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SYNERGISTIC ACTIVITY OF THE MONOLIGNOL DILLAPIOL AND VARIATION OF ITS PRODUCTION IN TWO PLANTS, *Anethum graveolens* AND *Piper aduncum*

by Anne-Sophie Belzile

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ABSTRACT

This study examined the plant derived monolignol dillapiol, a naturally occurring insecticide synergist. I initially compared dillapiol with the commercially-available piperonyl butoxide and showed similar synergistic activity of the two substances, combined with various natural insecticides, against the mosquito larvae (*Aedes atropalpus*). Subsequently, the activities of 19 synthetic dillapiol derivatives and analogues were assessed for synergism of the phototoxic activity of alpha-terthienyl towards *A. atropalpus*. While several promising new and patentable synthetic synergists were found, the naturally occurring lead molecule remained among the most active substances. The synergistic activity also was found to be predicted using the lipophilicity of the analogues, by Quantitative Structure-Activity Relationship analysis (QSAR).

Given the practical potential of dillapiol, the second part of the thesis concentrated mainly on environmental parameters affecting dillapiol production in two plants, Indian dill, *Anethum graveolens* and the Central American wild pepper, *Piper aduncum*. It was found that a higher nutrient availability affected positively the production of dillapiol in dill in a greenhouse experiment but negatively in wild accessions of *Piper aduncum*. Simulation of herbivory using methyl jasmonate did not succeed in increasing the concentration of dillapiol in dill. *Piper aduncum* is a good commercial source of dillapiol since it produces it in very high concentrations (4% d.w.) and can be easily propagated by vegetative means.

à mes parents, Christiane et Flavien

RÉSUMÉ

Cette étude a porté sur le monolignol dillapiol, une substance d'origine végétale reconnue pour ses propriétés synergiques avec divers insecticides.

Une première expérience a eu pour objet de comparer le synergisme du dillapiol avec celui du piperonyl butoxide, un composé synergique synthétique commercial. Les résultats ont montré une activité similaire des deux substances en présence de divers insecticides naturels, contre des larves de moustiques, *Aedes atropalpus*. Dans une seconde étude, le synergisme de 19 dérivés et analogues synthétiques du dillapiol a été mesuré en présence de la phototoxine alpha-terthienyl, sur des larves *d'A. Atropalpus*. D'une part les résultats ont révélé de nouveaux composés synergiques prometteurs qui pourraient être brevetés. D'autre part, la substance naturelle s'est avérée être parmi les plus actives. Il a par ailleurs été montré, par une analyse de la relation quantitative structure-activité (RQSA) , que le synergisme des analogues peut être prédit par leur lipophilicité.

Étant donné le potentiel d'application pratique du dillapiol, la seconde partie de la thèse a été consacrée à l'étude des paramètres qui affectent la production du dillapiol dans deux plantes, *Anethum graveolens*, l'aneth indien, et *Piper aduncum*, un poivrier sauvage d'Amérique centrale. Les résultats ont montré que la disponibilité des éléments nutritifs dans le milieu affecte positivement la production de dillapiol dans de l'aneth cultivé en serre, tandis qu'elle l'affecte négativement dans le poivre récolté à l'état sauvage. La simulation de l'herbivorie par l'ajout de jasmonate de méthyle n'a pas provoqué

d'induction de la production de dillapiol dans l'aneth. *Piper aduncum* serait une bonne source commerciale de dillapiol puisqu'il en produit en concentrations élevées (4 % m.s.) et qu'il pourrait être facilement cultivé par le biais de boutures.

ACKNOWLEDGMENTS

I would first like to thank Professor John Thor Arnason. He was an impressively knowledgeable, patient, open minded and tolerant supervisor! But also, while I had uncertainties and doubts about the program and my ability or will to pursue it, he understood and supported me throughout the years I spent in his laboratory and I will never thank him enough for it.

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LIST OF ABBREVIATIONS

α-T: alpha-terthienyl

ANOVA: analysis of variance

C. I.: confidence interval

JA: jasmonic acid

LC_{50}: Lethal concentration lethal to 50% of a population

MDP group: methylenedioxyphenyl group

MJ: Methyl jasmonate

PAL: phenylalanine ammonia lyase

PBO: piperonyl butoxide

PSMO: polysubstrate monooxygenase

SF: factor of synergism

I: Introductory Chapter

I. 1 GENERAL INTRODUCTION

Pest and pathogen resistance to chemicals, defined as the failure of a pesticide to control a pest with previously efficient doses and under the same conditions, is a constantly increasing phenomenon [Georghiou, 1990; Rotteveel, 1997]. Insecticide resistance was first reported in 1914 [Melander, 1914], and there are over 500 reported types of resistance at present. In human therapy, forms of resistance to chemical treatments are also common, for example, the efficiency of cancer and malaria treatments may fail because of multi-drug resistance of target cells [Gottesman and Pastan, 1993].

On the other hand, natural toxic defenses to pests in plants involve the use of combinations of phytochemicals with different modes of action rather than a single pure active principle [Guillet; 1997] which decreases the probability of occurrence of resistance or increases the efficacy of defenses. Thus recent research focuses on the effect of combinations of active principles together or with synergists or potentiators [Raffa et al., 1985; Clarke et al., 1997]. Various plant models of multifactorial defenses were the subject of a recently published conference entitled "Phytochemical diversity and redundancy in Ecological Interactions" [Romeo et al., 1996]. Among the effective plant combinations are mixtures of toxic secondary metabolites with synergists such as lignans.

Through chemical screening of various tropical plant species, the lignan dillapiol was isolated in our laboratory by Bernard et al. (1995) from an active fraction of Piperaceae plant extract (the Pepper family). Dillapiol showed promise in combination with naturally occurring insecticides in order to produce efficient botanical insecticides. The practical potential of the compound or its derivative is excellent yet many steps are necessary before possible commercialization. Among those steps which are addressed in this thesis are: 1) a better understanding of the efficiency of dillapiol in combination with various active agents, 2) a possible optimization of its activity by selection of the best analogues of the lead molecule, and 3) the understanding of its production by plants and an evaluation of potential recoveries. The objective of this thesis was to address some of the above aspects of dillapiol activity and production.

I. 2 LITERATURE REVIEW

I.2.1 Dillapiol, a lignan

Lignans and related monomeric phenylpropanoids have been the object of considerable interest because of their biological properties such as the antitumor [Dewick and Jackson, 1981] or antiviral activity [Kelly and Hartwell, 1954] of some podophyllotoxins first isolated in the resin of *Podophyllum*. Other properties have also been described such as antimicrobial, antifungal or antifeedant activities [Macrae and Towers, 1984; Whiting, 1990]. These secondary metabolites produced via the shikimic acid pathway in which the phenylalanine ammonia lyase (PAL) is a key controlling enzyme. Further enzymatic reactions lead to the production of the phenylpropanoid dimers known as lignans. Lignans may be broken down to produce simplified neolignans (monomeric phenylpropanoids) like dillapiol **(Figure I.1)**. The enzymatic system related to their biosynthesis is still relatively poorly understood [Davin and Lewis, 1991]. Dillapiol contains a methylenedioxyphenyl (MDP) group. This functionality in dillapiol, as well as all the other MDP-containing molecules such as apiol, sesamin or safrole, is responsible for inhibition of polysubstrate monooxygenases (PSMOs) and synergist properties of the molecule.

A synergist is a substance that increases the activity of another chemical, but that is inactive alone at the concentrations used [Metcalf, 1967]. The synergist may inhibit PSMO's and prevent metabolism of the active molecule or facilitate the penetration of the active molecule and ensure that it reaches its target [Bernard, 1995]. This first mode of action has previously been described for various insecticide synergists containing MDP groups and evidence suggests that dillapiol has this activity [Bernard et al., 1989]. MDP compounds bind specifically to the cytochrome P-450 of PSMO in insects which is responsible for enzymatic processes allowing insects to metabolize toxins into more hydrophilic and therefore more easily excretable substance. The best known MDP synergist is piperonyl butoxide (PBO) **(Figure I.2)**, a synthetic derivative of the naturally occurring safrole, a dillapiol analogue. Dillapiol has been previously shown to synergize a number of potent insecticides, was found to synergize a number of potent insecticides, organophosphates, carbamates and thiophenes [Singh et al., 1975; Lichtenstein et al., 1974; Mukerjee et al., 1979; Guillet, 1997]. Dillapiol has been identified in a variety of plants from different families. For example, it has been found to occur in Apiaceae, in dill

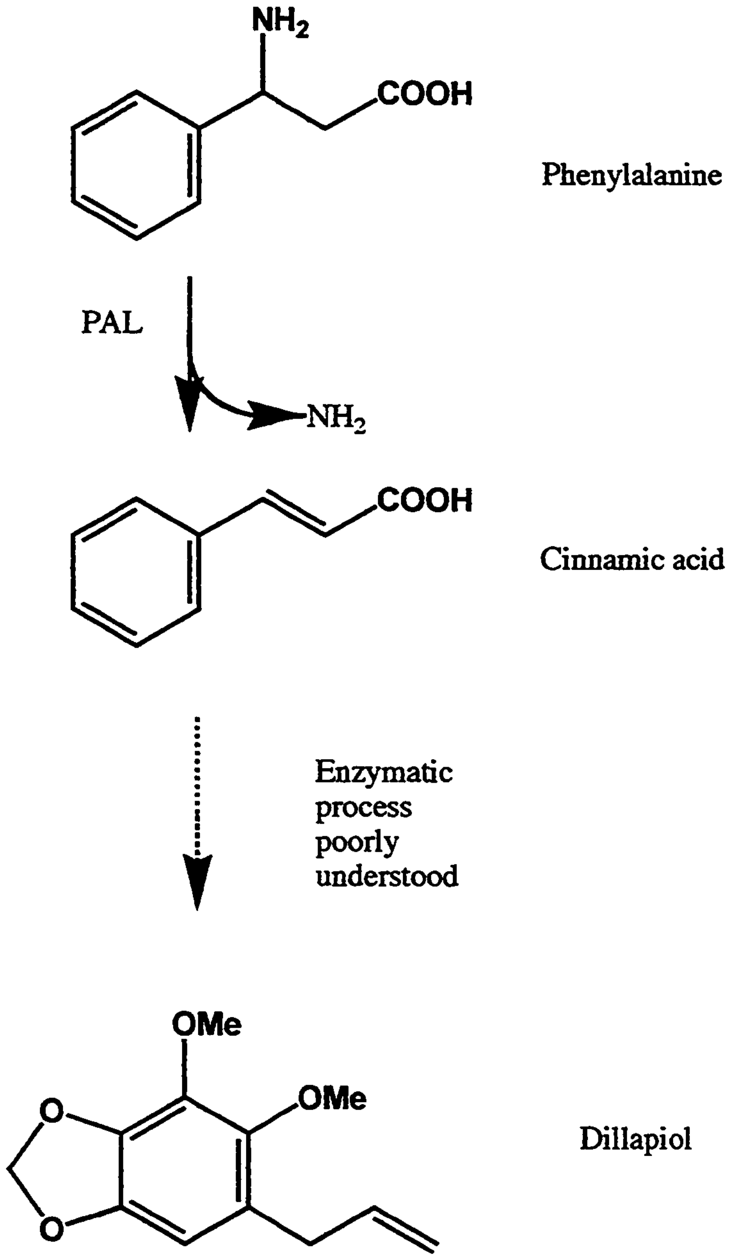


FIG. I.1: Dillapiol biosynthesis via the phenylpropanoid pathway

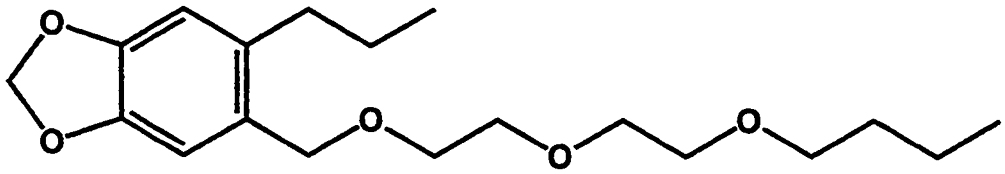


FIG. I.2 : Piperonyl butoxide

but also in *Pituranthos tortuosus* [Abdel Ghani and Hafez, 1995] of which it constituted 94% of the fruit essential oil and in seeds of *Liguisticum scotium* L. It was also found in *Perilla frutescens* from the Labiatae family which contains 0.35 % v.w. dillapiol in its oil [Honda et al., 1988], in *Erigeron* from the Asteraceae [Sørensen and Sørensen, 1969], in *Ocotea cymbarum*, a Lauraceae [Andrei et al., 1988] and in various Piperaceae such as *Piper auritum* [Castro et Poveda, 1983], *P. banksii* [Banerji and Das, 1989], *P. nigrum* [Russel and Jenning, 1969], *P. novae-hollandiae* [Loder et al., 1969], *P.umbellatum* [Chatrol, 1964], *P.hispidum* [Burke and Nair, 1986], *P. marginatum* [Ramos et al., 1986], *P. betle* [Amonkar et al., 1986*] and P.aduncum* [Gottlieb et al., 1981]. *Anethum graveolens* and *Piper aduncum* were chosen for the purpose of the present study for practical reasons mentioned in the following chapters.

I.2.2 Indian dill, Anethum graveolens (Apiaceae)

Dill, *Anethum graveolens*, is a common yellow-flowered annual herb about 50 cm tall, from the same family as carrot or other aromatic herbs such as coriander, fennel or parsiley. Althhough it probably originates from South-West Asia [Badoc and Lamarti, 1991], it has been cultivated for several centuries in different areas of the world from North America to Asia and Europe [Small, 1997]. Its popularity is due to the culinary and medicinal properties mainly of its leaves and fruits, commonly called seeds, from which an oil is also extracted.

Dill is propagated by seeds which germinate approximately 15 days after sowing. It can be easily grown on various types of soils although it prefers sandy loam and pH from 5.5 to 6.5. It requires full sunlight and irrigation during dry periods. The life cycle of the plant lasts about two and a half months.

Various environmental factors have been shown to affect dill growth and oil yield. For example, they have been shown to be positively related to light levels [Hälva et al. 1992]. Time to maturity was found to be shortened by long photoperiod and high temperatures [Hälva et al. 1993]. Treatments with sprayed ascorbic acid increased oil yield and quality [Pundarikakshudu and Bhavsar, 1991]. Nitrogen treatments increased the seed yield, while phosphorus treatments had no effect [Singh, 1991].

Few recent studies describing herbivory on dill are available. Populations of thrips, *Thrips flavus*, a common sap-sucking pest of fennel (Apiaceae), were recorded as pests on an Indian dill crop, in Punjab (India) [Sagar, 1986; Sagar and Mehta, 1991]. An outbreak of *Heliothis armigera* attacking flower buds and seeds was also reported on West Indian crops including dill [Judal and Upadhyay; 1989]. Dill was also found to be a potential host for the coriander aphid, *Hyadaphis coriandri*, in India [Kumar and Sagar, 1994]. Furthermore, it was found to be a major nectar plant source for the honeybee in middle Egypt [Atallah et al., 1988]. Since Apiaceae in general are highly attractive to some beneficial insects, a study was undertaken in which dill was found to be an efficient "companion plant". The role of companion plants is to attract predators or parasitoids of pests, as a source of pollen and nectar [Bowie et al., 1995].

From an experimental point of view, Su (1987) studied the long-term repellency of dill seed extract to confused flour beetles and concluded that it was efficient and could be used to protect grain in storage.

Dill has been part of recorded human culture for at least 5000 years. It was found to be mentioned on an Egyptian list of 3000 b.c. Mention of dill can also be found in texts from Plinus the Second, an author from the Antique Rome, or in the Bible where it is mentionned as an aromatic herb. Dill has been used in India as a spice, carminative and stomachic [Nadkarni, 1954]. Dill fruits have been used as diuretic agents, and decoctions as an antipoison [Turkmani, 1910; Boulos, 1983].

The phytochemistry of dill has been widely studied and found to vary qualitatively and quantitatively from one chemotype to the other [Badoc and Lamarti, 1991; Charles and Simon, 1995]. The main constituents in dill seed oil, present in all chemotypes, are the monoterpenes, carvone and limonene. Carvone, which co-occurs in similar quantities in caraway, is the main odorant component of dill seeds [Blank and Grosch, 1991] and is well known for its anticarcinogenic [Zheng et al., 1991], toxic [Lichtenstein et al., 1974] and "anti-tuberization in potato" properties [Van de Mheen and Muuse, 1993]. Phellandrene and dill ether are major constituents in dill herb oil [Brunke E.-J., 1991; Mahran et al., 1992]. Other important constituents are phenolic compounds such as the two analogues apiol and myristicin, an important odorant component of dill herb [Blank and Grosch, 1991] and an insecticide synergist [Lichtenstein et al, 1974]. Dillapiol, which is also very closely related to myristicin, is known to co-occur in various dill chemotypes but not in all of them . It is found in the highest amount in Indian dill , *Anethum graveolens* subsp. sowa., and is absent from European cultivars [Badoc and Lamarti, 1991]. Some studies have suggested that perhaps its presence or absence resulted from a phenotypic response to environmental conditions [Gupta, 1977].

Molecular structures of the main dill oil constituents are shown in **Figures I.3 and I.4**.

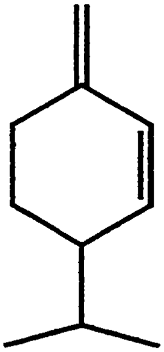
I.2.3 Piper aduncum **(Piperaceae)**

The Piperaceae family includes nine genera [Tripathi et al., 1996]. The genus *Piper* itself, represented by 700 described species, constitutes more than one third of the species found in the family. Most of them are successional tropical shrubs or small trees growing in various habitats, from shade to full light, from see level to 1500 m. Some propagate vegetatively, some propagate by seeds [Greig, 1993; Gartner, 1989]. The best known in developed countries is *Piper nigrum*, black pepper, which is widely used all around the world. Yet many other *Piper* species have been traditionnally used for their aroma, flavor or stimulant properties. *Piper betle* chewed with the betle nut in Southeast Asia and *Piper methysticum*, the hallucinogenic kawa, are just two examples.

Piper aduncum is a tropical shrub or small tree sometimes reaching 8 m in height. As a member of the *Piper* genus, it is characterized by stems with thick nodes, spicy aromatic vegetative parts, alternate entire leaves and solitary leaf-opposed densely flowered spikes. More specifically, *Piper aduncum* occurs in clones and can be recognized by its yellow-green foliage, its curved off-white spikes measuring from 6 to 16 cm and its

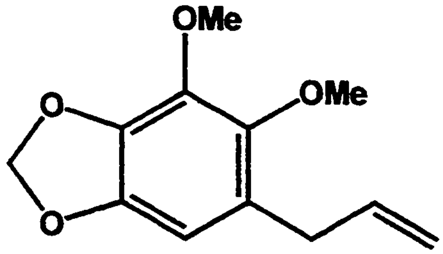
Limonene

Carvone

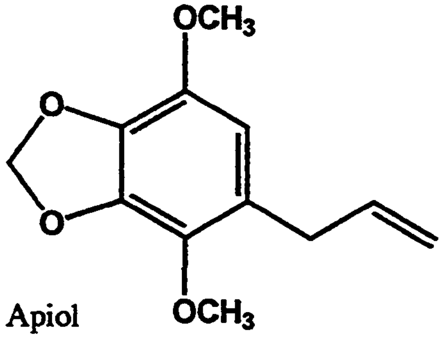


b-phellandrene

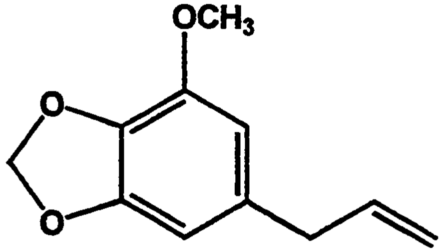
FIG. I.3: Monoterpenes occurring in *Anethum graveolens*



Dillapiol

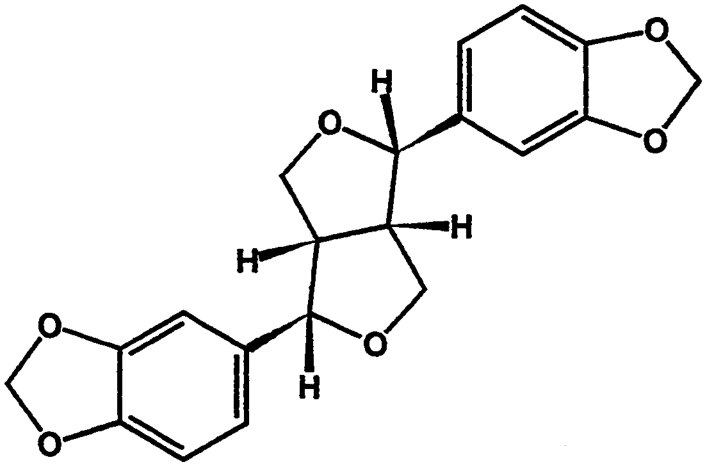


Apiol

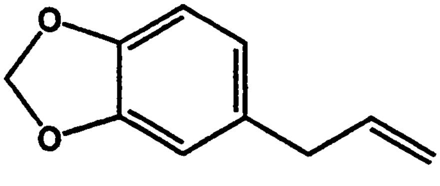


Myristicin

Monolignols occurring in *Anethum graveolens*



Sesamin



Safrole

Other examples of lignans

FIG. I.4: Dillapiol and other plant occurring lignans

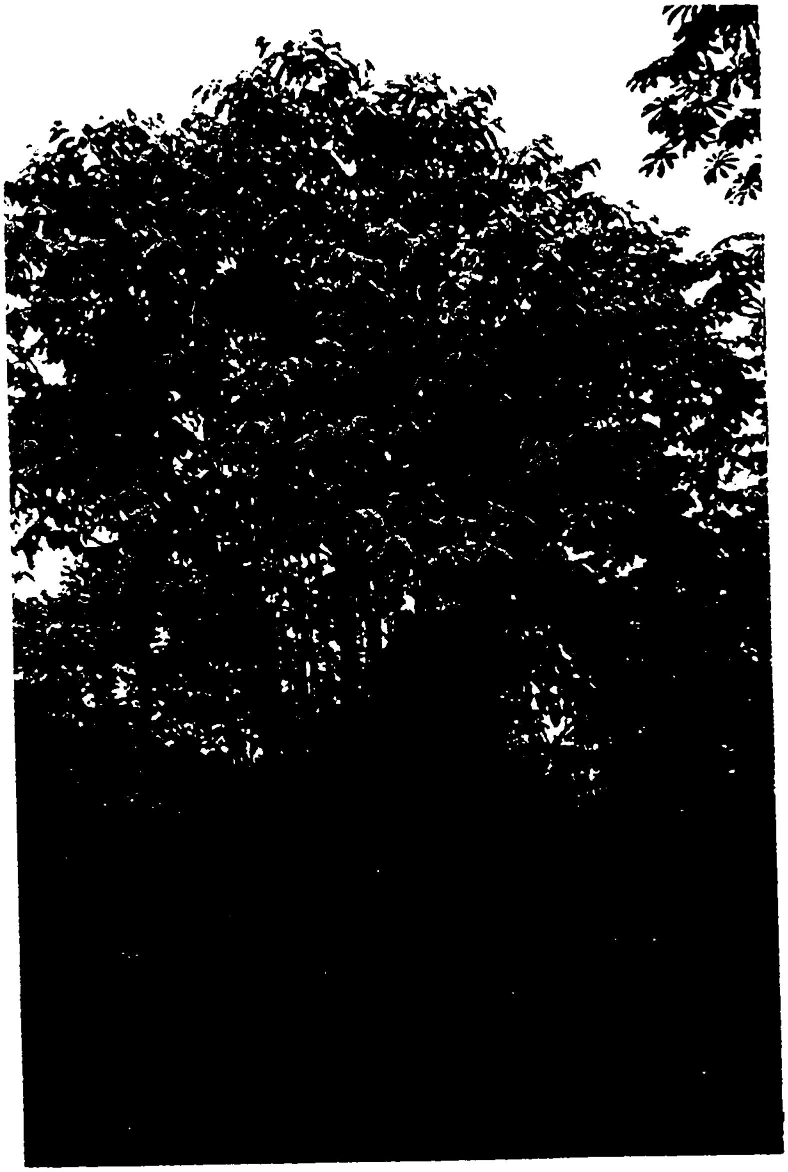
scabrous lanceolate to narrowly elliptic leaves 12 to 22 cm long [Burger, 1971] **(Figure I.5)**. The species is common in the Neotropics and can be found from 0 m to an altitude of 1500 m above sea level. It grows in disturbed, partly shaded or open areas and can be frequently seen along roads in Costa Rica.

