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Effect of Lignans in Associations with Naturally Occurring Allelochemicals from the Asteraceae on the Detoxification Enzymes and the Life Cycle of a Herbivorous Lepidoptera, Ostrinia nubilalis, Hubner

by

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"Thesis submitted to the School of Graduate Studies as partial fulfillment of the requirements for the degree of Master of Science"

at the

University of Ottawa

ISBN 0-315-53769-8

ABSTRACT

Lignans are phenylpropanoid secondary metabolites often containing a methylenedioxyphenyl group which can inhibit the cytochrome P-450 dependant oxidation enzymes (mixed function oxidases: MFOs) utilized by insects for the detoxification of insecticides. Such an inhibition of the MFOs in the European corn borer (ECB), Ostrinia nubilalis, by a selected lignan, dillapiol, was postulated to potentiate the insecticidal activities of representative allelochemicals from the family of the Asteraceae, a polyacetylene , phenylheptatriyne and a sesquiterpene lactone, tenulin.

In an in vitro system containing microsomes from the epithelial cells of the midgut wall of the ECB, three lignans, sesamolin, dillapiol and diasesartemin, which possess the methylenedioxy group, inhibited the activity of the ECB MFOs, as measured by aldrin epoxidase. The other allelochemicals had little effect in vitro. However, in vivo and in contrast to the in vitro resultslarvae fed during four days on diets containing dillapiol, PHT and tenulin at the concentrations of 100, 100 and 1000 ug/g respectively in a meridic diet, showed a strong induction of the epoxidase activity.

A study of the effects of the compounds on the entire life cycle of the insect revealed that, contrary to the hypothesis proposed, dillapiol did not synergize the activity of either of the two model secondary metabolites from the Asteraceae. Rather, dillapiol was toxic to the ECB larvae, and inhibited their growth. Highest mortality was observed with dillapiol alone while mixtures with other allelochemicals often caused less mortality. In growth of surviving insects, there were some additive but not synergistic effects of the allelochemicals. The induction of MFO evident after 4 days, appears to explain these results and could constitute a highly successful adaptation of this polyphagous species to these particular types of allelochemicals present in the host plants.

The results indicate that plant-insect interactions are complex and cannot be properly assessed on the basis of short term in vitro experiments. Allelochemical mixtures that are synergistic in some situations, may actually be counterproductive defense measures once insects have adapted to them.

RESUME

Les lignans sont des métabolites secondaires dérivés des phénylpropanes. Le groupe méthylènedioxyphenyl qu'ils contiennent est souvent un inhibiteur des enzymes d'oxidations multiples à cytochrome P-450 utilisées par l'insecte pour détoxifier de nombreuses substances étrangères. Notre hypothèse de travail était que l'inhibition des enzymes de détoxification chez pyrale du mais (ECB), Ostrinia nubilalis, par un lignan sélectionné, le dillapiole, , pouvait potentialiser l'activité insecticide de deux substances allélochimiques représentatives de la famille des Astéraceae, le polyacétylène phénylheptatriyne, et la lactone sesquiterpène ténuline.

Trois lignans, la sésamoline, le dillapiole et la diasésartemine, contenant le groupe méthylène-dioxyphenyl, sont des inhibiteurs de l'aldrin époxidase dans un système in vitro contenant la fraction microsomale des cellules épitheliales de la paroi de l'intestin moyen de la pyrale du mais. Les autres substances naturelles testées n'ont que peu d'effet sur ce système. Cependant, dans un système in vivo, on observe une forte induction de l'activité de l'epoxidase lorsque les larves d'insectes sont nourries pendant

quatre jours avec des concentrations de dillapiole, de phénylheptatriyne et de ténuline respectivement de 100, 100 et 1000 ug/g dans une diète semiraffinée, ce qui contraste avec les résultats in vitro.

Une étude de l'effet de ces composés sur le cycle vital de l'insecte montre que le dillapiole n'agit pas comme synergiste de l'activité insecticide de l'un ou l'autre des métabolites secondaires types des Astéraceae. Au contraire le dillapiole est en lui même toxique pour les larves de la pyrale du mais, en diminuant leur prise de poids et en retardant leur développement. Les effets sont plus importants avec le dillapiole seul plutôt qu'en association avec le PHT ou avec la ténuline. L'induction enzymatique observée au bout de quatre jours, paraît expliquer ces résultats et pourrait représenter une adaptation de la pyrale à un type particulier de composés chimiques rencontré dans ses plantes hôtes .

Ces résultats indiquent que les relations plante-insectes sont complexes et ne peuvent être évaluées par des expériences in vitro à court terme. Des mélanges de composés allélochimiques qui se potentialisent dans certaines situations, peuvent en fait avoir des effets inverses lorsque les insectes y sont adaptés.

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my supervisors, Drs J. Thor Arnason and Bernard J.R. Philogène, not only for their help, the constant and patient interest they showed in this work and the always constructive and friendly advice, but especially for introducing me to the fascinating field of plant-insect interactions. I greatly appreciated their availability and understanding during the course of these studies. I am also grateful to my advisors, Drs. Pearl Weinberger and D. Gardner for their guidance during committee meetings.

My studies have been greatly helped by the several unique compounds provided by Dr Jorgen Lam (Department of Chemistry, University of Aarhus, Denmark), thanks to whom the present work is original. Dr Tom Waddell (Department of Chemistry, University of Tennessee at Chattanooga, USA) provided the sesquiterpene lactone, while large quantities of PHT synthetized by A. MacEachern allowed me to perform part of the experiments. The hospitality of Drs. David Peakall and Sean Kennedy together with the technical help of Suzanne Trudeau (National Wild Life Research Center, Hull) was greatly appreciated.

The students in the laboratory, Sesh Iyengar, Robert Chenier, Cathy MacDougal and Elly Kourany among others, contributed towards creating a friendly and invigorating atmosphere. Special thanks go to Natalie Donskoff, both a dear friend and a great help in the laboratory, and to Dr Alok Sen who corrected this manuscript several times with attention and patience.

Finally, I would like to thank my husband Philippe who guided my first steps in laboratory research, as well as my children, Henri and Camille, for their help and patience in spite of randomly scheduled family activities. The moral support and stimulating interest of my parents overseas also contributed to my enthusiasm for the present work.

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LIST OF ABREVIATIONS USED IN THE TEXT

CUB.....Cubebin

DIA.....Diasesartemin

DIL.....Dillapiol

ECB.....European corn borer

EPI.....Epiyangambin

MDP.....Methylenedioxyphenyl

MFO.....Mixed function oxidases

PE.....Petroleum ether

PHT.....Phenylheptatriyne

PHE.....Phenobarbital

PIP.....Piperonyl butoxide

SES.....Sesamin

TEN.....Tenulin

1. INTRODUCTION

Plant characteristics such as trichomes or a hard cuticle have long been recognized as an evolutionary response to herbivory (Darwin, 1859). Since Fraenkel's landmark paper (1959) on plant-insect interactions, it has become generally accepted that chemicals play an important part in plant defense mechanisms. Ehrlich and Raven (1964) suggested a pattern of co-evolution in which plants and insects have continually adapted to one another. Through evolutionary time, chemical defense adaptations in plants involved the synthesis of secondary metabolites, i.e. chemicals that have no known role in the basic physiology of the plant, as opposed to the primary or intermediary metabolites which support the plant's vital processes. These secondary chemicals are also referred to as 'allelochemicals' when their deterrent properties have been defined. The diversity of

chemical structures (now estimated to be in the tens of thousands; Haslam, 1986), are produced via metabolic pathways, distinct from, but linked to the pathways used for primary metabolites. They may be found in all parts of the plant, where they are usually stored in specific tissues or organs such as the cuticle, vacuoles, canals or glands, and often in larger quantities at leaf margins or other locations possibly best suited for a first line protection (Swain, 1977).

Insect response to plant defence compounds may include behavioral adaptation (Dethier, 1937; Frazier,1986), acquisition of physiological counterstrategies such as the development of insensitivity in the target site (Brooks, 1976; Berenbaum, 1986), or special excretory and sequestration mechanisms (Rothschild & Edgar, 1978; Brattsten,1986; Mullin, 1986), all contributing to eliminate potential toxins from sensitive targets. However, the diversity of secondary metabolites suggests that polyphagous herbivorous insects encounter many new molecules, and that no particular detoxification can be forecast. In insects the detoxification of these foreign molecules is achieved through a complex of several enzymes catalysing general reactions such as oxidations, hydroxylations,

reductions or group transfers (Ahmad et al., 1986). By far the most important of these detoxification processes is a pool of oxidases with a cytochrome P-450 as prosthetic group, the mixed function oxidases (MFO) (Casida, 1970), or also known as polysubstrate monooxygenases (PMSO) (Ahmad et al., 1986). Numerous publications have appeared in the literature on this metabolic mechanism by which insects resist poisoning by synthetic or botanical insecticides (see reviews in Metcalf, 1967; Brattsten, 1979, 1986; Hodgson, 1985). It has been shown that persitency and effectiveness of the toxic agents are dependent on activity of the MFOs (Hodgson, 1985).

Some plants have developed, presumably through selective pressure of herbivory, a counter-strategy that involves the ability to synthesize inhibitors of the MFOs. These inhibitors usually occur in plants, together with other allelochemicals, and can possibly enhance the deterrent effect of these toxins (Berenbaum & Neal, 1985).

Plants often contain not only one class of allelochemicals, but many distinct biosynthetic groups. For instance, species of Asteraceae may contain

lignans, acetylenes and sesquiterpene lactones in a single plant. The relevance for a plant to involve energy in the synthesis of several molecules when a single one might sufficiently protect it is still largely uninvestigated. The allelochemical pools could be seen as remainders of separate attempts in plants to develop chemical defenses during evolution, resulting in strategies independent of one another. Alternately, an interaction between these defensive chemical compounds could occur, resulting in a synergistic effect between two or more compounds. Such a strategy would have the advantage of efficiently protect the plant at minimal energy cost, and avoid or delay the appearance of resistance in polyphagous insects.

1.1 HYPOTHESIS OF THE PRESENT STUDY

The present study was undertaken to test the hypothesis that in the plant family Asteraceae , several groups of natural allelochemicals can interact and achieve greater biological activity. Members of this plant family contains in particular lignans with a methylenedioxyphenyl group (MDP) that is similar to that of most insecticide synergists and known to inhibit

MFOs. Are then lignans in the Asteraceae also enhancing the toxicity to insects of other co-ingested allelochemicals? This hypothesis was investigated using representative compounds from the Asteraceae, including phenylheptatriyne (PHT), a characteristic polyacetylene, tenulin (TEN), a sesquiterpene lactone, and several lignans , with particular emphasis on dillapiol (DIL). The effects of these compounds was determined on a model insect, the European corn borer, Ostrinia nubilalis, which is a highly polyphagous herbivore feeding on many species of Asteraceae.

1.2. LITERATURE REVIEW

1.2.1 PLANT ALLELOCHEMICALS

1.2.1.1 LIGNANS

Lignans are a group of natural products widely distributed in plants. More than 200 lignans found in about 50 plant families and 146 species have been identified (MacRae & Towers, 1984), and new occurrences of these structures are continually being reported in plants, as well as recently, in human urine and blood

(Axelson et al., 1982). They may be found in Gymnosperms as well as in Angiosperms, and are most abundant in the Pinaceae, Podophyllaceae, Rutaceae and Lauraceae, with only one occurrence in the monocotyledons and a few in the ferns. The Asteraceae contain several lignans (Table 1), but they do not presently seem to be a predominant allelochemical of the family, when compared to the acetylenes or sesquiterpene lactones, for instance. However, in the Asteraceae, sesamin-type lignans occur in sufficient abundance and variety to constitute a useful taxonomic criterion within the tribe Artemisia (Greger, 1981). Lignans may constitute a large proportion of the total content of secondary metabolites in plants; for instance, in Sesamum angolense (Pedaliaceae), sesamin and sesangolin, were found to constitute up to 6.4% of the oil extract from fresh roots (Jones et al., 1962).

Lignans, strictly speaking, are dimers of phenylpropanoid units (C6-C3), linked by the central carbon of their side chain and are formed in plants from the C6-C3 amino acids phenylalanine or tyrosine (Hayworth, 1936). However, phenylpropanoid monomers such as dillapiol (Fig. 5) or myristicin (Fig. 4) will be considered, in this study, with the group of lignans with which they shre important structural features and

TABLE 1 : LIGNANS FROM THE ASTERACEAE

GENUS	SPECIES	LIGNAN	LOCATION IN PLANT	PHYSIOLOGICAL EFFECTS	REFERENCE
Anacylus	pyrethrum	(+)-sesamin		synergist	Burden & Crombie, 1969a.
Arctium	lappa	lappaol matairesinol arctiin arctigenin	fruits seeds	insecticide synergist antifungal	Shinoda & Kawagoye, 1929.
	minus loiospermun	arctiin	roots seeds	insecticide	Yakhontova & Kibal'chich, 1971
Artemisia	absinthium	sesartemin episesartemin diasesartemin	young shoots	insecticide acaricide	Greger, 1981.
	fragrans	sesamin-type			Bohlman et al., 1973.
Carphephorus	odoratissimus	eudesmin epieudesmin	all parts	-	Wahlberg et al., 1972.
Carthamus	tinctorius	tetracheloside matairesinoside	seeds	laxative tasteless	Nishibe et al., 1972.
		2-hydroxyarctiin	seeds		Palter et al., 1972.
Cardinae	spp.	arctiin-like	seeds	bitter	Haensel et al., 1964.
Centaurea	spp.	arctiin-like	seeds	bitter	Haensel et al., 1964.
Chrysanthemum cinerariaefolium		sesamin	flowers	insecticide	Doskotch & El-Feraly, 1969.
C.	fructescens	sesamin	roots	synergist	
Cnicus	benedictus	arctigenin arctiin trachelogenin nortracheloside 2-acetylnortrache- -loside salonitenolide	fruit leaves	antibacterial antitumoral	Vanhaelen & Vanhaelen, 1975.
Erigeron	pappochroma	dillapiol	all parts	antitumoral	Soeronsen & Soeronsen, 1969.
Heliopsis	scabra	helianthoidin helioxanthin	roots	insecticide	Burden et al., 1969.
Lappa	major minor tomentosa	arctiin arctiin arctiin			Lindner, 1948.
Sylybum	marianum	dihydrodiconife- -ryl alcohol silychristin	seeds	-	Weinges et al., 1970. Wagner et al., 1971.
	eburneum	silydianin silybin	seeds	-	Haensel et al., 1972. Koch, 1975.

biological activities (Mukerjee et al., 1979; Berenbaum and Neal, 1985). Deamination of the amino acid precursor by phenylalanineammonia lyase (often in the form of co-A esters), leads to various phenylpropanoids and phenols such as flavonoids, stilbenes, coumarins, cinnamyl esters, lignans, lignins, or polyphenols (Rao. 1978). The common biosynthetic pathway of these phenylpropanoids is shown in Fig. 1. The most abundant form of lignans found in nature, have their two phenylpropanoid units linked by the third carbon of their propane chain (Fig. 2). Permutations of the initial junction of the C6-C3 units may lead to structures such as norhydroguairaretic acid (Fig. 3a), bitetrahydrofuranoids such as pinoresinol or asarinin (Fig. 3b), or also dibenzylbutyrolactones such as cubebin (Fig. 5), savinin, hinokinin, podophyllotoxin, conidendrin or justicin (Fig 3c). Other natural phenols may be involved with C6-C3 units; the compound silychristin (Fig. 3d), from Silybum marianum (Asteraceae), for example, results from a C6-C3 unit and a flavononol unit. As it is often the case with other allelochemicals, such as flavones, for instance, lignans are sometimes linked to sugars, as in matairesinoside (Fig. 3d) and tetracheloside, both from Trachelospermum sp.(Apocynaceae), or from Carthamus tinctorius (Asteraceae).

FIG 1 : BIOSYNTHETIC PATHWAYS OF LIGNANS AND RELATED PHENYLPROPANOIDS.

(Adapted from Towers and Wat, 1979).

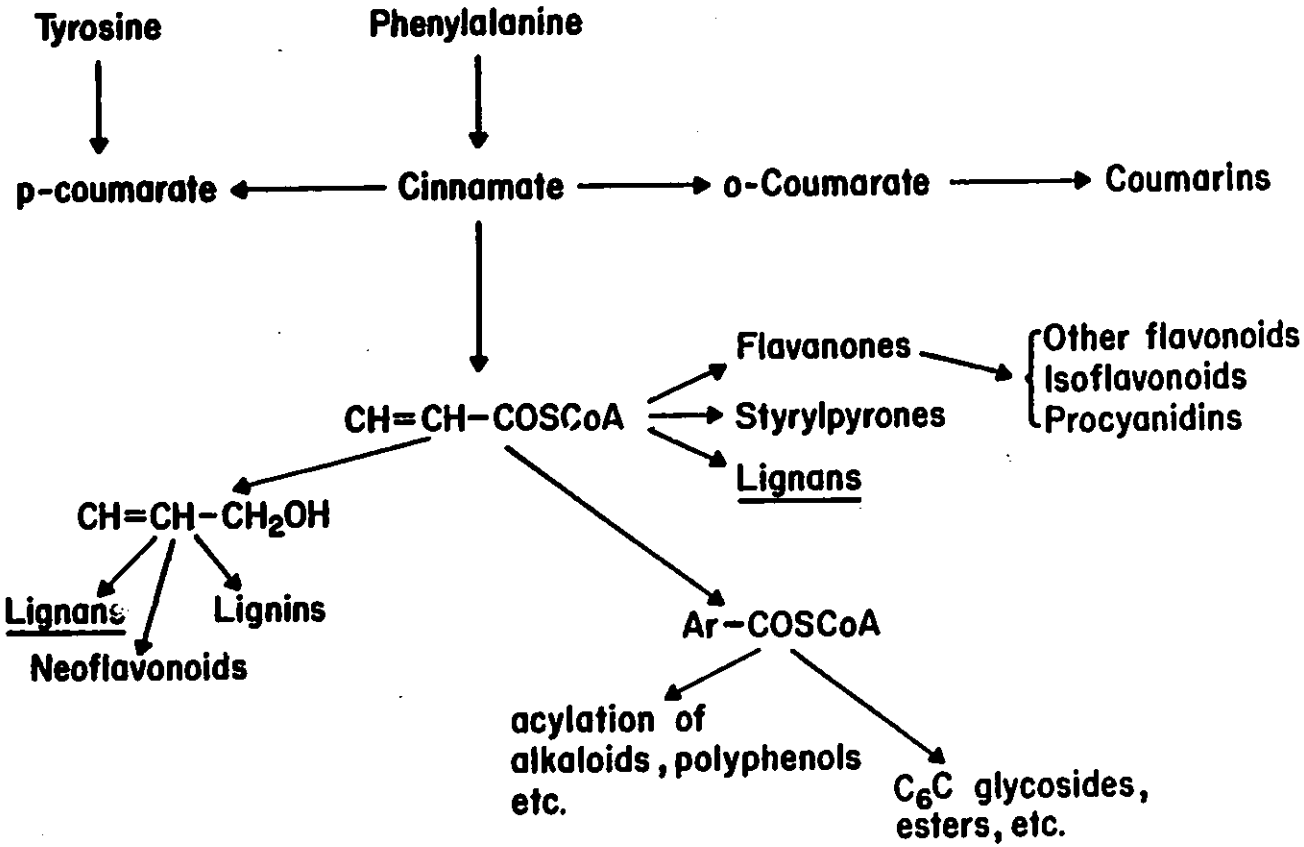

Tyrosine
Phenylalanine
p-coumarate
Cinnamate
o-Coumarate
Coumarins
Flavanones
Other flavonoids
Isoflavonoids
Procyanidins
CH=CH-COSCoA
Styrylpyrones
Lignans
CH=CH-CH2OH
Lignans
Lignins
Neoflavonoids
Ar-COSCoA
acylation of
alkaloids, polyphenols
etc.
C6C glycosides,
esters, etc.

FIG 2 : LIGNAN BASIC SKELETON: The two phenylpropanoid units are linked by the central carbons of their side chains. (Adapted from Rao, 1978).

