light regimes tested (D, VIS and VIS + UV) while the insecticidal effects of compounds **2** and **3** significantly differed between the light treatments (Table 1). The thiophene (**2**) was highly toxic to mosquito larvae only in presence of VIS + UV irradiation and the thiarubrine (**3**) generated the most lethal effects under both D and VIS + UV treatments (Table 1).

The distinct light-independent insecticidal effects of compound **3** compared to **2** were also confirmed with neonate *M. sexta* larvae. At a concentration of 50 µg/g diet, the thiarubrine (**3**) generated a lower weight gain and a higher mortality to *M.*

Table 1. LC_{50} values (μg/ml) for the three polyacetylene derivatives cited in Figure 1 against second instar *Aedes atropalpus* larvae under different light regimes.

Light regime	Tridecapentaynene[§] (1)	Thiophene[§] (2)	Thiarubrine[§] (3)
Dark	0.05^{a} (0.03-0.11)	5.51^{b} (2.84-6.92)	0.09^{a} (0.04-0.19)
Visible light	0.19^{a} (0.05-0.29)	2.59^{b} (1.08-4.97)	1.24^{b} (0.77-1.94)
Visible light + near-UV	0.03^{a} (0.01-0.07)	0.14^{a} (0.06-0.21)	0.18^{a} (0.085-0.27)

[§] Values in brackets represent the 95% fiducial limits as determined by probit analysis. For each column, values followed by different letters have non-overlapping 95% fiducial limits.

sexta larvae than either the control or the thiophene (**2**) treatments (Table 2).

These variations observed in the insecticidal properties between the three PADs investigated suggest that different mechanisms of toxicity are involved for each compound. Reisch *et al.* (1967) have reported that dark toxicity of straight chain polyines against bacteria and fungi is improved by decreasing saturation. As the tridecapentaynene (**1**) is the ultimate form of unsaturation in natural PADs, its dark antibiotic (Towers *et al.*, 1977) and insecticidal (Tables 1, 2) activities are not unexpected, although the mechanism of action is unknown and clearly different from light-activated toxicity.

It is also conceivable that a light-induced toxicity of **1** may be involved since straight chain polyacetylenes, which strongly absorb photons in the near-UV wavelengths, generate photoadducts with olefins when exposed to light (Chung *et al.*, 1993). Permeabilization of liposomes containing unsaturated lipids when irradiated under near-UV and in presence of straight chain polyacetylenes (McRae *et al.*, 1985) suggested that the generation of photoadducts may alter the integrity of cellular membranes and eventually lead to mortality as observed with *Escherichia coli* and *Saccharomyces cerevisiae* (McLachlan *et al.*, 1984). In support of such light-induced toxicity with straight chain polyacetylenes, Kagan *et al.* (Kagan *et al.*, 1992) showed that, under a near-UV radiation setup (320-420 nm) similar to the one used in the present study (300-400 nm), 2-chloro-3,11-tridecadiene-5,7,9-triyn-1-ol induces a photosensitized hemolysis of human erythrocytes under both aerobic and anaerobic conditions. Considering that no phototoxic effect was observed with compound **1** in the present study (Table 1), it is suggested that a competing photodegradation reaction may limit the impact of phototoxicity. This interpretation is supported by the observation of an approximately two orders of magnitude higher

Table 2. Effects on weight gain and mortality of neonate *Manduca sexta* larvae fed for 4 days in darkness with a meridic diet treated with the thiophene (**2**) or the thiarubrine (**3**).

Treatment	Larval weight gain[§] (mg)	Larval mortality[§] (%)
Control	18.9^{b} (4.1)	5.0^{a} (1.4)
Thiophene (**2**) (50µg/g)	15.3^{b} (2.1)	10.0^{a} (5.1)
Thiarubrine (**3**) (50µg/g)	7.2^{a} (1.6)	62.0^{b} (10.6)

[§] Values in brackets represent the standard deviation. For each column, values followed by different letters are significantly different ($P<0.05$, Tukey's test).

rate of photodegradation of the 2-chloro-3,11-tridecadiene-5,7,9-triyn-1-ol than the α-terthiophene when irradiated at 310 nm in 50% EtOH solutions (McLachlan *et al.*, 1984).

Enhancement of the toxicity of thiophenes in the presence of near-UV light has been reported for different classes of organisms (Towers Champagne, 1988) including some insects (Marles *et al.*, 1991; Downum *et al.*, 1984). This phototoxic process is due to the production of singlet oxygen when thiophenes are irradiated with near-UV light in an oxygenated medium (Scaiano *et al.*, 1987) and which ends up in a photooxidative peroxidation of lipids (Aucoin *et al.*, 1995).

The dark toxicity of the thiarubrine (**3**) against mosquito larvae was comparable to that observed for the thiophene (**2**) under photosensitizing wavelengths. This light-independent insecticidal properties of a thiarubrine is a new finding that corroborates those obtained with microorganisms (see review in Ellis *et al.*, 1995). Constabel and Towers (1989) have investigated the antibiotic properties of two different thiarubrines, among which one was compound **3**, and their related thiophenes. They found that toxicity of thiarubrines to *Saccharomyces cerevisiae*, *Escherichia coli* and *Pseudomonas flavescens*, was slightly lower or comparable in darkness to that under near-UV photosensitizing irradiation, although the two corresponding thiophenes were inactive unless irradiated with near-UV light. The toxicological similarity observed in the present study between the thiophene (**2**) and the thiarubrine (**3**) under both VIS and VIS + UV light regimes is also in agreement with the photo-conversion of thiarubrines to thiophenes through the extrusion of a sulfur atom (Constabel *et al.*, 1988). However, the mechanism for the dark toxicity of thiarubrine derivatives has not been elucidated.

The insecticidal results reported here shown different patterns of insecticidal action of the three PADs investigated when exposed under different light regimes. The chemical redundancy of the PADs **1** and **2** in the inflorescences of *R. hirta* could be linked to an evolutionary strategy designed to diversify the mechanisms of their chemical defenses. One potential advantage of such a strategy suggested by Berenbaum *et al.* (1991) is synergism, which allows a reduced investment in plant defense compounds. Alternatively, the synthesis of active compounds in the dark such as **1** and **3** may be necessary to counteract an adapted guild of insect herbivores that we have observed to avoid direct light exposure and phototoxic effects of PADs (Guillet *et al.*, 1995).

Acknowledgements

T. Durst and J. O'Meara (Department of Chemistry, University of Ottawa) synthesized the thiophene (**2**) and G.H.N. Towers (University of British Columbia, Vancouver) provided a sample of the thirabrine (**3**).

Chapter 8

Synergist properties of the volatile monoterpenes present in the glandular secretory cavities in leaves of *Porophyllum gracile* and *P. ruderale* (Asteraceae)

Introduction

The genus *Porophyllum* (Asteraceae) occurs naturally in an area extending from the south-west of the United States to South America (Rickett, 1966a,b). It is characterized by the presence of translucent foliar glandular secretory cavities (FGSCs) that are mainly located along the leaf margin but are also scattered throughout the lamina (Munz & Keck, 1973). The lumen of the FGSCs has a lysigenous origin and is delimited by a multilayered epithelium (Monteiro *et al.*, 1995). The FGSCs were also reported (Guillet *et al.*, 1997a) as an important feature that confers resistance against insect herbivory in *P. gracile* and *P. ruderale*. Under field conditions, individuals of *P. gracile* harbouring on average 4.9 FGSCs per leaf had only 4.3% of their leaves damaged by insect feeding while plants with only 2.5 FGSCs per leaf suffered a 5-fold higher damage by herbivores. The same study also confirmed under laboratory conditions that the volatile compounds emitted from the FGSCs of both *P. ruderale* and *P. gracile* are repellent against adults of the red-legged grasshopper, *Melanoplus femur-rubrum* (Orthoptera: Acrididae).

Phytochemical studies have identified a range of monoterpenes, phototoxic thiophene derivatives and sesquiterpenes, that occur in the genus *Porophyllum* (Lozoya & Gaspar, 1993; Downum *et al.*, 1985; Bohlmann *et al.*, 1980; Bohlmann & Zdero, 1979; Bohlmann *et al.*, 1983; Chan *et al.*, 1979; Downum & Towers, 1983). It is however not known which insecticidal compounds typically occur in the foliar GSCs of *Porophyllum* spp. since the chemicals reported were in most cases extracted from tissues of entire aerial parts, roots or whole plants.

In this study we investigated by gas chromatography-mass spectrometry (GC-MS) the volatiles emitted from the aerial parts of *P. gracile* and *P. ruderale* and confirmed their identity. The hypothesis that these volatiles exert a synergist effect on the insecticidal properties of α-terthienyl, a phototoxic polyacetylenic derivative also found in *P. gracile* and *P. ruderale*, was also tested against larvae of the European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Pyralidae).

Materials and methods

Chemical analysis

Seeds of *Porophyllum ruderale* (Jacq.) Cass. var. *macrocephalum* (DC.) were provided by Xavier Lozoya from Xochitepec (Mexico) while those of *Porophyllum gracile* Benth. were collected by Guillet in the area of Tucson (Arizona, USA) in August 1994. Seeds of both species were germinated in water and then transplanted in vermiculite. Plants were fertilized weekly with Hoagland's solution and grown under greenhouse conditions at 20 °C/8-h night and 25 °C/16-h day. Natural lighting supplied with high-pressure sodium lamps provided a daytime irradiance of 300 ± 50 µmol m^{-2} s^{-1} photosynthetic photon flux density.

Different techniques were used to extract the volatile compounds from plant tissues. A direct sampling of the liquid contained in the glands on leaves of *P. ruderale* was practiced using a Hamilton 10-ml syringe. A similar direct sampling was not performed with the foliar glands of *P. gracile* given their smaller dimensions.

Gas chromatography was performed using a Varian 6000 series Vista equipped with a flame ionization detector and a Varian DS 654 integrator. The columns used were a fused silica Durabond DB-5 (0.25 µm film thickness, 30 m x 0.25 mm i.d.), a fused silica Durabond DB-1 (1.0 µm film thickness, 30 m x 0.25 mm i.d.), and a fused silica Durabond DB-Wax (0.25 µm film thickness, 30 m x 0.25 mm i.d., J & W Scientific, Folsom, CA). For all analyses, the temperature program was as follows : 40 °C initial temperature, 2 °C/min to 250 °C with helium as the carrier gas. The injector and detector temperatures were 230 and 250 °C, respectively. Injections of 1 µl were made in the splitless mode and changed to the split mode after 0.60 min. Retention indices were calculated relative to n-alkane standards. Electron impact mass spectra (ca. 100 ng sample) were obtained using a Varian Saturn II mass spectra detector using an ionizing potential of 70 eV. Coumpounds were identified by comparing their retention in GC and mass fragmentation pattern in MS with those obtained with pure standards.

Insecticidal investigation

To verify if α-terthienyl and volatile monoterpenes, which co-occur in *P. ruderale* and *P. gracile*, exert additive insecticidal effects against the European corn borer, *Ostrinia nubilalis* Hübner (Lepidoptera: Pyralidae), a simple factorial experiment was designed. The following treatments were performed: non-exposed larvae (control), larvae exposed to either volatile monoterpenes or α-terthienyl, and larvae simultaneously exposed to both chemicals.

Newly molted 3rd instar *O. nubilalis* larvae obtained from a laboratory colony maintained as described in Arnason *et al.* (1985) were used. A meridic diet was prepared and warmed to 35°C before adding α-terthienyl at a concentration of 50 µg/g via an acetone solution (0.05% v/w). An equivalent amount of acetone was added in the diet for treatments without α-terthienyl. Diets were then incubated in darkness at room temperature for 2 h to let the agar solidify and the acetone evaporate.

The exposure of insects to volatile monoterpenes was controlled by inserting a leaf of *P. ruderale* harboring 5-7 FGSCs of 2-3 mm in length inside a Petri dish in which larvae were fed. Leaves were individually inserted under a fine metallic mesh of 25 mm of diameter that was glued on the inside of Petri lids. This protocol made the leaves inaccessible to *O. nubilalis* larvae although it did not prevent the volatiles from FGSCs from reaching the larvae. For treatments with exposure to volatiles, the FGSCs were physically crushed at the beginning of the experiment. The FGSCs were gently dissected out of leaves with a razor blade and discarded in treatments with no exposure to volatiles. The leaves from each dish were replaced every day.

At the beginning of the experiment, 15 third instar larvae were weighed together then transferred in a glass Petri dish containing 5 cubes of diet, approximately 0.9 cm^3 each, with or without α-terthienyl according to the respective treatment. For each treatment, 10 replicates were performed and insects were exposed to near-UV light as described in Guillet *et al.* (1995) to stimulate the photosensitization mediated by α-terthienyl (see review in Aucoin *et al.*, 1995). The experiment lasted 48 h. The final weights of insects were determined in order to calculate the relative growth rate (g/g/day).

In a second experiment, the effects of volatile monoterpenes released from the foliar GSCs of *P. ruderale* on the retention of α-terthienyl in *O. nubilalis* larvae were investigated. The exposure of actively feeding mid-fifth instar larvae to the volatiles released from the GSCs of a leaf of *P. ruderale* was controlled as above. Larvae were then fed for 24 h in darkness on a meridic diet prepared as above and containing α-terthienyl at a concentration of 50 μg/g. Before analyzing the amount of α-terthienyl present in the larvae, the peritrophic membrane was removed to avoid contamination from the α-terthienyl present in remains of the diet. This was done by cutting the head and the 2-3 last terminal abdominal segments of each larva so that the peritrophic membrane and its content could be pulled out using fine dissection tweezers. Insect tissues without the peritrophic membrane were then dipped in 10 ml of hexane to solubilize the α-terthienyl and to quantify its concentration using high pressure liquid chromatography as previously described (Guillet *et al.*, 1997b). Ten *O. nubilalis* larvae were used per replicate and six replicates were performed for each treatment.

Results

Chemical analysis

The volatile chemicals that were collected using the head-space technique were mostly composed of monoterpenes. Sabinene, myrcene and limonene constituted 91.1% of the total chemicals emitted from the leaves of *P. ruderale* and α-pinene, sabinene and myrcene added up to 83.9% of the volatile substances collected from the leaves of *P. gracile* (Tables 1, 2).

The steam distillation of leaves provided a similar profile of monoterpenes to the results obtained with the head-space technique for *P. ruderale* except that a small amount of 2,3-dihydro-1,8-cineole, 0.2% of the extract, was also detected (Table 1). As for *P. gracile*, the same monoterpenes were identified by both sampling techniques, but the relative proportions differed. This was especially evident for sabinene and α-pinene, which represented 3.5% and 17.7%, respectively, of the total extract obtained with the head-space sampling and 20.5% and 2.3% of the extract obtained through steam distillation (Table 2). Different fatty acid derivatives, 7-tetradecene, cis-4-decenal, pentadecanal and heptadecanal, were also detected in the extracts obtained by the steam distillation of leaves, which contributed 3.9% and 5.3% of the total extracts in *P. ruderale* and *P. gracile* respectively (Tables 1, 2). Finally, a sesquiterpene, β-cubebene, was identified in the steam distillation extract of *P. gracile* (Table 2).

The chemical analysis of the liquid directly sampled from the GSCs in the leaves of *P. ruderale* revealed a similar profile of monoterpenes to the one obtained with the head-space technique except that a small amount of trans-β-ocimene (0.1%) was found (Table 1). It is worth noting that the increase in the relative amount of the fatty acid derivatives was 24.6% of the total extract in the direct sampling compared to 3.9% and 0.0% in the steam distillation and head-space techniques respectively (Table 1).

One substance, 7-tetradecene, constituted 85.6% and 90.2% of the total extracts obtained from the stems of *P. ruderale* by steam distillation and head-space samplings, respectively (Table 3). Monoterpenes were not detected in the stems of *P. ruderale* (Table 3).

Table 1: Volatile constituents present in the leaves of *Porophyllum ruderale* (Jacq.) Cass. var. *macrocephalum* (DC.) according to different extraction techniques.

Chemical constituent	Sampling technique		
	Direct[¶][§] (% of the total extract)	Steam distillation[§] (% of the total extract)	Head-space[§] (% of the total extract)
Monoterpenes			
α-pinene	n.d.	n.d.	n.d.
sabinene	1.5	1.3	2.2
2,3-dihydro-1,8-cineole	n.d.	0.2	n.d.
myrcene	0.6	0.5	0.7
limonene	71.4	73.2	88.9
trans-β-ocimene	0.1	n.d.	n.d.
terpinolene	n.d.	n.d.	n.d.
Sesquiterpene			
β-cubebene	n.d.	n.d.	n.d.
Fatty acid derivatives			
7-tetradecene	n.d.	1.0	n.d.
cis-4-decenal	n.d.	0.7	n.d.
pentadecanal	1.6	2.0	n.d.
heptadecanal	23.0	0.2	n.d.
Total	98.2	79.1	91.1

[§] The abbreviation n.d. refers to not detected.

[¶] The extract was obtained from a direct sampling of the liquid secreted from the glandular secretory cavities present on the leaves.

Table 2: Volatile constituents present in the leaves of *Porophyllum gracile* Benth.according to different extraction techniques.

Chemical constituents	Sampling technique	
	Steam distillation [¶§] (% of the total extract)	Head-space[§] (% of the total extract)
Monoterpenes		
α-pinene	2.3	17.7
sabinene	20.5	3.5
2,3-dihydro-1,8-cineole	n.d.	n.d.
myrcene	40.6	62.7
limonene	n.d.	n.d.
trans-β-ocimene	n.d.	n.d.
terpinolene	n.d.	n.d.
Sesquiterpene		
β-cubebene	9.1	n.d.
Fatty acid derivatives		
7-tetradecene	1.4	n.d.
cis-4-decenal	n.d.	n.d.
pentadecanal	2.9	n.d.
heptadecanal	1.0	n.d.
Total	77.8	83.9

[§] The abbreviation n.d. refers to not detected.

[¶] The extract was obtained from a direct sampling of the liquid secreted from the glandular secretory cavities present on the leaves.

Table 3: Volatile constituents present in the stems of *Porophyllum ruderale* (Jacq.) Cass. var. *macrocephalum* (DC.) according to different extraction techniques.

Chemical constituents	**Sampling technique**	
	Steam distillation§ (% of the total extract)	**Head-space§ (% of the total extract)**
Monoterpenes		
α-pinene	n.d.	n.d.
sabinene	n.d.	n.d.
2,3-dihydro-1,8-cineole	n.d.	n.d.
myrcene	n.d.	n.d.
limonene	8.6	n.d.
trans-β-ocimene	n.d.	n.d.
terpinolene	n.d.	n.d.
Sesquiterpene		
β-cubebene	n.d.	n.d.
Fatty acid derivatives		
7-tetradecene	77.0	90.2
cis-4-decenal	n.d.	n.d.
pentadecanal	n.d.	n.d.
heptadecanal	n.d.	n.d.
β-cubebene	n.d.	n.d.
Total	85.6	90.2

§The abbreviation n.d. refers to not detected.

Insecticidal properties

The relative growth rate of *O. nubilalis* larvae exposed to FGSC volatiles from *P. ruderale* was not significantly different (P = 0.23) from the control treatment (Fig. 1). The larval relative growth rate was, however, reduced by 17% when α-terthienyl was added in a meridic diet at a concentration of 50 μg/g. Such a reduction of the relative growth rate mediated by α-terthienyl was moreover amplified by 2.4-fold, *i.e.* 41% vs 17%, when insects were concurrently exposed to the volatiles released from the FGSCs of the leaves of *P. ruderale* (Fig. 1). A nearly two-fold increase in the retention of α-terthienyl in larval tissues of the European corn borer mediated by volatiles was observed (Fig. 2). This doubling of α-terthienyl concentration in *O. nubilalis* larvae may be sufficient to explain the important decrease in growth rate observed with α-terthienyl and volatiles treatment (Fig. 1).

Discussion

Distribution of monoterpenes in *Porophyllum gracile* and *P. ruderale*

In plants, monoterpenes occur in secretory cavities (Gershenzon, 1994). The present study also suggests that the volatile monoterpenes released from the leaves of *P. gracile* and *P. ruderale* are sequestered in the FGSCs. There was no monoterpene detected in the stems of *P. ruderale* and a similar profile of monoterpenes was observed between the extracts obtained by either the head-space or the steam distillation techniques using whole leaves, and the direct sampling of the liquid directly secreted from the GSCs (Table 1). The results obtained with *P. gracile* remain partial since it was practically impossible to directly sample the content of the FGSCs due to their small size.

Figure 1: Additive effects of the volatile monoterpenes released from the glandular secretory cavities of the leaves of *Porophyllum ruderale* (Asteraceae) and α-terthienyl on the relative growth rate of 3rd instar *Ostrinia nubilalis* larvae. Treatments associated with distinct letters differ significantly (Tukey HSD multiple comparisons, $P<0.05$).