Few studies have been reported on the interactions between *Piper* species and insects or other animals. Marquis, (1992) studied the patterns of host specificity for two herbivore specialists on Piperaceae described, weevils from the subfamilies Bardinae and Rhyncophorinae and geometrid moths of the subfamily Larentiinae as two types of specialists on Piperaceae plants. The leaves of *Piper aduncum* were found to repel ants [Jacobson, 1990].

Humans have used *Piper aduncum* for various medicinal purposes. The dried leaves of *Piper aduncum* have been used as a styptic in Northern Amazonia [Stimson, 1971; Schultes, 1975]. In Colombia, the "cordoncillo" has been used in traditional medicine as an astringent, a tonic, a diuretic and a hemostatic agent [Garcia Barriga, 1974]. In Guatemala and in Mexico, the plant is used as a diuretic and a digestive stimulant. In Cuba, it is used as a hemostatic and as a remedy against various incommodities and in the Virgin islands, it is used as a sedative or a laxative. [Morton, 1981].

Piper aduncum from different areas of the world was found to contain dillapiol as a main constituent: the essential oil of plants from Fiji contained 58% dillapiol, 4% piperitone [Smith and Kassim, 1979]; two varieties from Brazil were found to contain

FIG. I.5: ***Piper aduncum***



respectively 74.5% and 88.4% dillapiol [Gottlieb et al., 1981], some from Panama contained 90% dillapiol [Gupta and Arias, 1983]. Diaz et al. (1984) extracted three main constituents from Colombian plants, dillapiol, myristicin and piperitone. The most recent study [Ciccio and Ballestero, 1997] is a general evaluation of the chemistry of *Piper aduncum* performed in Costa Rica. The results showed the occurrence of fifty three compounds among which are monoterpenes (15.5% of the oil), and phenolics, especially dillapiol: 32.9% in one population and 61.5% in another.

I.3 OBJECTIVES AND HYPOTHESES

The general purpose of the present study was to describe the production and the activity of dillapiol, quantitatively and qualitatively. First, the toxicology of dillapiol was considered **(Chapters II and III)**. The synergism of dillapiol with a variety of insecticides was tested. The possible optimization of its activity by structural modification of the lead molecule was investigated. The general hypothesis for this section was that the dillapiol analogues have a synergistic activity that can be predicted quantitatively based on the lipophilicity component of the molecule, according to toxicological models developed for use elsewhere.

Second, the dillapiol production was investigated. To address this, dillapiol concentrations were quantified by HPLC in two plant species belonging to different families, *Anethum graveolens* from the Apiaceae **(Chapters IV and V)** and *Piper aduncum*, from the Piperaceae under different nutrient regimes **(Chapters VI and VII)**. A greenhouse study was conducted with Indian dill and a field study with *Piper aduncum*. The effect of simulated herbivory on dillapiol concentrations also was tested on dill.

The following chapters are presented in the form of articles, each of them constituting an independent entity, with an introduction, a methodology, results, a discussion and a conclusion. A general conclusion will follow.

II: Note on the spectrum of activity of Dillapiol tested on various botanical insecticides against mosquito larvae (*Aedes atropalpus*).

II.1 INTRODUCTION

In order to assess the spectrum of activity of the monolignol dillapiol, its synergistic effect on the toxicity of different botanical insecticides was assessed. The activity of dillapiol was compared with the effect of piperonyl butoxide (PBO) **(Figure I.2)**, a commercial insecticide synergist. PBO, is also currently used in experimental protocols as an inhibitor of the polysubstrate monooxygenases (PSMOs) [Berenbaum and Neal, 1985] and dillapiol has been shown to have a similar mode of action [Bernard et al., 1989].

The natural insecticides used are biosynthesized through different pathways and have different modes of action. Alpha-terthienyl is a phototoxic thiophene isolated from the roots and leaves of *Tagetes erecta* (Compositae). Its activity is described in **Section III.1**. Piperine is a neurotoxic alkaloid isolated from various *Piper* species (Piperaceae) [Atal et al., 1975]. The toxic flavonoid rotenone occurs in various Leguminosae. It inhibits mitochondrial electron transport at site 1 and is well known for its use as a fish poison and also as an efficient insecticide. Asimicin is a lipid derived acetogenin occurring in *Asimina triloba* (Annonaceae) [Alkofahi et al., 1989] with a similar mode of action to rotenone. Finally, bakkenolide, a sesquiterpene lactone, is an antifeedant and insecticide operating by the Michael addition reaction in various Compositae [Harborne and Baxter; 1993] **(Figure II.1)**. The synergism by dillapiol and PBO of the toxicity of those botanical insecticides

Table II.1: Experimental concentrations of the different insecticides used to determine the LC_{50}.

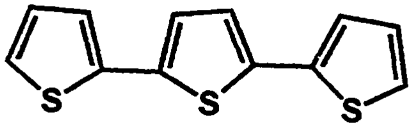
Insecticide	Experimental concentrations used (ppm)				
alpha-Terthienyl	0.0001	0.0002	0.0004	0.0008	0.0016
Bakkenolide	1.6	3.2	6.4		
Piperine	0.4	0.8	1.6	3.2	6.4
Asimicin	0.0001	0.0005	0.001	0.002	0.004
Rotenone	0.004	0.01	0.02	0.05	0.1

was tested with mosquito larvae (*Aedes atropalpus*, Culicidae, Diptera) (Section **III.2.2**). The hypothesis was that dillapiol had a synergistic effect on the different insecticides.

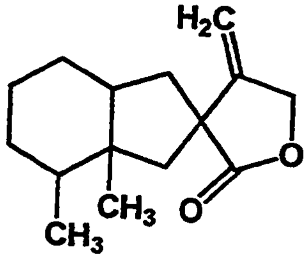
II.2 METHODS

Aedes atropalpus was reared under the laboratory conditions described in section **III.2.2** at the University of Ottawa. In order to assess whether dillapiol enhances the larvicidal activity of natural insecticides, the LC_{50}s of the different insecticides against *A. atropalpus* larvae were determined in presence or absence of a sublethal concentration of dillapiol or PBO.

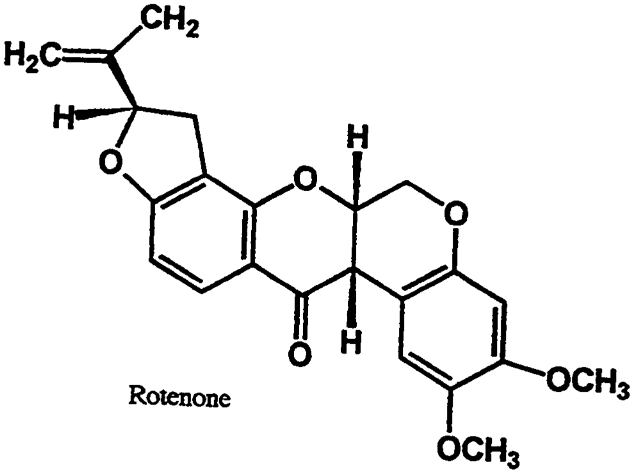
Twenty to twenty five 3rd instar larvae were pipetted in a 7 ml glass vial and the final volume of incubation was adjusted to 5 ml with distilled water. Dillapiol and PBO were separately dissolved in EtOH and a quantity of stock solution was added with a Hamilton syringe to obtain a final concentration of 1 ppm. Different ranges of concentrations were used for the different insecticides, according to preliminary trials **(Table II.1)**. Treatments containing the phototoxin alpha-terthienyl were placed under near UV light, according to the protocol described in **section III.2.3**. Mortality of larvae was assessed after 24 hours and LC_{50}s were computed as well as upper and lower confidence limits, using Probit analysis [Finney, 1964]. The factor of synergy was calculated as the ratio LC_{50} of the compound/ LC_{50} of the compound plus the synergist (see **section III.2.3**).



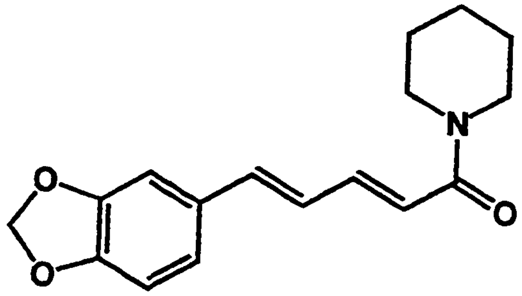
alpha-Terthienyl



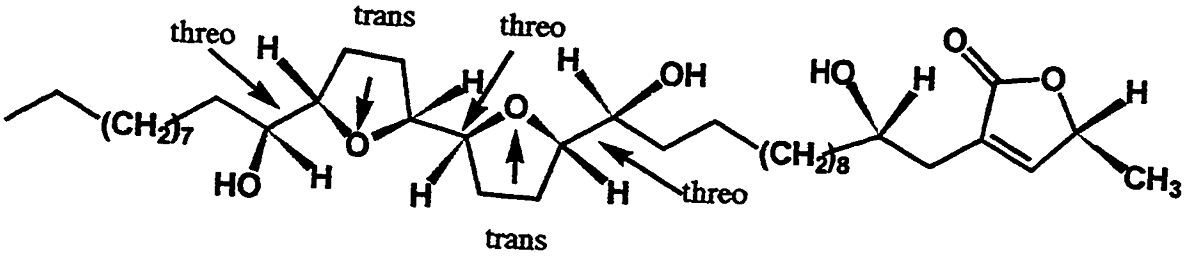
Bakkenolide



Rotenone



Piperine



Asimicin

FIG. II.1: Botanical insecticides

II.3 RESULTS AND DISCUSSION

The different LC_{50}s are reported in **Table II.2** with 95% Confidence Intervals. The factor of synergism also was calculated. In all cases except for bakkenolide it was found to be above 2 and was the highest for alpha-terthienyl for which it was found to be 6.

No synergistic effect was observed with bakkenolide or rotenone. Their structural types but not mode of action may be a restriction to the use of PSMO inhibitors such as dillapiol or PBO. Nevertheless, as can be seen, dillapiol and PBO significantly increased the activity of alpha-terthienyl, piperine and asimicin, three very distinct substances, in a similar manner.

II.4 CONCLUSION

Dillapiol was found to be as efficient as the commercial synthetic synergist piperonyl butoxide. This comparative experiment suggests the commercial potential of the naturally occurring substance and shows the wide spectrum of its activity on a variety of insecticides with different modes of action, an activity which was found to be the most important with the phototoxin alpha-terthienyl.

Table II.2: LC_{50}s for the different botanical insecticides, without addition of a synergist, with addition of 1 ppm dillapiol or with addition of 1 ppm PBO, with 95% C.I.; and associated factors of synergism (SF), with 90.25% C.I.

*For each insecticide, different letters represent significantly different results for the different treatments. (Confidence Intervals do not overlap).

	(-synergist)	**(+ dillapiol)**		**(+PBO)**	
	LC_{50} (ppm)	**LC_{50} (ppm)**	**SF**	**LC_{50} (ppm)**	**SF**
alpha-Terthienyl	0.0006 **a*** (0.0005-0.0008)	0.0001 **b** (0.0000-0.0001)	6.0 (5.00-10.00)	0.0002 **b** (0.0001-0.0002)	3.0 (2.50-4.00)
Piperine	8.6 **a** (5.90-33.25)	3.25 **b** (2.36-5.11)	2.6 (1.15-14.10)	4.27 **b** (3.01-7.52)	2.0 (0.78-11.05)
Bakkenolide	3.15 (0.001-9.46)	2.96 (0.001-9.52)	1.1 (0.00-9.46)	1.55 (0.00-7.38)	2.0 (0.00-9.46)
Rotenone	0.016 (0.013-0.021)	0.0013 (0.001-0.017)	3.7 (0.76-21.00)	0.0053 (0.0014-0.024)	4.5 (0.54-15.00)
Asimicin	0.0012 **a** (0.0009-0.0015)	0.0004 **b** (0.0004-0.0005)	2.7 (1.80-3.75)	0.0005 **b** (0.0004-0.0006)	2.7 (1.50-3.75)

III: Dillapiol derivatives as mosquito larvicide synergists: Structure–Activity Relationship Analysis.

III.1 INTRODUCTION

Dillapiol has synergistic properties when combined with a variety of insecticides such as DDT [Parmar and Tomar, 1983], carbaryl [Tomar et al., 1978], pyrethrins [Tomar et al., 1979], alpha-terthienyl (a phototoxic larvicide) [Assabgui et al., 1996] using different insect test species such as the flour beetle, *Tribolium castaneum*, or the mosquito larvae *Aedes atropalpus.*

Because of a renewed interest in botanical pesticides requiring synergists, we decided to investigate quantitative structure-activity relationships (QSAR) of dillapiol, analogues and derivatives **(Figure III.1)** as synergists for the phototoxic larvicide alpha-terthienyl **(Figure III.2)**. A quantitative structure-activity relationship measures the relationship that links physical properties of molecules with their biological activity. Hansch et al. (1989) suggested that the biological activity of a molecule was a function of its lipophilic, steric and electronic components. Lipophilicity appeared to be the most important parameter since it influences passive transport processes from a source to a target site, and since it plays a key role in regulating the interaction of bioactive substances with their bioreceptors. Marles et al. (1991) reported that the relationship between bioactive molecules and lipophilicity is frequently observed. They themselves observed this relationship for alpha-terthienyl analogues with mosquito larvae. Alpha-terthienyl is a naturally occurring phototoxin active against the mosquito *Aedes atropalpus*

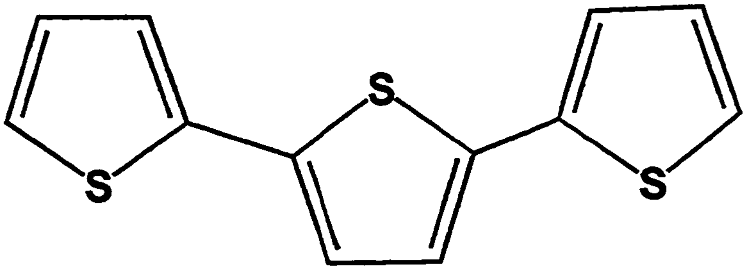
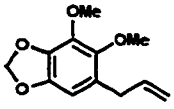
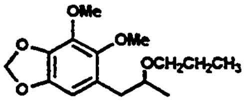


FIG. III.1 : Alpha-Terthienyl



Dillapiol



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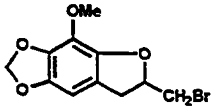

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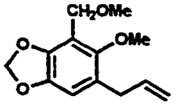

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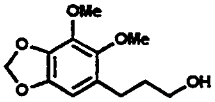

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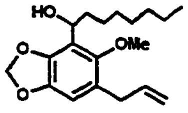

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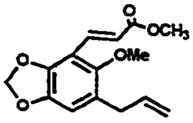

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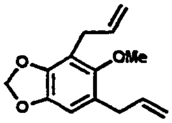

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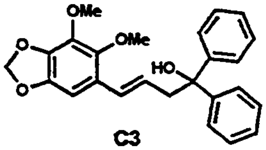

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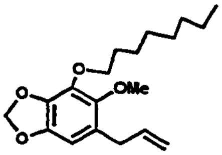

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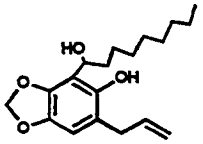

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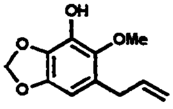

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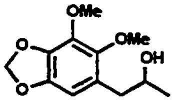

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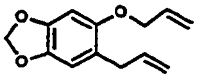

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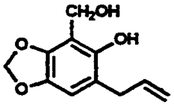

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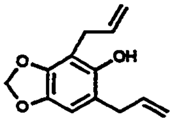

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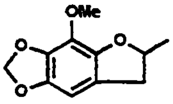

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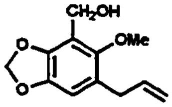

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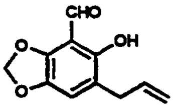

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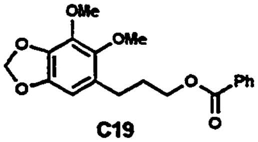


C19

FIG. III.2: Dillapiol and analogues

at levels in the nanomolar range [Hasspieler et al., 1988] when activated by natural light or UV light (300-400 nm) [Marles et al., 1991]. It acts by producing singlet oxygen which damages the anal gill membranes of larvae. The choice of this botanical photo-activated insecticide was based on the extensive work done on this compound in our laboratory and its potential commercial use in a more stable insecticide/synergist combination.

The objectives of the experiments described in this paper were first to assess the synergistic activity of dillapiol and the different dillapiol derivatives and subsequently to apply the quantitative structure-activity relationship model for dillapiol synergist.

III.2 MATERIALS AND METHODS

III.2.1 Compounds.

The synthesis of dillapiol analogues **(Figure III.1)** is described elsewhere [Majerus, 1997]. The dillapiol standard used (extracted from dill, *Anethum sowa*) was supplied by Dr. Krishnamurty (Department of Chemistry, University of Delhi, Delhi, India). Alpha-terthienyl was available from a stock previously synthesized as described in Philogène et al. (1985).

III.2.2 Test insect.

Aedes atropalpus L. *(Diptera: Culicidae)* is a common mosquito that can be found in the Ottawa River area. It is a good choice for bioassays since it does not carry diseases, it can be easily reared under laboratory conditions [Watt et al., 1981], and the female adults do not require blood meals. The mosquito undergoes four distinct aquatic larval stages over a period of two weeks after hatching, pupates and then the adults emerge. The whole life-cycle generally lasts four to five weeks.

III.2.3 Toxicity tests

Mosquito larvae (*Aedes atropalpus* Coq.*)* were reared in our laboratory under controlled photoperiod (16:8 h; L:D), temperature (23 °C), in trays filled with distilled water, and fed daily with TetraMin fish food. Tests were done according to an adapted World Health Organization procedure [Anonymous, 1963]. For each treatment and the control, 20 third instar or early fourth instar larvae were transferred into 10 ml vials, in a final volume of 5 ml distilled water. Different highly concentrated stock solutions of dillapiol, derivatives and of α-T were prepared in 95% ethanol . The various concentrations were added to the treatment vials with a Hamilton syringe. The final concentration of ethanol in all treatments and control was maintained at 5µl/ml, which is a non-toxic concentration. The final concentrations of α-T were 160, 120, 80, 60, 40, 20, 10, 5 ppb and the insecticide : derivative ratio was consistently 1:5 [Mukerjee et al., 1979]. Only α-T and the experimental concentration of ethanol were added to the controls. Larvae were then exposed during four hours to UV-A light from a bank of four

20W Westinghouse black light blue tubes as described previously [Arnason et al., 1981] and then kept in the dark for 20 more hours. Each treatment was replicated 4 times. All derivatives had been tested for toxicity at the highest concentrations, 800 ppb, under experimental conditions and no mortality had been found after 24 hours. Counts were then made to assess mortality and LC_{50}s were determined with 95% confidence intervals by Probit analysis [Finney, 1971]. The factors of synergism (SF), expressed by the ratio LC_{50} of α-T/ LC_{50} of α-T plus derivative were determined for each compound [Chadwick, 1963], with 90.25% confidence intervals.

III.2.4 Lipophilicity.

Lipophilicity was expressed as a function of the octanol/water partition coefficient (P) of the different molecules [Marles et al., 1991]. Those coefficients were determined according to the method described by Ellgehausen et al. (1981). They are a function of the retention time of the molecules, expressed by the capacity factor K', when eluted in a high pressure liquid chromatography mobile phase through a LiChrosorb RP-18 column. K' is defined as $K'=(t_R-t_0)/t_0$, t_R and t_0 being the retention time of the compound and the retention time of the non-sorbed solvent used, methanol in this case. To determine the equation relating partition coefficient and capacity factor, K' was determined for four standards with known partition coefficients [from Ellgehausen et al., 1981], metalaxyl, monuron, diuron and p,p'-DDT, and a linear regression was computed between log P and log K' and subsequently used to compute log P for all experimental compounds:

$\log P = 2.327 (\log K') + 2.689$ ($r^2 = 0.958$) **(Equation III.1)**. The conditions for chromatography analysis were the following: a 65:35 (ethanol:water) mobile phase with a flow rate of 1ml/min. produced by a Beckman High Pressure Liquid Chromatography pumping system connected to a 5x25 mm LiChrosorb C-18 column and a detector analysing at a wavelength of 254 nm. A Beckman autosampler was used for injection with a 20 µl loop. All compounds were dissolved in methanol.

III.2.5 Retention of alpha-terthienyl within the larvae tissue.

In order to assess the amount of α-T retained within the larval tissue (expressed in µg α-T / g of dry larval weight), 15 fourth instar *Aedes atropalpus* larvae were incubated in 5 ml distilled water. 10 µl of a 625 ppm stock solution of α-T in methanol and 10 µl of a 3125 ppm stock solution of dillapiol and/or derivatives (all except 9 and 19 which were no longer available) were added to the vials. Controls with only 10 µl α-T in methanol and 10 µl methanol were prepared as well as controls with only 20 µl methanol. The final concentrations in the treatments were 1.25 for α-T and 6.25 for dillapiol or derivatives (a 1:5 insecticide/derivative ratio). Each treatment was replicated four times. The experiment was performed in darkness to avoid photoxicity and mortality of insects. After 8 hours, the larvae were removed from the vials by pouring the contents on a doubled cheese cloth, they were gently rinsed with distilled water and then dried carefully with absorbant paper tissue. The larvae were then transferred to 1 ml hexane, sonicated 30 seconds to extract the α-T from the insects and filtered before analysis. The amounts of alpha-terthienyl in the hexane extracts were then analysed by HPLC. The mobile phase and the flow rate were respectively acetonitrile/water (70:30) and 1 ml/min. Retention time was 14.7 minutes. A reversed-phase C-18, Beckman column (4.6*250 mm, with 5 μ particle size) was used. Alpha-terthienyl concentrations were determined using the linear regression equation computed from a standard curve using pure α-T dissolved in hexane. All solvents were HPLC grade (BDH, Toronto).

TABLE III.1: LC_{50}s of combinations of α-T and dillapiol or derivatives combinations, on *Aedes atropalpus* larvae, with 95% C. I. and associated factors of synergism.

*The values followed by an asterisk denote situation where α-T + compounds are significantly different from α-T alone (Confidence Intervals are not overlapping). The confidence intervals for the Factors of Synergism (90.25%) were derived from the values of the 95% intervals computed for the LC_{50}. Factors of synergism that are followed by the same letter are not significantly different (Confidence Intervals are overlapping).

