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Finally, thanks to Dr. Pearl Weinberger and Dr. Anna Picman for their suggestions during committee meetings.

ABSTRACT

Phenylheptatriyne (PHT), a polyacetylene present in the genera Bidens, Coreopsis and Dahlia of the Asteraceae (Compositae) is a potent photosensitizer of microorganisms. A quantitative evaluation of its antifungal activity toward the model pathogen Fusarium culmorum was undertaken. In the presence of near-UV radiation (300-400 nm), the effective concentration for 50% inhibition (EC_{50}) of mycelial growth was 2.5 ppm. In addition, PHT under near-UV behaved as a fungicidal agent which inhibited germination and germ tube elongation. Inhibition of fungal growth was also apparent in the dark at higher concentrations of PHT. Endogenous concentrations of PHT at different stages of growth of Bidens pilosa (Beggar's Ticks) were at least an order of magnitude greater than concentrations required for inhibition of growth of F. culmorum suggesting that PHT may inhibit the growth of unadapted fungi in the host plant. Physiological damage induced by PHT in F. culmorum involves membranes as the site of action, as indicated by experiments on uptake of radioactive phenylalanine, potassium leakage and respiration.

RESUME

Phenylheptatriyne (PHT), un polyacétylène présent chez les genres Bidens, Coréopsis et Dahlia de la famille des Astéracées, est un puissant photosensibilisateur de microorganismes. Nous avons entrepris une évaluation quantitative de son activité fongicide chez le pathogène modèle Fusarium culmorum. En présence du proche UV (300-400 nm), la concentration effective pour 50% d'inhibition (CE_{50}) du mycélium était de 2.5 ppm. De plus, le PHT en présence de proche UV s'est comporté comme un agent fongicide qui inhibe la germination et l'élongation du tube de germination. L'inhibition de la croissance du champignon a été aussi détectable en l'absence de proche UV pour de fortes concentrations. Les concentrations endogènes du PHT à différents stades de croissance de Bidens pilosa ont été d'au moins un ordre de magnitude supérieur aux concentrations nécessaires pour l'inhibition de la croissance de F. culmorum, suggérant que le PHT pourrait inhiber la croissance de champignons non adaptés à la plante-hôte. Les dommages physiologiques induits par le PHT chez F. culmorum impliquent la membrane cellulaire comme site d'action, comme l'indiquent les expériences sur l'influx de phénylalanine radioactive, l'efflux de potassium et sur la respiration.

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LIST OF ABBREVIATIONS

A	absorbance
AIB	aminoisobutyric
BLB	black light blue
b.p.	boiling point
¹⁴ C-Phe	¹⁴ C-phenylalanine
°C	celsius
E.M.	electron microscopy
EtOH	ethanol
H.P.L.C.	high pressure liquid chromatography
h	hour
HCN	hydrogen cyanide
K	potassium
Log P	log of the water/octanol partition coefficient
m.p.	melting point
M.S.	mass spectra
μ	micron
min	minute
ε	molar extinction coefficient
near-UV	near-ultraviolet radiation
PDA	potato dextrose agar
P.E.	petroleum ether
PHT	phenylheptatriyne
ppm	parts per million
TLC	thin layer chromatography
w	watt

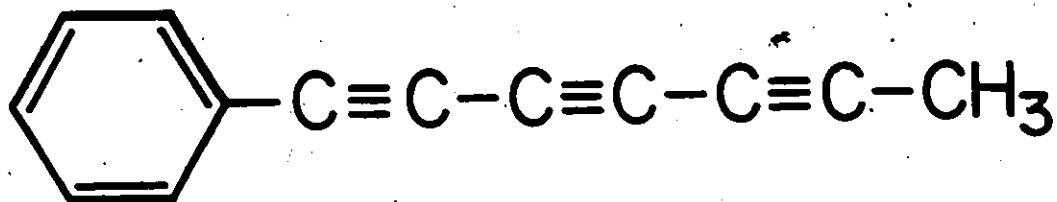
Chapter I

INTRODUCTION

Higher plants protect themselves from fungal attack by physical and chemical barriers to infection. Physical barriers inhibit the pathogens from gaining entrance and spreading through the plant. Preexisting physical barriers include the amount and quality of the waxy cuticle covering epidermal cells, the structure of epidermal cell walls, the size, location and shape of stomata and lenticels, and the presence on the plant of tissues made of thick-walled cells that hinder the advance of the pathogen (Agrios, 1978). Induced physical barriers include formation of cork layers, tyloses, papillae, and deposition of gums (Agrios, 1978). Other forms of physical barriers involve morphological changes in the plant cell wall leading to sheathing of penetrating hyphae or the hypersensitive (necrotic) defence reaction in which disintegration of the invaded cell's cytoplasm is believed to arrest the invading hyphae.

Another important aspect of resistance to fungal invasion is the production of allelochemicals generated by the "secondary metabolism" of higher plants. As described for physical barriers, chemical defence may be preformed or induced, resulting, in the case of induced substances, in the production of antifungal substances called phytoalexins.

Figure 1: The chemical structure of
7-phenylhepta-2,4,6-triyne (PHT)



The chemical structure of 7-phenylhepta-
2,4,6-triyne (PHT)

1.1 Preformed chemical defences.

Wood (1967) suggests four essential pieces of evidence before resistance can be attributed to a preexisting biochemical defence:

1. The substance must be present in those parts invaded by the pathogen.
2. In tissues invaded by the pathogen, the substance must be present in concentrations high enough to affect the pathogen seriously.
3. It must be demonstrated that the substance is present in plant tissues in a form available to the pathogen.
4. Where experimentally feasible, it should be demonstrated that an induced change in the concentration of an inhibitor in plant tissues results in a corresponding change in susceptibility to the pathogen.

There is a genuine lack of interest in preformed chemical defences which are regarded as "static" and "unspecific" as compared to phytoalexins (Schlösser, 1980). Wood's postulates also represent the only attempt to recognize the value of such chemicals as one important barrier offered by plants against fungal infection.

The main classes of preformed substances with specific examples (according to Schönbeck and Schlösser, 1976) are listed in Table 1 with chemical structures in Figure 2. Unsaturated lactones with cyclic or exocyclic double-bonding frequently occur in the plant kingdom, particularly in spe-

cies of Liliaceae, Ranunculaceae and Rosaceae.

Five-membered lactones seem to be more antifungal than six-membered lactones. Cyanogenic glycosides are present in more than 800 plant species representing about 70 families (Solemos, 1977). Antifungal sulfur compounds comprise thioethers (R-Sx-R') occurring as oils primarily in the genus Allium and isothiocyanates (R-N=C=S) occurring as glucosides primarily in Brassicaceae (Schönbeck and Schlösser, 1976). Antifungal phenolic compounds include simple phenols, coumarins, flavonoids and tannins. Saponins constitute a considerable fungicidal barrier and represent a very widely distributed class of plant secondary metabolites (Schlösser, 1976).

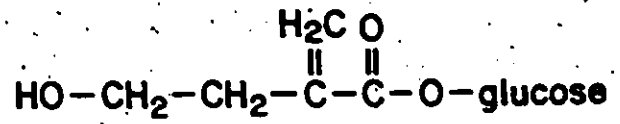
Table 1: The main classes of preformed antifungal substances

<u>Class of preformed antifungal substances</u>	<u>Example</u>	<u>Host Plant</u>	<u>Fungal pathogen</u>	<u>Reference</u>
Unsaturated lactone	Tuliposide A	Tulipa gesneriana	Botrytis tulipae	Schönbeck and Schroeder, 1972
Cyanogenic glycoside	Linamarin	Lotus corniculatus	Stemphylium loti	Millar and Hemphill, 1978
Isothiocyante	Sinigrin	Brassica nigra	Plasmodiophora brassicae	Parry et al. 1982
Phenolic compound	Catechol	Allium cepa	Colletotrichum circinans	Barazan et al. 1980
Saponin	Medicagenic acid	Avena sativa	Fusarium avenaceum	Luning et al. 1978

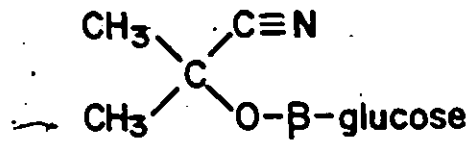
Table 1: The main class of preformed antifungal substances.

Figure 2: Chemical structures of preformed antifungal substances

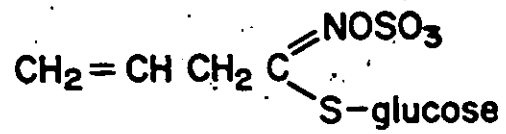
Tuliposide A



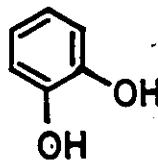
Linamarin



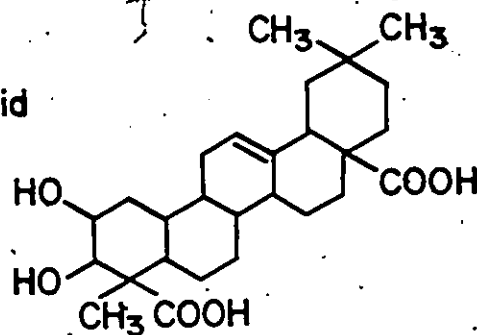
Sinigrin



Catechol



Medicagenic acid



A preëxisting biochemical defence may be stored in intracytoplasmic or extracytoplasmic compartments (Wiermann, 1981). Vacuoles and plastids represent intracytoplasmic storage of hydrophilic chemicals. The cell wall and waxy cuticle are sites of extracytoplasmic storage of lipophilic chemicals such as terpenoids, methylated flavonoids and sesquiterpene lactones. These lipophilic chemicals are sometimes referred to as preinfectional toxins, since they can exert their toxic action without further modification of the molecule. However, most preformed toxins are kept in a suitable bound form, frequently a glycoside, within a vacuole so that the toxin does not interfere with normal cellular processes. As soon as fungal penetration occurs, the toxin is liberated enzymatically from the bound non-toxic form and this acts on the fungal haustoria and prevents further colonization. Such a bound toxin is referred to as a post-infectional toxin (Ingham, 1973).

Investigations of the mechanism of action of preformed chemicals have focussed principally on cyanogenic glycosides and saponins. Cyanogenic glycosides can produce hydrogen cyanide (HCN), a known inhibitor of cytochrome oxidase and other metal-containing respiratory enzymes. There are no reports of fungal HCN detoxification. In insects and mammals, rhodanase (thiosulfate sulfur transferase) catalyses the conversion of HCN to thiocyanate

(Westley, 1973). The membrane-lytic action of saponins requires not only the presence of sterols in fungal cell membranes, but also of β -glycosidases, capable of converting saponins into the hydrophobic aglycones which are the active agent. Presence of β -glycosidases in fungal cell membranes (Segal and Schlösser, 1975) as well as the postulated aglycones has been confirmed. If either of the two prerequisites is lacking, saponins will not affect the fungal cell-membrane. Modes of saponin detoxification include lowering by the fungus of the pH at the infection site (Arneson and Durbin, 1967), release of inactivating enzymes and lack of membrane sterols for species of Pythium and Phytophthora (Schönbeck and Schlösser, 1976).

The role of preformed secondary metabolites in defence may be clarified through the use of mutants. For example, induced mutants of a number of fungi with lower membrane sterol content were less sensitive to the saponin tomatine (De Fago and Luescher, 1978). Recessive corn mutants with lower levels of preformed cyclic hydroxamates showed reduced resistance to corn pathogens (Day, 1974).

1.2 Phytoalexins

A second type of plant response to fungal penetration involves the rapid de novo synthesis of a fungitoxin, using simple starting material already available within the host tissue. These low molecular weight (usually between 200 and 500 daltons) toxins are called phytoalexins.

The basic postulates of the Phytoalexin Theory as originally proposed by Müller and Börger (1941) are:

1. A principle, designated as "phytoalexin", which inhibits the development of the fungus in hypersensitive tissue, is formed or activated only when the host cells come into contact with the parasite.
2. The defensive reaction occurs only in living cells.
3. This phytoalexin is non-specific in its toxicity towards fungi; however, fungal species may be differentially sensitive to it.
4. The basis of differentiation between a resistant and susceptible host is the speed of formation of the phytoalexin.

The first postulate of Müller and Börger implies that a phytoalexin response may be triggered only by fungi. Present workers consider that a phytoalexin response may be triggered by both abiotic (i.e. non-microbial) and biotic agents as long as the induced chemical has antifungal activity (Bailey and Mansfield, 1982).

There are about 100 known phytoalexins (Albersheim and Valent, 1978) distributed among at least 75 plant species representing 20 families (Yoshikawa, 1978). They include various classes of natural products (Figures 2 and 3, Table 2), with the notable exception of alkaloids. The majority of the phytoalexins so far known are isoflavonoids, produced mainly by members of the Fabaceae. The Solanaceae generally

produce diterpenes, the Convolvulaceae furanoterpenoids, the Orchidaceae dihydrophenanthrenes, the Apiaceae furanocoumarins, the Malvaceae naphthafurans and the Asteraceae polyacetylenes. Exceptions exist, such as the formation of the acetylenes wyerone acid in broad beans (Vicia faba) (Hargreaves et al., 1977) and falcarinol in tomato (Lycopersicon esculentum) (de Wit and Kodde, 1981).

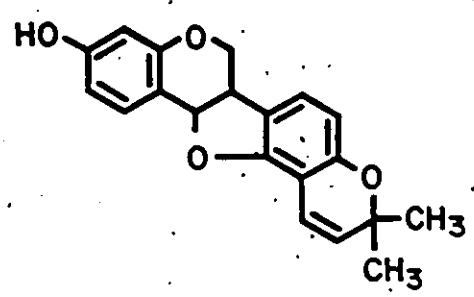
Table 2: Classes of phytoalexins

<u>Class of phytoalexin</u>	<u>Example</u>	<u>Plant source</u>	<u>Fungal pathogen</u>	<u>Reference</u>
Isoflavonoid	Phaseollin	Vicia faba	Fusarium solani	Perrin, 1964
Phenanthrene	Orchinol	Orchis militaris	Rhizoctonia repens	Skinnider, 1981
Furanoterpene	Ipomeamarone	Ipomoea batatas	Ceratocystis fimbriata	Ingham, 1972
Naphthafuran	Gossypol	Gossypium hirsutum	Verticillium albo-atrum	Stipanovic et al., 1975
Polyacetylene	Safynol	Carthamus tinctorius	Phytophthora drechsleri	Ichihara and Noda, 1977

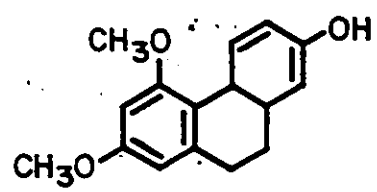
Table 2: Classes of phytoalexins.