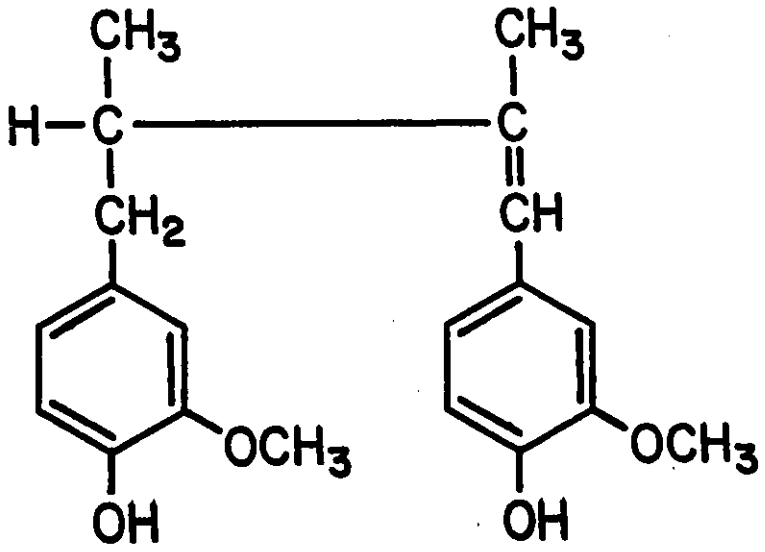

CH3
H
C
CH2
OCH3
OH
CH3
C
CH
OCH3
OH

FIG 3 : SOME REPRESENTATIVE LIGNANS

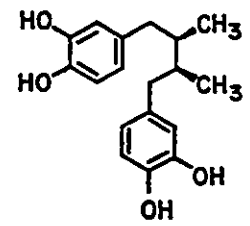
A. Lignan with the original C4 junction

B. Bitetrahydrofuranoids or substituted furans

C. Dibenzylbutyrolactones or diarylbutanes

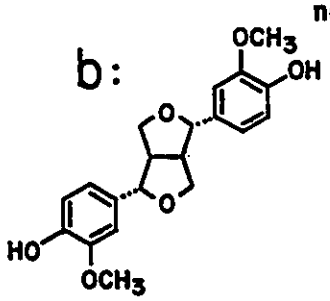
D. Lignans linked to other structures

a:

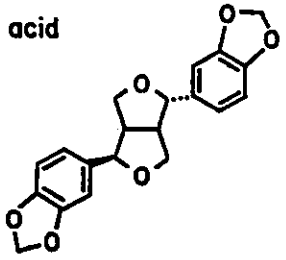


nor hydroguaiaretic acid

b:

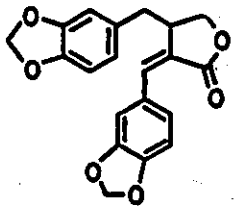


pinoresinol

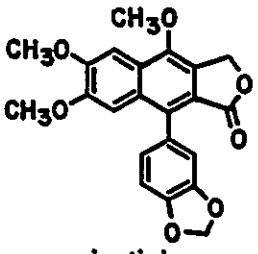


asarinin

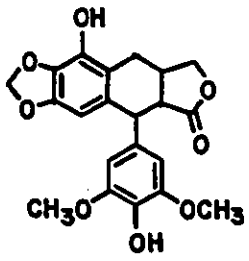
c:



savinin

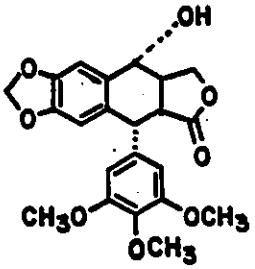


justicin



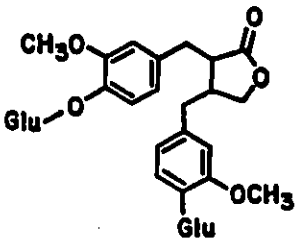
peltatin

c:



podophyllotoxin

d:



matairesinoside

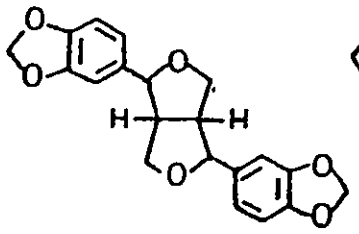
Lignans have a wide range of biological activities that have recently been reviewed by MacRae and Towers (1984). They constitute a useful tool for medicine as well as in the fight against crop pests. Podophyllotoxin (Fig. 3c), and some of its deoxy and demethylated derivatives from Hyptis spp. (Labiatae), and peltatin (Fig. 3c) from Linum album (Linaceae), are antimitotic agents (German, 1971), active against lymphocytic leukemia (Weiss at al., 1975), or against sarcoma 37 in mice (Kier et al., 1963). Norisoguaiacin showed antibacterial activity against several Strepcoccus species (Oliveto, 1972). The resinous exudate of creosote bush, Larrea tridentata (Zygophyllaceae) contains the potent antioxidant norhydroguaiaretic acid (Fig. 3a), a lignan that is used in commerce to prevent rancidity in animal or vegetable fats, as well as the oxidative degradation of vitamin A and E preparations (Mabry & Ulubelen, 1980). High yields of conidendrin, also an antioxidant, were extracted from Picea and Tsuga species through a commercial process developed in two Canadian laboratories, Forintek Canada Corp. and MacMillan Bloedel Ltd (reference in Mabry & Ulubelen, 1980).

Only a few biological activities have been reported in insects. The lignan peltatin A from Librocedrus bidwillii, and deoxypodophyllotoxin from several species of Podophyllum and Juniperus, were found to be toxic to the housefly Musca domestica, and the codling moth, Laspeyresia pomonella (Russell et al., 1976). Also, weak juvenile hormone activity was reported with sesamin and sesamolin towards the milkweed bug, Oncopeltus fasciatus (Bowers, 1968). Growth inhibition and antifeedant properties were observed with several lignans of the podophyllotoxin family, and the biepoxylignans kobusin and sesamin (Russell et al, 1976; Kamikado et al, 1975; Wada & Munakata, 1970).

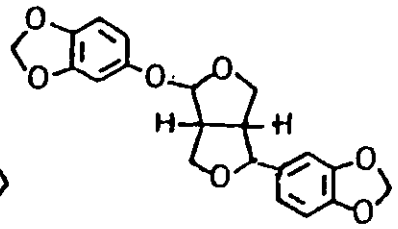
However, lignans alone have limited activity on insects, and a large number of them, e.g. myristicin, do not appear to have any noticeable activity at concentrations naturally occurring in plants (Berenbaum & Neal, 1985). Their role comes out when they are in association with synthetic pesticides or, in fewer reported instances, with other allelochemicals . Sesamin, sesamolin, sesangolin, asarinin, myristicin, savinin, hinokinin (Fig. 3 and 4), all of plant origin, are effective in enhancing a wide variety of insecticides (Casida, 1970), particularly pyrethrins and

FIG 4 : METHYLENEDIOXYPHENYL COMPOUNDS WITH SYNERGISTIC ACTIVITY ON INSECTICIDES

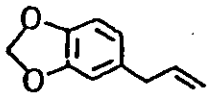
(Adapted from MacRae and Towers, 1984)



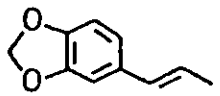
sesamin



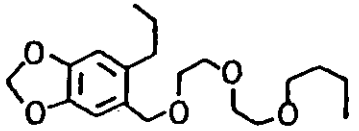
sesamolin



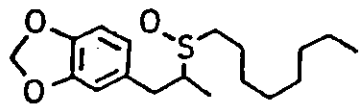
safrole



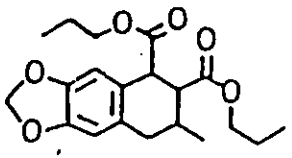
isosafrole



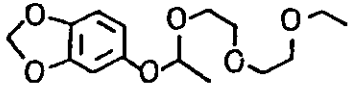
piperonyl butoxide



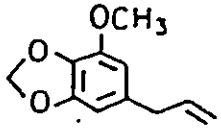
sulfoxide



propyl isome



sesamex



myristicin

carbamates, and led to the synthesis of the most active, numerous and economically important insecticide synergists.

Most of the lignans that are synergists of pesticides (Fig. 4), contain a methylenedioxyphenyl (MDP) group, a structure that appears to be responsible for the inhibition of the cytochrome P-450 detoxification system in animals, and in insects in particular (Metcalf, 1967). MDP compounds are widespread among plants (Newman, 1962). In several families, they occur together with other allelochemicals with known toxic, growth reducing, or otherwise fitness reducing properties towards insects (section 1.2.3). The possibility exists, then, that these MDP compounds act as natural synergists, interfering with MFOs in insects to increase the adverse effects of the other allelochemical(s) ingested simultaneously. If such a mechanism occurs in plants, it may allow them to reduce the quantity of allelochemicals produced, without affecting total efficacy of their defense strategy (Janzen, 1973). However, this aspect of plant defense is still largely uninvestigated. The large family Asteraceae appeared as a promising family to investigate since it contains , in addition to several

lignans (Table 1, Fig. 5), two groups of biologically active secondary metabolites with modes of action in insects fairly well understood, namely sesquiterpene lactones and polyacetylenes.

1.2.1.2 SESQUITERPENE LACTONES

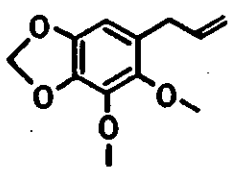
Sesquiterpene lactones are terpenoid compounds characteristic of the Asteraceae which account for 90% of the some 2000 known structures, with only a few occurrences in other angiosperm and non-angiosperm families (Seaman, 1982). They are formed by 3 five-carbon isoprene units (C-15), linked covalently, and the chains thus formed can cyclize to produce a bicyclic compound containing 2 double bonds, as seen for instance in biologically active sesquiterpene lactones (Fig. 6). Their biosynthetic pathway is related to that of squalene, triterpenoids, steroids, caretenoids and rubber.

Together with alkaloids, sesquiterpene lactones are usually the cause of bitterness in plants, and are mainly located in leaves and heads of the plant, where they can constitute up to 5% of the dry weight (Hegnauer, 1977). Picman (1986) recently reviewed the

FIG 5 : LIGNANS USED IN THE EXPERIMENTS OF THE PRESENT STUDY : Dillapiol (DIL), Diasesartemin (DIA), and Epiyangambin (EPI) are found in Artemisia spp. (Asteraceae). Cubebin (CUB) comes from Piper cubeba (Piperaceae), and sesamolin (SES) is a by product of sesame oil from Sesamum spp. (Pedaliaceae).

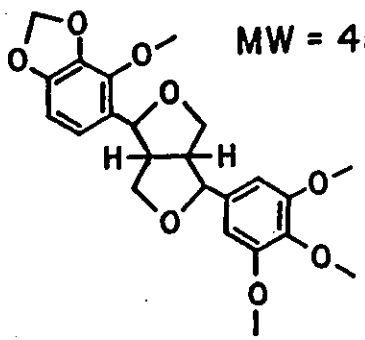
Dillapiol

MW = 222



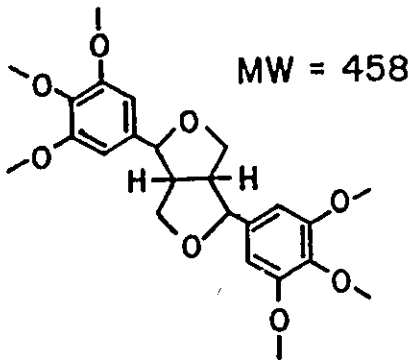
Diasesartemin

MW = 454



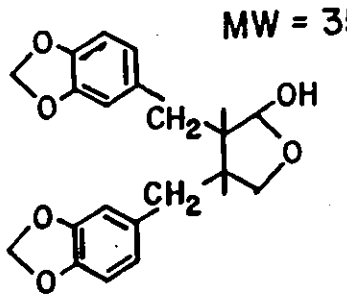
Epiyangambin

MW = 458



Cubebin

MW = 356



Sesamolin

MW = 370

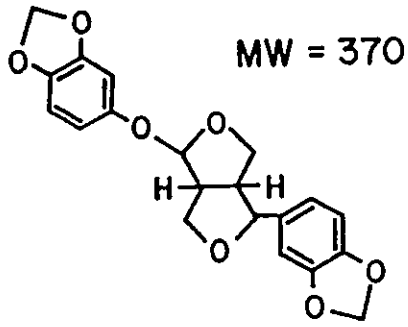
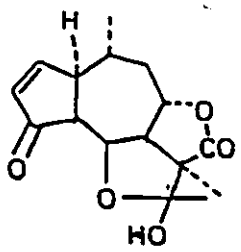
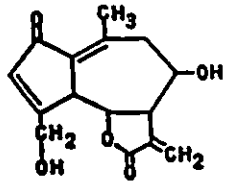


FIG 6 : STRUCTURES OF SOME BIOLOGICALLY ACTIVE SESQUITERPENE LACTONES.

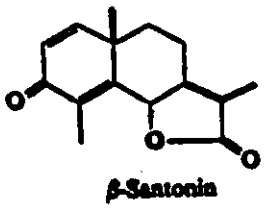
Helenalin, lactucin and tenulin (TEN) have pharmalological activity (sedative and analgesic), parthenin has allelopathic properties. Insect antifeedants include tenulin, santonin, parthenin and helenalin.



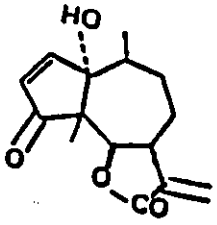
Tenulin



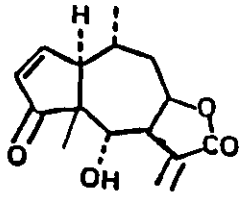
Lactucin



β-Santonin



Parthenin



Helenalin

numerous biological activities of sesquiterpene lactones, namely anti-tumor, anti-fungal, anti-bacterial, antiparasite, as well as adverse effects on insects. The antifeedant properties of this group of terpenes have recently been emphasized (Van Beek and de Groot, 1986), and exemples of their structures are given in Fig. 6. Their role in plant defense mechanisms against herbivores appears to be important, and the activities reported on 17 different insect species include feeding and oviposition deterrency, growth inhibition, lengthened development, reduction in pupal and adult weights and mortality (Picman, 1986; Arnason et al., 1987; Isman and Rodriguez, 1983). The increased concentration of sesquiterpene lactones found in the leaves of plants submitted to herbivory (Gershenzon et al, 1987) further shows the importance of sesquiterpene lactones in plant defense strategies.

Present in active sesquiterpene lactones such as parthenin, from Parthenium hysterophorus , coronopilin from Iva xanthifolia and Parthenium spp., and helenalin from Helenium spp. (Fig. 6), the α-methylene-γ-lactone moiety was found to be toxic to the confused flour beetle, Tribolium confusum (Picman and Picman, 1984), when the allelochemicals were included in its diet . In a survey of 12 sesquiterpene lactones from the

Asteraceae, Isman and Rodriguez (1983) found that toxicity to the larvae of the tobacco budworm, Heliothis zea increased with the degree of oxygenation of the molecule. Moreover, neutralization of the cardiac-inhibiting properties of parthenin in the migratory grasshopper, Melanoplus sanguinipes, by the addition of thiol agents such as cysteine, homocysteine and dithiothreitol (Picman et al, 1981), suggested the possible interference of sesquiterpene lactones with free sulfhydryl groups in the insect. The possibility of adduct formation between sesquiterpene lactones and thiol-containing proteins was demonstrated in-vitro (Hall et al., 1977), and recent investigations suggest that a similar mechanism probably occurs in-vivo (Arnason et al., 1987).

1.2.1.3 POLYACETYLENES

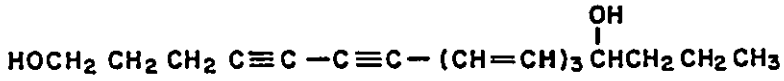
Polyacetylenes from plants are an unusual group of substances because of their considerable degree of unsaturation and high reactivity (Bohlman et al, 1973). In the Angiosperms, they are almost all restricted to 5 advanced plant families (Araliaceae, Campanulaceae, Asteraceae, Santalaceae and Apiaceae), with rare occurrences among other families such as Fabaceae

(wyerone), or Solanaceae (falcorindiol) (Hegnauer, 1977). Polyacetylenes from the Asteraceae, the most numerous of some 750 identified structures, distinguish themselves by a high degree of unsaturation and cyclization of the fatty acid precursors, forming aromatic, furanoid, thiophenic or spiroketal compounds. They are distributed in all parts of plants, although roots, stems, leaves, or fruits may each have a unique polyacetylene profile (Wrang and Lam, 1975).

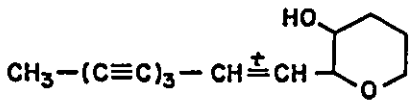
Polyacetylenes have a broad range of bioactivities including neurotoxicity to fish, livestock or humans (Bohlman et al., 1973) (Fig. 7), toxicity to nematodes (Uhlenbroek and Bijloo, 1958; Wat et al., 1981), fungi (Bourque et al., 1985) and several microorganisms (Towers, 1980; Arnason et al., 1981), or include phytoalexins such as safynol (Fig. 7) or wyerone (Hargraves et al., 1976). Studies on insects dealing with these secondary metabolites, originating mainly from two research centers, the University of British Columbia and the University of Ottawa, showed that polyacetylenes, as well as their thiophene derivatives, had a photosensitizing and a light independent toxicity (Arnason et al., 1980). One compound, alpha-terthienyl, is a potent phototoxin to mosquito and blackfly larvae as well as to the immature

FIG 7 : STRUCTURE OF SOME BIOLOGICALLY ACTIVE POLYACETYLENES AND THEIR DERIVATIVES.

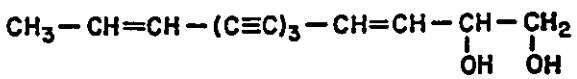
Neurotoxins include cicutonin and ichtyotherial, phytoalexins include wyerone and safynol, deydromatricaria ester is ovicidal. Alpha-terthyenyl and phenylheptatriyne (PHT) are phototoxic insecticides.



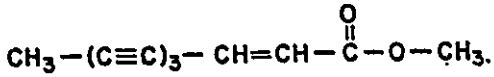
CICUTONIN



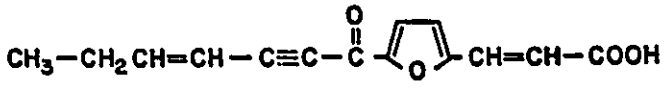
ICHTHYOTHERIAL



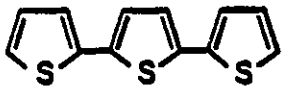
SAFYNOL



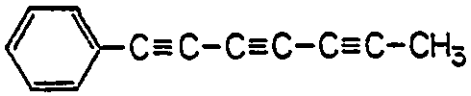
DEHYDROMATRICARIA ESTER



WYERONE



ALPHA-TERTHIENYL



7-phenylhepta-2,4,6-triyne (PHT)

stages of several species of Lepidoptera (Arnason et al., 1981; Philogene et al., 1985; Champagne et al., 1986), and its use as a possible pest control agent is presently under investigation.

1.2.2 THE EXPERIMENTAL ALLELOCHEMICALS

1.2.2.1 THE LIGNANS

Dillapiol (DIL: 3-[3,4-methylenedioxy-5,6-dimethoxyphenyl]-1-propene)(Fig. 5), sometimes called dimethoxysafrole, is commonly isolated from the essential oil of Piper novae-hollandiae Miq. (Piperaceae), a forest vine growing as a tree-top canopy (Loder et al., 1969), and also more recently from an Asteraceae, Artemisia scoparia (Greger, 1981). It is a major constituent of Indian dill, Anethum sowa, left as a waste product in the processing of this condiment. It occurs in P. novae-hollandiae together with at least 10 amides such as piperine, chavicine, fagaramide, already identified from other Piper species (Karrer, 1958). In traditional medicine, it was used to relieve sore gums and against gonorrhoea. The alcoholic wood extracts showed activity against implanted Lewis lung carcinoma in mice (references in Loder et al., 1969). In addition, DIL reflected significant synergism as

compared to piperonyl butoxide or safrole (Fig.4) when used in combination with N-methyl carbamate and pyrethrins against adult red flour beetles, Tribolium castaneum (Saxena et al., 1978; Mukerjee et al., 1979).

Diasesartemin (DIA: 2-[2-methoxy-3,4-methylenedioxyphenyl]-6-[3,4,5-trimethoxyphenyl]-3,7-dioxabicyclo {3,3,0} -octane)(Fig. 5), was isolated together with 13 other lignans from the roots of Artemisia absinthium, where it constitutes one of the major components of the resinous moeity. It accumulates in the schizogenous secretory canals of the plant (Greger, 1982). There is no record of its biological activity.

Epiyangambin (EPI: 2,6-[3,4,5-trimethoxyphenyl]-3,7-dioxabicyclo {3,3,0} octane)(Fig. 5), also called lirioresinol- A-dimethylether, was isolated from several species of Artemisia (Greger and Hofer, 1980), including A. absinthium (Lam, personal communication). In addition, it was described to be a component of Viriola peruviana (Lai et al., 1973). Its biological activity is unknown.

