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LA THÈSE A ÉTÉ
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STUDIES ON THE INDUCED ACCUMULATION, FUNGITOXICITY
AND METABOLISM OF THIOPHENES PRESENT IN
TARGETES ERECTA

by

ELLY KOURANY

A thesis
presented to the University of Ottawa
in fulfillment of the
thesis requirement for the degree of
MASTER OF SCIENCE
in
BIOLOGY

DEPARTMENT OF BIOLOGY
FACULTY OF SCIENCE AND ENGINEERING
UNIVERSITY OF OTTAWA

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ABSTRACT

The occurrence of thiophenes in Tagetes erecta (Asteraceae) in healthy plants as well as in plants exposed to biological or chemical stress was monitored using high pressure liquid chromatography. Severe treatment of 4 days old seedlings with mercuric chloride (0.001M) or with a highly pathogenic fungi (Alternaria tagetica) resulted in a general decrease in the amounts of the thiophenes 5-(4-acetoxy-1-butenyl)-2,2'-bithienyl (BBT-OAc), (5-buten-3-ynyl)-2,2'-bithienyl (BBT), alpha-terthienyl (alpha-T) and a compound tentatively identified as a polyine monothiophene. Infection of 2 week old seedlings by two different Fusarium oxysporum strains varying in their degree of virulence towards the host plant resulted in different patterns of thiophene kinetics in the infected tissues. The detected thiophenes 5-(4-hydroxy-1-butenyl)-2,2'-bithienyl (BBT-OH), BBT and alpha-T accumulated in roots and stems which had been partially damaged by the moderate pathogen F. oxysporum var callistephi race 2 (FOVC race 2), reaching maximum concentrations 12 days after inoculation (the combined yield of the 3 thiophenes was 0.9 and 3.4 µg/seedling for control and infected plants respectively). However, severe infection with F. oxysporum f.sp. radi-

cis lycoperisici (FORL) resulted in a decline in thiophene levels in the highly damaged tissues (the combined thiophene yield was 0.9 and 0.13 μg /seedling in control and infected plants respectively, 12 days after inoculation).

The antifungal activity of the naturally occurring thiophenes BBT-OH, BBT-OAc, BBT, α -T and 5-(3,4-diacetoxy-1-butynyl)-2,2'-bithienyl (BBT-2OAc) was tested against FOVC race 2 and FORL. All of these compounds completely inhibited in vitro spore germination of both fungi at a concentration of 5 $\mu\text{g}/\text{ml}$ in the presence of near UV light ($4 \text{ w}/\text{m}^2$). α -T was strongly phototoxic towards mycelial growth of both fungi in shake cultures with an EC_{50} of 0.06 and 0.03 $\mu\text{g}/\text{ml}$ for FOVC race 2 and FORL respectively. In the dark, only FOVC race 2 was inhibited by high concentrations of the compound. Tritiated α -T was not significantly metabolized by the pathogen FORL when grown in shake cultures containing 0.3 μg of α -T / ml. The role of thiophenes in disease resistance of T. erecta is discussed in relation to these findings.

RÉSUMÉ

La présence de thiophènes dans la plante Tagetes erecta (Asteraceae), est étudiée, chez les plantes saines comme chez celles qui sont exposées à un stress biologique ou chimique, au moyen de la chromatographie en phase liquide à haute pression (HPLC).

Sur des pousses de 4 jours, un traitement intensif au chlorure de mercure (0.001 M) ou avec un champignon hautement pathogène (Alternaria tagetica), provoque un abaissement général des taux de concentration des thiophènes 5-(4-acetoxy-1-butenyl)-2,2'-bithienyl (BBT-OAc), (5-buten-3-ynyl)-2,2'-bithienyl (BBT), alpha-terthienyl (alpha-T), ainsi que d'un composé qui est sans doute un monothiophène contenant plusieurs triples liaisons carbone-carbone.

L'infestation des pousses de 2 semaines par deux souches de Fusarium oxysporum de virulence différente vis à vis de la plante-hôte, révèle une cinétique différente des thiophènes dans les tissus infestés. Les thiophènes 5-(4-hydroxy-1-butenyl)-2,2'-bithienyl (BBT-OH), BBT et alpha-T, s'accumulent dans les racines et les tiges lorsque celles-ci sont partiellement endommagées par le pathogène modéré F. oxysporum var callistephi souche 2 (FOVC souche

2), et leur concentration maximale est observée 12 jours après l'inoculation (le rendement des 3 thiophènes combinés est de 0.9 et 3.4 μg / pousse respectivement chez les plantes contrôles et infestées). Cependant une infestation majeure par F. oxysporum f.sp. radicis lycopersici (FORL), a pour résultat un declin du taux des thiophènes dans les tissus affectés (le rendement global devient de 0.9 et 0.13 μg / pousse respectivement chez les plantes contrôle et infestées, 12 jours après inoculation).

Dans un deuxième temps, l'activité antifongique des thiophènes naturels BBT-OH, BBT-OAc, BBT, alpha-T et 5-(3,4-diacetoxy-1-butinyl) -2,2'-bithienyl (BBT-2OAc), est testée sur les pathogènes FOVC souche 2 et FORL. Tous ces composés, à une concentration de 5 $\mu\text{g}/\text{ml}$ et en présence d'UV-proche ($4 \text{ w}/\text{m}^2$), se montrent capable d'empêcher in vitro la germination des spores chez les deux espèces de champignons. L' alpha-T est fortement toxique pour le développement des deux mycelia pendant leur culture en agitation constante et la concentration effective est respectivement de 0.06 et 0.03 $\mu\text{g}/\text{ml}$ pour les pathogènes FOVC souche 2 et FORL. En absence de lumière, ce composé inhibe le développement de FOVC souche 2 seulement. L' alpha-T tritié n'est pas métabolisé significativement par le pathogène FORL si la culture en agitation constante contient 0.3 $\mu\text{g}/\text{ml}$.

En fonction de ces résultats, nous discutons du rôle des thiophènes dans la résistance de T. erecta aux maladies.

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Chapter I

INTRODUCTION

1.1 General introduction

Plants in nature are constantly challenged by a vast array of pathogenic microorganisms but resist almost all of them. The failure of most microorganisms to colonize plants has often been attributed to the presence of physical or chemical antagonists which inhibit or displace pathogens. One important phenomenon in this multicomplex interaction is the accumulation in many plants species of low molecular weight antimicrobial metabolites in response to microbial infection. During the past 25 years, the production of these toxic products, known as phytoalexins, has become the most extensively studied process of disease resistance in plants.

Many plant species belonging to 17 plant families produce phytoalexins. However, only two species from the large flowering plant family Asteraceae have been reported to possess the ability to accumulate phytoalexins. These phytoalexins are polyacetylenes and coumarin derivatives. However, many species of the Asteraceae also contain heterocyclic sulfur substances called thiophenes which have not yet been described as phytoalexins. These secondary metabo-

lites are toxic to a wide variety of organisms including microorganisms, insects and other animals in dark, but with substantially more activity in the presence of sunlight or artificial sources of near UV light (320-400 nm). The existence of chemicals of this sort within the plant tissues leads one to question their in situ significance. It is the role of thiophenes as phytoalexins that is the main topic of this thesis.

1.2 Literature review.

1.2.1 Plant disease resistance

Disease resistance is the ability of a plant to prevent, restrict or retard disease development and occurs at high, moderate or low levels. In contrast, virulence is the ability (low, moderate or high) of an infectious agent to overcome resistance (Bell, 1981).

Apart from the component of resistance conferred from the innate genetic characters of the host and the pathogen, the outcome of the host-pathogen interaction is the result of a complex system involving the coordinated action of many components. The plant can resist pathogenic invasion by the presence of constitutive passive barriers. But in many cases, resistance involves inducible dynamic defence mechanisms such as erection of structural barriers or accumulation of antimicrobial compounds. Failure of plants to invoke such defence reactions may result in disease susceptibility. The ability of the pathogen to bypass or degrade the preformed physical or chemical barriers and to actively suppress or avoid the active defence response mechanisms of its host may ultimately determine the outcome of the host-pathogen interaction (Bell, 1981).

1.2.1.1 Physical barriers

Preformed physical barriers are normally present in the plant prior to infection. For example, the cell wall is an efficient physical defence barrier. Cutin, suberin, lignin, cellulose and pectic substances are the components of the cell wall that may physically oppose the progress of the pathogens in the plant tissues (Schigeyasu and Fukutomi, 1977). They act by means of their impermeability to pathogen excretions, their insolubility to pathogen excretions and their impenetrability (Dickinson, 1960). In addition, superficial layers such as trichomes, root hairs, root caps, mucilage layers and seed coats may provide effective barriers against penetration by many pathogens (Campbell et al., 1977). Local saprophytic microflora composed of epiphytic microorganisms on aerial plant surfaces and ectomycorrhizae on roots may also assist the host defence since they can be antagonistic to many pathogenic fungi and bacteria (Campbell et al., 1977).

Active defense mechanisms exist that involve physical changes in plant structures and result in greater resistance. They include a) envelopment of parasites at the cell surface, b) hardening of plant cell walls by calcium accumulation, c) deposition (by means of vesicles) of callose in papillae at specific sites of infection or as a more generalized protective layer, d) deposition of additional wall layers, e) dedifferentiation, growth, division and rediffer-

entiation of cells to form protective tissues and f) vascular occlusion of xylem elements by the formation of tyloses and gels or gum plugs (Bell and Mace, 1981). In addition, all of these structures may become lignified by infusion and polymerization with stored or synthesized phenolic substances, producing tough walling-off structures that may minimize the effects of microbial invasions or other injuries due to environmental factors (e.g., chemicals and cold temperatures) and may function for rapid sealing-off of vascular infections (Beckman, 1977).

1.2.1.2 Chemical barriers

In addition to physical barriers, plants possess a large arsenal of biochemical defences against potential pathogens. Plant hydrolases, inhibitors of extracellular microbial enzymes, as well as a wide variety of antimicrobial secondary plant metabolites may offer a barrier to invasion by pathogens.

Plants possess hydrolases that can attack the cell walls of pathogens. Among these are chitinase, lysozyme and β -1,3-glucanase (Bell, 1981). Several studies have examined β -1,3-glucanase and chitinase in plants infected with fungi. In general, the activities of these enzymes were found to increase strongly in the course of infection (Boller, 1985). It has been clearly demonstrated that the two enzymes have the potential to attack and partially degrade fungal cell walls in vitro (Young and Pegg, 1982 Boller

et al., 1983), but it remains to be shown in what manner and to what extent this potential contributes to the resistance of a plant against an attacking pathogen in vivo.

Most microorganisms secrete hydrolytic enzymes that cleave the macromolecular constituents of host cell walls and bring about impaired host membranes (Bateman and Bas-ham, 1976). Some plants appear to have enzymes that inhibit the activity of these hydrolases. Phenolic compounds including tannins, orthoquinones as well as some unidentified proteins, appear to inactivate these extracellular microbial enzymes (Bell, 1981).

Many secondary plant products have antimicrobial properties and may play a role in plant defence reactions. Secondary plant metabolites are defined as all those compounds which are not part of the main metabolic pathways and cycles common to all plants (Stoessl, 1983). They include about 10,000 known compounds pertaining to a wide variety of chemical classes. Antimicrobial secondary plant products may function as preformed inhibitors. Some plants are protected from pathogenic attack by the simple mechanism of excreting toxic substances onto leaf surfaces. These substances are mainly lipophilic di- or triterpenoids (e.g., ursolic acid, lupeol and β -amyrin) and methylated flavanoids (e.g., eucaliptin and nobiletin) which can exert their toxic action without further modification of the molecule (Harborne and Ingham, 1978). Other preformed toxins (post-inhibitins) are

stored within plant cells in safe, inactive forms, frequently as glycosides, so that they do not interfere with normal cellular processes. As soon as microbial invasion occurs, the active toxin is liberated enzymatically from the bound non-toxic form (Swain, 1977). Glucosinolates, cyanogenic glycosides, lactonic glucosides and phenolic quinones are examples of such post-inhibitins. Enzymes concerned with the release of these toxins are mainly specific glucosidases but at least one oxidase and one amino acid lyase have also been implicated (Swain, 1977).

Antimicrobial substances can also accumulate to high concentrations in plant tissues in response to microbial invasion. These induced inhibitors, termed "phytoalexins", are believed to be major components of the dynamic defence reactions in plants.

1.2.2 Phytoalexins

For the purposes of this thesis, an appropriate definition for the molecules recognized as phytoalexins is proposed as follows: "Phytoalexins are low molecular weight (200-500 daltons) antimicrobial compounds that accumulate in plants in response to infection or stress" (Bailey and Mansfield, 1982). The chemistry, biosynthesis, toxicity and elicitation of these compounds, as well as their role in plant defence against pathogens is summarized below.

1.2.2.1 Structural and biosynthetic considerations

The accumulation of phytoalexins have been demonstrated in at least 17 plant families. They have been isolated mostly from the dicotyledons, rarely from the gymnosperms and monocotyledons, and have never been reported from lower plants.

The chemistry of these compounds is usually uniform within a plant family but is diverse within the plant kingdom. Plants from the Fabaceae, Solanaceae, Asteraceae and Convolvulaceae produce predominantly isoflavonoids, carbocyclic-sesquiterpenoids, polyacetylenes and furanoses-sesquiterpenoids respectively (Table 1) (Bailey and Mansfield, 1982). However, this is not an invariable rule and some structural classes as for example stilbenes, are represented by phytoalexins from a wide spectrum of unrelated plant families (Stoessl, 1982). Of the 102 phytoalexins reported from the Fabaceae, 84 are isoflavonoid derivatives, 8 are furanoacetylenes, 4 are stilbenes, 3 are benzofurans, 2 are chromones and 1 is a flavanone. Similarly, in the Solanaceae, 34 phytoalexins are terpenoid derivatives, 6 are phenylpropanoid phenols and 3 are acetylenes (Kuc and Rush, 1985). The sesquiterpenoid phytoalexins have not been reported in the Fabaceae nor the isoflavonoid phytoalexins in the Solanaceae.

Diversity of phytoalexin structures implies a complex integration of synthetic organization. The acetate-

mevalonate, acetate-malonate and shikimic acid pathways are the three main routes utilized in the biosynthesis of phytoalexins. The isoflavonoids produced by the Fabaceae are synthesized by a combination of precursors from the shikimate, the acetate-malonate and with some, the acetate-mevalonate pathway. The sesquiterpenes produced in the Solanaceae are synthesized by the acetate-mevalonate pathway. Figure 1 shows examples of phytoalexins produced by one of the pathways, or by a combination of 2 or 3 of the pathways.

Accumulation of phytoalexins may be due to an increase in the amounts of the enzymes catalyzing their appropriate biosynthetic pathways. Production of isoflavonoid phytoalexins appears to be the consequence of a specific coordinated increase in the rate of synthesis of many enzymes from the phenylpropanoid pathway including phenylalanine ammonia lyase (PAL), cinnamic acid 4-hydroxylase (C4H), 4-coumarate: CoA ligase (4CL), flavanone synthase, chalcone synthase and chalcone isomerase (Rathmell and Bendall, 1971; Dixon and Bendall, 1978; Dixon and Lamb, 1979; Cramer *et al.*, 1985). Increased synthesis of enzymes was also seen to be associated with accumulation of stilbenes (Fritzemeir and Kindl, 1981), furanoterpenes (Uritani *et al.*, 1976), sesquiterpenes (Corsini and Pavek, 1980) and furanocoumarins (Tietjen *et al.*, 1983 Hagmann *et al.*, 1983 Hauffe *et al.*, 1986). The induction of some enzymes was shown to be

based on rapid changes in amounts and activities of the corresponding messenger RNAs (Tietjen and Matern, 1983; Chap-
pell and Hahlbrock, 1984; Lawton et al., 1983) suggesting
that the control of phytoalexin biosynthesis lies at the
level of transcription or earlier.

TABLE 1

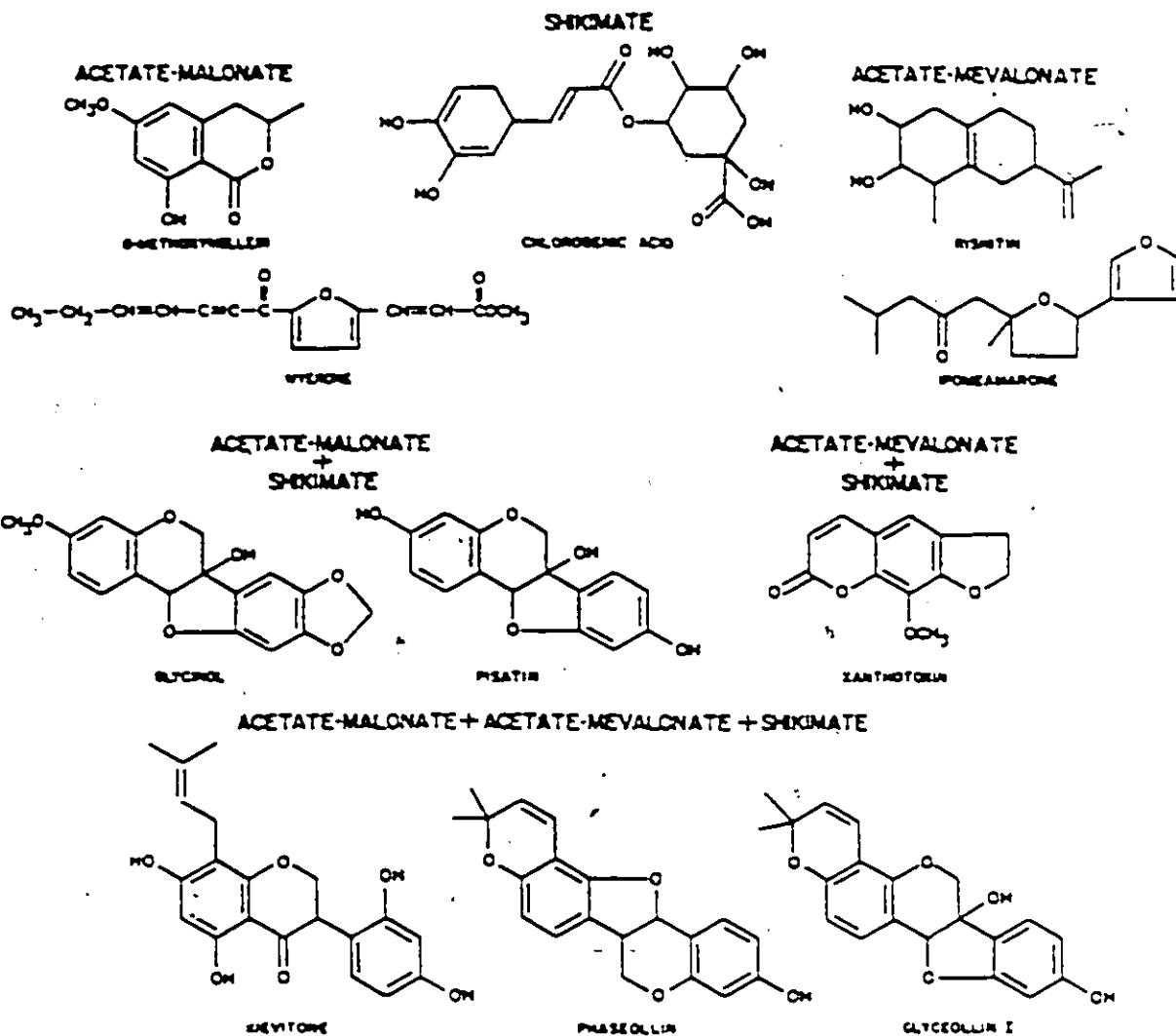
CHEMICAL NATURE OF THE PHYTOALEXINS ASSOCIATED WITH DIFFERENT PLANT FAMILIES

(Adapted from Harborne and Ingham, 1978)

FAMILY	CHEMICAL TYPE	EXAMPLE
GYMNOSPERMAE		
Pinaceae	Stilbene Lignan	Pinosylvin Hydroxymatairesinol
ANGIOSPERMAE		
Monocotyledones		
Gramineae	Diterpene	Momilactone A
Orchidaceae	Phenanthrene	Orchinol
Dicotyledones		
Chenopodiaceae	4'-Deoxyisoflavone	Betavulgarin
Compositae	Polyacetylene	Safynol
Convolvulaceae	Puranoterpene	Ipomeamarone
Euphorbiaceae	Diterpene	Casbene
Leguminosae	Isouavonoid	
	Isouavone	Wightone
	Isouavanone	Kievitone
	Pterocarpin	Medicarpin
	Isouavan	Vestitol
	Stilbene ^a	Resveratrol
	Benzofuran ^a	Vignafuran
	Furanosacetylene ^a	Wyerone acid
Linaceae	Phenylpropanoid	Coniferyl alcohol
Malvaceae	Naphthaldehyde	Gossypol
	Naphthafuran	Vergosin
Rosaceae	Phenolic acid	Benzoic acid
Solanaceae	Sesquiterpene	Rishitin
Umbelliferae	Chromone	Eugenin
	Dihydroisocoumarin	6-Methoxypellein
	Furanocoumarin	Xanthotoxin
Vitaceae	Stilbene oligomer	α -Viniferin

^a Very rare in this family where isoflavonoids predominate.