Figure 3: Chemical structures of phytoalexins

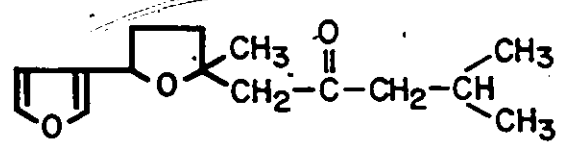
Phaseollin



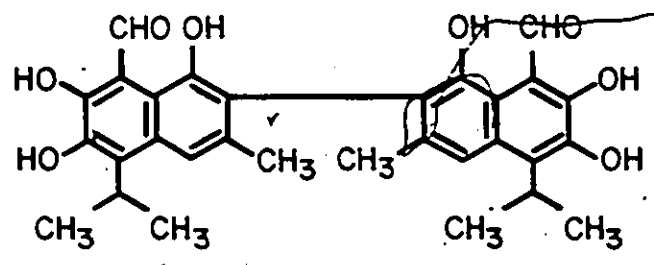
Orchinol



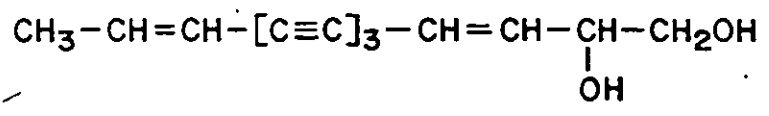
Ipomeamarone



Gossypol



Safynol



The mechanism by which phytoalexins stop the growth of fungal cells is not fully understood. Phytoalexins may alter the plasma membrane, inhibit oxidative phosphorylation (Oku et al., 1976) or cross-link DNA when exposed to ultraviolet light (Marciani et al., 1973).

Phytoalexins can be modified by fungi to less toxic or unstable compounds. The metabolic conversions include monooxygenation, reduction, hydration, oxidation and retroaldol cleavage (Bailey and Mansfield, 1982). The most common involves monooxygenases which catalyze the incorporation of one atom of molecular oxygen directly into a substrate. For example, phaseollin is oxidized to the more weakly fungitoxic hydroxyphaseollin by the fungus Fusarium solani, a pathogen of bean (Dewick, 1977). This fungus also releases extracellularly an acidic glycoprotein, now well characterized, which catalyses the conversion of the phytoalexin kievitone to the non-toxic kievitone hydrate (Ingham and Harborne, 1976). Other mechanisms of fungal response to phytoalexin which propose the release fungal phytotoxins or fungal inhibitors of host plant enzyme (Harborne and Ingham, 1978) are still speculative.

Elicitors are molecules responsible for the initial phytoalexin induction (Bailey and Mansfield, 1982). Biotic elicitors are of biological origin and the most thoroughly studied is the so-called Pms β -glucan elicitor obtained from Phytophthora megasperma var. sojae mycelial walls,

responsible for glyceollin accumulation in soybean (Albersheim and Valent 1978, Darvill and Albersheim 1984). Abiotic elicitors are of a physical or chemical nature. They include salts of heavy metals (mercury and copper), respiratory inhibitors and uncouplers (potassium cyanide and 2,4-dinitrophenol) and plant growth regulators (ethylene, 2,4-dichlorophenoxyacetic acid) (Yoshikawa, 1978). Constitutive (i.e. endogenous) elicitors have been obtained from soybean and tobacco cell-walls (Harborne and Ingham, 1978). Recently, sucrose has been recognized as a constitutive elicitor of the tropical legume Cajanus cajan (Cooksey et al., 1983). The accumulation of phytoalexin may be a response to stress and part of a general tissue repair mechanism (Kuc, 1976). It is now recognized that the traditional classification of elicitors as abiotic, biotic and constitutive is less physiologically meaningful. For example, abiotic elicitors may lead to the release of constitutive elicitors within the injured plant and thus operate through this mechanism.

1.3 Fungal pathogens.

Of the 100,000 species of fungi known, most are strictly saprophytic, living on dead organic matter which they help to decompose and only 8,000 species of fungi are recognized as causing diseases in plants (Schlösser, 1980). Not more than 1,500 species can claim attention as plant pathogens of

some importance. Examples are given in Table 3. Even when coincidence of location and time is provided and supplemented with optimum conditions for microbial development, most fungi cannot establish a parasitic relationship, or if they do so, it is only on plant tissues weakened by stress conditions (Schlösser, 1980). Generally, the physical and chemical barriers against fungal attack are efficient. A minority of fungi can colonize host tissues despite their high antifungal potential. They have adapted to these host plants and have acquired mechanisms to inactivate or detoxify antifungal compounds as described previously.

Table 3: Wide host range fungi

<u>Class of wide host range fungi</u>	<u>Genus</u>	<u>Infection site</u>	<u>References</u>
Myxomycete	Plasmodiophora -----	Root of cereals and grasses	Cook, 1982
Phycomycete	Pythium -----	Seed and root of turf grasses	Morton et al., 1982
Ascomycete	Botrytis -----	Tuber, root, stem and leaf of fruit trees	Sommer and Bearden, 1981
Deuteromycete fungi imperfecti	Fusarium -----	Root of cereals	El-Meleigi and Clafin, 1980
Basidiomycete	Puccinia -----	Stem of cereals	Hirata, 1980

Table 3: Wide host range fungi.
=====

1.4 Antifungal acetylenes

There are approximately 750 known acetylenes (Swain, 1977). They mostly occur in the Asteraceae (Compositae) and Apiaceae (Umbellifereae), but are also characteristic of the Araliaceae, Campanulaceae and Santalaceae. They are relatively non-polar hydrocarbons, methyl esters and acetates.

Biosynthetically, they are formed in the plant from oleic acid (Fig. 4 and 5). Tracer studies indicate that biosynthesis proceeds with the formation of triple bonds and chain-shortening reactions involving α - and β -oxidations from oleic acid via linoleic acid, crepenynic acid (9-octadecen-12-ynoic acid) and dehydrocrepenynic acid (9,14-octadecadien-12-ynoic acid) (Bohlmann et al., 1967 and 1969). While isolation of the enzyme systems for polyacetylene synthesis might give much information on the individual reactions, no such system has yet been isolated. The only experiment in vitro with a homogenate of a higher plant, Chysanthemum floculosum, showed that chloroplasts were probably necessary for triple bond formation, although labelled oleic acid was incorporated with low activity (Bohlmann and Schulz, 1968).

Figure 4: Metabolic pathways. Interrelationships between primary metabolism and synthesis of secondary plant substances. From Bailey and Mansfield, 1982.

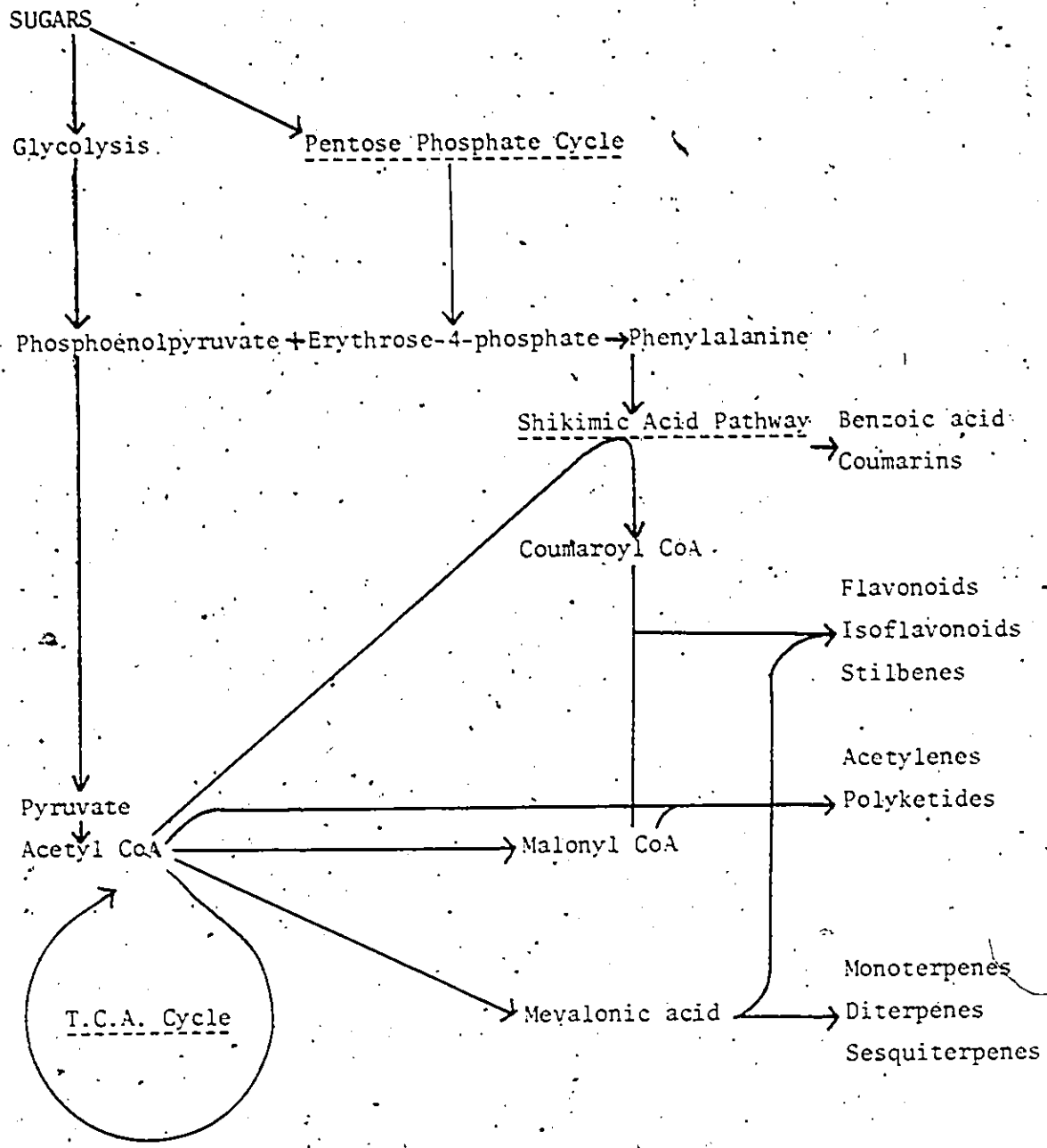
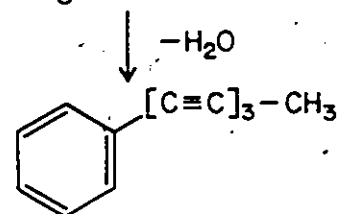
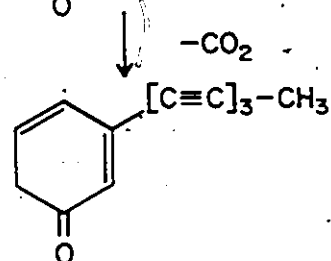
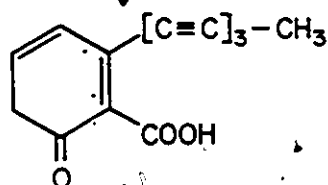
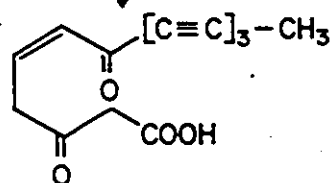
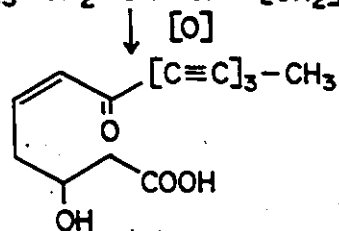
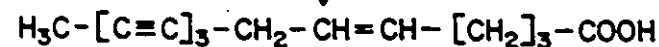
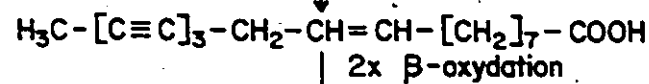
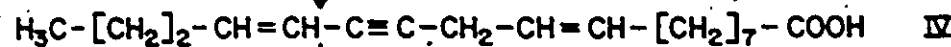
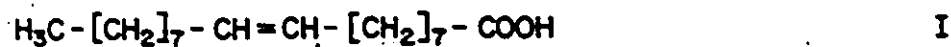


Figure 5: Biogenesis of PHT from oleic acid. I: Oleic acid, II: Linoleic acid, III: Crepenynic acid, IV: Dehydrocrepenynic acid, V: PHT. (Adapted from Bohlman et al., 1967 and 1969)



V

Polyacetylenes known to be antibiotic were not recognized first as polyacetylenes, then tested for activity, but rather the reverse. Anti-bacterial activity was observed in a fungal or plant extract, and when the active principle was isolated and characterized by its near-UV absorption spectrum, it proved to be a polyacetylene.

The polyacetylenes safynol (trans-trans -3,11-tridecadiene -5,7,9-triyne -1,2-diol) and dehydrosafynol (trans-11-tridecene-3,5,7,9-tetrayne-1,2-diol) (Allen and Thomas, 1971a and 1971b) accumulate in the first internodes of Carthamus tinctorius (Safflower) which have been wound inoculated with Phytophthora dreschleri. Additional antifungal polyacetylenes have also been found in other families. For example, wyerone, wyerone acid and wyerone epoxide (Rossal and Mansfield, 1984) are the major components of the response of tissues of Vicia faba (Fabaceae) to infection by Botrytis cinerea. Falcarindiol (cis-heptadeca-1,9-diene-4,6-diyne-3,8-diol) and falcarinol (cis-hepta-1,9-4,6-diyne-3-ol) accumulate in leaves and fruit of tomato, Lycopersicon esculentum (Solanaceae), after inoculation with Cladosporium fulvum (Kemp, 1978). None of these studies have considered the possibility that polyacetylenes may be light activated, as suggested in the present study.