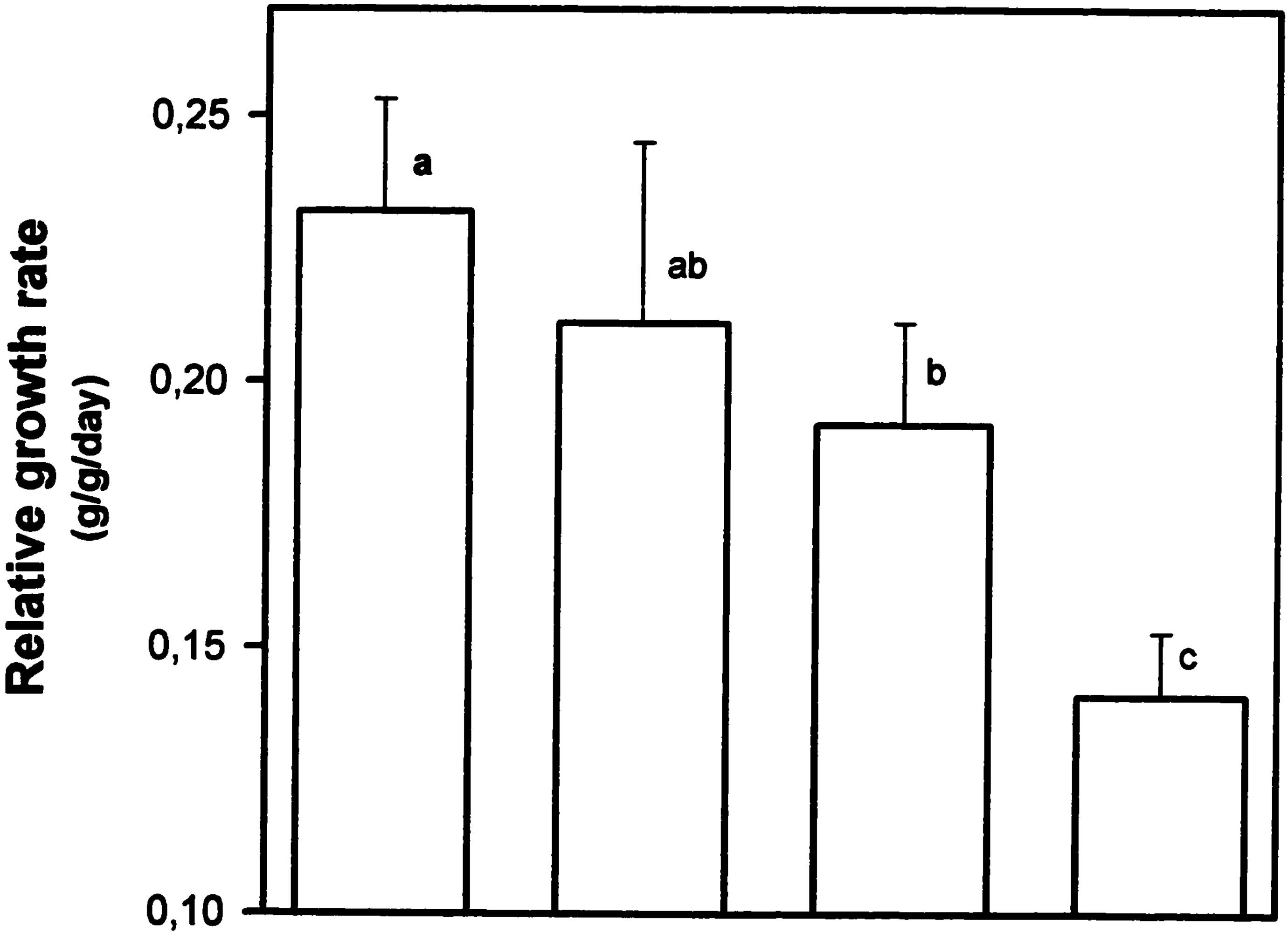
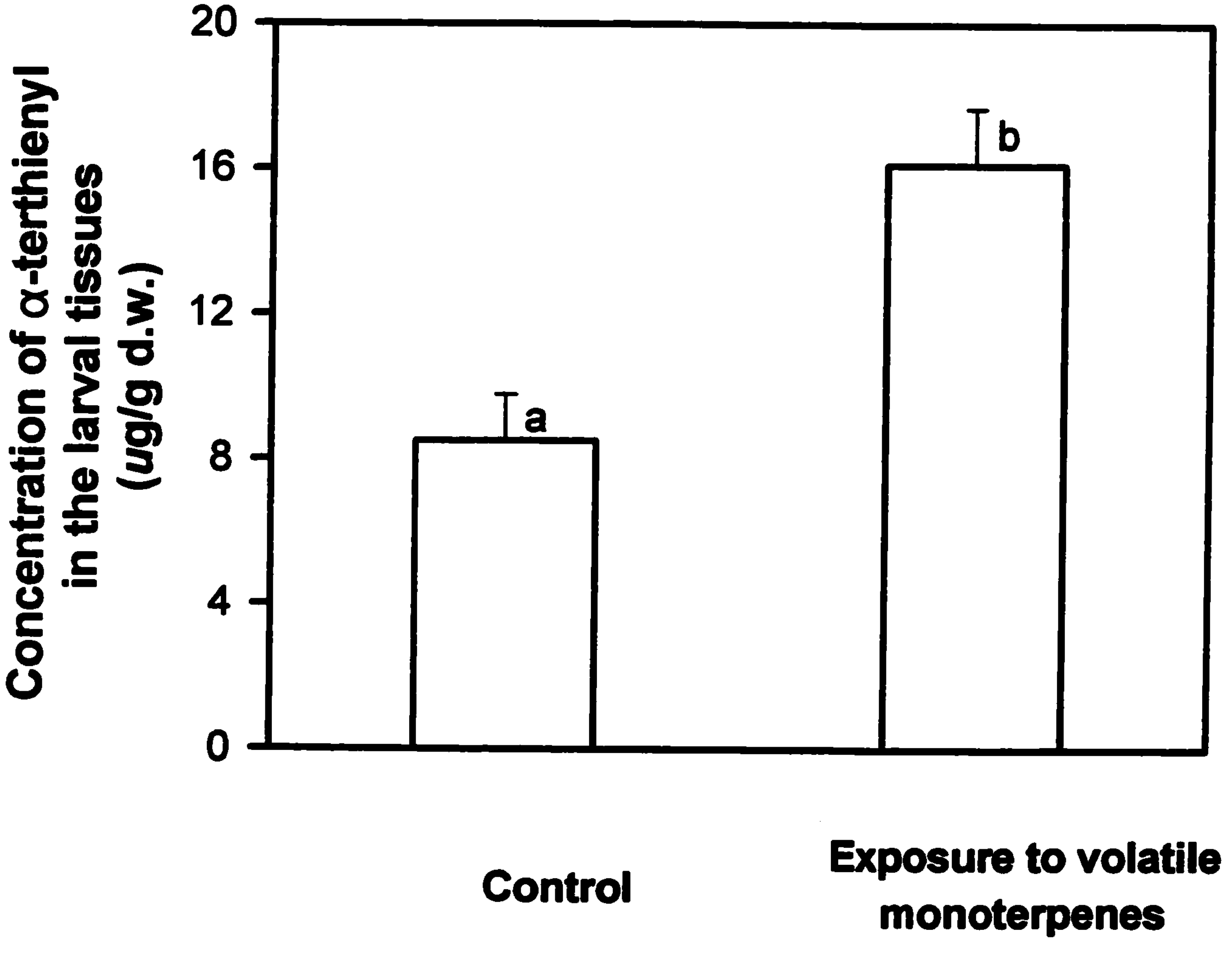

Relative growth rate
(g/g/day)
0,25
0,20
0,15
0,10
a
ab
b
c
Control
Volatiles
α-terthienyl
Volatiles and
α-terthienyl

Figure 2: Effects of the volatile monoterpenes released from the glandular secretory cavities of the leaves of *Porophyllum ruderale* (Asteraceae) on the retention of α-terthienyl in 5th instar *Ostrinia nubilalis* larvae fed with a meridic diet containing α-terthienyl. Treatments associated with distinct letters differ significantly (Tukey HSD multiple comparisons, $P<0.05$).


Concentration of α-terthienyl in the larval tissues (ug/g d.w.)
20
16
12
8
4
0
a
b
Control
Exposure to volatile monoterpenes

Insecticidal properties of the volatile monoterpenes released from the glandular secretory cavities of the leaves of *Porophyllum gracile*

It is likely that the monoterpenes, which constitute the major part of the chemicals emitted from the FGSCs (Tables 1,2), are responsible for the repellent activity previously reported for *P. gracile* and *P. ruderale* against red-legged grasshopper adults (Guillet *et al.*, 1997a). Although our results show that the volatile monoterpenes released from the GSCs of the leaves of *P. ruderale* did not reduce the relative growth rate of European corn borer larvae (Fig. 1), these monoterpenes sensitized the effects of α-terthienyl against the same insect (Fig. 1). This synergist effect of volatile monoterpenes on α-terthienyl-mediated effects on the relative growth rate of *O. nubilalis* was apparently related to an increase in the retention of α-terthienyl in the larvae (Fig. 2).

Other monoterpenes have also been shown to mediate an enhancement of the uptake of both hydrophilic and lipophilic chemicals into organic structures. Yamane *et al.* (1995a) showed that limonene and cineole enhance by 3.6- and 95-fold, respectively, the partitionning of a hydrophilic drug, 5-fluorouracil, from aqueous solutions into human stratum corneum. Ogiso *et al.* (1995) observed that cineole had a similar effect on the transdermal penetration of a lipophilic drug, indomethacin, in rat. Monti *et al.* (1995) also used different terpenes as penetration enhancers of a chemotherapeutic drug, dapiprazole, through hairless mouse skin.

The increase of the chemical uptake into organic tissues mediated by some monoterpenes appears to be related to an alteration of the lipid organization in cellular membranes or in intercellular lamellar structures (Yamane *et al.*, 1995a). In sophisticated studies involving results obtained from differential scanning calorimetry, small-angle X-ray diffraction and enhancer uptake in human stratum corneum, Cornwell *et al.* (1996) and Yamane *et al.* (1995b) have shown that some monoterpenes, including cineole and limonene, partially disrupt the lipid bilayer organisation of cellular membranes.

Acknowledgments

André Bélanger (Agriculture Canada, St-Jean-sur-Richelieu, Québec) performed the GC-MS analysis.

Chapter 9

How do sesquiterpene lactones strengthen the insecticidal activity of a phototoxin, α-terthienyl

Introduction

Glutathione (GSH), γ-glutamylcylteinylglycine, provides reducing equivalents to a set of GSH-dependent antioxidant enzymes including glutathione transferase (GST, EC 2.5.1.18) and glutathione peroxidase (GPOX, EC 1.11.1.9). GST and GPOX are involved in multiple functions including reduction of organic peroxides generated either by endogenous or xenobiotic metabolites (Iqbal *et al.*, 1996; see review in Ahmad, 1992). In insects, a single enzyme, the glutathione transferase-peroxidase (GSTPOX), mediates both GST and GPOX activities (Ahmad & Pardini, 1988).

The requirement of GSH to prevent an excessive accumulation of lipid peroxides was confirmed by experiments in which a partial depletion of GSH levels, which were induced via an addition of specific inhibitors of the GSH biosynthesis, resulted in an increase of lipid peroxides in rats (Torres *et al.*, 1989, 1991) and a herbivorous insect, *Manduca sexta* (Lepidoptera: Sphingidae) (Aucoin *et al.*, 1995). Alptekin *et al.* (1996) also showed that the increase in lipid peroxides observed in water-immersed rats was accompanied by a decrease in GSH content that was unrelated to a depression of GSH synthesis and/or an increase of GSH breakdown. These results agree with the hypothesis that GSH is consumed as reducing equivalents by GSH-depending antioxidant enzymes when oxidative stresses are induced.

The sulfhydryl group of the cysteine residue present in GSH makes this molecule particularly susceptible to nucleophilic attacks. The unsaturated double bonds in α-methylene-γ-lactone and cyclopentenone moieties that are present in some sesquiterpene lactones (SLs) are susceptible to form a conjugate with GSH following a Michael addition reaction (Lee *et al.*, 1977). A transient reduction in total GSH level in *M. sexta* larvae injected with helenalin, a SL with an α-methylene-γ-lactone and a cyclopentenone functions, corroborates *in vivo* formation of conjugates between an electrophilic group of a SL and GSH (Aucoin *et al.*, 1995). Another example is repin, a SL occurring in *Centaurea repens* (Asteraceae), that exerts cytotoxic effects to mouse astrocytes (Robles *et al.*, 1997). These toxic effects mediated by repin were accompanied with a partial depletion of GSH content and a rise in levels of both reactive oxygen species and oxidative damages to cellular membranes. These results strongly suggest that a diversion of the antioxidant GSH pool brought about by SLs may result in an exacerbated oxidative stress.

In the present study, the synergist insecticidal effects between a plant pro-oxidant compound, α-terthienyl (α-T), and different SLs with or without an α-methylene-γ-lactone and/or a cyclopentenone moieties were investigated. α-T is a phototoxin known for its ability to generate singlet oxygen ($^1O_2$) and to increase lipid peroxidation in the midgut of *M. sexta* larvae (Aucoin *et al.*, 1995). Given the requirement of GSH in enzymatic processes involved for the reduction of lipid peroxides (Ahmad, 1992), it was hypothesized that a depletion of GSH in insect larvae due to the formation of GSH-SL conjugates will exacerbate the lipid peroxidation induced by α-T, and thus the insecticidal activity of this natural phototoxin.

Materials and methods

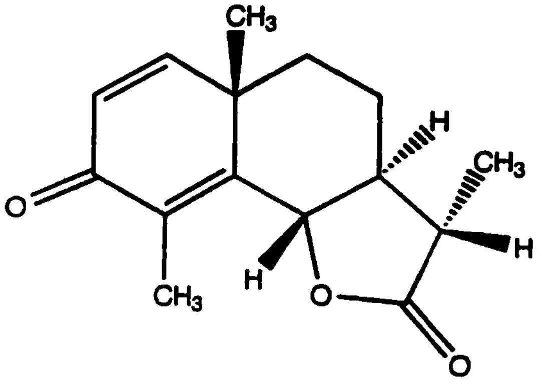
Feeding trials to determine the effects of sesquiterpene lactones on the total glutathione content in *Manduca sexta* larvae

The *in vivo* effects associated with ingestion of SLs on the total GSH levels in *M. sexta* larvae were determined. Newly molted third instar larvae obtained from a laboratory colony maintained as in Stewart & Philogène (1983) were fed for 48 h in plastic Petri dishes with a diet containing a concentration of 100 µg/g f.w. of one of the SLs illustrated in Fig. 1. Purity of each SL was verified by HPLC. The SLs were dissolved in acetone then added (10 µl of acetone/g of diet) and mixed into a warm meridic diet (35 °C). Diet of the control treatment received an equal amount of pure acetone. The experiment was performed in darkness and 20 larvae, which were placed in a 10 cm plastic Petri dish and offered with seven diet cubes of approximately 1 cm^3 each, were used for each replicate and 4-5 replicates were performed per treatment. At the end of the experiment, insects were removed and immediately analyzed for the total GSH content.

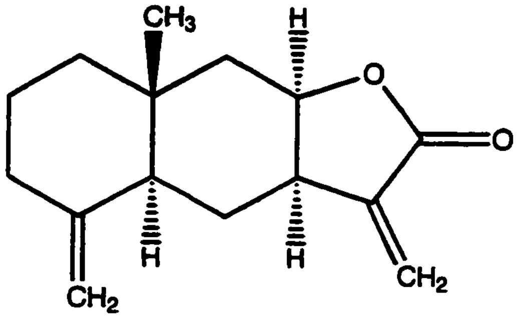
Measurement of the total glutathione content in *Manduca sexta* larvae

Total GSH levels were determined according to Griffith (1980). The 20 larvae of each sample, that were fed as described above, were used to determine the total GSH levels. Larvae were weighed then homogenized in 10 ml of cold phosphate buffer (100 mM , pH 7.4, 6.3 mM EDTA) containing 5% 5-sulfosalicylic acid using a Polytron. Preliminary observations showed that midgut remains and the peritrophic membrane did not significantly affect the level of total GSH in third instar *M. sexta* larvae so that whole insects were used for GSH measurements. An aliquot of 1 ml of the homogenate was transferred in a 1.5 ml Eppendorf tube and

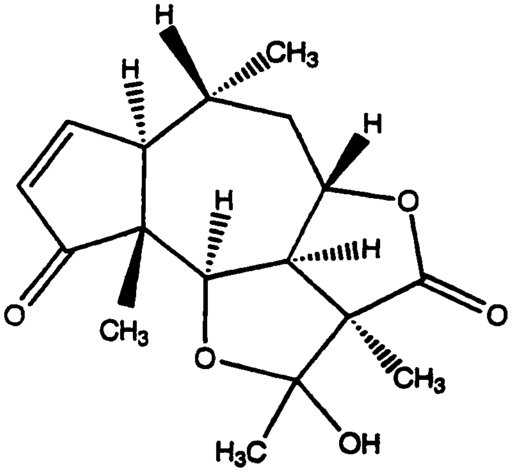
Figure 1. Molecular structure of the sesquiterpene lactones investigated in the present study.



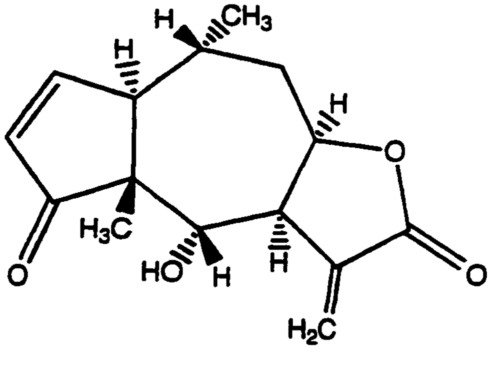
α–Santonin



Isohelenin



Tenulin



Helenalin

centrifuged at 16 000 *g* for 15 min.

Total GSH was determined using an enzymatic assay involving pure glutathione reductase (EC 1.6.4.2; Type III, bakers yeast, Sigma). A working buffer was prepared by adding 0.2 mM NADPH to the phosphate buffer. Two to 5 µl of the supernatant were combined in a quartz cuvette to 0.85 ml of working buffer and 0.1 ml of a 6 mM 5,5'-dithiobis(2-nitrobenzoic acid) phosphate buffer solution. The reaction mixture was mixed in a cuvette and equilibrated for 1 min at 25 °C. Fifty µl of glutathione reductase (20 units/ml) in phosphate buffer were then added to the reaction mixture which was mixed before to monitor the absorbance at 412 nm for 2 min (Beckman, model DU 640). Phosphate buffer solutions containing different concentrations of GSH, 0, 0.5, 1, 2, 4, 8, 16, 32 and 64 µg/ml, were analyzed as for experimental samples to establish a standard curve between mean slopes for the 412 nm absorbance signal and the concentrations of total GSH. The concentration of total GSH was expressed in µg GSH per g of insect f.w.

Feeding trials to determine the effects of sesquiterpene lactones on formation of lipid peroxides and mortality in *Manduca sexta* larvae fed with α-terthienyl

Newly molted third instar *M. sexta* larvae were fed as above except that α-terthienyl was co-added at a concentration of 20 µg/g f.w. with SLs in diets. Two independent sets of larvae were prepared involving each 20 larvae per replicate and five replicates for each of the five treatments as above. The first set of insects was kept for lipid peroxidation measurement and the other was reserved for larval mortality determination.

After feeding in darkness for 48 h as described above, larvae of the first set were exposed under a photosensitizing near-UV light regime provided by blacklight bulbs (Westinghouse F20T12/BLB, 1.0 mW/cm^2, 300-400 nm) for three h. This near-UV light exposure was performed to initiate the photosensitization oxidative stress mediated in larvae by the ingested α-T and that results in formation of lipid peroxides (Aucoin *et al.*, 1995). After this near-UV light exposure, dead larvae, which were discarded before measuring lipid peroxides, represented less than 10% in each individual replicate.

In the second set of insects reserved to determine the mortality rate, larvae were fed for 48 h in darkness then kept under near-UV photosensitizing light as above for 24 h to optimize photosensitization of α-T. Insect mortality was then determined for each replicate.