Figure 1: Examples of phytoalexins produced by major metabolic pathways (adapted from Kuc and Rush, 1985).



1.2.2.2 Elicitors

An extremely broad spectrum of compounds and environmental conditions causes phytoalexins to accumulate. Phytoalexin accumulation is induced by infection of plant tissues with bacteria, fungi, viruses, insects and nematodes; by exposure to cold and ultraviolet light, and also following treatment with many chemicals including heavy metal salts, polyamines, ethylene, antibiotics, metabolic inhibitors, fungicides and ribonucleases (Grisebach and Ebel, 1978; Bailey, 1982). Not all of the above induce or "elicit" phytoalexin accumulation in all plants, e.g., phytoalexin accumulation in the Fabaceae is elicited by many compounds and environmental conditions, but seems to be under much tighter metabolic regulation in the Solanaceae (Kuc and Rush, 1985).

There have been many investigations aimed at isolating and characterizing the products of microbial metabolism which may be responsible for the stimulation of phytoalexin accumulation. This has resulted in the identification of various microbial elicitors : a) monilicolin A, a low molecular weight peptide from Monilinia fructicola caused the production of phaseollin by French beans (Cruickschank and Perrin, 1968), b) glycopeptides isolated from various fungi, led to the accumulation of several phytoalexins such as rhisistatin in tomato, pisatin in pea and glyceollin in soybean (Bailey, 1982), c) cell wall degrading enzymes from both fungal (Lee and West, 1981) and bacterial (Davis

et al., 1984, 1986) sources induced phytoalexins to accumulate, an example being the induction of pterocarpan phytoalexins in soybean cotyledons by an endopolygalacturonic acid lyase from Erwinia carotovora (Davis et al., 1984), d) polysaccharides obtained from culture filtrates or from fungal cell walls (e.g., chitosan from cell walls of Fusarium solani) were active elicitors of phytoalexins such as pisatin in peas (Bailey, 1982), and finally e) two polyunsaturated fatty acids, arachidonic and eicosapentaenoic acids, isolated from Phytophthora infestans elicited the accumulation of sesquiterpenoid phytoalexins in potato tubers (Bosstock et al., 1981).

However, the fact that phytoalexins were able to accumulate in the absence of microorganisms or chemicals, especially after treatment with ultraviolet light, led to the suggestion that exogenous materials may not be required to initiate phytoalexin synthesis (Bailey, 1982). The discovery of materials originating within the plant tissues that were able to elicit phytoalexin accumulation in plants when released or activated resolved some of the difficulties encountered when considering the great diversity of phytoalexin elicitors. Several studies have indeed demonstrated that oligosaccharides released from plant cell walls by either partial hydrolysis (Hahn et al., 1981 Nothnagel et al., 1983) or treatment with pectin-degrading enzymes (Bruce and West, 1982; Davis et al., 1984, 1986 Kurosaki et al., 1985) were elicitors of phytoalexin accumulation.

Evidence that oligogalacturonides contribute to phytoalexin accumulation is provided by the observations that most, if not all, plants contain homogalacturonans within their cell walls (McNeil et al., 1984). Since all phytopathogenic microorganisms secrete pectin-degrading enzymes (Bateman et al., 1976), it is likely that elicitor-active oligogalacturonides are released from the cell wall during attempted infection.

1.2.2.3 Toxicity and metabolism

Phytoalexins exhibit toxicity across much of the biological spectrum. Fungi, bacteria, insects, nematodes, plants and animals are all susceptible to the action of individual phytoalexins (Smith, 1982).

Most of the research aimed at the elucidation of the molecular features of phytoalexins which confer toxic capabilities has concerned interactions between phytoalexins and fungi. The available evidence, based on cytological and physiological examinations is consistent with a) a multi-site toxic activity such as interference with respiration, non-specific binding to proteins, inhibition of enzymes and prevention of cell wall biosynthesis, and b) a rapid and substantial damage to the plasmalemma as reflected by cytoplasmic granulation, breakdown of the cell membrane, leakage of electrolytes and metabolites and dry weight loss. The wide variety of organisms detrimentally affected by phytoalexins and the apparent importance of a lipophylic nature

for these compounds would be consistent with such an activity (Smith, 1982).

The level of phytoalexin sensitivity varies significantly among fungal species and sometimes among fungal races (Smith, 1982). Apparently, pathogenicity is not always associated with a relative insensitivity to the host phytoalexin: while some studies have reported that pathogenic fungi were more tolerant to their host phytoalexin than non-pathogens (Cruickschank, 1962; Cruickschank and Perrin, 1971; Hutson and Mansfield, 1980; Mansfield, 1980), several exceptions also exist (Keen et al., 1971; Pueppke and Van Etten, 1974; Smith et al., 1975).

Detoxification of phytoalexins by pathogens has been frequently reported. Most of the known conversions of phytoalexins by fungi involve the creation of new hydroxyl groups by oxygenation, hydration, carbonyl reduction or ether cleavage (VanEtten et al., 1982). The metabolism of phytoalexins usually, though not always, yields products that are less toxic. Phytoalexin metabolism may play a rôle in pathogenicity of some fungi but many examples exist where no metabolism was detected. Examples of some metabolic studies performed in pure cultures are given in Table.2.

TABLE 2
EXAMPLES OF THE ABILITY OF SOME FUNGAL PATHOGENS TO METABOLIZE THEIR HOST'S PHYTOALEXINS

(Adapted from VanEtten et al., 1982)

PHYTOALEXIN	HOST	PATHOGEN	METABOLITE
Capsidiol	Pepper (<u>Capsicum frutescens</u>)	<u>Botrytis cinerea</u> <u>Fusarium oxysporum</u> f.sp. <u>vasinfectum</u> <u>Phytophthora capsici</u> <u>Cladosporium herbarum</u> <u>Phytophthora infestans</u>	capsenone capsenone no metabolism no metabolism no metabolism
Rishicin	Potato (<u>Solanum tuberosum</u>)		
Iponoxazonone	Sweet potato (<u>Ipomoea batatas</u>)	<u>Corticium rolfsii</u> <u>Botryodiplodia theobromae</u> <u>Ceratocystis fimbriata</u> <u>Botryodiplodia theobromae</u>	unidentified unidentified no metabolism unidentified
Iponoxazonol Wyerone acid	Broad bean (<u>Vicia faba</u>)	<u>Botrytis fabae</u>	reduced wyerone acid
Wyerone Wyerone epoxide		<u>Botrytis fabae</u> <u>Botrytis fabae</u>	wyerol wyerol epoxide
Phaseollin	French bean (<u>Phaseolus vulgaris</u>)	<u>Botrytis cinerea</u> <u>Fusarium oxysporum</u> f.sp. <u>phaseoli</u> <u>Thielaviopsis basicola</u> <u>Rhizoctonia solani</u> <u>Colletotrichum lindemuthianum</u>	6a-hydroxyphaseollin 6a,7-dihydroxyphaseollin no metabolism unidentified unidentified 6a-hydroxyphaseollin
Phaseollidin		<u>Fusarium solani</u> f.sp. <u>phaseoli</u> <u>Fusarium solani</u> f.sp. <u>phaseoli</u> <u>Colletotrichum lindemuthianum</u>	1a-hydroxyphaseollone phaseollidin hydrate unidentified
Phaseollinisoflavan		<u>Fusarium solani</u> f.sp. <u>phaseoli</u> <u>Colletotrichum lindemuthianum</u>	unidentified unidentified
Kievitone		<u>Fusarium solani</u> f.sp. <u>phaseoli</u> <u>Colletotrichum lindemuthianum</u>	unidentified kievitone hydrate
Medicarpin	Alfalfa (<u>Medicago sativa</u>)	<u>Fusarium solani</u> f.sp. <u>phaseoli</u> <u>Colletotrichum lindemuthianum</u> <u>Stemphylium botryosum</u>	unidentified unidentified vestitol
Sativan Pisatin	Pea (<u>Pisum sativum</u>)	<u>Nectria haematococca</u> <u>Ascochyta pisi</u> <u>Aphanomyces euteiches</u> <u>Sclerotinia trifoliorum</u>	3,6a-dihydroxy-8,9-methylenedioxypisatin (DMDP) DMDP DMDP 6a-hydroxymedicarpin
Medicarpin	Red clover (<u>Trifolium pratense</u>)	<u>Stemphylium saccharaeforme</u> <u>Phytophthora megasperma</u> var. <u>soiae</u>	6a,7-dihydroxymedicarpin unidentified no metabolism
Glyceollin	Soybean (<u>Glycine max</u>)		

1.2.2.4 Role of Phytoalexins in disease resistance

There can be little doubt that phytoalexins contribute to resistance in some, possibly in many plant-pathogen interactions. Demonstrations of the role of these antimicrobial compounds in disease resistance depend upon information on the accumulation of effective concentrations at appropriate times and places in tissues to cause the observed cessation of microbial growth.

Evidence has been provided by a number of studies that phytoalexins accumulate at the site of infection following penetration by the microbe, quickly enough and in sufficiently high concentration to inhibit the growth of bacteria and fungi (Bailey, 1974; Lyon and Wood, 1975; Rossal and Mansfield, 1980). Perhaps one of the best examples is the recent study by Hahn and his collaborators (1985) dealing with the quantitative localization of glyceollin I in relation to fungal hyphae in soybean roots infected with Phytophthora megasperma f.sp. glycinea using a specific radioimmunoassay for the phytoalexin and an immunofluorescent stain for the fungal hyphae. It was shown that local phytoalexin concentration in and around infection sites were high enough to inhibit fungal growth in the incompatible (host-resistant) but not in the compatible (host-susceptible) interaction.

There is evidence in many host-pathogen systems that the resistance response is accompanied by the hypersensitive

reaction in which some cells at the site of the infected plant tissues rapidly die and phytoalexins accumulate. Living and dead tissues existing together seem to be essential for phytoalexin accumulation. Phytoalexin accumulation is less pronounced and less rapid in the host-susceptible reactions (Grisebach and Ebel, 1978; Darvill and Albersheim, 1984). The difference in the phytoalexin yields between the compatible and incompatible interactions could be explained by any of several phenomena including a) secretion by the compatible pathogen of phytotoxins that injure or kill the plant cells in the vicinity of the pathogen, thereby making it difficult for the plant to accumulate sufficient phytoalexin concentration to stop the pathogen (Darvill and Albersheim, 1984), b) differential secretion by the pathogens of suppressors or inhibitors of phytoalexin synthetic enzymes (Oku *et al.*, 1980), c) more rapid phytoalexin detoxification by the compatible microbe, and d) differential elicitation of phytoalexin synthesis by the two pathogens, although the available evidence does not support this last possibility (Darvill and Albersheim, 1984).

The biosynthesis and accumulation of phytoalexins also appears to be a component of the resistance response seen in plant immunization in which infection with an avirulent pathogen stimulates resistance to subsequent infection by a virulent pathogen. The ability to immunize against some diseases provides strong support for the existence of a sys-

temic signal which sensitizes plants to recognize infections and respond rapidly (Kuc and Rush, 1985).

The interaction of hosts and pathogens appears to be a very complex phenomenon. Resistance mechanisms do not seem to be very specific with regard to their induction, the products produced and the specificity of the products to inhibit development of the attacking pathogens. In spite of this non-specificity, all these mechanisms are extremely important for the defence mechanisms in plants permitting survival and growth.

1.2.3 Thiophenes

1.2.3.1 Phytochemical aspects

Thiophenes are an important group of secondary metabolites which have been isolated, with the exception of a single microorganism, only from the family Asteraceae (Compositae) (Bohlmann and Zdero, 1985). These sulfur containing substances were first reported to occur in Tagetes erecta (Sease and Zechmeister, 1947). The first natural thiophene to be identified was alpha-terthienyl (alpha-T); this was followed by the identification of the closely related 5-(but-3-en)-2,2'-bithienyl (BBT) (Figure 2) (Sease and Zechmeister, 1947; Uhlenbroeck and Bijloo, 1958, 1959). Since then, the number of chemically distinct thiophene derivatives from this family has risen to more than 150.

Acetylenic thiophenes are useful chemotaxonomic markers for some tribes of the Asteraceae. Most genera that contain thiophenes belong to the tribes Heliantheae and Tageteae. The genera Dyssodia, Hymenanthrum, Poriphyllum and Tagetes are particularly rich in thiophene derivatives. No thiophenes have been isolated from members of the tribes Astereae, Calenduleae and Cichorieae. The occurrence of thiophenes in tribes of the family Asteraceae is described in Table 3.

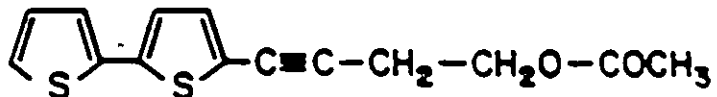
Figure 2: Chemical structures of the thiophene reference substances used in this study.

I



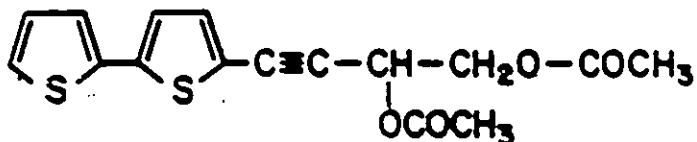
5-(4-hydroxy-1-butenyl)-2,2'-bithienyl
(BBT-OH)

II



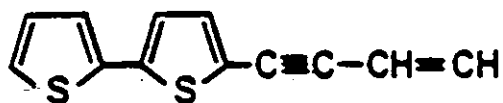
5-(4-acetoxy-1-butenyl)-2,2'-bithienyl
(BBT-OAc)

III



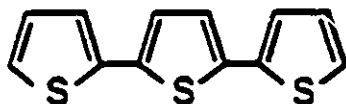
5-(3,4-diacetoxy-1-butenyl)-2,2'-bithienyl
(BBT-2OAc)

IV



5-(3-buten-1-ynyl)-2,2'-bithienyl
(BBT)

V



2,2':5,2''-terthienyl
(Alpha-T)

TABLE 3

THE OCCURRENCE OF THIOPHENES WITHIN THE ASTERACEAE

(Adapted from Sorensen, 1977; Bohlmann and Zdero, 1985)

TRIBE	MONOTHIOPHENES	DITHIOPHENES	TERTHIOPHENES
Anthemideae	+	-	-
Arctotideae	+	+	-
Asterace	-	-	-
Calenduleae	-	-	-
Cichorieae	-	-	-
Cynareae	+	+	-
Eupatorieae	+	-	-
Helentiae	+	+	+
Heliantheae	+	+	+
Inuleae	+	+	+
Lilae	+	-	+
Mutisieae	-	+	+
Senecioneae	+	+	-
Tageteae	+	+	+
Vernonieae	+	-	-

Naturally occurring thiophenes are heterocyclic compounds containing one, two or three aromatic rings per molecule. Most of them contain a linear moiety with conjugated double or triple bond systems, that give unique ultraviolet absorption spectra useful for rapid detection even in crude plant extracts. The content of thiophene derivatives varies in different organs during the vegetative growth (Sutfeld, 1982; Downum and Towers, 1983; Bohlmann and Zdero, 1985). For example, in Tagetes patula, maximum accumulation of thiophenes occurred within the first days of germination. BBT and acetoxybutynylbithiophene (BBT-OAc) (Figure 2) were the major components of the hypocotyls and roots. Alpha-T occurred exclusively in the cotyledons while the relative distribution of hydroxybutynylbithiophene (BBT-OH) (Figure 2) was quite variable among cotyledons, hypocotyls and roots (Sutfeld, 1982). It is not known whether translocation processes are involved in the accumulation of thiophenes in distinct organs, or if the different pattern of accumulation is due to separate sites of synthesis of the thiophenic compounds.

Natural thiophenic acetylenes are biosynthetically derived from oleic acid. Chain shortening of this eighteen-carbon fatty acid, by oxidation and decarboxylation through polyacetylenic intermediates, leads to the widespread thirteen-carbon intermediate tridecapentayne, the impor-

tant precursor of most of the naturally occurring thiophenes. The addition of methionine or its sulfur equivalent to this intermediate is believed to be the key step for the formation of the thiophenic ring(s), resulting in the various mono, di and terthienyl derivatives (Bohlmann et al., 1973).

The structural relationships between the natural thiophenes isolated from Tagetes, and results of feeding experiments with labelled precursors have suggested a plausible biosynthetic pathway (Figure 3). The biogenetic origin of alpha-T and BBT, both present in most Tagetes species has been established by feeding labelled tridecapentayne. It was suggested that in the route from the tridecapentayne to the naturally occurring thiophene derivatives, the pathway splits into two routes. While the first route yields the bithienyls, the other route in which one more bond becomes introduced into the intermediate, leads to the terthiophene alpha-T (Jente et al., 1981; Bohlmann and Zdero, 1985). BBT-OH and BBT-OAc would probably be formed through the epoxides of BBT (Bohlmann et al., 1973). Questions remain in this hypothesized biosynthetic pathway concerning the sequence of single steps. Various possibilities exist for the introduction of the required 1-2 acetylenic bond from tridecapentayne to the corresponding dihydrocompound. The stage at which the terminal methyl group is eliminated by oxidative decarboxylation also remains unknown (Bohlmann and Zdero, 1985).

Figure 3: Tentative biosynthetic pathway of the thiophenes BBT (2), BBT-OAc (3), BBT-OH (4) and alpha-T (5) present in Tagetes erecta from the tridecapentaynene precursor (1) (adapted from Bohlmann and Zdero, 1985; Jente et al., 1981).

Tracer studies are a preliminary guide to biosynthetic pathways, which can be established definitively only through enzymatic studies. Only two enzymatic studies have been undertaken. The first study involved the isolation of an enzyme from green parts of Tagetes patula. The enzyme, identified as being a highly specific acetoxylbutylbithiophene acetate esterase, catalyzed the transformation of BBT-OAc to BBT-OH (Sutfeld and Towers, 1982). Recently, experiments performed with crude enzyme preparations from Tagetes patula seedlings supported the existence of two position-specific dithiophene acetate esterases (Pensl and Sutfeld, 1985). It is not known whether these enzymes play a role in the biosynthetic or degradative step of thiophene metabolism. Further enzymological investigations are needed in order to elucidate this unresolved question.