Compound	LC_{50}(ng/ml) and 95% C.I.	Factor of synergism and 90.25% C.I.
αT + Dillapiol	41.1 (35.9-46.2)*	1.9 (1.5-2.4) ab
αT + C1	52.9 (47.2-58.5)*	1.5 (1.2-1.8) bcde
αT + C2	85.8 (78.2-94.0)	0.9 (0.7-1.1) fg
αT + C3	62.9 (57.0-68.8)*	1.2 (1.0-1.5) cdefg
αT + C4	84.6 (79.0-90.8)	0.9 (0.8-1.1) g
αT + C5	50.2 (44.6-55.8)*	1.5 (1.2-1.9) bcde
αT + C6	45.0 (40.0-49.9)*	1.7 (1.4-2.1) abc
αT + C7	64.3 (58.9-69.0)*	1.2 (1.0-1.5) cdefg
αT + C8	43.6 (38.4-49.2)*	1.8 (1.4-2.2) abc
αT + C9	60.6 (55.7-65.6)*	1.3 (1.1-1.3) bcd
αT + C10	64.8 (51.9-90.0)	1.2 (0.8-1.7) bcdefg
αT + C11	48.4 (41.1-64.6)*	1.6 (1.1-2.1) abcde
αT + C12	32.8 (29.6-35.4)*	2.4 (2.0-2.9) a
αT + C13	73.3 (64.9-84.8)	1.1 (0.8-1.3) defg
αT + C14	60.1 (52.7-64.4)*	1.3 (1.1-1.6) bcdefg
αT + C15	48.6 (43.1-53.9)*	1.7 (1.3-2.0) abcd
αT + C16	57.1 (51.1-63.3)*	1.4 (1.1-1.7) bcdef
αT + C17	63.1 (56.5-69.6)*	1.2 (1.0-1.5) cdefg
αT + C18	66.5 (59.7-73.1)	1.2 (1.0-1.4) cdefg
αT + C19	74.9 (67.4-82.4)	1.0 (0.8-1.3) efg
αT	77.7 (70.0-86.0)	---

III.3 RESULTS AND DISCUSSION

III.3.1 *Toxicity of alpha-terthienyl in combination with dillapiol derivative synergists, on Mosquito larvae, LC_{50} and Factor of synergism.*

The LC_{50} of α-T for third instar *Aedes atropalpus* larvae was found to be 77 ng/ml **(Table III.1)**. LC_{50} values for combinations with derivatives were significantly different when their confidence interval did not overlap with the confidence interval associated with the LC_{50} value for α-T. Therefore lethal concentrations computed were significant for dillapiol and compounds 1,3,5,6,7,8,11,12,14,15,16,17 and non-significant for compounds 2,4,10,13,18 and 19. When dillapiol was added in a 5 to 1 ratio, the LC_{50} dropped to 41.1 ng/ml. The SF associated with this result is 1.9 which is the second highest value. It can be compared to the value of 2.0 found by Tomar et al. (1979) for synergism of dillapiol with pyrethrum insecticides on the flour beetle, *Tribolium castaneum* Herbst. Yet a higher value had been obtained earlier with carbaryl insecticide on the same test insect (Tomar et al., 1978). The lowest LC_{50} found for the insecticide/synthetic derivative combination was 32.8 ng/ml for compound 12, which represents a SF of 2.4. Compounds 5, 6, 8, 11 and 15 also increased the toxicity of alpha-terthienyl by more than 50 %. The other molecules either had low or no synergistic activity.

For compounds that carried both methoxy groups of the parent dillapiol and for which only the side chain was modified (compounds 1, 2, 3, 4, 7 and 19), the synergistic activity was either reduced or lost. The most active compound among them (Compound1) had a significant synergism factor of 1.5. Tomar et al., in 1978, had found a higher synergistic activity for this molecule, which contains an ether substitution in its side chain, than for dillapiol with carbaryl insecticides, on the wheat flour beetle. In general, they found factors of synergism varying from 1.4 to 6.3; the level of significance was not mentioned. In their study, an ether group in a side chain of the right size (3 or 4-C) combined with alkoxy substitutions on the benzene ring enhanced activity. This was not observed in the present study, but only one similar compound was tested. Therefore, the unsaturated 3-carbon alkyl chain of dillapiol remained the optimal one. The most active molecules were found when this side chain was preserved and the substituent α to the methylenedioxy group was modified (8, 10, 11, 12, 15, 16 and 17). Among molecules with two aryl substitutions in addition to the methylenedioxy group (5, 6, 9, 13, 14, 15 and 18), the compounds possessing the (methyl cyclopentane) group (5 and 6) were the most active molecules, with factors of synergism of respectively 1.5 and 1.7. The bromination in compound 6 seems to increase the synergistic activity but the two values are not significantly different.

III.3.2 Lipophilicity.

The values obtained for the logarithm of the partition coefficient, log P, ranged between 1.7 and 6.6 **(Table III.2)**. Dillapiol had an average value of 3.5. Compound 8 was found to have a retention time, when eluted in the HPLC column, almost four times

Table III.2: Logarithms of the capacity factor (log K') and of the partition coefficient (log P) computed from Equation 1.

Compound	log K'	log P
Metalaxyl	-0.19	1.27a
Monuron	-0.21	1.95a
Diuron	0.05	2.89a
p,p'-DDT	1.37	5.94a
Dillapiol	0.34	3.50b
C1	0.42	3.68b
C2	-0.41	1.72b
C3	0.569	4.04b
C4	-0.38	1.78b
C5	0.01	2.72b
C6	0.28	3.35b
C7	1.03	5.09b
C8	1.67	6.57b
C9	0.91	4.80b
C10	0.2	3.15b
C11	0.51	3.87b
C12	0.34	3.48b
C13	1.22	5.53b
C14	0.1	2.92b
C15	0.62	4.14b
C16	0.83	4.63b
C17	0.15	3.05b
C18	0.38	3.57b
C19	0.66	4.23b

a Value obtained from Ellgehausen, H. (1981).
b Value computed from equation 1.

the average time observed for all other compounds (88 minutes). The resulting log P value was found to be higher than the standard DDT log P value which is considered to be extremely high and rarely exceeded. This could be explained either by the structure of compound 8, in which a long (9 carbons) side chain replaces one of the original methoxy groups of dillapiol, or may be a result of steric effects. For this reason, the data were shown for compound 8 but were not taken into consideration in the computation of regression curves.

III.3.3 Quantitative structure/activity relationship analysis.

Relationship between toxicity and lipophilicity.

A parabolic relationship **(Figure III.3)** between the logarithm of the toxicity of the combinations (alpha-terthienyl/dillapiol derivative) and the logarithm of the partition coefficient was found to be significant ($p=0.01$, $r^2=0.44$, $n=19$, with $\alpha=0.05$) and is described by the equation: $\log (1/LC_{50})=0.401 \log P - 0.054 (\log P)^2 - 2.456$ **(Equation III.2)**. More significant insecticide models have been previously observed for different sets of compounds. Marles et al. (1991), for example, found a highly significant ($r=0.73$, $p<0.05$, $\alpha=0.05$) bilinear relationship between the lipophilicity of α-T derivatives and toxicity to mosquito larvae. The lower r^2 found in the present study could perhaps be explained by the wide range of analogues studied or the fact that there was relatively low variation among the different synergistic activities of the different compounds due to low variation in the insect response toward the different treatments. The synergistic properties

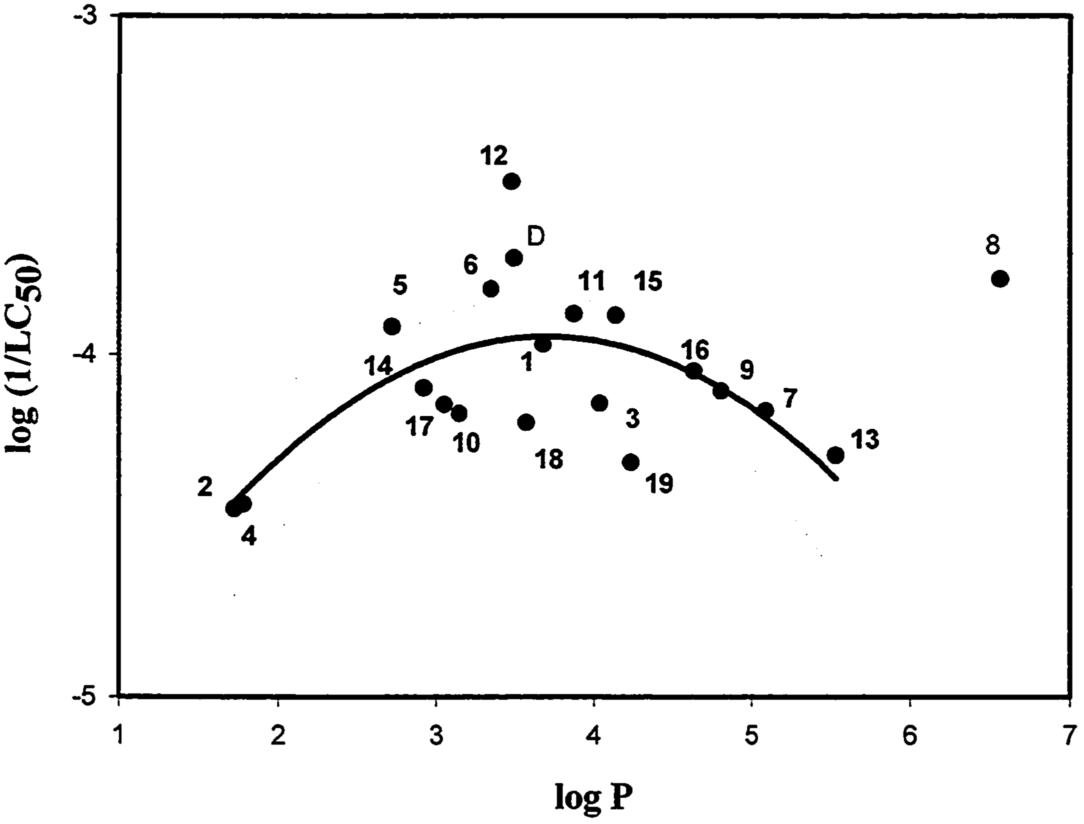


FIG. III.3: Parabolic relationship between Toxicity (log 1/LC$_{50}$) and Partition coefficients (log P) of dillapiol and derivatives, with 95% C. I.

Equation 2: log (1/LC5$_{50}$)= 0.401 log P - 0.054 (log P)^2 - 2.456

(p=0.01, r2=0.44, n=19)

Each compound is labelled according to its number, D stands for dillapiol.

The result for compound 8 is shown but not included in the model.

of several synthetic dillapiol derivatives were studied by Tomar et al. [1978, 1979], using carbaryl as the insecticide and the flour beetle *Tribolium castaneum* as a target. Their findings suggest that unsaturated side chains, when present, confer greater activity when the unsaturation is in conjugation with the methylenedioxyphenyl nucleus. They also observed that the length of those chains increased the activity up to an optimum (five C-atom) and then decreased it. Furthermore, the presence of some alkoxy groups (2 or 3 C-atoms) attached to the nucleus or within a side chain increased the activity. A relationship between the number of carbons in the side chains (linearly related to the lipophilicty of the molecules) and the synergistic activity of the compounds.

Dillapiol has recently been found to increase the retention of α-T within the tissues of mosquito larvae in a dose dependent manner [Guillet, 1997]. Therefore its mode of action is related to its ability to prevent the rapid excretion of the toxin by the target insect. Tomar et al. (1978) had found factors of synergism varying from 1.4 to 6.3 and the range in this study is only from 0.9 to 2.4. However the factor of synergism can vary depending on experimental conditions.

The model is significant and lipophilicity explains 44% of variation in toxicity of the α-T /derivative combination and of the synergistic activity of those derivatives. Considering optimal lipophilicity (under these experimental conditions a log P around 3.5) would therefore be part of a good strategy to follow in order to produce more active dillapiol derivatives.The second most active compound in this study is the naturally occurring dillapiol.

III.3.4 Relationship between lipophilicity and retention of alpha-terthienyl in the larvae.

A parabolic relationship **(Figure III.4)** between lipophilicity and retention of alpha-terthienyl in the insect tissue was found to be significant:

$\log [\alpha\text{-T}]=1.02 \log P - 0.2 \log P^2 + 3.82$ **(Equation III.3)**, with $r^2=0.12$ ($p<0.001$, $\alpha=0.05$, $n=63$).

The fact that a similar relationship existed between the lipophilicity of the synergist and both the retention of the insecticide in the larvae and the toxicity suggested that a relationship existed between the toxicity and the retention of the insecticide within the insect tissue. It is known that botanicals have short half-lives, for example α-T remains about 4 hours in the environment before being photodegraded [Philogène et al., 1985]. It is also rapidly eliminated from mosquito larvae [Hasspieler et al. 1988]. Thus a way to increase their efficiency is to ensure that they reach their target faster or remain longer within the organism before being metabolized. By preventing metabolism of the insecticide leading to detoxification or excretion, synergists stabilize botanical insecticides. Therefore, not surprisingly, toxicity and retention of the insecticide within the larval tissue were found to be correlated **(Figure III.5)**, with a coefficient of correlation $r=0.50$ ($p<0.001$, $\alpha=0.05$, $n=63$):

$\log (1/LC50)=0.16$ (log of concentration α-T remaining in the tissue) - 5.00 **(Equation III.4)**. Those results corroborate the findings of Guillet (1997) who suggested that the

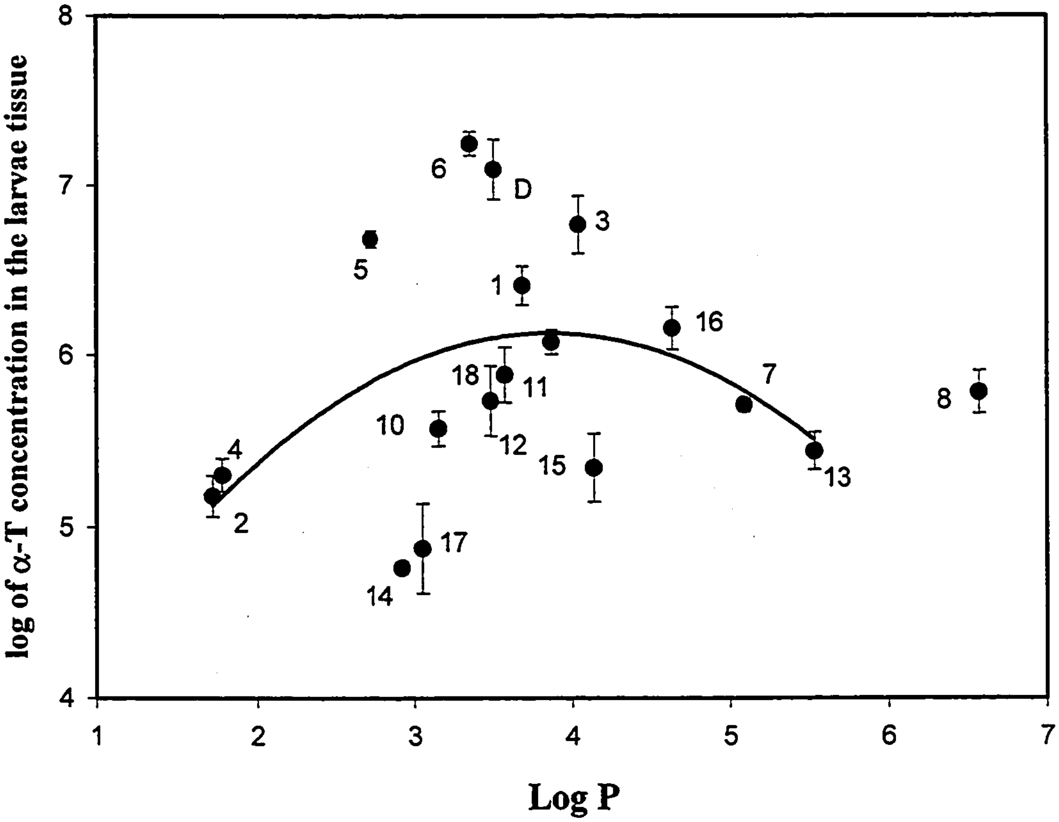


FIG. III.4 : Parabolic relationship between the retention of α-T in the larvae tissue and lipophilicity (log P), with 95% C.I.

Data for compounds 9 and 19 is not available. Compound 8 is not included in the model.

The equation 5 associated with the model is ($p<0.001$, $n= 67$ and $p<0.001$)

log (α-T concentration) = 1.02 log P -0.2 Log P2 + 3.82.

Bars represent the standard error of the mean.

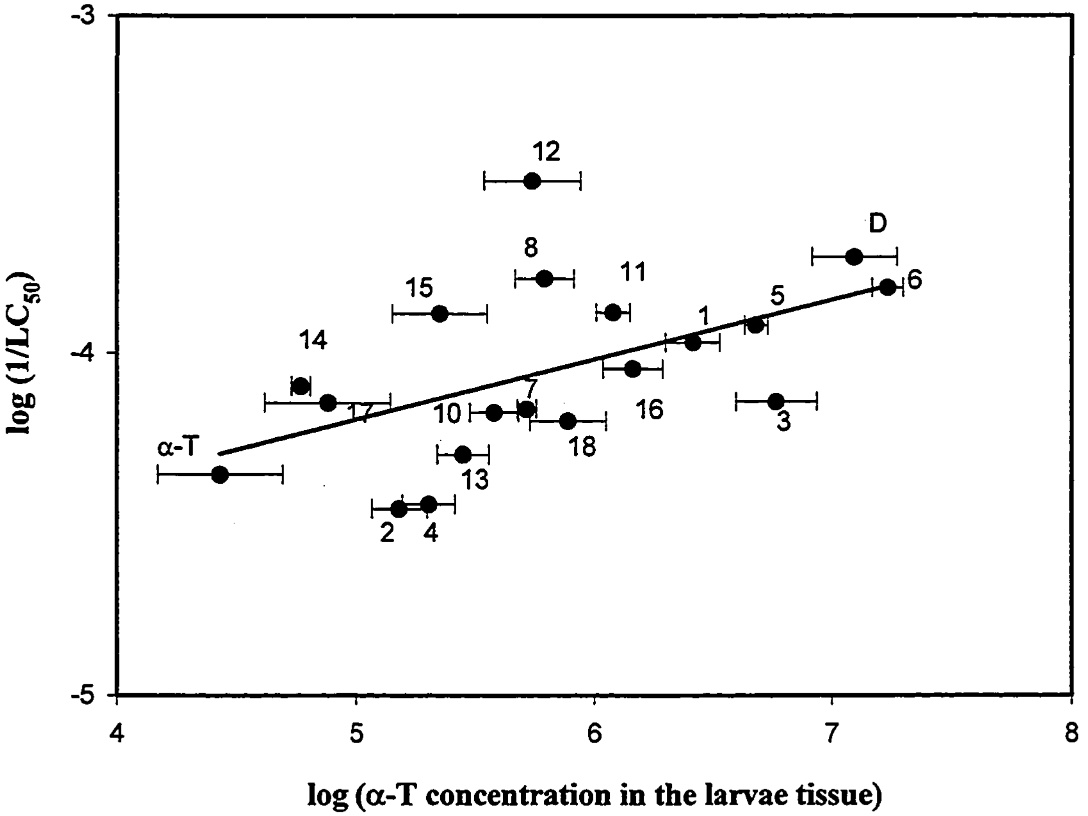


FIG III.5: Correlation between toxicity and retention of the insecticide within the larvae tissue, with 95% C.I.

Data for compounds 9 and 19 is not available. D stands for dillapiol.
The corresponding equation is: $\log(1/LC50) = 0.16 \log(\alpha\text{-T conc.}) - 5.00$; with $r = 0.503$, $p=0.000$ (Equation 4)

synergism of dillapiol was due to higher retention of the insectide within lepidopteran larvae.

III.3.5 Reanalysis for two subgroups:

After consideration of the different structural types, the initial set of derivatives was separated into two subgroups. All molecules containing four ether oxygen substitutions have similar electronic density in the aromatic ring and they were therefore placed in Subgroup I (Dillapiol and compounds 1, 2, 3, 4, 5, 6, 8, 17 and 19). The remaining molecules formed Subgroup II (Compounds 7, 9, 10, 11, 12, 13, 14, 15, 16 and 18). The analyses described previously were repeated for the two groups. The results and equations are presented in **Table III.3**. They show that by considering the electronic component of the molecules, the level of significance of the models is improved. Subgroup I which constitutes a coherent group has high correlation factors for the different models (up to $r^2=0.77$, with $p<0.001$), while Subgroup II formed by remaining compounds shows lower significance levels (up to $r^2=0.37$, with $p<0.001$).

TABLE III.3: Equations and statistical significance for Subgroup 1 with analogues containing two oxygens attached to the benzene ring (Dillapiol and Compounds 1, 2, 3, 4, 5, 6, 17, 19) and Subgroup 2 with remaining derivatives (Compounds 7, 9, 10, 11, 12, 13, 14, 15, 16, 18).

	Equations	r or r^2	n	p-value
Subgroup I	$\log(1/LC_{50}) = 1.58 \log P - 0.24 \log P^2 - 6.47$	$r^2 = 0.77$	9	<0.001
	$\log[\alpha\text{-T}] = 0.75 \log P + 0.001 \log P^2 + 3.92$	$r^2 = 0.43$	31	0.001
	$\log(1/LC_{50}) = 0.20 \log[\alpha\text{-T}] - 5.31$	$r = 0.80$	31	<0.001
Subgroup II	$\log(1/LC_{50}) = 1.45 \log P - 0.19 \log P^2 - 6.71$	$r^2 = 0.37$	11	<0.001
	$\log[\alpha\text{-T}] = 4.39 \log P - 0.52 \log P^2 - 3.17$	$r^2 = 0.24$	44	0.007
	$\log(1/LC_{50}) = 0.17 \log[\alpha\text{-T}] - 4.99$	$r = 0.50$	44	0.001

III.4 CONCLUSION

It is interesting to note that the second most active compound in this study is the naturally occurring dillapiol, suggesting that natural selection may have played a role in designing this molecule as an effective natural synergist. Only compound C12 showed a greater synergism and is therfore a starting point for future syntheses. Other physico-chemical parameters could be used to improve the models, such as the electronic or steric components of the molecules.

IV: Effect of Nitrogen treatment on the production of the neolignan dillapiol in dill.

IV.1 INTRODUCTION

Considering the results of different bioassays with dillapiol and analogues **(Chapter III)**, and the comparison with the common commercial synergist Piperonyl butoxide **(Chapter II)**, the naturally occurring molecule appears to have a real economic potential. Its synthesis is very costly and requires several reaction steps [Majerus; 1997]. One main concern therefore is whether it would be appropriate and less costly to extract dillapiol from plant sources and use it under a botanical label suitable for organic growers. Thus, the purpose of this study was to consider dillapiol concentration in plant tissue and determine optimal conditions for recovery.

There is a great deal of interest recently in the scientific literature regarding environmental parameters affecting the production of secondary metabolites in plants. The Growth-Differentiation Balance Hypothesis (first proposed by Loomis in 1932 and described by Herms and Mattson in 1992), suggests that differentiation, including secondary metabolism, is negatively correlated with growth and primary metabolism, for they both require the same resources. Generally, plants that grow fast, as opposed to slow-growing plants, will produce less defenses. Within a population, this corresponds to less defended plants in more nutrient-rich environments. Coley's et al. (1985) hypothesis suggests that the nature and the quantity of defenses are determined by the resources available in the local habitat. Along this line of thought, Bryant et al. (1985) proposed the Carbon/Nutrient Balance hypothesis which suggests that plant chemical defense is affected by nutrient availability. It predicts that when nutrients are limiting, carbon-based compounds should be found in higher concentration, while nitrogen-based compounds, for example, should be found in higher concentration when carbon is limiting. Carbon is rarely limiting and photosynthesis usually provides enough precursors for carbon-based compounds [Bazzaz et al., 1987; Tuomi et al., 1988]. On the other hand, plant growth is known to be most often limited by nutrient, especially nitrogen, availability in the soil [Salisbury and Ross, 1992].

The purpose of the present study was to investigate and describe the effect of nutrient availability on the production of dillapiol by plants. Indian dill was used for this greenhouse experiment. It was chosen for various reasons. It has been domesticated and is grown widely under continental climates, although it originates from tropical regions. It is of reasonable height in an experimental context, has a relatively short life-cycle (about three months) and is easily available.

The consideration of plant part and plant age as factors influencing secondary metabolism is also important from both economical and scientific point of views. In order to optimize the recovery of a secondary metabolite, it is necessary to know in what part or at what stage the production is the highest and it is worth harvesting. Secondary chemistry is known to vary through time, along the different stages of plant growth and also in the different plant structures according to strategic resource allocation [Bazzaz et al., 1987]. For example, tender tissues have been shown to contain more qualitative highly toxic defenses such as alkaloids and terpenes while older tissues contain more structural defenses or growth reducers such as tannins [Feeny, 1976; Baumann and Gabriel, 1984; Mihalaik and Lincoln, 1989]. Feeny (1976) also suggested that valuable reproductive tissues such as seeds contain more secondary metabolites than vegetative tissues. Flower and seed chemistry is known to be essential in survival, pollination and dispersal processes which involve insects or larger animals, attracted by or feeding on the plant [Harborne, 1993]. Therefore, part and age of the plant were introduced in the study, as well as their interactions.