Measurement of lipid peroxides

The procedure of Hermes-Lima *et al.* (1995) was used to estimate the lipid peroxide concentrations. The principle of this assay is based on the formation of a complex between malondialdehyde (MDA), the oxidative degradation of lipid peroxides yielding to production of MDA, and thiobarbituric acid (TBA) (Bird & Draper, 1984). This MDA-TBA complex can be quantified according to its peak absorbance at 532-535 nm. TBA was dissolved in a solution containing 50 mM NaOH and 10 mM butylated hydroxytoluene (BHT). For each analysis, 20 third instar *M. sexta* larvae were pooled, weighed and homogenized in 4 ml of 1.1 % phosphoric acid using a Polytron apparatus. From the homogenate, 0.4 ml were transferred in each of two 15-ml glass tubes. One tube served as a control and contained 0.2 ml of 7% phosphoric acid and 0.4 ml of 3 mM HCL whereas 0.2 ml of 7% phosphoric acid and 0.4 ml of TBA solution had been added to the second sample tube. A styrofoam lid was put on each tube before boiling them for 20 min.

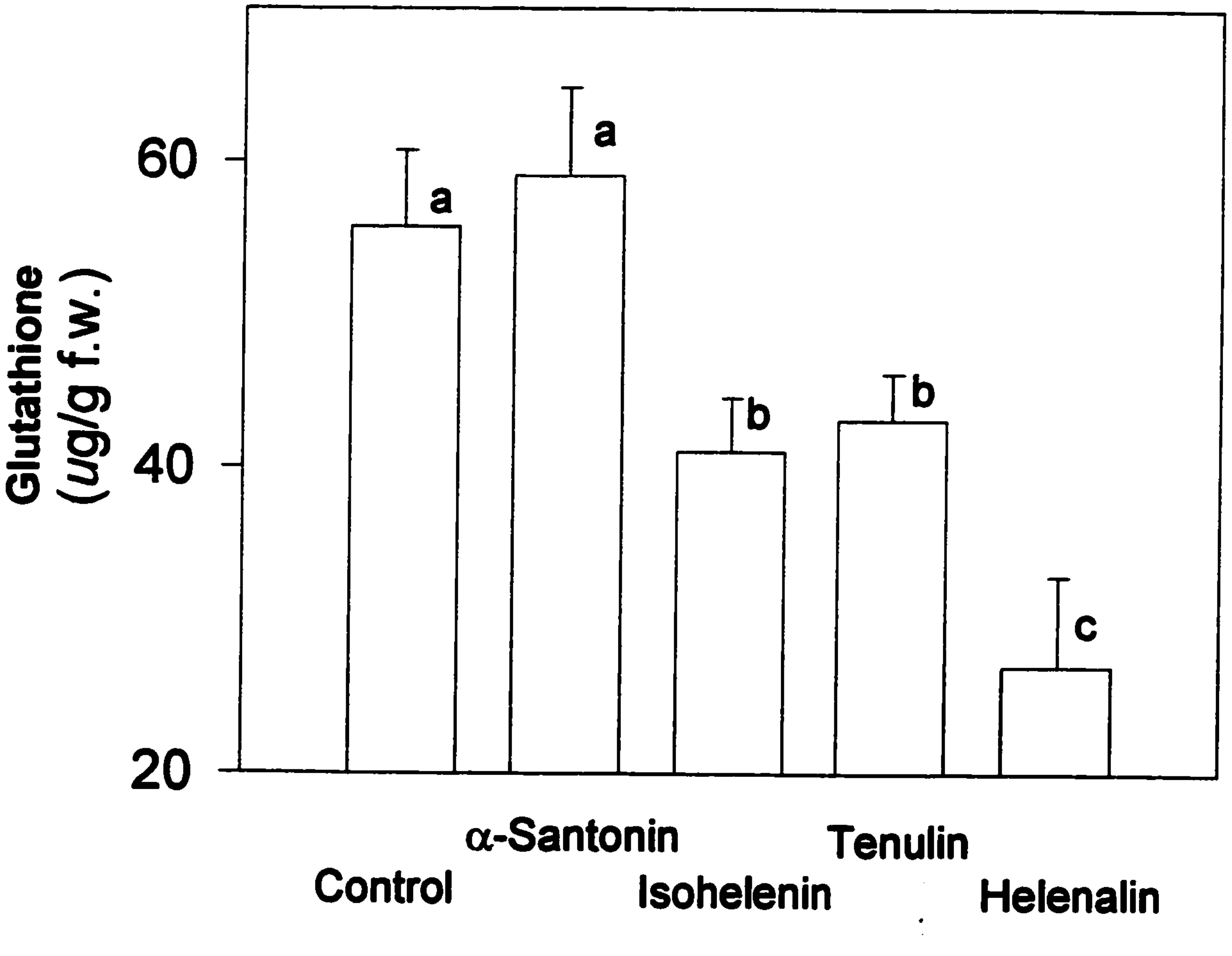
After cooling the tubes at room temperature, 1.5 ml butanol were added to each of these tubes. The glass tubes were vortexed for 15 sec and left on a bench for 2 h to resolve the two phases. The organic layer was then transferred in 1.5 ml Eppendorf tubes and centrifuged at 16 000 *g* for 5 min. The butanol supernatant was transferred into 1 ml quartz cuvette to determine the absorbance at both 532 nm and 600 nm (Beckman, model DU 640). The net absorbance of thiobarbituric acid reactive substances (TBARS) was determined as the difference between readings at 532 nm and 600 nm to remove background signals. The final concentration of TBARS was derived by substracting the net TBARS absorbance of the control tubes from the corresponding sample tubes and by considering a molar absorption coefficient for TBARS of 156 000 $M^{-1}cm^{-1}$. Lipid peroxides were expressed in nmole of TBARS per g of insect. Four to five replicates were performed for each of the five treatments, that is control and each of the four SLs tested (Fig. 1).

Results

Effects of sesquiterpene lactones on the total glutathione levels

All SLs except α-santonin significantly reduced the total GSH levels in third instar *M. sexta* larvae compared to the control treatment (Fig. 2). Isohelenin and tenulin exerted a similar effect by reducing by approximately 25% the total GSH level compared to either the control or the α-santonin treatments. Helenalin, however, exerted a more pronounced effect with a nearly 50% reduction in total GSH compared to control (Fig. 2).

Figure 2. *In vivo* effects of ingested sesquiterpene lactones on the total glutathione levels in third instar *Manduca sexta* larvae. The error bars represent the standard deviation and treatments with distinct letters differ significantly (Tukey, $P<0.05$).


Glutathione
(*u*g/g f.w.)
60
40
20
a
a
b
b
c
Control
α-Santonin
Isohelenin
Tenulin
Helenalin

Effects of sesquiterpene lactones on the lipid peroxidation stress induced by α-terthienyl

A two-fold variation was observed in concentrations of TBARS in *M. sexta* larvae fed with a diet containing α-T alone or α-T and one of the four SLs illustrated in Fig. 1. The lowest concentration of TBARS was obtained for the treatment with α-santonin while treatment with helenalin induced the highest level, that is a nearly two-fold higher value compared to α-santonin or control treatments (Fig. 3). Concentrations of TBARS for isohelenin and tenulin treatments were intermediate compared to those values obtained for α-santonin and isohelenin (Fig. 3).

Effects of sesquiterpene lactones on insecticidal properties of α-terthienyl

No mortality was induced in third instar *M. sexta* larvae when all four SLs (Fig. 1) were individually added at a concentration of 100 μg/g in diets lacking α-T (data not shown). However, the co-addition of some SLs with α-T in diets enhanced by up to two-fold the mortality of third instar *M. sexta* larvae compared to control treatment that contained only α-T (Fig. 4). Helenalin was particularly efficient in increasing the α-T induced mortality since its co-addition with α-T in diets resulted in a 85% higher mortality than α-T alone (Fig. 4). The effects of isohelenin and telunin were similar with an approximate increase of mortality of 50% (Fig. 4). α-santonin was the only SL tested to not significantly affect the α-T induced mortality in third instar *M. sexta* larvae.

Discussion

Michael addition occurring between an oxidized group conjugated to a

Figure 3. Lipid peroxidation in third instar *Manduca sexta* larvae fed with a meridic diet containing α-terthienyl (20 µg/g diet) with or without a sesquiterpene lactone (100 µg/g diet). The error bars represent the standard deviation and treatments with distinct letters differ significantly (Tukey, $P<0.05$).

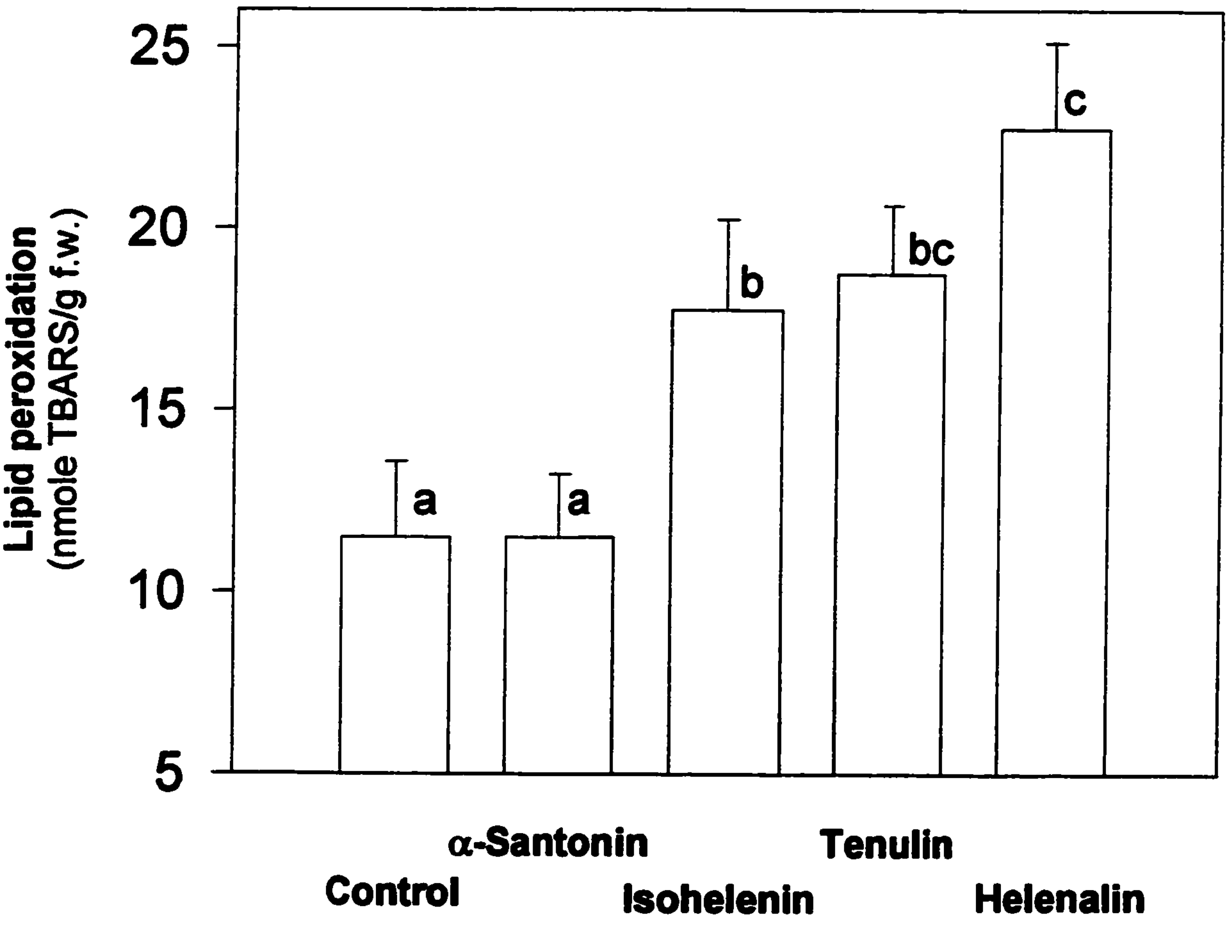
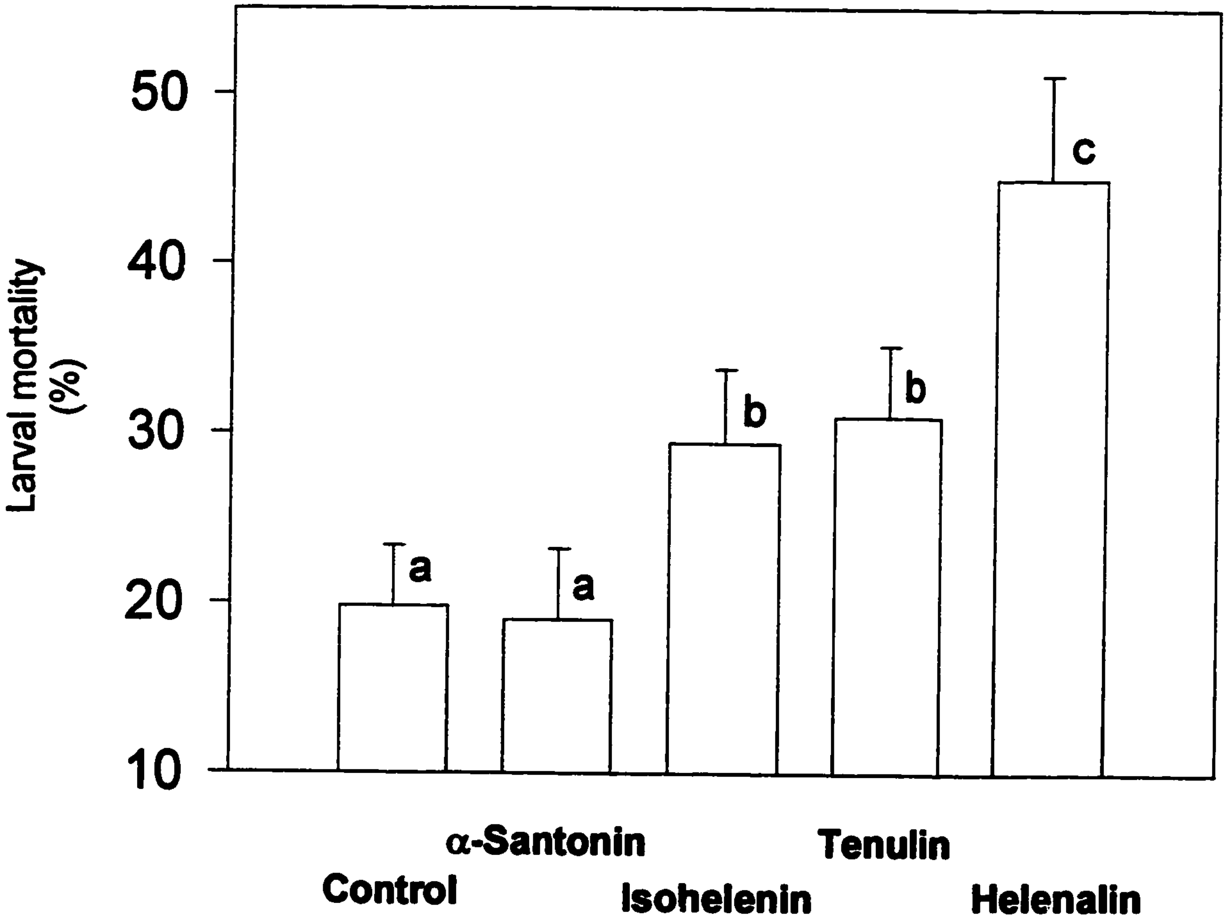

Lipid peroxidation
(nmole TBARS/g f.w.)
25
20
15
10
5
a
a
b
bc
c
Control
α-Santonin
Isohelenin
Tenulin
Helenalin

Figure 4. Mortality of third instar *Manduca sexta* larvae fed with a meridic diet containing α-terthienyl (20 μg/g diet) with or without a sesquiterpene lactone (100 μg/g diet). The error bars represent the standard deviation and treatments with distinct letters differ significantly (Tukey, $P<0.05$).


50
40
30
20
10
Larval mortality (%)
a
a
b
b
c
α-Santonin
Tenulin
Control
Isohelenin
Helenalin

double bond and a sulfhydryl nucleophilic moiety may explain the toxicity of SLs (see reviews in Mabry & Gill, 1979, and Nawrot & Harmatha, 1994). Amino residues of proteins are particularly susceptible to formation of such SL conjugates that may inhibit enzymatic activities as it was reported in *in vitro* studies for GST (Zheng *et al.*, 1996). Huacuja *et al.* (1993) also reported that binding of SLs to sulfhydryl groups present on human sperm surface reduces sperm motility and sperm nuclear decondensation up to 85% and 81%, respectively.

The present study confirmed that total GSH levels may be reduced in *M. sexta* larvae when SLs are added into diets. The results also corroborated previous studies which suggested that the *in vivo* depletion of GSH induced by SLs is correlated with the presence of electrophilic centers, that is the α-methylene-γ-lactone and the cyclopentenone moieties (Lee *et al.*, 1977; Picman *et al.*, 1979; see review in Macías *et al.*, 1992 and Ketterer *et al.*, 1983). Only the SL lacking both electrophilic centers, α-santonin, did not reduce the total GSH content. On the other hand, helenalin, which was the only SL to possess both α-methylene-γ-lactone and cyclopentenone electrophilic centers, induced the most important reduction of total GSH in *M. sexta* larvae compared to other SLs tested (Fig. 2).

Results of the present study also provided information regarding the insecticidal mode of action of α-T and the way this toxicity is enhanced by SLs. The significant positive correlation between lipid peroxide levels and mortality in third instar *M. sexta* larvae (Fig. 5) corroborates that oxidative damages induced by α-T (Scaiano *et al.*, 1989; MacRae *et al.*, 1980; McRae *et al.*, 1985) are responsible for the remarkable insecticidal activity of this phototoxin (see reviews in Arnason *et al.*, 1987, 1989, 1995). But since the lipid peroxidation was also negatively correlated with the total GSH contents (Fig. 6), it is concluded that synergist

Figure 5. Relationship between lipid peroxidation and larval mortality in third instar *Manduca sexta* larvae fed with a meridic diet containing α-terthienyl (20 μg/g diet) with different sesquiterpene lactones ($r^2 = 0.910$, $P = 0.042$). Each point represents a different sesquiterpene lactone and its value was obtained from the mean of 4-5 replicates for both axis. The error bars refer to ± 1 standard deviation.