1.2.3.2 Photobiological aspects

Plants of the genus Tagetes have been extensively used in Central and South America as well as in other parts of the world as medicinal plants, insect repellents and to discourage soil nematodes (Neher, 1968). It was only in the late 1950's that the nematocidal principles were first isolated from the roots of the marigold Tagetes erecta and identified as alpha-T and the structurally related BBT (Uhlenbrock and Bijloo, 1958, 1959). It was subsequently observed that irradiation with near-ultraviolet light (UV-A) strongly enhanced the nematocidal activity of alpha-T (Gom-

mers, 1972, Gommers and Geerligs, 1973). Since then, thiophenes have been demonstrated to be physiologically active against a wide variety of organisms, possessing greater activity in the presence of sunlight or artificial sources of UV-A light (320-400 nm) than in the dark. In addition to nematodes, these "photosensitizers" are phototoxic against microorganisms including fungi (DiCosmo *et al.*, 1982), bacteria and yeasts (Camm *et al.*, 1975 Chan *et al.*, 1975 Arnason *et al.*, 1981a Downum *et al.*, 1982, 1983), various insects (Arnason *et al.*, 1981b, 1983 Wat *et al.*, 1981 Kagan and Chan, 1983; Champagne *et al.*, 1983 (Champagne, 1984; Downum *et al.*, 1984) and other organisms including fish (Towers, 1980), marine and fresh water algae (Arnason *et al.*, 1981c Sinclair and Arnason, 1982) as well as other plant species (Campbell *et al.*, 1982). In addition, alpha-T can cause skin damage (Towers *et al.*, 1979) and lysis of human erythrocytes (Wat *et al.*, 1980).

The mechanisms by which thiophenes exert their photobiological activity in the presence of UV-A irradiation is a topic of considerable interest. A photosensitizer is described as a molecule that can be excited to a new electronic state by absorption of a quantum of light and which, in its excited state, reacts with itself or with some other suitable molecule in a given system (Spikes, 1977). Two types of photosensitizations are known: those requiring oxygen, known as photodynamic sensitizations and those occur-

ring without oxygen. The latter are most frequently reactions involving photoinduced modifications of DNA, mediated by plant products such as furanocoumarins and furanquinolines (Spikes, 1977). Photodynamic sensitizations can occur as either type I or type II processes. Type I photodynamic reactions yield superoxide radicals $O_2^{\bullet-}$ through a series of electron transfer processes following light absorption. Type 2 photodynamic reactions involve the production of singlet oxygen $^1O_2$ as a result of transfer of the excitation energy. The two types of activated species of oxygen are highly reactive and can cause biological damage by oxidation of biological molecules such as sterols (Suwa et al., 1978), proteins (Lamola and Doleiden, 1980) and nucleic acids (Spikes, 1977).

Evidence has accumulated that thiophenes are efficient generators of singlet oxygen (Bakker et al., 1979 Arnason et al., 1981a Gommers et al., 1982 Mc Lachlan et al., 1984 Reyftmann et al., 1985) and that the primary mechanism of action is the photodynamic disruption of membranes and does not involve DNA (MacRae et al., 1980 Downum et al., 1982 Yamamoto et al., 1984). Photosensitization involving both oxidative and non-oxidative (production of free radicals) mechanisms have also been described for polyacetylenes (Mc Lachlan et al., 1984 Weir et al., 1985).

The phototoxicity of certain secondary plant substances leads one to question their in situ significance. It is now generally accepted that these endogenous photosensitizers confer advantages as natural protective agents to the plant that possesses them (Spikes, 1977). Similarly, the existence of toxic chemicals such as thiophenes in the Asteraceae, that have no known metabolic function in the plant, suggests their role as protective agents against fungal, bacterial or viral pathogens, insects or other animals. It is this defence role of thiophenes against fungal infection that is the main topic of this thesis.

1.3 Plan of study

Although in vitro toxicity of thiophenes towards various organisms is well documented, no information exists concerning the in situ role of these compounds in the plant defence reactions, probably due to the lack of a well defined host-pathogen system for study.

This research was an effort to evaluate the possible involvement of these powerful photosensitizers in plant resistance to fungal pathogens. The main purpose was to investigate whether thiophenes play a role as phytoalexins. For this, the changes in thiophene levels occurring in plant tissues following microbial infection or exposure to abiotic stress, as well as the in vitro toxicity of these compounds were examined. An attempt was also made to assess a) wheth-

er the physiological growth stage affects the ability of the host to respond to applied stress in terms of thiophene production, b) whether the severity of the fungal infection affects the yield of thiophenes in the infected tissues and c) whether differences in fungal pathogenicity can be associated with differential sensitivity of mycelium to the toxic thiophenes produced by the host. An effort was also made to determine if a naturally occurring thiophene can be metabolized in vitro by a virulent fungus. It was hoped that information concerning these aspects of the host-pathogen interaction would help elucidate the role of thiophenes in plant defence reactions.

But before in situ studies could be conducted, it was essential to design a suitable host-pathogen system, to characterize thiophenes present in the host plant and to find a sensitive method for accurate quantification of thiophenes in the plant tissues.

1.3.1 The host

The African marigold Tagetes erecta (Tageteae, Asteraceae) was chosen as a suitable host because it was reported to contain a relatively broad spectrum of biochemically related thiophenes including BBT-OH, BBT-OAc, BBT and alpha-T (Figure 2) (Bohlmann et al., 1973 Hogstad et al., 1984). In addition, many fungal pathogens exist for this plant including species of Alternaria (Cotty et al., 1983), Septoria (Changsri and Weber, 1958), Fusarium (Olsen, 1965), Botrytis and Phytophthora (Pirone, 1978).

Tagetes is a diverse genus, comprising strongly scented species, some of which have become well established horticulturally. Their natural range extends from southeast Mexico into Argentina and the area of their greatest diversity is in south-central Mexico (Neher, 1968).

Only four phytoalexins have been isolated from members of the family Asteraceae. These are safynol and dehydrosafynol from safflower (Allen and Thomas, 1971a,b), as well as ayapin and scopoletin from sunflower (Tal and Roberson, 1985). The widespread occurrence of phytoalexins in many plant species makes it probable that other plant species from the Asteraceae family also produce phytoalexins in response to stress. Thiophenes, characteristic of this family, may be possible candidates.

To test the effect of the physiological growth stage on the thiophene yield in the plant tissues exposed to stress, young germinating seedlings (4 days old) were used as compared to older less vulnerable seedlings (2 weeks old) to set up two different experimental systems. Seedlings were used instead of older plants because they were easier to grow and to manipulate.

1.3.2 The pathogens

Alternaria tageticae (Deuteromyces) was the fungus used to infect young germinating seedlings of Tagetes erecta. This organism is the causal agent of stem, leaf and flower blight of marigolds (Cotty et al., 1983). Other Alternaria

species cause leaf spots of crucifers and cucurbits or early blight of potato (Hawksworth et al., 1983).

The second host-pathogen system used was the root and stem rot of older Tagetes erecta seedlings caused by Fusarium oxysporum. This fungus is the most important member of the genus Fusarium (Deuteromyces), and causes severe vascular wilts in many agricultural crops and many ornamentals (Armstrong and Armstrong, 1973).

Two Fusarium oxysporum strains, differing in their pathogenicity towards Tagetes erecta were tested for their ability to initiate accumulation of thiophenes in Tagetes erecta root and stem tissues during the course of infection development.

Chapter II

MATERIALS AND METHODS

2.1 Chemical analyses of thiophenes

2.1.1 Chemicals

Acetonitrile and water used for high performance liquid chromatography (HPLC), were of HPLC grade (Fisher). All other solvents used for extraction and chromatography were of analytical quality and were twice distilled before use.

Alpha-terthienyl (alpha-T) was prepared by our collaborators Drs L.C. Leitch and P. Morand (Department of Chemistry, University of Ottawa) by a large-scale version of a Grignard synthesis developed in their laboratory (Philogene et al., 1984). Samples of 5-(4-hydroxy-1-butenyl)-2,2'-bithienyl (BBT-OH), 5-(4-acetoxy-1-butenyl)-2,2'-bithienyl (BBT-OAc), 5-(3,4-diacetoxy-1-butenyl)-2,2'-bithienyl (BBT-2OAc) and (5-buten-3-ynyl)-2,2'-bithienyl (BBT) (Figure 2), were kindly supplied by Dr. R. Sutfeld, University of Munster, F.R.G.. Reference compounds were checked for purity by HPLC, UV and mass spectroscopy (M.S.).

2.1.2 Qualitative analysis

2.1.2.1 Extraction

Fresh plant tissue (0.3-1.5 g) was ground in 95% ethanol (20 ml) using a motor driven homogenizer (Lourdes, Model MM1) and the homogenate was filtered under vacuum on a Buchner funnel using Whatman #1 filter paper. The filter containing the plant residue was washed with an equal volume of 95% ethanol. The resulting filtrate was then refiltered using 0.45 μ , 47 mm HPLC nylon filter (Schleicher and Schuell, Inc., N.H) to remove any residual particulate matter, before being reduced to a small volume (2 ml) on a rotary evaporator (30°C). The concentrated fraction was stored at -20°C until HPLC analysis. The procedure was conducted under reduced lighting to avoid photodegradation of thiophene compounds.

2.1.2.2 High pressure liquid chromatography

HPLC, used for qualitative and quantitative analysis of thiophenes, was performed by isocratic elution with acetonitrile-water (70:30, v/v) at a flow rate of 1 ml/min (Beckman 110A pump and 420 controller). A reversed-phase Ultrasphere 5 μ ODS 4.6 mm x 25 cm (RP-C₁₈) column was used for analytical work (injection volume: 20 μ l). A Beckman 165 Variable Wavelength detector set at 250 nm was used to detect eluted compounds. The eluate was scanned (220-400 nm) on-line with a high speed linear spectrophotometer, allowing rapid identification of the compounds.

Peaks corresponding to thiophene compounds were recognized by comparison of their retention time and UV spectrum with those of reference material. The identity of each substance was later confirmed by isolation, purification and spectroscopic identification of each thiophene derivative.

2.1.2.3 Isolation and purification.

Identification of thiophenes was conducted after isolation from crude plant extracts. Spectral analysis of pure samples (UV spectra and mass spectra) and comparison with published data were used for structural elucidation of compounds.

Bulk extraction of Tagetes erecta was achieved by homogenizing fresh tissue (500 g) in 95% ethanol (1 l) and filtering twice as previously described (see Extraction, section 2.1.1). After evaporation of the resulting filtrate to dryness on a rotary evaporator (30°C), the residue was dissolved in hexane (2 ml). This solution was subjected to spinning plate thin layer chromatography (Chromatotron Model 7924T, Harrison Research Inc.). The rotor was coated with a 1 mm thick absorbent layer made of Silica Gel GF-Gypsum containing 13% binder ($\text{CaSO}_4 \cdot 1/2\text{H}_2\text{O}$). Following complete saturation of the absorbent layer with hexane and partial equilibration, the concentrated plant extract was introduced into the Chromatotron. Gradient elution, starting with a low polarity solvent to increasing amounts of a polar solvent was used to develop the chromatogram. This was con-

ducted at a flow rate of 3 ml/min in 5 consecutive steps with solvent systems consisting of 100% hexane (for step 1, 10 min elution time) and 2%, 6%, 10% and 20% ethyl acetate in hexane (for steps 2 to 5 respectively, elution time: 5 min each). This was followed by an acetonitrile wash to remove residual polar compounds.

Collected fractions were monitored for thiophenes with a Pye-Unicam SP8-100 spectrophotometer. Fractions containing thiophenes were further purified using preparative reverse-phase HPLC. The column used for preparative work was a Beckman ultrasphere 5 μ ODS 10 x 25 cm equipped with a 200 μ l sample loop. The samples were eluted with acetonitrile-water (70:30, v/v) at a flow rate of 2 ml/min with the UV detector set at 250 nm. Fractions containing thiophene derivatives were collected and extracted once with hexane and once with ethyl acetate after the addition of water. The two extracts were then pooled together, evaporated to dryness using a stream of nitrogen and the residue concentrated in ethyl acetate before sending to M.S. analysis. All M.S. analyses (direct probe) were performed by the Chemistry Dept., University of Ottawa, using a VG 7070-E mass spectrometer.

2.1.3 Quantitative analysis

Peaks attributable to thiophenic compounds detected on the HPLC chromatograms of crude plant extracts were quantified by reference to standard concentration curves. The concentration curves were determined by preparing various dilutions of the thiophene reference substances in ethanol. Each dilution (20 μ l) was then injected into the reverse-phase HPLC column. The peak heights of the resulting chromatograms were estimated and concentration plots were drawn.

2.2 Abiotic and biotic treatments of 4 day old seedlings of *Tagetes erecta*

2.2.1 Plant material

Seeds of *Tagetes erecta* var "Crackerjack" purchased from Stokes Seeds Inc., Buffalo, N.Y., were imbibed between sheets of moist filter paper (Whatman #1) at 25°C for 96 hrs. The pericarps were then carefully removed from germinating seeds, the seedlings separated and washed with distilled water. These could be prepared and handled without inflicting severe damage.

2.2.2 Treatment with an abiotic agent

Lots of 200 seedlings were submerged in 100 ml solutions of HgCl_2 (0.001 M) and left for different periods of time (10, 30, 60 and 120 min). After treatment, germinated seedlings were washed 5 times with 100 ml distilled water.

Treated and control plants, which had been immersed in water not containing HgCl_2 , were placed in 10 cm plastic Petri plates lined with Whatman #1 filter paper (10 seedlings per plate) and incubated for 1, 2 and 3 days in a controlled environment chamber (Conviron S10h) at 25°C, 70% R.H., 16:8 hr light:dark with illumination provided by solar simulating Vita Lights (Durotest 48T12). The light intensity at plate level was estimated to be 17 w/m^2 using a Yellow Springs Instrument 65-A radiometer. At the end of each incubation period, triplicate samples from each treatment (50 seedlings per sample, 1-1.5 g fresh weight) were extracted and stored at -20°C until thiophene analysis by HPLC (as described in section 2.1).

2.2.3 Treatment with a biotic agent

2.2.3.1 Fungal cultures

Alternaria taquetica, isolate 16 MV obtained from material collected in Los Mochis, Sinaloa, Mexico, was kindly supplied by Dr. P. Cotty, College of Agriculture, University of Arizona, and was chosen as the pathogenic agent. The culture was maintained on a modified V-8 medium containing 10% (v/v) V-8 vegetable juice solidified with 2% agar (w/v), at 25°C, 10 cm below a cool-white fluorescent tube (20 w) using a 16 hr light, 8 hr dark photoperiod (light intensity at plate level = 2 w/m^2). Spores were obtained from 2 weeks old cultures grown under these conditions.

2.2.3.2 Preparation of monospore cultures

Fungal cultures derived from single spores were prepared for use as stock cultures. Sterile distilled water (5 to 10 drops) was spread onto the surface of the sporulating fungus. After agitation, a loopful of this suspension was transferred using a flamed broach to a plate containing the appropriate fungal growth medium and mixed with 0.5 ml of sterile distilled water. One loop of the resulting mixture was uniformly dispersed into 0.5 ml of sterile distilled water added to another Petri dish containing the suitable medium. The process was repeated until various dilutions of the fungal conidia were obtained. Dilution plates were then incubated at 25°C, 10 cm below a cool-white fluorescent tube (20 w) with continuous illumination and examined after 24 hrs under a stereoscopic microscope at 50x magnification to check for germination of the conidia. Small pieces of agar containing single germinating conidia were transferred to Petri dishes containing the suitable fungal medium using a flamed broach (size XX Fine, Union Broach Corporation). Five plates, each containing one germinating conidia, were prepared for each fungal isolate used in this study. The plates were incubated at 25°C with continuous fluorescent illumination. After 5 days, a small piece of agar containing mycelia was transferred from each monoconidial culture to a dozen of 2% agar slants. These were kept for 24 hrs under the conditions just described prior to storage at 4°C for use as stock monosporal cultures.

2.2.3.3 Inoculation of plants

For the production of the inoculum, sterile distilled water was pipetted onto a 2 weeks old A. tagetica culture grown from single spores on the modified V8 medium. The surface of the culture was scraped using a sterile glass microscope slide and the suspension was passed through a layer of cheesecloth to collect mycelial fragments. The resulting mycelial mat was washed with sterile deionized water prior to fragmentation in a Waring blender for 90 s. The suspension was diluted to a final concentration of 1 mg mycelial dry weight per ml of water and was used to inoculate plants. Plastic Petri dishes (10 cm) lined with moist Whatman #1 filter paper, each containing 10 T. erecta seedlings prepared as previously described (see section 2.2.1, plant material) were sprayed to runoff with the fungal inoculum. Control plants were sprayed with distilled water. All plates were incubated for 0, 1, 2, 3 and 4 days in a controlled environment chamber (Conviro S10h) at 25°C, 70% R.H., under solar simulating Vita lights (Durotest 48T12) with a 16:8 hr light:dark cycle. After the required period of incubation, plants were examined for the presence of disease symptoms and triplicate samples from each treatment (50 seedlings per sample, 1-1.5 g fresh weight) were extracted and stored at -20°C until thiophene analysis and quantification by HPLC (as described in section 2.1).

2.2.3.4 Reisolation from diseased plants

To check for the presence of the pathogen in diseased tissue obtained following fungal treatment, segments 0.5 cm in length were cut from different parts of the infected plant using a razor blade. The excised pieces were then surface-sterilized in 70% ethanol for 3 min and then rinsed in sterile distilled water (3 x 10 min rinses). The margins of each excised piece were then cut out and the remaining 2 mm central strip plated in a sterile medium containing 53 mg/l of Rose Bengal and 20 mg/ml of Difco agar. The plates were then incubated for 5 days at 25°C, 10 cm below a white-cool fluorescent tube (2 w/m² at plate level) with continuous illumination prior to examination and isolation of the fungus for comparison with starting material.

2.3 Biotic treatment of 2 weeks old seedlings of *Tagetes erecta* under aseptic conditions.

2.3.1 Plant material

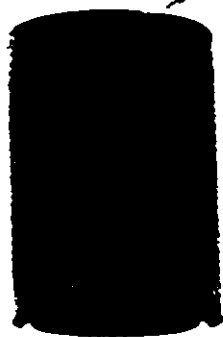
T. erecta var "Crackerjack" (from Stokes Seeds Inc., Buffalo, N.Y.) was also used in this study. Seeds were surface sterilized before germination.

Lots of approximately 500 seeds were soaked for 3 hrs in distilled water (500 ml) containing 0.04% powdered detergent (Sparkleen, Fisher scientific). The detergent was added to decrease surface tension and thus facilitate imbibition of the seeds. Following hydration, the seeds were

submerged in 500 ml of a 10% Javex solution for 6 min and then rinsed 3 times with 500 ml sterile distilled water. For germination, seeds were plated on 2% agar (10 seeds per Petri plate) and incubated in the dark for 96 hrs at 25°C.

Following the required period of incubation, each surface sterilized seedling (5 to 6 cm) was aseptically transferred to a sterile glass tube (25 x 200 mm) containing 35 g of 2 mm glass beads covered with 10 ml of a 75% Hoagland's solution (Appendix 1). The bottom of each tube was fitted with a black plastic sleeve to prevent entry of light into the root zone. All the culture tubes (Figure 4) (in total: 600 tubes) were incubated for 10 days in an environmental growth chamber (Conviroon S10h) at 25°C, under solar simulating Vita light (Durotest 48T12) with a 16:8 hr light:dark photoperiod. The light intensity reaching the plants inside the tubes was 15 w/m² as measured using a YSI-Kettering model 65A radiometer.