Cubebin (CUB: 3,4-[3,4-methylenedioxyphenyl]-dimethyl-2-furanol)(Fig. 5), was identified

as early as 1839 as one of the components of the unripe berries of cubeb, Piper cubeba (Piperaceae) (Capitaine and Soubeiran, 1839), and its structure was later confirmed by Hearon and MacGregor (1955). More recently Burden et al. (1969) completed a total synthesis. In addition to cubebin, P. cubeba may contain some 18 other lignans or neolignans such as ashantin, dillapiol, sesamin, hinokinin, yatein, cubebinin, magnosalin, heterotropan (Badheka et al., 1987), together with several amides (Loder et al, 1969). Plants from Piper spp. have long been known for their insecticidal activity. However, the possible contribution of cubebin or other lignans to the recognized toxicity of the amides that are frequent in this family (Miyakado et al., 1983), has not been investigated.

Sesamolin (SES: 2-[3,4-methylenedioxyphenoxy]-6-[3,4-methylenedioxyphenyl]-cis-3,7-dioxabicyclo {3.3.Ø} octane)(Fig. 5), a better known lignan, is obtained from the flowers of Chrysanthemum cinerariaefolium (Asteraceae) (Beroza, 1954), but the extracts used commercially are derived from the seeds of Sesamum angolense and S. indicum (Pedaliaceae) (Jones et al., 1962). The high synergistic activity of the oil from S. angolense can be accounted for by the simultaneous

presence of sesamolin and sesangolin in these species (Budowski, 1964). Beroza (1954) reported sesamolin to be 5 times as potent as sesamin when tested as a synergist for pyrethrin against houseflies. Together with sesamin, from which it differs only by the presence of a connecting oxygen atom between tetrahydrofurfurofuran nucleus and one of the MDP groups, sesamolin has given rise to several insecticide synergists such as piperonyl butoxide, sulfoxide and sesamex, among others, all active synergists of pyrethrins, carbamates, or chlorinated hydrocarbons. In addition, sesamol, a phenolic constituent formed from sesamolin under certain processing conditions, was found to be a potent antioxidant, possibly increasing the efficiency of use of carotene and counteracting fish oil toxicity in feed for poultry (Ershoff, 1971).

1.2.2.2 THE TWO MODEL ALLELOCHEMICALS FROM THE ASTERACEAE

Tenulin (TEN, Fig. 6), a sesquiterpene lactone, is present in large quantities in several Helenium species, and has a wide range of biological activities (Picman, 1986). It has been used commercially as a fish poison, and has mild sternutatory action (Hall et al., 1977). Its activity against various types of carcinomas is due to the inhibition of cellular enzyme activity and

metabolism that it induces (Lee et al., 1977). The mode of action of sesquiterpene lactones was further elucidated by studies on insects, with TEN in particular (Arnason et al., 1985a, 1987). The retention of the cyclopentenone moiety in TEN was required for antifeedant and growth reducing activity on the larvae of Ostrinia nubilalis. Adduct formation between the α-β-unsaturated ketone in TEN and SH groups in free haemoproteins, as demonstrated in vitro (Hall et al., 1977), was probably involved (Arnason et al., 1987).

Phenylheptatriyne (PHT, Fig. 7), the polyacetylene used in this study, has both a phototoxic and an oxygen independant (non-photodynamic) activity (MacLachlan et al., 1982). The particularly large quantity of this compound contained in the subtropical weed Bidens pilosa (Asteraceae), where it can constitute up to 700ug/g of the fresh weight in the leaves, may contribute to the protection of this plant from insect herbivory. In laboratory as well as under field conditions, it inhibits growth and has antifeedant properties, at the ground state, against three Lepidoptera species, Manduca sexta, Euxoa messoria, and Ostrinia nubilalis, (Champagne et al., 1986), and Heliothis virescens (Iyengar, 1988). A previous investigation with PHT fed

at 100ug/g in the diet, showed larval growth inhibition in the European corn borer, O. nubilalis, limiting the frequency of pupation.

1.2.3 CO-OCCURRENCE OF ALLELOCHEMICALS

Up to now, only a few reports on lignans cooccurring with other allelochemicals are available, and even fewer studies have investigated the bioactivity of lignans in association with other bioactive naturally occurring compounds.

The identification of the synergist sesamin in pyrethrum flowers, Chrysanthemum cinerariaefolium (Asteraceae), also containing the insecticide pyrethrum (Jones et al., 1962), triggered new interest in the possible interaction between the numerous secondary metabolites synthesized by plants. Thus in other families or species, including Argyranthemum frutescens, Anacycluss pyrethrum, Otanthus maritimus, and in some Achillea species , sesamin was found to co-occur with olefinic and acetylenic amides (Greger,1981, Burden & Crombie, 1969). In fact, lignans such as sesamin, seldom occur as the only allelochemicals in plants; some examples of these associations are given in Table 2.

TABLE 2 : EXAMPLES OF COOCCURRENCE OF LIGNANS AND OTHER ALLELOCHEMICALS IN THE ANGIOSPERM.

	LIGNAN	OTHER ALLELOCHEMICALS	REFERENCES
PIPERACEAE			
Piper longum	sesamin	amides (4, e.i. pipartine) alkaloid (piperlonguminine)	Burden & Crombie, 1969a
P.peruloides	diaeudesmin		Burden & Crombie, 1969a
P. cubeba	cubebin	amides (with additive toxic effects)	
	18 others		Miyakado et al., 1983
	hinokinin,yatein, magnosalin...		Badleka et al.,1987
P. nigrum		amides; pipercide	Miyakado et al., 1983
P. novae-hollandiae		alkaloids; piperstachine ongostachine	Miyakado et al.,1983
P. guineense	guineensine	alkaloid (visanine)	
ASTERACEAE			
Artemisia maritima		polyacetylenes, sesquiterpene lactones	Pathak & Khanna, 1987
A. glutinosa		polyacetylenes(glutinosol	Gonzalez et al., 1981
A. tridentata	14 sesamin-type lignans	coumarins(10 i.e.esculin, umbelliferone, skimmin, chicoriin...) flavonoids (4, i.e. axillarin, eupafolin, dihydropeltone)	Bohlman, 1973 Greger, 1981
A. absynthium	sesartemin	flavonoids (4,i.e. axillarin, luteo)	
	diasesartemin	sesquiterpenes lactones(42, i.e. absinthine, belenolide	Greger, 1981 Fraga, 1986
	epiyangambin	polyacetylenes (10, i.e. artelin, esculin), thiophenes amides	Wada & Munakata, 1984 Bohlman, 1973 Greger, 1981
Anacyclus pyrethrum	sesamin	amides (5, i.e. anacyclin, pellitorin,tyramine)	Burden & Crombie, 1969a
Chrysanthemum cinerariaefolium	sesamin, sesamolin	sesquiterpene lactones (pyrethrosin)	
Elephantopus augustifolius	guaianolide lignans	sesquiterpenes lactones (elephantopin) polyacetylenes	Bohlman et al., 1980 Jakupovic et al., 1987
Erigeron philadelphicus	dillapiol	matricaria esters fatty acids diterpenes (erigerol)	Waddell et al., 1983
Heliopsis scabra	helioxanthin	amides (2, i.e.scabrin,heliopsin)	Burden et al., 1969
Ambrosia spp.	helianthoidin	sesquiterpenes lactones(50) several thiophenes	Seaman, 1982
Otanthus maritimus	sesamin	olefinic and acetylinic amides	
Achillea spp.	sesamin	amides epoxipolyacetylenes	Greger,1981
PODOCARPACEAE			
Librocedrus bidwillii	deoxypodophyllotoxin, peltatin	terpene lactones i.e. (nagilactone)	Russel et al., 1976
UMBELLIFERAE			
Atriscus spp.	deoxypodophyllotoxin	polyacetylenes	Newman, 1962
Pasticana sativa	myristicin	coummarins, furanocoumarins terpenes, flavonoids linear polyacetylenes	Newman. 1962 Harborne & al., 1969

Yet, while the importance of mixtures of allelochemicals occurring together in the same plants for the choice of the host plant by the insect seems now well established, the role of these mixtures in plant defense has received little attention. In particular, there have been few investigations on the interactions of lignans with other biologically active allelochemicals. Only recently has such an interaction between plant secondary metabolites been reported in the family Apiaceae, where the lignan myristicin clearly synergized the activity of xanthotoxin, a representative allelochemical of this family (Berenbaum and Neal, 1985). Also, Dr Lam suggested that lignans might be protective agents of the polyacetylenic compounds against photooxidation, in a manner similar to the known electronic interactions between polyacetylenes or aromatic compounds and caffeine (Lam, pers. comm.). However, the possibility that each allelochemical synthesized by a plant has a separate biological activity cannot be rejected, especially in view of the various biological activities found in lignans (MacRae & Towers, 1984).

1.2.4 INSECT RESPONSE TO PLANT ALLELOCHEMICALS

During their evolution, herbivores including phytophagous insects had to deal with the numerous plant secondary metabolites many of which were unsuitable to their physiology, but obligatorily ingested with their food. As their environment changed and new plant defense compounds appeared, insect herbivores had to develop mechanisms to survive exposure to such chemicals, a general process referred to as plant-insect coevolution (Ehrlich and Raven, 1964). Examples of herbivorous insect adaptations to plant allelochemicals (see review in Brattsten, 1979) include behavioral avoidance (Hsiao, 1985), sequestration of the toxin away from sensitive metabolism, facilitated excretion (Duffey, 1980), temporary binding with a carrier protein, or modification of target sites (Morris, 1984), and one step beyond, the ability to utilize plant allelochemicals for their own defense against predators, pathogens or parasites (Price et al., 1980). However, these processes can be assumed to evolve fairly slowly, since they involve the fine tuning of many molecular interactions (Frazier, 1986).

1.2.4.1 INSECT MONOOXYGENASES

The principal adaptation that has allowed insects to survive and eventually evolve the finer counter strategies mentioned above, is an array of detoxification enzymes performing such general reactions as oxidation-reduction, side group removal, or conjugation with other molecules, all resulting in an excretable product (Brattsten, 1979; Dowd et al., 1983). The five major enzymatic systems identified in the detoxification of plant allelochemicals all share a requirement of high degree of substrate lipophilicity. They are: the cytochrome P-450 dependant monooxygenases, often called mixed function oxidases (MFO) or also polysubstrate monooxygenases (PMSO), reductases, esterases, epoxide hydrolases and glutathione or other group transferases (Ahmad et al., 1986). The detoxification steps have been conveniently grouped into primary (phase I) and secondary (phase II) metabolic processes (Hodgson, 1985). Generally the resultant product is a metabolite more polar than the parent compound (I), which is further conjugated with an amino acid, glutathione, sugars, phosphates or sulfates by specific transferases (II), to finally produce an excretable metabolite.

The MFOs are by far the most important of these detoxification systems, because they detoxify a wide range of foreign chemicals, natural or synthetic. Localized in insects mainly in the smooth endoplasmic reticulum of the cells of the midgut walls, this enzymatic system has 3 major components (Fig 8): the cytochrome P-450, the NADPH-cytochrome-P-450-reductase and a phospholipid (Capdevila et al., 1975). The cytochrome P-450 moiety is a simple polypeptide containing an iron protoporphyrin group located in a so-called hydrophobic pocket in the apoprotein. The NADPH-cytochrome P-450 reductase interferes in the transport of electrons to the cytochrome P-450. The basic reaction catalysed by these enzymes is:

$$RH + O_2 + NADPH = ROH + NADP + H_2O$$

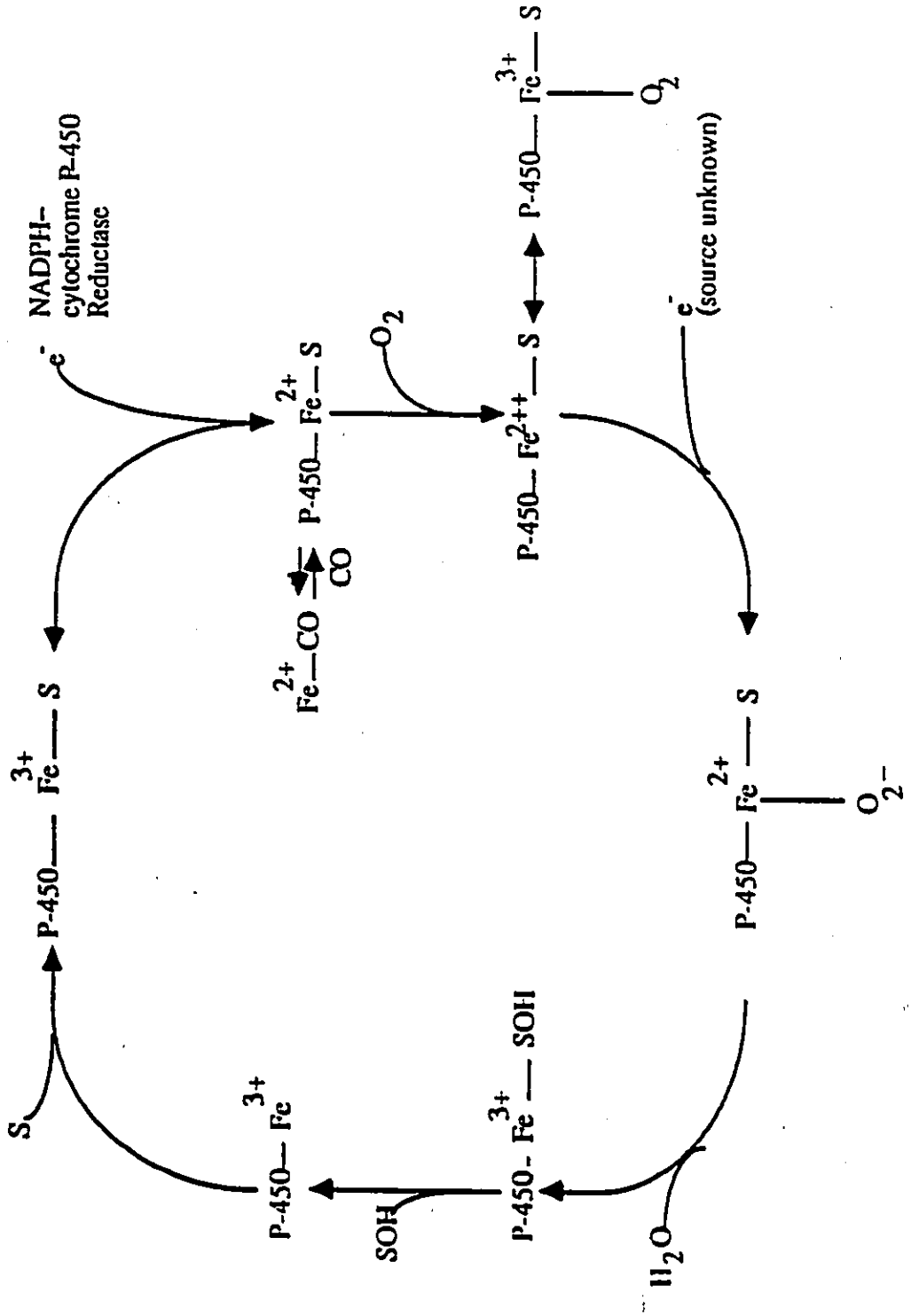
where the usually lipophilic substrate (RH) , in the presence of molecular oxygen and a reducing equivalent (NADPH), results in the formation of alcohol or phenol (ROH) a more hydrophilic metabolite.

1.2.4.2 ROLE OF MONOOXYGENASES IN THE DETOXIFICATION OF ALLELOCHEMICALS.

Numerous studies have been conducted on the role of MFOs in drug and insecticide metabolism. In insect pest management, the importance of these enzymes is

FIG 8 : REACTIONS CATALYSED BY THE CYTOCHROME P-450 DEPENDANT MONO-OXYGENASE SYSTEM (MFO).

(Adapted from Hodgson, 1985 and Iyengar, 1988).


NADPH-
cytochrome P-450
Reductase
e⁻
P-450— Fe3+ — S
S
P-450— Fe3+ — SOH
SOH
H2O
P-450— Fe2+ — S
O2 2−
e⁻
(source unknown)
P-450— Fe2++ — S
O2
P-450— Fe3+ — S
O2
P-450— Fe2+ — S
O2
CO
P-450— Fe2+ — CO

great, as it was revealed that the appearance of resistance to pesticides in pest populations correlated closely with high levels of MFOs (Metcalfe, 1967). Although most of the studies on MFOs have dealt with their potential in the detoxification of synthetic pesticides, the role of these enzymes in plant-insect interactions is now well documented (Krieger et al., 1971; Brattsten 1979).

MFOs play a major role in the detoxification of the chemicals contained in the daily food of herbivores. Allelochemicals known to be metabolized by MFOs include phytoecdysones (Bowers, 1981), precocenes (Ellis-Pratt, 1983), monoterpenes, pugelone (Brattsten, 1983; Gunderson et al., 1986)), indole, safrole, flavone, estragol, digitoxin, coumarins, a phenylpropane (eugenol), alkaloids (atropine, strychnine, caffeine), 13 monoterpenes, 2 sesquiterpenes, a diterpene (phytol), steroids (stigma-, sito- and ergosterol), squalene, β-carotene (see Yu, 1987). The lignans myristicin, sesamin and sesamolin are known to bind to MFOs and probably are metabolized by them, but the metabolites have not been identified. Probably, a much larger number of natural compounds are also metabolised by the MFOs, but metabolism studies

have been limited by the small quantities of the allelochemicals available.

Krieger et al. (1971), first related the activity in the guts of larvae of Lepidopteran species to their feeding habits, suggesting that since polyphagous insects were in contact with a large diversity of plant allelochemicals, they had acquired through evolution the high MFO levels that were observed. In contrast, monophagous insects were adapted to their host's specific defensive allelochemicals with lower MFO levels (Rhoades and Cates, 1976). This theory was further supported by studies reporting that ingested plant allelochemicals could, in fact, differentially affect MFO levels in insects. For instance Brattsten (1979) observed that when fed on Apiaceae such as carrots, parsley and coriander, the southern armyworm, Spodoptera eridania, had significantly greater induction of gut MFOs than with its usual host plant, the lima bean . Similarly, in the variegated cutworm, Peridroma saucia, peppermint induced higher levels of gut MFOs than the snap bean (Yu et al., 1979). Furthermore, the pattern of induction obtained after 24 hours with diets of various complexity paralleled the observation of Krieger et al. (1971). The induction was highest in field

collected Japaneese beetles, Popillia japonica, intermediate in laboratory-reared insects on a limited number of plant species, and lowest on single plant species (Ahmad, 1983). The close relationship between food intake and MFOs was also reflected in the variation of the enzyme levels during the insect life cycle. In the gypsy moth, Lymantria dispar, highest MFO activity was recorded during active feeding periods, whereas a reduction of the enzyme activity was observed just before molts and pupation (Ahmad, 1982).