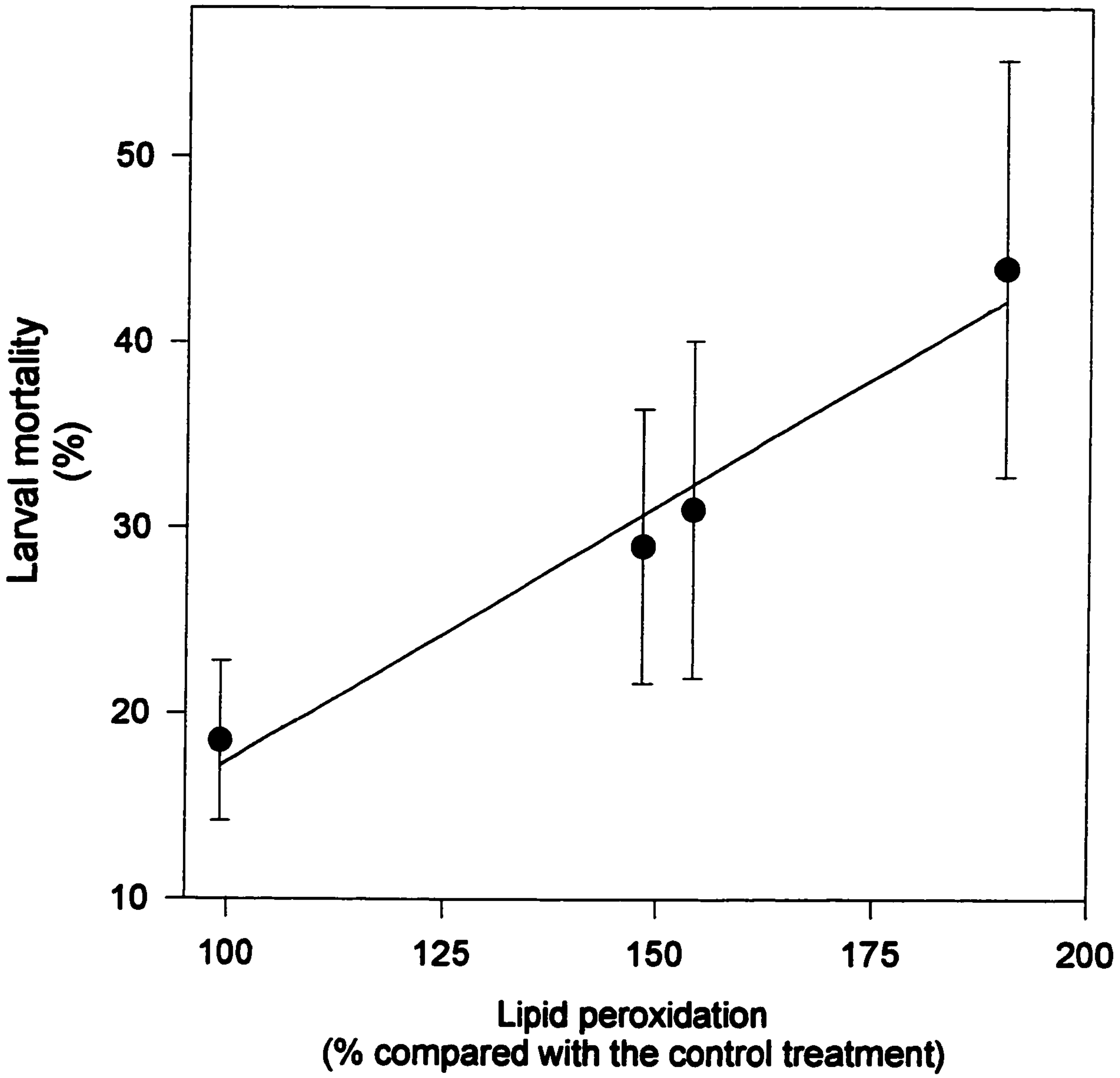
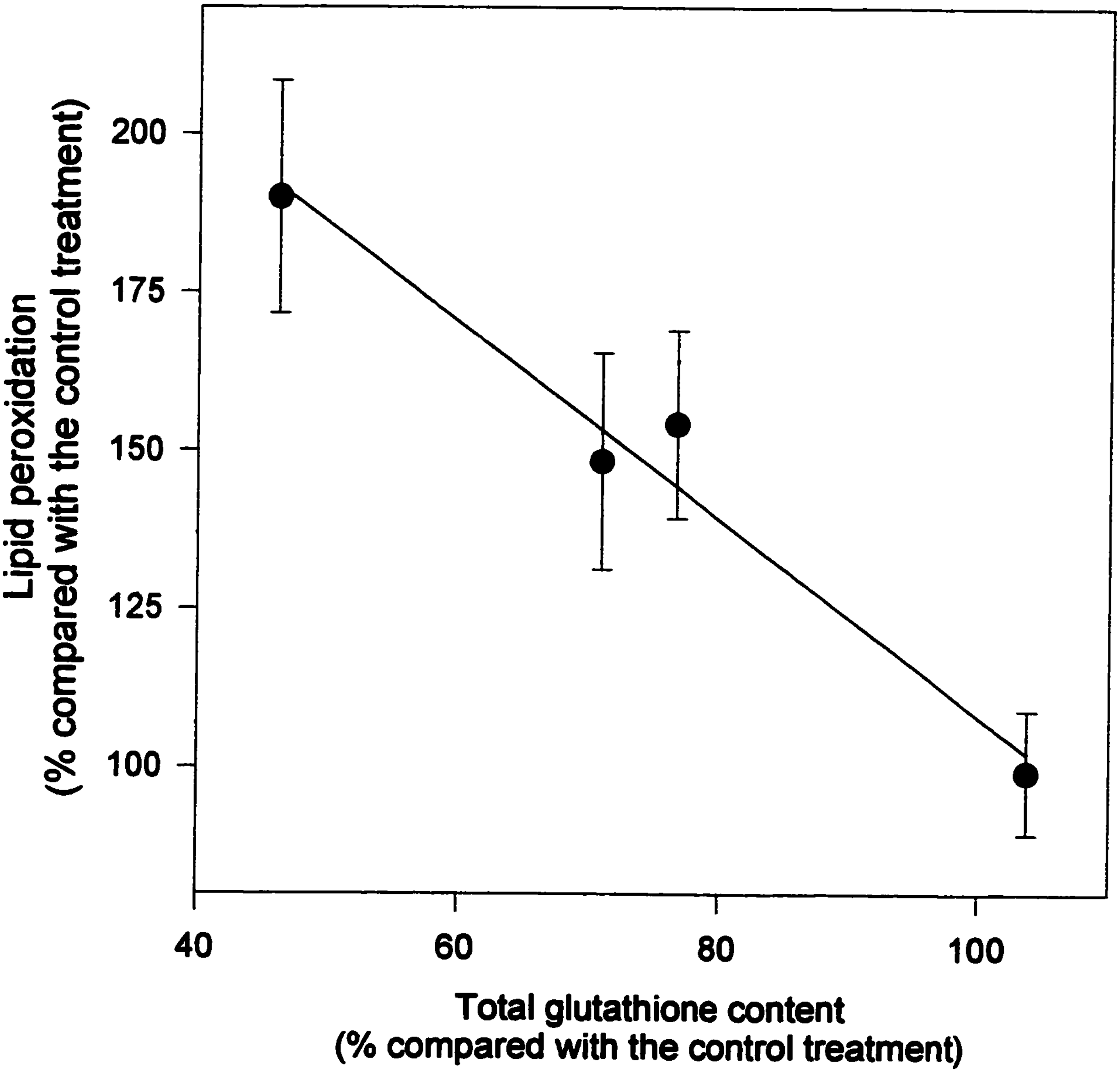

50
40
30
20
10
Larval mortality (%)
100
125
150
175
200
Lipid peroxidation (% compared with the control treatment)

Figure 6. Relationship between lipid peroxides and total glutathione levels in third instar *Manduca sexta* larvae fed with a meridic diet containing α-terthienyl (20 μg/g diet) with different sesquiterpene lactones ($r^2 = 0.952$, $P = 0.016$). Each point represents a different sesquiterpene lactone and its value was obtained from the mean of 4-5 replicates for both axis. The error bars refer to ± 1 standard deviation.


Lipid peroxidation
(% compared with the control treatment)
200
175
150
125
100
40
60
80
100
Total glutathione content
(% compared with the control treatment)

insecticidal effects between SLs and α-T are mediated via a depletion of the total GSH content. These results may be explained by an impairment of GSH-dependent enzymes, such as GSTPOX and glutathione reductase (GR) that are important in reducing organic peroxides (see review in Ahmad, 1992), under conditions of low GSH levels. Previous results (chapter 4) showed the importance of these two enzymes, GSTPOX and GR, to larvae of a leaf beetle, *Zygogramma continua* (Coleoptera: Chrysomelidae), that was observed to feed innocuously on leaves of *Viguiera annua* (Asteraceae) that contain high concentrations of phototoxins biosynthetically related to α-T.

The synergist effects of SLs on the insecticidal properties of PTs may be ecologically relevant in nature given the ubiquitous distribution of both classes of compounds in the Asteraceae (Seaman, 1982; Bohlman et al., 1977). The concentrations used in the present study were representative of those occurring in plants. Vasquez *et al.* (1990) reported the occurrence of different SLs in *Rudbeckia* spp. at concentrations generally over 10 mg/g d.w.of total SLs. On the other hand, the concentration of α-T used in the present protocol, that is 20 μg/g diet, is lower than levels of phototoxins in Asteraceae (see Table 1 in chapter 2).

Acknowledgments

J. Harmatha, Institute of Organic Chemistry and Biochemistry, Prague The Czech Republic, provided the sesquiterpene lactones.

Chapter 10

General discussion

Some results of the present study are in contradiction with the assumptions and predictions of the classical coevolutionary model proposed by Ehrlich and Raven (1964, see chapter 1). In an attempt to integrate the different results in a more consistent framework, I here present a new evolutionary hypothesis called the plant integrated chemical defenses (PICDs). The PICD hypothesis is discussed in relation to the ecophysiological constraints acting on plants relying on phototoxic defenses. It is argued that phototoxic plants may compensate for ecophysiological constraints acting on them by making use of synergist chemical defense involving phototoxins (PTs) and other classes of secondary chemicals (SCs).

Summary of the results of the present study

Despite the noticeable insecticidal properties of PTs reported under laboratory conditions (see chapter 1), the field investigation reported in chapter 2 showed that plants producing PTs do not benefit from a lower herbivore pressure. The total herbivory damage in leaves and inflorescences was found to be unrelated to the phototoxic activity of hostplant tissues, although the specific pattern in which the tissues were removed by herbivores was affected by phototoxicity.

An explanation for why herbivore pressure was not much reduced in phototoxic plants is the different behavioral and biochemical adaptations noticed in herbivorous insects (chapters 3, 4, 6). Even the generalist insects, which are commonly assumed to be sensitive to "qualitative" toxins present in plants, were shown to possess adaptive behavioral and biochemical features protecting them from phototoxicity (chapter 3).

Another significant contribution of the present study was to emphasize that plant defenses against herbivorous insects rely on the interrelated insecticidal properties of different classes of SCs. The resulting diffuse chemical protection conferred by such a mixture of active SCs was shown to involve a diversification in the insecticidal modes of action (chapter 7) and synergist interactions between the different components (chapters 8, 9). Results obtained with *Carthamus tinctorius* (Asteraceae) indicated that different SCs provide a wider protection against herbivores because of the relative susceptibility of different insects to the different classes of SCs found in this hostplant. For example, the fitness of *Melanoplus femurrubrum femurrubrum* adults was positively correlated with both the constitutive and inducible concentrations of phototoxins but negatively correlated with the content of soluble phenolics in *Carthamus tinctorius* seedlings whereas the reproductive output of *Myzus persicae* was negatively correlated with these two phytochemical traits (chapter 5). It was furthermore found that variation in the constitutive or inducible levels of PTs and the level of soluble phenolics in *C. tinctorius* seedlings did not affect the relative growth rate of *Ostrinia nubilalis* larvae (chapter 5).

The present study thus contradicts the two following assumptions of the chemical arms race evolutionary scheme (Table 1):

1. Only specialist insects can feed on hostplants containing "qualitative" chemical defenses because they possess the specific co-evolved adaptations allowing them to feed ingest inocuously the tissues.
2. Plants have responded to the aggressions exerted by different major pests, *i.e.*

the specialists, by diversifying independent lines of phytochemicals (Fig. 1).

To reconcile the results obtained in a more consistent evolutionary scheme, I describe in the following section an alternative evolutionary hypothesis, the plant integrated chemical defenses (PICDs). The particular benefit to phototoxic plants relying on a PICD strategy will then be discussed on a subsequent section.

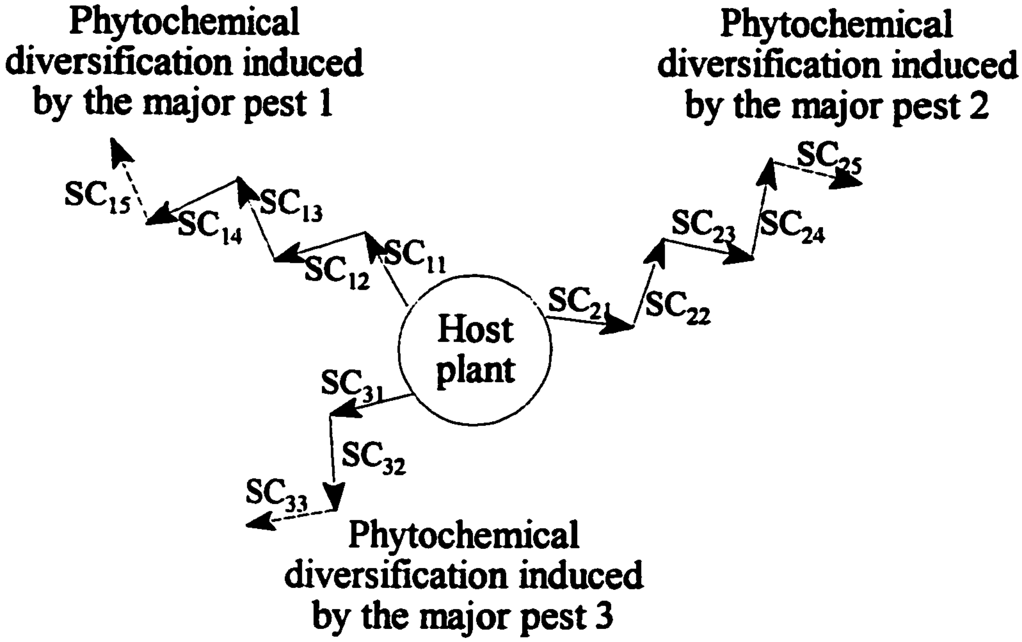
Plant integrated chemical defenses

Definition

Many results of the present study indicate that the toxic effect of a particular SC may be increased by the presence of other SCs. Synergist insecticidal activities, especially those between PTs and monoterpenes or PTs and sesquiterpene lactones (Chapters 8, 9), are likely to co-occur widely in nature because of the ubiquitous distribution of these SCs in the Asteraceae (Bohlmann *et al.*, 1973; Harborne, 1991; Gershenzon & Croteau, 1992; Gershenzon, 1994; Bernays & Chapman, 1994; Seaman, 1982). I use this argument of the probable ecological importance of synergist interactions between different SCs in the Asteraceae to justify an evolutionary view of plant-insect relationships in which synergism is emphasized. I designate this hypothesis as the PICD.

By integrated chemical defenses, I refer to a protection that is mediated via the synergist toxic effects of a mixture of active compounds synthesized by a given plant. According to the definition of synergism used here, a mixture of active compounds exerts a higher toxicity than the summation of the individual toxic effects generated by each of the components of a mixture. The term "integrated" is

Figure 1. Coevolutionary arms race in ecological biotic relationships: divergent independent lines of reciprocal genomic interactions between a plant and its major pests. The circle represents a hostplant that is attacked by three major pests as indicated by the three independent lines of diversification. Each arrow represents an advancement in a new radiative zone that is subsequent to the production of new secondary compounds, SC_{xy}, where y is for the nth derivative and x is for a particular class of secondary compound. The outcome of a particular line of reciprocal interaction is independent of the others.


Phytochemical diversification induced by the major pest 1
Phytochemical diversification induced by the major pest 2
Phytochemical diversification induced by the major pest 3
Host plant
SC_{11}
SC_{12}
SC_{13}
SC_{14}
SC_{15}
SC_{21}
SC_{22}
SC_{23}
SC_{24}
SC_{25}
SC_{31}
SC_{32}
SC_{33}

used to emphasize the appreciation in plant chemical protection that is conferred via the synergist defenses.

The establishment of the PICD, which implies the preferential evolutionary retention and production of those SCs exerting synergist toxic effects, is possible only if a diversification of SCs in a given plant has previously occurred. This preliminary diversification of SCs could be mediated via the classical reciprocal coevolutionary interactions between a hostplant and its major pests as predicted by the chemical arms race model (Fig. 1; Berenbaum, 1996). The PICD is thus not an exclusive evolutionary hypothesis since it is partially compatible and dependent on other evolutionary processes. It is the originality of the PICD to provide both a functional explanation for the diversity of SCs in plants (Romeo *et al.*, 1996) and a reconciliation between different evolutionary models.

The integration step, which would be initiated only when a sufficient phytochemical diversity has been generated in a plant, would involve an evolutionary "running-in" of the plant genome in order to obtain an optimal expression of the genes coding for the SCs harboring synergist toxic effects. In other words, the PICD strategy would involve a genotypic "effort" to integrate and optimize the production of SCs to make it as profitable as possible to hostplants. Although the "gene mechanics" purportedly involved in such fine tuning processes remain an elusive notion, similar concepts have already been proposed. For example, it is accepted that oviposition preference by insect females on specific hostplants relies on a dynamic fine tuning of coadapted gene complexes (Charlesworth *et al.*, 1987; Thompson, 1994). Jarvis and Miller (1996) recently presented experimental evidence for "... self-organizing driving forces operating ..." on genomes of plants to optimize their chemical defenses against fungi and insects.

The review provided by these researchers focuses on the importance for plants to cluster the different genes involved in a given defensive pathway to optimize its general efficiency.

In summary, the PICD strategy involves two successive and possibly overlapping sequences: (1) a diversification of phytochemicals occurring in plants and (2) a functional integration for a preferential biosynthesis of few SCs harboring synergist properties.

Plant integrated chemical defenses: comparisons with the chemical arms race model

The chemical arms race hypothesis indirectly predicts that the number of major pests is related with the number of classes of SCs occurring in a plant. In other words, "... the selection pressures exerted by any particular herbivore on a plant population is independent of the presence or absence of other herbivore species" (Hougen-Eitzman & Rausher, 1994) (Fig. 1). It is easily conceivable, due to the ecophysiological constraints limiting the ability of a plant to produce SCs (chapters 5 and 6; see review by Zangerl and Bazzaz, 1992), that such independent reciprocal coevolutionary processes can not occur widely in nature especially for plants that are attacked by a diversified range of pest organisms (Fox, 1981). It is unlikely, however, that corn, *Zea mays*, which may be severely damaged by about 90 different phytophagous insects (Dicke & Guthrie, 1988), could maintain an equivalent number of diverse metabolic pathways to produce as many independent classes of SCs. Costs related to biosynthesis of SCs may then "put the brakes" on reciprocal coevolutionary processes.

The PICD hypothesis specifically takes into consideration these trade-offs related to the costs required for the production of SCs. The integration step of the PICD strategy is indeed assumed to be driven by the excessive costs required for biosynthesis of SCs. The PICD strategy can thus be seen as the ultimate outcome to dead end reciprocal coevolutionary interactions that have become too expensive for hostplants to maintain any longer given the increasing diversity of SCs that requires more and more energy for their synthesis.

The benefits of a plant relying on a PICD strategy would not only relate to lower costs associated with the production of synergist SCs (see discussion in Berenbaum & Zangerl, 1996), but also to delay the onset of insect resistance (Isman *et al.*, 1996; Berenbaum, 1985). It is well established in agriculture that "... the use of mixtures is always more effective in delaying the onset of resistance, often by many orders of magnitude" (Mani, 1985). The benefit of relying on an active mixture of insecticidal compounds, as is the case for a PICD, to delay insect resistance was clearly demonstrated by Feng and Isman (1995). These researchers observed a 9-fold resistance to pure azadirachtin in the green peach aphid, *Myzus persicae*, over 40 generations, although no resistance was noticed in the colony treated with a seed extract of *Azadirachta indica* (Meliaceae) containing a complex mixture of SCs including azadirachtin. If, as Darwin argued, natural and artificial selection are really analogous processes (Darwin, 1859), the above results would corroborate the benefits of a PICD strategy to delay the appearance of insect resistance.

The above considerations regarding the major differences between the chemical arms race model and the PICD hypothesis are summarized in Table 1. It is suggested that these distinctions between the chemical arms race and the PICD hypothesis may simply originate from different "pictures" taken at different

Table 1. Differences between the chemical arms race and the integrated chemical defenses hypotheses.

	Chemical arms race hypothesis	Integrated chemical defenses
Aspects related to herbivore pressure		
Major pest insects	Few specialist insects	A diversified suite of specialist and generalist insects
Selective pressure exerted on herbivorous insects	Hard because of an oligogenic resistance	Soft because of a polygenic resistance
Delay for insects to evolve adaptations	Low	High
Aspects related to phytochemical diversity		
Evolutionary effects of herbivore pressure	Independent diversification of few classes of secondary chemicals	Interrelated diversification of different classes of secondary chemicals
Costs to production of chemical defenses	High because each major specialist pest involves an independent defensive strategy	Low because of synergist interactions

evolutionary steps of a common pattern of plant-insect interactions. The chemical arms race processes would constitute the initial responses of hostplants to their major pests and thus provide the suitable phytochemical diversity that is required for PICD to eventually take place.

Plant integrated chemical defenses: comparisons with the diffuse resistance model

There are two fundamental distinctions between the PICD and the diffuse coevolution model as proposed by Fox (1981). First, Fox assumed that only apparent plants, those that are easier to be found by herbivores like the perennials, must rely on a diffuse chemical resistance mediated by a mixture of "digestibility-reducing defenses" because of the multiplicity of interacting pests that are likely to take place. Unapparent plants, those that occur in a heterogeneous spatio-temporal pattern and that are difficult for herbivores to locate, are the only plants that may evolve accordingly to a stepwise coevolutionary process given the limited herbivores occurring on these plants (Fox, 1981). The PICD hypothesis, however, assumes any restriction either for the mode of action or the kind of hostplant relying on a diffuse, or generalized, chemical defense. The observations of the present study were indeed performed on unapparent plants – asteraceous species are opportunist often annual plants occupying disturbed areas such as roadsides or abandoned fields – *i.e.*, those that should not count on a diffuse chemical defense according to Fox (1981). Second, in Fox's model, there is no reference to any synergist chemical defense, although it is implicit to the PICD hypothesis. Despite these two differences, both the diffuse model and the PICD hypothesis fundamentally emphasize that "... coevolution must be considered in a community context and not simply as an isolated two-species interaction" (Fox, 1988).