Figure 4: Sterile culture tube used for the growth of T. erecta. Each 4 days old seedling was aseptically transferred to a tube containing 75% Hoagland's solution (Appendix 1) and incubated for 10 days in a controlled environment chamber at 25°C, 70% R.H. with a 16:8 hr light:dark photoperiod (light intensity reaching the plants = 15 w/m²), prior to fungal infection.



2.3.2 Fungal cultures

Two isolates of Fusarium oxysporum differing in their virulence towards T. erecta were chosen as the fungal pathogens: a) F. oxysporum var callistephi race 2 (FOVC race 2), described by Olsen (1965) as causing a vascular wilt of the African marigold, was obtained from the Commonwealth Mycological Institute, England (culture # 141122), and b) F. oxysporum f.sp. radicis lycopersici (FORL) was obtained from Dr. W. Jarvis, Plant Pathology, Harrow Research Station. This organism was not known previously to infect marigolds.

Monospore cultures of FOVC race 2 and FORL were grown on potato sucrose agar (PSA) prepared as described in Appendix 1, 10 cm below a daylight fluorescent lamp (2 w/m² reaching the plates) with continuous illumination. After 10 days of growth, conidia were harvested by flooding plates of the sporulating fungi with sterile distilled water and agitating the surface of the culture using 2 mm sterile glass beads. The conidial suspension was filtered twice through sterilized muslin to remove mycelial fragments. The filtered spores were centrifuged for 15 min at 1,000 x g and washed twice with 10 ml of sterile distilled water before adjusting the concentration to approximately 5×10^9 spores/ml as estimated using a Klett densitometer previously standardized from haemocytometer counts.

2.3.3 Inoculation of plants

Two weeks old T. erecta seedlings grown under aseptic conditions as previously described (section 2.3.1) were inoculated with 1 ml of a 7 day old hyphal solution in Fries medium (Appendix 1). This fungal solution was prepared by inoculating 200 μ l of the 5×10^9 spores/ml suspension in a 250 ml Erlenmeyer flask containing 200 ml of the sterilized Fries medium, and growing the fungus with continuous shaking at 100 rpm in the dark at 25°C. Fries medium (1 ml) was administered to control seedlings.

All culture tubes were returned to the growth cabinet after treatment and incubated for 0, 4, 8 and 12 days. After each incubation period, plants were examined for the presence of disease symptoms, and the root and stem portions below the point of attachment of the cotyledons were excised from each plant using a razor blade. For each treatment (control plants, plants infected with FOVC race 2 and plants infected with FORL), triplicate samples consisting of roots and stems excised from 15 plants (0.3-0.5 g fresh weight) were extracted and stored at -20°C until thiophene analysis by HPLC (section 2.1).

2.4 Fungitoxicity and metabolism of thiophenes

2.4.1 Effects of alpha-terthienyl on fungal growth

The liquid bioassay method used to determine the biological activity of alpha-terthienyl (alpha-T) against two fungal isolates in the absence and in the presence of near UV light was essentially that of Bourque *et al.* (1985). The fungi used were Fusarium oxysporum var callistephi race 2 (FOVC race 2) and F. oxysporum f.sp. radicis lycopersici (FORL) previously described (section 2.3.2).

Erlenmeyer flasks (25 ml), each containing 10 ml of sterilized Fries medium, were inoculated with 100 μ l of a spore suspension (5×10^9 spores/ml) prepared from 10 day old fungal cultures grown on PSA as described in section 2.3.2. Appropriate amounts of alpha-T were then aseptically added to each flask in 10 μ l of 95% ethanol to achieve final concentrations of 0.001, 0.01, 0.1, 1, 10, 50 and 100 μ g/ml. Controls received 10 μ l of 95% ethanol without alpha-T. Each treatment consisted of 5 replicates. The conidial cultures were then incubated at 25°C with continuous shaking at 150 rpm in the dark or in the presence of near UV radiation (16:8 hr, light:dark photoperiod). The source of UV-A irradiation consisted of a bank of 4 Westinghouse blacklight blue tubes (Westinghouse F 20T12/BLB, emission in the 300-400 nm range). The light intensity of less than 400 nm reaching the fungal cultures was estimated to be 4 w/m^2 using a YSI 65-A radiometer and a Kodak Wratten filter # 2A. The Erlenmeyer flasks used were then checked for

UV absorption and were found to be transparent to wavelengths above 300 nm.

Following a 48 hr incubation period, 100 μ l aliquots were removed from control and from alpha-T treated samples (1-100 μ g/ml, +UV-A) and examined under phase contrast microscopy to check for any symptom of toxicity. After 72 hrs, the mycelial dry weights of the fungal cultures were determined. The fungi were collected on Whatman # 2 filter paper (4.25 cm) using a vacuum filtration apparatus and weighed after 24 hrs at 75°C. Each experiment was repeated twice. The results presented are the adjusted mycelial dry weights obtained after subtracting the weight of the culture medium.

2.4.2 In vitro metabolism of alpha-terthienyl by fungi

Fusarium oxysporum f.sp. radicis lycopersici (FORL), described previously (section 2.3.2), was used in this study.

To obtain mycelial growth, 100 μ l of a spore suspension (3×10^9 spores/ml), prepared from 10 day old cultures of FORL grown on PSA (as described in section 2.3.2) was inoculated into 300 ml of sterile Fries medium contained in 500 ml erlenmeyer flasks to give a final concentration of 10^6 spores/ml. The culture was then incubated at 25°C on a reciprocal shaker (150 rpm) in the dark for 48 hrs.

Uniformly labelled ^3H -labelled alpha-T with a specific activity of 1.3 mc/nmole was prepared by our collaborator

Dr. Werstiuk, Chemistry Dept., University of Mc Master, by a high temperature dilute acid procedure developed in his laboratory. The purity of ^3H -alpha-T was assessed by cochromatography with pure unlabelled alpha-T using various solvent systems (see below) and was found to be above 97%. The labelled alpha-T (10,000 disintegrations min / μg) was aseptically added to the culture medium in 30 μl of 95% ethanol to obtain a concentration of 0.3 $\mu\text{g}/\text{ml}$. This concentration was observed to have no inhibitory effects on the growth of FORL in the dark (see results, section 3.4.1). Two types of controls accompanied this experiment and consisted of ^3H -alpha-T or unlabelled alpha-T (final concentration: 0.3 $\mu\text{g}/\text{ml}$) added to flasks containing sterile Fries medium only. Three replicates were used for each treatment.

After the media had been shaken with alpha-T at 25°C for 24 hrs, 50 ml of acetone were added to each flask to halt metabolic activity. The mycelium was then filtered on Whatman # 2 filter paper and washed with acetone (50 ml) to extract alpha-T and its derivatives from the mycelia. Acetone was removed from the filtrate by evaporation under reduced pressure. The aqueous fraction remaining was extracted once with hexane (300 ml) and once with ethyl acetate (300 ml). Both extracts were pooled and evaporated to dryness using a rotary evaporator. The residue was suspended in 200 μl of 95% ethanol. Control samples were extracted and concentrated in a similar way. In order to estimate

the quantitative recovery of the labelled material using this extraction procedure, filters containing the mycelial mat, as well as 20 μ l samples from each of the water and the combined hexane and ethyl acetate fractions, were placed in 10 ml scintillation fluid (Scintiverse 2, Fisher) and kept for radioactivity determination.

Thin layer chromatography (TLC) was used to identify possible metabolic products of alpha-T. The concentrated ethanolic extracts (50 μ l) from control and treated samples were spotted on 250 μ m Silica gel TLC plates (Baker Si 250F, UV₂₅₄ TLC plates). Three chromatographic solvent systems were used to develop the chromatograms: a) hexane:ethyl acetate (1:1 v/v), b) hexane:ethyl acetate (25:3 v/v) and c) petroleum ether:acetone (95:5 v/v). The chromatograms were subsequently examined under UV light. To determine radioactivity incorporated into the UV-visible spots, these were scraped off the plates and eluted with distilled acetone (10 ml) into scintillation vials, the silica gel was removed by filtration through cotton. The acetone was subsequently evaporated from the scintillation vials using a stream of nitrogen prior to the addition of scintillation fluid (Scintiverse 2, Fisher) and determination of radioactive counts. All counting was performed using a Beckman LS-150 liquid scintillation counter. Quench corrections were made using quench curves derived from external standards. The efficiency of counting was found to be between 30% and 35%.

2.4.3 Fungitoxicity of thiophene standards

The thiophene reference substances BBT-OH, BBT-OAc, BBT-2OAc and BBT (Figure 3) were examined for their ability to inhibit germination of conidia of the two pathogenic fungi FOVC race 2 and FORL described in section 2.3.2.

A 20 μ l aliquot of each stock solution of the test compound in ethanol containing 25 μ g/ml was pipetted into the cavities of microscope well slides. Following evaporation of the solvent, the dried deposits were then covered with 100 μ l of a spore suspension of 5×10^4 spores/ml in Fries media. Control spores received 20 μ l ethanol in absence of the test compounds. The slides were placed in covered Petri plates lined with moistened filter paper and incubated for 18 hrs at 25°C in the presence of UV-A light, with illumination provided by a bank of 4 Westinghouse blacklight blue tubes (Westinghouse F 20T12/BLB). The light intensity reaching the plates was estimated to be 4 w/m² using a YSI 65-A radiometer. After the required period of incubation, the percentage germination was determined by examining more than 100 spores per sample by phase contrast microscopy. All treatments were duplicated and each experiment carried out twice.

2.5 Statistical analysis of results

Statistical analysis performed on the data obtained in this study (Appendices 3 to 6) was achieved using an Amdahl 470/V7A processor running the IBM VM 370/CMS System (University of Ottawa) and the statistical package for social sciences (SPSS).

Chapter III

RESULTS

3.1 Chemical analysis of thiophenes

Thiophene derivatives present in Tagetes erecta were separated using reverse phase liquid chromatography (HPLC). This has been shown to be a very efficient and sensitive method for qualitative and quantitative analysis of thiophenes in crude plant extracts, as well as for preparative work (Downum and Towers, 1983).

Three studies involved the use of HPLC. The first two studies entailed the examination of thiophene levels in T. erecta at various times after treatment of young germinating seedlings (4 days old) with an abiotic agent (mercuric chloride) and with a biotic agent (Alternaria tagetica). Changes in thiophene concentrations occurring in roots and lower stems of T. erecta grown aseptically at different times after inoculation of 2 weeks old seedlings with fungal pathogens were determined in the third study.

3.1.1 Qualitative analysis

3.1.1.1 Analytical HPLC

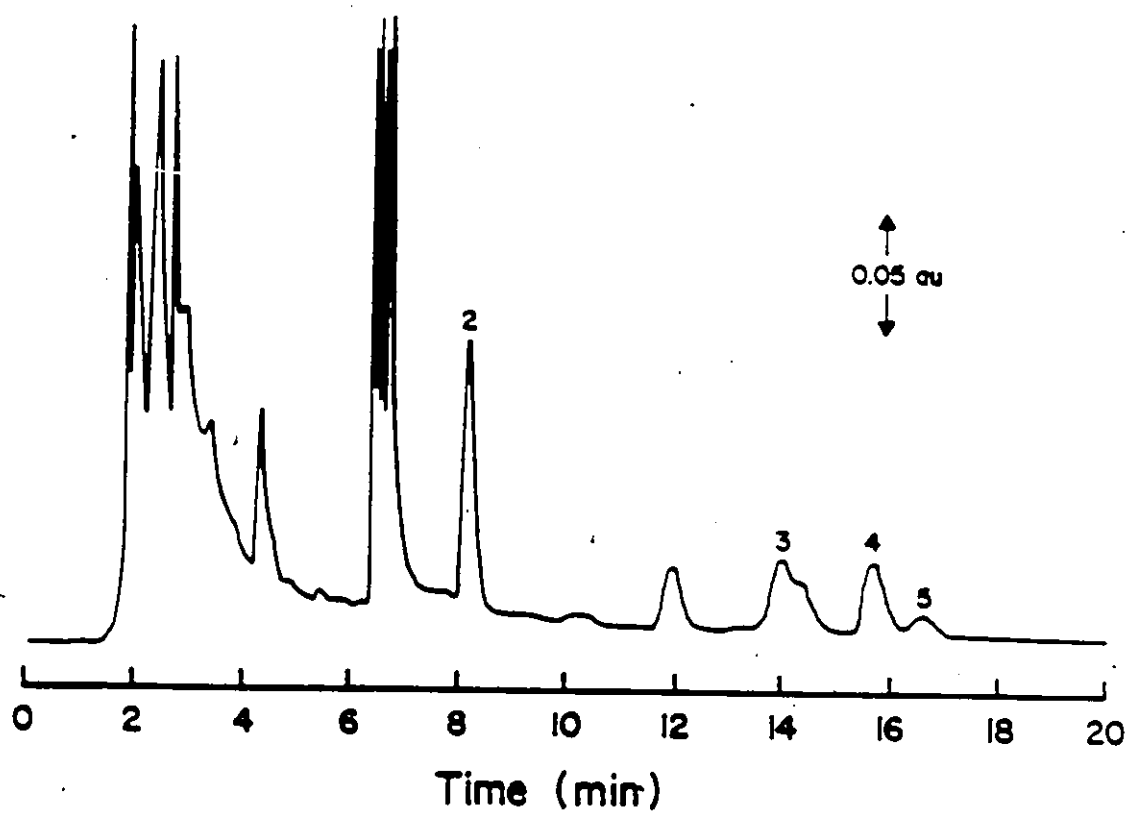
The HPLC chromatograms showing compounds absorbing at 250 nm in extracts of T. erecta are presented in Figure 5. In the extracts of whole young germinating seedlings, 4 different substances were detected which displayed characteristic UV spectra of thiophenes (Figure 5a). Extracts from roots and lower stems of older seedlings grown in Hoagland's solution showed a different thiophene pattern (Figure 5b), two of the detected thiophenes were the same as those present in younger seedlings, as shown by their coinciding peaks in the 2 chromatograms and their similar UV spectra. All of these substances, except one, had comparable retention times on the chromatograms to those of authentic reference substances (Table 4). The purity and identity of these reference compounds had been confirmed by spectroscopy (MS and UV) and by comparison with spectral data described in the literature (Appendix 2).

Compound 1, which eluted first ($R_f = 4.5$ min) had the same retention time as the most polar thiophene standard 5-(4-hydroxy-1-butenyl)-2,2'-bithienyl (BBT-OH). The peaks of compounds 2 and 3 coincided with those of authentic 5-(4-acetoxy-1-butenyl)-2,2'-bithienyl (BBT-OAc) ($R_f = 8$ min) and 5-(3-buten-1-ynyl)-2,2'-bithienyl (BBT) ($R_f = 13.8$ min) respectively. The peak of substance 4 did not correspond with any of the reference compounds. Compound 5,

retained the longest, on the column ($R_T = 16.3$ min) was comparable to synthetic standard 2,2':5',2''-terthienyl (alpha-T), the least polar compound. The structures of the thiophene standards are shown in Figure 2.

Figure 5: HPLC chromatograms of compounds absorbing at 250 nm present in ethanolic extracts of T. erecta (a) from 1 week old seedlings and (b) from roots and stems of 3 weeks old seedlings. Plant growth conditions and HPLC parameters are described in Materials and Methods (Section 2).

a)



b)

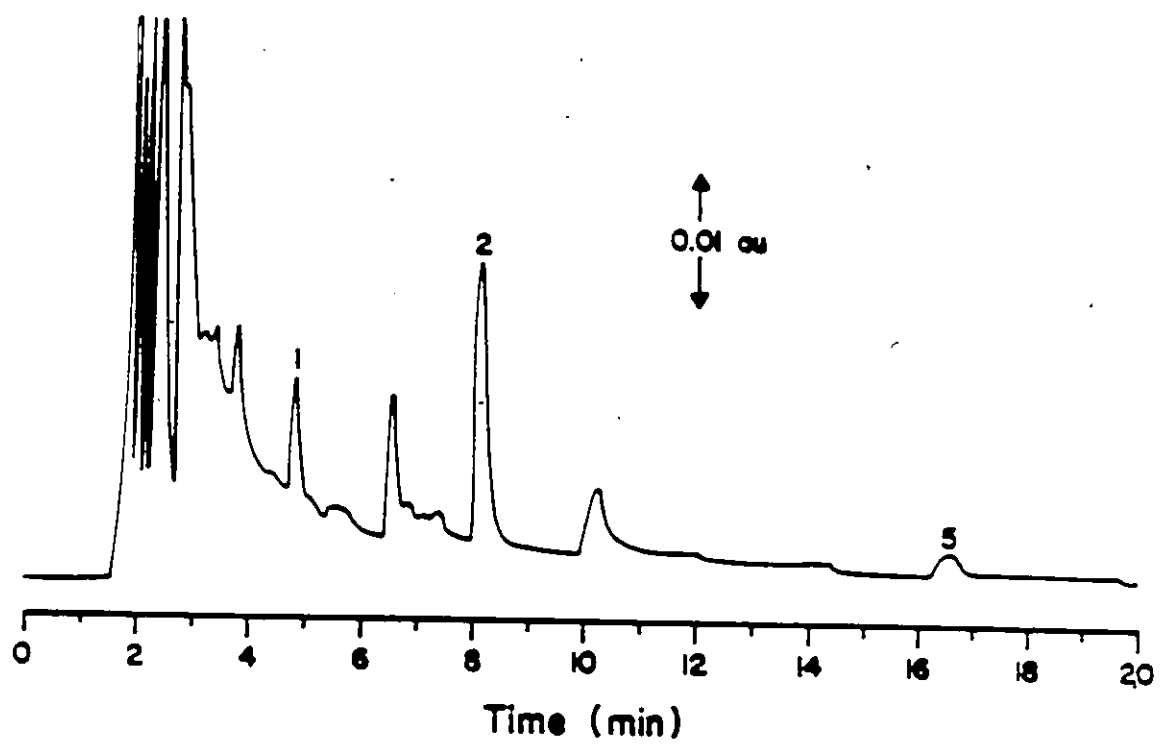


TABLE 4

RETENTION TIMES (t_R), ADJUSTED RETENTION TIMES (t'_R)
AND MAXIMUM ABSORBANCE WAVELENGTHS (λ_{MAX}) OF COMPOUNDS
1 TO 5 separated by HPLC ($t_0 = 1.6$ min)

COMPOUND	t_R (min)	t'_R (min)	λ_{MAX} (nm)
1	4.7	3.1	335
2	8.0	6.4	335
3	13.8	12.2	345
4	15.5	13.9	335
5	16.3	14.7	350

3.1.1.2 Purification and chemical identification of thiophenes in T. erecta

In order to confirm the identity of compounds 1 to 5 detected in HPLC chromatograms of T. erecta extracts, each substance was isolated and subjected to combined GC-MS analysis. Separation was achieved using spinning plate TLC combined with preparative reverse phase HPLC.

Spinning plate TLC was a relatively efficient and rapid method for pre-purification of samples. Gradient elution with increasing amounts of a polar solvent (0% ethyl acetate in hexane increasing to 20% ethyl acetate in hexane during a 30 min time period) yielded a relatively discrete separation of bands. Eluted fractions collected from the Chromatotron were examined for the presence of thiophenes by UV spectroscopy. Compounds 3, 4 and 5 were not resolved by this method and were eluted together ($R_f = 8$ min). Compound 2 was eluted when the percentage of the polar solvent was increased to 15-20% ethyl acetate in hexane ($R_f = 26$ min). The more polar substance (1) was removed from the absorbent layer by cleaning up with a UV transparent polar solvent (acetonitrile) and had a retention time of 38 min.