The appearance in plants of inhibitors of MFOs may constitute a next step in the coevolutionary scenario of Ehrlich and Raven (1964) . Focussing on the insect's principal enzymatic adaptation to overcome toxicity from allelochemicals, plant MFO inhibitors appear to be subtle countermeasures to offset insect chemical defenses. The most numerous and potent MFO inhibitors contain a methylenedioxyphenyl (MDP) ring in their structure (Casida, 1970), a structure that is most frequently encountered in lignans (see section 1.2.1.1), as well as in other phenylpropanoid compounds and in amides. The overall mechanism of action of these inhibitors involves the formation of stable adducts between the planar MDP ring of the lignan and the heme of the cytochrome P-450 terminal oxidase of the MFO

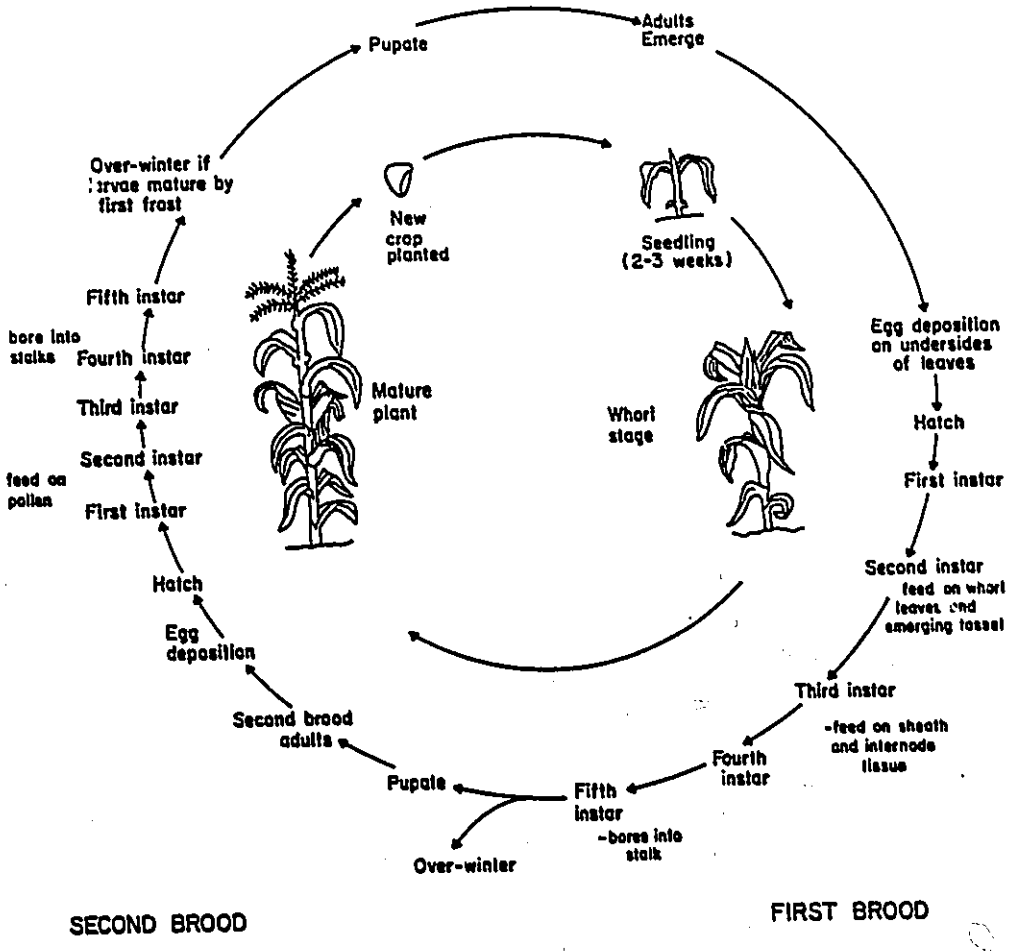
system (Metcalfe, 1967), possibly following the formation of free radical species such as a carbocation, a carbanion or a carbene (Hodgson, 1985). The decrease in the enzyme availability for other substrates such as insecticides or toxic allelochemicals, results in an increase or a lengthening of the effects of the toxin applied simultaneously. Such a mechanism was proposed for the synergism of xanthotoxin, a secondary metabolite detoxified by the MFO system in the tobacco budworm, H. zea (Ivie et al., 1983), and its natural synergist the lignan, myristicin (Berenbaum & Neal, 1985).

1.2.4.3. THE TEST INSECT : THE EUROPEAN CORN BORER (ECB), Ostrinia nubilalis (Hubner) Lepidoptera : Pyralideae.

The European corn borer (ECB) Ostrinia nubilalis, was introduced into North America at the beginning of this century (Davidson and Lyon, 1979), spread rapidly and presently ranges over all temperate corn producing areas. It now also attacks other economically important crops such as sweet pepper, beet, bean, potato, oat, soybean, and wheat (Davidson and Lyon, 1979). Its

FIG 9 : LIFE CYCLE OF OSTRINIA NUBILALIS (Hubner) IN RELATION TO ONE OF ITS HOST PLANT, ZEA MAYS.

(Adapted from Guthrie, 1971)


Pupate
Adults Emerge
Over-winter if larvae mature by first frost
New crop planted
Seedling (2-3 weeks)
Fifth instar
bore into stalks
Fourth instar
Third instar
Second instar
feed on pollen
First instar
Mature plant
Whorl stage
Egg deposition on undersides of leaves
Hatch
First instar
Second instar
feed on whorl leaves and emerging tassel
Third instar
-feed on sheath and internode tissue
Fourth instar
Fifth instar
-bores into stalk
Pupate
Over-winter
Second brood adults
Egg deposition
Hatch
SECOND BROOD
FIRST BROOD

history on corn is however relatively recent and the approximately 100 wild host plant species of this highly polyphagous species include species from the Asteraceae which are known to contain large amounts of the compounds studied in this wor', polyacetylenes (up to 600 ug/g PHT in the leaves of Bidens pilosa), sesquiterpene lactone (santonin in Artemisia sp.), or lignans (sesamolin in Chrysanthemum flowers, matairesinol in Arctium lappa), together with other biologically active allelochemicals (Table 3).

The general biology of the ECB is best documented in relation to its most economically important host plant, corn (Fig. 9). Eggs are deposited in masses on the underside of leaves. They hatch 4 or 5 days later after entering a stage where the cephalic capsules of the embryo melanize and become visible through the chorion, a condition referred to as the 'black head' stage. There are normally 5 larval instars. The first and second instar larvae feed on corn leaves. Subsequently, larvae enter the corn stalk, feeding and excavating tunnels that weaken the plant and may cause it to fall over. These last two instars are the most damaging to the plant. If weather and photoperiodic conditions are favorable , larvae enter pupation and emergence of adults occurs approximately a week later

TABLE 3 : HOST PLANTS OF OSTRINIA NUBILALIS AND SOME OF THEIR ALLELOCHEMICAL CONTENT.

	POLY ACETYLENES	SESQUITERPENES LACTONES	LIGNANS
1. PLANTS SEVERELY ATTACKED			
Amaranthus retroflexus L.			
*Ambrosia spp.	+	+	+
*Bidens spp.	+		
Cannabis sativa L.			
Chenopodium spp.		+	
*Dalhia spp.	+		
Echinochola crus-galli L. (Beauv.)			
Holcus sorghum L.			
Humulus japonicus Sieb. & Zucc.			
*Iva xanthifolia Nutt.	+		
Pheum rhaponticum L.			
Phytolacca decandra L.			
Polygonum spp.		+	
Rumex spp.			
*Xanthium spp.	+	+	
Zea mays L.			
2. PLANTS FREQUENTLY ATTACKED			
*Arctium spp.			+
*Artemisia spp.	+	+	+
Beta vulgaris			
*Callistema chinense			
Capsicum annum L.			
Celeri graveolens L. (Britton)			
*Chrysanthemum spp.	+		+
Datura spp.			
Euchlaena mexicana Schrad.			
*Erigeron canadensis L.	+		+
Gossypium hirsitum L.			
*Helianthus spp.		+	
Holcus sorghum L.			
Hordeum vulgare L.			
Panicum miliaceum L.			
Phaseolus spp.			
Polygonum sieboldii De Vries			
Solanum tuberosum L.			
*Tanacetum vulgare L.	+	+	
Vigna sinensis L. Endl.			
Zinnia elegans Jacq.		+	
3. PLANTS OCCASIONALLY ATTACKED			
Abutilon theophrasti Medic.			
*Achillea millefolium L.	+	+	
Althaca rosea Cav.			
*Aster spp.	+		
Atriplex spp.			
Avena sativa L.			
*Boltonia asteroides L. L'Her.	+		
Brassica spp.			
Calendula officinalis L.			
Canna spp.			
Colosia spp.			
Chenopodium album L.	+		+
*Cirsium spp.	+		
Coleus spp.			
*Cosmos bipinnatus Cav.			
*Erechites hieracifolia L. Raf.			
Fagopyrum vulgare Hill			
*Galinsoga spp.	+		
Gladiolus spp.			
*Helianthus tuberosus L.		+	+
Helichrysum bracteatum Andr.			
Humulus lupulus L.			
*Lactuca spp.			
Leonurus cardiaca L.			
Lycopersicon esculentum Mill.			
Malva spp.			
Portulaca oleracea L.			
*Rudbeckia laciniata L.			
Salvia splendens Ker-Gawl.			
Solanum melongena L.			
*Solidago spp.	+		
*Sonchus spp.			
Syntherisma sanguinalis L. Bernh.			

* Members of the Asteraceae

Note: This table presents known occurrences according to current reviews. It may not however contain very recently identified material.

(Adapted from Caffrey and Worthley, 1927; Hodgson, 1928; Willson, 1980; Tedders et al, 1981; Weires & Straub, 1982).

(Bonnemaison, 1978). Otherwise, winter is spent as mature larvae, in diapause, within the corn stalks. The number of generations per year varies with location and latitude (Davidson and Lyon, 1979). In our laboratory environment, the proportion of larvae entering diapause was kept very low by the use of a non-diapause inducing photoperiod and temperature schedule (see section 2.2).

1.3 OBJECTIVES OF THE STUDY

Given the hypothesis outlined in 1.1.1 (p. 4) and the previous work outlined in the literature review, the general objective of the present study was to determine the participation of a selected lignan (DIL) in plant defence against a polyphagous herbivore, Ostrinia nubilalis, as well as to determine the interactions of this lignan with the sesquiterpene lactone (TEN) and the polyacetylene (PHT), all allelochemicals from the Asteraceae. To achieve this, it was necessary to:

1. Characterize the MFO system and to optimize the activity of these enzymes in O. nubilalis,

2. Determine the in vitro variations of the MFO

activity of O. nubilalis in the presence of five different lignans at various concentrations, and select an adequate MFO inhibitor, the lignan DIL,

3. Relate the in vitro inhibitory effect of DIL, the most active lignan assayed, with its in vivo effect on the MFOs of O. nubilalis,

4. Investigate in vivo effects of DIL, in double or triple associations with 2 other characteristic allelochemicals from the family of the Asteraceae, TEN and PHT, on the entire life cycle of O. nubilalis. Observations on the insects included mortality, growth and developmental time of larvae, pupae and adults.

The association of DIL with TEN and PHT was chosen as representative of the type of allelochemicals encountered in the diet of O. nubilalis (Table 3), e.g. in the tribe Artemisia, which contains these three classes of allelochemicals. In addition, the mode of action of TEN and PHT on this insect species has been previously investigated (see sections 1.2.1.2 p. 20 and 1.2.1.3 p. 26). The choice of these three compounds was also directed by their availability in quantities necessary to perform in vitro and in vivo experiments.

2 MATERIAL AND METHODS

2.1 TEST COMPOUNDS

Structures of the lignans, the polyacetylene PHT and the sesquiterpene lactone TEN, with their plant origins, as well as the structure of the synthetic compounds used in the present work, are given in Fig. 5, 6 and 7. Table 4 lists some physical constants of these compounds, as well as the reference to the method used for their extraction or synthesis as the case may be. Evidence of purity was obtained for each compound as described below.

The lignans were all of plant origin and provided by Dr J. Lam, Department of Organic Chemistry, University of Aarhus (Denmark), either as crystals of the pure compound (diasesartemin: DIA, sesamolin: SES, cubebin: CUB, epiyangambin: EPI), or as a solution of 1,9 mg/ml of the lignan in 95% ethanol (dillapiol: DIL).

The polyacetylene phenylhepta-1,3,5-triyne (PHT) (fig 7), was obtained from mature leaves of Bidens pilosa grown from seeds collected in Miami, Florida. After steeping in ethanol (95%) for at least two weeks, the extract was treated by the modified method of Wat & al. (1979): dilution with an equal amount of

TABLE 4 :

SOME PHYSICAL CONSTANTS OF THE COMPOUNDS USED IN THE BIOLOGICAL ASSAYS OF THE PRESENT STUDY.

Compound	Molecular weight (g/mole)	Melting point (C)	λ max. (nm)	E λ max. (e-mole.cm)	Reference
Lignans					
Dillapiol: DIL	223	30	285	2320	Bernhard and Thiele, 1978.
Diasesartemin: DIA	432	98-102			Greger, 1981.
Epiyangambin: EPI	448	118-120			Rao, 1978.
Cubebin: CUB	369	127-128	287		Batterbee et al.,1969.
Sesamolin: SES	371	92			Beroza, 1954.
Polyacetylene					
Phenylheptatriyne: PHT	164		310	17320	McLachlan, 1982.
Sesquiterpene lactone					
Tenulin: TEN	306	196			Hertz et al., 1975.
Others					
Phenobarbital: PHE	232	174	240		Chao, 1978.
Piperonyl butoxide: PIP	338	37			Blum, 1955.

water, extraction in petroleum ether (PE) three times in a separatory funnel. The combined PE fractions were dried with sodium sulfite and the volume reduced to 1 ml on a rotary evaporator at 30 C. Elution of PHT was performed on a Silica Gel Chromatography column (Baker 40-140), under low nitrogen pressure with glass distilled PE as eluent. PHT eluted just behind the solvent front and just before the first carotenoid band, as identified by spectrophotometry (see Table 4 for PHT constants). The PHT containing fraction was concentrated to almost dryness in a rotary evaporator (30 C), and allowed to crystallize at -40 C. Evidence of purity (over 99%) of the PHT solution was established on the HPLC by elution with acetonitrile-water (70:30, v/v) at a flow rate of 1 ml/min (Beckman 110a pump and 420 controller), with a reverse phase column (RP C 18, pore size 5 um, Ultrasphere R octadecylsilicate (ODS) of internal diameter of 4.6 mm x 25 cm). A single peak with retention time of 3.1 min was observed. Concentrations of the solution of PHT in 95% ethanol to be used for the bioassays was verified on a Pye-Unicam SP8-100 spectrophotometer.

Alternatively, PHT was synthesized in the Department of Chemistry of the University of Ottawa, following a procedure described by McEachern (1987),

which provided larger quantities of the compound.

The sesquiterpene lactone tenulin (TEN), extracted from the leaves and stems of Helenium amarum (Asteraceae) (Lucas et al., 1964), was provided as a pure compound in powder form by T. Waddell, Department of Chemistry, University of Tennesse at Chattanooga, Tennessee. It was used without further purification.

The synthetic compounds phenobarbital (PHE) and piperonyl butoxide (PIP), were purchased from Sigma chemical and were purified by column chromatography on silica gel prior to their utilization by J. Kaminski, Department of Chemistry, University of Ottawa.

For in vitro studies, 10 ul of the test compound were put in the assay vial prior to the addition of 1 ml of the enzyme source (see section 2.3.1). A control without test compound but containing ethanol was also included in the assay.

For in vivo studies, stock solutions of the allelochemicals and the synthetic compounds were made up in ethanol (10 mg/ml), and incorporated in the diet for a final concentration of 1% ethanol. To facilitate

solubility, SES and PHT had to be dissolved in ethanol by sonication immediately before incorporation in the diet. The concentration of the solution of DIL and PHT were verified on a Pye-Unicam SP8-100 spectrophotometer. The other compounds were weighed and made up to the proper concentration.

2.2 INSECTS

2.2.1 Stock culture

Eggs and larvae of Ostrinia nubilalis were obtained from a stock culture maintained in the laboratory, which was started using eggs supplied by M. Hudon (Agriculture Canada, St. Jean, P.Q.) and periodically boosted with eggs supplied by G. McLeod (Agriculture Canada, London, Ont.).

Larvae were reared in mass culture according to the method of Guthrie (1971), in a controlled environment chamber (Conviron cabinet E7, photoperiod of 16/8 light/dark, 26 C during photophase, 19 C during scotophase, constant relative humidity of 80%). Lighting was provided by four 20 W solar simulating fluorescent lamps (Vitalight R 48T12 U.H.O.), separated from the chamber by a 3mm thick plexiglass sheet. The

resultant light intensity in the chamber was 40 W/m2, as measured by a Yellow Springs Radiometer. The meridic diet used was essentially that of Guthrie (1971), with the omission of corn cob grits during the time of the experiments to achieve a homogenous distribution of the allelochemicals (Appendix A).

Adults were maintained in a plexiglass and aluminium screen cage (inner dimensions 45 x 45 x 61 in height), in a controlled environment chamber similar to that for larvae (same setting except for the constant relative humidity of 85%). Lighting was provided by four 60 W fluorescent lamps (Coolwhite F48T12/CW/HO) and one 40 W incandescent bulb, separated from the chamber by a 3 mm plexiglass sheet. The resultant light intensity inside the cage was 50 W/m2. Water was constantly available to the moths through the mist from a cool-spray vaporizer positioned in the chamber, adjacent to the cage, with the outlet port facing one side of the screen cage.

2.2.2 Preparation of test diets

The meridic diet used for the stock culture was prepared as explained above. The appropriate amount of the compound, dissolved in 95% ethanol, was deposited

in a small beaker and the liquid meridic diet at approximately 40 C (to prevent degradation of the compounds), was then added. The content of the beaker was quickly and vigorously mixed and poured into 1 x 1 x 0.6 cm moulds to cool. The amount of solvent did not exceed 1% in the diet.

2.3 ENZYME ASSAYS

2.3.1 Isolation of the microsomal fraction.

Extracts were prepared from fifth instar actively feeding larvae of homogenous weights (90 mg ±15), which was usually the third day of this larval stage. Larvae were opened longitudinally, the alimentary canal drawn out and the gut content cleared with a syringe filled with ice cold Insect Ringer solution. The midgut was then freed from the rest of the insect and rinsed again in the cold Ringer. The microsomal fraction was then isolated according to the method described in Gould and Hodgson (1980) and modified for the ECB. Midguts were homogenized in groups of 10 in Tris-HCl buffer (0.05 M), pH 7.6 or 7.8, containing KCl (1.15% w/v), in a hand driven Ten Broek homogenizer previously cooled to 4 C. The homogenate was centrifuged for 15 minutes at 1000 g

in a Beckman L5-50 ultracentrifuge refrigerated at 4 C. Sediments consisting of cellular debris and nuclei were discarded. The supernatant was then centrifuged at 100 000 g in the same preparative ultracentrifuge to obtain the microsomal fraction (100 000 g pellet). Protein content of the homogenate was determined by the Lowry method (Lowry et al., 1951), using purified bovine serum albumen as a standard. Because of the short life time of the microsome preparations, protein concentration was determined after the recording of the difference spectra (section 2.3.2) or the epoxidation experiment (section 2.3.3).

2.3.2 Determination of the MFO content by cytochrome P-450 difference spectrum

The microsomal pellet was gently washed, then resuspended in the Tris-HCl buffer to make a final concentration of 2 to 2.5 mg protein / ml. The suspension was separated in two 1 ml quartz cuvettes and the difference spectrum was determined between the wavelengths 500-400 nm, on a Cary 2020 spectrophotometer. The microsomal suspension had an approximate absorbance of 1.6 at 450 nm, as measured against air. In a first step, a baseline was recorded with two identical cuvettes of microsomal suspension.

Then both cuvettes were removed. Carbon monoxide was bubbled in the sample cuvette for 1 minute under the fumehood, at the approximate rate of 5ul per second. The reducing agent sodium dithionite (approximately 0.1 mg), was then added to both cuvettes and the difference spectrum was recorded repetitively from 500 nm to 350 nm until the peak at 450 nm attained its highest value. Subsequently, the peak at 450 nm diminished and the peak at 420 nm increased (Fig. 11).

The cytochrome P-450 content was estimated as the absorbance (peak at A[450]nm minus A[490]nm, or peak A[420]nm minus A[490]nm) of the dithionite-reduced cytochrome P-450 carbon monoxide difference spectrum according to the method of Omura and Sato (1964). The guidelines given by Kulkarni and Hodgson (1976) were used to minimize errors in the interpretation of the spectra, and the concentrations of cytochrome P-450 were calculated using the equation provided by Guengerich (1982).

2.3.3 *In vitro* and *in vivo* MFO assays: aldrin used as a substrate for epoxidation.

Standard incubation mixtures were prepared at 4 C, following the method described by Gould & Hodgson

(1980). The pellet obtained from the 100 000g ultracentrifugation of 40 to 60 guts was resuspended by several passages through the needle of a hypodermic syringe, in 10 to 14 ml of Tris (0.05 M) -KCL (1.15%) at pH 7.6, and containing NADPH (5 x 10-6 M, final concentration). One ml of the mixture was frozen immediately for later determination of the protein content. The remaining microsomal suspension was allowed to equilibrate 4-5 minutes in a water bath at 30 C.

Each assay performed in an open Erlenmeyer flask included 10 ul of the tested allelochemical at the desired concentration (in ethanol), 1ml of the microsomal suspension, and, to start the reaction, 1 ul of a solution of aldrin (10 mg/ml) in ethanol. The reaction was allowed to run for 15 minutes, with constant agitation, in the 30 C water bath (conditions during which the reaction rate remained linear), and was terminated by the addition of 2 ml of acetone. The flasks were then closed with a glass cap and immediately placed in the refrigerator.