1.5 PHT as an antifungal agent for *Fusarium culmorum*.

Recently PHT (Fig. 1), the polyacetylene investigated in the present study, was shown in tests with treated filter paper disks to be much more toxic to a variety of microorganisms in the presence of sunlight or near-UV radiation (300-400 nm) (Wat et al., 1979). These authors have suggested that PHT may act as a natural protective agent in vivo against plant pathogens. However, no information is at present available on the phototoxic effects of PHT on germination, growth and development of a phytopathogenic fungus.

Fusarium culmorum, an aggressive pathogen of cereal crops, does not infect *Bidens pilosa* in the field (Weiss, 1960) and cannot be induced to do so under laboratory conditions (cf. Section 3.2). Therefore it was chosen as a model unadapted pathogen for the present study since it would not be expected to contain detoxifying enzymes specifically for this substance.

PHT is a natural antimicrobial compound found in high concentration in the cuticle of leaves and stems of the weed *B. pilosa*, a member of the Asteraceae (Wa et al., 1979). *B. pilosa* is a persistent pan-tropical weed which is extremely abundant in agricultural and disturbed sites. Bohlmann et al., (1973), investigated the distribution of PHT in the roots or leaves of species belonging to the following genera of the Asteraceae: *Heliopsis*, *Coreopsis*,

Dahlia, Zoegea and Bidens. The three main Bidens species in Canada are B. cernua, B. frondosa and B. vulgata. PHT is absent in B. frondosa and B. vulgata was not investigated. PHT is present in the leaves and roots of B. cernua, an aggressive weed present from Prince Edward Island to British Columbia (Scoggin, 1978). The antimicrobial activity of PHT extracted from this species against gram-positive bacteria, dermatophytes, certain yeasts, yeast-like and mould fungi, was investigated in 1968 by Bondarenko (Bondarenko et al., 1968).

A systematic study of the effects of the polyacetylene PHT has been undertaken in some procaryotes and eucaryotes. Studies of photosensitization of Escherichia coli and Saccharomyces cerevisiae by PHT (Arnason et al., 1980 and McLachlan et al., 1984) suggest a dualistic mechanism of action in which PHT produces both toxic singlet oxygen and free radicals. This mode of action is clearly distinct from that of furanocoumarins, another group of photosensitizers forming DNA cross links in the presence of near-UV light (Wat et al., 1979). At 10 ppm, PHT is toxic to the freshwater alga Euclena (Arnason et al., 1981) and has antifeedant activity on the cutworm Euoxia messoria (McLachlan et al., 1982). PHT does not induce chromosome aberration in Syrian hamster cell (MacRae et al., 1980) which makes it a possible candidate for use as a pesticide or topical antibiotic.

Many studies of secondary compounds have been limited to a description of chemical structures and effective concentration for 50% inhibition of growth (EC_{50}) in a single test of biological activity. Little quantitative data on the variation of their concentrations in plants, on their mode of action, synergistic or antagonistic effects and detoxification are available (Swain, 1977).

This thesis is an examination of the antifungal activity and mechanism of action of the secondary metabolite phenylheptatriyne (PHT, Fig. 5) from the pan-tropical weed and persistent agricultural pest Bidens pilosa (Beggar's Ticks). This polyacetylene has not yet been classified as either a pre-formed substance or a phytoalexin and the present study focuses on the inhibitory effects of this natural product on the growth and development of a model pathogen Fusarium culmorum and its mode of action.

In the first part of this study, a quantitative evaluation of the antifungal activity of PHT in a liquid bioassay was made. This assay is perceived as having more credibility than the classical agar bioassay (Bailey et al., 1976). In addition, it was decided to investigate if PHT behaves as a fungistatic or fungicidal agent as well as an inhibitor of germination and germ tube elongation of F. culmorum. The growth inhibiting effects of PHT are related to concentrations of this secondary metabolite at different stages of growth of B. pilosa. Because no mode of

action study is available on any of the antifungal acetylenes, a second objective of this work is to characterize the physiological events leading to toxicity. This study began with a careful microscopic examination of cell damage during treatment. Because previous work has suggested a membrane site of action of PHT in other organisms (Wat et al., 1979), three membrane related events were monitored: potassium leakage, transport of radioactive phenylalanine and respiration.

Chapter II

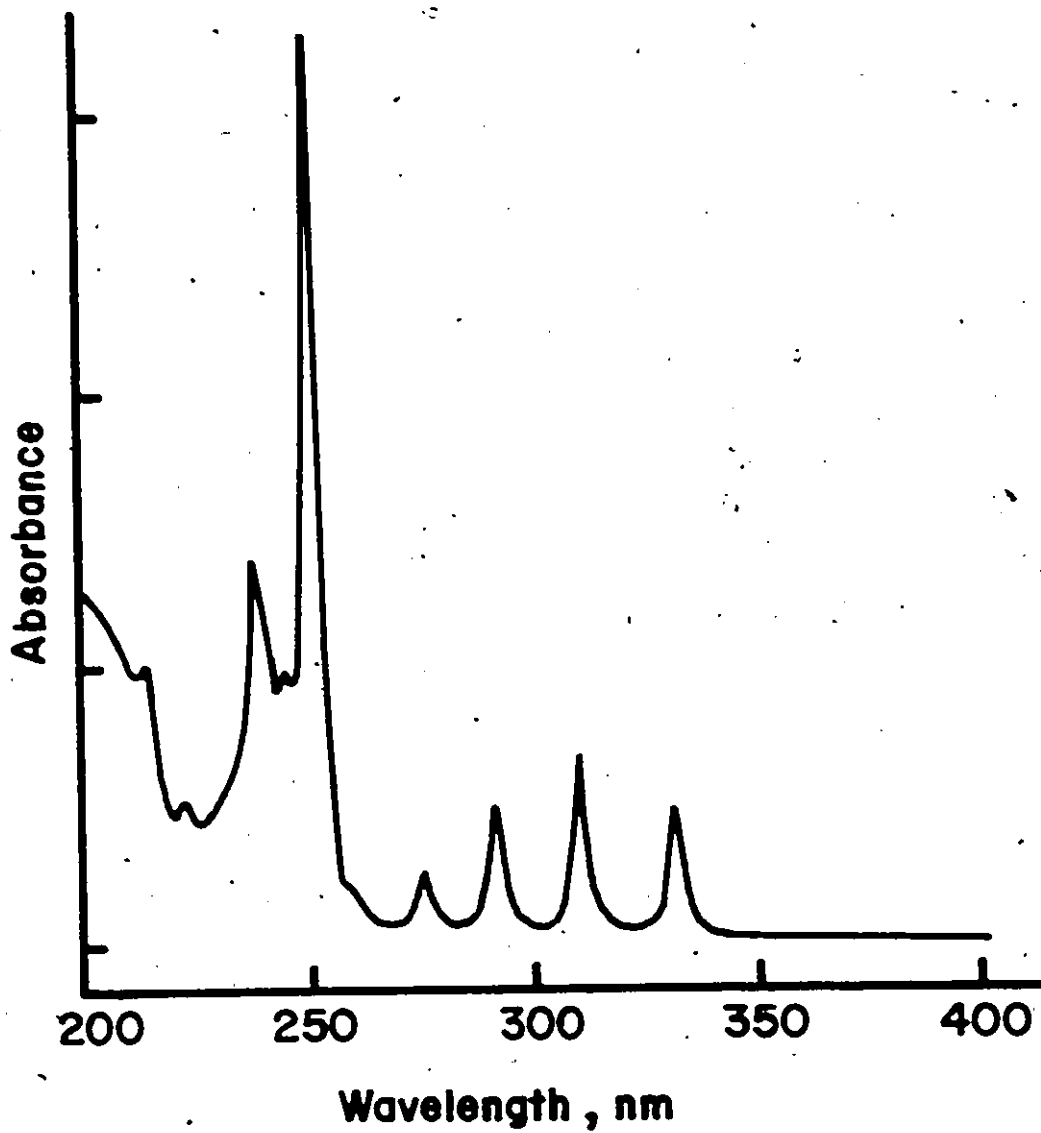
MATERIALS AND METHODS

2.1 Isolation of PHT

PHT was extracted from Bidens pilosa leaves according to a modified method of Wat et al., (1979). B. pilosa seeds were collected by Dr. J.T. Arnason in Miami, Florida. Plants were grown from seeds in greenhouses at the University of Ottawa and the Chemistry and Biology Research Institute, Agriculture Canada, Ottawa. This species was used since it is known to possess a high concentration of PHT in the leaf, up to 600 µg/g fresh weight at maturity (Towers and Wat, 1978). Leaves were harvested at maturity and immediately placed in 95% ethanol (EtOH). Leaves were ground in a Waring blender with 95% EtOH to a slurry. This mixture was filtered using a Buchner funnel with Whatman no.1 filter paper under vacuum to remove particulate matter. The leaf residue was discarded. The resulting filtrate was partition-extracted three times with petroleum ether (P.E., boiling point: 30-60°C). This was performed in a large separatory funnel after the addition of an equal volume of H₂O (1 EtOH filtrate:1 H₂O:2 P.E.). The three fractions were then combined and dried with anhydrous sodium sulfite for 24 h.

The P.E. extract was evaporated to dryness under vacuum on a rotary flash evaporator fitted with a continuous feed. The residues sticking to the flask were redissolved in a small amount of P.E. This concentrated extract was then applied to the top of a 2 x 50 cm P.E. saturated silica gel column (Baker: 40-140 mesh) and eluted with glass distilled P.E. The eluant (5 ml/min) was collected and monitored for PHT using a Pye Unicam SP8-100 double-beam spectrophotometer (Fig. 6).

Figure 6: UV-absorption spectrum of PHT. ^{max}
(petroleum ether) ($\log \epsilon$) 238, 250, 259,
274, 291, 310, 330 (4.9, 5.2, 4.0, 4.2,
4.4, 4.5, 4.3).

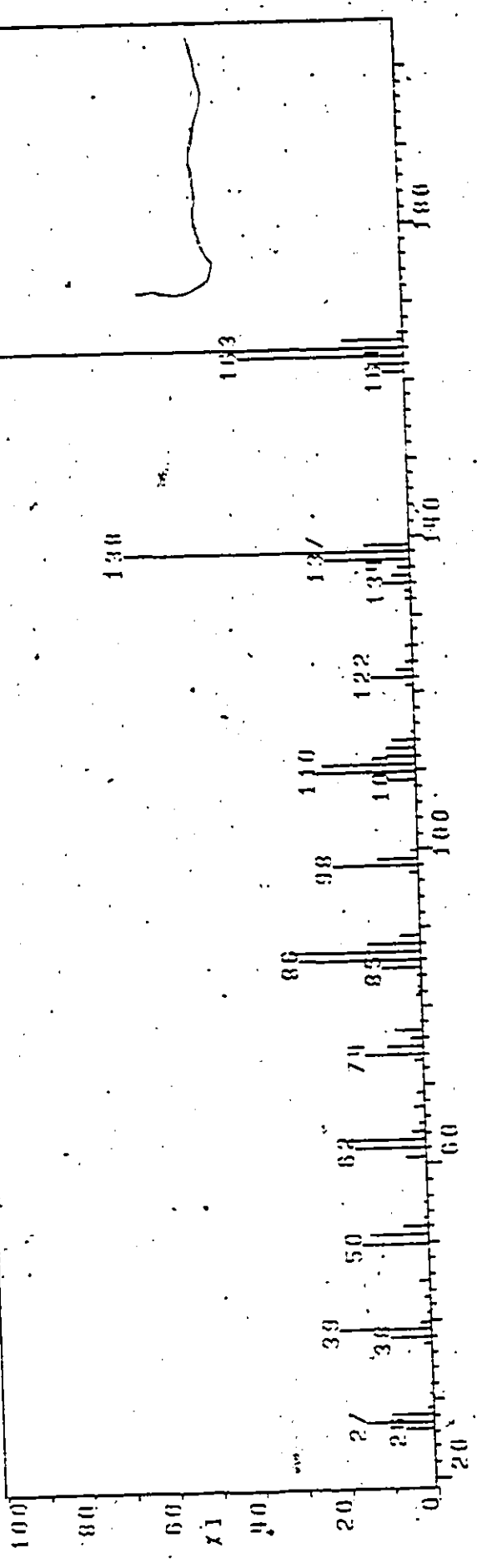


The column fraction eluting ahead of the first carotenoid band contained PHT. It was reduced in volume on the rotary flash evaporator. Crystals were obtained by reducing the PHT fraction further using a nitrogen airstream to volatilize the P.E. and subsequently lowering the temperature of the remaining fraction to -25°C . The long white needle-like crystals were recovered and then recrystallized in a small volume of glass distilled 95% EtOH.

Colorless prisms recrystallisation give: mp. $55-56^{\circ}\text{C}$., U.V. spectrum: max (P.E.) (log extinction coefficient) 238, 250.2, 259, 274, 291, 310, 331.5, (4.9, 5.2, 4.0, 4.2, 4.4, 4.5, 4.3). Mass spectra (M.S.) for ions with a relative abundance greater than 10%: m/e 164.0 (100.00%), m/e 163.0 (47.29%), m/e 138.0 (46.51%), m/e 165.0 (14.31%), m/e 86.0 (11.57%), m/e 87.0 (11.00%), m/e 137.0 (10.78%) (Fig. 6). The M.S. was performed by J.R. Kraus (Department of Chemistry, University of Ottawa) on a VG 7070-E mass spectrophotometer.