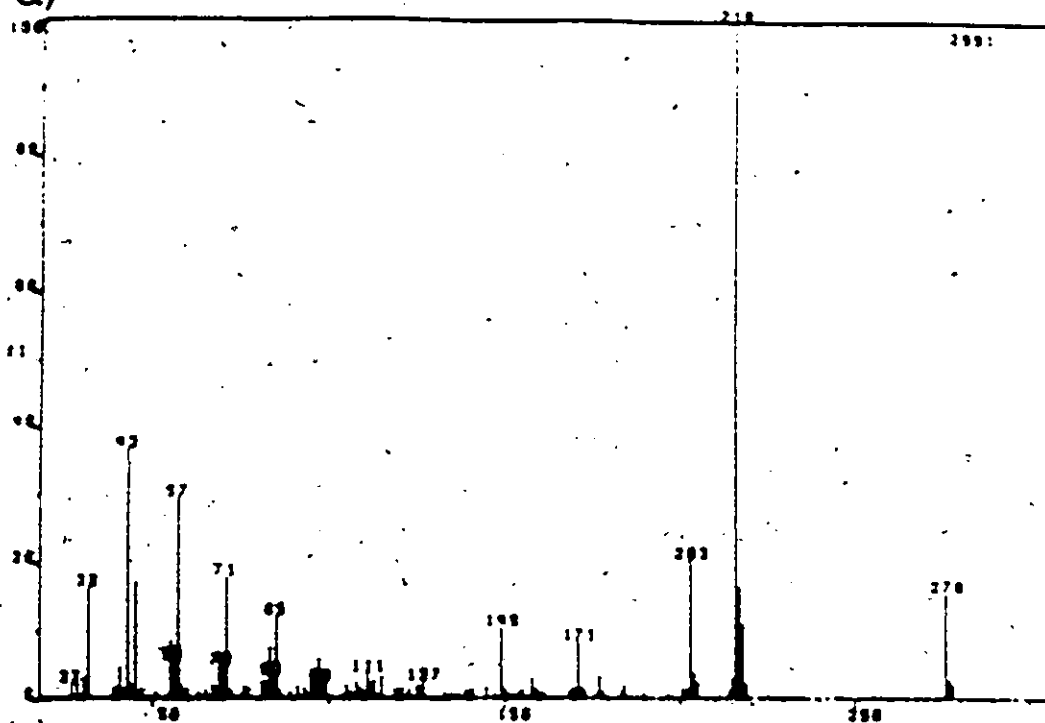
Compounds separated by spinning plate TLC were then subjected to preparative reverse phase HPLC for further purification. This was achieved by elution with an isocratic solvent system (Acetonitrile:Water (70:30, v/v)). Each thiophene eluted from the preparative column and correspond-

ing to one peak on the HPLC chromatogram, was collected separately. The purity of each thiophene derivative was checked by reinjection onto the preparative column which ultimately yielded one pure peak on the HPLC chromatogram. Isolated thiophenes were then submitted to direct probe MS analysis. Analysis of the mass spectra (Figure 6) and comparison with the spectral properties of the reference compounds and with published data (Appendix 2) permitted the identification of compounds 2, 3 and 5 as BBT-OAc, BBT and alpha-T respectively. The elemental composition of compound 4 was determined by accurate mass measurement with the VG 7070E mass spectrometer which indicated that the molecular formula was $C_{13}H_8S$. This compound was tentatively identified as being one of the polyine monothiophenes shown in Figure 7, based on UV spectral comparisons.

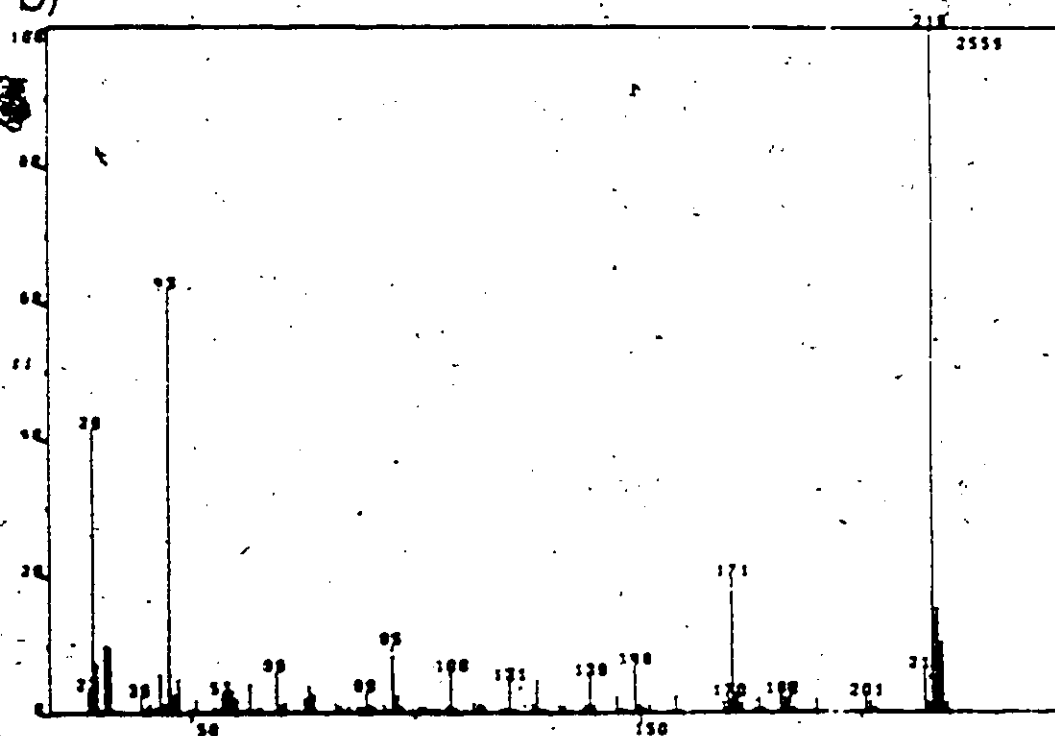
Structural verification of compound 1, expected to be BBT-OH was difficult because of its unstable nature. Identification was achieved by alkaline hydrolysis of BBT-OAc (saturated ethanolic KOH, 5 min incubation at room temperature) which yielded BBT-OH. The conversion of BBT-OAc to BBT-OH as detected by analytical reverse phase HPLC is depicted in Figure 8. It can be clearly seen from the chromatograms that the product of the alkaline hydrolysis: BBT-OH, has an identical retention time to that of compound 1. Samples of (1) were collected and were shown to display a UV absorption spectrum identical to that of the authentic BBT-OH prepared by hydrolysis.

Figure 6: Mass spectra of compounds isolated from seedlings of T. erecta (a) compound 2 (BBT-OAc), (b) compound 3 (BBT), (c) compound 4 (C H S) and (d) compound 5 (alpha-T) (see Figure 2 for structural formulae). Relative abundance is plotted against m/z.

a)



b)



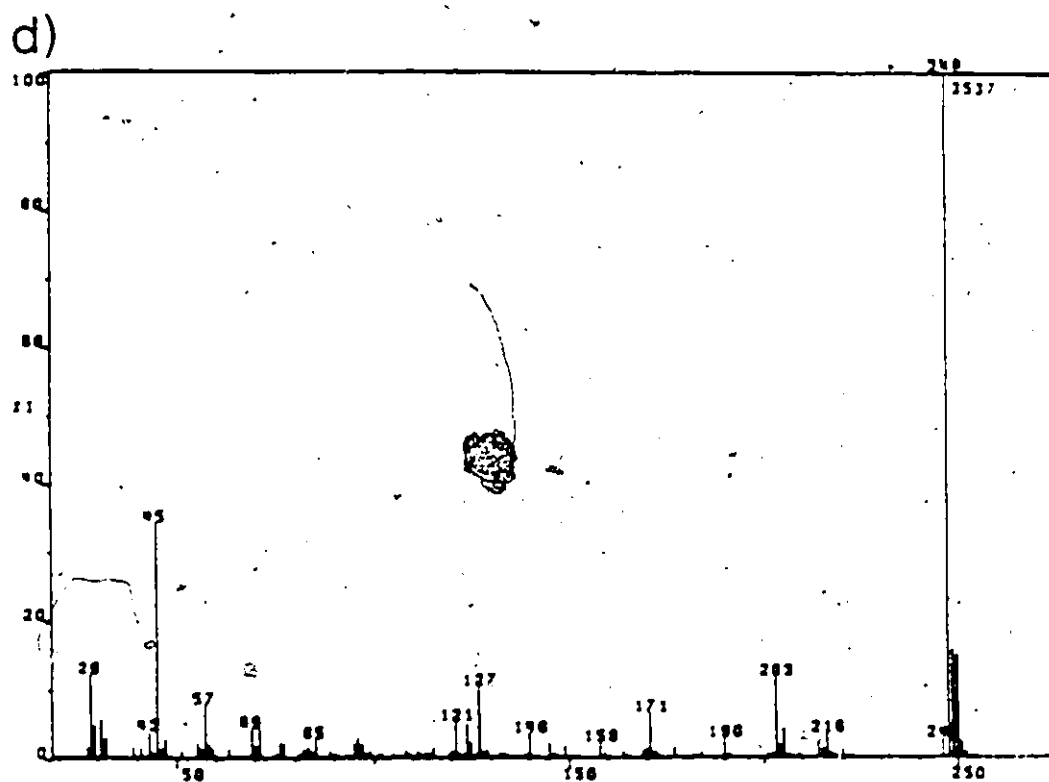
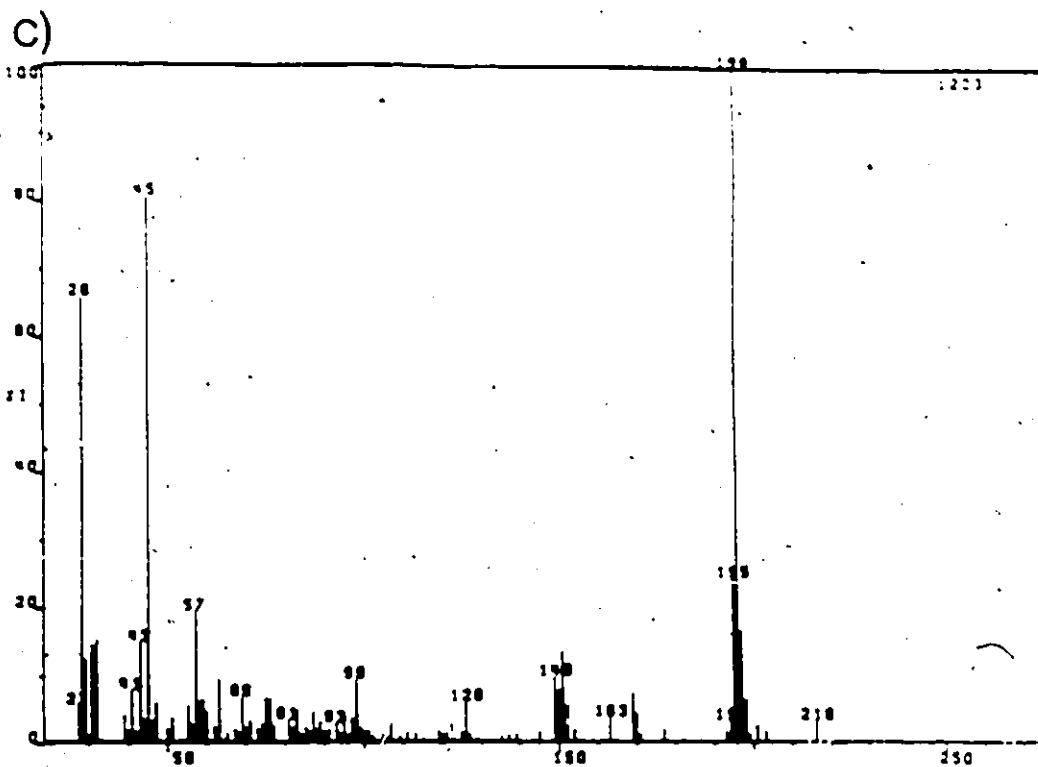
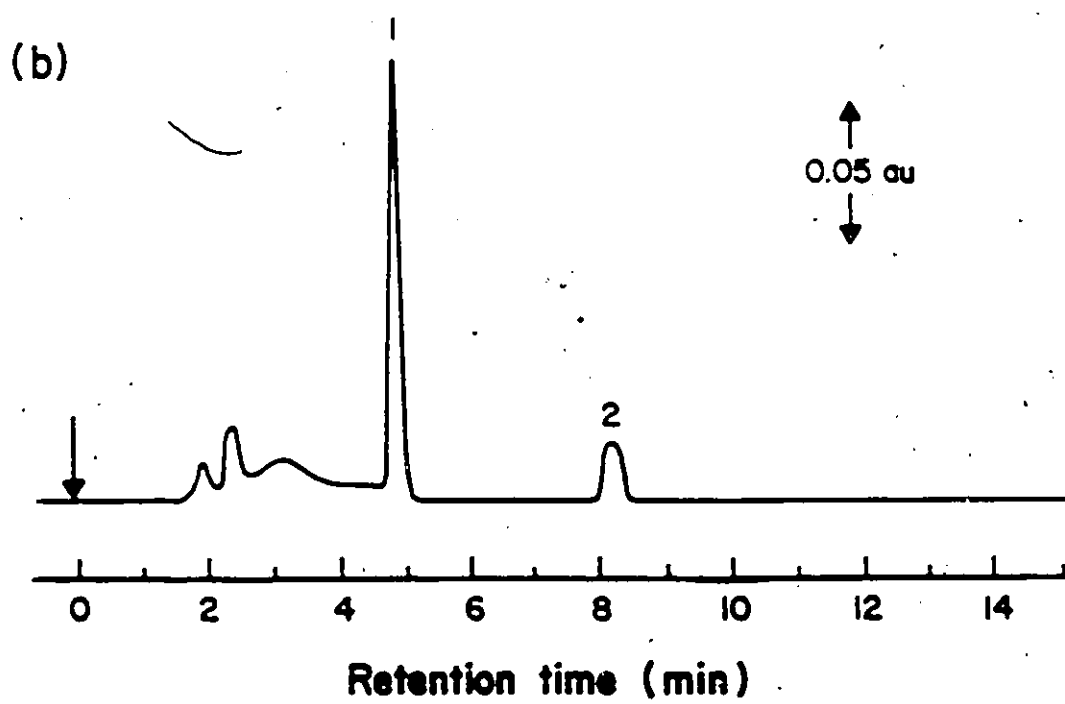
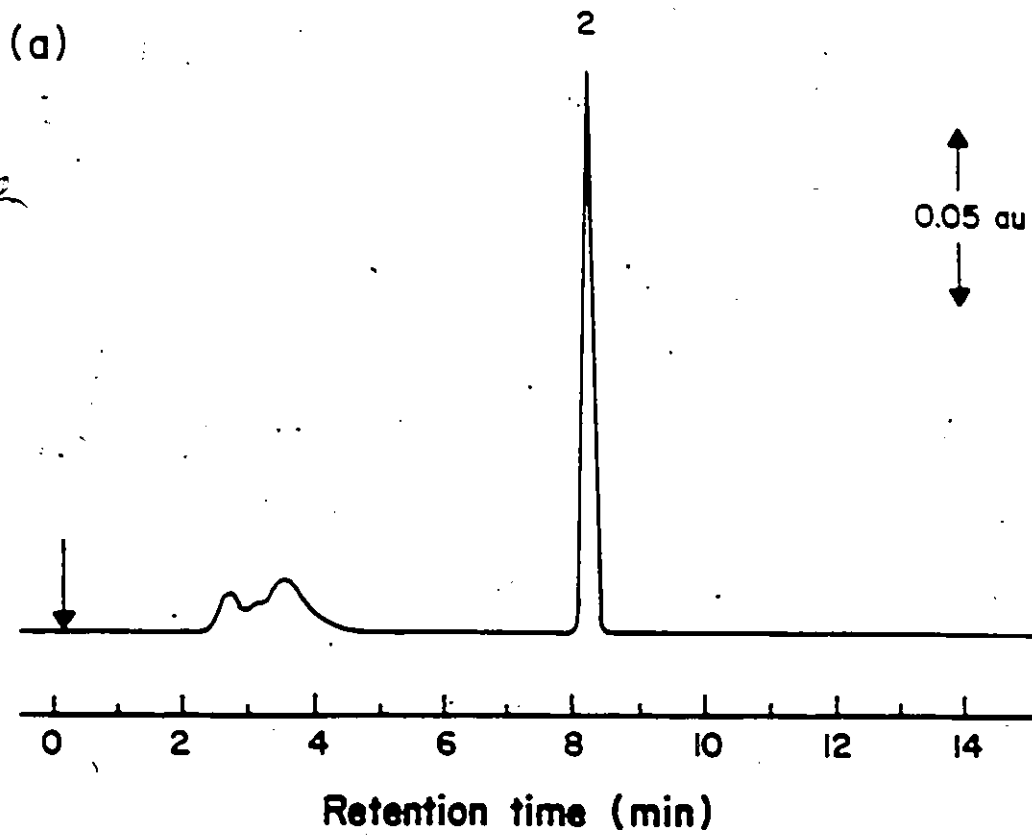


Figure 7: Tentative identification of compound 4 from extracts of T. erecta seedlings. The UV absorption maxima (nm) are followed by published extinction coefficients in parentheses.

FORMULA	MOLECULAR WEIGHT	POSSIBLE CHEMICAL STRUCTURE	UV-MAXIMA (nm)
a) $C_{13}H_8S$	196	$H_3C[C\equiv C]_2$  $C\equiv C-CH=CH_2$	332 (30300) 357 (26200)
b) $C_{13}H_8S$	196	$H_3C-C\equiv C$  $[C\equiv C]_2-CH=CH_2$	338 (31600) 358 (22400)

Figure 8: Conversion of BBT-OAc (compound 2) to BBT-OH (compound 3) as detected by analytical HPLC. The chromatograms show the changes in the levels of the compounds occurring (a) before and (b) after alkaline hydrolysis of BBT-OAc.



3.1.2 Quantitative analysis

Quantitation of thiophene peaks obtained in the HPLC chromatograms of *T. erecta* extracts was achieved by direct comparison with the standard concentration curve of the corresponding thiophene. When various dilutions of the thiophene standards were examined, the HPLC detector response (recorded as peak height from the HPLC chromatogram) / concentration plots were linear for all thiophenes with correlation values (r) > 0.99 (Figure 9). A calibration curve was determined for every reference compound, since the molar extinction coefficient at the UV-A λ_{max} differed for each of them (Appendix 2). Because of the lack of an authentic reference sample for substance 4 tentatively identified as being a polyine monothiophene (Figure 7), quantitation was achieved by reference to the alpha-T standard concentration curve since the molar extinction coefficients of the 2 probable structures are comparable to that of alpha-T at the monitoring wavelength (350 nm) (Figure 7, Appendix 2).

Previous work demonstrated that HPLC was a very sensitive method, able to detect nanomolar quantities of thiophenes (Downum and Towers, 1983). This was confirmed in this study where even lower amounts of thiophenes could be quantified. The minimal detectable levels were 17 ng/seedling for BBT-OH, 27 ng/seedling for BBT-OAc and 31 ng/seedling for alpha-T and compound 4. The minimal instant detector response was 0.02 mm peak height on the HPLC chromatogram.