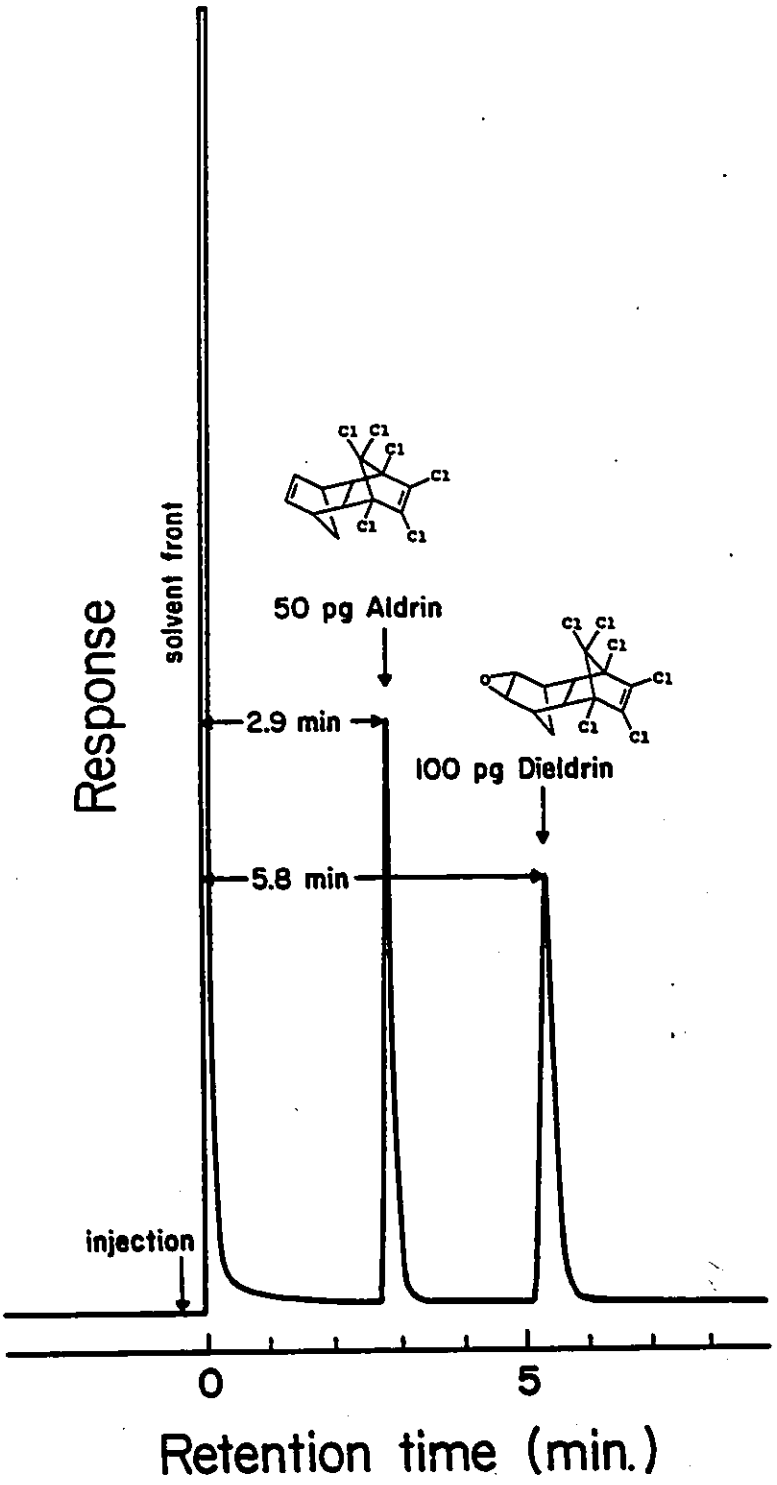
Usually the next day, aldrin and dieldrin (produced by the epoxidation of aldrin by the MFOs; Fig. 10), were extracted in 1 ml of PE two times at room

temperature. Each extraction included a vigorous mixing of the mixture followed by 10 minutes centrifugation for an accurate phase separation. The organic phase was diluted 2 to 5 fold with PE to obtain the appropriate dieldrin concentration for gas chromatography (Hewlett-Packard 5730A chromatograph with Electron Capture Detector; temperature of oven: 180 C, injection port: 250 C; detector: 300 C; column: mixed phase packing; mobile phase: argon 95%, methane 5%; flow rate: 33 ml/min). Under these conditions, the retention time for aldrin and dieldrin was 2.9min and 5.8min respectively (Fig. 10). The linearity of detector response (peak area) versus dieldrin concentrations was established (correlation coefficient: $r = 0.97$), and later checked at the beginning of each session with 1 ul of at least 3 dieldrin standards at different concentrations. After injecting 1 ul of the control sample, the dieldrin standard giving the closest response was selected and subsequently injected after every second injection of the samples.

In vivo assays of MFO activity (aldrin epoxidase activity) was determined in larvae entering their 5th instar fed with the appropriate test allelochemical(s) for 4 days prior to the assay (20 larvae per treatment;

FIG 1Ø : GAS CHROMATOGRAM OF A STANDARD MIXTURE OF ALDRIN AND DIELDRIN.

(Hewlett-Packard 5730A chromatograph with Electron Capture Detector; 'mixed phase' column packing; mobile phase argon/ methane 95%/5%; temperatures: oven 180°C, injection port 250°C and detector 300°C)


Response
solvent front
50 pg Aldrin
2.9 min
100 pg Dieldrin
5.8 min
injection
0
5
Retention time (min.)

mean larval weight = 80 ±15mg). Ten guts of the same group were pooled, 0.5 ml of the microsomal suspension was set aside for protein determination, and the microsomal assays performed as in section 2.3.1.

2.4 GROWTH AND DEVELOPMENT STUDIES

Egg masses at the 'black head' stage (approximately 6 hours before hatching) were placed on a single cube of the treated meridic diets in 15 ml scintillation vials capped with cotton wool. The total number of eggs used per treatment was 150, distributed in 3 vials, in the first experiment and 250 in the second one, distributed in 5 vials. Vials were kept during the course of the experiment in the controlled environment chamber at 85% RH. After eight days, 30 larvae were randomly chosen from each treatment group, weighed and individually placed on approximately 0.4 g of the appropriate diet. The remaining larvae from the original 3 or 5 vials were counted, then discarded (except for one vial of each treatment which was kept until day 13). Subsequently, larvae were weighed on days 8, 13, 17, 22, with the diets changed at the same time. The days when the head capsules were shed were noted for each larva throughout its development.

Pupation day as well as pupal weight were recorded

and each pupa was then placed in a clean vial with a strip of moist filter paper to maintain high humidity within the vial.

Adults were weighed and sexed on the day of their emergence. Adults from the same treatment were placed in a mating enclosure consisting of a dessication chamber with a wax paper suspended within the chamber to provide support for egg laying. New adults were added as emergence occurred. Adult pairs and eggs laid were recorded every day.

Mortality was recorded in larvae, pupae and adults. When larvae died before day 13, they were replaced with new larvae from the reserve vial of the same treatment. This was taken into account in the calculation of the cumulative mortality.

Experiments were set up as two trials of 30 insects per treatment of a single or an association of allelochemicals. Each trial had to be separated in 2 parts (each including a control group) since the number of insect groups was too large to handle simultaneously. Analysis of variance with Tukey-HSD Multiple Range Test (Tukey, 1949) was performed on weight data using the SPSSX General Linear Models Procedure. Time measurements were subjected to the Kruskal-Wallis Test

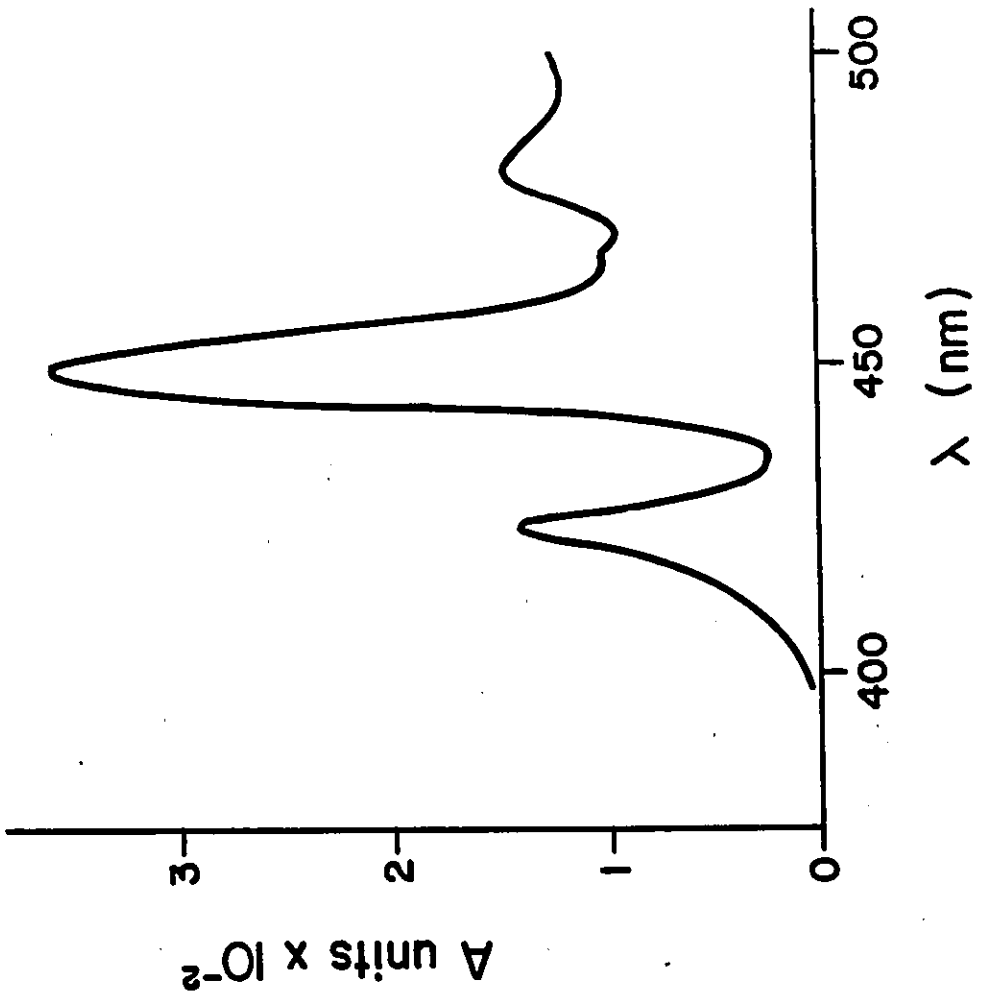
(Kruskal and Wallis, 1952; SPSSX). The level of significance was P = 0.05.

3. RESULTS

3.1 Evidence for the presence of a cytochrome P-450 monooxygenase in microsomes from Ostrinia nubilalis.

The characteristic difference spectrum of the reduced cytochrome P-450 produced with or without carbon monoxide was obtained with a microsomal preparation containing 20 guts per ml (Fig. 11). The maximum P-450 peak was obtained approximately 30 minutes after resuspension. The amount of cytochrome P-450 in the larvae reared on the standard meridic diet estimated from the P-450 peak alone was 0.096 nanomoles of cytochrome P-450/mg protein (SD = 0.01, n = 3). As the peak obtained at 420 nm (which represents the inactivated form of the cytochrome, and is present to some extent in all reported preparations)was not negligible, a theoretical total amount of the enzyme present in the suspension was estimated from a calculation of the amounts given by the P-450 and the P-420 peaks (Guengerich, 1982). It was 0.114 nanomoles cytochrome P-450 / mg protein (SD = 0.02, n = 3).

FIGURE 11 : DIFFERENCE SPECTRUM OF A CO DITHIONITE-REDUCED CYTOCHROME P-450 FROM THE MICROSOMAL FRACTION OF THE MIDGUT OF O. NUBILALIS.
(see section 2.3.2 for methodology)
The peak at 450 nm and 420 nm represent respectively the active and inactive forms of the cytochrome.


A units x 10^{-2}
0
1
2
3
400
450
500
λ (nm)

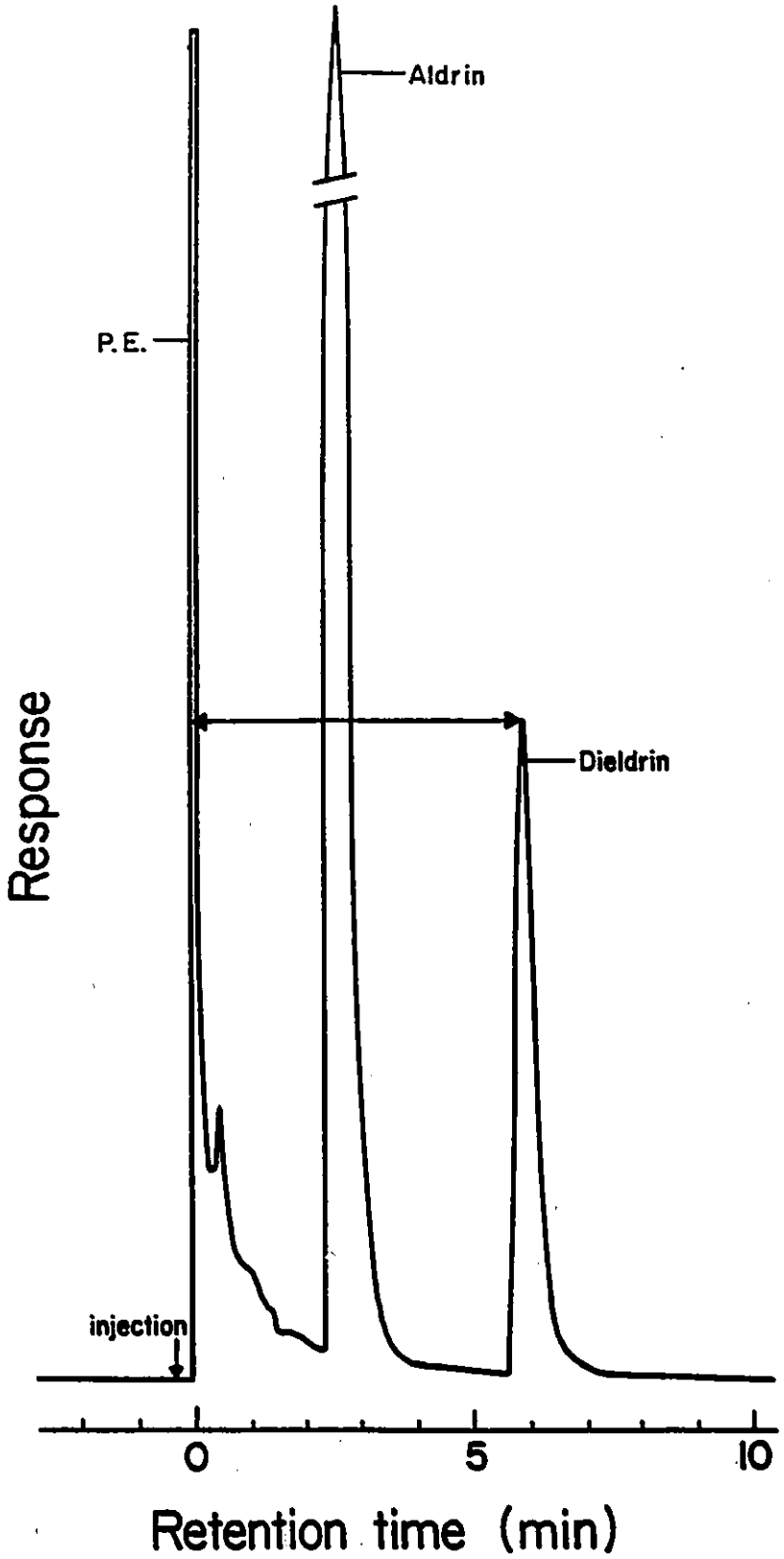
3.2 *In vitro* activity of the aldrin epoxidase.

3.2.1 Dieldrin formation in controls (Fig. 12).

A typical chromatogram of the aldrin and dieldrin extracts in petroleum ether (PE) of the microsomal suspension in control groups showed the excellent separation of product (dieldrin) and precursor (aldrin) (Fig. 12). Within a pH range of 7.0 to 8.0 the enzyme activity remained at its maximum and the general bell shape of the curve was maintained (data not shown). Dieldrin formation increased in a linear pattern with respect to time in the range examined (5 to 20 min). The mean value of aldrin epoxidase activity per gut in controls was 0.06 nanomoles dieldrin / min / gut (SD = 0.02, n = 8), and the specific activity (activity expressed per mg of microsomal protein) was 0.11 nanomoles dieldrin formed / min / mg of protein (SD = 0.08, n = 8).

FIGURE 12 : GAS CHROMATOGRAM OF A MICROSOMAL FRACTION FROM THE MIDGUT OF O.NUBILALIS LARVAE.

(See Fig. 10 for experimental conditions)


Aldrin
P.E.
Dieldrin
Response
injection
0
5
10
Retention time (min)

3.2.2 Influence of allelochemicals on *in vitro* aldrin epoxidase activity (Fig. 13, 14 and 15).

Percent activities in Fig. 13, 14 and 15 are based on the amount of dieldrin produced /min / mg protein of controls from the same tissue preparation.

Piperonyl butoxide (PIP) inhibited almost totally the MFO activity of *O. nubilalis in vitro* with concentrations ranging from 10 -3 M to 10 -6 M (Fig. 13). This result established the similarity of the MFO response in the present assay conditions with data found in the litterature.

The five lignans tested affected the activity of the aldrin epoxidase in various ways (Fig. 14): dillapiol (DIL) clearly decreased the activity of the epoxidase by 50% or more at all the concentrations assayed (Fig. 14). Suprisingly, sesamolin (SES) was only a moderate inhibitor at low concentrations but was a good inhibitor at higher concentrations (50% inhibition at 10 -4 M ; Fig. 14). Diasesartemin (DIA) was also a moderate inhibitor (20% to 39% inhibition; Fig. 14), except at the highest concentration assayed

FIGURE 13 : IN-VITRO ACTIVITY OF ALDRIN EPOXIDASE IN THE MIDGUT MICROSOMAL FRACTION OF O. NUBILALIS: Effect of the synthetic MDP compound piperonyl butoxide (PIP).

The reaction medium contained ten ul of the allelochemical (in ethanol), one ml of the microsomal suspension (0.5mg protein), and 10ug of aldrin. It was performed in a shaking bath at 30 C, pH 7.6, during 15 minutes under aerobic condition. The reaction was stopped with acetone.