Figure 7: Mass spectrum of PHT

E/420 0 14-NOV-83 COL:COL STD:G.D. 80 SCOR: 95 J: 8
0-11-83. PHT -- 93/0



2.2 Growth of the fungus

F. culmorum (W.G. Smith) Sacc. isolate CT440 obtained from R. Tinline, Agriculture Canada, Saskatoon, was initially isolated from diseased wheat plants. Cultures of F. culmorum were grown in potato dextrose agar (PDA) at 25°C, 10 cm above 2 daylight fluorescent lamps (350-700 nm range: 50 w·m⁻²) with continuous illumination. Potato dextrose agar was prepared by suspending 39 g PDA powder (Canlab, F5DMND) per liter distilled H₂O. Heat with stirring was required to completely dissolve the PDA powder. The preparation was sterilized by autoclaving at 121 °C for 15 min. In the laminar flow, 20 ml liquid PDA were poured into each 15X100 mm Petri dishes and allowed to solidify at 45°C. Every 7 days, 3 PDA plates were inoculated with an 8 mm diameter agar disc from the previous plate covered with mycelia. Inoculation was performed under sterile conditions in a laminar flow.

Macroconidia from 3 PDA plates were obtained by surface agitation of the cultures with sterile glass beads in sterile distilled water (3 ml water, 5 beads/ml) and the suspension was collected. The spore suspension was centrifuged for 10 min at 1,500 x g, the supernatant discarded and the pellet resuspended in sterile water and adjusted to 10¹¹ macroconidia/ml. Macroconidia density was estimated on a Klett densitometer previously standardized from haemocytometer counts.

2.3 Effects of PHT on fungal growth and development.

From the concentrated suspension, an inoculum of 100 μ l of 10^{11} spores/ml was prepared and dispensed in 10 ml sterile Fries medium (Madhosingh and Orr, 1978) in sterile 25 ml beakers. The final concentration was 10^7 macroconidia/ml Fries medium.

The spore cultures were treated in triplicate with the following concentrations of PHT in Fries medium: 1.8, 4.5, 9.1 and 18.2 ppm. The PHT was dissolved in 10 μ l EtOH and an EtOH control was included. Each beaker was covered with plastic film (Saran Wrap) during incubation under appropriate photosensitizing radiation.

The light source consisted of a bank of four horizontal lamps. The two central lamps were 20 w Black Light Blue (Westinghouse F20T12 BLB, wavelength range: 300-400 nm) and the two flanking 20 w solar simulating Vita lamps (Duro Test, wavelength range: 300-700 nm). A 16 h light, 8 h darkness schedule was used. Intensity in the near-UV inside the beakers was estimated at $5 \text{ w}\cdot\text{m}^{-2}$ with a Yellow Spring Instrument radiometer and near-UV cutoff filter (Kodak Wratten Filter CB2P). Two 30 x 30 cm filters were placed over the beakers when elimination of the photosensitizing wavelengths less than 400 nm was required.

The beaker cultures were incubated in a water bath, with shaking at 150 revolutions per minute (r.p.m.). The temperature was controlled at 30°C inside the beakers.

Microscopic examination of samples from the liquid culture assay was undertaken after 16 h incubation. An aliquot (100 μ l) of the liquid culture was removed from the incubation medium and examined by phase contrast microscopy. Each data point in the Fig. 8 represents the mean of germination (%) of three groups of 100 macroconidia. A spore was considered germinated if the germ tube was one-half the width of the spore. This assay was based on primary macroconidia; microconidia and chlamydoconidia were not considered in this study. Length of the germ tube at 16 h was estimated on a haemocytometer in similar experiments. After 48 h, the mycelium was filtered and dried at 110°C for 24 h to determine dry weight.

For the fungicidal test, macroconidia prepared in an identical fashion were given the minimum treatment for complete arrest of germination (9.1 ppm PHT and 16 h near-UV). These were then 3X centrifuged and transferred to fresh medium without PHT. Germination and germ tube extension were monitored during the next 20 h (without near-UV) and compared to untreated spores (no PHT, no near-UV) freshly prepared at the time of washing.

Other methods of assaying growth effects of PHT were considered but not used because of special problems associated with them. For example, one antifungal assay for secondary metabolites present in minimal quantity is to spray a spore solution on a thin layer chromatography (TLC)

plate of a plant extract. The active molecule corresponding to an area of inhibition can be characterized by conventional chemical methods. This assay is unsuitable for polyacetylenes which generally are prone to oxidation on silica gel which has a very high surface area. Another method is to incorporate in agar the antifungal agent and measure the diameter of growth of a mycelial plug.

2.4 Endogenous concentrations of PHT in *Bidens pilosa*.

PHT levels in the *B. pilosa* plant grown in sterilized soil were monitored at 2 week intervals during growth under controlled conditions of humidity: 60%, temperature: 25°C, light regime: 16 h light, 8 h dark under solar simulating Vita lights (intensity 200 w·m⁻²). Weighed samples (1 g fresh weight) of roots, leaves and stems, pooled from several plants were homogenized in a Polytron (Brinkman, Model PT-10-20-3500) in 10 ml EtOH, extracted 3X into 20 ml P.E. (30-60°C), filtered and chromatographed on a 5 x 50 mm column of silica gel (BAKER: 40-140 mesh) eluted with P.E. The PHT was collected before the first carotenoid band and its concentration determined spectrophotometrically at 310 nanometers (log ϵ = 4.5) (Fig. 6). Recovery of PHT by this method was estimated to be better than 85%.

2.5 Photomicrography

Photomicrography of stained *F. culmorum* cells was performed to observe any cytological disruption after PHT and near-UV treatment. The stain Sudan Black B, a lipophilic stain, was chosen because the high partition coefficient of PHT suggests its absorption in cell membranes.

Macroconidia were prepared and incubated under the same condition as for the liquid bioassay (Section 2.3). PHT was added at the time of inoculation (concentration: 10 ppm). Near-UV irradiation periods of 3, 6, 12, 16 and 24 h were initiated immediately after incubation, then held in the dark for the balance of treatment period. The 24 h samples were stained for 15 min with Sudan Black B in 70% EtOH. They were then washed three times with 5 ml 50% EtOH to remove unbound stain (10 min centrifugation at 1,000 x g). Aliquots of this stained and washed preparation were dispensed on a microscope slide or haemocytometer and observed under phase contrast microscopy. Counts of macroconidia were made as either normal, granulated or lysed. Three sets of 100 macroconidia each were examined, from which means of triplicates were determined.

2.6 Respiration

Macroconidia were prepared in Fries medium as described in Section 2.3. PHT was added at the beginning of the

incubation period to give the following concentrations in Fries medium: 0, 5, 10, 15, 20 ppm. The samples were incubated for 16 h in Fries medium in the presence of near-UV. The oxygen uptake of mycelia was measured at 25°C with a Clark oxygen electrode (Model 53, Yellow Spring Instruments) and results recorded on a flat-bed chart recorder (Canlab, R2970-3). The respiration rate was related to the weight of mycelia dried to constant weight as described in Section 2.3. The sample chamber was surrounded by a water jacket thermostated at 25 ±0.1°C. The electrode was calibrated assuming that air saturated water contains 0.26 moles oxygen per ml at 25°C (Weast, 1970).

2.7 Potassium leakage

The study of leakage of potassium ions from F. culmorum cells was performed according to a modified method of Wat et al., 1980. An inoculum of 200 μ l of 10^{11} macroconidia/ml was dispensed in 20 ml sterile Fries medium (Madhosingh and Orr, 1978) and incubated for 48 h. After incubation, the mycelium was centrifuged at 1,000 x g for 10 min. The Fries medium supernatant was discarded and the pellet resuspended in 20 ml deionized H₂O. Deionized H₂O was prepared by filtering distilled H₂O through a demineralizer cartridge (Barnstead Combination Demineralizer, 10 megahoms/cm purity). In order to remove any traces of potassium from the initial 20 ml Fries medium preparation,

three washes were performed by centrifugation at 1,000 x g for 10 min, and replacing the supernatant each time with 20 ml deionized H₂O. 1 ml of this final preparation was dispensed into each of twelve flasks. These 25 ml Erlenmeyer flasks were each washed before use with deionized H₂O to remove any traces of potassium. Then 9 ml deionized H₂O was added in each flask to obtain a total volume of 10 ml. Six samples were treated with PHT (10 ppm) and 6 without PHT but an equal volume of EtOH (10 µl) was added. The samples were irradiated for 0, 20, 40, 60, 80 and 100 min intervals under near-UV conditions, then held in the dark for the balance of the treatment period. After 100 min, the mycelia of each sample were filtered. The filtrate volume was determined and the concentration was determined in a flame photometer (Carl Zeiss PMQ2). Potassium emission was calculated at 768 nm and related to a standard curve. Because of the difficulty of handling more than 12 samples at once, there were no replicates in each experiment, but the experiment was repeated in total three times.

2.8 ¹⁴C-Phe transport

The uptake study was originally attempted with ¹⁴C-aminoisobutyric acid (AIB), an analogue of alanine, which presents the advantage of being transported inside cells without being metabolized (Hamaide, 1984). The percentage uptake of ¹⁴C-AIB by F. culmorum cells was low

(~500 cpm, which was approximately the background level of the scintillation counter used), indicating that this amino acid analogue was not undergoing significant transport in this species. ^{14}C -Phe was then tried, since it is known to be actively transported inside F. culmorum macroconidia (Madhosingh and Orr, 1981).

The transport study of L-(U- ^{14}C) phenylalanine (specific activity: 494.5 mCi/mol, New England Nuclear) into the cells was performed according to a modified method of Wat et al., 1979. An inoculum of 200 μl of 10^{11} spores/ml was dispensed in 20 ml sterile Fries medium. This spore culture was incubated for 48 h in the dark at 30°C. After 48 h, the culture was centrifuged at 1,000 x g for 10 min. The supernatant was discarded and the pellet (mycelium) was resuspended in 15 ml M63 transport medium (13.6 g/l KH_2PO_4 , 2 g/l $(\text{NH}_4)_2\text{SO}_4$, 2 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.0005 g/l $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 10 g/l sucrose, 28.1 mg/l cycloheximide. The pH of the transport medium was previously adjusted to 7.0 with concentrated KOH.

In order to remove any traces of Fries medium, three washes were performed by centrifuging at 1,000 x g, discarding the M63 medium and resuspending with 15 ml fresh M63 medium each time. PHT in 10 μl EtOH was added to obtain a concentration of 10 ppm. Aliquots (1 ml) of this PHT-treated preparation were dispensed into twelve 5 ml test tubes and irradiated with near-UV ($5 \text{ w}\cdot\text{m}^{-2}$) for 20, 40, 60,

80, 100 min then held in the dark for the balance of the treatment period. After 100 min, all samples were removed and 2 μ l of the 14 C-Phe added to each 1 ml sample.

Incubation time with 14 C-Phe was 40 min. The mycelia were filtered and the filters (Amicon microporous, 2 mm diameter, pore size .45 μ) were placed in 10 ml scintillation fluid (Scintiverse 2, Fisher). Counting was performed on a Beckman LS-150 (Liquid Scintillation System) for a period of 10 min/sample.

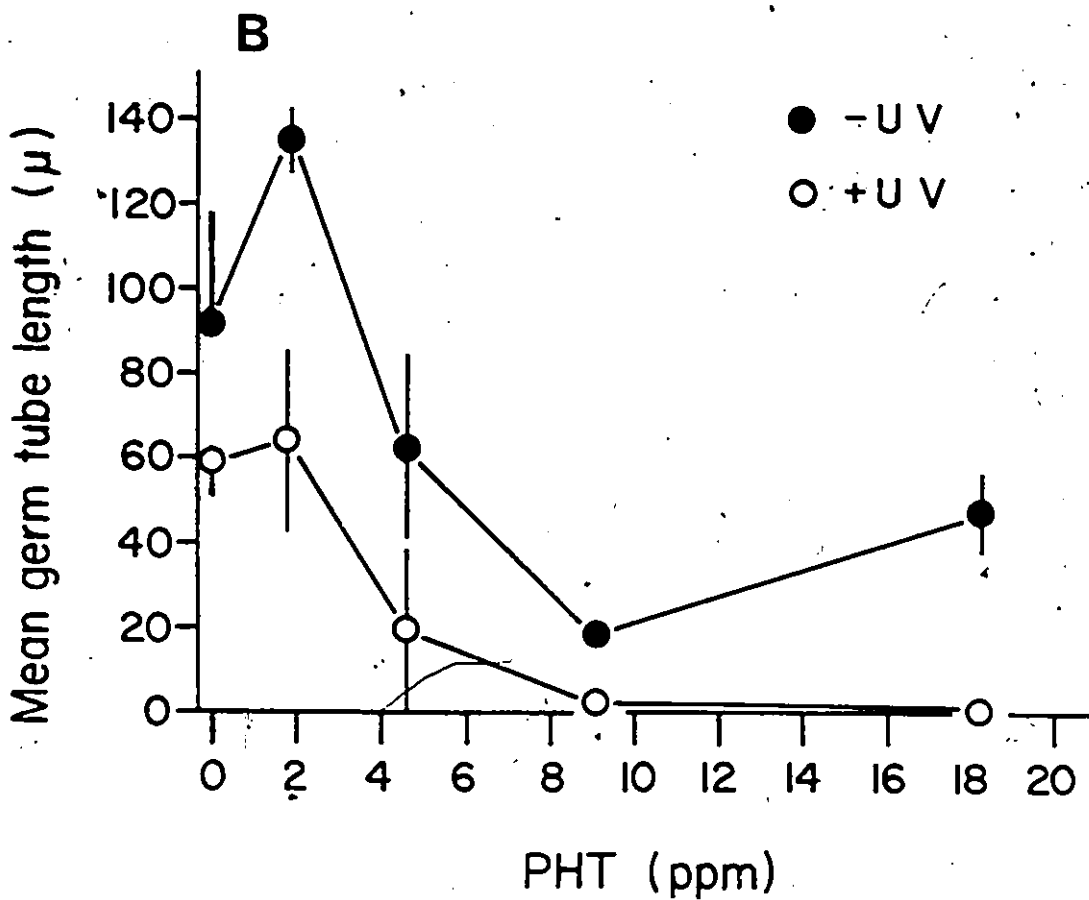
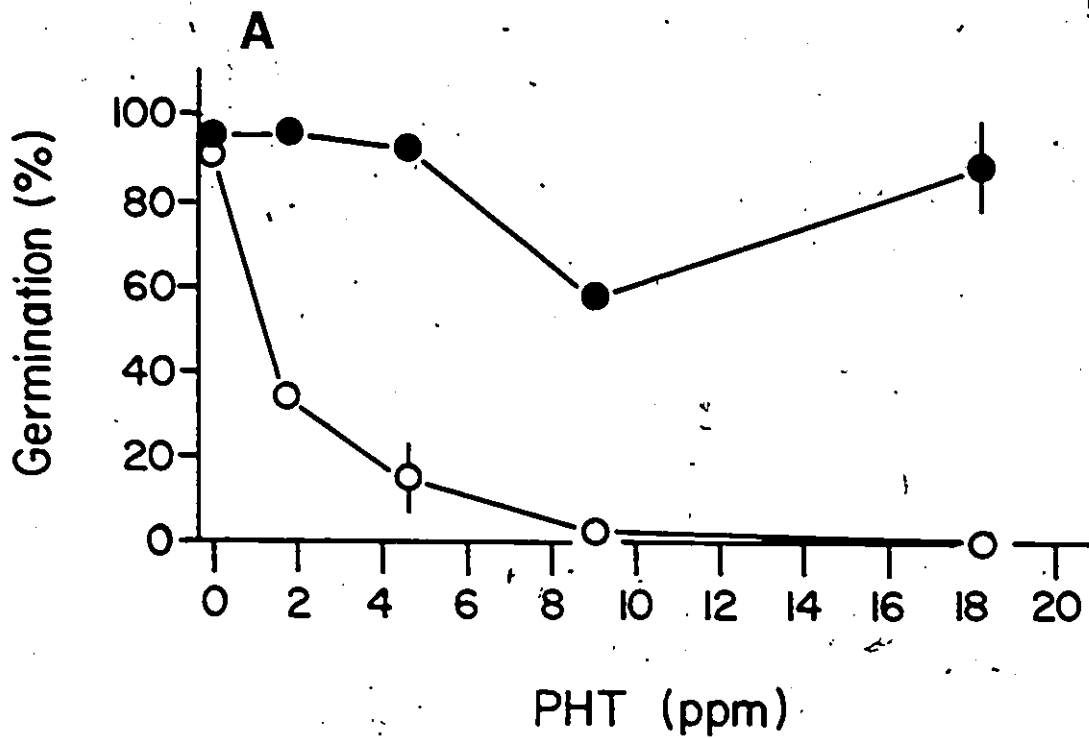
Chapter III