Why plants producing phototoxins rely on an integrated chemical defenses

The remarkable toxicity of PTs and their occurrence in plant tissues at concentrations sufficient to generate acute insecticidal toxicity (chapter 2) led to the hypothesis that "... photosensitization has real ecological significance in plant-pest interactions under natural conditions" (Champagne *et al.*, 1986). The results of the present investigation have, however, identified some flaws for plants that rely on phototoxic defenses. I here describe the major gaps related to a phototoxic defense in plants, and I then argue that these drawbacks may have compelled phototoxic plants, via natural selection, to the elaboration of an integrated chemical defense strategy.

Ecophysiological constraints related to phototoxic defenses in plants

By definition, phototoxic defenses depend on the availability of light. All the polyacetylenic derivatives, from which many are phototoxic, occurring in more than 1000 species of Asteraceae (see intro chapter 4) specifically rely on near-UV wavelengths to exert their full toxicity. But in nature this light-dependent toxicity may be severely compromised for plants located in the shade. For example, the range of polyacetylenic photosensitizing wavelengths between 320 nm to 360 nm represent only 1% of the total solar radiation (value derived from Larson & Berenbaum, 1988) and over 90% of the wavelengths under 650 nm are absorbed by a single leaf of a plant (Fitter & Hay, 1987). This clearly points out that near-UV light may be a restrictive factor to phototoxic defenses for hostplants located in the shade or even for the lower canopy strata of plants established in open habitats. The efficiency of phototoxic defenses may be furthermore compromised by insects having either some light-avoidance responses or those able to build an opaque shelter with the tissues of their hostplants (Fig. 2, chapters 3 and 4).

Light also affects another important characteristic of PTs in plant-insect relationships, that is the ability of plants to synthesize these SCs. The volume of the glands containing PTs in leaves of *Porophyllum* spp. was improved by two orders of magnitude when plants were grown at 380 μmole photon m^{-2} s^{-1} compared to 50 μmole photon m^{-2} s^{-1} (chapter 6). Results from chapter 5 also exemplified the tremendous importance of light intensity for the inducible production of PTs following a simulated herbivory aggression on leaves of *Carthamus tinctorius*. This inducible pattern suggests that *C. tinctorius* individuals occurring in the shade in nature would be lacking such an inducible production of PTs, and thus unable to strengthen their phototoxic chemical defenses when they are attacked by herbivores. Since inducible responses to herbivores represent an important aspect of the defensive strategies of plants (Tallamy & Raupp, 1991), it is suggested that phototoxic plants growing in the shade may be more vulnerable to herbivores and other pest organisms.

In the present study, field observations have demonstrated that phototoxic plants are colonized by a diversified guild of generalist and specialist insects that are making use of different strategies to avoid phototoxicity. It was shown in chapters 3 and 6 that generalist grasshoppers were selectively feeding between the glands containing PTs and other SCs (Fig. 4) whereas specialist larvae of *Cloantha perspicillaris* were attracted by the glands containing hypericin in leaves of *Hypericum perforatum*. Such opposite responses to SCs by herbivorous insects harboring different lifestyles cause a dilemma for hostplants since they suffer damages no matter the level of chemical defense produced (Fig. 3). It is hypothesized that PICD represents an elegant way for hostplants to face a diffuse

Figure 2. Behavioral adaptation of a case maker insect observed to feed on the phototoxic leaves of *Hypericum perforatum* L. (Guttiferae).

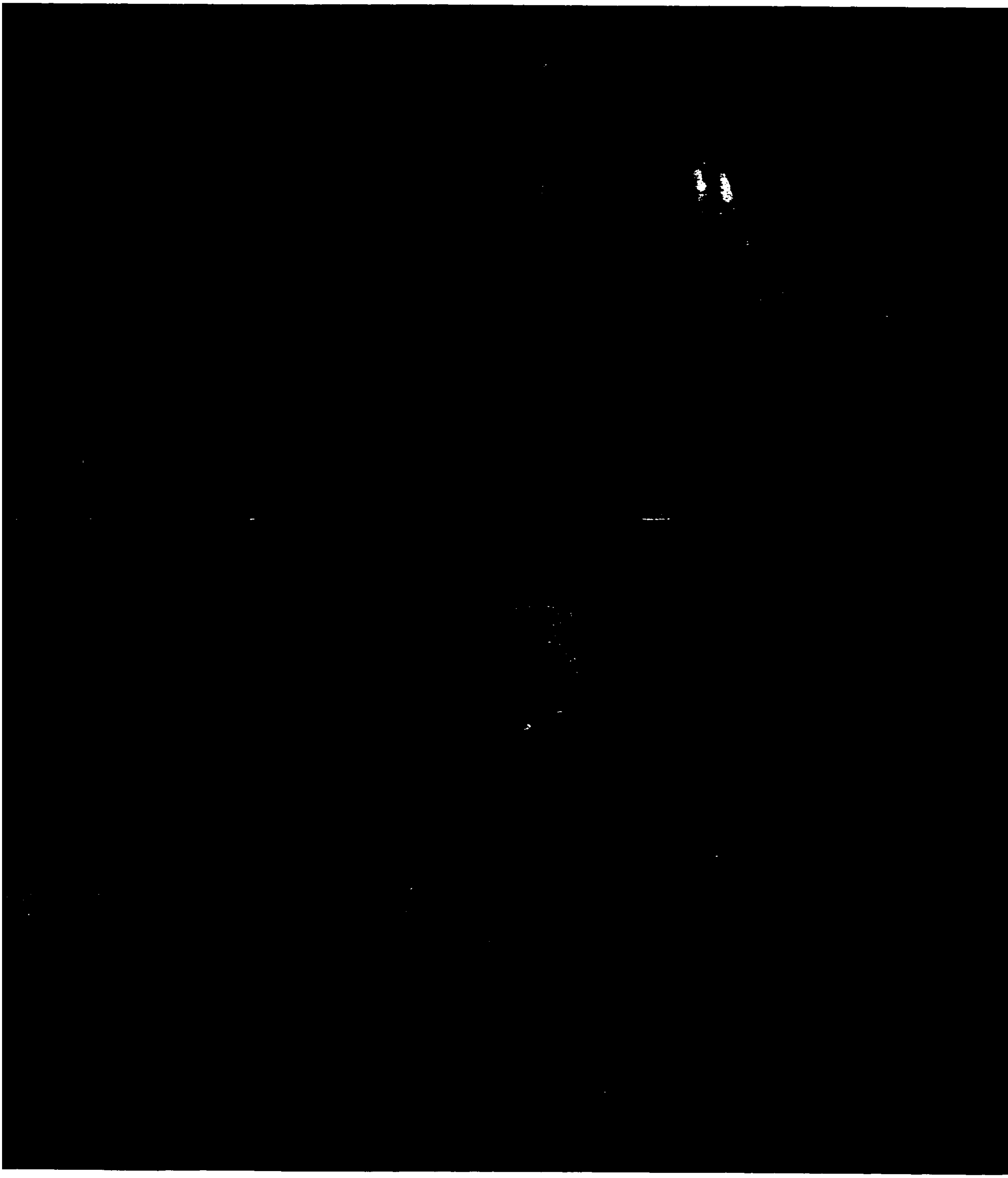


Figure 3. Effects on the concentration of a "qualitative" defense substance on both herbivore pressure (A) and plant fitness (B and C): distinct effects of specialist and generalist herbivores (Adapted from van der Meijden, 1996).

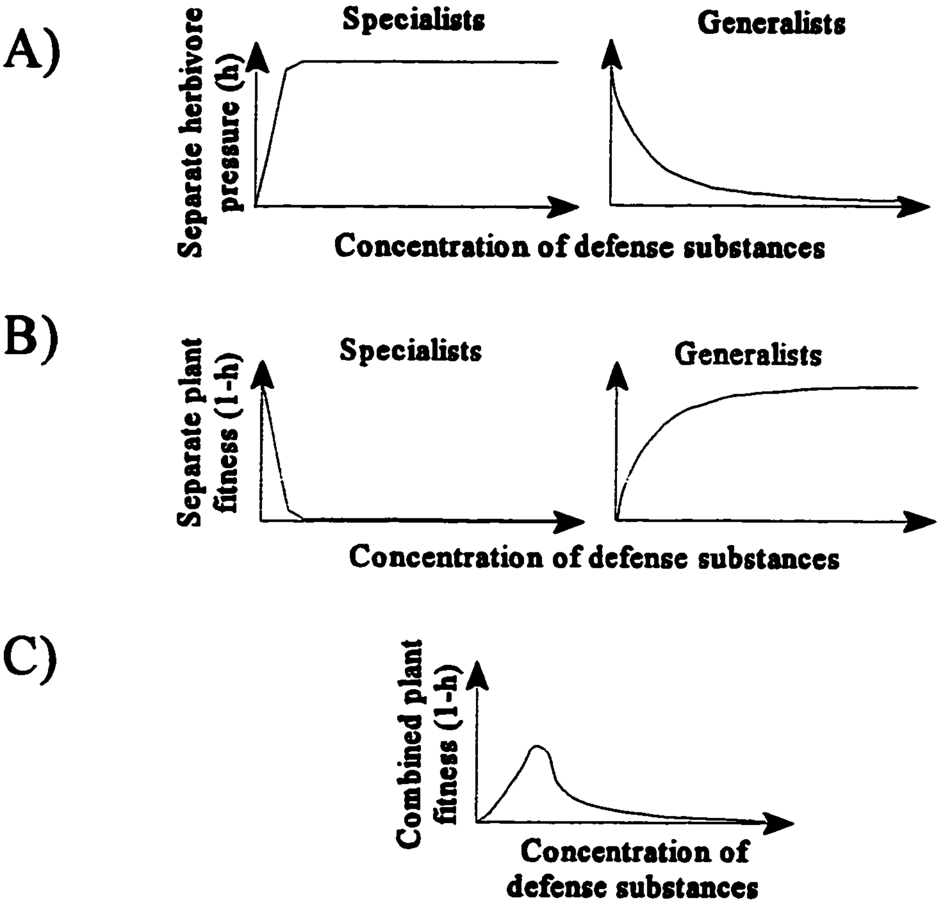

A)
Specialists
Generalists
Separate herbivore pressure (h)
Concentration of defense substances
B)
Specialists
Generalists
Separate plant fitness (1-h)
Concentration of defense substances
C)
Combined plant fitness (1-h)
Concentration of defense substances

Figure 4. A *Melanoplus femurrubrum femurrubrum* (Orthoptera: Acrididae) adult feeding selectively between the glands in a leaf of *Porophyllum ruderale*. These glands contain phototoxins and monoterpenes which exert synergist insecticidal effects.

herbivore pressure involving specialist and generalist pests because synergist defenses confer resistance to a broader range of organisms.

Finally, it is possible that the efficiency of phototoxic defenses may be further limited due to the non-specific oxidative mechanism of action of PTs. All living organisms are submitted to an endogenous production of toxic forms of oxygen that may involve a chain event which may cascade into a widespread chain reaction (Fridovich, 1983; Ahmad, 1992). It is likely that many herbivorous insects are exposed to different sources of more or less efficient pro-oxidant chemicals. Dihydroxy-phenolic compounds as well as quercetin are widely distributed in plants and may all induce an oxidative stress (see discussion in Ahmad, 1992). I hypothesized that the wide distribution of other classes of pro-oxidants in nature may have favored the maintenance of higher activities for antioxidant enzymes in generalist grasshoppers since they are more likely to be exposed to different dietary sources containing some pro-oxidants. This interpretation would explain why higher constitutive activities of GSTPOX and GR were observed in generalist grasshoppers compared to specialist insects feeding specifically on leaves of *Hypericum perforatum* (chapter 3). But if the few insect species selected for the measurement of antioxidant enzymes in this study are really representative of other insects in nature, these results would mean that the oxidative stress imposed by PTs may be tolerated by a wider range of herbivorous insects than previously expected. In other words, PTs would not confer a generalized chemical protection to hostplants.

It is expected that the light-conditional toxicity, the light requirement for the constitutive and inducible production of PTs in plants, the behavioral strategies used by herbivores to circumvent phototoxicity, the diversity of insect herbivorous lifestyles likely to occur in nature, and the non-specific oxidative mode of action of PTs, represent five major ecophysiological limitations to phototoxic defenses in nature. These limitations are likely to explain, at least in part, why phototoxic plants are not better protected than non-phototoxic plants in nature even if PTs are remarkably toxic to insects under laboratory conditions (chapter 2).

How phototoxic plants may complement their chemical defense

Over 750 polyacetylene derivatives which are potentially phototoxic occur in the Asteraceae (Bohlmann et al., 1973). Such phytochemical diversity of PTs involves molecules with different physico-chemical properties affecting the stability of PTs, the efficiency of the phototoxic processes as well as the hydrophobicity of PTs (McRae *et al.*, 1985; Marchant & Cooper, 1987; Marles *et al.*, 1991a,b; Marles *et al.*, 1992). But this variation in the molecular structures of PTs may also represent an adaptation to target different tissues or cellular components of pest organisms (Santus *et al.*, 1983; Bunting, 1992). It has even been argued that differences in hydrophobicity of PTs may be useful to target different pest organisms with anatomical dissimilarities as reported for the integuments between mosquito and brine shrimp (Marles *et al.* 1991a).

Biosynthetically related polyacetylene derivatives may also lead to distinct modes of insecticidal action as observed in *Rudbeckia hirta* (chapter 7). It is hypothesized that the mixture of the three polyacetylene derivatives investigated and having distinct modes of insecticidal action is more efficient in protecting against the different specialist phytophagous insects feeding on *R. hirta* in the Ottawa/Hull area (Guillet & Arnason, 1995) as well as other pest organisms. The thiarubrine derivative that was shown to be active against insects even in darkness is likely to confer a particular benefit to phototoxic plants to get rid of phytophagous insects harboring light-avoidance strategies (chapters 3-4; Guillet *et al.*, 1995).

However, an integrated chemical protection of plants relying on synergist interactions between PTs and other classes of SCs may be of particular interest to counteract the ecophysiological flaws of a phototoxic defensive strategy. The synergist insecticidal properties observed between α-terthienyl, a classical PT, and monoterpenes (chapter 8) and between α-terthienyl and sesquiterpene lactones (Guillet *et al.*, 1996), clearly corroborate the concept that plants may rely on an integrated defense as described above. The results obtained in chapter 5 also support the requirement for phototoxic plants to rely on complementary SCs, and thus the PICD hypothesis, to face generalist herbivores. It was particularly shown that physical (water content) and chemical (soluble phenolics) defensive traits must compensate for the inefficiency of PTs to confer a resistance to safflower plants that were offered to generalist phytophagous insects under light conditions representative of the shade in nature.

Some predictions based on the plant integrated chemical defenses hypothesis

If the PICD hpothesis is ecologically relevant in nature, some specific patterns should be detectable. First, a crude phytochemical extract of a plant should possess a higher biological activity than the mean value of the different fractionated, or purified, extracts of the same plant. This appears as the easiest way to test the PICD hypothesis since the identification of the different SCs present in plants is not required.

Another prediction of the PICD hypothesis is that the different SCs acting in synergism should be produced in a synchronized way. In other words, for synergist integrated defenses to be functional, there should be a spatio-temporal convergence in the production of the different SCs acting in synergism. This involves that synergist SCs should be produced in plants at the same phenological stage of development and in the same parts. By considering an evolutionary time scale basis, it is also predicted that the phylogenetic distribution of synergist SCs should also converge. If the synergist insecticidal effects observed between PTs and the sesquiterpene lactones having an α-methylene γ-lactone and/or a cyclopentenone groups (chapter 9) is ecologically relevant in conferring a better chemical protection to plants against insects and other pests, then a convergence in the phylogenetic distribution of both SCs should be detected.

Bibliography

Ågren J. & Schemske, D.W., 1993. The cost of defense against herbivores: an experimental study of trichome production in *Brassica rapa*. *American Naturalist* **141,** 338-350.

Ahmad S, 1994. *Oxidative stress and antioxidant defenses in biology*. New York, Chapman & Hall.

Ahmad S, 1992. Biochemical defence of pro-oxidant plant allelochemicals by herbivorous insects. *Biochemical Systematics and Ecology* **20,** 269-296.

Ahmad S & RS Pardini, 1988. Evidence for the presence of glutathione peroxidase activity towards an organic hydroperoxide in larvae of the cabbage looper moth, *Trichoplusia ni*. *Insect Biochemistry* **18,** 861-866.

Allen EH & CA Thomas, 1972. Relationship of safynol and dehydrosafynol accumulation to *Phytophthora* resistance in safflower. *Phytopathology* **62,** 471-474.

Allen EH & CA Thomas, 1971. *Trans-trans*-3,11-tridecadiene-5,7,9-triyne-1,2-diol, an antifungal polyacetylene from diseased safflower (*Carthamus tinctorius*). *Phytochemistry* **10,** 1579-1582.

Alptekin N, S Seckin, S Dogruabbasoglu, F Yelkenci, N Kocaktoker, G Toker & M Uysal, 1996. Lipid peroxides, glutathione, gamma-glutatmylcysteine synthetase and gamma-glutamyltranspeptidase activities in several tissues of rats following water-immersion stress. *Pharmacological Research* **34,** 167-169.

Andreoni A, A Colasanti, P Colasanti, M Mastrocinque , P Riccio & G Robert, 1994. Laser photosensitization of cells by hypericin. *Photochemistry and Photobiology* **59,** 529-533.

Armbruster WS, 1992. Phylogeny and the evolution of plant-animal interactions. *BioScience* **42,** 12-20.

Arnason JT, RJ Marles & RR Aucoin, 1991. Isolation and biological activity of plant derived phototoxins. In: Valenzeno DP, RH Pottier, P Mathis & RH Douglas (Eds.), *Photobiological techniques*. NATO ASI Series, Vol. 216, Plenum Press, New York, 187-196.

Arnason J.T., BJR Philogène, N Donskov, M Hudon, C McDougall, G Fortier, P Morand, D Gardner, J Lambert, C Morris, & C Nozzolillo, 1985. Antifeedant and insecticidal properties of azadirachtin to the European corn borer *Ostrinia nubilalis*. *Entomologia Experimentalis et Applicata* **38,** 29-34.

Arnason JT, BJR Philogène & GHN Towers, 1992. Phototoxins in plant-insect interactions. In:

Rosenthal G & M Berenbaum (Eds.), *Herbivores: their interactions with secondary plant metabolites*, 2^{nd} ed., Vol. II: *Evolutionary and ecological processes.* Academic Press, New York, 317-341.

Arnason JT, RJ Marles & RR Aucoin, 1991. Isolation and biological activity of plant derived phototoxins. In Valenzeno DP, RH Pottier, P Mathis & RH Douglas (Eds). Photobiological techniques. NATO ASI Series, Vol. 216. Plenum Press, New York, 187-196.

Arnason T, T Swain, C-K Wat, EA Graham, S Partington & GHN Towers, 1981b. Mosquito larvicidal activity of polyacetylenes from species in the Asteraceae. *Biochemical Systematics and Ecology* 9, 63-68.

Arnason JT, BJR Philogène, P Morand, K Imrie, S Iyengar, F Duval, C Soucy-Breau, JC Scaiano, NMH Werstiuk, B Hasspieler & AER Downe, 1989. Naturally occuring and synthetic thiophenes as photoactivated insecticides. In: Arnason JT, BJR Philogène & P Morand (eds.). *Insecticides of plant origin.* ACS Symposium Series 387, American Chemical Society, 164-172.

Arnason T, GHN Towers & JDH Lambert, 1983. The role of natural photosensitizers in plant resistance to insects. In PA Hedin (ed.), *Plant resistance to insects.* American Chemical Society Symposium Series 208. ACS, Washington, D.C., 139-151.

Arnason JT, BJR Philogène, P Morand, JC Scaiano, N Werstiuk & J Lam, 1987. *Thiophenes and acetylenes: phototoxic agents to herbivorous and blood-feeding insects.* In: Heitz JR & KR Downum (eds.), *Light-Activated Pesticides.* ACS Symposium Series 339, American Chemical Society, Washington DC, 255-265.

Arnason T, JR Stein, E Graham, C-K Wat & GHN Towers, 1981a. Phototoxicity to selected marine and freshwater algae of polyacetylenes from species in the Asteraceae. *Canadian Journal of Botany* 59, 48-54.

Arnason JT, BJR Philogène & GHN Towers, 1992. Phototoxins in plant-insect interactions. In Rosenthal G & MR Berenbaum (Eds.), *Herbivores: Their interactions with secondary plant metabolites. Vol. II: Evolutionary and Ecological Processes*, 2nd ed., Academic Press, New York, 317-341.

Arnason JT, RJ Marles & RR Aucoin, 1991. Isolation and biological activity of plant derived phototoxins. In Valenzeno DP, RH Pottier, P Mathis & RH Douglas (Eds.), *Photobiological techniques*, Series A: Life Sciences, Vol. 216, Plenum Press, New York, 187-196.