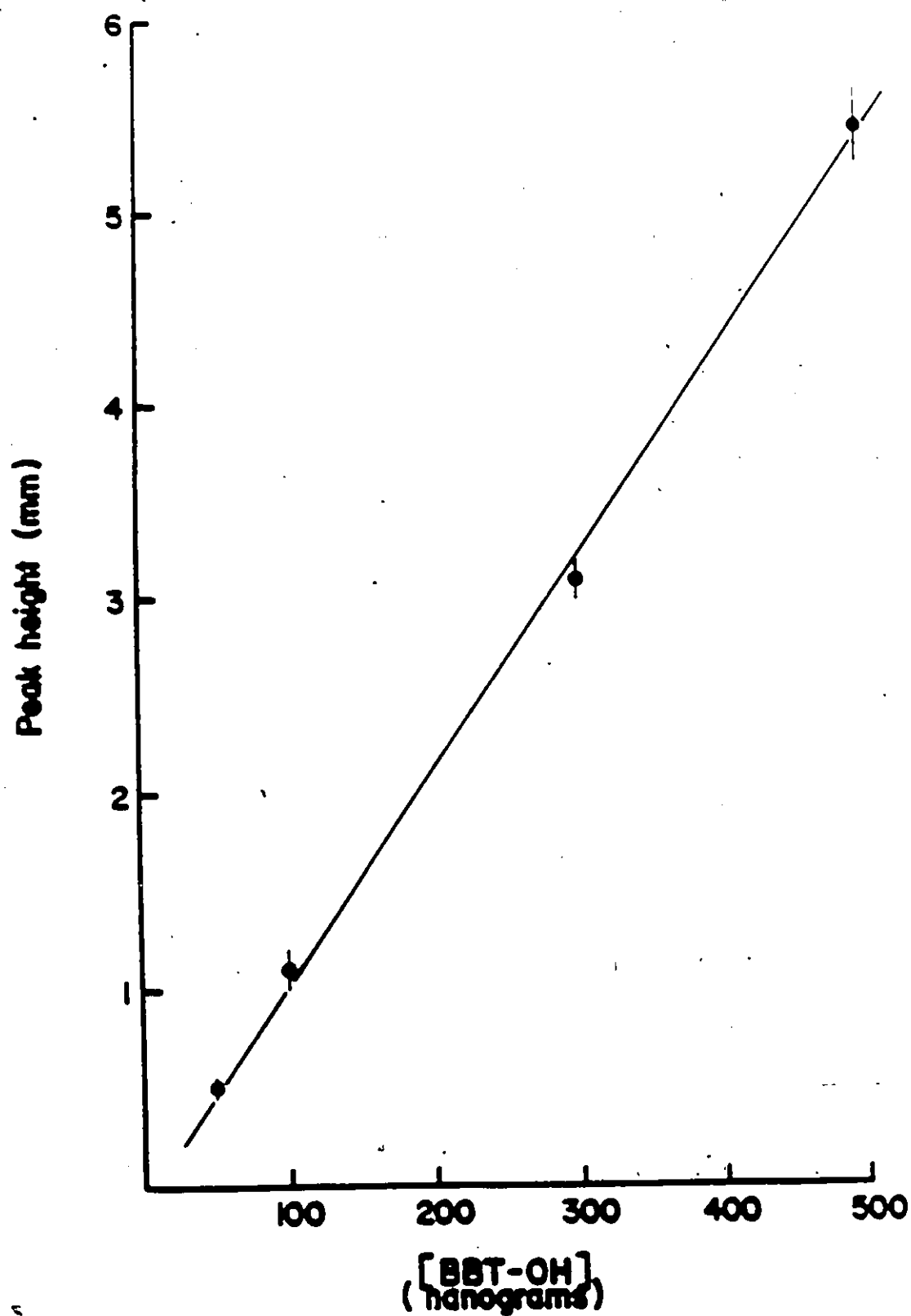
Quantitative work indicated that BBT-OAc was the most strongly concentrated compound (0.35 $\mu\text{g}/\text{seedling}$) in 4 day-old germinating seedlings of T. erecta. BBT and compound 4 were also present in substantial quantities (0.16 and 0.13 $\mu\text{g}/\text{seedling}$ respectively). Alpha-T was present in only trace amounts (0.06 $\mu\text{g}/\text{seedling}$).

BBT-OAc was the most prominent compound present in the roots and lower stems of T. erecta (at 2 weeks) grown under aseptic conditions, with a concentration of 0.97 $\mu\text{g}/\text{seedling}$. BBT-OH and alpha-T were also present but at a much lower concentration, the amounts being insufficient for accurate quantitation when small samples were analyzed. BBT, reported to be present in roots of T. erecta (Uhlenbrock and Bijloo, 1959 Bohlmann et al., 1973 Hogstad et al., 1984) was not detected in roots and lower stems of the seedlings under the environmental conditions used here.

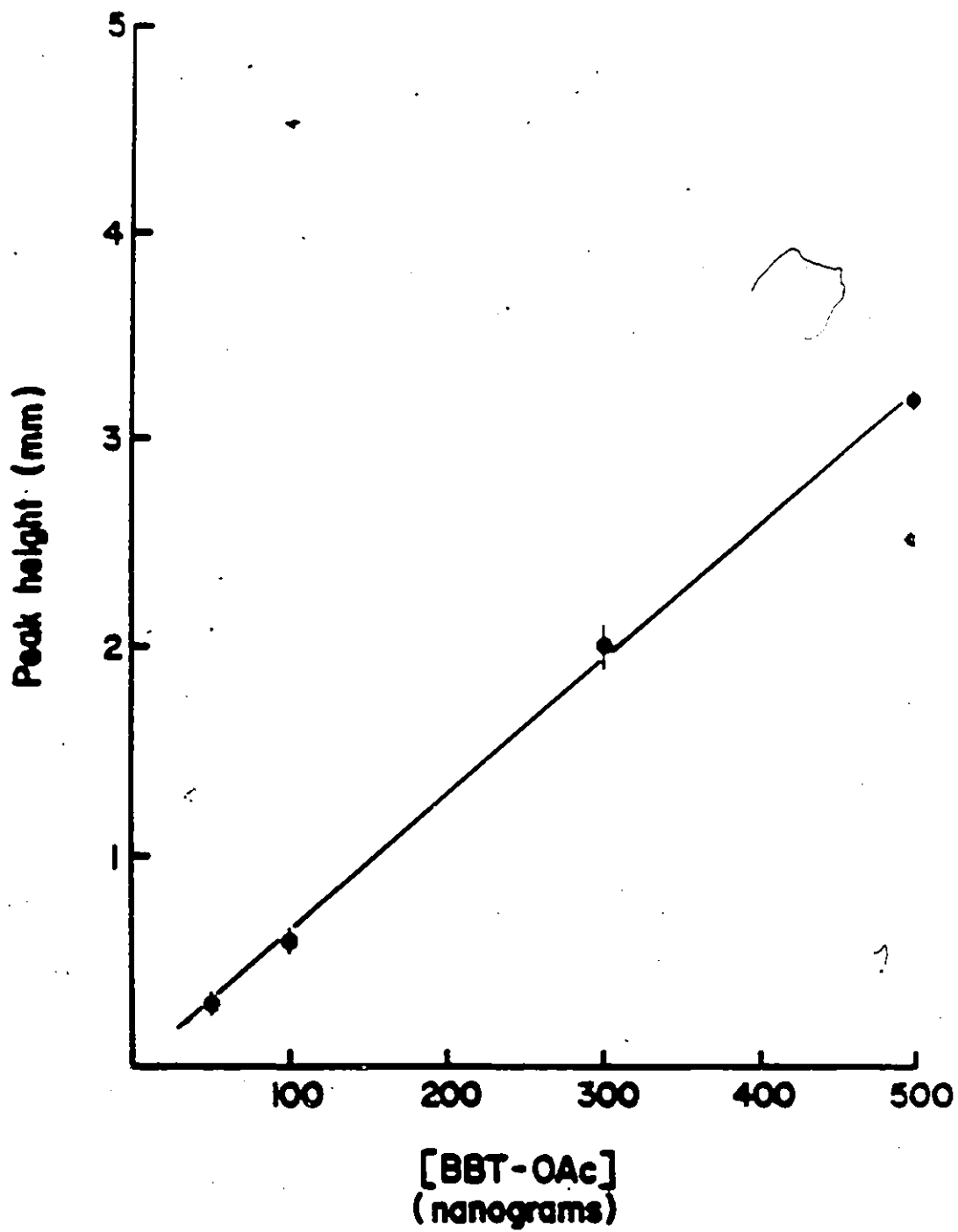


Figure 9: Standard concentration curves for thiophenes (a) BBT-OH, (b) BBT-OAc, (c) BBT and (d) alpha-T relating HPLC peak height (mm) to amount of standard injected (nanograms). The results shown are means of triplicate samples. Bars represent standard deviations. The correlation values (r) are > 0.99 for all plots.

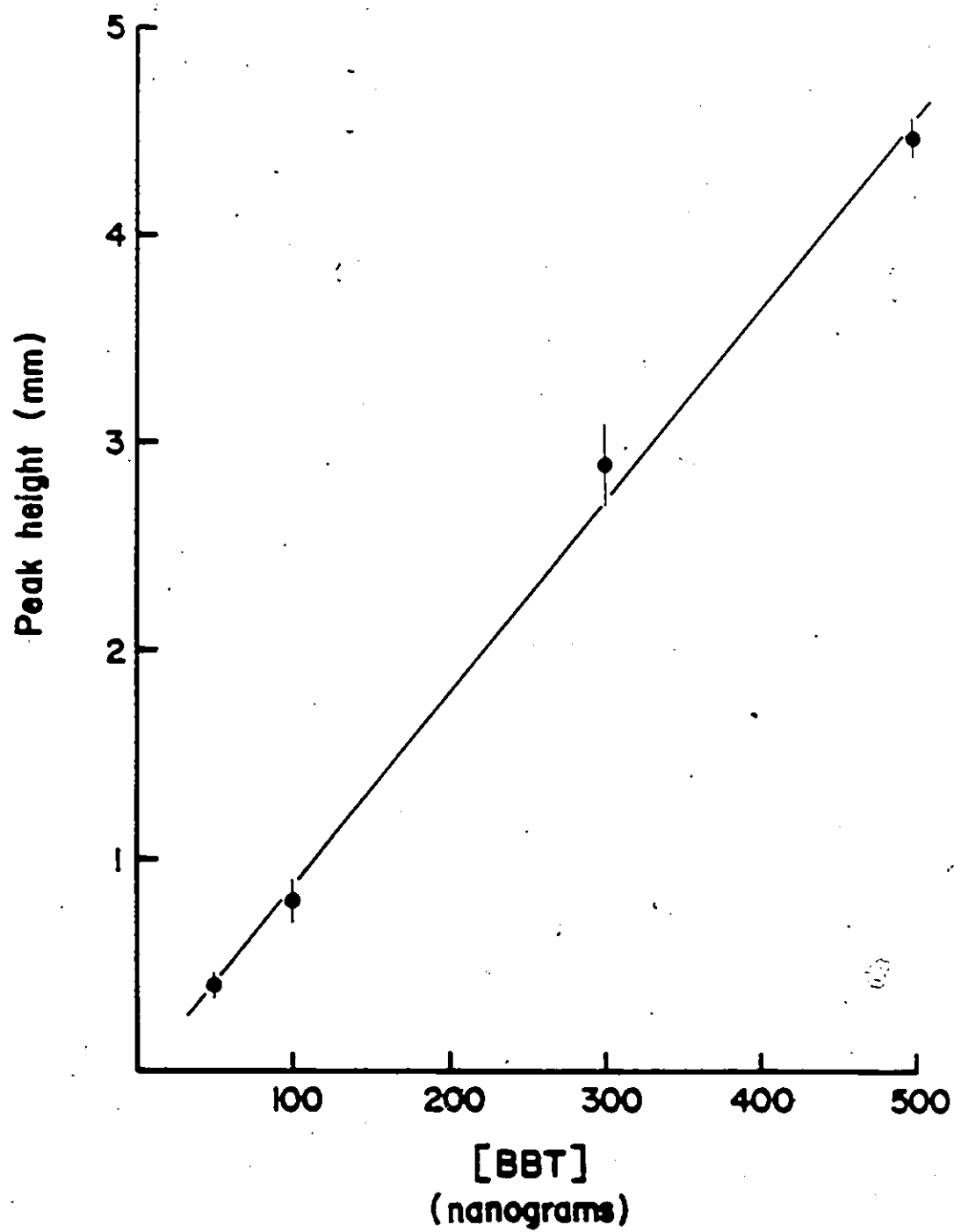
a)

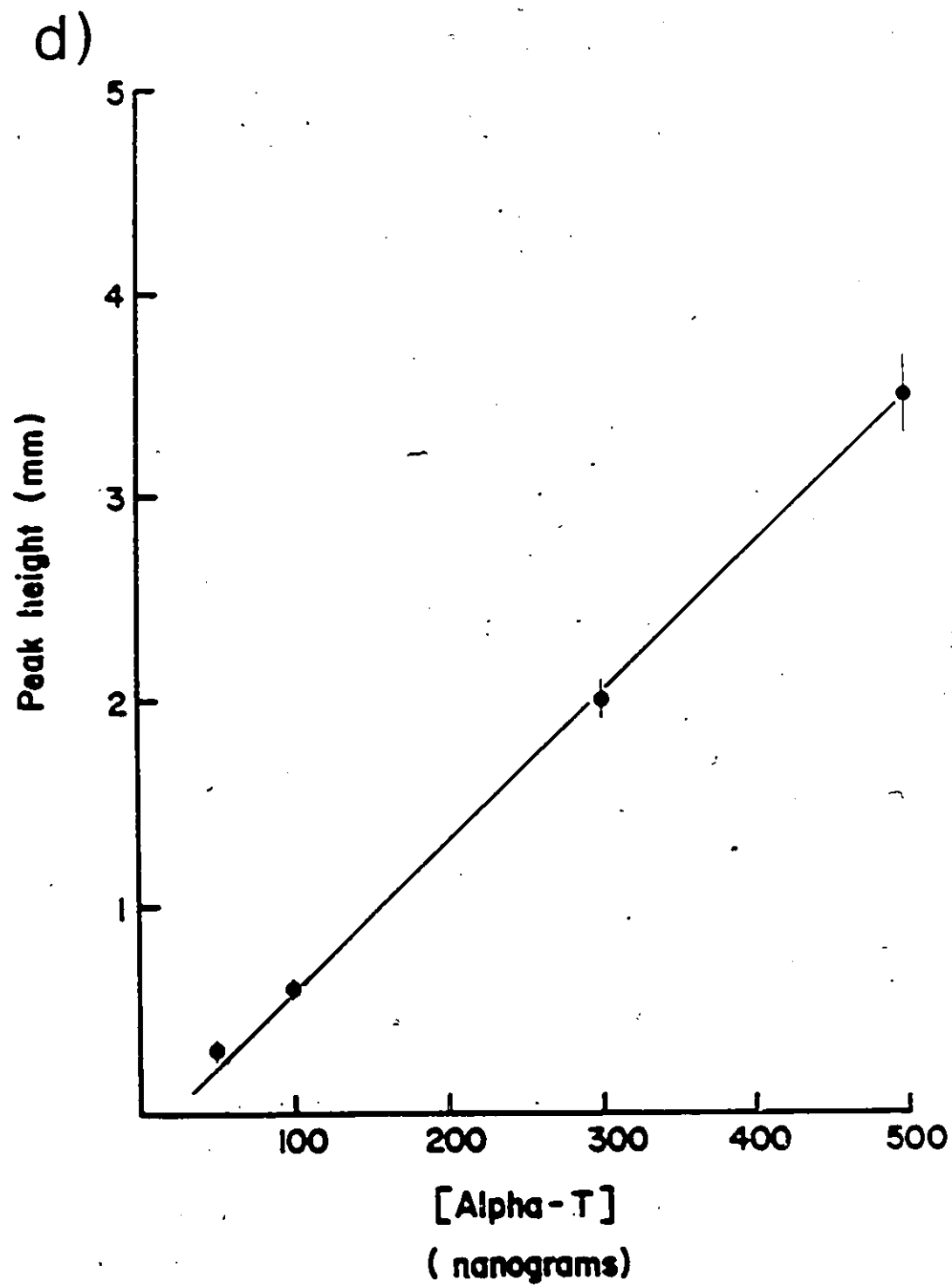


b)



C)





3.2 Abiotic and biotic treatment of 4 day-old germinating seedlings

3.2.1 Treatment with an abiotic agent

Tissue damage was clearly evident in young seedlings after treatment with HgCl_2 (0.001 M). Gradual increase in the length of treatment (from 10 min to 120 min) resulted in a progressive increase in browning and discolouration of the plants. Most of the seedlings collapsed when treated with HgCl_2 for 60 min or more.

BBT-OAc, BBT, alpha-T and compound 4 were detected in HPLC chromatograms of all tissue extracts. Quantitative analysis showed that the highest levels of thiophenes were present in control plants treated with distilled water. Treatment with mercuric chloride caused a reduction in the combined yields of the 4 thiophenes; increasing the treatment period resulted in a decrease in the thiophene content of the seedling (Figure 10a,b,c)

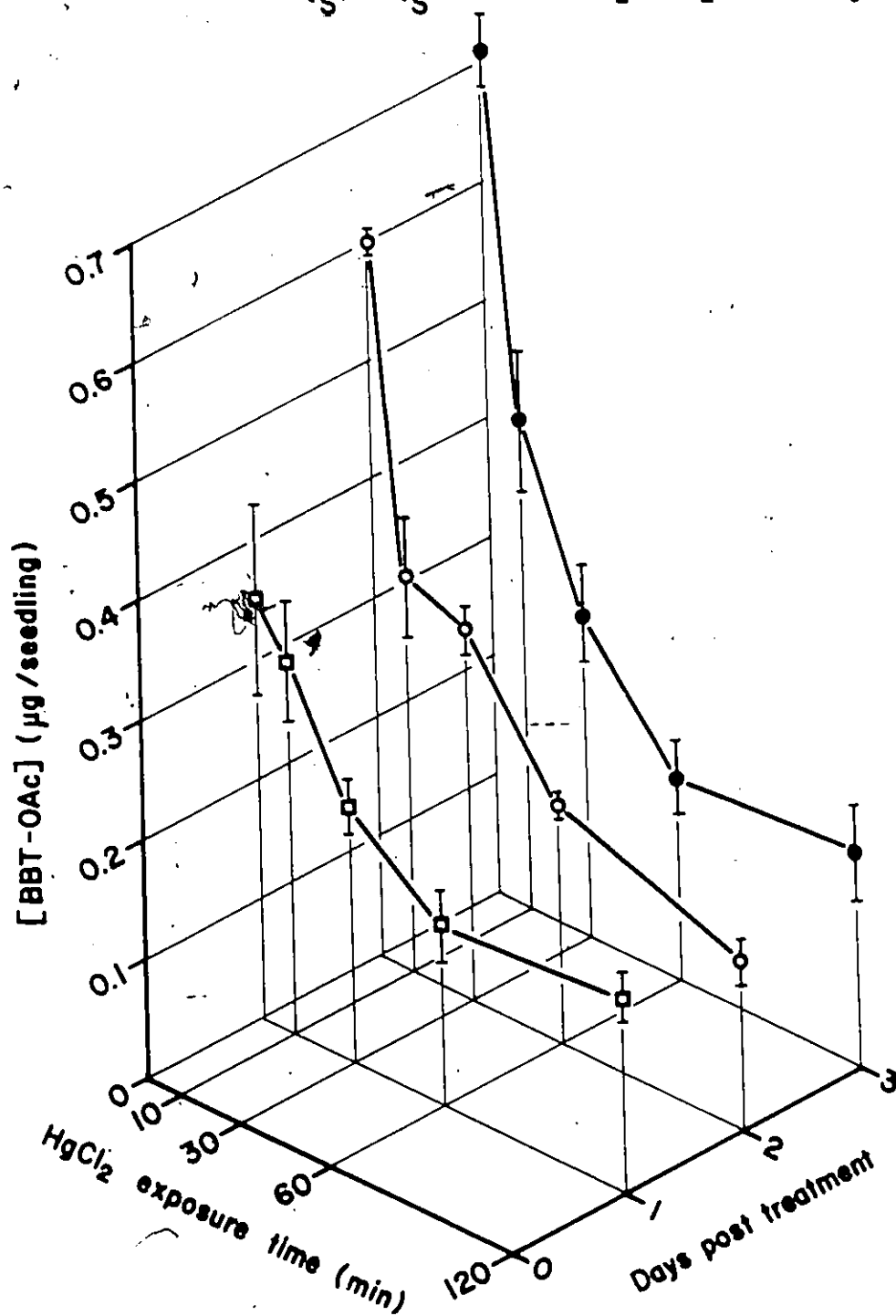
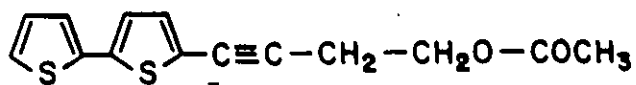
Analysis of variance of the data (Appendix 3) showed that seedlings treated with HgCl_2 for 30 min or more had significantly less BBT-OAc ($P = 0.05$) than untreated control plants, beginning from the first day post-treatment (Figure 10a). The concentration of BBT-OAc in healthy marigolds increased continuously with time until it reached 0.71 $\mu\text{g}/$ seedling by the end of the experiment. At this time, much smaller quantities of this thiophene were detected in HgCl_2 treated seedlings (0.41 $\mu\text{g}/$ seedling for the 10 min treatment and 0.18 $\mu\text{g}/$ seedling for the 120 min treatment).