RESULTS

3.1 Photosensitizing effects

The photosensitizing effects of PHT on germination of F. culmorum are clearly evident (Figure 8A). Germination declined rapidly with increasing concentration of PHT ($EC_{50} = 5.6$ ppm) in the presence of near-UV radiation. The PHT and near-UV treated samples were significantly ($P = .05$) different from the controls at concentrations of 2 ppm and greater. Statistical analysis was performed by Duncan's Multiple Range Test and is presented in Appendix A. There was little effect on germination without near-UV except at 9 ppm. Near-UV treatments alone without PHT had no significant effect on germination of conidia.

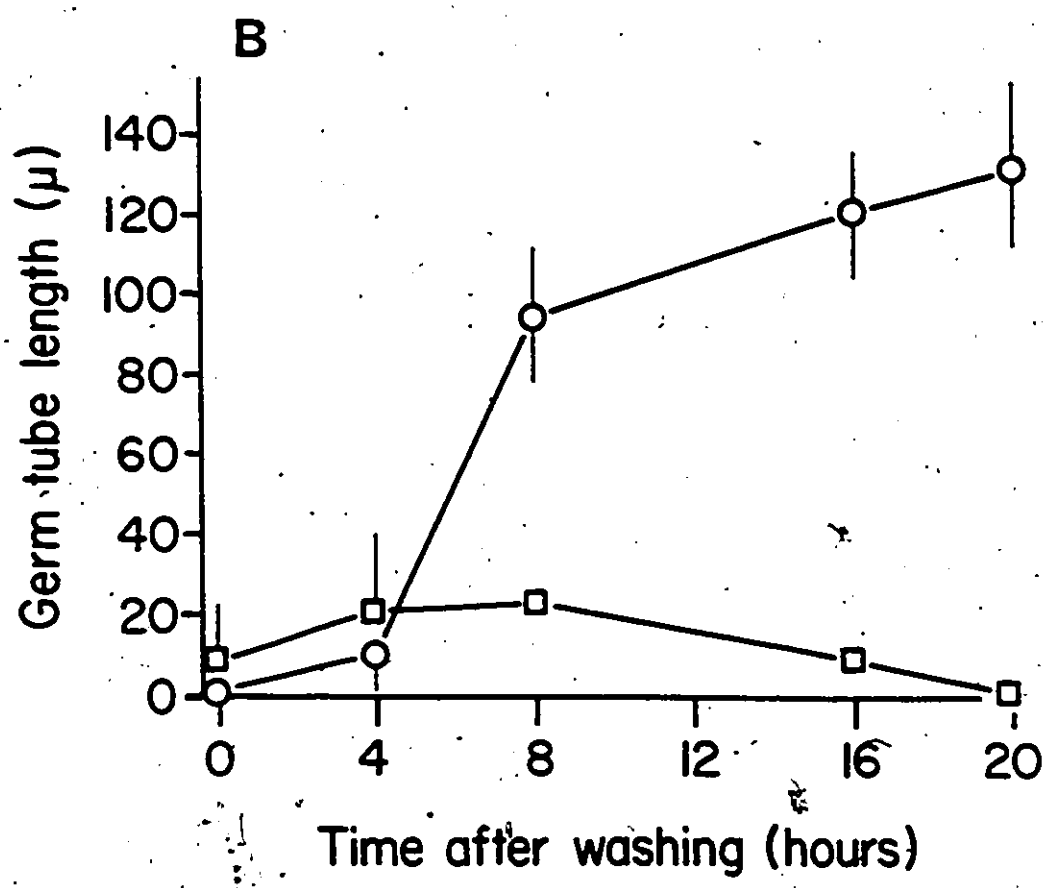
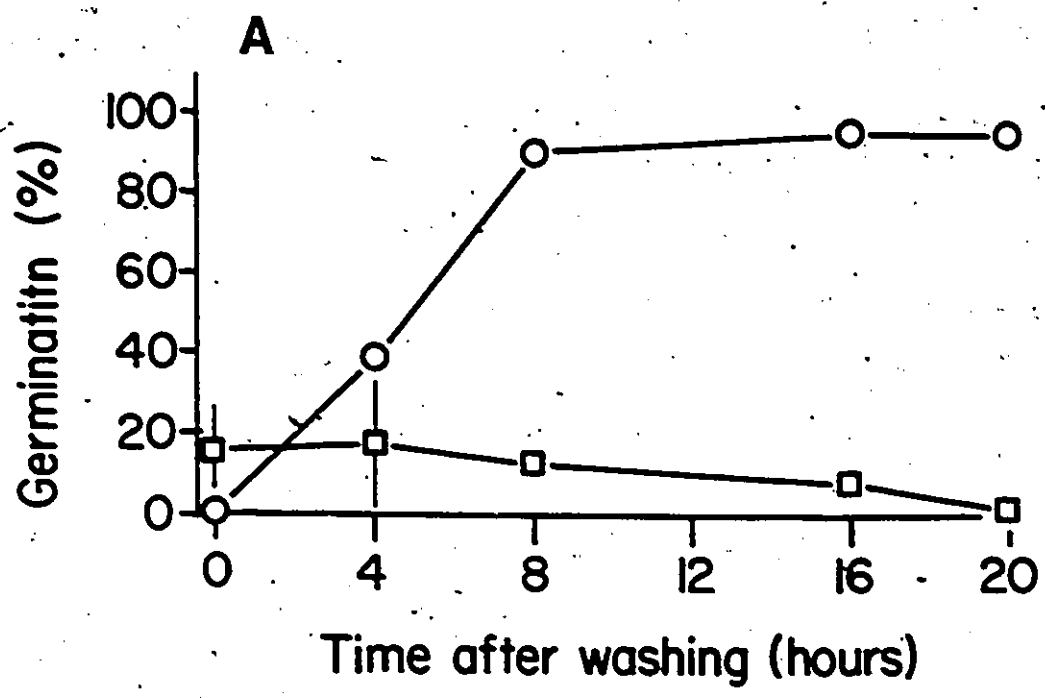
Figure 8: Germination and elongation experiment. The effect of PHT treatments on primary macroconidia germination (A) and elongation (B) of 16 h cultures in Fries liquid medium. Germination and elongation were evaluated in the presence (open circles) and absence (closed circles) of near-UV wavelengths (300-400 nm, $5 \text{ w}\cdot\text{m}^{-2}$). Bars represent standard deviation. Where omitted, standard deviations were $< 5\%$.



At concentrations above 4 ppm, PHT strongly inhibited germ tube elongation (Fig. 8B) with and without near-UV treatment, although some stimulation was observed at low concentrations (2 ppm). The near-UV and control groups are significantly different at all concentrations except 9.1 ppm. Partial but not statistically significant inhibition with respect to controls of elongation in treatments with near-UV but without PHT suggest that the combined effects of PHT and near-UV were not highly synergistic. Thus, photosensitization effects in germ tube elongation were less evident than in the germination study.

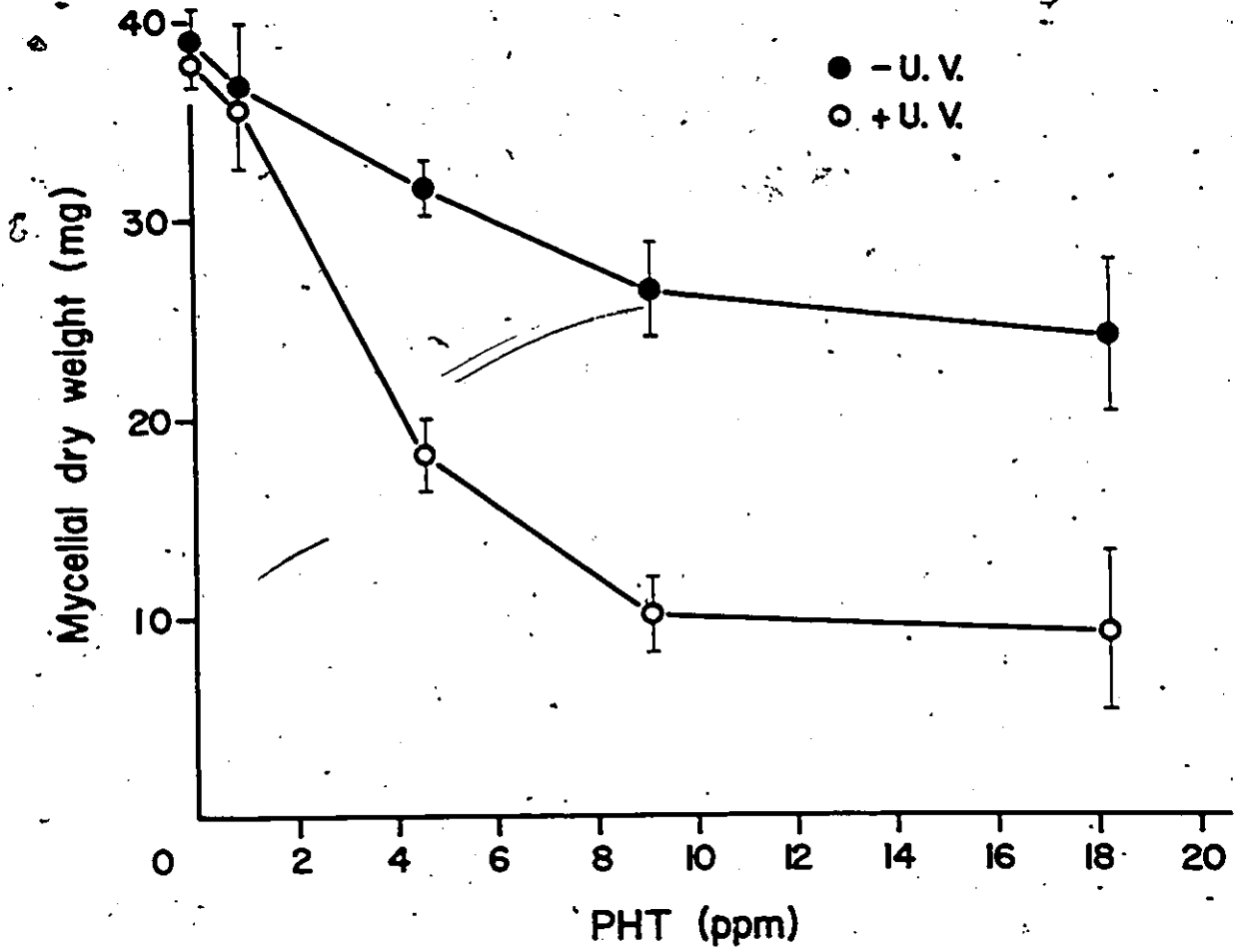
In a subsequent experiment (Fig. 9), we investigated whether the effect of PHT photosensitization on macroconidia was fungistatic or fungicidal. Macroconidia given the minimum treatment for complete arrest of germination (9.1 ppm PHT, 16 h near-UV) were washed 3X in PHT free medium and incubated without further near-UV treatment. These had a low germination rate and no germ tube extension took place over the next 20 h, while control macroconidia (no PHT, no near UV and freshly prepared at the time of washing) germinated and extended the germ tube normally. Thus PHT photosensitization is clearly a fungicidal effect from which there is no recovery.

Figure 9: Fungistatic-fungicidal experiment. The fungicidal effects of PHT on primary macroconidia germination (A) and elongation (B) in Fries liquid medium. Circles represent control (0 ppm) and squares represent cultures previously treated for 16 h with 9.1 ppm PHT and near-UV, then transferred to fresh medium with no PHT. Other conditions as in Fig. 8.