Arnason JT, T Durst, M Kobaisy, RJ Marles, E Szenasy, G Guillet, S Kacew, B Hasspieler & AER Downe, 1995. Fate of phototoxic terthiophene insecticides in organisms and the environments. In Heitz JR & KR Downum (Eds.), *Light-activated pest control.* ACS Symposium Series 616, American Chemical Society, Washington DC, 144-151.

Arnon DI, 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. *Plant Physiology* **24**, 1-15.

Asthana A & RW Tuveson, 1992. Effects of UV and phototoxins on selected fungal pathogens of *Citrus*. *International Journal of Plant Science* **155**, 442-452.

Atkinson RE & RF Curtis, 1965. *Tetrahedron Letters* 297.

Aucoin R, G Guillet, C Murray, BJR Philogène & JT Arnason, 1995. How do insect herbivores cope with the extreme oxidative stress of phototoxic host plants? *Archives of Insect Biochemistry and Physiology* **29**, 211-226.

Aucoin RR, P Fields, M Lewis, BJR Philogène & JT Arnason, 1990. The protective effect of antioxidants to a phototoxin-sensitive insect herbivore. *Journal of Chemical Ecology* **16**, 2913-2924.

Aucoin RR, BJR Philogène & JT Arnason, 1991. Antioxidant enzymes as biochemical defences against phototoxin-induced oxidative stress in three species of herbivorous lepidoptera. *Archives of Insect Biochemistry and Physiology* **16**, 1-13.

Bell G, 1997. *Selection: The mechanism of evolution.* Chapman & Hall, New York.

Bennett KD, 1997. Evolution and ecology: the pace of life. Cambridge University Press, Cambridge, UK.

Bennett JP, 1996. Floristic summary of *Manual of vascular plants of northeastern United States and adjacent Canada*, second edition. *The Botanical Review* **62**, 203-206.

Berenbaum M, 1981. Patterns of furanocoumarin distribution and insect herbivory in the Umbelliferae: plant chemistry and community structure. *Ecology* **62**, 1254-1266.

Berenbaum MR, 1983. Coumarins and caterpillars: a case for coevolution. *Evolution* **37**, 163-179.

Berenbaum MR & AR Zangerl, 1996. Phytochemical diversity: Adaptation or random variation? In Romeo JT, JA Saunders & P Barbosa (Eds.), *Phytochemical deversity and redundancy in ecological interactions*. Recent advances in phytochemistry, Vol. 30, Plenum Press, New York, 1-24.

Berenbaum MR, 1990. Evolution of specialization in insect-umbellifer associations. *Annual Review of Entomology* **35**, 319-343.

Berenbaum MR, 1996. Introduction to the symposium: on the evolution of specialization.

American naturalist **148**, S78-S83.

Berenbaum M & D Seigler, 1992. Biochemicals: Engineering problems for natural selection. In: Roiberg BD & MB Isman (Eds.), *Insect Chemical Ecology: an Evolutionary approach*. Chapman and Hall, New York, 89-121.

Berenbaum MR & AR Zangerl, 1992. Quantification of chemical coevolution. In Fritz RS & EL Simms (Eds.), *Plant resistance to herbivores and pathogens: Ecology, evolution, and genetics*. The University of Chicago Press, Chicago, 69-90.

Berenbaum MR, 1995. Phototoxicity of plant secondary metabolites: insect and mammalian perspectives. *Archives of Insect Biochemistry and Physiology* **29**, 119-134.

Berenbaum MR, 1995. The chemistry of defense: theory and practice. *Proceeding of the National Academy of Sciences* (USA) **92**, 2-8.

Berenbaum MR, 1994. Metabolic detoxification of plant prooxidants. In Ahmad S (Ed.), *Oxidative stress and antioxidant defenses in biology*. New York: Chapman and hall, 181-209.

Berenbaum MR & AR Zangerl, 1994. Costs of inducible defense: protein limitation, growth, and detoxification in parsnip webworms. *Ecology* **75**, 2311-2317.

Berenbaum M, 1985. Brementown revisited: interactions among allelochemicals in plants. *Recent Advances in Phytochemistry* **19**, 139-169.

Berenbaum MR, 1991. Coumarins. *n* Rosenthal GA & Berenbaum MR (eds), *Herbivores: Their interactions with secondary plant metabolites*, Vol 1. Academic Press, San Diego, CA, pp: 221-249.

Berenbaum MR, JK Nitao & AR Zangerl, 1991. Adaptive significance of furanocoumarin diversity in *Pastinaca sativa* (Apiaceae). *Journal of Chemical Ecology* **17**, 207-215.

Bergvinson DJ, JT Arnason & LN Pietrzak, 1994a. Localization and quantification of cell wall phenolics in European corn borer resistant and suseptible maize inbreds. *Canadian Journal of Botany* **72**, 1243-1249.

Bergvinson DJ, JT Arnason, RI Hamilton, JA Mihm & D Jewell, 1994b. Determining leaf toughness and its role in maize resistance to the European corn borer (Lepidoptera: Pyralidae). *Journal of Economic Entomology* **87**, 1743-1748.

Bernays EA & RF Chapman, 1994. *Host-plant selection by phytophagous insects*. Chapman & Hall, New York.

Bernays EA, 1991. Relationship between deterrence and toxicity of plant secondary compounds for the grasshopper *Schistocerca americana*. *Journal of Chemical Ecology* **17**, 2519-2526.

Bernays EA, 1990. Plant secondary compounds deterrent but not toxic to the grass specialist acridid *Locusta migratoria*: Implications for the evolution of graminivory. *Entomologia Experimentalis et Applicata* **54**, 53-56.

Bernays EA & M Graham, 1988. On the evolution of host specificity in phytophagous arthropods. *Ecology* **69**, 886-892.

Bernays EA, K Bright, JJ Howard, D Raubenheimer & D Champagne, 1992. Variety is the spice of life: frequent switching between foods in the polyphagous grasshopper *Taeniopoda eques* Burmeister (Orthoptera: Acrididae). *Animal Behavior* **44**, 721-731.

Bird RP & HH Draper, 1984. Comparative studies on different methods of malonaldehyde determination. *Methods in Enzymology* **105**, 299-305.

Bohlmann F, T Burkhardt & C Zdero, 1973. *Naturally Occurring Acetylenes*. Academic Press, London.

Bohlmann F, C Zdero, RM King & H Robinson, 1983. Thymol derivatives from *Porophyllum riedelii*. *Phytochemistry* **22**, 1035-1036.

Bohlmann F & C Zdero, 1979. Über dimere terpenketone aus *Tagetes gracilis*. *Phytochemistry* **18**, 341-343.

Bohlmann F, J Jakupovic, H Robinson & RM King, 1980. Polyacetylene compounds. Part 258. Dithienylacetylene from *Porophyllum ruderale*. *Phytochemistry* **19**, 2760.

Bohlmann F, S Köhn & C Arndt, 1966. Polyaceylenverbindungen. CXIV. Die polyine der Gattung *Carthamus* L. *Chem. Ber.* **99**, 3433-3436.

Bombardelli E & P Morazzoni, 1995. *Hypericum perforatum*. *Fitoterapia* **66**, 43-68.

Bourett TM, RJ Howard, DP O'Keefe, & DL Hallahan, 1994. Gland development on leaf surfaces of *Nepeta racemosa*. *International Journal of Plant Sciences* **155**, 623-632.

Bowers JE, 1982. The plant ecology of inland dunes in western North America. *Journal of Arid Environments* **5**, 199-220.

Broadway RM, 1996. Dietary proteinase inhibitors alter complement of midgut proteases. *Archives of Insect Biochemistry & Physiology* **32**, 39-53.

Brossart JL & JM Scriber, 1995. Maintenance of ecologically significant genetic variation in the tiger swallowtail butterfly through differential selection and gene flow. *Evolution* **49**, 1163-1171.

Brown JR, 1996. Preparing for the flood: evolutionary biology in the age of genomics. *Trends in Ecology and Evolution* **11**, 510-513.

Bryant JP, FS Chapin III, & DR Klein, 1983. Carbon/nutrient balance of boreal plants in relation to vertebrate herbivory. *Oikos* **40**, 357-368.

Bunting JR, 1992. A test of the singlet oxygen mechanism of cationic dye photosensitization of mitochondrial damage. *Photochemistry and Photobiology* **55**, 81-87.

Bussell BM, JA Considine, & ZE Spadek, 1995. Flower and volatile oil ontogeny in *Boronia megastigma*. *Annals of Botany* **76**, 457-463.

Camm EL, GHN Towers & JC Mitchell, 1975. UV-mediated antibiotic activity of some Compositae species. *Phytochemistry* **14**, 2007-2011.

Carpenter S & GA Krauss, 1991. Photosensitization is required for inactivation of equine infectious anemia virus by hypericin. *Photochemistry and Photobiology* **43**, 677-680.

Carroll M, A Hanlon, T Hanlon, AR Zangerl & MR Berenbaum, 1997. Behavioral effects of carotenoid sequestration by the parsnip webworm, *Depressaria pastinacella*. *Journal of Chemical Ecology* (submitted).

Cerezke HF & RE Holmes, 1986. Control studies with carbofuran on seed and cone insects of white spruce. *Canadian Forest Services*, Northern Forestry Centre, Inf. Rep. NOR-X-280.

Champagne DE, JT Arnason, BJR Philogène, P Morand & J Lam, 1986. Light-mediated allelochemical effects of naturally occurring polyacetylenes and thiophenes from Asteraceae on herbivorous insects. *Journal of Chemical Ecology* **12**, 835-857.

Champagne DE, JT Arnason, BJR Philogène, G Campbell & DG McLachlan, 1984. Photosensitization and feeding deterrence of *Euxoa messoria* (Lepidoptera: Noctuiidae) by α-terthienyl, a naturally occurring thiophene from the Asteraceae. *Experientia* **40**, 577-578.

Chan GFQ, MM Lee, J Glushka & GHN Towers, 1979. Photosensitizing thiophenes in *Porophyllum*, *Tessaria* and *Tagetes*. *Phytochemistry* **18**, 1566.

Charlesworth B, JA Coyne & NH Barton, 1987. The relative rates of evolution of sex chromosomes and autosomes. *American Naturalist* **130**, 113-146.

Chopard L, 1951. *Orthoptéroïdes*. Faune de France #56. Paul Chevalier, Paris, 107-109.

Christensen LP & J Lam, 1991. Acetylenes and related compounds in Heliantheae. *Phytochemistry* **30,** 11-49.

Christensen LP & J Lam, 1991. Acetylenes and related compounds in Astereae. *Phytochemistry* **30,** 2453-2476.

Christensen LP & J Lam, 1990. Acetylenes and related compounds in Cynareae. *Phytochemistry* **29,** 2753-2786.

Cohen MB, MA Schuler & MR Berenbaum, 1992. A host-inducible cytochrome P450 from a host-specific caterpillar: molecular cloning and evolution. *Proceedings of the National Academy of Sciences (USA)* **89,** 10920-10924.

Coley P, 1983. Herbivory and defensive characteristics of tree species in a lowland tropical forest. *Ecological Monographs* **53,** 209-233.

Coley PD, JP Bryant & FS Chapin III, 1985. Resource availability and plant antiherbivore defense. *Science* **230,** 895-899.

Constabel CP & GHN Towers, 1989. The complex nature of the mechanism of toxicity of antibiotic dithiacyclohexadiene polyines (Thiarubrines) from the Asteraceae. *Planta Medica* **55,** 35-37.

Constabel CP, F Balza & GHN Towers, 1988. Dithiacyclohexadienes and thiophenes of *Rudbeckia hirta*. *Phytochemistry* **27,** 3533-3535.

Cornwell PA, BW Barry, JA Bouwstra & GS Gooris, 1996. Modes of action of terpene penetration enhancers in human skin: differential scanning calorimetry, small-angle X-ray diffraction and enhancer uptake studies. *International Journal of Pharmaceutics (Amsterdam)* **127,** 9-26.

Cottee PK, EA Bernays & AJ Mordue, 1988. Comparisons of deterrency and toxicity of selected secondary plant compounds to an oligophagous and a polyphagous acridid. *Entomologia Experimentalis et Applicata* **46,** 241-247.

Croes AF, M Bosveld and GJ Wullems, 1988. Control of thiophene accumulation in *Tagetes*. In: Lam J, H Breteler, T Arnason and L Hansen (Eds.), *Chemistry and biology of naturally-occurring acetylenes and related compounds* (NOARC), Elsevier, New York, 255-265.

Darwin, [1859] (1979). *The origin of species*. Avenel, New York.

Dethier VG, 1954. Evolution of feeding preferences in phytophagous insects. *Evolution* **8,** 33-54.

Dicke FF & WD Guthrie, 1988. The most important corn insects. In Sprague GF & GW Dudley (Eds.), *Corn and corn improvement*. Madison WIS, American Society of Agronomy, 767-817.

Dirzo R & JL Harper, 1982. Experimental studies on slug-plant interactions. IV. The performance of cyanogenic and acyanogenic morphs of *Trifolium repens* in the field. *Journal of Ecology* **70,** 119-138.

Dobler S, P Mardulyn, JM Pasteels & M Rowell-Rahier, 1996. Host-plant switches and the evolution of chemical defense and life history in the leaf beetle genus *Oreina*. *Evolution* **50,** 2373-2386.

Downum KR, LA Swain & LJ Faleiro, 1991. Influence of light on plant allelochemicals: a synergistic defense in higher plants. *Archives of Insect Biochemistry and Physiology* **17,** 201-211.

Downum KR & S Nemec, 1987. Light-activated antimicrobial chemicals from plants: their potential role in resistance to disease-causing organisms. In Heitz JR & KR Downum (Eds.), *Light-activated pesticides*. ACS Symposium Series 339, American Chemical Society, Washington D.C., 281-294.

Downum KR & GHN Towers, 1983. Analysis of thiophenes in the Tageteae (Asteraceae) by HPLC. *Journal of Natural Products* **46,** 98-103.

Downum KR, DJ Keil & E Rodriguez, 1985. Distribution of acetylenic thiophenes in the Pectidinae. *Biochemical Systematics and Ecology* **13,** 109-113.

Downum KR, LA Swain & LJ Faleiro, 1991. Influence of light on plant allelochemicals: a synergistic defense in higher plants. *Archives of Insect Biochemistry and Physiology* **17,** 201-211.

Downum KR, GA Rosenthal & GHN Towers, 1984. Phototoxicity of the allelochemical, α-terthienyl, to larvae of *Manduca sexta* (L.) (Sphingidae). *Pesticide Biochemistry & Physiology* **22,** 104-109.

Dubois C-F & A Dubois Fils, 1880. *Les lépidoptères de la Belgique: leurs chenilles et leurs chrysalides*. Bruxelles & Leipzig, Bruxelles.

Duffey SS & JM Pasteels, 1993. Transient uptake of hypericin by chrysomelids is regulated by feeding behariour. *Physiological Entomology* **18,** 119-129.

Duffey SS, 1986. Plant glandular trichomes: Their partial role in defence against insects. In Juniper B & TRE Southwood (Eds.), *Insects and the Plant Surface*, Edward Arnold, 151-172.

Duke SO, RN Paul & SM Lee, 1988. Terpenoids from the genus *Artemisia* as potential herbicides. *American Chemical Society Symposium Series* **380**, 318-334.

Duke MV, RN Paul, HK ElSohly, G Sturtz SO & Duke, 1994. Localization of artemisinin and artimesitene in foliar tissues of glanded and glandless biotypes of *Artemisia annua*. *International Journal of Plant Sciences* **155**, 365-373.

Duriyaprapan S & E Britten, 1982. *Journal of Experimental Botany* **33**, 810-814.

Dussourd DE & RF Denno, 1991. Deactivation of plant defense: Correspondence between insect behavior and secretory canal architecture. *Ecology* **72**, 1383-1396.

Dussourd DE & T Eisner, 1987. Vein-cutting behavior: insect counterploy to the latex defense of plants. *Science* **237**, 898-901.

Dussourd DE & RF Denno, 1994. Host range of generalist caterpillars: Trenching permits feeding on plants with secretory canals. *Ecology* **75**, 69-78.

Ehrlich PR & PH Raven, 1964. Butterflies and plants: a study in coevolution. *Evolution* **18**, 586-608.

Eigenbrode SD & KE Espelie, 1995. Effects of plant epicuticular lipids on insect herbivores. *Annual Review of Entomology* **40**, 171-194.

Eisenstark A, P Yallaly, A Ivanova & C Miller, 1995. Genetic mechanisms involved in cellular recovery from oxidative stress. *Archives of Insect Biochemistry & Physiology* **29**, 159-173.

Ellis SM, F Balza, P Constabel, JB Hudson & GHN Towers, 1995. Thiarubrines: novel dithiacyclohexadiene polyyne photosensitizers from higher plants. In Heitz JR & KR Downum (Eds.), *Light-activated pest control*. ACS Symposium Series 616, American Chemical Society, Washington, 164-178.

Farrell BD & C Mitter, 1990. Phylogenesis of insect/plant interactions: have *Phyllobrotica* leaf beetles (Chrysomelidae) and the Lamiales diversified in parallel? *Evolution* **44**, 1389-1403.

Farrell BD, C Mitter & DJ Futuyma, 1992. Diversification at the insect-plant interface. *BioScience* **42**, 34-42.

Feeny P, 1976. Plant apparency and chemical defense. *Recent Advances in Phytochemistry* **10**, 1-40.

Felton GW & CB Summers, 1995. Antioxidant systems in insects. *Archives of Insect Biochemistry and Physiology* **29**, 187-197.

Feng R & MB Isman. 1995. Selection for resistance to azadirachtin in the green peach aphid, *Myzus persicae*. *Experientia* **51,** 831-833.

Fiedler K, 1996. Host-plant relationships of lycaenid butterflies: large-scale patterns, interactions with plant chemistry, and mutualism with ants. *Entomologia Experimentalis et applicata* **80,** 259-267.

Fields PG, JT Arnason & RG Fulcher, 1989. The spectral properties of *Hypericum perforatum* leaves: the implications for its photoactivated defences. *Canadian Journal of Botany* **68,** 1166-1170.

Fields PB, JT Arnason & BJR Philogène, 1990. Behavioural and physical adaptations of three insects that feed on the phototoxic plant *Hypericum perforatum*. *Canadian Journal of Zoology* **68,** 339-346.

Fields PG, JT Arnason, BJR Philogène & RR Aucoin, 1991. Phototoxins as insecticides and natural plant defences. *Memoirs of the Entomological Society of Canada* **159,** 29-38.

Finney DJ, 1964. *Probit Analysis: A statistical treatment of the sigmoid response curve* (2nd ed.). University Press, Cambridge.

Firn RD & CG Jones, 1996. An explanation of secondary product "redundancy". In Romeo JT, JA Saunders & P Barbosa (Eds.), *Phytochemical deversity and redundancy in ecological interactions*. Recent advances in phytochemistry, Vol. 30, Plenum Press, New York, 295-312.

Fitter AH & RKM Hay, 1989. *Environmental physiology of plants*. Academic Press Inc., San Diego, CA, 29.

Fitter AH & RKM Hay, 1987. *Environmental physiology of plants* (2nd ed.). Academic Press, New York.

Foote CS, 1987. Type I and type II mechanisms of photodynamic actin. In Heitz JR & KR Downum (Eds.), *Light-activated pesticides*. ACS Symposium Series 339, American Chemical Society, Washington D.C., 22-38.

Foote CS, 1991. Definition of type I and type II photosensitized oxidation. *Photochemistry and Photobiology* **54,** 659.

Fox LR, 1981. Defense and dynamics in plant-herbivore systems. *American Zoologist* **21,** 853-864.

Fox LR, 1988. Diffuse coevolution within complex communitites. *Ecology* **69,** 906-907.

Fox LR & PA Morrow, 1981. Specialization: Species property or local phenomenon? *Science* **211**, 887-893.

Fraenkel G, 1959. The raison d'être of secondary plant substances. *Science* **129**, 1466-1470.

Franco-Vizcaino E, RC Graham & B Alexander, 1993. Pland species diversity and chemical properties of soils in the central desert of Baja California Mexico. *Soil Science* **155**, 406-416.

Fridovich I, 1983. An endogenous toxicant. *Annual Review of Pharmacology and Toxicology* **23**, 239-257.

Genin D & AP Pijoan, 1993. Seasonality of goat diet and plant acceptabilities in the coastal scrub of Baja California, Mexico. *Small Ruminant Research* **10**, 1-11.

Gershenzon J, 1994. Metabolic costs of terpenoid accumulation in higher plants. *Journal of Chemical Ecology* **20**, 1281-1328.