Mean activity in controls (no allelochemical but ten ul of ethanol) was 0.11 nanomoles of dieldrin formed/min/mg of microsomal protein. Bars represent the standard deviation

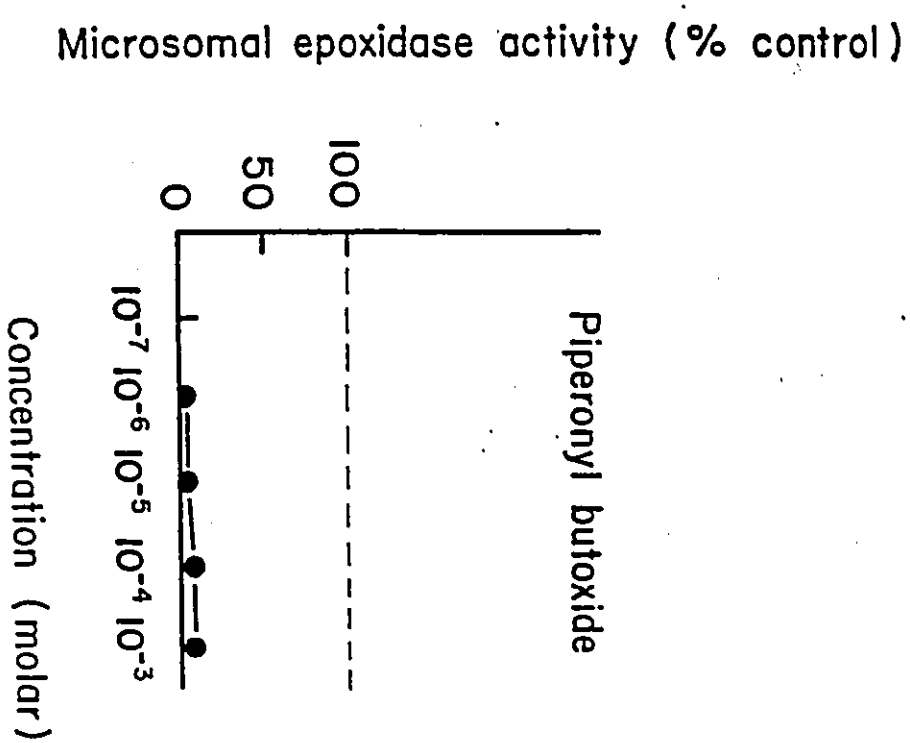

Microsomal epoxidase activity (% control)
0
50
100
Piperonyl butoxide
Concentration (molar)
10-7 10-6 10-5 10-4 10-3

FIGURE 14 : IN-VITRO ACTIVITY OF THE ALDRIN EPOXIDASE IN THE MIDGUT MICROSOMAL FRACTION OF O. NUBILALIS: Effect of the five lignans used in the present study, dillapiol (DIL), diasesartemin (DIA), cubebin (CUB), epiyangambin (EPI) and sesamolin (SES).
(See Fig. 13 for assay conditions)

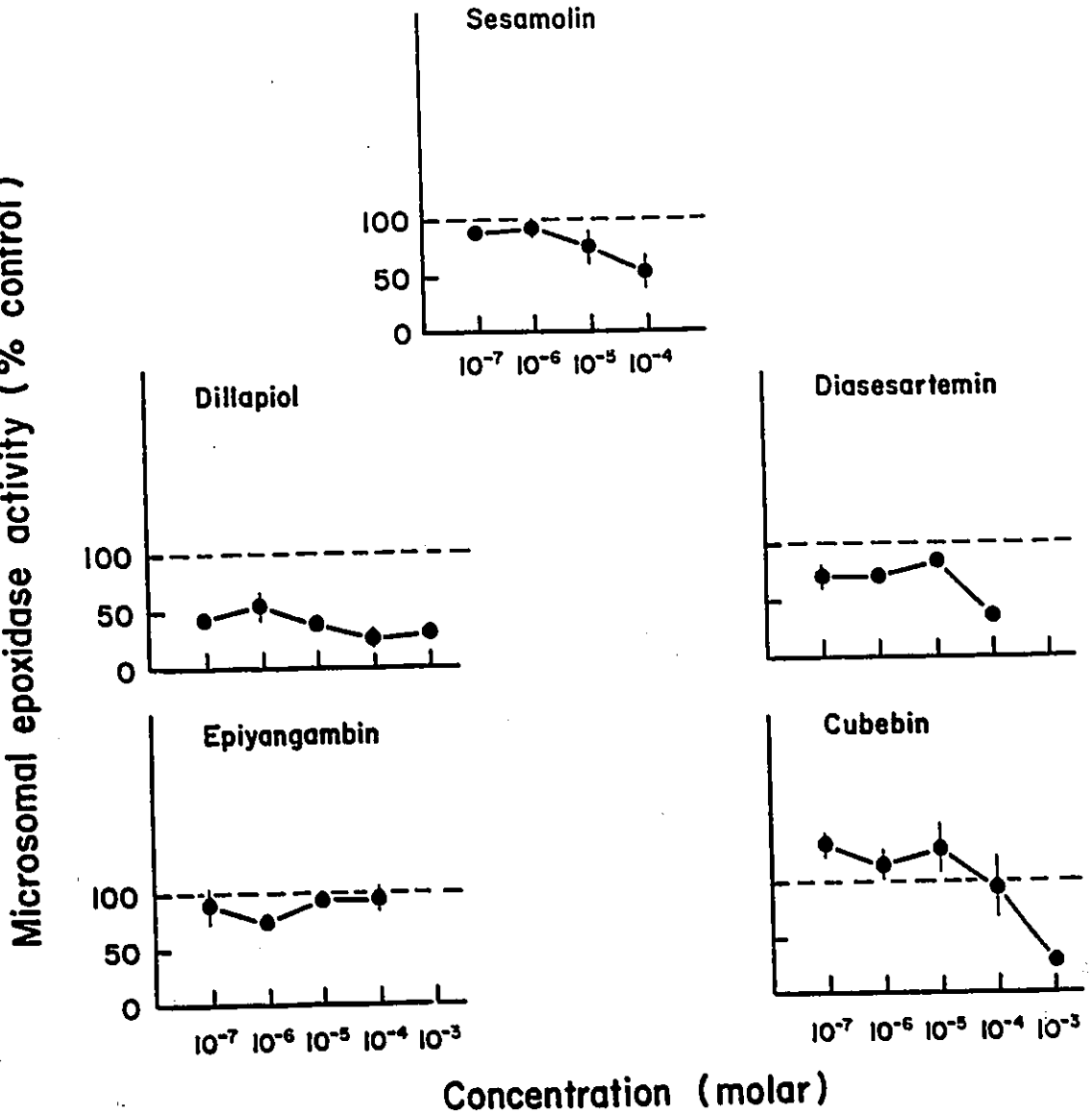
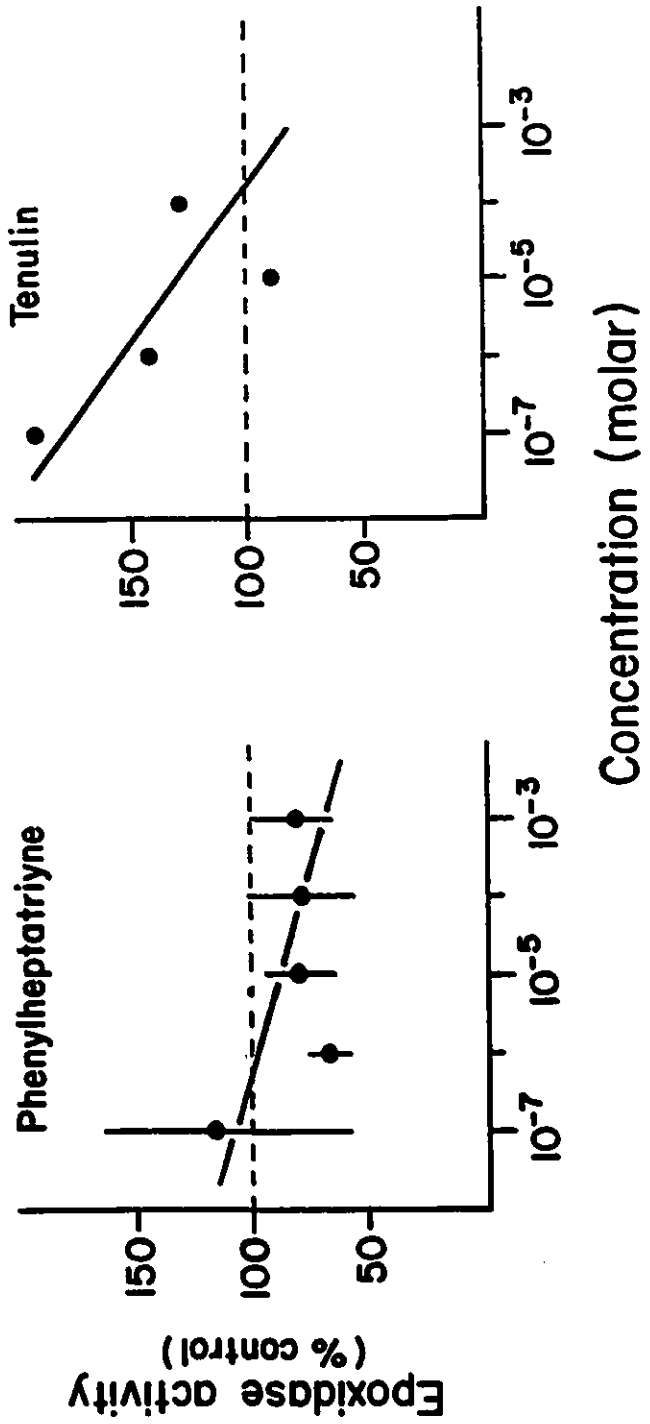

Sesamolin
Dillapiol
Diasesartemin
Epiyangambin
Cubebin
100
50
0
10^{-7} 10^{-6} 10^{-5} 10^{-4}
10^{-7} 10^{-6} 10^{-5} 10^{-4} 10^{-3}
Microsomal epoxidase activity (% control)
Concentration (molar)

FIGURE 15 : IN-VITRO ACTIVITY OF THE ALDRIN EPOXIDASE IN THE MIDGUT MICROSOMAL FRACTION OF O. NUBILALIS: Effect of two model allelochemicals from the Asteraceae, the polyacetylene, phenylheptatriyne (PHT) and the sesquiterpene lactone, tenulin (TEN).
(See Fig. 13 for assay conditions)


Tenulin
Phenylheptatriyne
150
100
50
10^{-7}
10^{-5}
10^{-3}
Concentration (molar)
Epoxidase activity
(% control)

(70% inhibition at 10 -3 M). Cubebin (CUB) also was an inhibitor only at a high concentration of 10 -3 M; Fig. 14). Epiyangambin (EPI) had no significant effect at any of the concentrations assayed (Fig. 14). I-50 values (concentration of the allelochemical inducing 50% inhibition of the enzyme activity, in vitro in the present case), were calculated from probit curves (log [% inhibition / % of control] versus log concentration of allelochemical), using values between 20% and 80% of controls (Table 5). DIL appeared to be the most potent inhibitor of the MFOs from the ECB, with an I-50 value of 2 x 10 -7 M, followed by DIA , CUB and SES in decreasing order.

Effects of the polyacetylene phenylheptatriyne (PHT), and the sesquiterpene lactone tenulin (TEN) were minimal (20% inhibition with PHT at 10 -5 to 10 -3 M). However TEN increased the activity of the epoxidase at the lowest concentrations (Fig. 15).

3.3 Effect of allelochemicals in vivo on the activity of the aldrin epoxidase (Fig. 16).

Phenobarbital (PHE) was used as a standard inducer, to test the response of the MFOs in the present system.

FIGURE 16 : EFFECT OF ALLELOCHEMICALS FROM THE ASTERACEAE ON THE IN-VIVO ACTIVITY OF THE ALDRIN EPOXIDASE OF O. NUBILALIS.

Allelochemicals were incorporated to the diet of third instar larvae during 4 days prior to the assay. Concentrations of the allelochemicals in the diet were:

- Control=CTR : solvent only
- Dillapiol=DIL : 100 u/g
- Tenulin=TEN : 1000 ug/g
- DIL + TEN : 100 + 1000 ug/g respectively
- Phenylheptatriyne=PHT : 100 ug/g
- Phenobarbital=PHE : 100 ug/g
- Piperonyl butoxide=PIP : 100 ug/g.

(See Fig 13 for assay conditions)

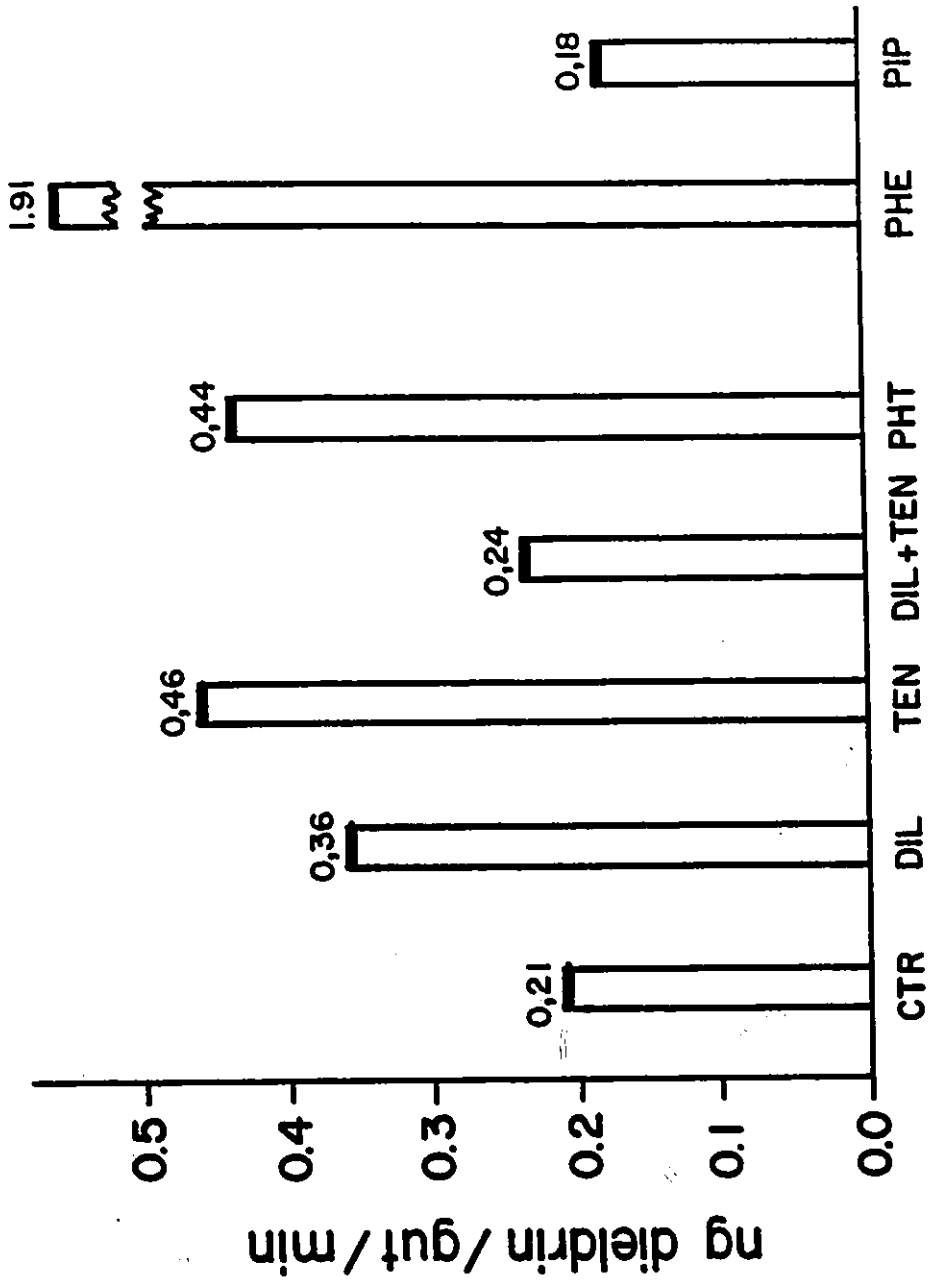

ng dieldrin /gut / min
0.0
0.1
0.2
0.3
0.4
0.5
0,21
0,36
0,46
0,24
0,44
1.91
0,18
CTR
DIL
TEN
DIL+TEN
PHT
PHE
PIP

Table 5: IN VITRO INHIBITORY ACTIVITY OF LIGNANS

LIGNAN	I_{50} (M)*
DILLAPIOL	2×10^{-7}
DIASESARTEMIN	6×10^{-5}
CUBEBIN	5×10^{-4}
SESAMOLIN	3×10^{-4}

* I_{50} values were calculated from probit curves using inhibitions values between 20% and 80% of controls.

(See Fig. 13 for assay conditions)

Four days feeding on PHE increased MFO activity by almost ten fold. Inhibition by piperonyl butoxide (PIP), a standard inhibitor, was not great (0.18ng dieldrin / gut / min. = 86% of control). All three types of allelochemicals were *in vivo* inducers of the MFO activity to varying extents. The smallest effect was observed with the association DIL+TEN (0.24ng dieldrin / gut / min. = 114 % of control), while the increases of activity with the lignan DIL, the polyacetylene PHT and the sesquiterpene lactone TEN were as follows: DIL 0.36 ng dieldrin / gut / min = 170% of control; PHT 0.44 = 200%; and TEN 0.46 = 220%). However, data should be interpreted with caution since limited amount of phytochemicals permitted only one trial. Furthermore, the amount of food ingested during the experiment was not measured.

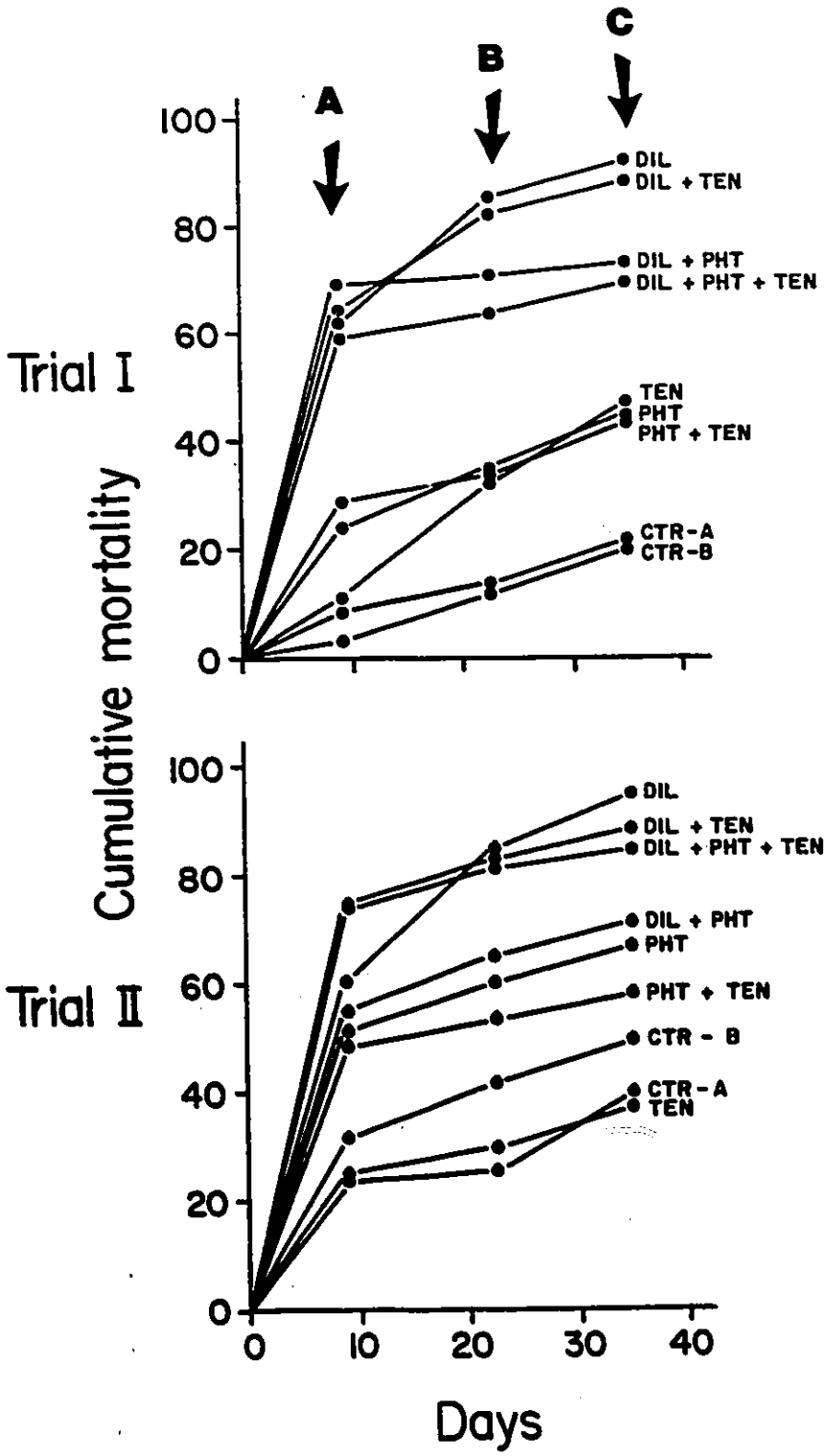
3.4 Effect of allelochemicals on growth and development

Acute toxicity was expressed by the cumulative mortality induced by the allelochemicals (Fig. 17). Mortality was recorded in the neonates (17a) throughout the larval development (17b) and in the pupal and adult stages (17c). The two trials are plotted separately for clarity and data from both trials are given below.

FIGURE 17 : CUMULATIVE MORTALITY IN O. NUBILALIS EXPOSED TO SINGLE, DOUBLE, OR TRIPLE ALLELOCHEMICALS FROM THE ASTERACEAE.

Mortality was assessed at day 10 after hatching (A), at the end of the larval stage (B) and at the end of the pupal stage (C).

(See Fig 16 for concentration of the allelochemicals in the diet)


A
B
C
Trial I
Trial II
Cumulative mortality
100
80
60
40
20
0
DIL
DIL + TEN
DIL + PHT
DIL + PHT + TEN
TEN
PHT
PHT + TEN
CTR-A
CTR-B
DIL
DIL + TEN
DIL + PHT + TEN
DIL + PHT
PHT
PHT + TEN
CTR - B
CTR-A
TEN
0
10
20
30
40
Days

In all treatments, mortality was maximal in the neonates, as seen at day 8 (Fig. 17a). During larval development (Fig. 17b) and the pupal and adult stages (Fig. 17c), toxicity was generally less pronounced. However, in all groups containing DIL, mortality remained high throughout the life cycle. DIL alone was the most toxic treatement with, respectively in trial I and II, 86-85% of the larvae dying before reaching the pupal stage, as compared to 12-26% in the control groups. Subsequent increase of mortality was observed in pupae, resulting in 92-95% of the insects failing to successfully reach the adult stage. The mortality curve for the double associations including DIL fell slightly beneath the curve of DIL alone, with a cumulative mortality at the end of development being 89-87% and 73-71% respectively with DIL+TEN and DIL+PHT. Surprisingly, the triple association was not always the most toxic of these DIL treatments, and resulted in a final cumulative mortality of 70-85%. Treatments with PHT, TEN or PHT+TEN, were slightly detrimental to O. nubilalis development. Although cumulative mortality differed between the two trials, probably because of the different times in the year at which the experiments were performed, total mortality remained low in these three last groups.