IV.2 METHODS

IV.2.1 Greenhouse conditions.

The experiment was conducted in the greenhouse at the University of Ottawa (Ottawa, Ontario, Canada). Temperature was 23±3 °C, RH 45% and a photoperiod 14:10h (L:D) provided by 14 h of artificial light to supplement 10 h of natural daylight (which increased slightly over the two months of the experiment, from January to March).

IV.2.2 Sowing and fertilization.

Indian dill seeds, *Anethum graveolens,* obtained from the Pioneer Company (USA) were planted in 10x10cm pots containing neutral vermiculite. Seven seedlings were kept per pot. The plants were watered daily in the late afternoon. Once a week starting at day 15 after sowing, the plants were fertilized with 50 ml Hoagland solution per pot

[Hoagland, 1950]. Three levels of nitrogen were chosen: the optimal nitrogen level in the Hoagland solution (N=1), half of this level (N=1/2) and one-twentieth of this level (N=1/20). This choice of concentrations, which were used in a first trial, was found to be suitable since growth was significantly reduced but not stopped by low nitrogen treatments. These treatments were randomly assigned to the pots at the beginning of the experiment.

IV.2.3 Harvesting.

Throughout the life cycle, the following harvests were undertaken. In a preliminary trial, three sampling dates were chosen: days 38, 58 and 78. At day 78, the plants were senescing without giving fruits (data not shown). In a following main study, more harvests were done, every ten days starting at days 28 (28,38,48, 58 and 68) after sowing. Day 28 corresponds to an early stage (3-4 leaves), days 38 and 48 correspond to a stage of exponential growth, day 58 and 68 correspond to a reproducing stage, with the presence of flowers. At each harvest, 24 pots were randomly selected for each nitrogen treatment. Among them, eight were analysed for the leaf, eight for the vegetative stem and eight for the roots.

The pots were brought back to the laboratory. Plant parts were separated with a razor blade. The roots were gently taken out of the vermiculite medium, cleaned and rinsed with distilled water and the excess water was removed with absorbent tissues.

Each part was separately weighed and a maximum of one gram (to keep the mass more or less constant) of tissue was randomly taken from the total amount to give an accurate estimate of the concentration , weighed with a Sartorius BP 210 (USA) scale, and put aside for dillapiol extraction. The whole harvest and processing were always started in the morning around nine and lasted 2.5-3h.

IV.2.4 Extraction.

Each sample was then extracted in 10 ml hexane in a 20 ml glass vial, homogenized with a Polytron (Switzerland, distributed by Brinkmann Instruments, USA) for 30 seconds at speed 6, capped and left to extract overnight with moderate mechanical agitation (about 120 rotations/min.) under the fume hood. The next day, the extracts were homogenized with a sonicator (Ultrasonic Cleaner from Branson, model 200) for 30 seconds, and 2 ml were removed with a Hamilton syringe, filtered with Alltech filters (Illinois, USA, Nylon 66 Membranes, 13 mm, 0,2 micron) and bottled in 1.5 ml Chromatographic Specialties HPLC vials in preparation for analysis.

IV.2.5 HPLC analysis.

HPLC analysis was performed with a Beckman gold chromatographic system (from Beckman Instruments Inc., CA) consisting of a solvent pumping system, Module 126, a photodiode array detector, Module 168, and an auto-sampler, Module 502, with a 20 µL injection loop. The column and precolumn used were Beckman Ultrasphere ODS, 250 x 4.6 mm, 5 µm Column, and Beckman ODS, 45 x 4.6 mm, 5 µm Precolumn. The mobile phase consisted of a combination of water and acetonitrile (30:70), with a flow of 1 ml/min. Detection was at 254 nm. The retention time for pure dillapiol was 5,8 minutes. A standard curve was established from peak areas and the concentration of dillapiol per fresh weight was calculated.

IV.2.6 Recovery.

Recovery analyses for this method were estimated by comparing the concentration of dillapiol after processing in half of an homogenized plant tissue extract with no dillapiol added with the concentration present in the other half of the homogenized plant tissue extract where a known amount of dillapiol had been added. This was repeated three times and the average recovery for the method was found to be 95.5%.

IV.3 RESULTS and DISCUSSION

IV.3.1 Effect of time and nitrogen availability on plant growth.

Tissue growth was exponential for all parts of the plant from the date of germination to day 48 when it reached a plateau. At day 68, the plant had started to senesce **(Figures IV.1, IV.2 and IV.3).** Data for fresh weight at day 68 were not available (Only analysed samples were weighed and they do not represent the total mass of tissue available). The total biomass of tissue varied according to the nitrogen treatment. As nitrogen input decreased, the total biomass of tissue decreased, and the aerial part (leaves and stem):root ratio decreased. At day 48 **(Figure IV.4)** for example, at the peak of vegetative growth, aerial parts represented 62 % of the whole plant treated with complete Hoagland's solution while it represented only 34 % of the total mass of plants treated with 1/20 nitrogen. At this day, the average weight of the whole plant treated with complete Hoagland's solution was 12.5 g, while it was 7.5 g for plants treated with N=1/2 and about 4.75 g for plants treated with N=1/20. Growth reduction was also accompanied by some chlorosis, another nitrogen deficiency symptom [Salisbury and Ross, 1992].

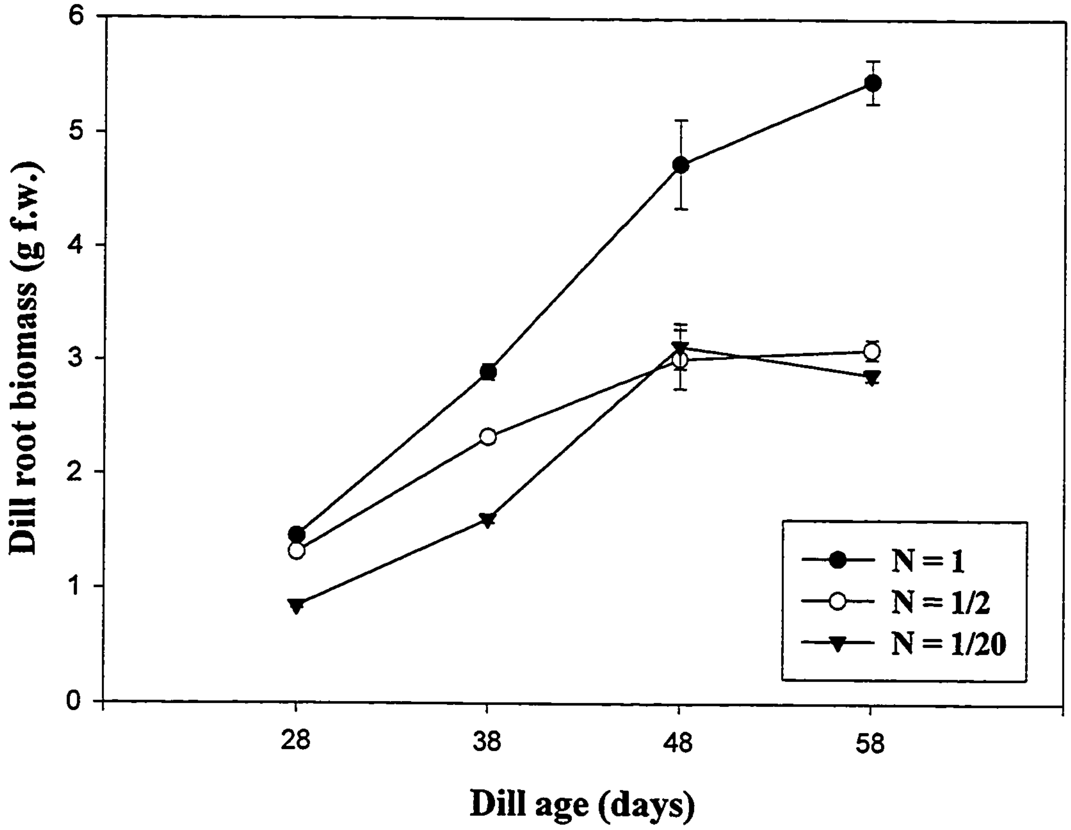


FIG. IV.1: Biomass (g f.w.) of dill roots through time, for different nitrogen treatments. Bars represent the standard error of the mean.

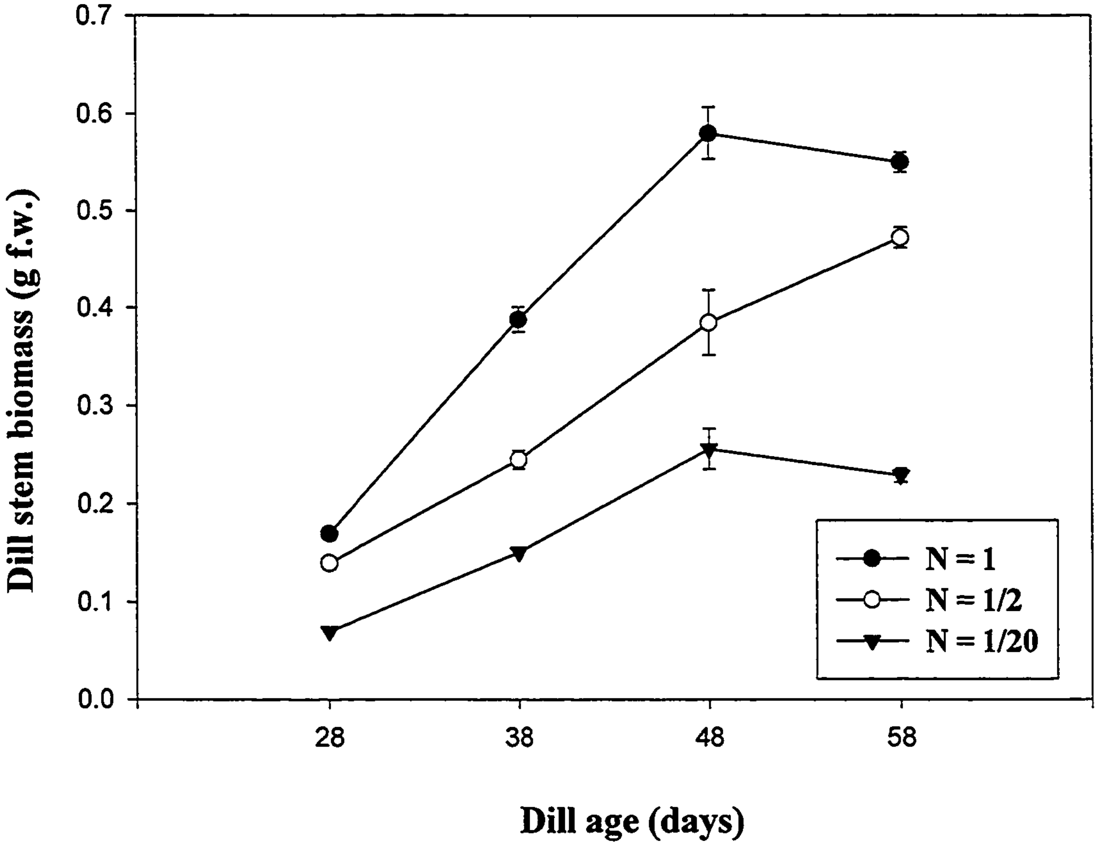


FIG. IV.2: Biomass (g f.w.) of dill stem through time, for different nitrogen treatments. Bars represent the standard error of the mean.

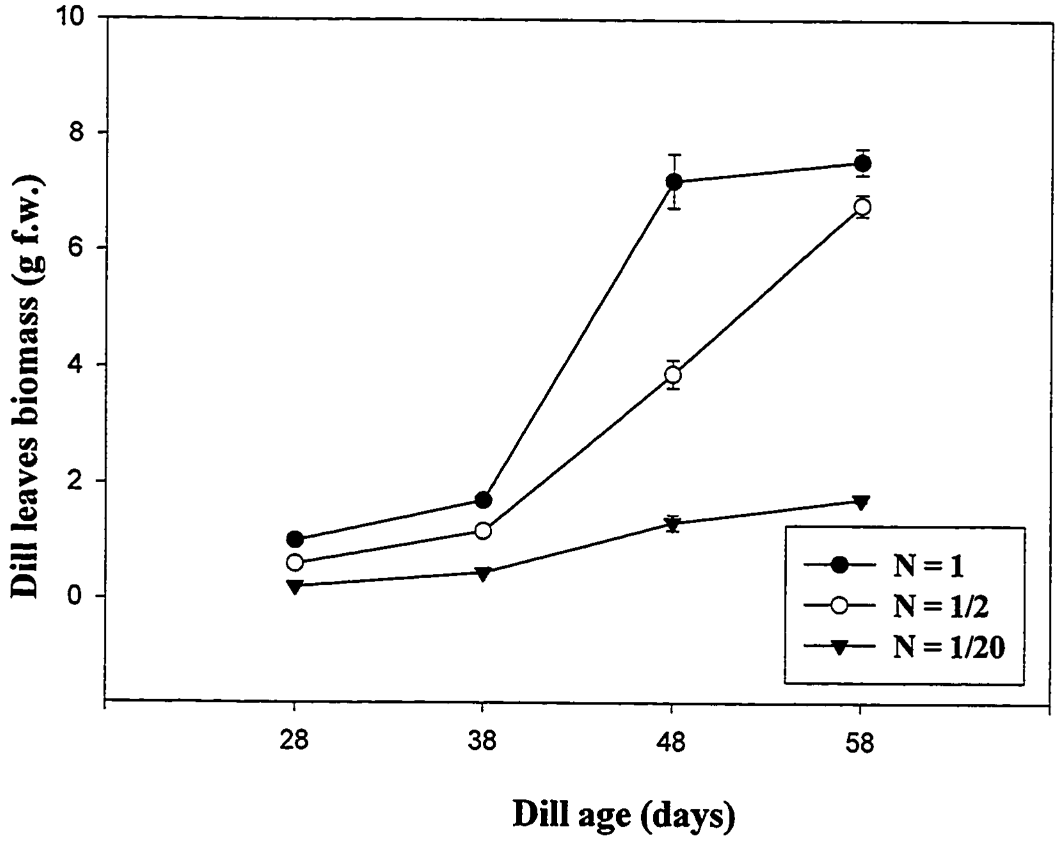


FIG. IV.3: Biomass (g f.w.) of dill leaves through time, for different nitrogen treatments. Bars represent the standard error of the mean.

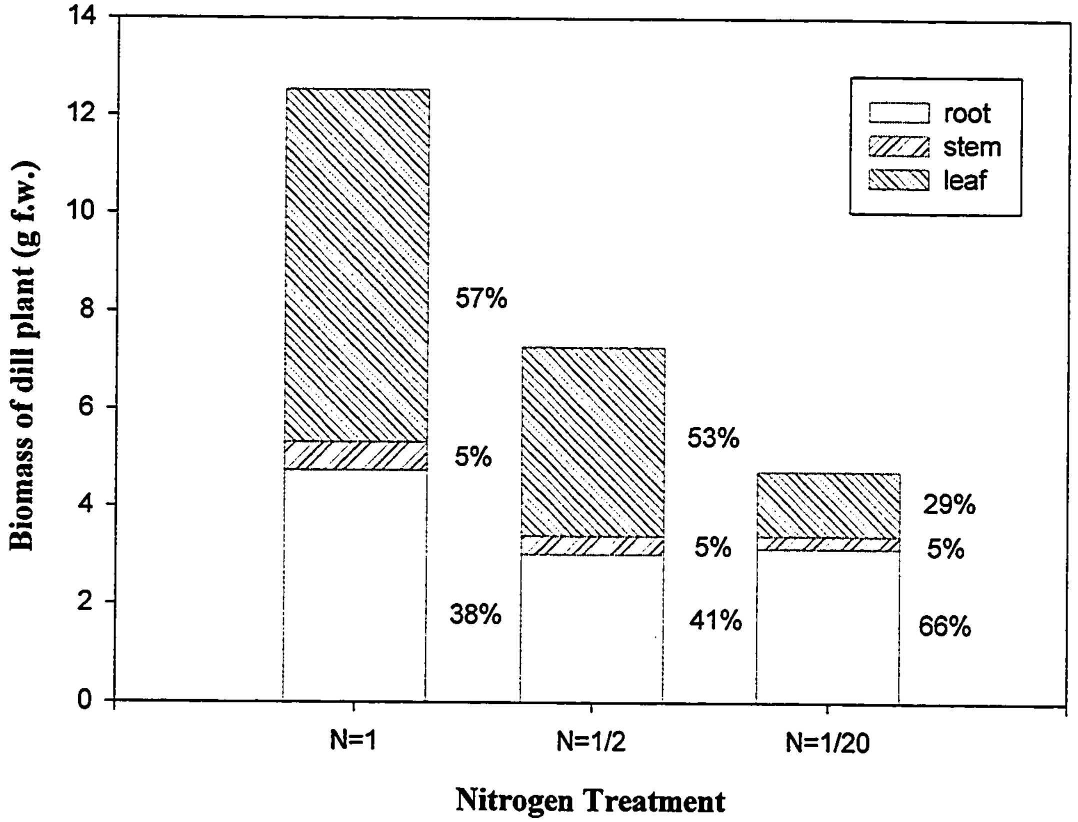


FIG. IV. 4: Average total and partial masses of dill plants treated with three levels of nitrogen, 48 days after sowing.

Percentages represent the relative importance of each part in the total weight of the plant.

IV.3.2 Concentration of dillapiol in the different parts of Anethum graveolens.

The three-term interaction with stage, part and nitrogen treatment as categorical independent factors and the ln-transformed concentration of dillapiol as dependent factor was highly significant (3-way ANOVA, $p < 0.001$; F= 4.074, n=360). Because of the difficulty of analysing a 3-way interaction model and since the most important parameter was the nitrogen treatment in this study, each part of the plant was considered separately and the data were reanalysed. It should be noted that, for each treatment, dillapiol concentration (per fresh weight) was generally lower in the root, with an average concentration of about 400 ppm, than in the aerial parts, where the average is about 700 ppm, at the beginning of the life cycle (days 28, 38 and 48; $p<0.001$, n=24). When flowers were produced, the concentration in the leaf decreased and reached the same concentration as in the roots **(Figures IV.5, IV.6, IV.7)**. The concentration of dillapiol was found to be the highest in the flowers, harvested at days 58 and 68, with concentrations reaching up to 2800 ppm or 0.28 % f.w. **(Figures IV.8 and IV.9)**. This can be generalized for all three nitrogen treatments.

Aerial parts were found to be better defended than underground parts. Yet this study only considered one compound and other chemicals, for which the concentration was not measured, are known to be present in the plant. They are mainly monoterpenes such as carvone and limonene, and other lignans such as apiol and myristicin [Badoc and Lamarti, 1991; Brunke et al., 1991; Pino et al, 1995; Charles et al, 1995]. Since above ground and under ground are two distinct environments, the plants are susceptible to

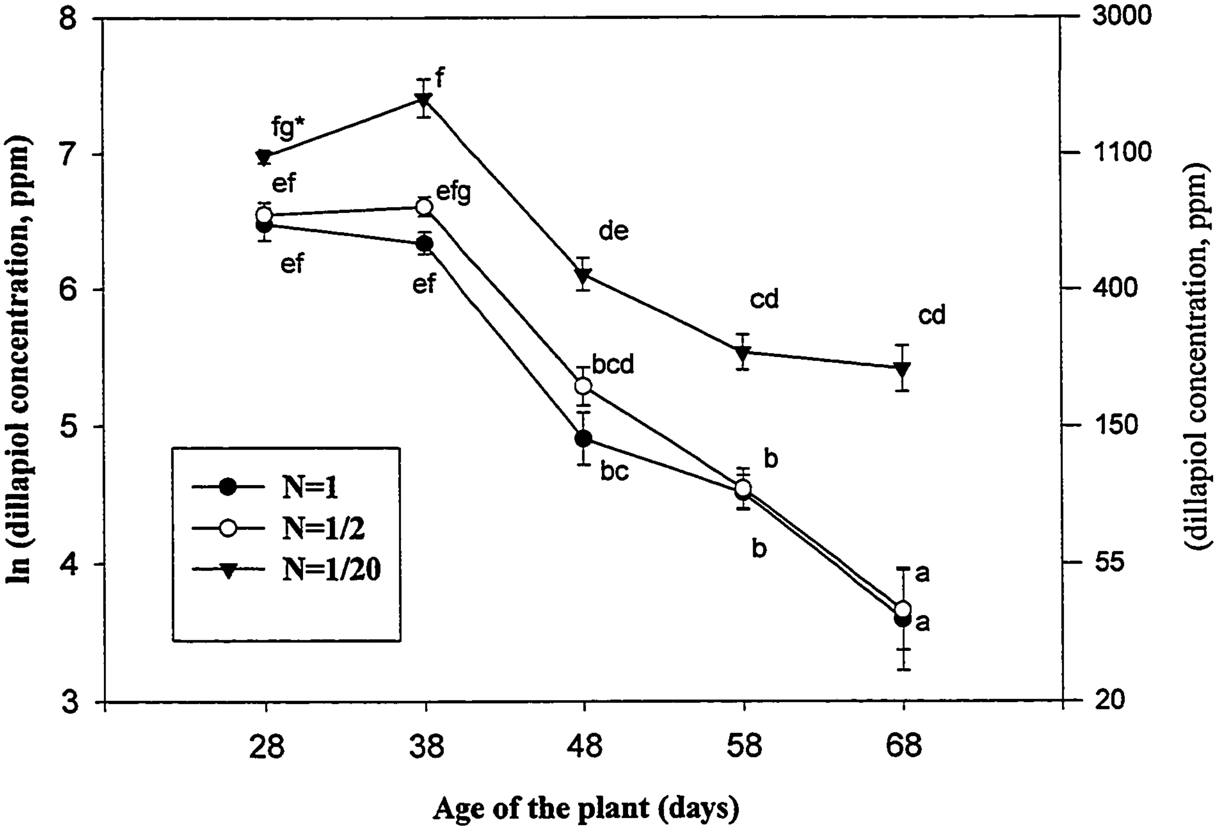


FIG. IV.5: ln of dillapiol concentration in the leaves of *Anethum graveolens* at different harvesting dates and for different nitrogen treatments.

*treatments with the same letter are not significantly different.
(Tukey Test of Multiple comparison, $\alpha = 0.05$)
The 2-way ANOVA (age of plant x nitrogen treatment) was significant, with $p = 0.005$, $F = 2.92$, $n = 120$.
Bars represent the standard error of the mean.

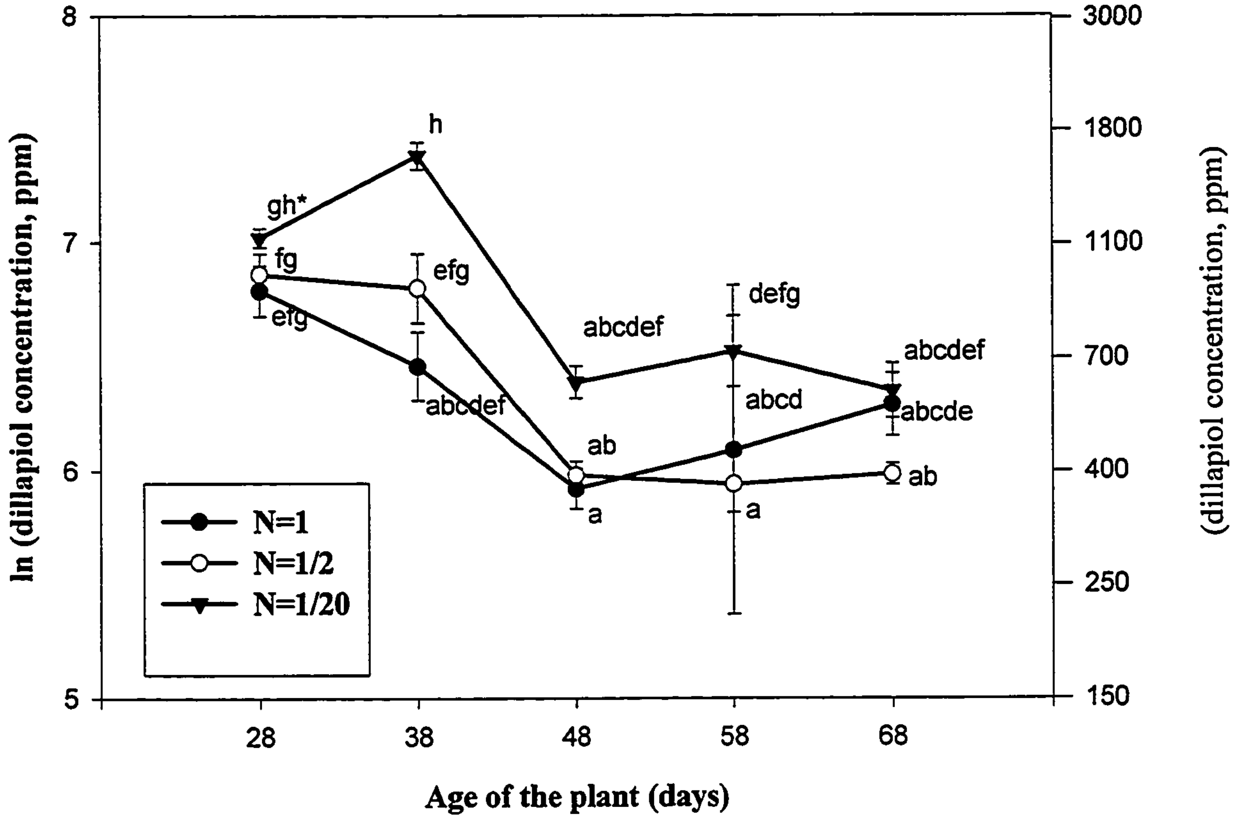


FIG. IV.6: ln of dillapiol concentration in the stem of *Anethum graveolens* at different harvesting dates and for different nitrogen treatments.