The concentration of BBT and compound 4 decreased in HgCl_2 treated plants 24 hrs after the observed fall in the concentration of BBT-OAc, while they remained constant in control seedlings until the termination of the experiment (Figure 10. b,c). The drop in the amount of compound 4 occurred gradually with time, reaching its lowest value 3 days after treatment with the toxic agent. The decrease in BBT content was significant ($P = 0.05$) relative to the controls in 6 and 7 day-old seedlings treated for 120 min with HgCl_2 (0.18-0.19 $\mu\text{g}/\text{seedling}$ in controls and 0.09-0.11 $\mu\text{g}/\text{seedling}$ in treated plants) but shorter treatment periods did not significantly affect ($P = 0.05$) the concentration of the compound.

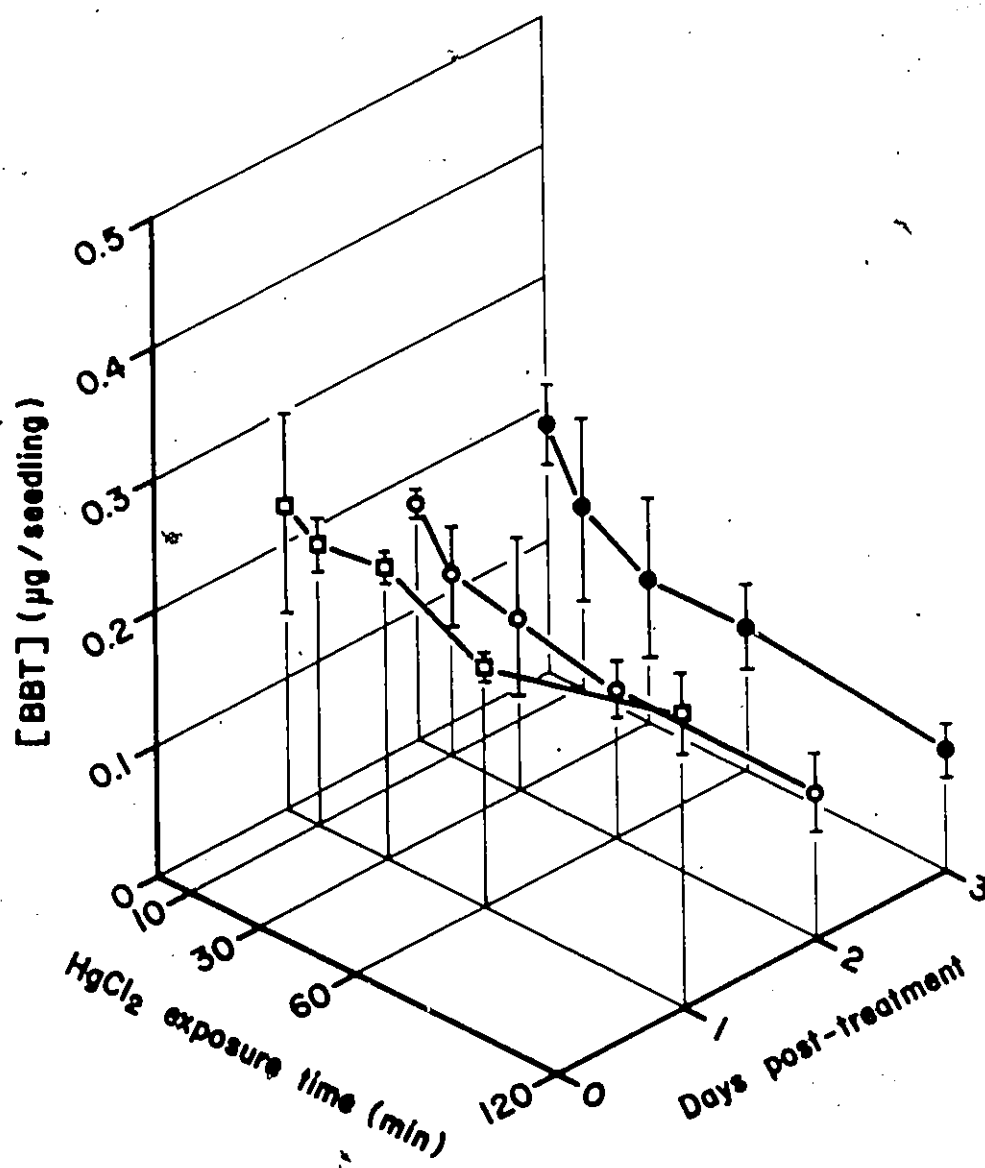
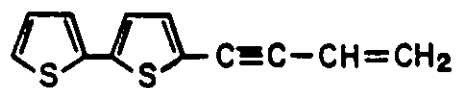
Alpha-T₁ was present in small concentrations (Figure 10d). The slight variabilities encountered in the levels of alpha-T between the different treatments did not appear to be related to the degree of damage done to the plant by the abiotic agent.

Figure 10: Yield of thiophenes (a) BBT-OAc, (b) BBT, (c) compound 4 and (d) alpha-T in seedlings of *T. erecta* (4 day-old) treated with HgCl_2 (0.001 M) for different lengths of time and incubated for various days in a controlled environment chamber at 25°C, 70% R.H., with a 16:8 hr light:dark photoperiod (light intensity = 17 w/m²). Each point represents the mean of at least 3 replicates. Bars represent standard deviations. Where omitted, standard deviations were < .5%. Control plants and plants treated with HgCl_2 for 10, 30, 60 and 120 min grew by 9.4%, 4.4%, 1.9%, 2% and 0% respectively, from day 1 to day 3 post-treatment.

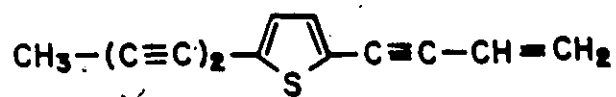
a)



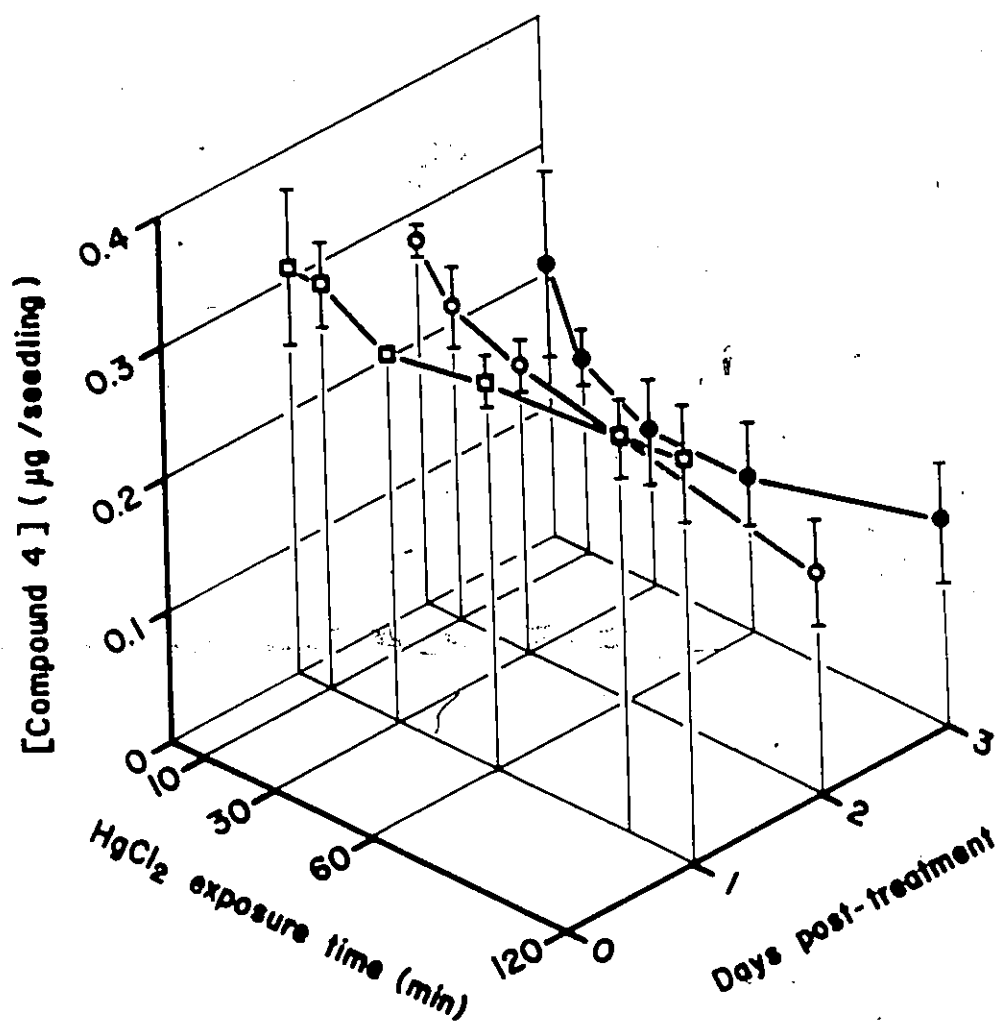
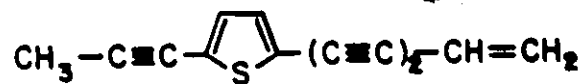
b)



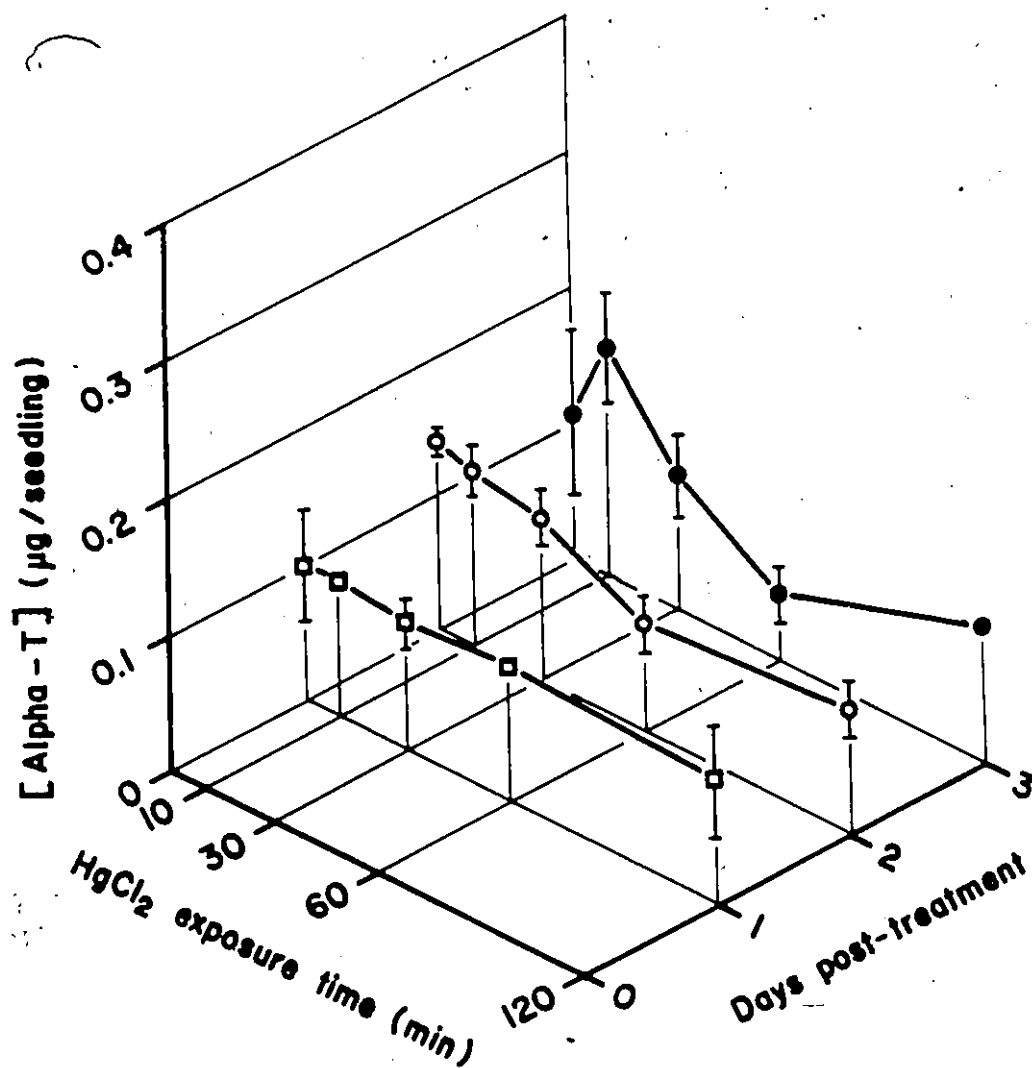
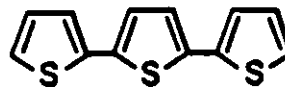
c)



or



d)



3.2.2 - Treatment with a biotic agent

This study involved the quantitative determination of thiophenes (using reverse phase HPLC) at various times after inoculation of 4 days old seedlings with A. tagetica.

Within 2 days of inoculation, dark-brown necrotic spots were observed on stems and leaves of diseased plants. The infection progressed rapidly causing browning and drying of the seedlings and culminating in plant mortality 4 days after fungal treatment. A. tagetica could be recovered from all portions of diseased plants.

BBT-OAc, BBT, alpha-T and compound 4 were detected in HPLC chromatograms of all tissue extracts. Quantitative estimation of the results revealed a general decline in thiophene content of plants infected with A. tagetica, the total thiophene yield being much higher in normal healthy plants than in diseased plants (0.87 and 0.27 µg/seedling in control and treated plants respectively at the termination of the experiment).

A clear reduction in the BBT-OAc content of infected plants was apparent, beginning the first day after inoculation and continuing so until the termination of the experiment (Figure 11a). At this time (4 days post-inoculation), dead seedlings contained only 0.12 µg/seedling of BBT-OAc, a 4.5 fold decrease in concentration as compared to normal plants. Analysis of variance of the data (Appendix 4) showed that the observed differences in the amounts of BBT-

OAc. between healthy and diseased plants were significant ($P = 0.05$).

A significant decline in the concentrations of BBT and compound 4 was evident 3 days after inoculation with the microbial pathogen and after 4 days, only 0.05 $\mu\text{g}/\text{seedling}$ of each compound was left in the severely damaged marigold, as compared to 0.13 $\mu\text{g}/\text{seedling}$ present in healthy plants (Figure 11 b,c).

Alpha-T was present in low concentrations in all plant tissue examined. Its levels remained constant (Figure 11c) and did not differ significantly ($P = 0.05$) between healthy and diseased plants (Appendix 4).

These results show that thiophenes did not accumulate in very young T. erecta seedlings (4 to 8 days old) exposed to severe growth and infection conditions, with the invading pathogen causing severe tissue damage at a very fast rate.

Figure 11: Changes in concentrations of (a) BBT-OAc, (b) BBT, (c) compound 4 and (d) alpha-T in control *T. erecta* seedlings and in seedlings infected with *A. tagetica* at various days after inoculation. Plants were maintained in a controlled environmental chamber at 25°C, 70% R.H., with a 16:8 hr light:dark photoperiod (light intensity = 17 w/m²), until the termination of the experiment. Each point represents the mean of at least 3 replicates. Bars represent standard deviations. When omitted, standard deviations were < 5%.



3.3 Biotic treatment of 2 weeks old seedlings grown under aseptic conditions.

Changes in thiophene levels were monitored (using reverse phase HPLC) in roots and lower stems of T. erecta grown aseptically in Hoagland's solution 0, 4, 8 and 12 days after inoculation of 2 weeks old seedlings with 2 fungi showing different degrees of pathogenicity toward the plant.

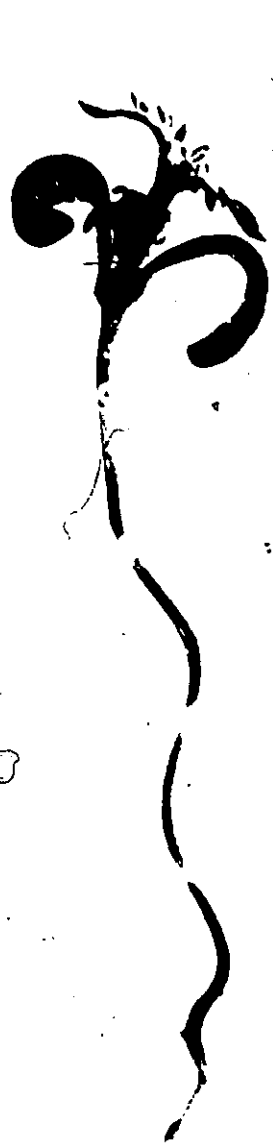
3.3.1 Description of disease symptoms

T. erecta seedlings were moderately susceptible to Fusarium oxysporum var callistaphi race 2 (FOVC race 2). Initial disease symptoms were apparent within 4 days of inoculation with this pathogen, as evidenced by the browning of the roots at the site of inoculation beneath the mycelium which had developed on the surface of the Hoagland's solution. A discolouration of lateral root junctions and lateral root tips was observed after 8 days, and within 12 days, a more extensive decomposition of the roots had occurred along with foliar chlorosis of some leaves (Figure 12). Reisolation of viable FOVC race 2 was achieved from the roots and stems of infected seedlings using standard methods (section 2.2.2.4).

F. oxysporum f.sp. radicis lycopersici (FORL) was highly pathogenic toward T. erecta seedlings. This fungus is not reported to infect African marigolds but the experimental conditions used possibly induced decreases in host resistance and development of the fusarium wilt. Among the factors that could have influenced the outcome of the inter-

action were: the use of a very virulent FORL strain, inoculation of the plants at a susceptible growth stage and maintenance of the plants in aseptic cultures at favorable temperature and humidity conditions. FORL rapidly invaded the susceptible marigold. Zones of discolouration of the roots were first visible within 4 days after inoculation, especially below the point of contact with the culture medium, the surface of which was completely covered with mycelial growth. The hyphae continued to proliferate and after 8 days, extensive unchecked fungal colonization had resulted in seedling death as evidenced by complete necrosis of stems, roots and leaves. The appearance of the seedling 12 days after inoculation with FORL is shown in Figure 12. At this time, viable FORL were recovered from all portions of the plant. Neither fungus was recovered from healthy control seedlings which showed no symptoms of disease.