Photosensitization of mycelia in liquid culture was also clearly apparent (Fig. 10) and significantly different from the controls at 4.5 ppm and above (see Appendix A). The EC_{50} obtained from a probit analysis of the data was 5.6 ppm. In the absence of photosensitizing wavelengths (less than 400 nm) growth inhibition was considerably reduced but still evident. These results are significantly different from the controls and the near-UV treated group at 4.5 ppm and above. Near-UV radiation alone without PHT had no significant effect on mycelial growth.

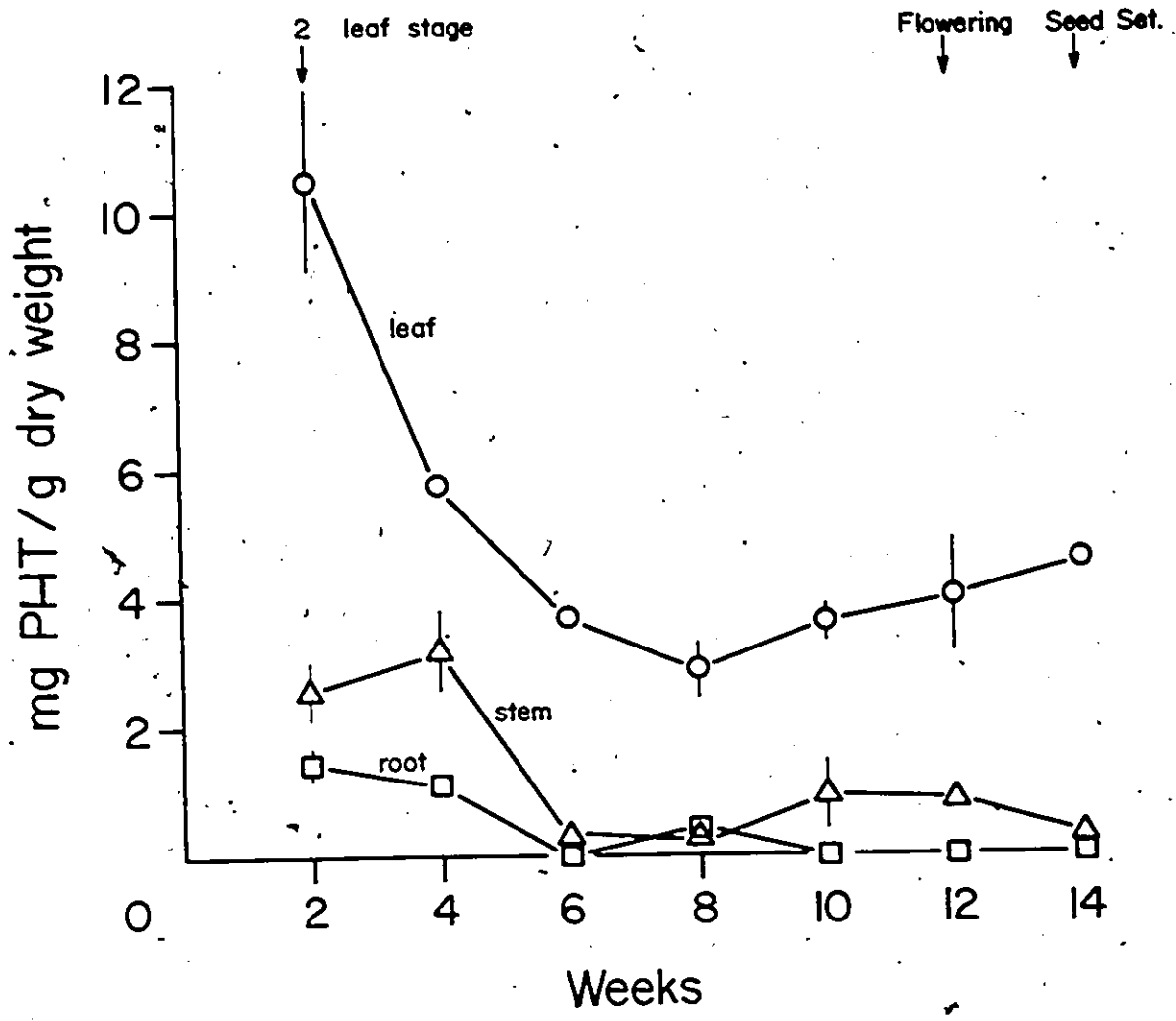
Figure 10: Mycelial dry weight experiment. The effect of PHT on mycelial dry weights of *F. culmorum*. Open circles represent incubation in presence of near-UV radiation ($5 \text{ w} \cdot \text{m}^{-2}$) and closed circles, incubation in absence of near-UV radiation. Bars represent standard deviation.



3.2 Endogenous levels of PHT in *Bidens pilosa*.

In order to assess the possible role of PHT as a natural protectant in its host plant, *B. pilosa*, endogenous levels of the substance were monitored during growth and development of the plant. Concentrations were highest in all plant parts at the seedling stage (Fig. 11). They declined with age in leaves and stems and reached zero in roots. Levels in the leaf increased slightly at flowering. Values are reported on a dry weight basis of plant tissues to reduce variance attributable to water content in the plants. At 6 weeks, the values correspond to 450 µg/g fresh weight of leaves, a value falling in the range of PHT concentrations determined by Towers and Wat (1978) for *B. pilosa*.

Figure 11: PHT levels in *Bidens pilosa*. Endogenous concentrations of PHT in *B. pilosa* during growth at 25°C under solar simulating Vita lights (16 h light, 8 h dark cycle 200 $\text{w}\cdot\text{m}^{-2}$). Circles, triangles and squares represent concentrations of PHT in the leaves, stems and roots respectively. Bars represent standard deviation and where omitted were $<.4$ mg PHT/g dry weight.



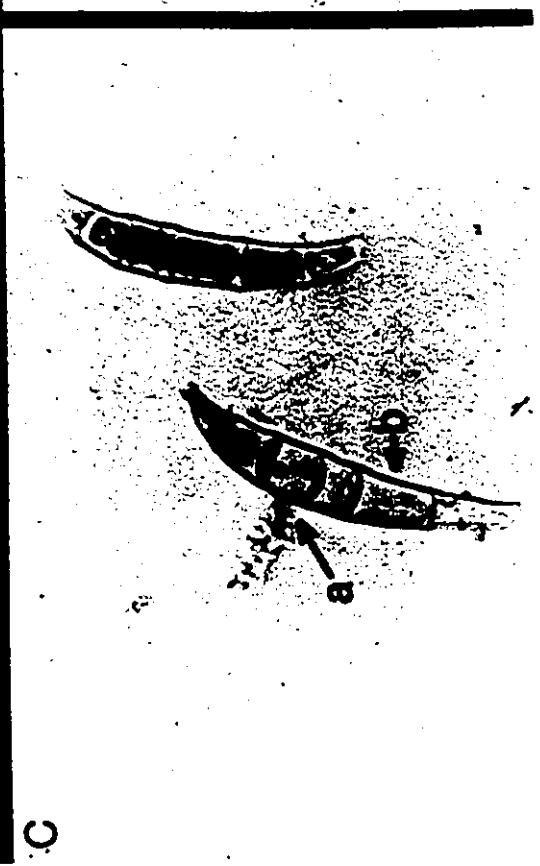
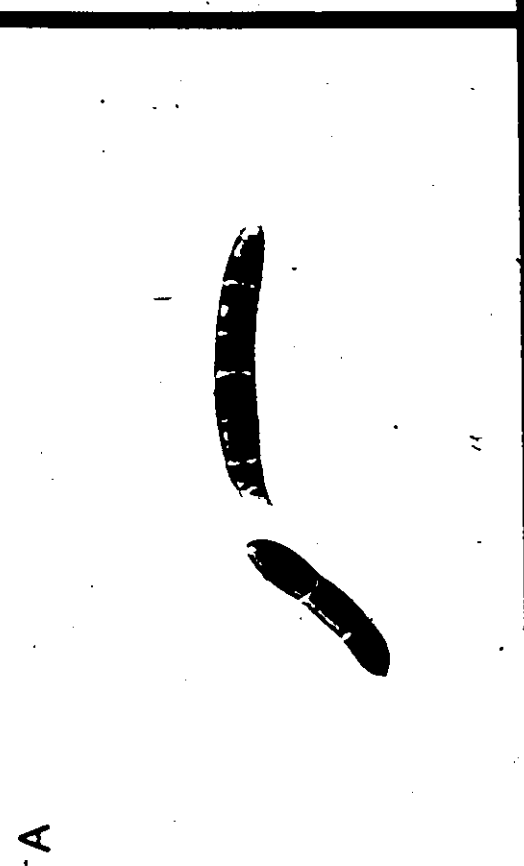
Some elicitation studies were carried out with B. pilosa at different stages, in order to detect any possible increase of levels of PHT in response to F. culmorum or mechanical damage i.e. a phytoalexin response. Several methods were used to attempt to achieve successful infection with F. culmorum. A plate of dilute PDA agar (1.5%) preparation was streaked with fungal spores of F. culmorum and seeds of B. pilosa were placed over it. Germinated seeds were not infected. A second method was to prick B. pilosa seedlings (2 weeks) with the tip of a Hamilton microsyringe along the main vein of leaf. A spore solution was then sprayed on the abaxial side of the leaves. Similar infection techniques were tried with B. pilosa plants at the 6 week stage. Also, a range of spore concentrations was used with all attempts but none of the above led to successful infection. Mechanical damage of B. pilosa tissue and any of the attempted infections, whether alone or in combination, did not affect PHT levels in B. pilosa on the basis used.

3.3 Photomicrography

After PHT and near-UV treatment, macroconidia appeared under the phase contrast microscope as either normal, granulated or lysed (Fig. 12). Normal macroconidia had a uniform bluish tint. Granulated macroconidia were scored as having 3 or more visible black dots, indicating aggregation of cytoplasmic lipids into discrete globules. Lysed macroconi-

dia had a visible membrane disruption which led to complete loss of cell contents.

Figure 12: Photomicrography. Photomicrographs of F. culmorum macroconidia stained with Sudan Black B after 24 h near-UV incubation with 10 ppm PHT. Normal (A), granulated (B), partly lysed (C) and completely lysed (D) macroconidia are shown. Percentages of (A), (B) and (D) are recorded in Fig. 13.



B

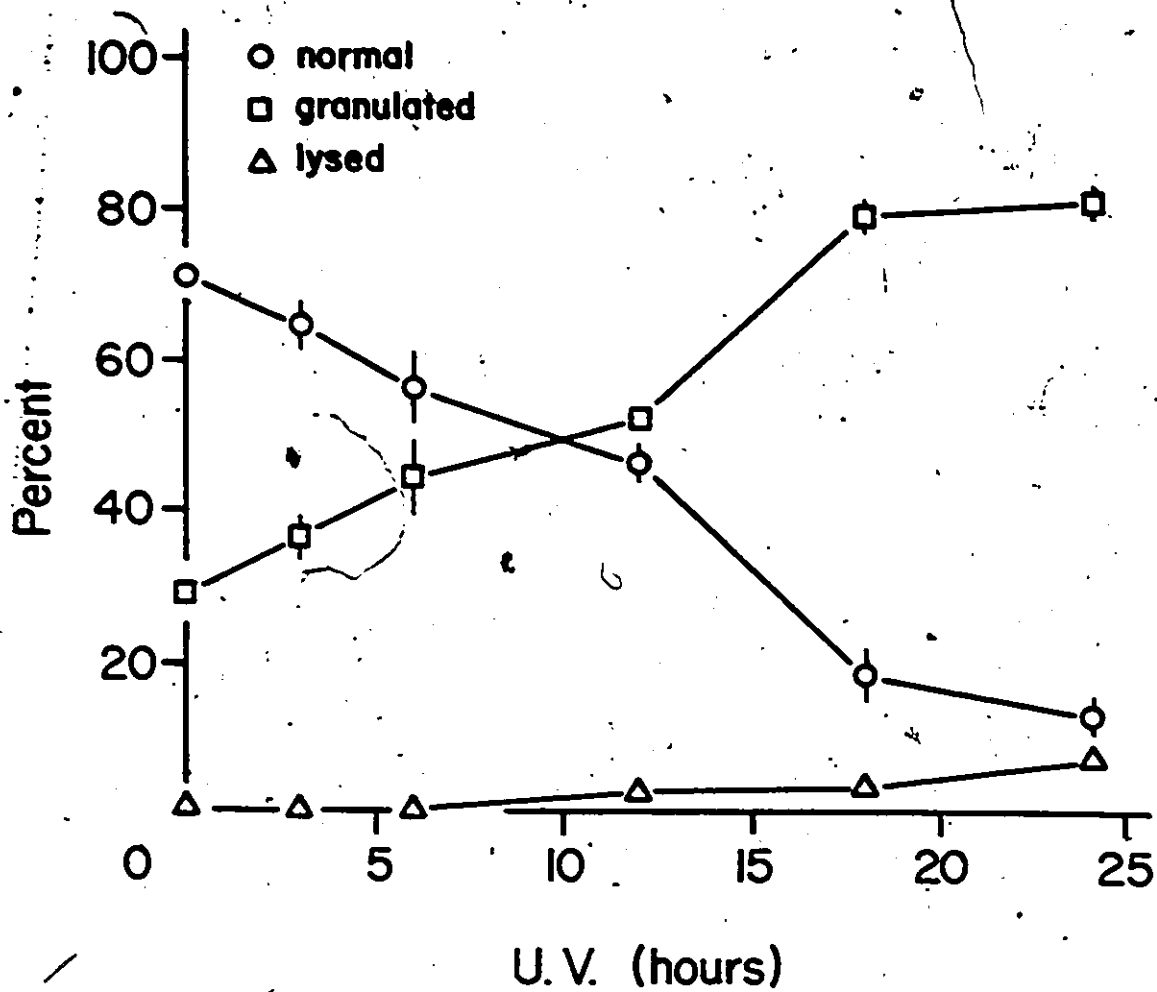
D

A

C

With 10 ppm PHT but no near-UV treatment, 30% of macroconidia became granulated (Fig. 13). With photosensitizing near-UV and PHT treatment, this value increased to 80% whereas the percent of normal cells steadily decreased to 15%. After 12 h, a minority of macroconidia in this latter group became lysed. Phase contrast microscopy of 16 h treated mycelia showed also presence of granulation, but more extensively.