*treatments with the same letter are not significantly different.

(Tukey Test of Multiple comparison, $\alpha = 0.05$)

The 2-way ANOVA (age of plant x nitrogen treatment) was significant, with $p = 0.006$, $F = 2.92$, $n = 120$.

Bars represent the standard error of the mean.

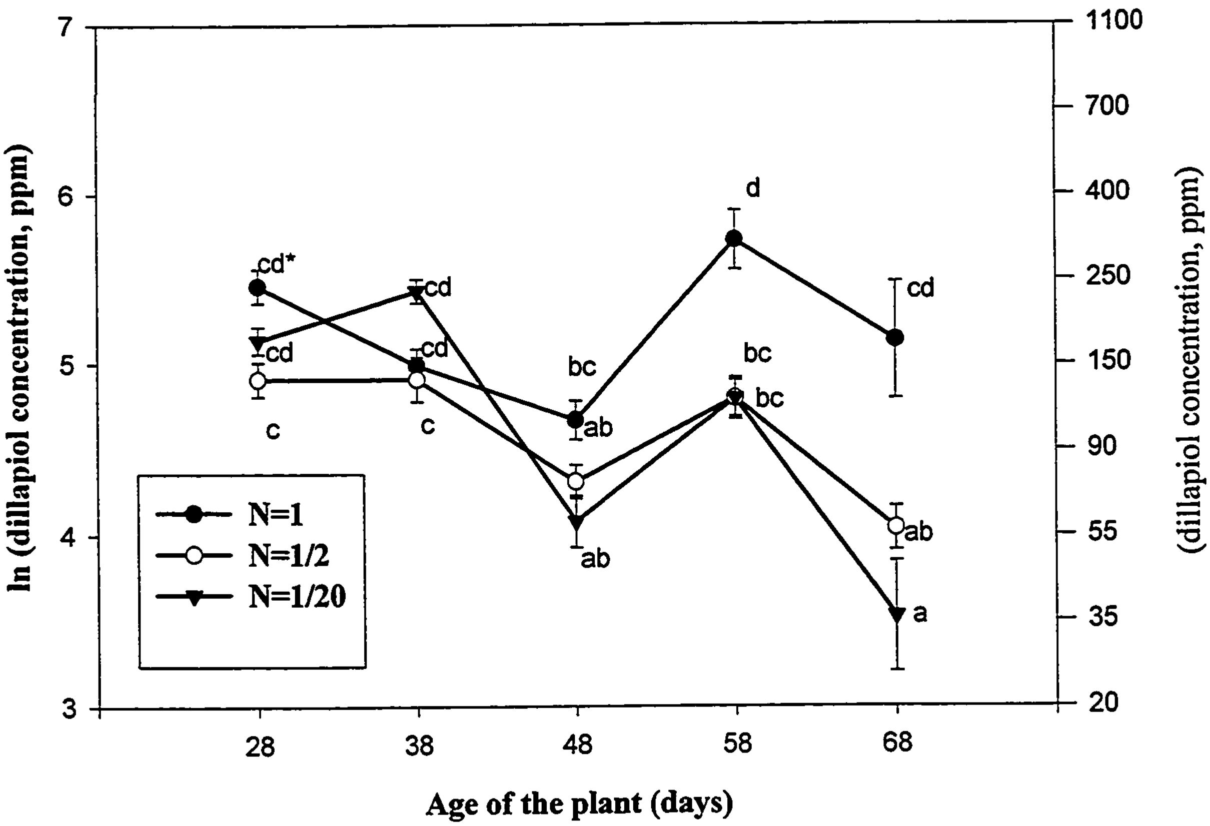


FIG. IV.7: ln of dillapiol concentration in the roots of *Anethum graveolens* at different harvesting dates and for different nitrogen treatments.

*treatments with the same letter are not significantly different.

(Tukey Test of Multiple comparison, $\alpha = 0.05$)

The 2-way ANOVA (age of plant x nitrogen treatment) was significant, with $p<0.001$, $F = 5.65$, $n = 120$.

Bars represent the standard error of the mean.

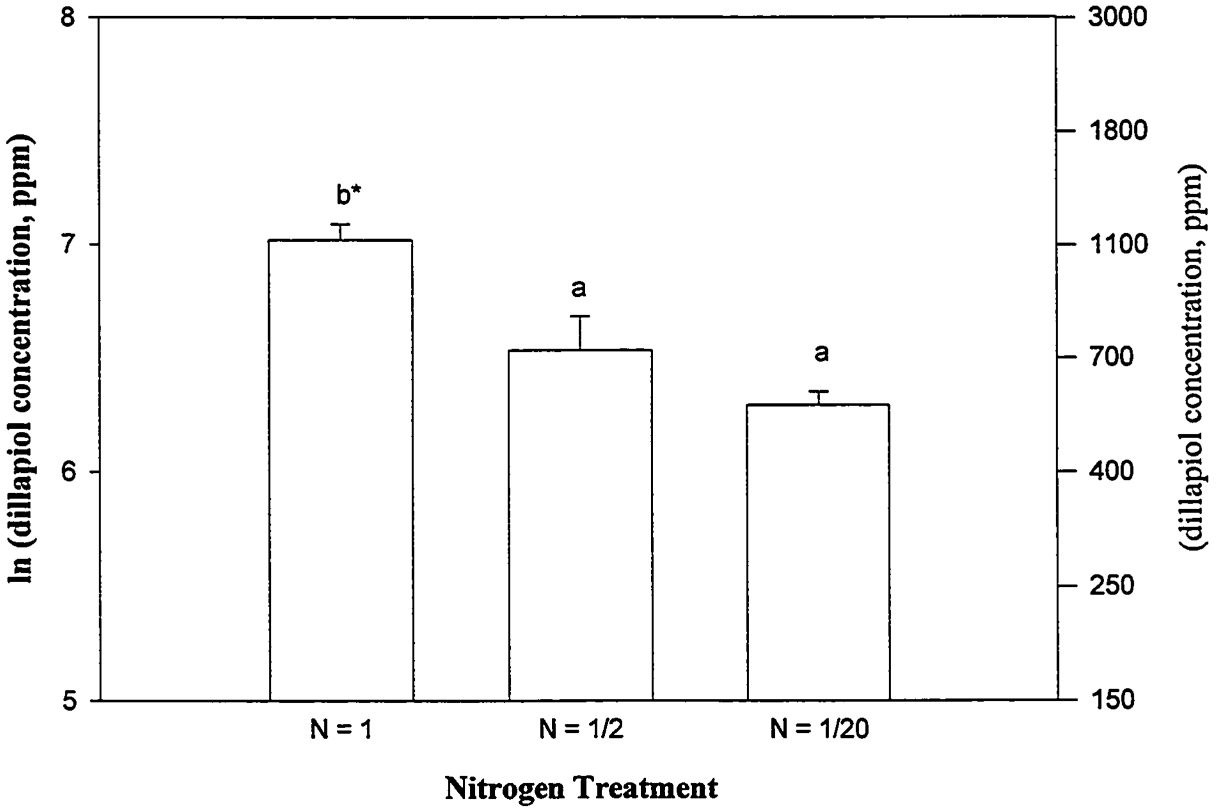


FIG. IV.8: ln of dillapiol concentration in the flowers of *Anethum graveolens* at day 58, for different nitrogen treatments.

*Treatments with the same letter are not significantly different (ANOVA, $p < 0.001$, $F = 13.96$, $n = 24$; Tukey Test of multiple comparison, $\alpha = 0.05$). Bars represent the standard error of the mean.

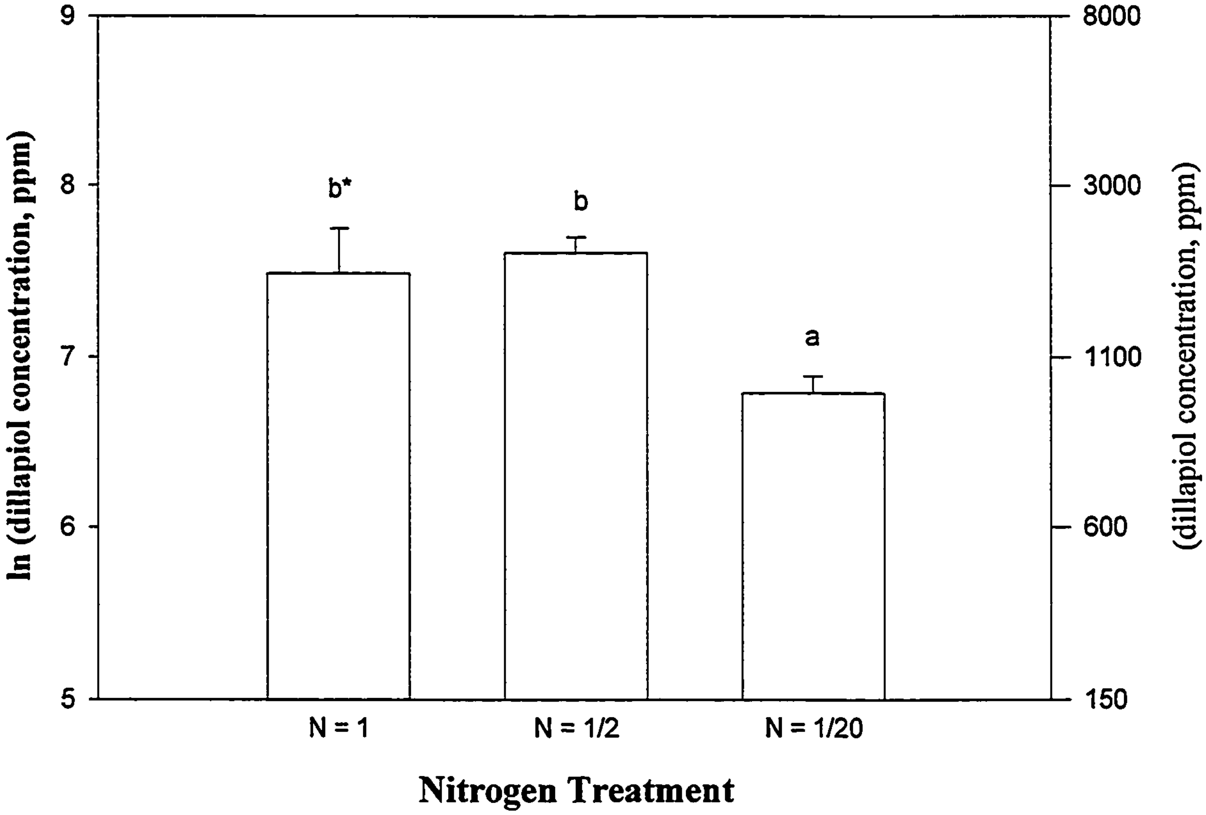


FIG. IV.9: ln of dillapiol concentration in the flowers of *Anethum graveolens* at day 68, for different nitrogen treatments.

*Treatments with the same letter are not significantly different (ANOVA, $p < 0.027$, $F = 6.95$, $n = 9$; Tukey Test of multiple comparison, $\alpha = 0.05$). Bars represent the standard error of the mean.

different herbivores or pathogens and it is likely that their strategy of defense would be different [McKey, 1979]. The fact that flowers were found to contain more dillapiol than other parts agrees with the theory that more valuable reproductive parts should be better or differently defended than vegetative parts [Feeny, 1976; Bazzaz et al., 1987].

IV.3.3 Effect of age on dillapiol concentration.

Generally, the concentration was lower in all parts and for most treatments in older plants. **(Figures IV.5, IV.6 and IV.7)**. This supports the conclusion that dillapiol could be a constitutive defense with early synthesis in young tissue followed by growth dilution or metabolism [Tuomi et al., 1988].

IV.3.4 Effect of nitrogen treatment on dillapiol concentration.

When considering different parts separately, all interactions again were found significant, indicating that the concentration of dillapiol varies according to both stage and treatment ($p<0.006$; $n=120$) (see **Figures IV.5, IV.6 and IV.7** for leaves, stem and roots). It was observed that, for the highest nitrogen input, dillapiol concentration per fresh weight increased in the leaves, remained stable in the vegetative stem and decreased in the roots. **(Figures IV.5, IV.6 and IV.7)**. The difference between leaves and roots was surprising. A phenomenon of dilution of dillapiol in the growing biomass appeared to be a possible explanation, as well as a new allocation of resources based on dillapiol translocation. To clarify this, the total quantity of dillapiol present in the plant tissues was analysed.

IV.3.5 Effect of nitrogen on total quantity of dillapiol in plant tissues.

To investigate whether the variation in dillapiol concentration was related to plant growth and involved dilution of a constant quantity in the plant tissue or reallocation of this constant quantity in different parts of the plant, the total quantity of dillapiol in each tissue was computed and analysed. The concentration for each sample was multiplied by the total weight of the tissue. The results are shown in the **Figures IV.10, IV.11 and IV.12**, for each part of the plant separately.

It was found that the quantity of dillapiol did not vary significantly in the stem (No statistical significance was found in the 2-way ANOVA with age of plant and nitrogen treatment as categories and ln (Total dillapiol, μg) as the dependent variable; $p = 0.22$, $F = 1.42$, $n = 96$). It can be noted that it peaked for all treatments at day 38 **(Figure IV.11)**. In the leaves **(Figure IV.10)**, the logarithm (ln) of the Total quantity of dillapiol significantly increased ($p = 0.002$, $F = 3.8$, $n = 96$, Tukey Test of multiple comparison with $\alpha = 0.05$) from day 28 to day 38, and then decreased from day 38 to 58, for all treatments. In the roots **(Figure IV.12)**, it significantly increased from day 28 to day 58, with a peak at day 38 for $N = 1/2$ and $N = 1/20$ nitrogen treatments. ($p < 0.001$, $F = 8.7$, $n = 96$). For each nitrogen treatment, it can be generalized that for all parts and at all ages,

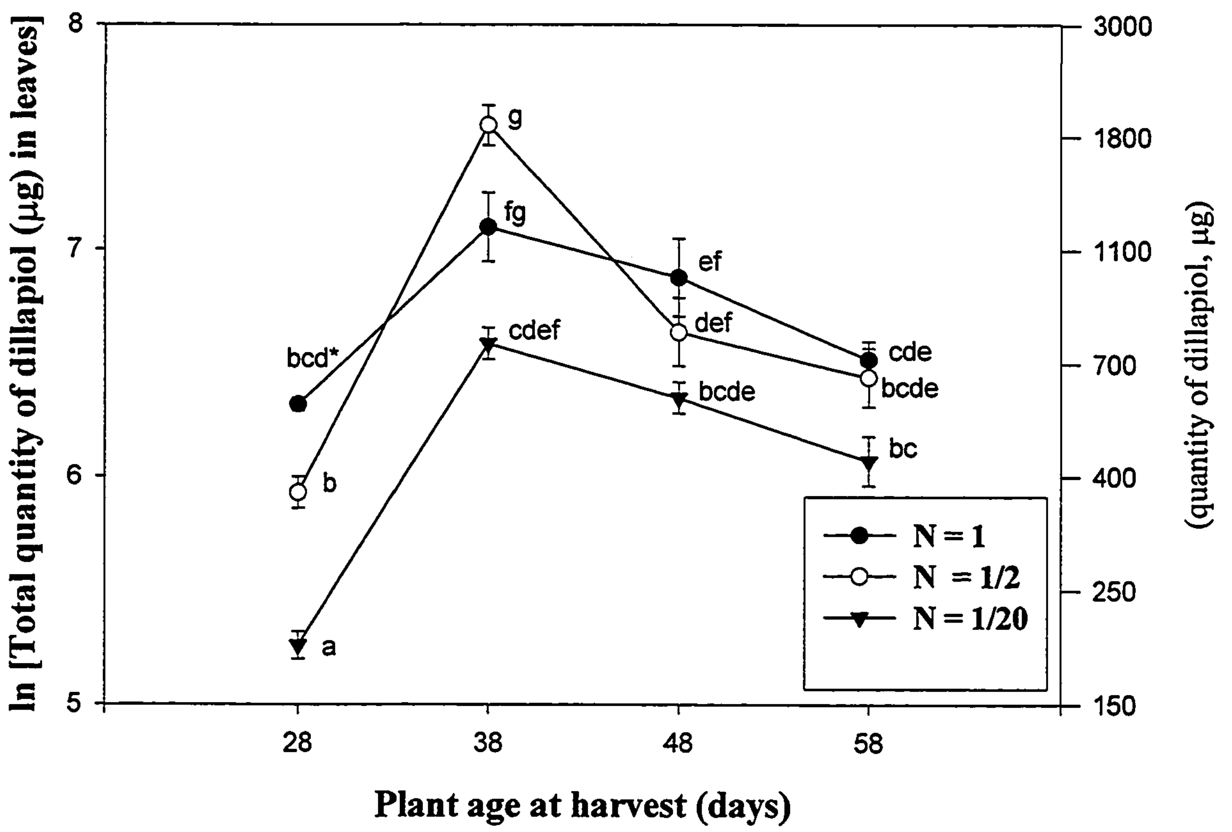


FIG. IV.10: ln of the total quantity of dillapiol present in the dill leaves, *Anethum graveolens*, at different stages and for different nitrogen treatments.

*Treatments with the same letter are not significantly different

(Tukey Test of multiple comparison, $\alpha = 0.05$).

The 2-way ANOVA (age of plant x plant part) was significant,

with $p = 0.002$, $F = 3.8$, $n = 96$.

Bars represent the standard error of the mean.

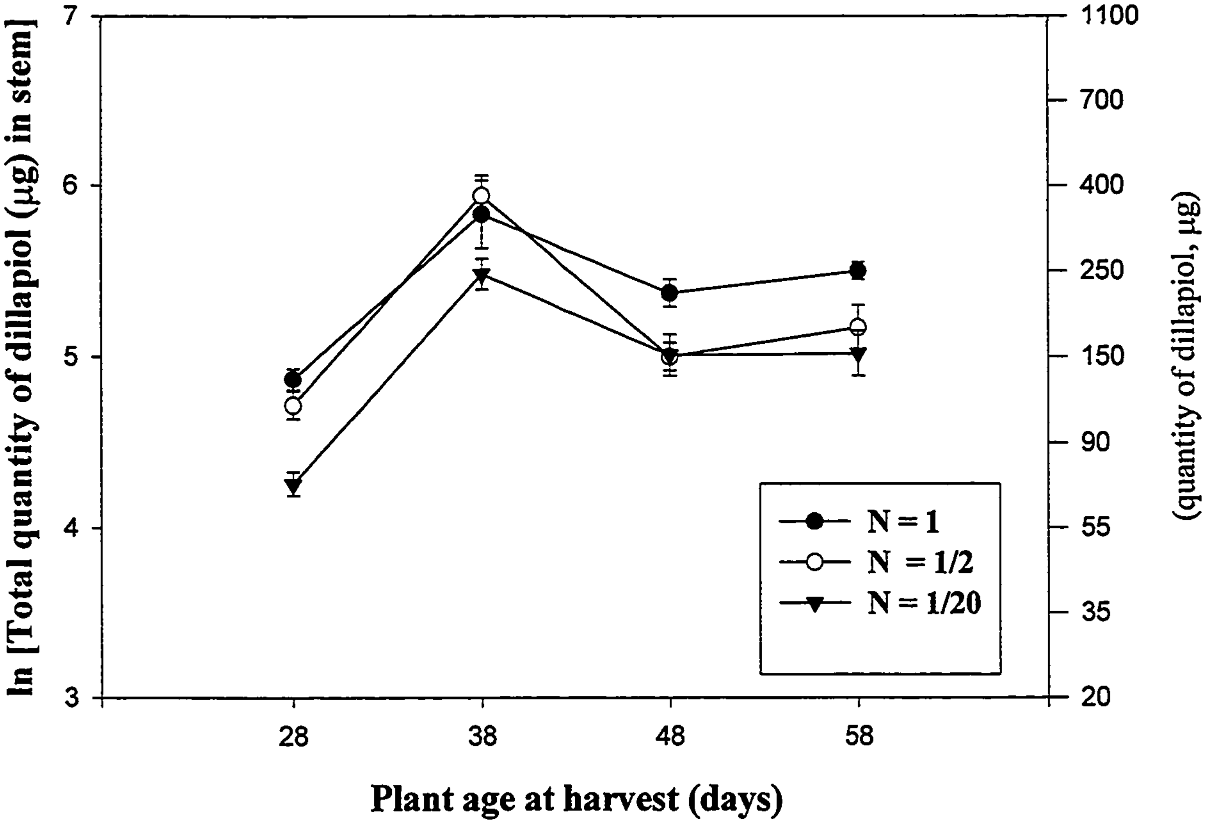


FIG. IV.11: ln of the total quantity of dillapiol present in the dill stem, *Anethum graveolens*, at different stages and for different nitrogen treatments.

No statistical difference between treatments was observed (Two-way ANOVA with age of plant and nitrogen treatment as categorical variables; $p = 0.216$, $F = 1.422$).

Bars represent the standard error of the mean.