Table 6 :

EFFECT OF DIETARY ALLELOCHEMICALS ON THE LARVAL, PUPAL, AND ADULT WEIGHTS (mg) OF OSTRINIA NUBILALIS.
Trial I.

ALLELO-CHEMICALS *	MEAN LARVAL WEIGHT (mg) # Day 8	Day 13	Day 17	Day 22	MEAN PUPAL WEIGHT # (mg)	MEAN ADULT WEIGHT # (mg)
Trial I-A						
CTR-A	1.13c	8.51b	30.58c	78.46c	71.09a	39.91a
DIL	0.55ab	5.55a	19.76a	58.94a	75.07a	45.89a
TEN	1.00bc	8.35b	27.76bc	74.88abc	82.14a	47.27a
DIL+TEN	0.43a	5.38a	20.64ab	58.88a	79.82a	49.00a
Trial I-B						
CTR-B	1.69de	9.79b	34.09c	78.48c	77.80a	43.00a
PHT	1.83e	9.57b	29.21c	73.69ac	76.30a	40.90a
PHT+DIL	1.12cd	5.65a	20.83ab	60.53ab	77.90a	44.10a
PHT+TEN	1.94e	9.18b	27.78bc	70.40ac	75.80a	41.21a
PHT+DIL+TEN	1.25c	5.42a	19.41a	60.41a	83.50a	44.70a

* Concentrations in the diet were: DIL = 100 ug/g; TEN = 1000ug/g; PHE = 100 ug/g. CTR and treated diets contained the same amount of the solvent (EtOH=1%).

Values were subjected to Tukey's Multiple Range Test. Means in columns followed by the same letter are not significantly different at P = 0.05.

Table 7 :

EFFECT OF DIETARY ALLELOCHEMICALS ON THE LARVAL, PUPAL, AND ALDULT WEIGHTS (mg) OF OSTRINIA NUBILALIS.
Trial II

ALLELO-CHEMICALS*	MEAN LARVAL WEIGHT (mg)# Day 8	Day 13	Day17	Day 22	MEAN PUPAL WEIGHT # (mg)	MEAN ADULT WEIGHT # (mg)
Trial II-A						
CTR-A	1.04bc	12.07d	34.86c	102.44d	87.05a	53.62a
DIL	0.78ab	6.47abc	20.30ab	69.29abc	74.93a	45.84a
TEN	1.14c	8.92c	26.50bc	88.51cd	92.88a	51.61a
DIL+TEN	0.58a	4.32a	15.52a	56.65a	86.40a	45.84a
Trial II-B						
CTR-B	1.21c	7.94bc	32.01c	86.05bcd	80.71a	43.53a
PHT	1.19c	6.39abc	27.10bc	88.72cd	90.08a	51.46a
PHT+DIL	0.95bc	5.07ab	20.47ab	65.69ab	82.32a	46.57a
PHT+TEN	1.15c	6.04abc	21.53ab	64.63ab	91.85a	48.83a
PHT+DIL+TEN	0.77ab	4.05a	14.98a	48.08a	93.61a	52.14a

* Concentrations in the diet were: DIL = 100 ug/g; TEN = 1000ug/g; PHE = 100 ug/g. CTR and treated diets contained the same amount of the solvent (EtOH=1%).

Values were subjected to Tukey's Multiple Range Test. Means in columns followed by the same letter are not significantly different at P = 0.05.

Table 8:

EFFETS OF DIETARY ALLELOCHEMICALS ON GROWTH PARAMETERS OF *OSTRINIA NUBILALIS*@

* ALLELO-CHEMICALS	# MEAN TIME (days) TO PUPATION		# MEAN PUPAL WEIGHT (mg)		# MEAN TIME (days) TO ADULT EMERGENCE		# MEAN ADULT WEIGHT (mg)	
	M	F	M	F	M	F	M	F
CRTs-A	26.3bc	27.6a	66.9a	90.9a	34.8d	35.2d	33.9a	56.2a
DIL	28.6ab	29.9b	69.5a	87.6a	38.0ab	38.1abc	36.7a	55.0a
TEN	27.7abc	28.8ab	73.7a	103.2a	36.6bcd	36.8bcd	36.8a	62.5a
DIL+TEN	30.5a	30.7b	76.7a	91.2a	39.8a	38.5ab	37.5a	59.3a
CTRs-B	25.8c	26.0a	72.6a	87.4a	35.5cd	35.1d	35.3a	52.2a
PHT	26.4c	27.9a	72.1a	95.9a	35.6cd	36.6cd	35.2a	58.7a
PHT+DIL	27.3abc	30.1b	69.6a	93.8a	37.2abc	38.8abc	33.7a	58.1a
PHT+TEN	27.4abc	29.6b	73.6a	100.3a	37.0bc	38.3ab	34.2a	62.2a
PHT+DIL+TEN	29.4a	30.4b	77.2a	98.6a	39.0a	38.9a	35.9a	58.3a

@ Mean of trial 1 and trial 2.
* See table 6
# Time values were subjected to the Kruskal-Wallis test. Weight values were subjected to Tukey's Multiple range test. Means in columns followed by the same letter are not significantly different at p = 0.05.
M : males ; F : females.

Chronic toxity was evaluated by the weight gains and developmental times. Results are shown in Table 6, 7 and 8, and in Fig. 18a to 18d.

At the larval stage, in both trials, there was a reduction in the growth rate when DIL was present in the diet (Table 6 and 7). As a result, weights were significantly lower (P = Ø.Ø5, Tukey's multiple range test) than weights of both controls and otherwise treated groups, in groups treated with DIL, DIL+TEN, DIL+TEN+ PHT, and sometimes DIL+PHT. PHT and TEN alone slightly reduced weight gains during the larval stage when compared to controls, although this was not significant at P = Ø.Ø5. The double treatment PHT+TEN usually had a more important growth reducing effect than each compound alone, but here again, data are not significantly different from either control or single treatment except in a few instances (day 8 - trial I different from TEN, day 17 - trial I different from CTL, day 22 -trial II different from single treatments).

At the pupal and adult stages, the effect was reversed. Allelochemical treated groups, including single double and triple treatments, often reached a higher weight, although data were not significantly different at p = Ø.Ø5 (table 6 and 7).

FIGURE 18a TO 18d :

LARVAL GROWTH CURVES FOR O. NUBILALIS FED WITH SINGLE, DOUBLE, OR TRIPLE ASSOCIATIONS OF ALLELOCHEMICALS FROM THE ASTERACEAE.
(Weights are expressed as % of control. Bars represent the range of the mean value of the 2 separate trials - Trial I and Trial II - of 30 individuals per group).
Data are from Table 6 and 7.

18 - A : Dillapiol and tenulin: DIL and TEN

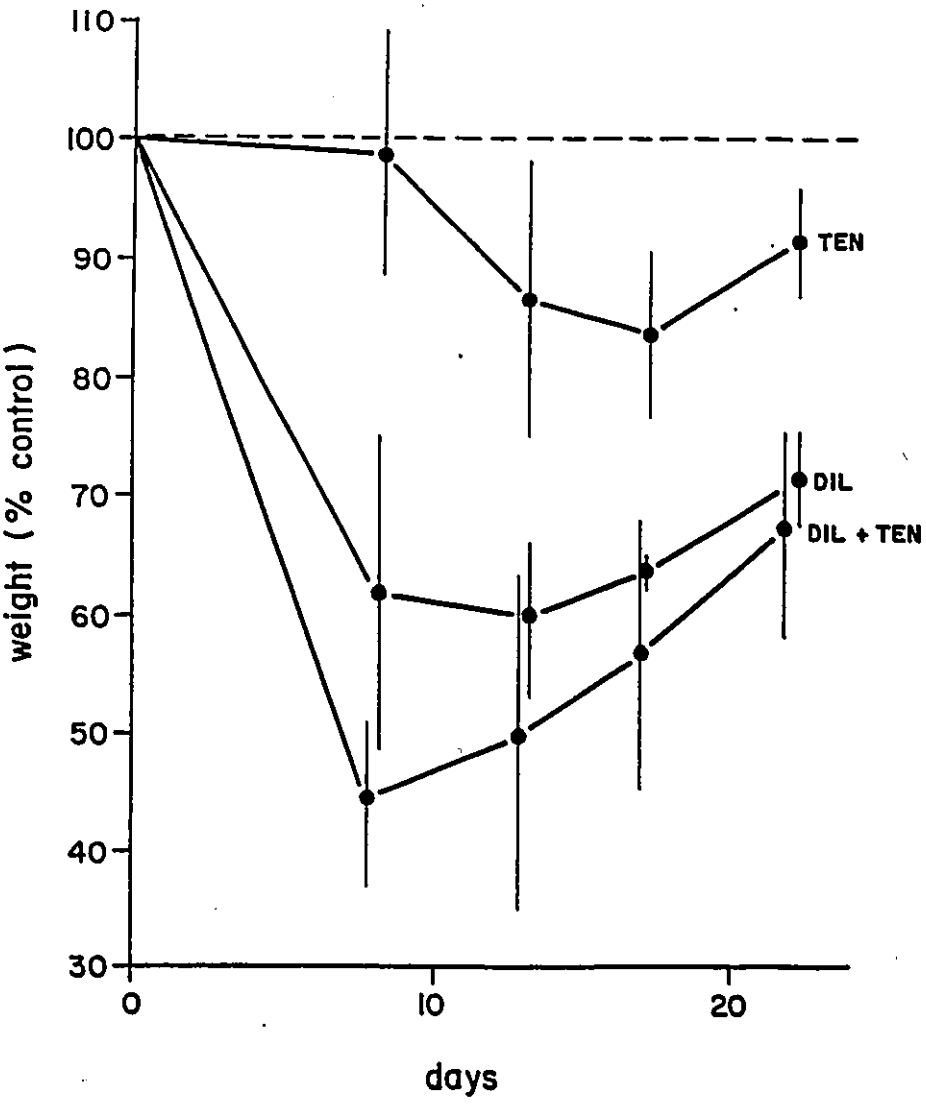
18 - B : Dillapiol and Phenylheptatriyne: DIL and PHT

18 - C : PHT AND TEN

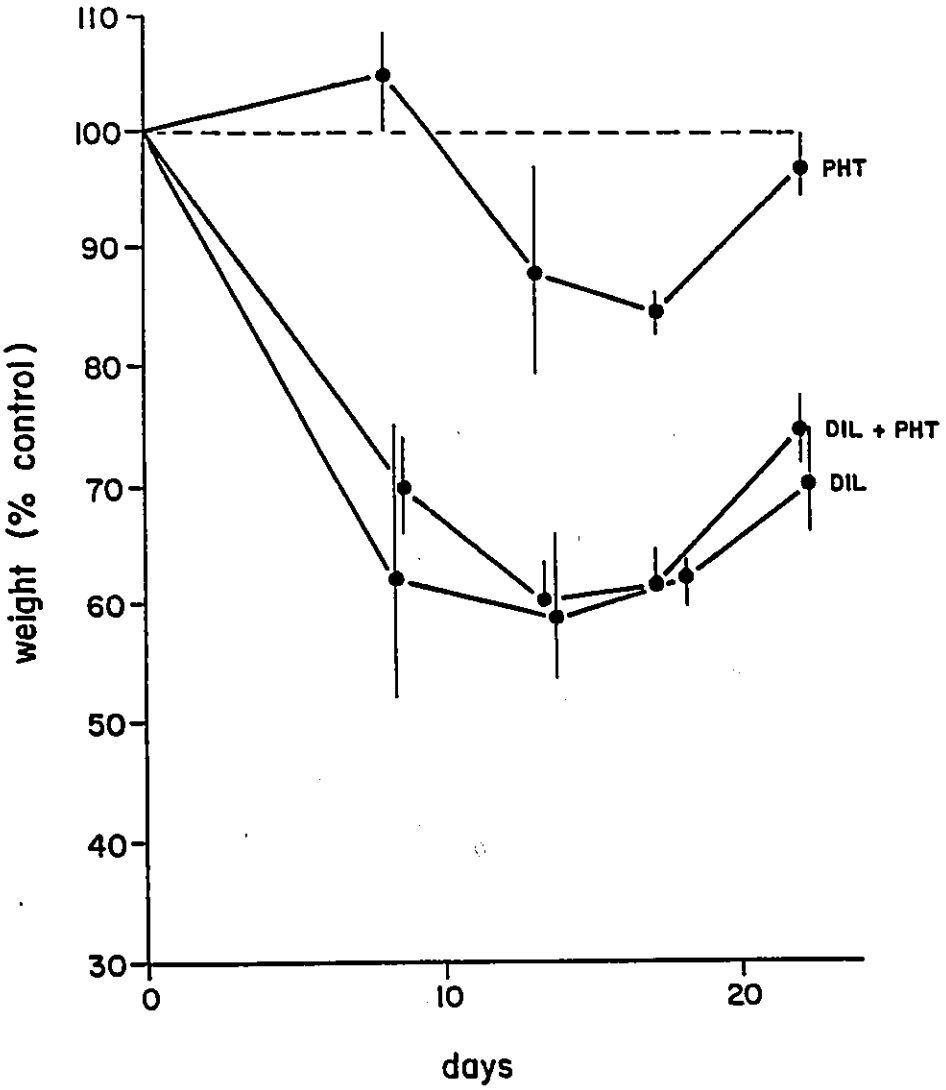
18 - D : PHT, DIL AND TEN

(See Fig. 16 for concentrations of the allelochemicals in the diet)

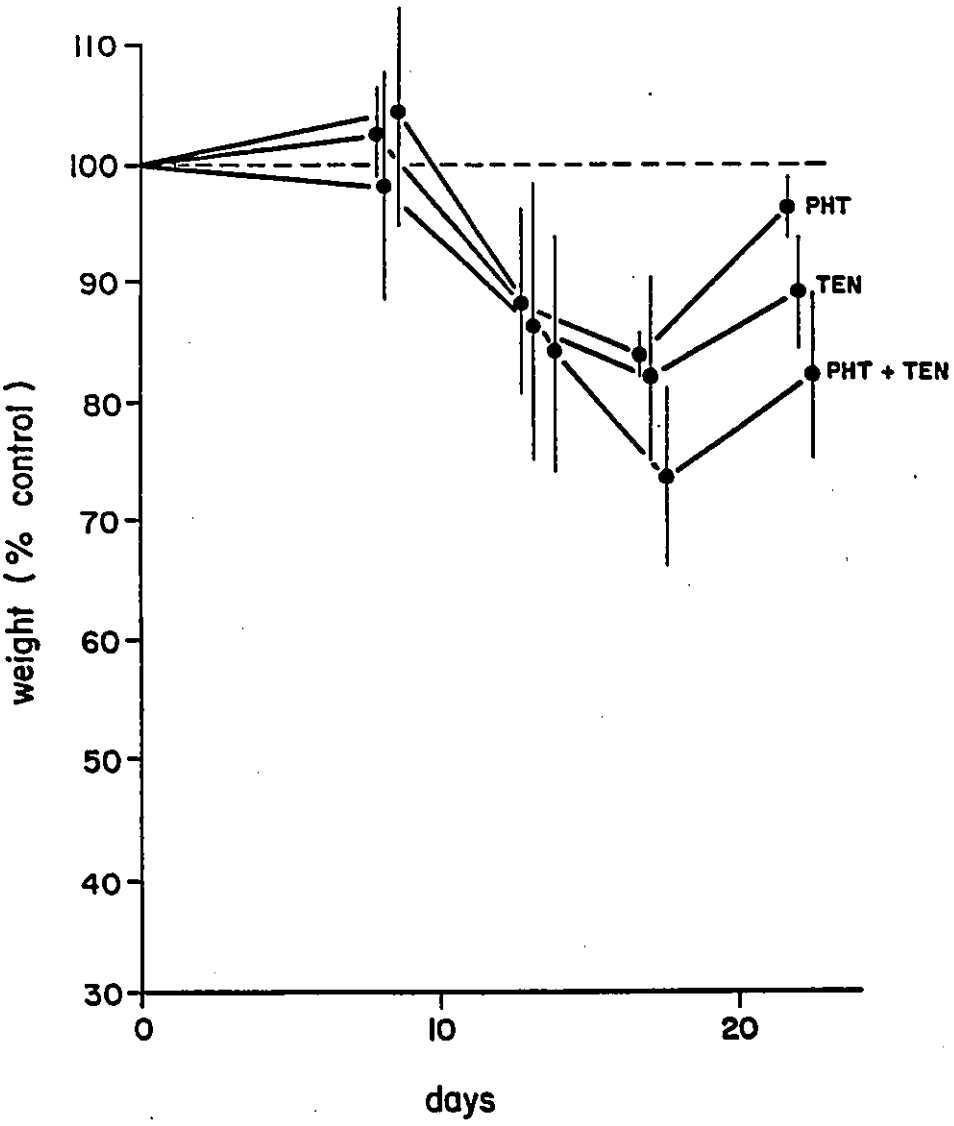
A :


110
100
90
80
70
60
50
40
30
0
10
20
days
weight (% control)
TEN
DIL
DIL + TEN

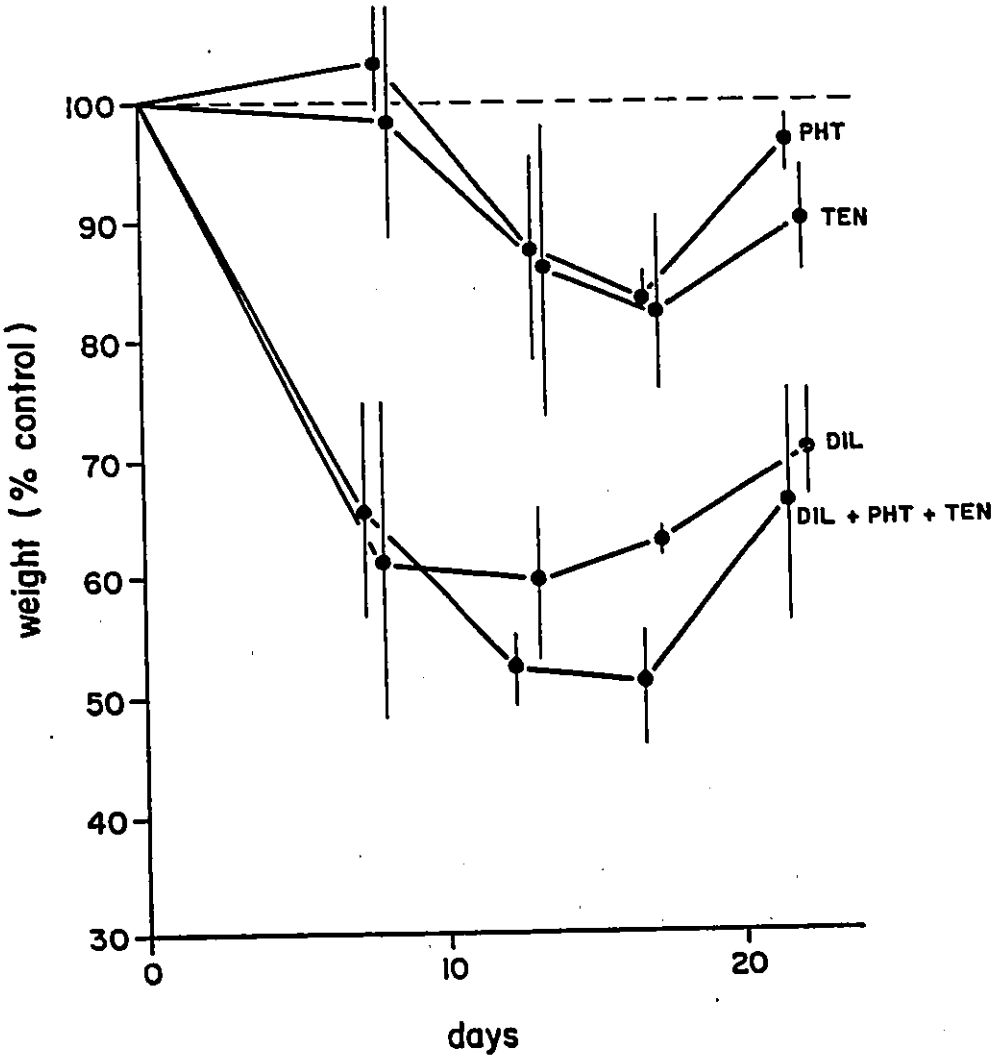
B :


110
100
90
80
70
60
50
40
30
weight (% control)
0
10
20
days
PHT
DIL + PHT
DIL

c :


110
100
90
80
70
60
50
40
30
0
10
20
days
weight (% control)
PHT
TEN
PHT + TEN

D :


100
90
80
70
60
50
40
30
0
10
20
days
weight (% control)
PHT
TEN
DIL
DIL + PHT + TEN

Since females reached a higher weight than males and the difference was significant in all groups (T-test, P = Ø.ØS), growth parameters at the pupal and adult stages were analysed separately by sex (Table 8). However, this did not yield significant differences in weights (Tukey's multiple range test, P = Ø.ØS) when the effect of one type of treatment was compared to the next. The time to reach pupation (Table 8) was however significantly affected (Kruskal-Wallis test followed by T test, P= Ø.ØS)) by treatments containing DIL, alone or in associations (a mean of 28.6 days for DIL and 3Ø.5 7days for DIL+TEN after hatching is significantly different from CRTs-A: 26.3 days; 29.4 days for PHT+DIL+TEN is significantly different from 25.8 days for CRTs-B). A similar trend of 2-5 days delay to reach adult emergence was observed in groups containing DIL and 1-2 days delay in those with PHT or TEN (Table 8).