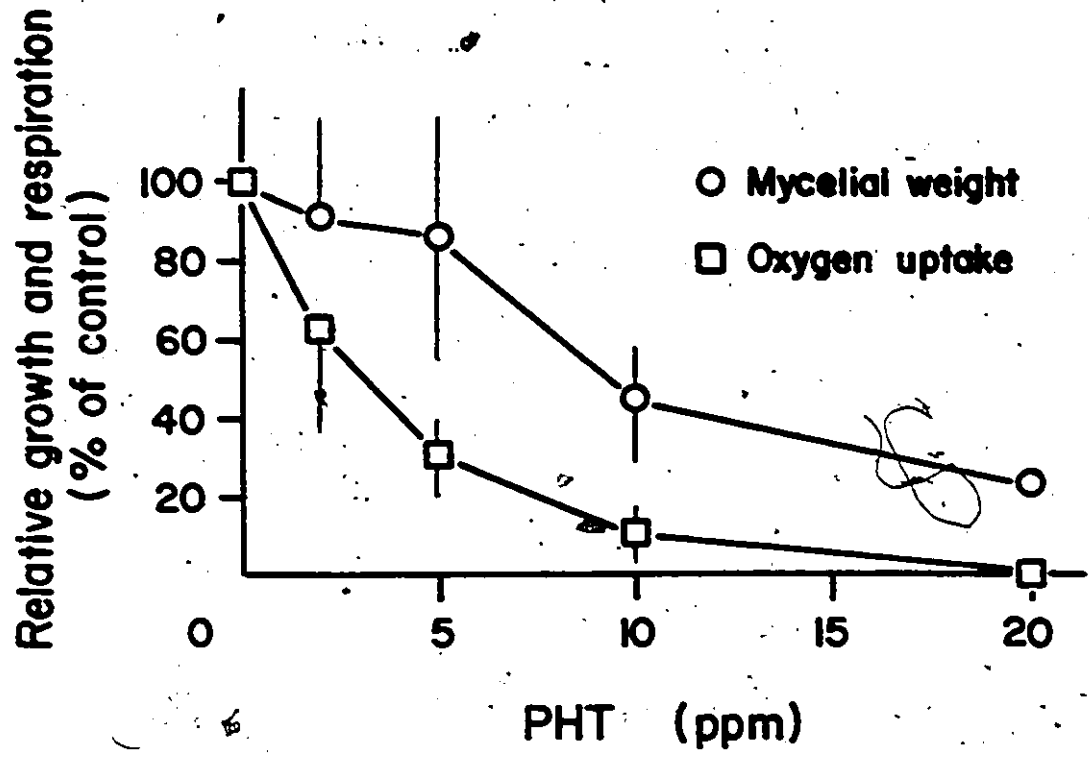
Figure 13: Quantitative data from photomicrography. The effect of near-UV radiation ($5 \text{ w}\cdot\text{m}^{-2}$) on macroconidia treated with 10 ppm PHT for 24 h. Circles, squares and triangles represent normal, granulated and lysed macroconidia respectively. Bars represent standard deviation.



3.4 Respiration

The effect of addition of 10 ppm PHT at time of inoculation on the respiration and growth of 16 h mycelia grown in the presence of near-UV is shown in Fig. 14. Both respiration and relative growth decreased steadily as the concentration of PHT was increased (Fig. 14). However, the decline of the respiration rate appeared to be a primary inhibitory effect since it was inhibited at lower concentrations than was mycelial growth. At 10 ppm for instance, the respiration rate was 10% of control while growth was 30% of control. There was no difference in respiration rate of controls (-PHT,-UV) or near-UV treated mycelia (-PHT,+UV).

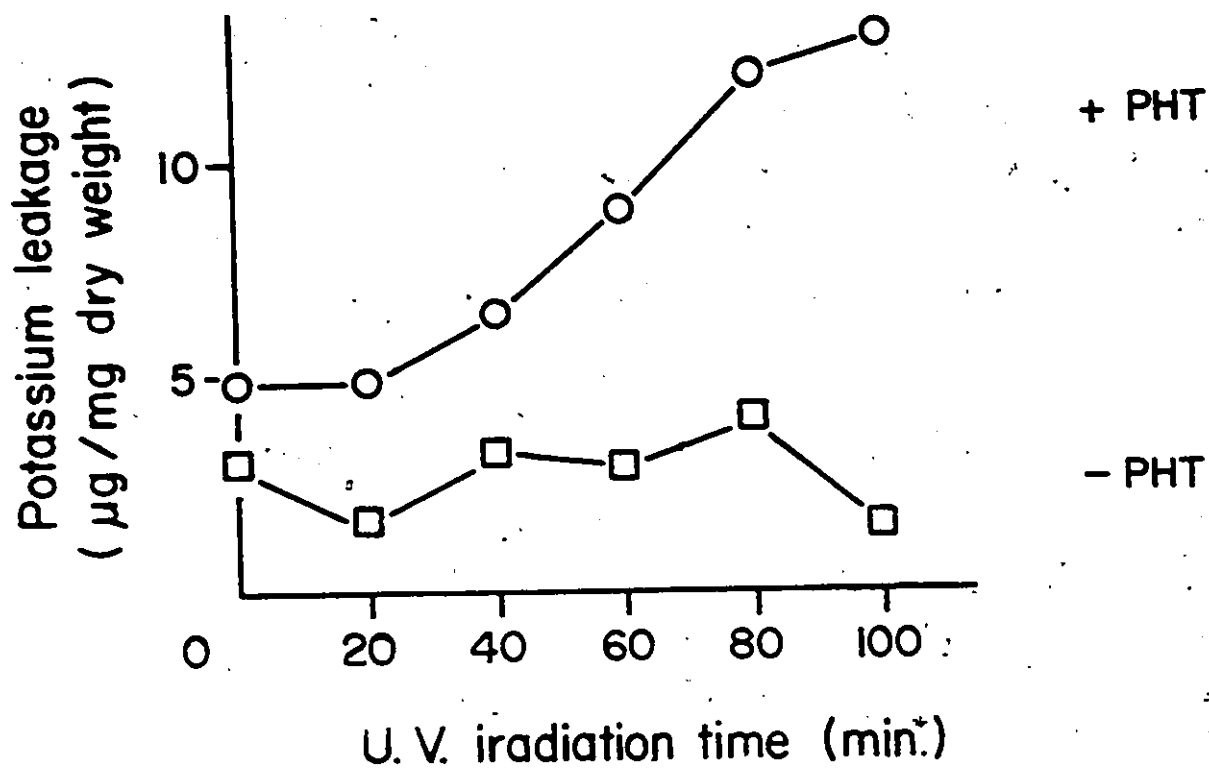
Figure 14: Respiration experiment. The effect of PHT treatments on mycelial weight (circles) and oxygen uptake (squares) of *F. culmorum*. Macroconidia were incubated for 16 h under near-UV radiation ($5 \text{ w}\cdot\text{m}^{-2}$) with varying PHT concentrations. Bars represent standard deviation and where omitted were $< 5\%$.



3.5 Potassium leakage

The results of a typical K leakage experiment are shown in Fig. 15. During the 100 min holding period in deionized water, control mycelia released approximately 3 μg K/mg dry weight. This value did not increase with near-UV treatment (without PHT). For 10 ppm PHT treated samples, the potassium leakage of mycelia was initially 5 μg K/mg dry weight and gradually increased with near-UV irradiation time to a maximum of 13 μg K/mg dry weight at 100 min. This near-UV irradiation time also represented the maximal inhibition of ^3C -Phe uptake. The experiment was repeated 4X indicating each time the same trends as shown in Fig. 15.

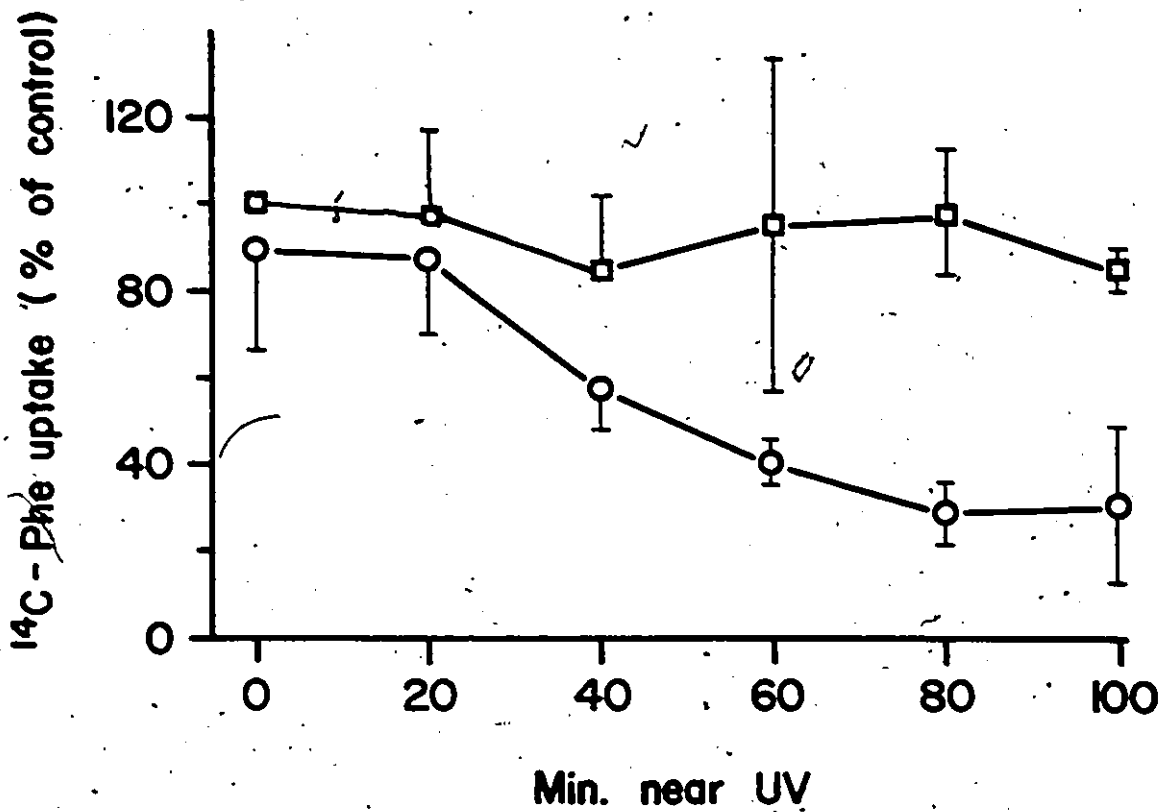
Figure 15: Potassium leakage experiment. The effect of near-UV irradiation on potassium leakage of F. culmorum cells incubated without (squares) and with (circles) PHT (10 ppm).



3.6 ¹⁴C-Phe uptake

With ¹⁴C-Phe, substantial incorporation (~10,000 cpm in control cells) permitted measurement of transport of PHT treated cells. For control samples, near-UV irradiation slightly decreased ¹⁴C-uptake, the lowest value being at 40 min near-UV (84% of control). For 10 ppm PHT treated samples, increasing near-UV irradiation time steadily inhibited ¹⁴C-uptake. The ¹⁴C-uptake of F. culmorum cells irradiated with near-UV for a period of 100 min has decreased to a value of 31% of the control (-PHT, -near-UV).

Figure 16: ^{14}C -Phe uptake experiment. The effect of near-UV irradiation ($5\text{w}\cdot\text{m}^{-2}$) on ^{14}C -Phe uptake by F. culmorum mycelia incubated in absence (squares) and presence (circles) of PHT (10 ppm).



Chapter IV

DISCUSSION

4.1 Overview

The present study clearly establishes the photosensitizing and non-photosensitizing growth inhibiting effects of PHT on germination, germ tube elongation and mycelial growth. It also strongly suggests that fungal membranes are the principal targets of photosensitization. Endogenous levels and location of PHT in the plant B. pilosa suggest that PHT is a preformed fungal inhibitor and could inhibit the growth of this and possibly other adapted phytopathogenic fungi.

4.2 Growth studies

The study of the effect of PHT on F. culmorum suggests that this acetylene is a potent antifungal substance. However, the comparison of the toxicity of PHT with values previously reported for other acetylenes must be limited to dark studies since there are few reports on the effects of electromagnetic radiation in the process (Table 4). For example, de Wit and Kodde (1981) found an EC_{50} for inhibition of germination of Cladosporium cucumerinum of 24 ppm for falcarindiol and for cis-tetradeca-6-ene-1,3-diyne-5,8-diol

of 36 ppm. The EC for inhibition of F. culmorum by PHT in the presence of near-UV is considerably lower (1.7 ppm), but when near-UV is excluded, the EC exceeds 20 ppm. The only study relating to photosensitizing effects is that of DiCosmo et al., (1982a) on a biosynthetically related group of substances, the thiophenes. Alpha-terthienyl was the most active of three substances tested with an EC_{50} of 0.1 ppm for inhibition of growth of Pythium aphanidermatum on agar.

Table 4: Selected toxicity data

EC₅₀ for PHT, related acetylenes, thiophenes and other fungicidal agents.