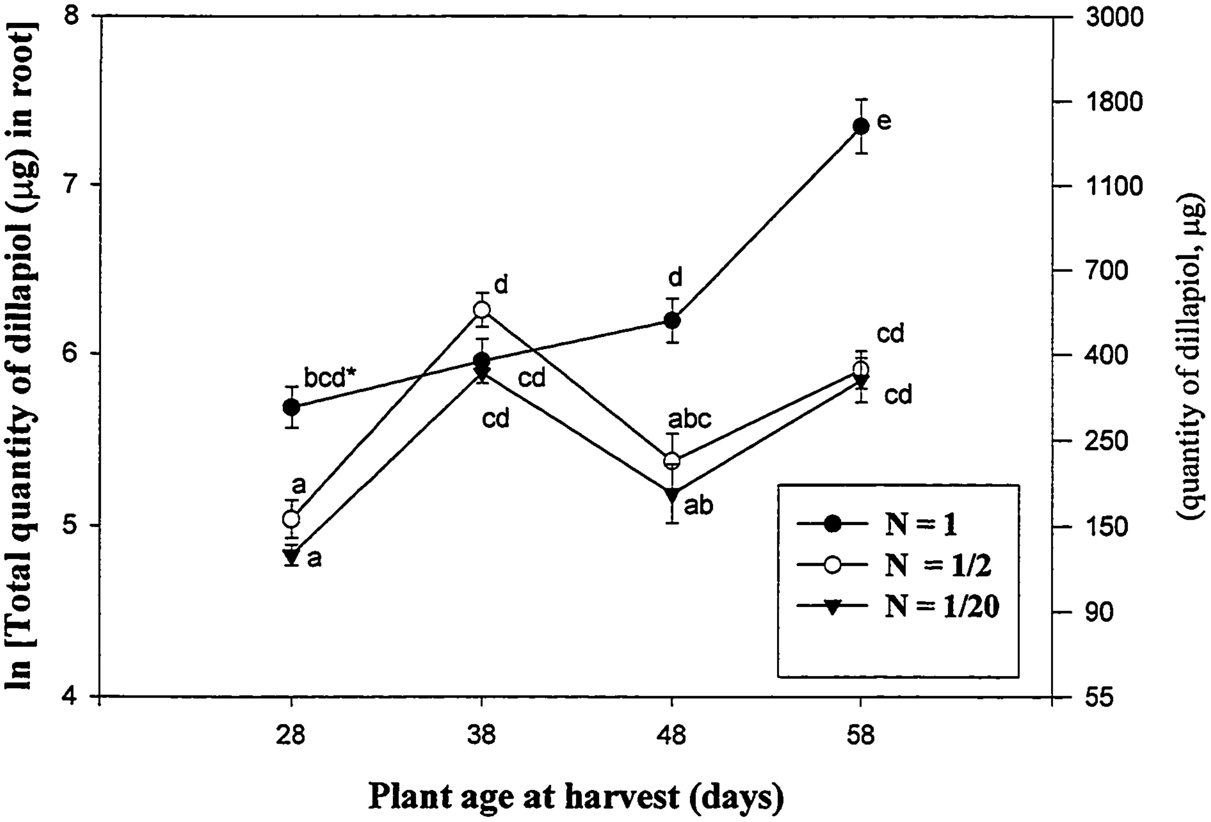


FIG. IV.12: Ln of the total quantity of dillapiol present in the dill root, *Anethum graveolens*, at different stages and for different nitrogen treatments.

*Treatments with the same letter are not significantly different

(Tukey Test of multiple comparison, $\alpha = 0.05$)

The 2-way ANOVA (age of plant x plant part) was significant

($p < 0.001$, $F = 8.7$, $n = 96$). Bars represent the standard error of the mean.

the quantity of dillapiol decreased, often significantly, with a decrease in nitrogen input **(see Figures IV.10, IV.11 and IV.12)**.

A more visual summary of all the results follows **(Table IV.1)**. During the exponential phase of growth, the quantity of dillapiol increased and then decreased as the growth rate lowered. Dillapiol could be produced in early phases of growth and as the plant tissues get older growth dilution or metabolism could occur leading to depletion of the dillapiol stock. The recycling of secondary metabolites into primary metabolism as the plant ages has been observed for example with the alkaloid caffeine in the coffee plant [Frischknecht et al., 1986]. The results observed support the consideration of dillapiol as a constitutive or immobile [Coley, 1985] defense and the theory that younger plants are chemically better defended than older ones, since they are more vulnerable and lack structural defenses [Feeny; 1976]. Furthermore, the smaller biomass produced when less nitrogen is available and the premature senescence could explain the lower quantity of dillapiol observed at low nitrogen levels. As the physiology of the plant is compromised by low N, less dillapiol is produced. This result does not follow the predictions of Bryant's Carbon-Nutrient Balance Hypothesis. It can also be noted that the resurgence of high concentration of dillapiol in the flowers probably results from a new and localized biosynthesis related to new tissue expansion. This, along with the fact that dillapiol stocks decreased gradually in all tissues with time does not support the possibility of a new allocation with translocation of dillapiol within the plant.

Table IV.1: General summary of the findings of the greenhouse experiment on *Anethum graveolens*.

LEAVES

	[dillapiol]	Total dillapiol	Biomass
N ↘	↘	↘	↘↘
Age ↗	↘	↗↘	↗↗

STEM

	[dillapiol]	Total dillapiol	Biomass
N ↘	↘	=	=
Age ↗	↘	=	↗

ROOTS

	[dillapiol]	Total dillapiol	Biomass
N ↘	↘	↘	↘
Age ↗	↘	↗↘	↗

IV.4 CONCLUSION

Dillapiol was found to be a constitutive defense, with quantities building up in the tissues during early vegetative growth and then decreasing as it slows down. It was found to be present mainly in the aerial parts, especially in the flowers. Lower nitrogen input reduced significantly the quantity of dillapiol present in the tissues as it reduced plant growth. The results do not follow the predictions of Bryant's C/N Balance Hypothesis.

V: Induction of dillapiol production by methyl jasmonate

V.1 INTRODUCTION

It has been shown that in some plants, specific enzymatic processes are activated when tissues are damaged or under certain types of stresses such as herbivory or pathogen attack [Ryan, 1987; Bol, 1990; Cutt and Klessig, 1992; Ryals et al., 1996]. Those processes lead in some cases to increased accumulation of secondary metabolites within the plant tissue [Baldwin, 1989; Paré and Tumlinson, 1997]. Those secondary metabolites have been considered to be induced chemical defense that confer a certain level of protection to the plant by their toxicity or their deterrent activity [Zangerl et al., 1997; Wold and Marquis, 1997].

Based on current knowledge, some specific signalling molecules are responsible for the regulation of the pathways involved in secondary metabolism. Among them are salicylic acid [Doares et al., 1995], ethylene [Xu et al., 1994], systemin [Constabel, 1995], and the jasmonates (the most studied being Jasmonic acid (JA) and its methylated form, methyl jasmonate (MJ)), derived from linolenic acid [Vick and Zimmerman, 1984]. Methyl jasmonate was first described as a major aroma component in jasmine [Demole et al., 1962] and is now widely used in the perfume industry. MJ and JA were, along with related compounds, characterized in many tissues of other plants from a wide diversity of families, among them soybean, apple and Douglas fir [Meyer et al., 1984], in concentrations varying from 30 μg/kg fresh weight (in the tea family, *Camellia* and *Mimosa* species) to 4 mg/kg fresh weight in the pericarp of soybean (*Glycine max*) seeds.

At the physiological level, the jasmonates were found to be significant regulators of growth and metabolism in plants: they were found to inhibit growth [Yamane et al, 1981], to induce senescence [Ueda and Kato, 1980], and to be partly responsible for many other physiological processes such as tuberization [review by Koda, 1992; Koda, 1997]. Recently, new properties were associated with this lipid-derived compound. It has been found that when a tomato, a soybean or a potato tissue is wounded, the concentration of methyl jasmonate increases within this tissue [Creelman et al., 1992; Peña-Cortes et al. 1993, Koda and Kikuta, 1994]. It is now recognized that JA is an intermediary messenger necessary to activate genes for the chemical defence processes [Farmer E.E., Ryan C.A., 1992]. Therefore, methyl jasmonate is now regarded as one of the regulators of resource allocation toward secondary metabolism [review by Creelman and Mullet, 1997].

It has been shown that different pathways of secondary metabolism are activated under herbivore damage or wounding in plants. The phenylpropanoid pathway is one of them [Halbrock and Sheel, 1989]. The activation of this pathway results in the production of phenolic secondary metabolites via the transformation of L-phenyalanine into cinnamic acid by the biosynthesis of the phenylalanine ammonia lyase (PAL) and other enzymes. Wounding activates a lipase that initiates the conversion of linolenic acid to JA via the octadecanoic acid pathway. JA then activates transcription of the PAL and other genes for phenolic biosynthesis. For example, exposure to 30 µM methyl jasmonate was shown to increase the production of furanocoumarins from 0. 15µM/g to 0.8 µM/g fresh weight in parsley cell cultures (Apiaceae) [Ellard-Ivey and Douglas, 1996]. The neolignan dillapiol is a phenolic derived from the phenylpropanoid pathway. In our effort to thoroughly understand and optimize dillapiol production in Indian dill, *Anethum graveolens,* and the possible induction of dillapiol by methyl Jasmonate was tested. In the current investigation, a time study of the induction of dillapiol production by 200 ppm of MJ was undertaken. Dillapiol occurs both in the leaves and roots, as seen previously **(Chapter IV)**. Since induction has been previously found to be systemic [Turlings and Tumlinson; 1992], and not only to occur in the stressed tissue, both treated and untreated parts were considered. The hypothesis were that the level of dillapiol within dill leaves exposed to MJ is significantly different from the level of dillapiol within non-exposed leaves; and that the level of dillapiol within dill roots exposed to MJ is significantly different from the level of dillapiol within non-exposed roots.

In a second and third experiments the dose dependent effect of methyl jasmonate on the production of dillapiol in the dill was assessed. Some studies have shown that induction of chemical response may be dose-dependent. Guillet (1997) found that the induction by MJ of polyacetylenes in *Carthamus* sp. was dose dependent and that the highest accumulation was found for the highest level of sprayed MJ (1000 ppm). Other studies have shown that dose dependence of induction is not necessarily linear. For example Ellard-Ivey and Douglas (1996) found that furanocoumarin concentration increases, reaches a maximum and then decreases in parsley (Apiaceae) cell culture treated with increasing concentrations of methyl jasmonate. Therefore, two experiments were conducted using a wider range of concentrations. The new hypothesis was that the concentration of dillapiol in the leaves of *Anethum graveolens* varies in a methyl-jasmonate dose-dependent manner.

V.2 METHODS

V.2.1 Greenhouse conditions.

The dill was grown under the same conditions as described in **Chapter IV.**

V.2.2 Age of the plant and time of experimentation.

Induction and high production of secondary metabolites are known to occur when the plants are young and more susceptible to herbivory or other types of stresses [Feeny, 1976]. For this reason the experiments were conducted from day 17 to day 19 after sowing, which corresponds to an early stage of the life cycle (two-leaves). Chosing this growth stage is a common procedure [Farmer and Ryan, 1990].

The time necessary for induction and accumulation of secondary metabolites has been shown to vary among different plants, but it is generally noticeable around 24 hours after exposure [Paré and Tumlinson, 1997; Farmer and Ryan, 1990, Farmer and Ryan, 1992]. Indeed, while MJ or JA accumulation is known to occur within the first few hours after wounding or simulated herbivory [Creelman et al., 1992; Peña-Cortés et al. 1993; Koda and Kikuta, 1994], more time is necessary for the production of secondary metabolites. In order to obtain a profile of a possible induction in the first experiment, measurements were made at different times after exposure. t=2h, t=6h, t=12h, t=24h, t=48h and t=72h. The experiments on the effect of MJ concentration on dillapiol induction were conducted with 17 days old plants, over a period of 24 hours, time at which induction is found to be the highest.

V.2.3 *Chemicals and concentrations used.*

Methyl Jasmonate (95%) was obtained from Aldrich Chem.Co. In the first experiment, an aqueous solution was prepared with 40 µl of MJ, 40 µl of DMSO (to solubilise the MJ) and 200 ml of distilled water. The final concentration of the solution was 200 ppm. This concentration was chosen according to results of previous experiments by Guillet (1997) in which he found an induction by methyl jasmonate of polyacetylenes in *Carthamus tinctorius* (Asteracea). In the second experiment, the concentrations used were 0, 10, 100 and 1000 ppm. As MJ is known to be a growth inhibitor, concentrations higher than 1000 ppm were not used since senescence of the young plant would have been detrimental (Guillet, 1997). In the third experiment, the concentrations used were 0 (control), 0.001, 0.01, 0.1, 1, 10 and 100 ppm . The ANOVA both for roots ($p=0.1$, $F=1.952$, $\alpha=0.05$, $n=42$) and leaves ($p=0.703$, $F=0.632$, $\alpha=0.05$, $n=42$) were found to be non-significant.

The MJ solution was sprayed on the aerial parts of the treated plants in a separate growth chamber, left to dry for two hours in a separate and closed growth chamber [Guillet, 1997], and then put back with the untreated plants. In the first experiment, at each different sampling time, 10 replicates or pots were randomly chosen among controls and 10 among treatments. In the second and third experiments, 6 replicates were used per treatment.

V.2.4 Extraction.

The leaves (cotyledons at this stage) and the roots (in experiments 1 and 30) were separated from the rest of the plant using a razor blade, weighed (per pot) and each sample was homogenized with a Polytron apparatus for 30 seconds and extracted in 5 ml hexane for 24 hours. The solution was filtered and 2 ml samples were prepared for High Pressure Liquid Chromatography (HPLC) analysis.

V.2.5 HPLC Analysis.

The HPLC method used was the same as described in **Chapter IV**.

V.2.6 Statistical analysis.

Data were analysed with Systat using ANOVA models. In the first experiment, two two-way ANOVAs were performed, for the leaves and for the roots. The dependent variable was the ln-transformed concentration of dillapiol in the plant tissue (data had to be transformed to meet homoscedasticity and normality assumptions). The different categories were: Harvest in time and methyl jasmonate treatment (+ or -). In the second and third experiments, simple ANOVAs were performed, with dillapiol concentration as the dependent variable and MJ concentration as the categorical variable.

V.3 RESULTS

V.3.1 Time study of the induction of dillapiol production by 200 ppm of sprayed MJ.

The two-way models were found to be non-significant for the leaves ($p=0.12$, $F=1.90$, $\alpha=0.05$) and for the roots ($p=0.25$, $F=1.38, \alpha=0.05$). Those results show the absence of dillapiol induction by Methyl Jasmonate when using a concentration of 200 ppm **(Figures V.1 and V.2)**. Yet there seems to be for both tissues a trend toward an increase in dillapiol concentration at 24 hours.

V.3.2 Dose dependent effect of Methyl Jasmonate on the production of dillapiol in the dill.

In the experiment with a high range of MJ concentrations **(Figure V.3)**, the analysis of variance was non significant ($F=0.757$, $p=0.607$ with $\alpha=0.05$). The assumptions of homoscedasticity and normality were met and therefore no transformation of the data was necessary. The concentrations of dillapiol were consistently around 1000 ppm (μg/g fresh weight) in each treatment. This is consistent with the values obtained in the previous experiment.

In the experiment with a low range of MJ concentrations **(Figure V.4)**, the ANOVA both for roots ($p=0.1$, $F=1.952$, $\alpha=0.05$, $n=42$) and for leaves ($p=0.703$, $F=0.632$, $\alpha=0.05$, $n=42$) were found to be non significant.

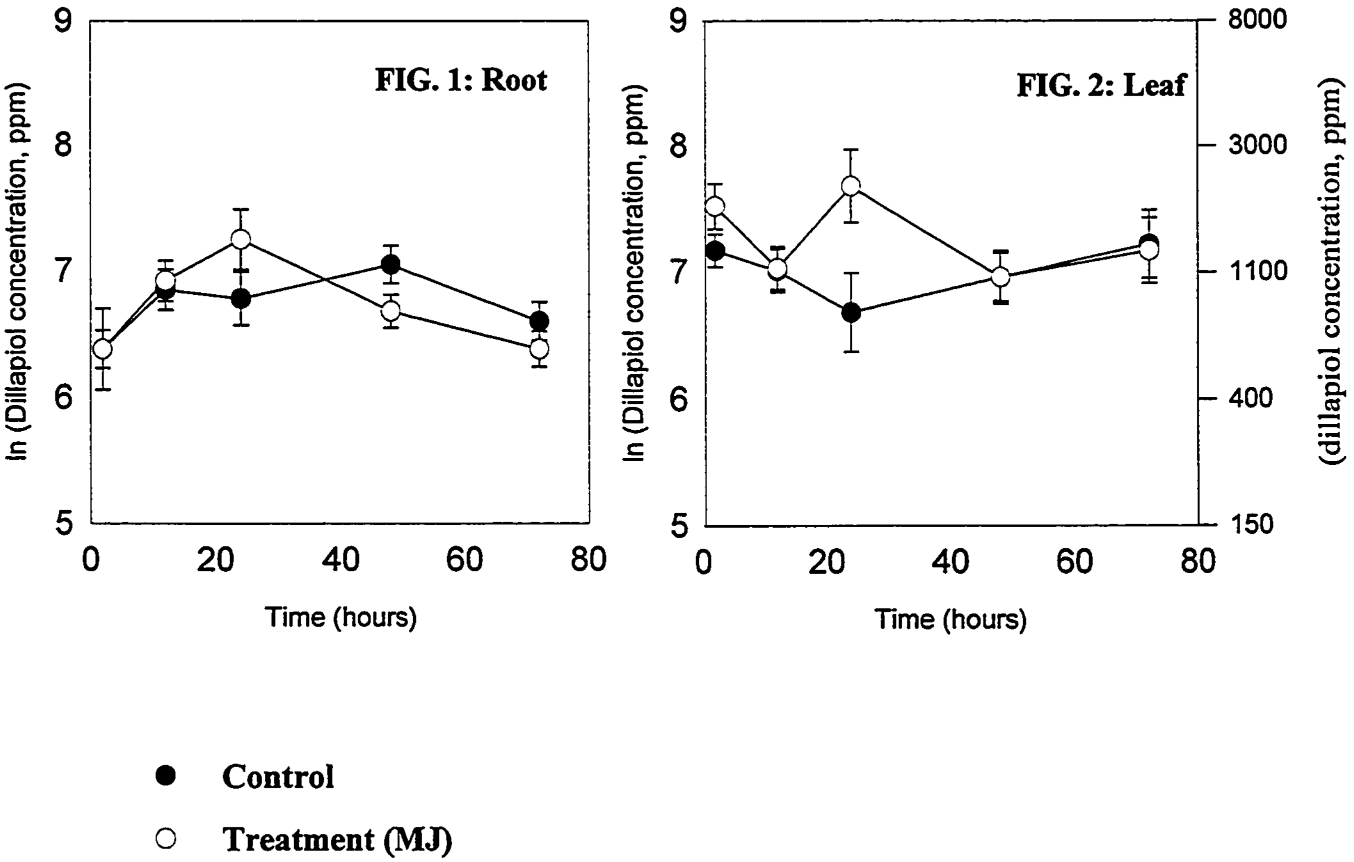


FIG. V.1 and V.2: Effect of MJ Treatment on dillapiol concentration in Indian dill, *Anethum graveolens*, in the roots and the aerial parts.

The 2-way ANOVA model (harvest*treatment) was found to be non-significant for both tissues. In the roots, $p = 0.25$ ($F = 1.38$, $\alpha = 0.05$) and in the leaves, $p = 0.12$ ($F = 1.9$, $\alpha = 0.05$).

Bars represent the standard error of the mean.

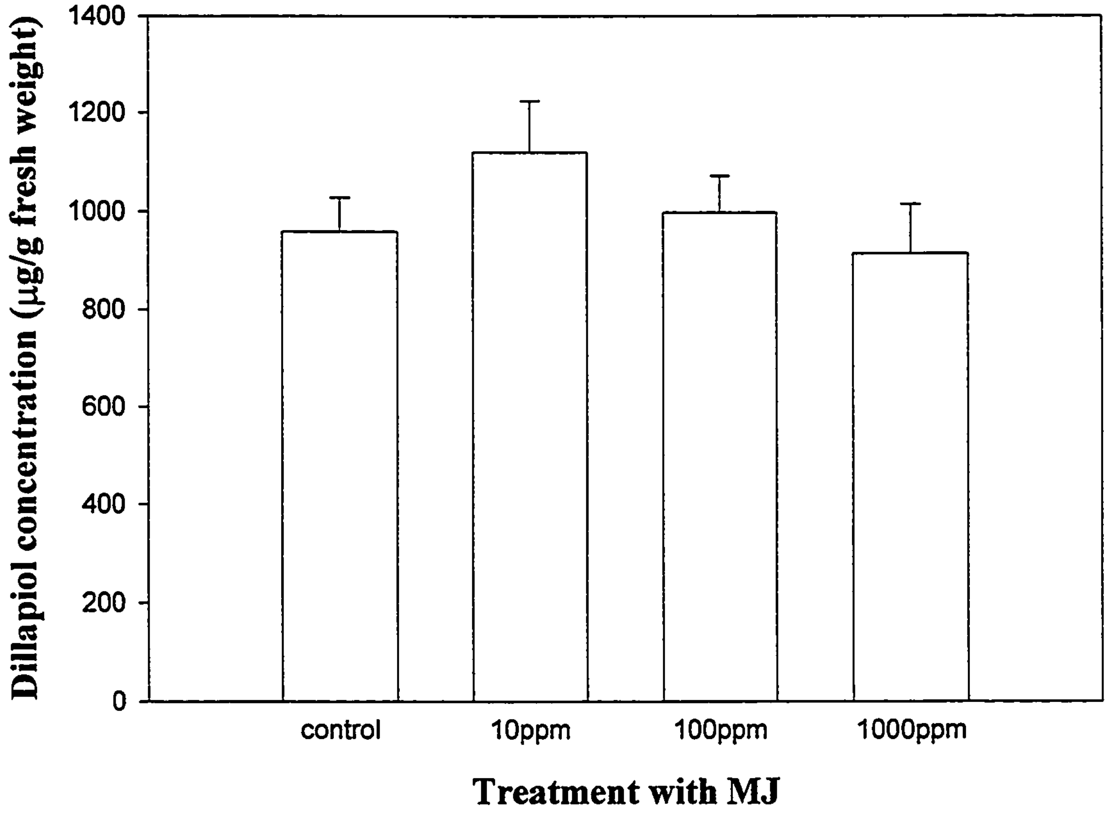


FIG. V.3: Concentration of dillapiol in 20 day old dill leaves (*Anethum graveolens*) 24 hours after treatment with different concentrations of Methyl Jasmonate.

The ANOVA (Methyl Jasmonate concentration) was found to be non-significant: $p = 0.607$ ($F = 0.757$, $\alpha = 0.05$).

Bars represent the standard error of the mean.

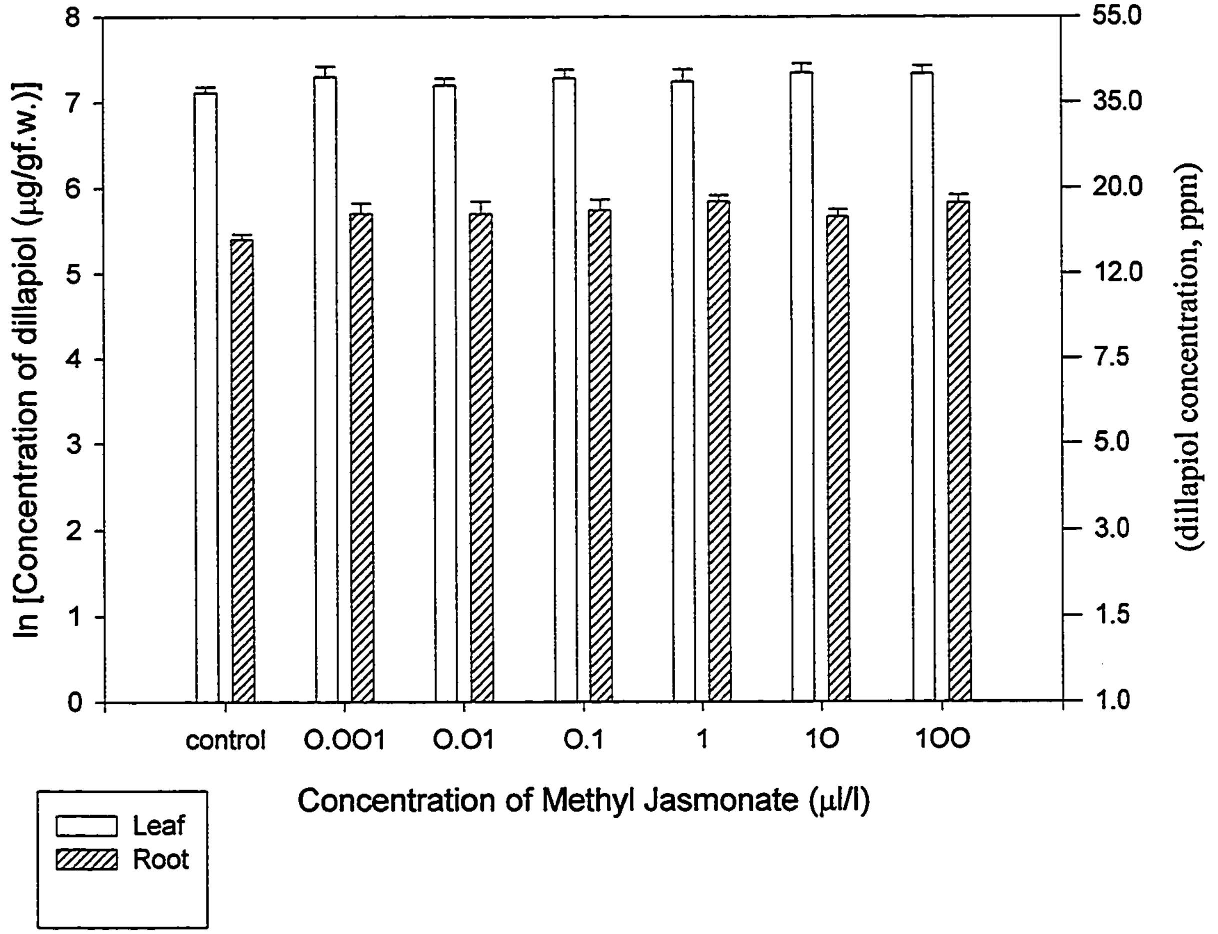


FIG. V.4: ln of the concentration of dillapiol in 17 day old dill leaves and root (*Anethum graveolens*) 24 hours after a treatment with various concentrations of Methyl Jasmonate.