Gershenzon J & R Croteau, 1992. Terpenoids. In Rosenthal GA & MR Berenbaum (Eds.), *Herbivores: Their interactions with secondary metabolites*, vol. 1, Academic Press, New York, 165-220.

Girotti AW, 1990. Photobiology school: photodynamic lipid peroxidation in biological systems. *Photochemistry and Photobiology* **51**, 497-509.

Gomez-Barrios ML, FJ Parodi, D Vargas, L Quijano, MA Hjortso, HE Flores & NH Fisher, 1992. Studies on the biosynthesis of thiarubrine A in hairy root cultures of *Ambrosia artemesiifolia* using ^{13}C-labelled acetates. *Phytochemistry* **31**, 2703-2707.

Gommers FJ & J Bakker, 1988. Mode of action of α-terthienyl and related compounds may explain the suppressant effects of *Tagetes* species on populations of free living endoparasitic plant nematodes. In Lam J, H Breteler, T Arnason & L Hansen (Eds.), *Chemistry and biology of naturally-occurring acetylenes and related compounds (NOARD)*. Elsevier Science Publications, Vol. 7, Amsterdam, 61-69.

Gowda JH, 1996. Spines of *Acacia tortilis*: what do they defend and how? *Oikos* **77**, 279-284.

Gray JT, 1982. Community structure and productivity in *Ceanothus* chaparral and coastal sage scrub of southern California. *Ecological Monography* **54**, 415-435.

Griffith OW, 1980. Determination of glutathione and glutathione disulphide using glutathionereductase and 2-vinylpyridine. *Analytical Biochemistry* **76**, 5606-5610.

Grossweiner LI, 1989. Photophysics. In KC Smith (Ed.), *The Science of Photobiology*, Plenum Press, New York, 1-45.

Guillet G, D Chauret & JT Arnason, 1997. Phototoxic polycetylenes from *Viguiera annua* and adaptations of a chrysomelid beetle, *Zygogramma continua*, feeding on this plant. *Phytochemistry* **45**, 695-699.

Guillet G, M-E Lavigne, BJR Philogène & JT Arnason, 1995. Behavioral adaptations of two phytophagous insects feeding on two species of phototoxic Asteraceae. *Journal of Insect Behaviour* **8**, 533-546.

Guillet G, F Lorenzetti, A Bélanger, JT Arnason & EA Bernays, 1997a. Production of glands in leaves of *Porophyllum* spp. (Asteraceae): Ecological and genetic determinants, and implications for insect herbivores. *Journal of Ecology*, in press.

Guillet G, C Bourret-Bernard, C Podeszfinsky, J Harmatha, T Durst, S Delorme, BJR Philogène, JT Arnason, 1996. How terpenes, lignans and sesquiterpene lactones strengthen the insecticidal properties of thiophenes in Asteraceae? Proceedings of the 13th annual meeting of the International Society of chemical Ecology held in Prague.

Guillet G, A-S Belzile & JT Arnason, 1997b. Synergism of dillapiol on natural and commercial insecticides. In preparation.

Guillet G, 1994. *Ecological importance of phototoxins in plant-insect interactions.* M.Sc. thesis, University of Ottawa.

Guillet G & JT Arnason, 1995. Phytophagous insects found on *Rudbeckia hirta* and *Chrysanthemum leucanthemum* in the Ottawa/Hull area. *Proceedings of the Entomological Society of Ontario* **126**, 1-3.

Guthrie WD, JC Robbins & JL Jarvis, 1985. *Ostrinia nubilalis.* In Singh P & RF Moore (Eds.), *Handbook of Insect Rearing*, Elsevier, New York, 407-415.

Hadjur C & P Jardon, 1995. Quantitative analysis of superoxide anion radicals photosensitized by hypericin in a model membrane using the cytochrome c reduction method. *Journal of Photochemistry & Photobiology. B - Biology* **29**, 147-156.

Harborne JB (Ed.), 1991. *Ecological chemistry and biochemistry of plant terpenoids.* University Press, Oxford.

Harper JL, 1977. *Population biology of plants.* Academic Press, London.

Harshman LG, JA Ottea & BD Hammock, 1991. Evolved environment-dependent expression of detoxication enzyme activity in *Drosophila melanogaster*. *Evolution* **45**, 791-795.

Hartley RD & CW Ford, 1989. Phenolic constituents of plant cell walls and wall biodegradability. In Lewis NG & MG Paice (Eds.), *Biogenesis and biodegradation*, ACS Symposium serie 399, 137-145.

Hartmann T, 1996. Diversity and variability of plant secondary metabolism: a mechanistic view. In Städler E, M Rowell-Rahier & R Bauer (Eds.), *Proceedings of the 9th International Symposium on Insect-Plant Relationships*. Kluwer Academic Publishers, Boston, 177-188.

Hasspieler BM, JT Arnason AER & Downe, 1990. Modes of action of the plant derived phototoxin, α-terthienyl in mosquito larvae. *Journal of the American Mosquito Control Association* **4**, 79-84.

Heitz JR & KR Downum (Eds.), 1995. *Light-activated pest control*, American Chemical Society Symposium Series 616, ACS, Washington, D.C.

Hermes-Lima M, WG Willmore & KB Storey, 1995. Quantification of lipid peroxidation in tissue extracts based on Fe(II) xylenol orange complex formation. *Free Radical Biol. Med.* **19**, 271-280.

Herms DA & WJ Mattson, 1992. The dilemma of plants: to grow or defend. *The Quaterly Review of Biology* **67**, 283-335.

Hoffmann MP, FG Zalom, LT Wilson, JM Smilanick, LD Malyj, J Kiser, VA Hilder & WM Barnes, 1992. Field evaluation of transgenic tobacco containing genes encoding *Bacillus thuringiensis* δ-endotoxin or cowpea trypsin inhibitor: efficacy against *Helicoverpa zea* (Lepidoptera: Noctuidae). *Journal of Economic Entomology* **83**, 2516-2522.

Hougen-Eitzman D & MD Rausher, 1994. Interactions between herbivorous insects and plant-insect coevolution. *The American Naturalist* **143**, 677-697.

Hu SA & QL Zhao, 1995. Studies on epidermal hairs of *Gossypium*. *Acta Botanica Sinica* **34**, 311-314.

Huacuja RL, A Carranco, SA Guzman & C Guerrero, 1993. Inactivation of SH groups with sesquiterpene lactones: Effects on nuclear decondensation pattern-motility induced by heparin in human spermatozoa. *Advances in Contraceptive Delivery Systems* **9**, 97-106.

Hudon M, EJ LeRoux & DG Harcourt, 1989. Seventy years of European corn ober (Ostrinia nubilalis) research in North America. In Russell GE (Ed.), *Biology and Population Dynamics of Invertebrate Crop Pests*. Intercept Ltd., Andover, Hampshire, U.K., 1-44.

Hudson JB, F Balza, L Harris & GHN Towers, 1993. Light-mediated activities of thiarubrines against human immunodeficiency virus. *Photochemistry and Photobiology* **57**, 675-680.

Hudson JB, I Lopez-Bazzocci & GHN Towers, 1991. Antiviral activities of hypericin. *Antiviral Research* **15**, 101-112.

Hudson JB, EA Graham & GHN Towers, 1994. Antiviral assays on phytochemicals: the influence of reaction parameters. *Planta Medica* **60**, 329-332.

Hughes MA, 1996. *Plant Molecular Genetics.* Addison Wesley Longman Limited, Edinburgh, England.

Igrc J, CJ DeLoach & V Zlof, 1995. Release and establishment of *Zygogramma suturalis* F. (Coleoptera: Chrysomelidae) in Croatia for control of Commo Ragweed (*Ambrosia artemisiifolia* L.). *Biological Control* **5**, 203-208.

Iqbal M, SD Sharma SD, H Rezazadeh, N Hasan, M Abdulla& M Athar, 1996. Glutathione metabolizing enzymes and oxidative stress in ferric nitrilotriacetate mediated hepatic injury. *Redox Report* **2**, 385-391.

Isman MB, H Matsuura, S MacKinnon, T Durst, GHN Towers & JT Arnason, 1996. Phytochemistry of the Meliaceae. In Romeo JT, JA Saunders, P Barbosa (Eds.), *Phytochemical diversity and redundancy in ecological interactions.* Recent Advances in Phytochemistry, Vol. 30, Plenum Press, New York, 155-178.

Ivie GW, DL BUll, RC Beier, NW Pryor & EH Oertli, 1983. Metabolic detoxification: mechanism of insect resistance to plant psoralens. *Science* **221**, 374-376.

Iyengar S, JT Arnason, BJR Philogène, P Morand & NG Werstiuk, 1990. The comparative metabolism of α-terthienyl in three species of insect herbivores. *Pesticide Biochemistry and Physiology* **37**, 154-164.

Iyengar S, JT Arnason, BJR Philogène, P Morand, NH Werstiuk & G Timmins, 1987. Toxicokinetics of the phototoxic allelochemical alpha-terthienyl in three herbivorous lepidoptera. *Pesticide Biochemistry and Physiology* **29**, 1-9.

Jansen M, AJ Baars & DD Breimer (1984). Cytosolic glutathione S-transferases in *Drosophila melanogaster*. *Biochemical Pharmacology* **33**, 3655-3659.

Janzen DH, 1988. Ecological characterization of a Costa Rican dry forest caterpillar fauna. *Biotropica* **20**, 120-135.

Jarvis BB & JD Miller, 1996. Natural products, complexity, and evolution. In Romeo JT, JA

Saunders & P Barbosa (Eds.), *Phytochemical diversity and redundancy in ecological interactions*. Recent Advances in Phytochemistry Vol. 30, Plenum Press, New York, 265-294.

Jayanth KP & PNG Visalakshy, 1996. Succession of vegetation after suppression of *Parthenium* weed by *Zygogramma bicolorata* in Bangalore, India. *Biological Agriculture and Horticulture* **12**, 303-309.

Jensen KIN, SO Gaul, EG Specht & DJ Doohan, 1995. Hypericin content of Nova Scotia biotypes of *Hypericum perforatum* L. *Canadian Journal of Plant Science* **75**, 923-926.

Jente R, E Richter, F Bosold & GA Olatunji, 1988. In Lam J, T Breteler, T Arnason & L Hansen (Eds.), Bioactive Molecules Vol. 7, Elsevier Science Publishing Company Inc., New York, 188.

Kagan J, TP Wang, IA Kagan, RW Tuveson, G-R Wang & J Lam, 1992. Photosensitization by 2-chloro-3,11-tridecadiene-5,7,9-triyn-1-ol: damage to erythrocyte membranes, *Escherichia coli*, and DNA. *Photochemistry and Photobiology* **55**, 63-73.

Ketterer B, B Coles & DJ Meyer, 1983. The role of glutathione in detoxication. *Environmental Health Perspectives* **49**, 59-69.

Kingsbury JM, 1964. *Poisonous plants of the United States and Canada*. Pentice-Hall, Inc., Englewood Cliffs, NJ.

Knox JP, RI Samuels & AD Dodge, 1987. Photodynamic action of hypericin. In: JR Heitz & KR Downum (Eds.), *Light-Activated Pesticides*. American Chemical Society symposium ACS series 339, 265-270.

Knox JP & AD Dodge, 1985. Isolation and activity of the photodynamic pigment hypericin. *Plant, Cell and Environment* **8**, 19-25.

Kokkini S, R Karousou & D Vokou, 1994. Pattern of geographic variation of *Origanum vulgare* trichomes and essential oil content in Greece. *Biochemical Systematics and Ecology* **22**, 517-528.

Köpf A, N Rank & H Roininen, 1996. Geographic variation in feeding and mating preferences in the *Phratora tibialis* complex. *Entomologia Experimentalis et Applicata* **30**, 311-314.

Koziel MG, GL Beland & C Bowman, 1993. Field performance of elite transgenic maize plants expressing an insecticidal protein derived from *Bacillus thuringiensis*. *Bio/Technology* **11**, 194-200.

Larcher W, 1995. *Physiological Plant Ecology* (3rd ed.). Springer-Verlag, New York.

Larson RA, 1995. Plant defenses against oxidative stress. *Archives of Insect Biochemistry and Physiology* **29**, 175-186.

Larson RA & MR Berenbaum, 1988. Environmental phototoxicity: solar ultraviolet radiation affects the toxicity of natural and man-made chemicals. *Environment, Science and Technology* **22**, 354-360.

Larson RA, KA Marley, RW Tuveson & MR Berenbaum, 1988. β-carboline alkaloids: mechanisms of phototoxicity to bacteria and insects. *Photochemistry and Photobiology* **48**, 665-674.

Lee K & MR Berenbaum, 1990. Action of antioxidant enzymes and cytochrome P450 monooxygenases in the cabbage looper in response to plant phototoxins. *Archives of Insect Biochemistry and Physiology* **10**, 151-162.

Lee K, 1991. Glutathione S-transferase activities in phytophagous insects: induction and inhibition by plant phototoxins and phenols. *Insect Biochemistry* **21**, 353-361.

Bradford MM, 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248-254.

Lee K-H, IH Hall, E-C Mar, CO Starnes, SA ElGebaly, TG Waddell, RI Hadgraft, CG Ruffner & I Weidner, 1977. Sesquiterpene antitumor agents: inhibitors of cellular metabolism. *Science* **196**, 533-536.

Lerdau M, 1992. Future discounts and resource allocation in plants. *Functional Ecology* **6**, 371-375.

Levin DA, 1975. The role of trichomes in plant defense. *The Quaterly Review of Biology*, **58**, 3-15.

Lopez-Bazzocchi I, JB Hudson & GHN Towers, 1991. Antiviral activity of the photoactive plant pigment hypericin. *Photochemistry and Photobiology* **54**, 95-98.

Loveys BR, SP Robinson, JJ Brophy & EK Chacko, 1992. Mango sapburn: Components of fruit sap and their role in causing skin damage. *Australian Journal of Plant Physiology* **19**, 449-457.

Lozoya MM & I Gaspar, 1993. Phototoxic effect of methanolic extracts from *Porophyllum macrocephalum* and *Tagetes erecta*. *Fitoterapia* **64**, 35-41.

Luquet G & H Bellman, 1995. *Guide des sauterelles, grillons et criquets d'Europe occidentale*. Editions de Lachaux et Niestlé, Paris.

Mabry TJ & JE Gill, 1979. Sesquiterpene lactones and other terpenoids. In Rosenthal GA & DH Janzen (Eds.), *Herbivores: their interaction with secondary plant metabolites*, Academic Press, New York, 501-537.

Machlis L & JG Torrey, 1956. *Plants in Action: A Laboratory Manual of Plant Physiology*. W.H. Freeman, San Francisco.

Macías FA, JCG Galindo & GM Massanet, 1992. Potential allelopathic activity of several sesquiterpene lactone models. *Phytochemistry* **31**, 1969-1977.

MacRae WD, DAJ Irwin, T Bisalputra & GHN Towers, 1980. Membrane lesions in human erythrocytes induced by the naturally occurring compounds alpha-terthienyl and phenylheptatriyne. *Photochemistry and Photobiology* **1**, 309-318.

Maffei M, F Chialva & T Sacco, 1989. *New Phytologist* **111**, 707-716.

Malcolm SB, 1991. Cardenolide-mediated interactions between plants and herbivores. In GA Rosenthal & MR Berenbaum (Eds.), *Herbivores: Their interactions with secondary plant metabolites*, 2nd edition, Vol. I: *The chemical participants*. Academic Press, San Diego, 251-296.

Mani GS, 1985. Evolution of resistance in the presence of two insecticides. *Genetics* **109**, 761-783.

Marchant YY & GK Cooper, 1987. Structure and function relationships in polyacetylene photoactivity. In Heitz JR & KR Downum (Eds.), *Light-activated pesticides*. ACS Symposium series 339, American Chemical Society, Washington, D.C., 241-254.

Marles RJ, RL Compadre, CM Compadre, C Soucy-Breau, RW Redmond, F Duval, B Mehta, P Morand, JC Scaiano & JT Arnason, 1991b. Thiophenes as mosquito larvicides: structure-toxicity relationship analysis. *Pesticide, Biochemistry and Physiology* **41**, 89-100.

Marles RJ, JT Arnason, RL Compadre, CM Compadre, C Soucy-Breau, B Mehta, P Morand, RW Redmond & JC Scaiano, 1991a. Quantitative structure-activity relationship analysis of natural products: phototoxic thiophenes. In Fischer NH, MB Isman & HA Stafford (Eds.), *Modern Phytochemical methods*. Recent Advances in Phytochemistry, Vol. *25*, Plenum Press, New York, 371-396.

Marles RJ, JB Hudson, EA Graham, C Soucy-Breau, P Morand, RL Compadre, CM Compadre, GHN Towers & JT Arnason, 1992. *Photochemistry and Photobiology* **56**, 479-487.

Marles RJ, T Durst, M Kobaisy, C Soucy-Breau, M Abou-Zaid & JT Arnason, 1995. Toxicity pharmacokinetics and metabolism of the plant derived phototoxin α-terthienyl. *Pharmacology* *and Toxicology* **77**, 164-168.

Marquis RJ, 1992. The selective impact of herbivores. In Fritz RS & EL Simms (Eds.), *Plant resistance to herbivores and pathogens: Ecology, evolution, and genetics*. The University of Chicago Press, Chicago, 195-215.

Mattson WJ, 1978. The role of insects in the dynamics of cone production of red pine. *Oecologia* **33**, 327-349.

McCloud ES, MR Berenbaum & RW Tuveson, 1992. Furanocoumarin content and phototoxicity of rough lemon (*Citrus jambhiri*) foliage exposed to enhanced ultraviolet-B (UVB) irradiation. *Journal of Chemical Ecology* **18**, 1125-1137.

McKey D, 1979. The distribution of secondary compounds within plants. In Rosenthal GA & DH Janzen (Eds.), *Herbivores: Their Interaction with Secondary Plant Metabolites*, Academic Press, New York, 55-133.

McLachlan D, T Arnason & J Lam, 1984. The role of oxygen in photosensitization with polyacetylenes and thiophene derivatives. *Photochemistry and Photobiology* **39**, 177-182.

McRae DG, E Yamamoto & GHN Towers, 1985. The mode of action of polyacetylene and thiophene photosensitizers on liposome permeability to glucose. *Biochimica Biophysica Acta* **821**: 488-496.

Mendelsohn R & M Balick, 1995. The value of undiscovered pharmaceuticals in tropical forests. *Economic Botany* **49**, 223-228.

Menken SBJ, 1996. Pattern and process in the evolution of insect-plant associations: *Yponomeuta* as an example. *Entomologia Experimentalis et Applicata* **80**, 297-305.

Metzger FW & DH Grant. Repellency to the Japanese beetle of extracts made from plants immune to attack. *USDA Technical Bulletin* **299**: 1932.

Mitich L W, 1994. Common St. John's-wort. *Weed Technology* **8**, 658-661.

Mitter C, B Farrell & DJ Futuyma, 1991. Phylogenetic studies of insect-plant interactions: insights into the genesis of diversity. *Tree* **6**, 290-293.

Monteiro WR, MD Castro, A Fahn & W Caldeira, 1995. Observations on the development of the foliar secretory cavities of *Porophyllum lanceolatum* (Asteraceae). *Nordic Journal of Botany* **15**, 69-76.

Monti D, Saettone MF, Giannaccini B & Galli Angeli D, 1995. Enhancement of transdermal penetration of dapiprazole through hairless mouse skin. *Journal of Controlled Release* **33**, 71-77.

Mopper S & D Simberloff, 1995. Differential herbivory in an oak population: the role of plant phenology and insect performance. *Ecology* **76**, 1233-1241.

Munz PA & DD Keck, 1983. *A California Flora*. University of California Press, Berkeley.

Murray CL, 1996. A p-glycoprotein-like mechanism in the nicotine-resistant insect, *Manduca sexta*. Ph.D. thesis, University of Ottawa.

Murray CL, M Quaglia, JT Arnason & CE Morris, 1994. A putative nicotine pump at the metabolic blood-brain barrier of the tobacco hornworm. *Journal of Neurobiology* **25**, 23-34.

Mutikainen P & M Walls, 1995. Growth, reproduction and defence in nettles: responses to herbivory modified by competition and fertilization. *Oecologia* **104**, 487-495.

Muzika R-M, 1993. Terpenes and phenolics in response to nitrogen fertilization: a test of the carbon/nutrient balance hypothesis. *Chemoecology* **4**, 3-7.

Myers JH & D Bazely, 1991. Thorns, spines, prickles, and hairs: are they stimulated by herbivory and do they deter herbivores? In: Tallamy, DW & MJ Raupp (Eds), *Phytochemical induction by herbivores*. Wiley, New York, 325-344.