In order to compare the effect of a single allelochemical to that of a combination of allelochemicals in the diet, weight gain curves in larvae were expressed as % of their control (using data from tables 6 and 7), and plotted on 4 different graphs, where the bars stand for the range of the means of 2 trials (Fig. 18a to 18d).

The double treatment DIL+TEN (Fig. 18a) reduced growth approximately twice as much as each compound alone. With PHT and TEN, the same effect was observed although growth reduction appeared at the end of the development only (Fig. 18c). PHT+DIL had an effect similar to DIL alone during the course of larval development (Fig. 18b). It is possible that the stimulating effect of PHT alone on growth (probably phagostimulatory), antagonized DIL's growth inhibiting effect particularly in the early stages.

When the three allelochemicals were combined in the diet, the resulting growth inhibition was more important than that induced by any single treatment (Fig. 18d), although this was not always significant (Table 6 and 7). However, a double treatment with DIL+TEN (Fig. 18a) was more detrimental to the larvae especially in the early stages than the triple treatment (Fig. 18d). The stimulating, or at least neutral, effect of both PHT and TEN up to the third instar (day 10 - 13) acted in a way opposite to the inhibitory effect of DIL alone. It resulted in weight gains higher with DIL+PHT+TEN than with DIL alone (Fig. 18d). A similar effect was noted with DIL+TEN (Fig. 18a), and was also observed to a lesser extent with DIL+PHT (Fig. 18b), when compared to DIL alone.

4. DISCUSSION

The results of the present study showed that, *in vitro*, the lignan DIL was a strong inhibitor of MFOs in *O. nubilalis*. However, no synergism occurred *in vivo* between DIL and either the polyacetylene or the sesquiterpene lactone, suggesting that *O. nubilalis* responded to even short time inhibition of MFOs by induction of higher levels of activity.

The results also indicated that *in vitro* inhibition of detoxification enzymes by a compound does not necessarily lead to synergism of insecticide by this compound. It is suggested that an adapted herbivore such as *O. nubilalis* can circumvent the plant's chemical defenses involving MFO inhibition.

In the present system, the lignan DIL also acted as an allelochemical in its own right, with a noticeable toxicity to the ECB. The results suggest that characterization of lignans as synergists is simplistic.

4.1 *In vitro* variations of MFO levels and activity in *O. nubilalis*

The cytochrome P-450 dependent MFOs from *O. nubilalis* are comparable to those of other insect species in their ability to produce characteristic difference spectra which, in other organisms, have been demonstrated to be associated with carbon monoxide adducts. In addition, MFO levels in the gut of *O. nubilalis* (section 3.1), are consistent with the data reported in the literature for other lepidopteran species with similar feeding habits. For example, it was 0.133 and 0.180 nanomoles/ mg protein respectively in the last larval instar of the tomato budworm, *Heliothis zea* and the southern armyworm, *Spodoptera eridania* (Neal, 1987; Murray, 1987). Iyengar (1988) also reported a similar level of cytochrome P-450 in *O. nubilalis* (0.313 nanomoles/mg protein).

The mean activity of 0.449 nanomoles of dieldrin formed /min /mg protein (SD= 0.20, n=7), obtained with the sensitive and reproducible test for MFO activity, namely the epoxidation of aldrin into dieldrin (Wolf et al., 1979), was very close to the value of 0.35 nanomoles /min /mg protein reported by Krieger et al. (1971). Williamson and Schechter (1970), using

premolting larvae and individuals in diapause (i.e. non feeding stages) of O. nubilalis, reported an activity in the gut epoxidase eight times smaller than in the present study. In several Lepidoptera species, there is indeed a close relationship between feeding and MFO activities, within a larval stage and from one instar to the next (Brattsten, 1986). Recently in H. zea (Neal, 1987), the MFO titre was reported to drop from 0.133 to 0.090 nanomoles /mg protein of cytochrome P-450, from the third to the fourth day of the last instar, while feeding is reduced as part of the pre-molting process.

The MFO system from the ECB is sensitive to a variety of lignans from the Asteraceae. In vitro screening revealed that four of them, DIL, DIA, CUB, and SES, mono or double methylenedioxyphenyl compounds (MDPs), were inhibitors of the microsomal epoxidase of O. nubilalis to various degrees. Current evidence indicates that inhibition by MDP derivatives, in mammals as well as in insects, results from the formation of a MDP - cytochrome P-450 complex (Testa & Jenner, 1981). The formation of such a complex may have occurred with some of the lignans used in this study (DIL, DIA, CUB and SES), as suggested by low levels of enzyme available for the substrate, aldrin (section 3.2.2). The

formation of cytochrome P-450 complexes could have been detected directly by a Soret peak at 455 nm and a type III difference spectrum of the dithionite reduced microsomal suspension (Kulkarni & Hodgson, 1976). However, this experiment was not performed in the present study, since emphasis was placed on inhibition of an MFO mediated conversion of a substrate.

I-50 values (Table 5), indicate that inhibition occurs with low concentrations of DIL (I-50 = 2 x 10-7), but that larger concentrations of DIA, CUB and SES are needed to obtain a similar inhibition of the epoxidase. When compared to the synthetic insecticide synergist PIP, whose mode of action is known to involve the formation of a MDP - cytochrome P-450 complex, the four lignans were less potent inhibitors of the ECB epoxidase. However, I-50 values of the lignans of the present study are comparable to those of some groups of insecticide synergists, as well as in vitro inhibitors of the aldrin epoxidase. For instance, 6-butyl-1,2,3-benzothiadiazole had an I-50 of 4.9 x 10 -7 M, and 6-methoxy-1,2,3-benzothiadiazole an I-50 of 6,5 x 10 -5 M using the gut enzyme preparation of the 6th larval instar of S. eridania (Gil & Wilkinson, 1977).

The only non-MDP lignan of this study, EPI, was an *in vitro* inhibitor of the epoxidase only at high concentrations. However, the MDP ring is not the only structure in lignans that is known to inhibit enzyme activities. EPI contains a biepoxyfuran ring, a structure that appears important in the biological activity of other lignans such as syringaresinol , pinoresinol, or dehydrocaffeic acid dilactone, all inhibitors of cAMP phosphodiesterase (MacRae & Towers, 1984). The cytochrome P-450 complex, however, does not appear to be very sensitive to the biepoxyfuran structure in the present experiments.

The insensitivity of the MFO preparation to PHT is surprising since a broad range of monoacetylenes, including phenylacetylenes, have been shown to destroy the prosthetic heme of the cytochrome P-450 by alkylation in a self catalysed reaction *in vitro* (Ortitz de Montellano and Correira, 1983), which probably explains their use as synergists of pesticides (Casida, 1970). At the concentrations of the present assays, however, no destruction was observed. Rather an enhancement of the activity occurred with the lowest concentration of PHT. Possible reasons for the lack of the previously described activity are that triacetylenes act differently from monoacetylenes, or that ECB MFOs

are insensitive to this type of inhibitor. Such an adaptation would be a distinct advantage to a polyphagous insect such as the ECB which feeds on many acetylene producing plants.

In other systems, the mode of action of TEN, *in vitro* as well as *in vivo* (Hall et al., 1977; Arnason et al., 1987), probably involves the formation of covalent complexes with sulfhydryl groups of proteins through electrophilic type addition at the site of the cyclopentenone ring . The moderately low inhibition by TEN may be due to alkylation of S-H groups present in the microsomal preparation.

4.2 *In vivo* variations of MFO activity with allelochemicals

In 4 day feeding trials (section 3.3), all allelochemicals tested alone induced MFO activity to various extents. This is in apparent contradiction to the *in vitro* results. However, it is now clearly established that the *in vivo* effects of MDP compounds and some other synergists are biphasic. The initial inhibition subsequent to the formation of MDP - cytochrome P-450 stable adducts, is often followed by an

induction of the titer of MFOs, in liver microsomes (Fisher et al., 1981), as well as in insects (Yu & Terriere, 1974; Thongsinthusac & Krieger, 1974). Thus, the initial inhibition of the MFO activity observed with DIL added to the ECB microsomes *in vitro*, appears to be masked by the induction of the enzyme during the 4 day feeding period. Although *de novo* synthesis is probably involved, this remains to be demonstrated in the present system. Moreover, the effectiveness of the monooxygenase may be improved, possibly through induction of specific cytochrome P-450. Increased enzyme effectiveness was observed in *Musca domestica* with several substrates, including aldrin, where the amount of cytochrome P-450 in pesticide resistant strains was 3 times that of susceptible strains, but the epoxidase activity was 30 times higher (Agosin, 1985). While inhibition of MFOs is one defense strategy available to plants in the Asteraceae, successful herbivores such as the ECB can overcome these defenses by induction of MFOs.

The association of DIL and TEN in the diet of *O. nubilalis* had apparently little effect on the gut MFO activity. This is unexpected in view of the induction observed with each of the allelochemicals separately.

However, the consistency of these results will appear in the subsequent discussion of life cycle studies (section 4.3.1).

4.3 Influence of allelochemicals on the life cycle of O. nubilalis

One of the questions raised in this study was whether DIL inhibition, and possibly secondary induction of MFO activity, affected the overall tolerance of O. nubilalis to the other allelochemicals.

This study confirms the toxicity of PHT and TEN to insects but is the first report of DIL toxicity to insects. Although we have information on the mode of action of PHT and TEN (Arnason et al., 1983 and 1987), which explains the mortality observed in the present study by these compounds, we have little information on the mode of action of DIL. However, MFO patterns of inhibition and induction shown in this study may bring some light on the mechanisms of toxicity of this allelochemical.

4.3.1 Acute and chronic toxicities.

Both acute and chronic effects are important to insect development and they were especially evident ECB with DIL. Acute toxicity of allelochemicals is most often evaluated in terms of induced mortality. However, impairment of growth , a prolonged development period, or a carry-over effect on imaginal stages, all signs of chronic toxicity, may have drastic long term consequences for insect herbivores. Increased duration of the sensitive larval stages can increase exposure to predators and parasitoids (Pryce et al., 1980), or may cause failure to reach the appropriate life stage before the onset of adverse climatic conditions.

All treated groups of neonate larvae exhibited high mortality. In effect, highest sensitivity to xenobiotics in early larval instars of lepidoptera is common (Brattsten, 1979; Yu, 1983; Ahmad, 1986). For instance, in two strains of ECB, the LC-50 (concentration inducing 50% mortality in a population), for ingested carbaryl was 10 times smaller in 3rd than in 5th instar larvae (Kuhr & Davis, 1975). PHT , several other polyactylenes and TEN also induced highest

mortality in early stages of the ECB (Champagne, 1984; Arnason et al., 1987). Susceptibility to xenobiotics correlates well with low MFO activity in several insect species (review in Hodgson, 1985), and the lack of MFO activity is a well known fact in fetal and newborn mammals or birds (Testa & Jenner, 1981). Although data for neonate insect larvae are lacking, hatching and young insect larvae most likely also lack the enzymatic machinery necessary to detoxify certain compounds. MFO specific activity was in fact lowest in second instar gypsy moth , Lymantria dispar (Ahmad, 1986), the earliest measurement, as compared to later instars. From these data and previously reported ones mainly on the southern army worm, S. eridania (Krieger & Wilkinson, 1969), and the Japanese beetle, Popilia japonica (Ahmad, 1983), feeding appears to trigger, or at least to increase, the MFO activity throughout the insect developing stages, so that activity in hatching larvae, before feeding begins, should be minimal, and result in a high toxicity of ingested xenobiotics.

Furthermore, feeding on MFO inducers would affect the overall survival of the larvae. In fact, in groups fed with TEN and PHT, both important inducers of MFOs (section 3.3), mortality was similar to that of control

groups (section 3.4). Previous studies showed that PHT had both an important photodynamic action together with a low 'dark' toxicity in several insect species, including the ECB (Arnason et al., 1981; Champagne , 1986), and this study confirms the low 'dark' toxicity of this compound at the level of 100 ug/g in the diet. Similarly, the adverse effects of TEN on insects include growth reduction, longer developmental periods and reduced pupal and adult weights (Arnason et al., 1987), which are signs of chronic toxicity . In the case of the ECB, chronic toxicity rather than acute toxicity of PHT and TEN at the levels used in this study, may have limited the negative effects observed in early and at later instars, as it allowed time for MFO induction.

In contrast, the high mortality observed throughout the insect's life cycle in the four groups containing DIL may result from the inherent toxicity of the compound itself , combined with the minimal induction of MFOs observed with DIL. DIL fed larvae had the highest mortality of all groups, but when either TEN or PHT , or both together were added, mortality decreased. It is possible that MFO induction by PHT and TEN had favored the rapid elimination of DIL ingested together with these allelochemicals, and partially

prevented DIL toxicity. Such an induction by PHT and TEN acts as an antagonist of the inhibition of the MFOs by DIL. Similarly, reduction of acute toxicity of the insecticides carbaryl and diazinon was observed when larvae of Heliothis virescens and H. zea were fed on plants containing secondary substances with MFO inducing capacity, such as certain monoterpenes in peppermint leaves or 2-tridecanone in tomato leaves (Riskallah et al., 1986).

DIL also had a negative effect on growth and development time of the ECB larvae (section 3.5). Growth inhibiting activity of lignans on insects has been documented in only one case: kubosin and sesamin, both from Magnolia kobus inhibited growth in silkworm larvae, Bombyx mori (Kamikado et al., 1975). The role played by feeding deterrency or decreased utilization of food in the chronic toxicity with DIL remains unknown. Although no feeding deterrency was observed in a preliminary study where DIL was incorporated into the meridic diet and fed to third and fourth instar larvae, further studies on antifeedant action need to be performed using fresh leaf material treated with the allelochemical. In fact, feeding inhibition may remain hidden due to the obstruction of the pores of the

chemosensory papillae by the meridic diet. This phenomenon was first reported in Manduca sexta (Schoonhoven, 1967) and was also observed in ECB larvae with azadirachtin, a well known antifeedant, that lost this activity when incorporated in an artificial diet (Arnason et al., 1985b).

In contrast to what was observed for mortality, TEN, when added to DIL, further inhibits growth in the ECB. The individual negative effect of TEN as well as that of several other sesquiterpene lactones on insect herbivores includes growth inhibition and lengthened development time (Picman, 1986; Arnason et al., 1987), and a potentiation effect between DIL and TEN occurred while double stress was applied to the insect. Conversely, PHT at levels similar to those used in the present study, was reported to have a phagostimulant effect on young larvae of Euxoa messoria (Champagne, 1984). Such an effect on the ECB, may have partially antagonized the growth inhibiting activity of DIL, resulting in a growth curve with PHT+DIL slightly above that of DIL alone. The role of DIL in these associations of allelochemicals is not constant. In the red flour beetle, Tribolium castaneum, DIL as well as some of its ester derivatives was reported to synergize

the activity of pyrethrins and N-methylcarbamates (Mukherjee et al., 1979). The activity of DIL observed in the present study could perhaps be compared to that of closely related compounds, safrole and iso-safrole, differring from DIL by two methoxy groups in place of the two hydrogens in the phenol ring. These MDP lignans were also inhibitors of MFOs in mammals and insects (Marcus et al., 1987; Wilkinson et al., 1984), while in rats, they altered cholesterol levels and were hepatocarcinogenic (Hagan et al., 1965). The mechanism of toxicity was believed to involve metabolic and pharmacokinetic factors as much as intrinsic inhibitory activities (Testa & Jenner, 1981).

Growth inhibition results suggested that each of the allelochemicals in the present system contributed to the toxity of the mixture, featuring a simultaneous attack at different or similar targets in the insect, rather than one non toxic compound synergizing the activity of the others.

Both an additive stress and true synergism have been described in the interaction between plant allelochemicals. For instance, the insecticidal amides pellitorine and piperine, or also pipercide and

guineensine, all present in the black pepper, Piper nigrum, had a substantially increased toxicity to the Adzuki bean weevils, Callosobruchus chinensis, when administered in pairs rather than individually (Miyakado et al., 1983). Also, several furanocoumarins from the seeds of the parsnip, Pastinaca sativa, exerted synergistic activity on the corn earworm, H. zea, through both a photodynamic action (xanthotoxin, bergapten) and via a probably completely different non-photodynamic mechanism (isopimpinellin, imperatirin) (Berenbaum, 1984). In addition, multiple attack may result from the action of chemicals synthesized by plants through unrelated biosynthetic pathways, as in the system assayed in the present study. The parsnip, Pasticana sativa, contains among other allelochemicals furanocoumarins and large amounts of the MDP lignan myristicin, which acts as an even more potent synergist of the furanocoumarin xanthotoxin than piperonyl butoxide (Berenbaum and Neal, 1985), probably through the impairment of the detoxification processes of H. zea. The lack of synergism in this study as compared to other studies presented above, appears to be related to the insect response in inducing MFOs.

4.3.2 General conclusions: Adaptability of insects to plant allelochemicals.

Plant systems to circumvent insect resistance mechanisms through the synthesis of MDP compounds, inhibitors of the detoxification enzymes in insects, represent a sophisticated and a potentially very successful defense strategy. In fact, the MDP compounds studied did inhibit MFO activity *in vitro* and potentially could facilitate the activity of already existing defense chemicals. The presence of such a system in plants could be very effective towards non adapted insect species.

However, the ECB appears well adapted. This species is commonly exposed to at least 2 MFO inhibitors, DIA in *Artemisia* spp. and DIL in *Erigeron* spp., in addition to several polyacetylenes and sesquiterpene lactones. Selection may have favoured the individuals capable of MFO induction with these compounds, a situation paralleling the selection of strains with high MFO levels in resistance to pesticides. Other studies with the ECB also showed high adaptative capacities of this particular insect to plant allelochemicals. Avoidance of polyacetylene

phototoxicity through concealed feeding and spinning of large amounts of silk (Champagne, 1984), together with a fast clearance of the allelochemical (Iyengar, 1988), may be preadaptations of the ECB to a group of allelochemicals abundant in its host plant. These specific types of enzymatic, metabolic or behavioural adaptations contribute to the success of the ECB as a pest.

4.4. Future work

A few questions need to be addressed with regard to the activity of lignans against phytophagous insects:

Firstly, can synergism between the same allelochemicals occur in insects probably unadapted to MDP lignans, such as in the Solanaceae specialist Manduca sexta?

Secondly, the physiological and molecular basis of lignan activity in the insect, should be further investigated. What are the primary or the most sensitive sites in insects, and how is it distributed in the body? What physiological processes other than MFO are altered?

Thirdly, the role of MFOs in insect adaptation appears important. How does induction and inhibition occur in the ECB? Does it also exist to a similar extent in other insect species? Is it specific to certain allelochemicals? Knowledge on these enzymatic activities could help to further evaluate the mechanisms of plant defenses.

In view of the potential use of selected lignans as insecticide synergists, investigations on how these inhibitors affect the third trophic level should be carried out. The question of whether MFO inhibitors are passed on to the parasitoid, or metabolized first by the insect, appears important to optimize natural control mechanisms in an integrated pest management strategy.

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APPENDIX: A

Composition of the semi-defined diet of Ostrinia nubilalis. (Adapted from Guthrie, 1971).

INGREDIENTS	WEIGHT (g)
Distilled water	70.00 ml
Agar	1.50 g
* Dry mix (prepared in advance)	9.80 g
Methyl-p-hydroxybenzoate	0.40 ml
Propionic acid	0.46 ml
Formaldehyde	0.04 ml
Fulmidil B	0.04 g
Corn cob grits	2.20 g

* Dry mix composition:

wheat germ (defatted)	27.7 g
dextrose (anhydrous)	21.3 g
casein	23.4 g
cholesterol (USP, Bioserv)	1.7 g
salt mix W (Wesson, Bioserv)	7.7 g
Vanderzant-Adkisson vitamin mix	4.9 g
ascorbic acid (USP, Bioserv)	6.4 g
aureomycin (14.1%)	1.4 g
sorbic acid (Bioserv)	3.8 g

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