The ANOVA was found to be non-significant for both leaves ($p = 0.70$, $F = 0.632$, $n = 42$) and roots ($p = 0.1$, $F = 1.95$, $n = 42$). Bars represent the standard error of the mean.

V.4 DISCUSSION and CONCLUSION

The time study led to the conclusion that with a MJ concentration of 200 ppm, there was no significant induction. The dose dependent study with a range of MJ concentrations including 200 ppm confirmed the absence of induction although a visible increase of dillapiol concentration for a MJ concentration of 10 ppm suggested that perhaps induction occured at lower MJ concentrations. Yet the dose dependent experiment with lower MJ concentrations confirmed once again the absence of induction.

From the experiments described above, it can be concluded that, in Indian dill, dillapiol is not-inducible with MJ alone. This suggests that it is probably regulated by a combination of factors possibly including MJ or by other factors related to plant growth, as it was suggested by the life cycle study **(Chapter IV)**. Perhaps other compounds in the dill may be induced such as the terpenes carvone and limonene which are present in larger amounts in the plant. As suggested by Berenbaum and Neal (1985) who found that the neo-lignan myristicin, widely occurring in the Apiaceae, synergizes the co-occurring phototoxic xanthotoxin at natural levels against *Heliothis zea*, dillapiol which caught our interest for its applied synergistic properties probably acts as a synergist within the plant. In that case, its induction could have been one possible strategy for the plant, but not the only one. More theoretically, dill can be considered a fast growing plant since it is an annual herb and has a short life cycle. We would therefore have expected, according to Coley et al. (1985), to find within the plant mainly inducible or mobile defenses which represent a lower cost than constitutive ones. The results of the current experiments do not support that hypothesis but confirm the role of dillapiol as a constitutive defense and agree with the findings of the previous section.

VI: Variation in the concentration of dillapiol in wild accessions of *Piper aduncum,* according to plant tissue and soil nutrient availability.

VI.1 INTRODUCTION

The potential use of dillapiol in agriculture as a synergist for natural insecticides has lead us to investigate potential natural sources for great scale production of the botanical. *Piper aduncum* from Costa Rica was known to produce dillapiol [Bernard, 1995] in appreciable amounts. The production of dillapiol in different plant tissues and according to their level of maturity has not been previously assessed in the different tissues (lignified stem, new leaf, mature leaf, new fruit, mature fruit). According to Feeny (1976) and Mc Key (1979) we would have expected the following pattern for dillapiol concentrations: new fruits > mature fruits > new leaves > mature leaves > stem, according to the qualitative value of the tissue (fruits being the most valuable tissues since they are involved in propagation) and the presence or absence of other types of defense such as tannins (in the old leaf more than in the new leaf) or structural defenses such as lignins (in the stem).

Furthermore, as in **Chapter IV**, the effect of environmental factors, such as nutrient availability in the soil, was considered to be important on dillapiol production in *Piper aduncum*. According to the Carbon/Nutrient Balance hypothesis [Bryant, 1983], the carbon-based defense dillapiol should be produced in larger amounts in nutrient poor environments. Coley's (1985) hypothesis predicts similar results in that fast growing plants will produce more defense in nutrient-poor environments. Since growth is limited by low-nutrients, resources are allocated to differentiation including secondary metabolism or defense. Since nitrogen, phosphorus and potassium are the main growth limiting factors, and are commonly deficient in soil [Peck and Soltanpour, 1990] they were all measured, as well as pH which is also limiting in plant growth since it affects plant nutrient availability, especially between the range of 4 and 6 [Chandrabose et al., 1988].

VI.2 METHODS

VI.2.1 Collecting

Nine accessions of *Piper aduncum* were collected in different areas of Costa Rica: Horquetas, Sarapiqui (10°22'-84°00'; 2 sites); Puerto Viejo, Sarapiqui (10°27'-84°02'; 1 site); Fortuna, Arenal Volcano National Parc (10°31'-84°48'; 2 sites); Herradura, Talamanca (9°38'-83°33'; 1site) and Quepos, Manuel Antonio National Parc (9°25'-84°09'; 2 sites). Sites were chosen for the presence of accessible fully grown plants (at least 3 m tall) of *Piper aduncum*, generally along low traffic roads or trails, where it is common.

At each sampling site, 10 g (fresh weight) of different tissues of the plant were cut and reduced to small pieces with secateurs and preserved in leak proof 60 ml plastic (Nalgene, USA) bottles, with 40 ml 95% ethanol until processing. Three replicates were taken per sample from the same tree and averaged.

The different parts were freshly grown leaves, mature leaves, freshly grown spikes, mature spikes and wood taken from secondary branches (1 cm diameter). At each sampling site, soil samples were also collected for nutrient analysis. The soil was spread to air dry on a flat surface and then sealed in Plastic bottles for transportation.

Vouchers from each sampling site were pressed and brought back to the University of Ottawa Herbarium (Department of Biology, Vanier Building, room 415). Processing and analysis were completed at Department of Biology, University of Ottawa, Ottawa, Ontario.

VI.2.2 Processing

The plant tissues were homogenized with a Polytron (Switzerland, distributed by Brinkmann Instruments, USA) at speed 6 for 2 min. in the preservation solvent, 95% ethanol . The solution was then vacuum filtered with No. 4 Whatman filter paper in an Erlenmeyer and the filtrate was collected and refrigerated. The process was repeated twice with 40 ml ethanol 95 %, with 48 hours between each extraction and the three resulting ethanolic phases were combined. The volume was then rounded up to 150 ml with 95% Ethanol. Two ml samples were removed with a Hamilton syringe, filtered with Alltech filters (Illinois, USA, Nylon 66 Membranes, 13 mm, 0.2 micron) and bottled in 1.5 ml HPLC vials (Chromatographic Specialties). Samples were preserved in a refrigerator for a maximum of four days at 4°C until analysis. The dry weight of the samples was measured after extraction. The tissues were dried in an oven at 50°C for 48 hours and weighed on a Sartorius BP 210 (USA) scale.

VI.2.3 Analysis

Analysis by HPLC was done using the same method as described for dill tissue analysis **(Chapter IV)**. Concentrations of dillapiol, expressed in ppm, were obtained from the standard curve equation determined at 254 nm: Concentration of dillapiol in solution=9.27 (μg/ml/unit area) x peak area observed on chromatograms. Appropriate conversions were then done according to the volume of solvent and the dry weight of tissues. The final concentrations were expressed in μg dillapiol/ g dry weight.

VI.2.4 Recovery Tests

The method of extraction and analysis described above was tested for samples of leaf, fruits and wood. Since no fresh material was available, for each dry tissue, a known amount similar to the weight of two collected samples, was homogenized and separated in two different 60 ml Nalgene plastic bottles. Two final ethanolic extracts were evaporated down to 40 ml, mixed and homogenized. They were then separated into two 40 ml fractions and a known amount of dillapiol was added to one of them. The extraction procedure and chromatography analysis were done and the recovery, expressed by the percentage of dillapiol recovered compared with the dillapiol expected (known to be present) gave the following results: for the wood sample: 84.7%; for the fruit sample: 97.6%; for the leaf sample: 77%.

VI.2.5 Nutrient in Soil analysis

Soil analysis was done for the three main nutrients involved in growth, nitrogen, phosphorus and potassium. The pH was also measured.

Nitrogen analysis: A KCl extraction for nitrogen was performed. Ten g of dry soil were weighed in a flask, 50 ml of 2 N KCl solution were added and the mixture was then shaken for 60 minutes at 160 oscillations per minute. The solution was then filtered through Whatman #2 filter paper, collected in 50 ml scintillation vials and refrigerated until analysis, the next day [Bremner, 1965; Quinn et al., 1973]. The samples were analysed with a Technicon auto-analyser for nitrogen (ammonium, nitrite and nitrate) present in the soil, at Agriculture Canada, by Ms. Viviane Deslauriers, Eastern Cereal and Oilseed Research Center (Ottawa, Ontario, Canada). A standard colorimetric determination method was used, according to the Industrial methods of Technicon Industrial Systems (Tarrytown, New York, USA) for nitrite/nitrate and ammonia in water and wastewater referenced respectively as Industrial Methods No. 824-87T and No. 780-86T. Ammonium and nitrite/nitrate concentrations were given in ppm (µg/g of soil) and were added to obtain total nitrogen.

Phosphorus analysis: The samples were analysed for total phosphorus (P) at Agriculture Canada, again by Viviane Deslauriers. Samples of 1 grams of dry soil were digested in 250 ml calibrated tubes, using block digestor, model BD-20. The total phosphorus was measured using automatic colorimetric determination, and an Auto-Analyser II system. Phosphorus concentrations were given in ppm (µg/g of soil). The extraction of inorganic available phosphorus or total P has been shown to be related to the organic P fraction, which is itself a significant source of plant available P [Fixen and Crove; 1990].

Potassium analysis: Total potassium in soil was extracted with neutral normal NH_4 OAc (Ammonium acetate) [Knudsen et al., 1982; Subash Chandrabose; 1988]. This procedure is accurate and does not require, like for nitric acid digestion, special equipment such as a perchloric acid fumehood. Five g of soil were weighed and transferred to 50 ml beakers previously cleaned with deionized water. Twenty-five ml of neutral N NH_4 OAc were added and the solution was shaken with a mechanical reciprocating shaker for 5 minutes at 160 oscillations per minute. It was then filtered with No. 40 Whatman filter paper and the filtrate was collected in clean, dried capped Nalgene bottles and refrigerated until analysis.

The concentration of potassium was analysed by atomic emission, using a Thermo Gerrall Ash Inductively coupled argon plasma atomic emission sequential spectrometer (model Atomscan 25). The analysis was performed in the laboratory of Dr. J. Loop in the Ottawa Carleton Geoscience Center, Geochemistry Laboratory, University of Ottawa (Ottawa, Ontario, Canada). Potassium concentrations in the soil were given in ppm (µg/g of soil).

pH measurements: For each sample, 5 g of soil were weighed and transferred to a test tube. Five ml of distilled water [Van Lierop, 1990] were added and the solution was homogenized with a sonicator for 30 seconds. The pH was measured using a standard pH-meter (Fischer; USA).

VI.2.6 Statistical analysis

The concentrations of dillapiol in the different tissues were compared with a one way ANOVA, with tissue as the categorical variable and ln of dillapiol concentration as the dependent variable. ln transformations were necessary to meet the normality assumptions. Regressions were used to assess the effects of the different nutrients on the production of dillapiol. The software SYSTAT 5.0 for Windows was used for those analyses.

VI.3 RESULTS AND DISCUSSION

The concentrations of dillapiol found in the different tissues generally followed the predictions **(Figure VI.1)**. The best defended tissues were in order of dillapiol content in the dry tissue: new fruits (3.5% d.w.)≥ mature fruits (3% d.w.) > new leaves (1.5% d.w.)≥ mature leaves (1% d.w.) > stem (0.3% d.w.). As a comparison, dill was found to contain 0.1% f.w. **(Chapter IV)**. The one way ANOVA showed the dillapiol

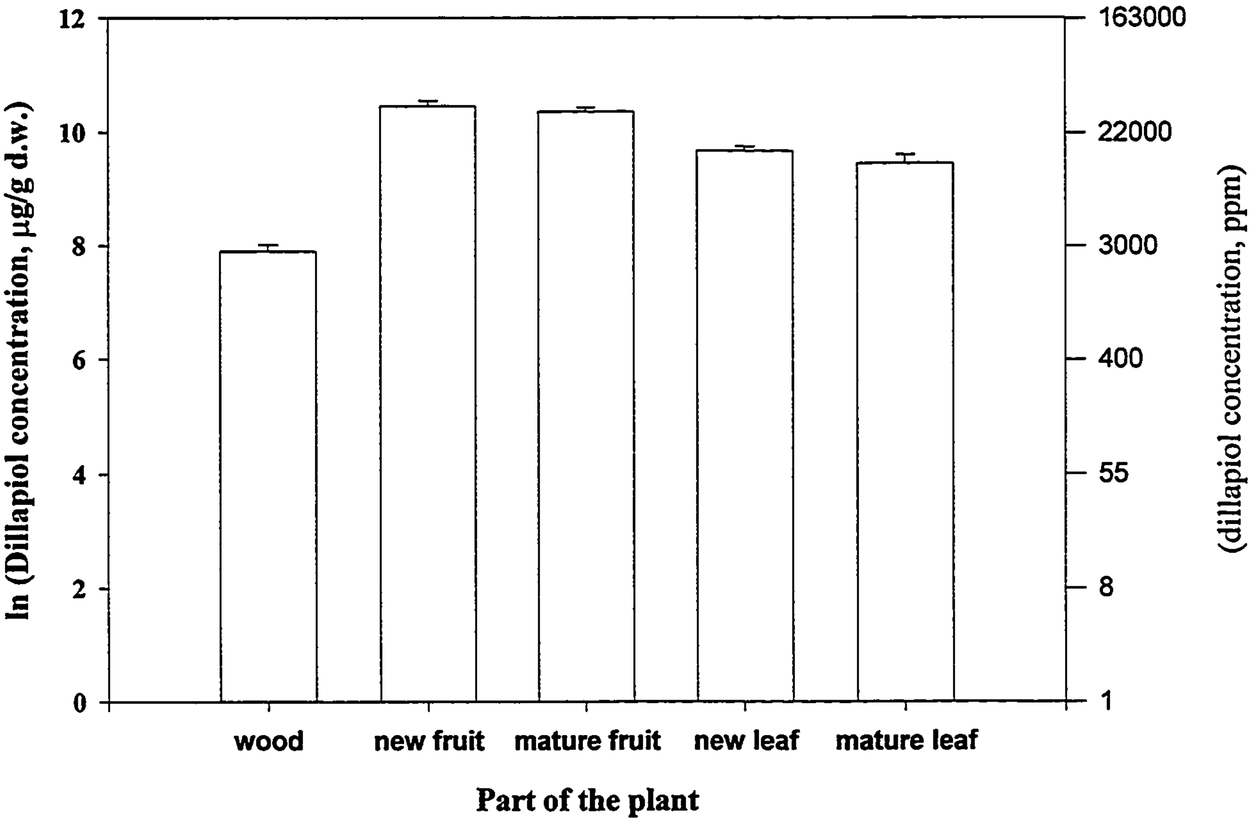


Fig. VI.1: ln of the concentration of dillapiol within the different tissues of *Piper aduncum.*

The one way ANOVA ln [Dillapiol] by plant part was found to be significant ($p < 0.001$, $F = 88.01$, $n = 45$).
Bars represent the standard error on the mean.
*Treatments with same letteres are not significantly different
(Tukey Test of multiple comparison, $\alpha = 0.05$).

concentration by plant part to vary significantly (p<0.001, F=88.012, n=45) and a Tukey Test of multiple comparison showed that the three different tissues have different concentrations of dillapiol, but that the age of the tissue does not matter: new and mature leaves as well as new and mature fruits do not have significantly different concentrations. The results for the concentrations in the different tissues corroborate the theory of Feeny (1976) since more apparent and more valuable tissues are better defended. Mc Key (1979) observed that the type of defense found in different tissues vary according to the lifespan of that tissue. In short-lived tissues like leaves low molecular weight compounds, recyclable and easy to translocate, are more profitable for the plant while in long lived tissues such as wood, higher molecular weight compounds were a better investment. Dillapiol is a low molecular weight compound and the results of this study support Mc Key's observations.

The results observed for young and mature leaves suggest that the quantity of dillapiol increases with increasing biomass, as was found in dill, *Anethum graveolens*, in early stages. In dill, measurements for young and old tissues were done at different life stages and represent an average for the whole plants, while in *Piper*, the measurements were done at once on tissues with different stages of maturity within a plant. Mc Key (1979) suggested that during senescence of a tissue, the plant would gain by recycling its defenses, either by translocating them toward other tissues or by converting them back to primary metabolites. This author added that nitrogen-based compounds would be more likely to be recycled than other types of compounds since they often involved a high biosynthesis cost. Studies have shown that phenolics like tannins are found in high amounts in senescing or shedded leaves [Towers, 1964; Sheen, 1969] and that they constitute an important source of humic acids in soils [Janzen, 1974]. Dillapiol, as a carbon-based constitutive defense, may remain in mature leaves. In mature fruits, it may confer specific cues to dispersal agents, as suggested by Janzen (1975).

Regarding the effect of nutrient concentrations and pH in the soil **(Figures VI.2 to VI.6)**, regression analysis showed a significant relationship of dillapiol concentration and pH in soil. **(Figures VI.5 and VI.6)**. For potassium, in the new leaves,

ln (dillapiol concentration)=-0.214 ln K + 10.38 **(Equation VI.1)**,

($p=0.017$, $r^2=0.58$, $n=9$) and in the old leaves,

ln (dillapiol concentration)=-0.46 ln K + 10.98 **(Equation VI.2)**,

($p=0.014$, $r^2=0.602$, $n=9$) and for pH, in the new leaves,

ln (dillapiol concentration)=-1.097 ln pH + 11.53 **(Equation VI.3)**,

($p=0.011$, $r^2=0.625$, $n=9$), in the old leaves,

ln (dillapiol concentration)=-2.62 ln pH + 13.90 **(Equations VI.4)**,

($p=0.002$; $r^2=0.754$, $n=9$).

Potassium concentrations were also found to be a covariate of pH,

ln (K conc.) =3.95 ln pH - 3.41 **(Equation VI.6)**

($p=0.009$, $r^2=0.650$, $n=9$) (data not shown). The nutrient concentrations are reported in **Table VI.1**.

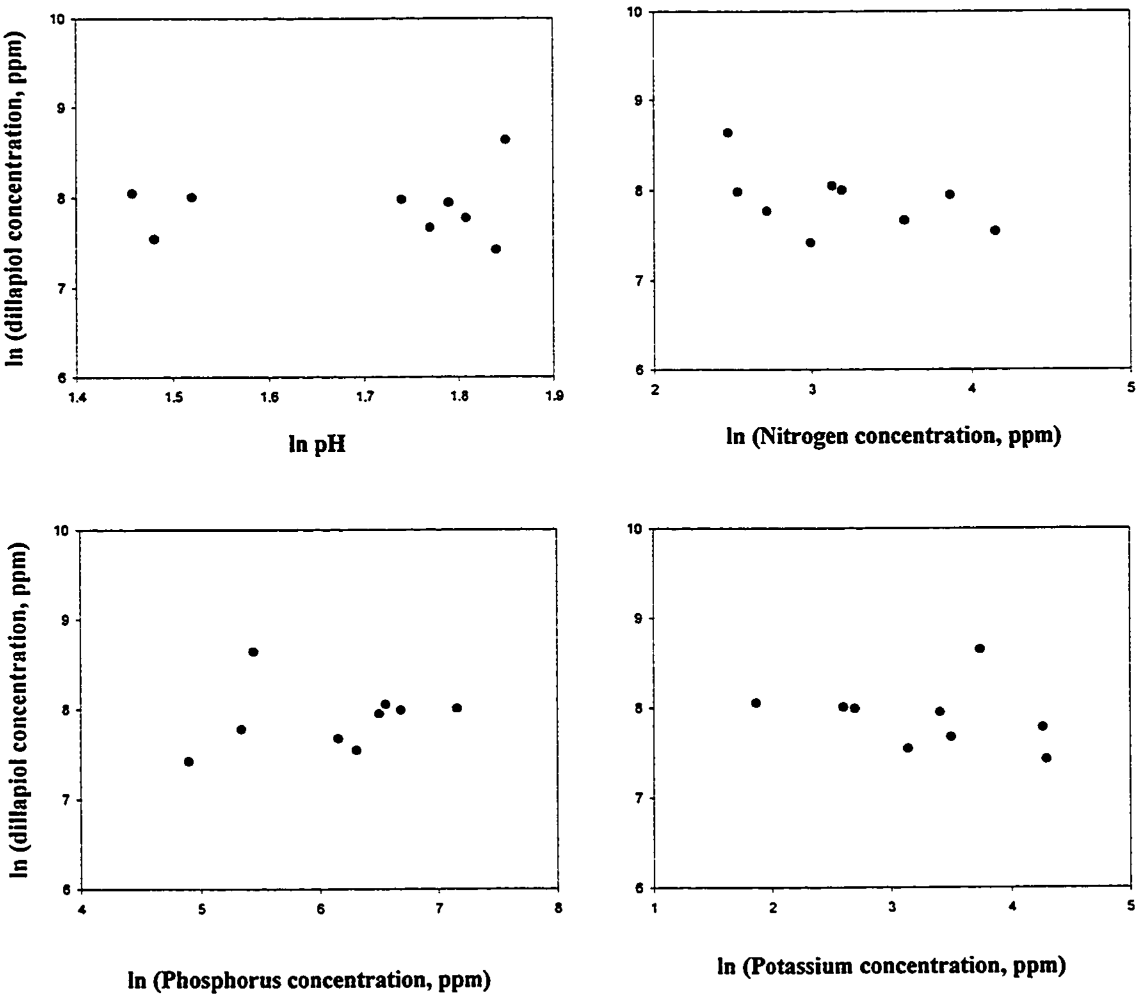


FIG. VI.2: Effect of different nutrients and pH on dillapiol concentration (ppm) in the wood of *Piper aduncum*.

No significant regressions were found. ($p > 0.05$; $n = 9$).

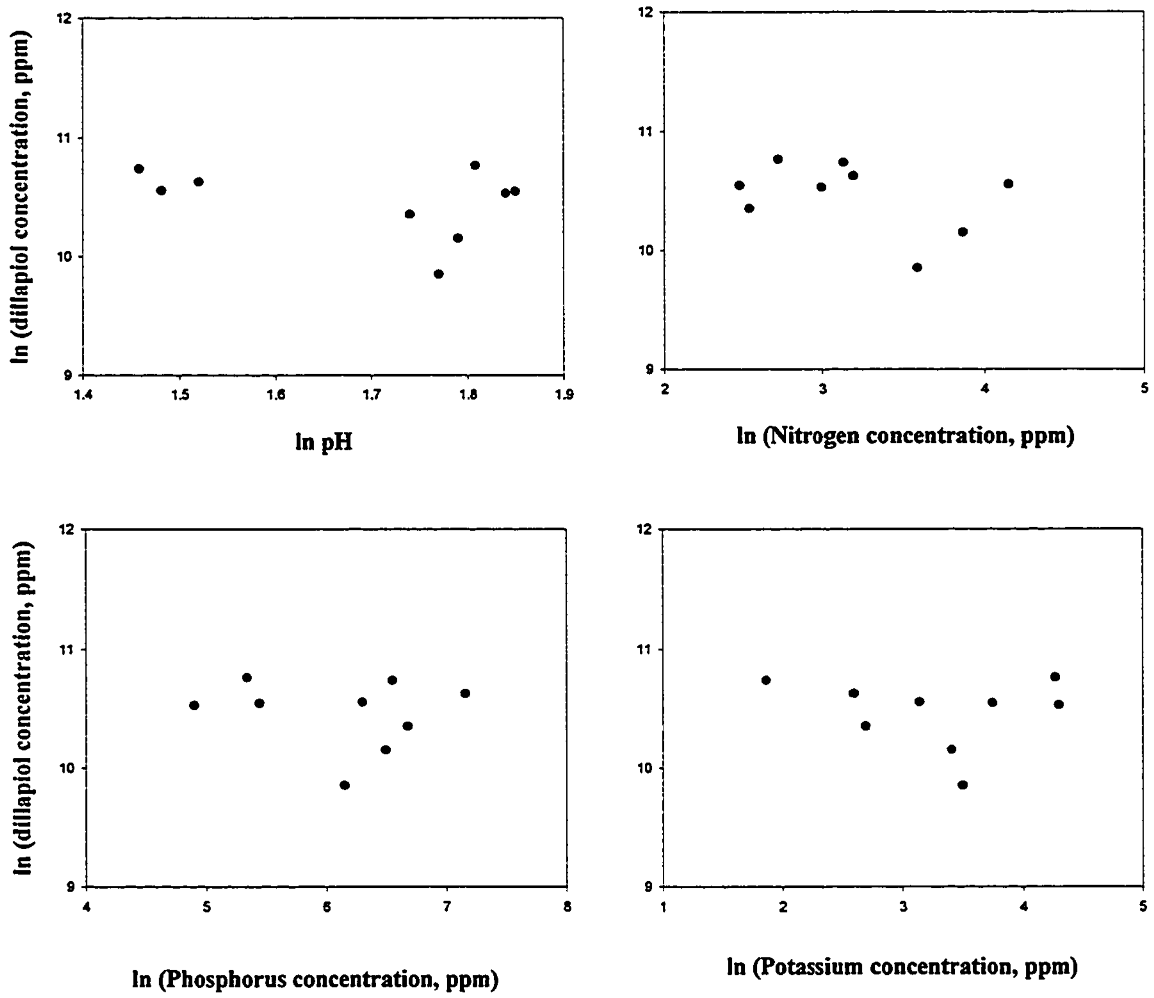


FIG. VI.3: Effect of different nutrients and pH on dillapiol concentration (ppm) in the new fruits of *Piper aduncum*.