Table 4: Selected toxicity data for PHT, related acetylenes and thiophenes and other fungicidal agent

Type of compound	Substance	Target organism	EC ₅₀ (ppm)			Reference
			Liquid Media	Agar	Germination	
Acetylene	PHT	Fusarium culmorum	48 (-UV) 5.6 (+UV)		>20 (-UV) 1.9 (+UV)	This study
	Myerone acid	Botrytis fabae			26	Letcher et al., 1970
Hypocholesteremic agent	Falcarindiol	Cladosporium cucumerinum			24	de Wit and Kodde, 1981
	cis-1,3,11-trideca-1,3,7,9-tetrayne	Puccinia carthami			0.15	Binder et al., 1977
	cis-tetradeca-6,8-diol	Cladosporium cucumerinum			36	de Wit and Kodde, 1981
Carbamate fungicide	Clotribrate	Fusarium culmorum	0.16			Madhosingh and Orr, 1978
	Benomyl	Fusarium culmorum		1-2.5		Van Der Hoeyen and Bollen, 1980
Thiophene	Alpha-terthienyl	Pythium aphanidermatum		>1000 (-UV) 0.1 (+UV)		Di Cosmo et al., 1982
	5-(but-3-enynyl)-2,2-bithienyl	Pythium aphanidermatum		20 (-UV)		Di Cosmo et al., 1982
	2-chloro-4-(5-penta-1,3-dienyl)-2-thienyl but-3-ynylacetate	Pythium aphanidermatum		8 (-UV) 0.1 (+UV)		Di Cosmo et al., 1982

PHT has fungicidal activity towards both spores and hyphae, as does the furanoacetylene wyerone (Rossal et al., 1980) and the dihydrophenanthrene orchinol (Ward et al., 1974). However some secondary plant metabolites, such as phaseollin, have a fungicidal activity against spores but fungistatic activity against hyphae (VanEtten and Bateman, 1970).

Comparison of the values of PHT inhibition of F. culmorum with the fungicide benomyl (EC_{50} = 1-2.5 ppm on agar) (Van der Hoeven and Bollen, 1980) and the hypocholesteremic agent, clofibrate (EC_{50} = 0.16 ppm in liquid medium) (Madhosingh and Orr, 1978), indicate that PHT is a potent inhibitor, but not active enough to be considered as a control agent at this time. Preparation of analogues or investigation of other natural acetylenes may produce useful substances for control purposes.

4.3 PHT levels during growth of B. pilosa.

The present study is the first report of the production of PHT in roots of B. pilosa. Previous reports have dealt only with a single analysis of mature plants (Bohlmann et al., 1973 and Wat et al., 1979). The physiological levels in the plant indicate that the highest concentration of this fungal inhibitor occurs during its most critical stages of growth, namely the seedling and flowering stages.

Growing leaves contain higher levels of the polyacetylene PHT than do mature tissue. Differences between older and younger leaf tissue may be a result of the loss of compounds by : 1) biochemical or photochemical degradation in older tissues 2) volatilization from glands or 3) dilution in older tissue. Conversely, younger tissue may be the site of polyacetylene synthesis. A study of the accumulation of hydrocarbons and epoxide polyacetylenes per seedling in safflower was performed over a two weeks period following germination (Ichiara and Noda, 1977). Within the first 7 days following germination, polyacetylenes increased rapidly, mainly in the cotyledons rather than in hypocotyls and roots.

PHT segregation in the waxy cuticle (Wat et al., 1979) suggests the high lipophilicity of this molecule. This has been confirmed by measuring its Octanol/Water partition coefficient ($\log P = 5.82$) (McLachlan et al., 1984). The concentration of PHT required for photosensitizing effects on *F. culmorum* eg. $EC_{50} = 1.7$ ppm for spore germination and $EC_{50} = 5.6$ ppm for mycelial growth inhibition, are far below the concentrations found in the various plant parts. In addition, the levels of near-UV used ($3 \text{ w}\cdot\text{m}^{-2}$) were well below those measured in natural sunlight ($10 \text{ w}\cdot\text{m}^{-2}$ in June at midday in Ottawa 45° N latitude). These data suggests that PHT on leaf and stem surface could inhibit the growth of this and possibly other unadapted plant pathogens.

throughout the various phases of their life cycle. Even in the roots where no photosensitization is possible, the non-photosensitizing effects of PHT are sufficient to inhibit growth.

4.4 PHT: preformed defense or phytoalexin ?

The lack of response to mechanical injury suggests that PHT does not behave as a constitutive phytoalexin in the plant. Also, F. culmorum did not elicit synthesis but this experiment does not rule out that additional possibility that PHT may be elicited by other adapted pathogens.

DiCosmo et al., (1982) failed to produce PHT with Bidens callus tissue under a variety of culture techniques including environmental stress. However, they were ultimately able to elicit PHT synthesis using cell free fungal-culture filtrates of Pythium aphanidermatum. This experiment suggests that the B. pilosa genome does possess the capacity for a phytoalexin response, although synthesis of PHT in leaves appears to be derepressed under normal growth conditions in the intact plant.

The high levels of PHT in leaves and its location in the cuticle suggests instead its role as a pre-infectious inhibitor. In the case of an aerial pathogen which would try to infect B. pilosa leaves, PHT meets the first three criteria of Wood's postulate (1967). The fourth criterion could be verified by comparing the rate of fungal infection

through roots and leaves, which have different levels of PHT. However, a mutant of B. pilosa, with no PHT, might be preferable for such a study.

4.5 . Photomicrography

The granulation observed in the cytoplasm of PHT treated macroconidia or hyphae is known to be a characteristic stress response of Fusarium cells (Madhosingh and Orr, 1981). The dark blue color of the globules in our experiment indicates that there was aggregation of cytoplasmic lipids. No such dark and discrete globules were present in the controls.

Other antifungal plant secondary metabolites induce visible aggregation inside treated cells. They include kievitone (Smith, 1976), orchinol (Ward et al., 1974) and phaeollin (Slayman and VanEtten, 1974). Most studies used phase contrast microscopy to visualize toxic effects including protoplast lysis (Lyon and Mayo, 1978), cessation of cytoplasmic streaming (Smith, 1976) and effect on germ tubes (Higgins, 1978). Electron microscopy (E.M.) studies have been carried out to visualize effects on organelles such as mitochondria (Higgins, 1978) and the endoplasmic reticulum (Hargreaves, 1980).

When observing photomicrographs of PHT treated macroconidia, we should be aware of the danger of relating morphological and physiological damage. There are no clear

benchmarks by which a severely stressed but still normal cell can be distinguished from a cell that has been taxed to the point of injury. Similarly, there are no certain parameters by which the injured but still viable cell can be differentiated from one that is fatally injured. This is why experiments on fungistatic or fungicidal activity and careful dose response experiments are the only ones which give an indication of the degree of damage.

Respiration and transport represent vital processes contributing to the disease potential of F. culmorum cells. Both require the physical and physiological integrity of the fungal cell membrane.

4.6 Potassium leakage

Many PHT studies have indicated that the cytoplasmic membrane is a primary site of attack (Wat et al., 1979 and Yamamoto et al., 1979). The potassium leakage study of PHT treated mycelia cells confirms that this is the case in F. culmorum, since leakage of potassium occurred within the initial 100 min of incubation. Leakage of potassium may impair the K dependent enzyme phosphofructokinase in the glycolytic pathway and upset the osmotic balance of the cell. In addition potassium ions efflux from damaged F. culmorum cells, other substances which normally do not readily penetrate the cytoplasmic membranes, may enter or leave the cell readily.

It is difficult to compare my studies of leakage induced by PHT with those of other antifungal substances, since the few studies have monitored parameters other than potassium efflux; for example ^{14}C -glucose (Higgins, 1978), betacyanin (Hargreaves, 1980) and conductivity of electrolytes (Hargreaves, 1980).

4.7 ^{14}C -Phe transport

The active transport of amino acids across cellular membranes is performed by carrier proteins embedded within the bilayer of lipids (Stryer, 1981). PHT in the presence of near-UV significantly affected the uptake of the amino acid phenylalanine into F. culmorum hyphae. This inhibition of transport study represents a second confirmation of the membrane site of action of PHT.

It is difficult to compare % inhibition of Phe uptake with other known antifungal secondary metabolites since these studies were often performed with different substances and species. For example, the studies of transport inhibition by phaseollin (VanEtten and Bateman, 1971) and maackian (Higgins, 1978) were performed with ^{14}C -glucose instead of ^{14}C -Phe.

A possible mechanism of PHT action is the polymerization of F. culmorum membrane lipids following treatment (McLachlan, 1984). This may affect the configuration or position of the embedded protein responsible for phenylalanine active transport.

4.8 Respiration

The effect of PHT on fungal respiration represents a third confirmation of a membrane site of action. This experiment suggested that the photosensitization effect on mitochondria is a primary one since the decrease in respiration rate, as measured with the oxygen electrode, occurred at lower concentrations of PHT than did the decrease in dry weight of mycelia. The significance of this result is that it suggests that PHT can cross the cytoplasm into other lipophilic structures such as mitochondria and that respiration may be one of the primary sites of inhibition as has been observed with other lipophilic toxins (Healy et al., 1971, Helenius and Simons, 1975).

Unfortunately, it was not always possible to relate the leakage and transport studies with mycelial dry weight since the incubation time of 100 min was too short for measuring a significant change in dry weight. Again, it is difficult to compare respiration inhibition studies since they have been performed with different concentrations and times of incubation. The thiophene, alpha-terthienyl, also inhibited respiration in the green alga Chlorella (Sinclair and Arnason, 1982).

4.9 Molecular mechanism of damage

These experiments indicate that PHT damage occurs on the plasma membranes. To explain the mode of inhibition of

F. culmorum by PHT, we have considered two properties of this molecule. First, PHT is a very non-polar lipid. Therefore it initially binds to the lipid in the plasma membrane of the pathogen. It's highly linear and inflexible structure may change the fluidity of the membrane, and contribute to the non-photosensitizing effects of the molecule in darkness. Secondly, PHT forms singlet O_2 and possibly free radicals in the presence of near-UV radiation. A chain reaction triggered by free radical formation would lead to polymerization or alteration of membrane lipids of F. culmorum. This could create point rigidities and eventually small ruptures in the membrane of the pathogen, leading to leakage of ions. Polymerization or alteration of cellular membrane lipids could also distort the conformation or orientation of transport proteins.

Chemicals with modes of action similar to that of PHT include polyene antibiotics (Kinsky, 1967) and carbon tetrachloride (Chopra et al., 1972). The former binds with sterols whereas the latter generates free radicals which react with unsaturated membrane lipids.

4.10 Conclusion

Despite recent progress, relatively little is known of the biological function of polyacetylenes in plant tissue. It is surprising that of the approximately 600 acetylenes known to occur in higher plants (mainly in the Asteraceae), only a few have been credited with fungitoxic activity.

To understand in greater depth the fungicidal activity of PHT on F. culmorum, additional experiments should be performed. For example, electron spin resonance studies would provide more information on the free radical generation of PHT. A study of the lipids, especially sterols in target fungi before and after photosensitization might reveal the molecular nature of the damage. A biochemical study could determine if PHT inhibits the main enzymes in lipid metabolism such as cholesterol ester hydrolase and pyruvate dehydrogenase. Investigation of adapted fungi known to parasitize B. pilosa could also lead to information on fungal response to plant phototoxins.

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Appendix A.

STATISTICAL DATA ANALYSIS OF FIG. 8, FIG. 10,

FIG. 14 AND FIG. 16.

Note: means followed by the same letter in a column are not significantly different ($P = 0.05$) in Duncan Multiple Range Test.

Analysis of germination data (Fig. 8)

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Treatment groups	Mean of germination (%)	0.9 ppm	4.6 ppm	9.1 ppm	18.2 ppm
-PHT -UV	96.3 ^a	96.3 ^a	96.3 ^a	96.3 ^a	96.3 ^a
-PHT +UV	91.0 ^a	91.0 ^a	91.0 ^a	91.0 ^a	91.0 ^a
+PHT -UV	96.3 ^a	93.3 ^a	58.3 ^b	89.3 ^a	89.3 ^a
+PHT +UV	34.3 ^b	15.0 ^b	02.0 ^c	00.0 ^b	00.0 ^b

Analysis of mycelial weight data (Fig. 10):

Mean of mycelial weights (mg) for PHT concentrations:

Treatment groups	0.9 ppm	4.6 ppm	9.1 ppm	18.2 ppm
-PHT -UV	39.1 ^a	39.1 ^a	39.1 ^a	39.1 ^a
-PHT +UV	37.9 ^a	07.9 ^a	37.9 ^a	37.9 ^a
+PHT -UV	36.8 ^a	31.7 ^b	26.4 ^b	24.1 ^b
+PHT +UV	35.6 ^a	38.0 ^c	10.1 ^c	09.2 ^c

Analysis of respiration and mycelial weight data (Fig. 14)

Treatment groups	Mean of respiration and mycelial weight (%) for PHT concentrations:			
	2 ppm	5 ppm	10 ppm	20 ppm
-PHT +UV (Res.)	100.0 ^a	100.0 ^a	100.0 ^a	100.0 ^a
-PHT +UV (Wei.)	100.0 ^a	100.0 ^a	100.0 ^a	100.0 ^a
+PHT +UV (Res.)	62.0 ^a	64.9 ^a	9.7 ^b	0.0 ^b
+PHT +UV (Wei.)	91.7 ^a	86.1 ^a	44.7 ^c	22.6 ^b

Analysis of ¹⁴C-Phe uptake data (Fig. 16).

Mean of ¹⁴C-Phe uptake (%) for near-UV irradiation:

Treatment groups 20 min. 40 min. 60 min. 80 min. 100 min.

-PHT -UV	100.0 ^a	100.0 ^a	100.0 ^a	100.0 ^a	100.0 ^a
-PHT +UV	97.6 ^a	84.3 ^{a,b}	94.9 ^a	97.9 ^a	84.7 ^a
+PHT -UV	89.8 ^a	89.8 ^a	89.8 ^a	89.8 ^a	89.8 ^a
+PHT +UV	86.9 ^a	57.4 ^b	40.1 ^b	29.4 ^b	31.2 ^b

Value of P for COCHRAN test

Variable	0.9 ppm	4.6 ppm	9.1 ppm	18.2 ppm
Mycelial weight	0.326	1.000	0.921	0.619
Germination	0.200	0.496	0.083	0.142
Germ tube growth	0.291	0.592	0.002	0.012

Variable	2 ppm	5 ppm	10 ppm	20 ppm
Respiration	0.379	0.239	0.035	-

Variable	20 min	40 min	60 min	80 min	100 min.
¹⁴ C-Phe	0.359	0.079	0.007	0.143	0.027