Nawrot J & J Harmatha, 1994. Natural products as antifeedants against stored products insects. *Postharvest News ans Information* **5**, 17N-21N.

Newman RM, WC Kerfoot & Z Hanscom III, 1996. Watercress allelochemical defends high-nitrogen foliage against consumption: effects on freshwater invertebrate herbivores. *Ecology* **77**, 2312-2323.

Nihoul P, 1993. Do light intensity, temperature and photoperiod affect the entrapment of mites on glandular hairs of cultivated tomatoes? *Experimental and Applied Acarology* **17**, 709-718.

Nivsarkar M, PG Kumar, M Laloraya & MM Laloraya, 1991. Superoxide dismutase-inactivation by α-terthienyl: a novel observation in thiophene photochemistry. *Pesticide Biochemistry and Physiology* **41**, 53-59.

Ogiso T, M Iwaki & T Paku, 1995. Effect of various enhancers on transdermal penetration of indomethacin and urea, and relationship between penetration parameters and enhancement factors. *Journal of Pharmaceutical Sciences* **84**, 482-488.

Ottea JA, LG Harshman & BD Hammock, 1987. Patterns of epoxide metabolism by epoxide hydrolase and glutathione S-transferase associated with age and genotype in *Drosophila melanogaster*. *Mutation Research* **177**, 247-254.

Pardini RS, CA Pritsos, SM Bowen, S Ahmad & GJ Blomquist, 1989. Adaptations to plant prooxidants in a phytophagous insect model: Enzymic protection from oxidative stress. In Simic MG, KA Taylor, JF Ward & C von Sonntag (Eds.), *Oxygen radicals in biology and medicine*. Plenum Press, New York, 725-728.

Parrott WL, JN Jenkins & JC McCarty, 1983. Feeding behavior of first-stage tobacco budworm (Lepidoptera: Noctuidae) on 3 cotton cultivars. *Annals of the Entomological Society of America* **76**, 167-170.

Phillipson JD, 1995. A matter of some sensitivity. *Phytochemistry* **38**, 1319-1343.

Philogène BJR, JT Arnason, CW Berg, F Duval & P Morand, 1986. Efficacy of the plant phototoxin α-terthienyl against *Aedes intrudens* and effects on nontraget organisms. *Journal of Chemical Ecology* **12**, 893-898.

Philogène BJR, G Guillet & F Lorenzetti, 1997. Protection intégrée des récoltes par la résistance variétale, les ennemis naturels et les biopesticides. In Bouguerra L (Ed.), *Développement durable et introduction aux enjeux environnementaux: Principes directeurs et applications*, submitted.

Philogène BJR & JT Arnason, 1995. La résistance du maïs aux insectes phytophages: une question de molécules. *Cahiers Agricultures* **4**, 85-90.

Pickett CB, CA Telakowski-Hopkins, GJ-F Ding, L Argenbright & AYH Lu, 1984. Rat liver glutathione S-transferases: Complete nucleotide sequence of a glutathione S-transferase mRNA and the regulation of the Ya, Yb and Yc mRNAs by 3-methylcholanthrene and phenobarbital. *Journal of Biological Chemistry* **259**, 5182-5188.

Picman AK, E Rodriguez & GHN Towers, 1979. Formation of adducts of parthenin and related sesquiterpene lactones with cysteine and glutathione. *Chemical-Biological Interactions* **28**, 83.

Pimentel D, L McLaughlin, A Zepp, B Lakitan, T Kraus, P Kleinman, F Vancini, J Roach, E Graap, W Keeton & G Selig, 1991. Environmental and economic impacts of reducing US agricultural pesticide use. In Pimentel D (Ed.), *Handbook on pest management in agriculture*. CRC Press, Boca Raton, 679-718.

Pollard AJ & D Briggs, 1984. Genecological studies of *Urtica dioica* L. III. Stinging hairs and plant-herbivore interactions. *The New Phytologist* **97**, 507-522.

Pritsos CA, J Pastore & RS Pardini, 1991. Role of superoxide dismutase in the protection and tolerance to the prooxidant allelochemical quercetin in *Papilio polyxenes*, *Spodoptera eridania*, and *Trichoplusia ni*. *Archives of Insect Biochemistry and Physiology* **16**, 273-282.

Raubenheimer D & SJ Simpson, 1996. Meeting nutrient requirements: the roles of power and efficiency. In Städler E, M Rowell-Rahier & R Baur (Eds.), *Proceedings of the 9th International Symposium on Insect-Plant Relationships*, Kluwer Academic Publishers, Boston, 65-68.

Rausher MD & EL Simms, 1989. The evolution of resistance to herbivory in *Ipomoea purpurea*. I. Attempts to detect selection. *Evolution* **43**, 563-572.

Reisch J, W Spitzner & KE Schulte, 1967. *Arzneimittel Forschung/Drug Research* **17**, 816-825.

Richard C & G Boivin, 1994. Maladies et ravageurs des cultures légumières au Canada. Société d'Entomologie du Canada, Ottawa, Canada.

Rickett HW, 1966a. *Wild Flowers of the United States, Vol. 4: The southwestern states.* McGraw-Hill Book Company, New York.

Rickett HW, 1966b. *Wild Flowers of the United States, Volume 3: Texas*. McGraw-Hill Book Company, New York.

Robinson H, 1981. A revision of the tribal and subtribal limits of the Heliantheae (Asteraceae). *Smithsonian Contribution to Botany* **51**, 1-102.

Robinson T, 1974. *Science* **184**, 430-435.

Robles M, N Wang, R Kim & BH Choi, 1997. Cytotoxic effects or repin, a principal sesquiterpene lactone of russion knapweed. *Journal of Neuroscience Research* **47**, 90-97.

Rosner B, 1995. *Fundamentals of biostatistics (4th ed.)*. Duxbury Press, Washington.

Ruohomäki K, FS Chapin III, E Haukioja, S Neuvonen & J Suomela, 1996. Delayed inducible resistance in mountain birch in response to fertilization and shade. *Ecology* **77**, 2302-2311.

Sandberg Sl & MR Berenbaum, 1989. Leaf-tying by tortricid larvae as an adaptation for feeding on phototoxic *Hypericum perforatum*. *Journal of Chemical Ecology* **15**, 875-885.

Santus R, C Kohen, E Kohen, JP Reyftmann, P Morliere, L Dubertret & PM Tocci, 1983. Permeation of lysosomal membranes in the course of photosensitization with methylene blue and hematoporphyrin: study by cellular microspectrofluorometry. *Photochemistry and Photobiology* **38**, 71-77.

Saule M, 1991. *La grande flore illustrée des Pyrénées*. Éditions Milan-Randonnées Pyrénéennes, France.

Scaiano JC, C Evans & JT Arnason, 1989. Characterization of the alpha-terthienyl radical cation: evidence against electron transfer to oxygen in vitro. *Journal of Photochemistry and Photobiology B: Biology* **3**, 411-418.

Scaiano JC, A MacEachern, JT Arnason, P Morand & D Weir, 1987. *Photochemistry and Photobiology* **46**, 193.

Schultz JC, 1988. Many factors influence the evolution of herbivore diets, but plant chemistry is central. *Ecology* **69**, 896-897.

Scriber JM, 1984. Larval foodplant utilization by the world Papilionidae (Lep.): Latitudinal gradients reappraised. *Tokurana (Acta Rhopalocerologica)* **6/7**, 1-50.

Scriber JM, 1988. Tale of the tiger: Beringial biogeography, binomial classification, and breakfast choices in the *Papilio glaucus* complex of butterflies. In Spencer KC (Ed.), *Chemical mediation of coevolution*. Academic Press, New York, 241-301.

Scriber JM, 1996. A new "cold pocket" hypothesis to explain local host preference shifts in *Papilio canadensis*. *Entomologia Experimentalis et Applicata* **80**, 315-319.

Seaman FC, 1982. Sesquiterpene lactones as toxonomic characters in the Asteraceae. *The Botanical Review* **48**, 123-177.

Shower I & Y Erner, 1989. The nature of oleocellosis in Citrus fruits. *The Botanical Gazette* **150**, 281-288.

Shure DJ & LA Wilson, 1993. Patch-size effects on plant phenolics in successional openings of the southern appalachians. *Ecology* **74**, 55-67.

Simms EL, 1992. Costs of plant resistance to herbivory. In Fritz Rs & El Simms (Eds.), *Plant Resistance to Herbivores and Pathogens: Ecology, Evolution, and Genetics*, The University of Chicago Press, Chicago, 392-425.

Simms EL & MD Rausher, 1992. Uses of quantitative genetics for studying the evolution of plant resistance. In Fritz RS & EL Simms (Eds.), *Plant resistance to herbivores and pathogens: ecology, evolution, and genetics*. The University of Chicago Press, Chicago, 42-68.

Simpson SJ & D Raubenheimer, 1996. Feeding behaviour, sensory physiology and nutritional feedback: a unifying model. In Städler E, M Rowell-Rahier & R Baur (Eds.), *Proceedings of the 9th International Symposium on Insect-Plant Relationships*, Kluwer Academic Publishers, Boston, 55-64.

Singh P, 1988. Naturally-occurring thiophene derivatives from *Eclipta* species. In Lam J, H

Breteler, T Arnason and L Hansen (Eds.), *Chemistry and biology of naturally-occurring acetylenes and related compounds* (NOARC), Elsevier, New York, 179-186.

Smirle MJ & MB Isman, 1992. Metabolism and elimination of ingested allelochemicals in a homometabolous and a hemimetabolous insect. *Entomologia Experimentalis et Applicata* **62**, 183-190.

Snyder MJ, E-L Hsy & R Feyereisen, 1993. Induction of cytochrome P-450 activities by nicotine in the tobacco hornworm, *Manduca sexta*. *Journal of Chemical Ecology* **19**, 2903-2916.

Stewart JG & BJR Philogène, 1983. Reproductive potential of laboratory reared *Manduca sexta* as effected by sex ratio. *Canadian Entomologist* **10**, 81-84.

Stoner KA, 1990. Glossy leaf wax and plant resistance to insects in Brassica oleracea under natural infestations. *Environmental Entomology* **19**, 730-739.

Stryer L, 1988. *Biochemistry* (3rd ed.). Freeman and Company, New York.

Tallamy DW & MJ Raupp, 1991. *Phytochemical induction by herbivores*. John Wiley & Sons Inc., New York.

Tanaka S, T Yamaura, R Shigemoto & M Tabata, 1989. Phytochrome-mediated production of monoterpenes in thyme seedlings. *Phytochemistry* **28**, 2955-2957.

Thomas C, RS MacGill, GC Miller & RS Pardini, 1992. Photoactivation of hypericin generates singlet oxygen in mitochondria and inhibits succinoxidase. *Photochemistry and Photobiology* **55**, 47-53.

Thompson JN, 1994. *The coevolutionary process*. The University of Chicago Pres, Chicago.

Torres AM, JV Rodriguez & MM Elias, 1989. Rat kidney function related to tissue glutathione levels. Effects of different glutathione depletors. *Comprehensive Biochemistry and Physiology* **94C**, 581.

Torres AM, JE Ochoa & MM Elias, 1991. Role of lipid peroxidation on renal dysfunction associated with glutahtione depletion. Effects of vitamin E. *Toxicology* **70**, 163-172.

Tosi B, D Lodi, F Dondi & A Bruni, 1988. Thiophene distribution during the ontogenesis of *Tagetes patula*. In Lam J, H Breteler, T Arnason and L Hansen (Eds.), *Chemistry and biology of naturally-occurring acetylenes and related compounds* (NOARC), Elsevier, New York, 209-216.

Towers GHN & DE Champagne, 1988. Medicinal chemistry of the Compositae: the activities of selected acetylenes and their sulfur derivatives. In Lam J, H Breteler, T Arnason & L Hansen

(Eds.), *Chemistry and Biology of Naturally-Occurring Acetylenes and Related Compounds (NOARC)*, Bioactive Molecules, Vol. 7, Elsevier, Amsterdam, 139-149.

Towers GHN, C-K Wat, EA Graham & RJ Bandoni, 1975. Ultraviolet-mediated antibiotic activity of species of Compositae caused by polyacetylenic compounds. *Lloydia* **40**, 487-498.

Towers GHN & JT Arnason, 1988. Photodynamic herbicides. *Weed Technology* **2**, 545-549.

Turlings TCJ, JH Loughrin, PJ McCall, USR Röse, WJ Lewis & JH Tumlinson, 1995. How caterpillar-damaged plants protect themselves by attracting parasitic wasps? *Proceedings of the National Academy of Sciences* (USA) **92**, 4169-4174.

Upadhyaya MK & NH Furness, 1994. Influence of light intensity and water stress on leaf surface characteristics of *Cynoglossum officinale*, *Centaurea* spp., and *Tragopogon* spp. *Canadian Journal of Botany* **72**, 1379-1386.

Van Damn NM, TJ de Jong, Y Iwasa & T Kubo, 1996. Optimal distribution of defences: are plants smart investors? In: Städler E, M Rowell-Rahier & R Baur (eds.), *Proceedings of the 9th International Symposium on Insect-Plant Relationships*, Kluwer Academic Publishers, Boston, 307-310.

Van der Meijden E, 1996. Plant defence, an evolutionary dilemma:contrasting effects of (specialist and generalist) herbivores and natural enemies. *Entomologia Experimentalis et Applicata* **80**, 307-310.

Vasquez M, L Quijano, FR Fronczek, FA Macias, LE Urbatsch, PB Cox & NK Fischer, 1990. Sesquiterpene lactones and lignanes from *Rudbeckia* species. *Phytochemistry* **29**, 561-565.

Via S, R Gomulkiewicz, G De Jong, SM Scheiner, CD Schlichting & PH Van Tienderen, 1995. Adaptive phenotypic plasticity: consensus and controversy. *Tree* **10**, 212-217.

Vicari M & DR Bazely, 1993. Do grasses fight back? The case for antiherbivore defences. *TREE* **8**, 137-141.

Vickery VR, DE Johnstone & DK McKevan, 1974. The orthopteroid insects of Quebec and the atlantic provinces of Canada. Lyman Entomological Museum and Research Laboratory Memoir No. 1, Special Publication No. 7, 96-98

Werker E, E Putievsky, U Ravid, N Dudai & I Katzir, 1993. Glandular hairs andessential oil in developing leaves of *Ocimum basilicum* L. (Lamiaceae). *Annals of Botany* **71**, 43-50.

Whitham TG & S Mopper, 1985. Chronic herbivory: Impacts on architecture and sex expression of pinyon pine. *Science* **228**, 1089-1091.

Wilcox JA, 1983. In: Arnett RH Jr (Ed.), *Checklist of the beetles of North and Central America and the West Indies*. Vol. VIII, Flora and Fauna Publications, Gainesville.

Wilkens RT, GO Shea, S Halbreich & NE Stamp, 1996. Resource availability and the trichome defenses of tomato plants. *Oecologia* **106**, 181-191.

Williams MC, 1990. Control of annual goldeneye (*Viguiera annua*) on rangeland. *Weed Technology* **4**, 661-662.

Williams MC, 1989. Accumulation of nitrate by annual goldeneye and showy goldeneye. *Journal of Range Management* **42**, 196-198.

Wilson EO, 1992. *The diversity of life*. Belknap Press, Cambridge, MA.

Witham TG, J Maxchinski, KC Larson & KN Paige, 1991. Plant responses to herbivory: the continuum from negative to positive and underlying physiological mechanisms. In Price PW, TM Lewinsohn, GW Fernandes & WW Benson (Eds), *Plant-animal interactions: Evolutionary ecology in tropical and temperate regions*. Wiley, New York, 227-256.

Yamane MA, AC Williams & BW Barry, 1995b. Terpene penetration enhancers in propylene glycol/water co-solvent systems: Effectiveness and mechanism of action. *Journal of Pharmacy & Pharmacology* **47**, 978-989.

Yamane MA, AC Williams & BW Barry, 1995a. Effects of terpenes and oleic acid as skin penetration enhancers towards 5-fluorouracil as assessed with time: Permeation, partitioning and differential scanning calorimetry. *International Journal of Pharmaceutics* **116**, 237-251.

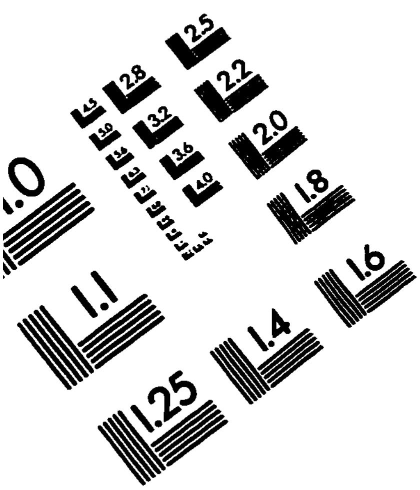
Yamaura T, S Tanaka & M Tabata, 1989. Light-dependent formation of glandular trichomes monoterpenes in thyme seedlings. *Phytochemistry* **28**, 741-744.

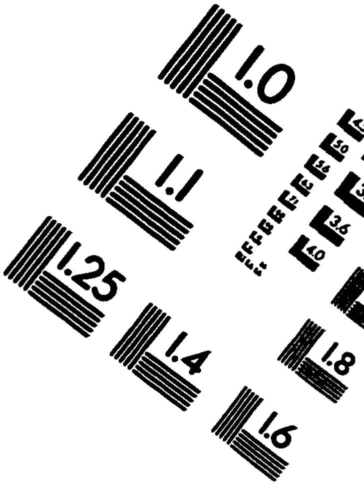
Yao K, V De Luca & N Brisson, 1995. Creation of a metabolic sink for tryptophan alters the phenylpropanoid pathway and the susceptibility of potato to *Phytophthora infestans*. *The Plant Cell* **7**, 1787-1799.

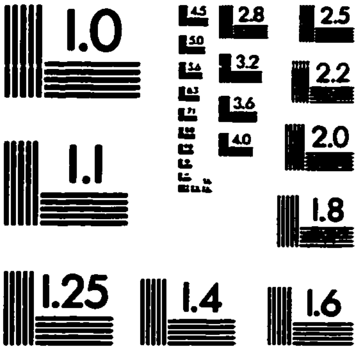
Zangerl AR & FA Bazzaz, 1992. Theory and pattern in plant defense allocation. In RS Fritz & EL Simms (Eds.), *Plant Resistance to herbivores and pathogens: Ecology, evolution, and genetics*. The University of Chicago Press, Chicago, 363-391.

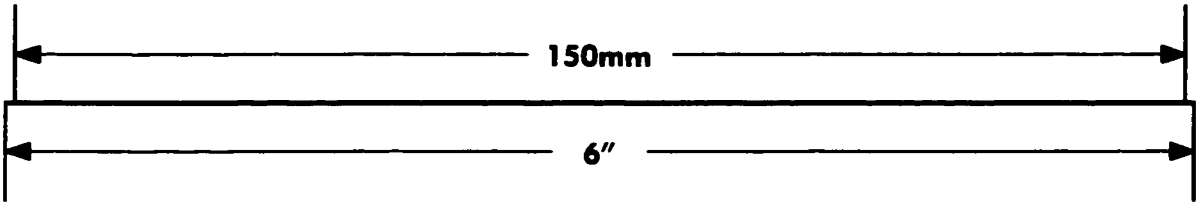
Zheng JA, AE Mitchell, AD Jones & BD Hammock, 1996. Haloenol lactone is a new isozyme-selective and active site-directed inactivator of glutathione S-transferase. *Journal of Biological Chemistry* **271**, 20421-20425.

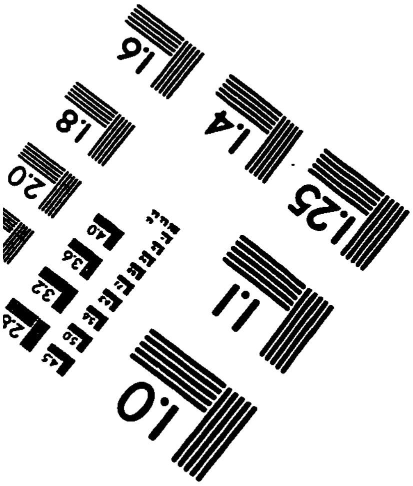
IMAGE EVALUATION
TEST TARGET (QA-3)

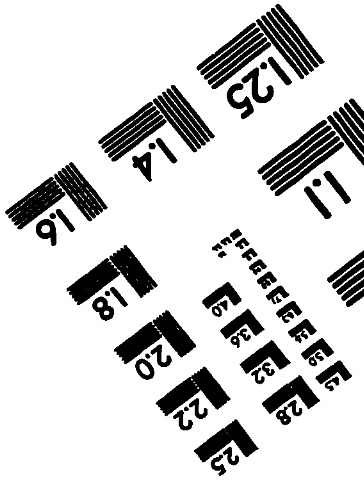












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