No significant regressions were found. ($p > 0.05$; $n = 9$).

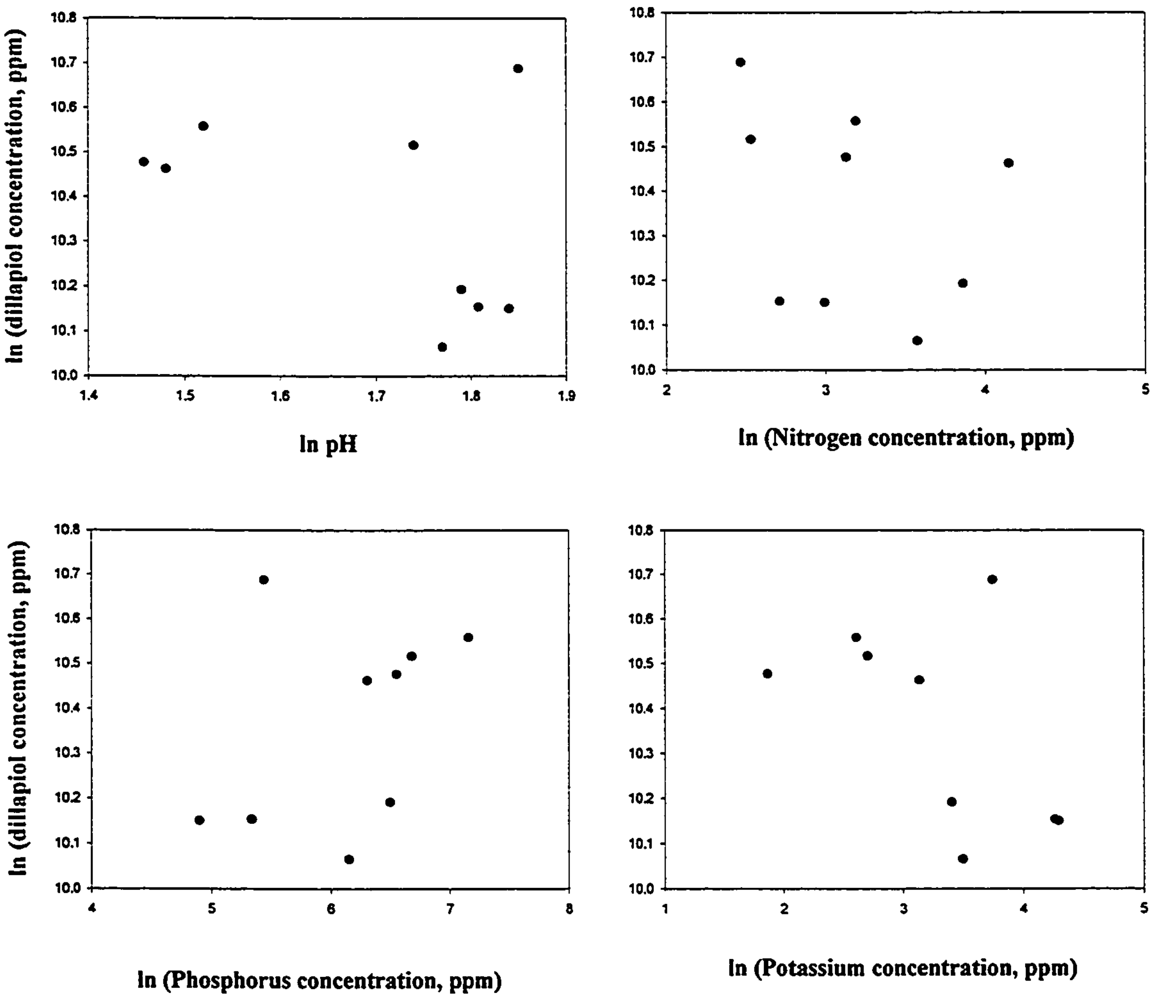


FIG. VI.4: Effect of different nutrients and pH on dillapiol concentration (ppm) in the mature fruits of *Piper aduncum*.

No significant regressions were found. ($p > 0.05$; $n = 9$).

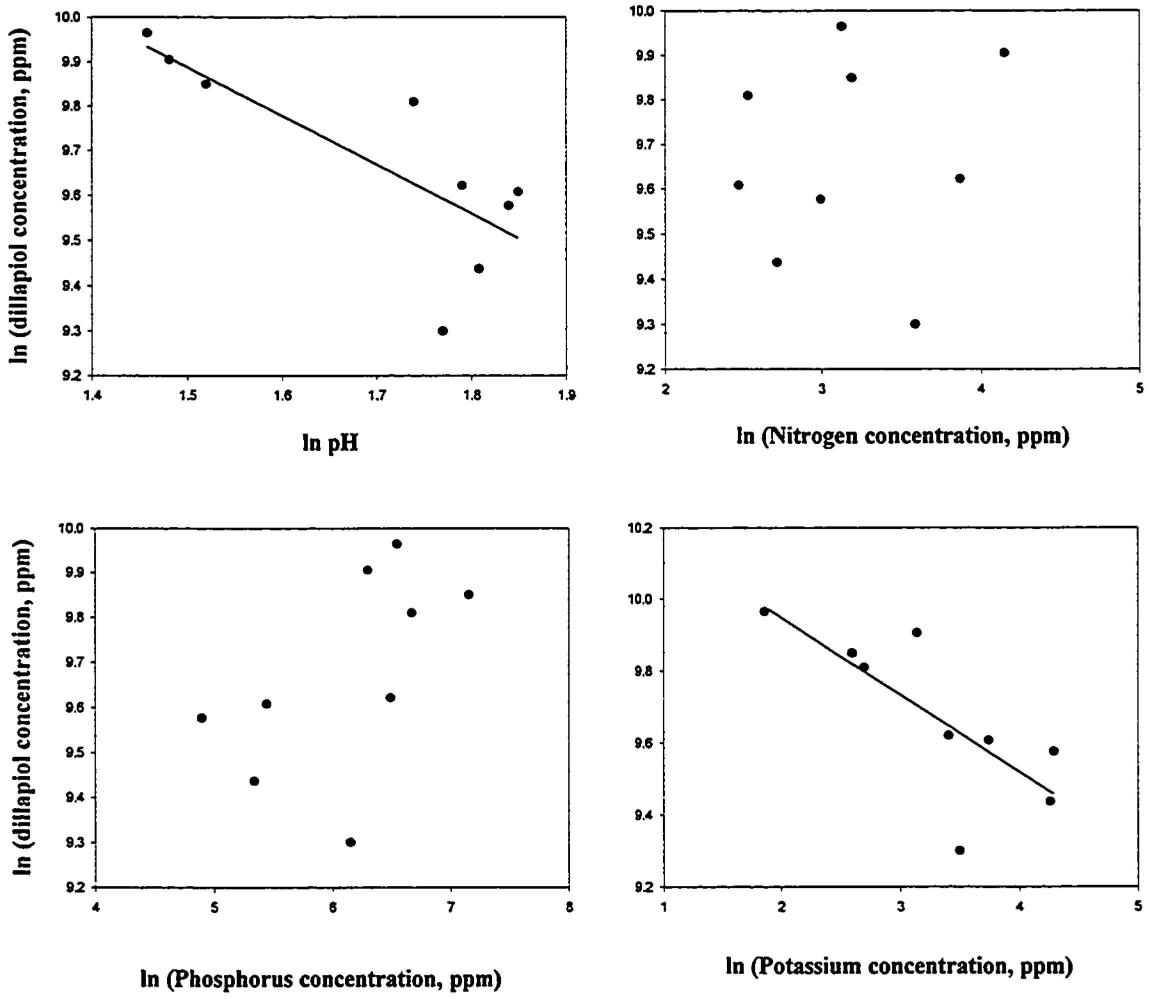


FIG. VI.5: Effect of different nutrients and pH on dillapiol concentration (ppm) in the new leaves of *Piper aduncum*.

A significant effect of pH and available potassium was found:

Equation 1: ln (dillapiol conc.) = -1.097 ln pH + 11.53 ($p = 0.011$, $r^2 = 0.625$, $n = 9$).

Equation 2: ln (dillapiol conc.) = -0.214 ln K + 10.38 ($p = 0.017$, $r^2 = 0.58$, $n = 9$).

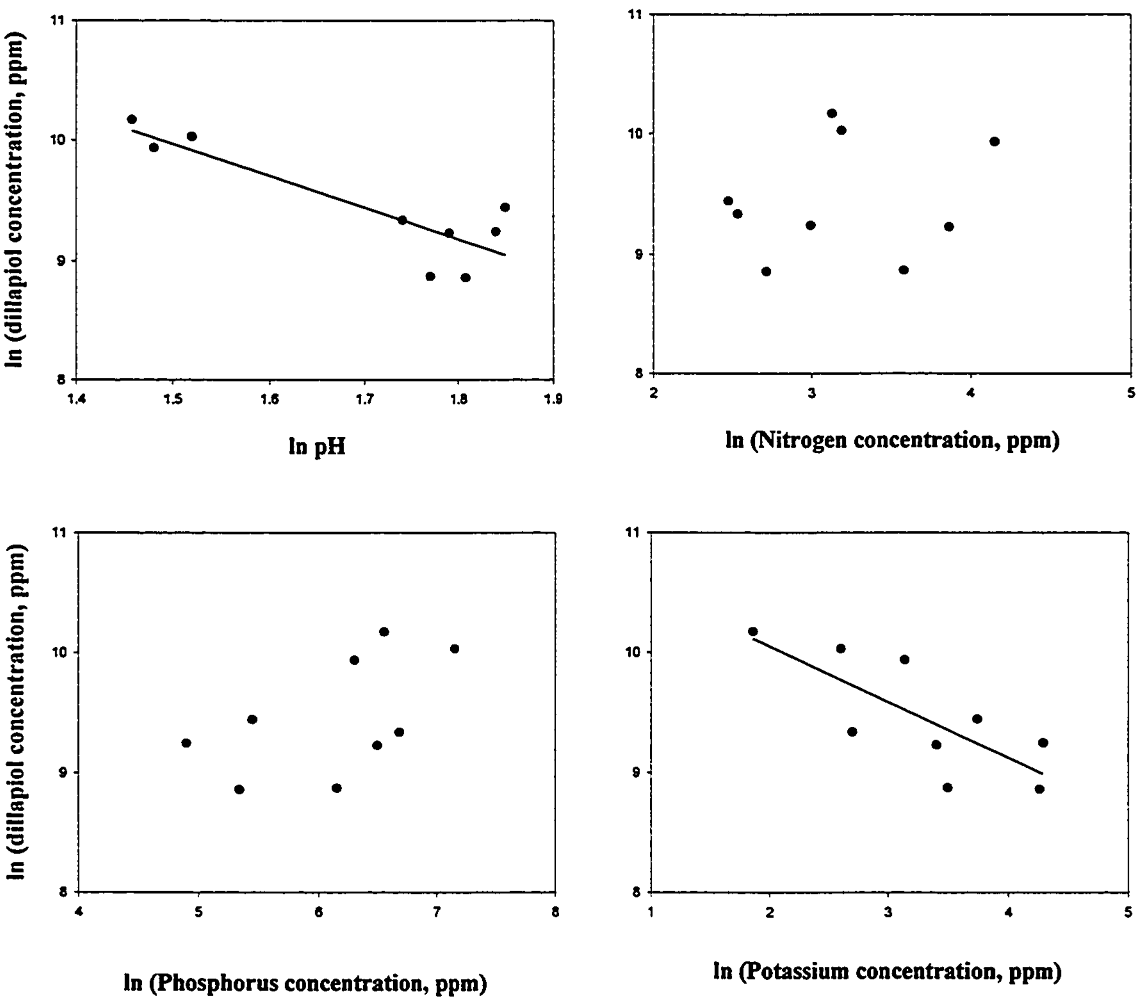


FIG. VI.6: Effect of different nutrients and pH on dillapiol concentration (ppm) in the old leaves of *Piper aduncum*.

A significant effect of pH and available potassium was found:

Equation 3: ln (dillapiol conc.) = -2.62 ln pH + 13.9 ($p = 0.014$, $r^2 = 0.60$, $n = 9$).

Equation 4: ln (dillapiol conc.) = -0.46 ln K + 10.98 ($p = 0.002$, $r^2 = 0.754$, $n = 9$).

Table V1.1: Nutrient concentrations and pH at the different sampling sites.

*Type of soil, description taken from Vasquez M.A. (1983):
A-1: well drained alluvial soils, rich in organic matter, dark, fertile (Udolls, Ustolls, Tropepts).
B-5: Soils developed over ancient eroded terraces, reddish, heavy textured, with low fertility (Humults, Udults).
D-1: Volcanic ash derived soils on montainous relief, dark, rich in organic matter, medium textured, moderately fertile, excessively drained (Andepts, Humods).
D-2: residual soils on steeply dissected relief, excessive external drainage, very low fertility, (Udults, Ustults, Tropepts).

SITE	Location in Costa Rica	Type of soil*	Total N (ppm)	Total P (ppm)	Available K (ppm)	pH
1	Horquetas, Sarapiqui	B-5	64	545	23	4.4
2	Horqueats, Sarapiqui	B-5	23	699	6	4.3
3	Horquetas, Sarapiqui	B-5	24	1280	14	4.6
4	Fortuna, Arenal Volcano	D-1	13	795	16	5.7
5	Fortuna, Arenal Volcano	D-1	48	661	30	6
6	Herradura, Cordillera of Talamanca	D-2	36	469	33	5.9
7	Quepos, Pacific Coast	A-1	12	232	42	6.4
8	Quepos, Pacific Coast	A-1	15	208	71	6.1
9	Puerto Viejo, Sarapiqui	B-5	20	134	73	6.3

It was predicted that for low nutrient concentrations, according to the Carbon/Nutrient Balance hypothesis, higher dillapiol concentrations would be observed and that the trend would have been especially clear in mature leaves (since leaf nutrients are redistributed, with priority given to flowers and seeds [Salisbury and Ross; 1992, p131]. No trend was found for nitrogen or phosphorus although this trend was found for the effect of potassium on the concentration of dillapiol in the leaf. In Costa Rica, the hot temperature and humidity level favor the activity of microorganisms and nitrogen is rarely found to be limiting. Phosphorus is important mainly in energy metabolism and production of reproductive structures and therefore may not be limiting in a context where fruits are not compulsory for propagation since vegetative reproduction is possible **(see Chapter VII)**. Potassium is important during expansion of green tissues and for maintaining cell osmotic pressure [Salisbury and Ross; 1992, p131]. Since *Piper aduncum* is a fast growing plant with an important green tissue biomass, potassium probably was the limiting nutrient in the context of this study. The findings therefore agree with Coley's (1985) resource availability hypothesis since the plants were better defended under low nutrient conditions. Those results disagree with the observations from the greenhouse study on the production of dillapiol in Indian dill **(Chapter IV)** which suggested that dillapiol concentrations in dill tissues were positively related to nitrogen availability during highest tissue expansion.

VI.4 CONCLUSION

It can be concluded from this study that dillapiol is produced in very high amounts in *Piper aduncum*, especially in the fruits, and that the concentrations observed do not vary according to the age of the tissue. Furthermore, the results of the study on the effect of nutrients on the production of dillapiol suggest a negative relationship with potassium availability in the soil.

VII: Note on the agronomy of *Piper aduncum*

VII.1 INTRODUCTION

Preliminary work was done on the agronomy of *Piper aduncum* in Costa Rica. The plant grows wildly and no information was available on the possibililties of propagating the plant whether by pollination or vegetative reproduction. It is known that both strategies are effective for some *Piper* spp. [Greig and Mauseth, 1991] and that others propagate mainly through one mode or the other. Greig (1993) has suggested that the fitness associated with both strategies depended on some ecological factors such as light. Shade tolerant species were found to reproduce preferably by vegetative means, including stem sprouting, root sprouting and fragmentation. Shade-intolerant species reproduced mainly by seeds. But Greig (1993) also observed that no seedling survived in experimental conditions, whether the species were shade-tolerant or shade-intolerant. According to those observations, it was thought that *Piper aduncum,* an early succession species which grows in partly shaded or open areas would naturally propagate by seedlings but that in an experimental context, vegetative reproduction should be possible and give a better rate of success. The was verified by using cuttings of *Piper aduncum.*

VII.2 METHOD

The experiments were conducted in the field station of Bioforesta (Horquetas, Sarapiqui, Costa Rica). Cuttings were removed from a local clone with a sharp machete. In a first experiment, 280 cuttings were planted directly in the soil, in an open field.

A second and more controlled experiment was performed. This time, 40 cm long cuttings were used, from stems of 0.5 cm (containing about 7 nodes), 1 cm (containing about 5 nodes) and over 1 cm in diameter (containing about 3 nodes). 20 cuttings were used for each diameter. They were planted in plastic containers containing local soil, placed in a shaded area and kept moist.

VII.3 RESULTS and DISCUSSION

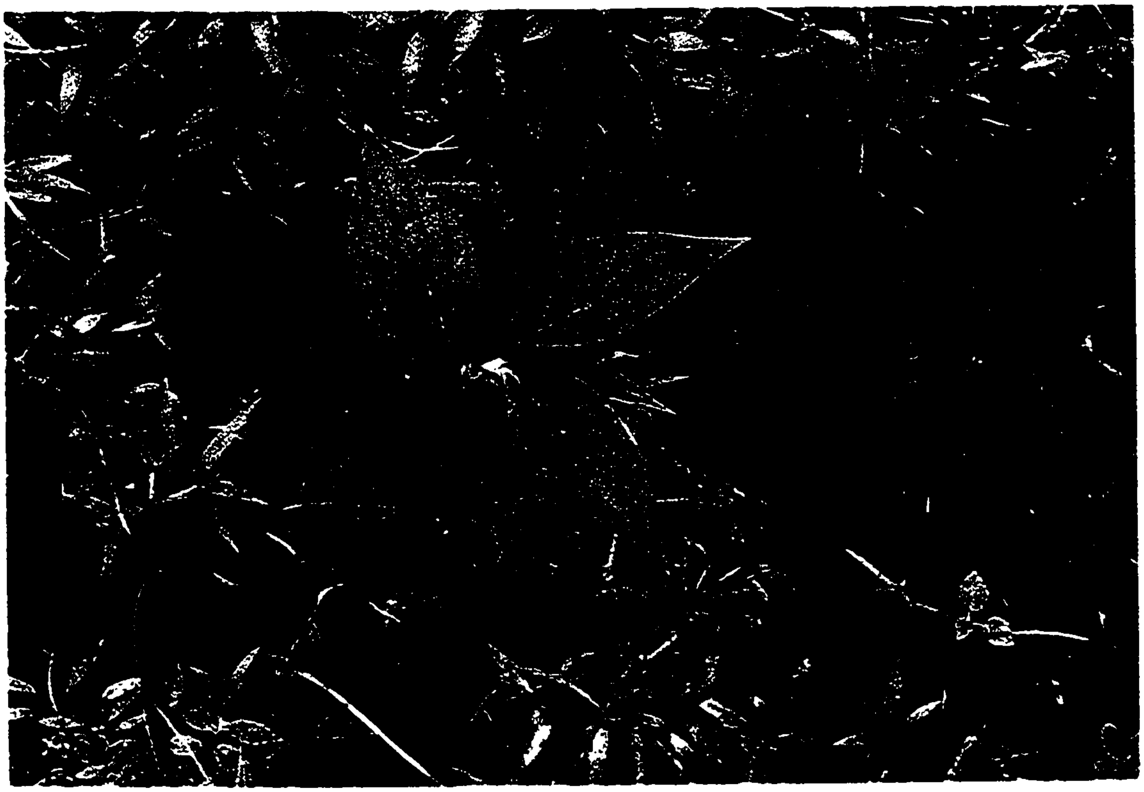
In the first experiment, after one month, 14/280 or 5% of the cuttings showed new leaf growth. No presence of roots was observed. The remaining cuttings had dried, probably due to the unstable climatic conditions (long periods of heavy rain followed by long periods of dry heat).

In the second experiment, after 14 days, 10% of the 0.5cm cuttings, 95% of the 1 cm cuttings and 12.5% of the cuttings over 1 cm showed new leaves. Roots were found to have grown on only one cutting, two months its removal from the plant. The experiment was repeated in the same conditions and similar results were observed.

VII.4 CONCLUSION

It was shown by those observations that *Piper aduncum* can be vegetatively propagated **(Figure VII.1)** and that, although it can be found in open areas, shade and high humidity are necessary initially for the cuttings to survive. The size of the cutting is

FIG. VII.1: *Piper aduncum*; mature leaves and fruits (above); newly grown leaves on a cutting (under)



also important in the success of growth. Further transplantation should probably be done when roots are developed.

VIII: General conclusion

Various conclusions can be drawn from the results presented in this thesis. From a toxicological point of view, it was found that several of the synthetic dillapiol derivatives have a comparable synergistic activity to the one of the naturally occurring lead compound. This provides avenues for further investigation and patenting. They could be used to synergize synthetic insecticides, for example as a replacement of piperonyl butoxide which is now under toxicological review. Furthermore, dillapiol was found to be one of the most active compounds in the series tested and this supports its potential as a botanical synergist, an alternative for organic growers. These synergists may also find application in medicine in formulations of drugs with costly active principles, such as the new generation of protease inhibitors used in HIV treatment.

From a production point of view, it was found that *Piper aduncum* produces very high amounts of dillapiol, especially in its fruits. That, combined with the fact that *P. aduncum* can be vegetatively propagated and is fast growing, makes the plant an ideal source for larger scale production. Since synthesis is very costly, it is a good opportunity for tropical countries such as Costa Rica to benefit from this finding. Since the plant is a wild species, very little is currently known about its agronomy and further investigations of propagation and cultivation techniques should be undertaken.

The production of dillapiol in Indian dill is much lower than in *P. aduncum* and therefore dill does not appear to be a good source for economic purposes. Yet it was a useful plant in the context of a chemical ecology study. It was found that dillapiol production in *Anethum graveolens* is not inducible with Methyl Jasmonate alone and that its variation within the plant is not directly related to the nitrogen input in the growth medium. It is a constitutive defense, programmed to be produced in a specific amount, independently of external factors such as pathogen attack or nutrient availability. The opposite trend was observed in *Piper aduncum* in which dillapiol is one of the main constituents, which suggests that the strategy of defense may vary from one plant to the other.

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