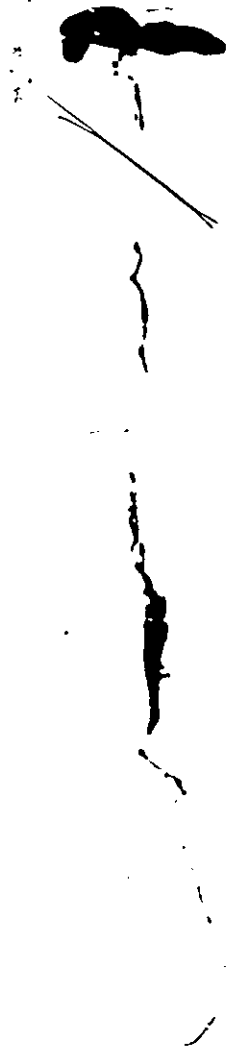
Figure 12 T. erecta seedlings 12 days after inoculation with F. oxysporum var callistephi race 2 (FOVC race 2) and F. oxysporum f.sp. radicis lycopersici (FORL). Two week-old seedlings grown aseptically in Hoagland's solution were inoculated with a mycelial solution of the appropriate fungus in Fries medium. Control plants were treated with sterile Fries medium.



Control



**FOVC
race 2**



FORL

3.3.2 Thiophene content during infection

Three thiophenes: BBT-OH, BBT-OAc and alpha-T were detected (using reverse phase HPLC) in healthy T. erecta seedlings and in seedlings infected with FOVC race 2 or FORL. Quantitative analysis showed striking differences in the patterns of thiophene kinetics (Figure 13). In the first pattern associated with moderate susceptibility to the invading pathogen (FOVC race 2 infection), thiophenes tended to accumulate during the infection process. In the second pattern associated with high susceptibility to the attacking fungus (FORL infection), the opposite tendency was observed.

In uninfected control plants, levels of BBT-OH increased slightly with time and were significantly different ($P=0.05$) from initial levels at day 12 (Appendix 5).

In FOVC race 2 infected plants, there was a much more dramatic accumulation of BBT-OH with time. By 4 days post-inoculation, levels were significantly different ($P = 0.05$) from 0 day levels and uninfected control levels at 4 days. By 8 days post-treatment, the yield of BBT-OH was significantly higher ($P = 0.05$) than the yields at days 0 and 4 or the yield of uninfected controls at day 8 (Appendix 5). Thereafter, the concentration went up to 464 ng/seedling at 12 days, which represents 27 times the day 0 concentration or 12 times the healthy control concentration at day 12 (Figure 13a). The fate of BBT-OH was different in seedlings

infected with FORL, reaching its maximum concentration (70 ng/seedling) 4 days after treatment and decreasing afterward (Figure 13a). The differences in the yields of BBT-OH between control and infected plants were significant ($P = 0.05$) 4 and 12 days following FORL treatment (Appendix 5).

Amounts of BBT-OAc in control healthy marigolds at day 4 were significantly lower ($P = 0.05$) than initial levels (day 0). In both groups of 4 day-infected plants, a still greater decline below initial concentrations (day 0) was observed (Figure 13b). These levels were significantly different from day 0 levels or from uninfected controls at day 4.

In FORL infected seedlings, BBT-OAc continued to decrease with time, attaining low values (0.08 $\mu\text{g}/\text{seedling}$) by the end of the treatment period (12 days) (Figure 13b). The levels of BBT-OAc reached in tissues challenged with FORL for 4, 8 and 12 days were significantly different ($P = 0.05$) from the levels encountered in normal healthy plants (Appendix 5).

In FOVC race 2 infected seedlings, original concentration of BBT-OAc was re-established by 8 days post-inoculation. This concentration was not significantly different ($P = 0.05$) from the initial concentration at day 0 or from the concentration present in the controls at day 8. Thereafter, BBT-OAc increased to 2.64 $\mu\text{g}/\text{seedling}$ at day 12 post-treatment which represents 2.7 times the amount present

at day 0 or 3.2 times the amount in uninfected controls at day 12 (Figure 13b).

In normal control marigolds, alpha-T concentration initially remained constant with time (0-8 days) followed by a significant increase over the initial levels at 12 days (Figure 13c)

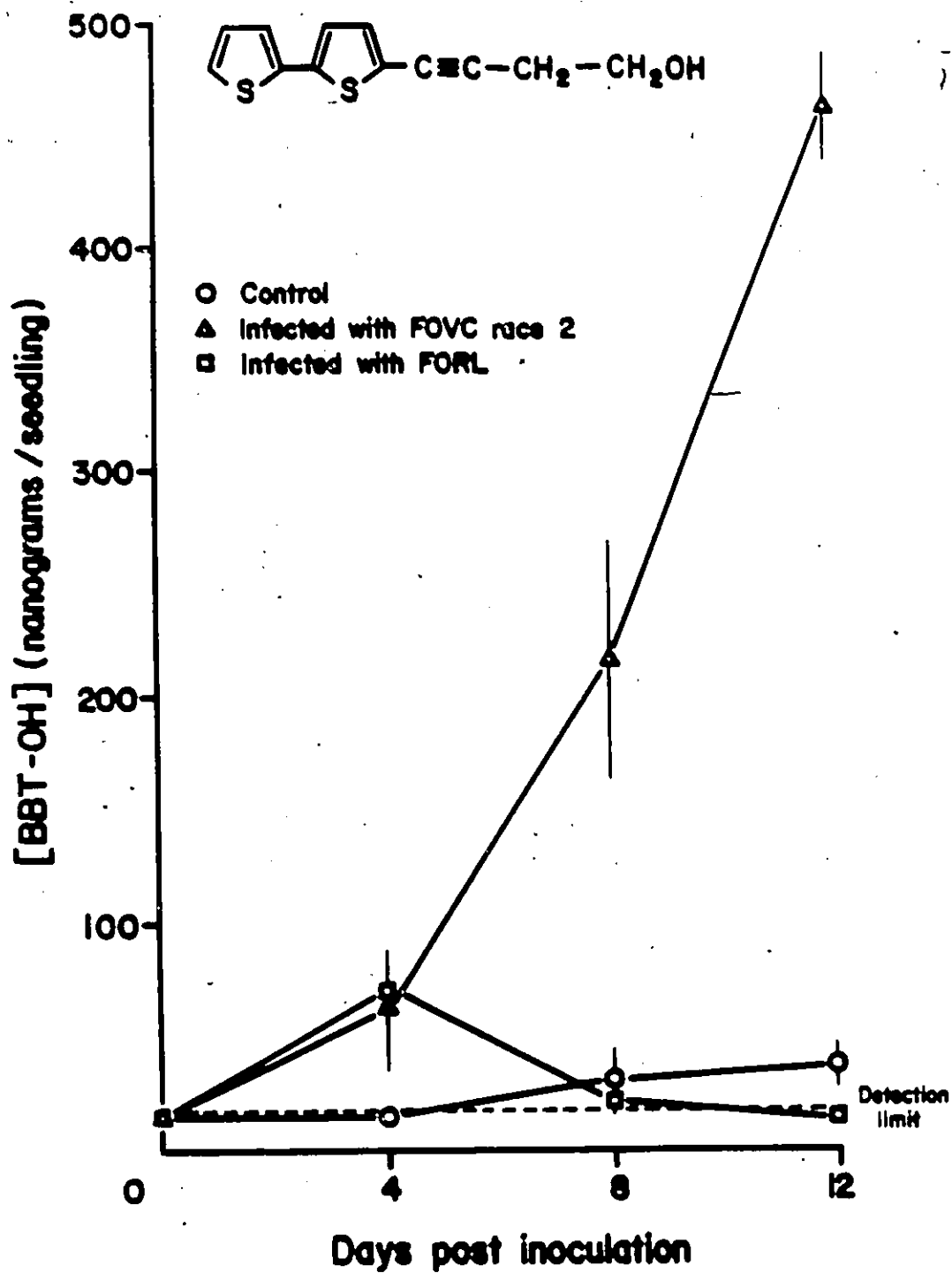
In marigolds infected with the more virulent FORL, a very slight but significant increase in the yield of alpha-T occurred after 4 days of treatment, but the levels fell thereafter. At day 12, no alpha-T was detected in the dead infected seedlings by the assay method utilized, in contrast to 100 ng of alpha-T / seedling detected in control plants at day 12 (Figure 13c).

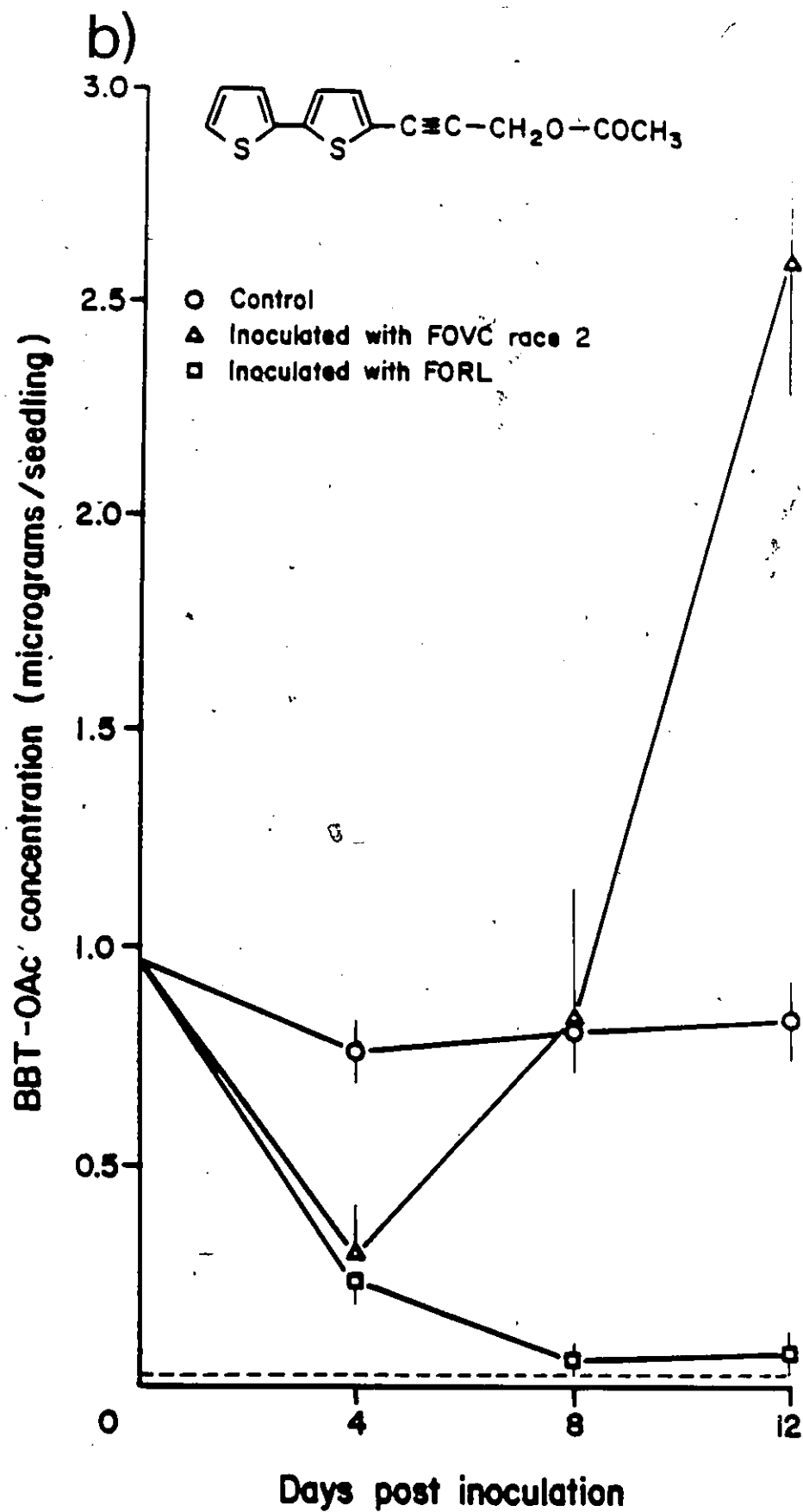
In the marigolds infected with the less virulent FOVC race 2, the yield of alpha-T increased over time. By 8 days post-inoculation, levels were significantly different ($P = 0.05$) from initial levels (day 0) or from control levels at 8 days. Subsequently, alpha-T reached its maximum value of 267 ng/seedling at day 12, which represents more than 8.6 times its value at day 0 or 2.7 times its value in uninfected control plants at day 12 (Figure 13c).

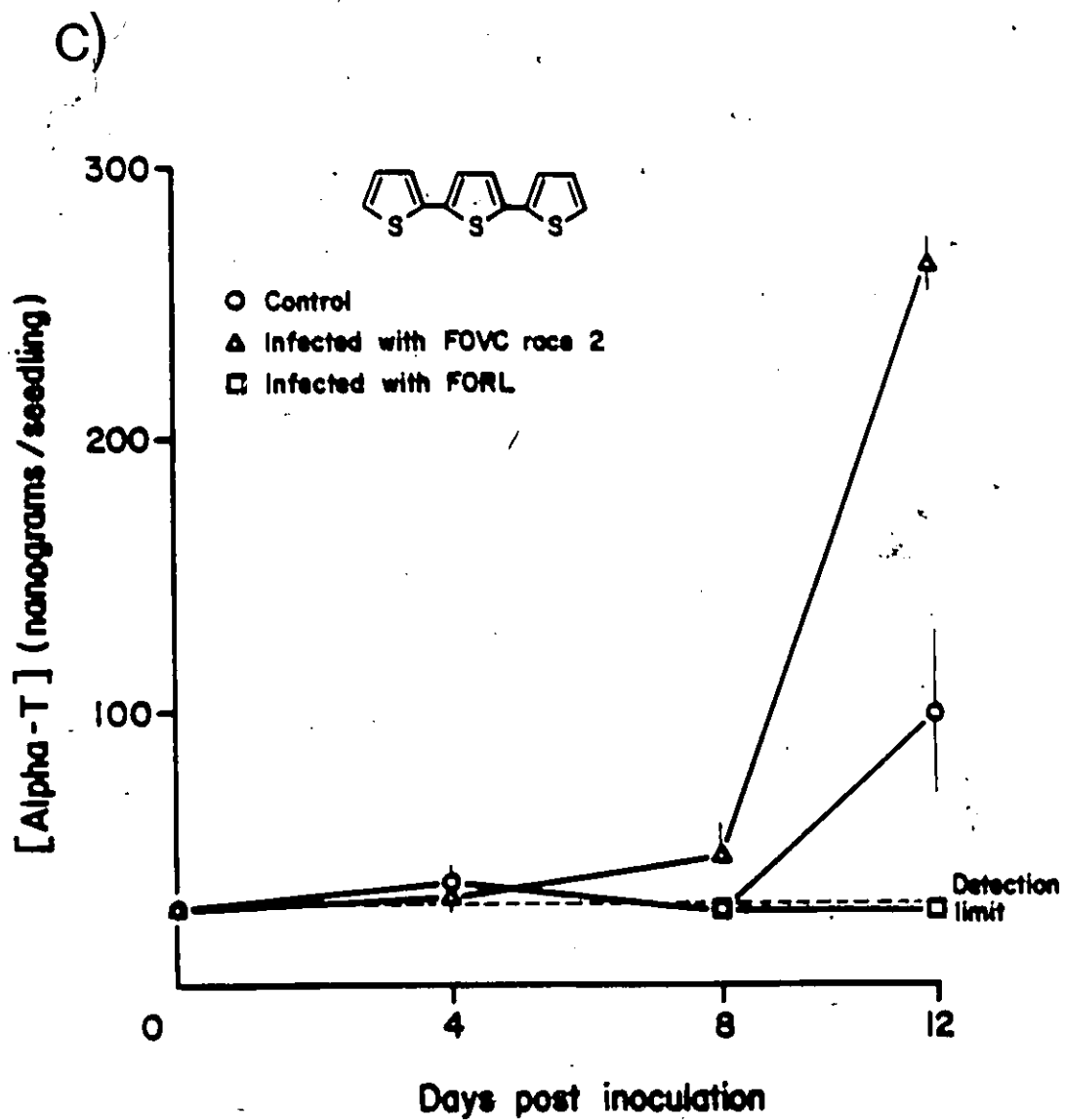
An increase in the levels of thiophenes occurring in T. erecta seedlings partially damaged by FOVC race 2 was clearly apparent in these results. Tissues severely damaged by FORL infection did not accumulate any thiophenes.

Figure 13: Changes in the concentrations of (a) BBT-OH, (b) BBT-OAc and (c) alpha-T in roots and stems of *T. erecta* in control seedlings and in seedlings infected with *F. oxysporum* var *callistephi* race 2 (FOVC race 2) or *F. oxysporum* f.sp. *radicis lycopersici* (FORL), at various days after inoculation. Plants, which were 2 weeks old at the time of inoculation, were grown in aseptic culture in a controlled environment chamber at 25°C, 70% R.H., with a 16:8 hr light:dark photoperiod (the light intensity reaching the plants was 15 w/m²). Each point in the graphs represents the mean concentration of at least 3 replicates. For concentrations which are below the limit of sensitivity of the HPLC assay method, the maximum possible values are plotted. Bars represent standard deviations.

a)







3.4 Fungitoxicity and metabolism of thiophenes

3.4.1 Effect of alpha-T on fungal growth

The liquid shake culture bioassay technique was selected to carry out a comparative toxicity test of the thiophene alpha-T towards 2 isolates of *F. oxysporum* (FOVC race 2 and FORL) exhibiting different degrees of pathogenicities toward the host plant (*T. erecta*), in the dark or in the presence of near UV irradiation (Figures 14 and 15).

When no alpha-T was present, approximatively 50% of the spores germinated 12 hrs after inoculation of the liquid medium with either fungus. After 24 hrs, extensive branched mycelia grew in culture. Hyphae with conidiophores bearing conidia were seen after 48 hrs, many conidia were also present in the medium at that time. Near UV irradiation did not have any effect upon germination of conidia.

Addition of alpha-T (0.01-100 µg/ml) to cultures of FORL grown in the dark did not have any significant effect ($P = 0.05$) upon mycelial growth of the fungus (Appendix 6). No significant ($P = 0.05$) dry weight loss was evident in cultures of FOVC race 2 incubated in the dark and containing 0.01 µg/ml of alpha-T or less. Higher levels of alpha-T tended to cause a significant reduction in fungal growth (Appendix 6) reaching a value of 46% growth inhibition at the highest concentration of treatment (100 µg/ml) (Figure 14). The effects of high alpha-T concentrations (0.1 µg/ml or more) on FORL and FOVC race 2 cultures incubated in the dark were significantly different (Appendix 6).

When combined with near UV irradiation, alpha-T had a greatly enhanced toxic effect on mycelial growth, causing a considerable reduction in the dry weights of the two fungal colonies. The growth of FORL was significantly affected at concentrations of 0.01 $\mu\text{g/ml}$ or more, during treatment (Appendix 6). But the pattern of response of this fungus to alpha-T in the presence of near UV light varied between replicate samples at concentrations around 0.1 $\mu\text{g/ml}$, as reflected by the high standard deviation obtained (Figure 15). Higher concentrations of the thiophene gave consistently strong inhibition of the fungus. Probit plot analysis of the data gave a reasonably linear transformation (Figure 15) and showed that a 50% mycelial dry weight loss (EC_{50}) was induced upon exposure to 0.03 $\mu\text{g/ml}$ of alpha-T.

FOVC race 2 grew well in shake cultures incubated in the presence of UV-A light and containing relatively low amounts of alpha-T (up to 0.01 $\mu\text{g/ml}$). Further increase in the concentration of this substance led to progressively less dense mycelial growth, and at 1 $\mu\text{g/ml}$ of alpha-T or more, growth of FOVC race 2 was completely prevented. The EC_{50} of alpha-T on FOVC race 2 was 0.06 $\mu\text{g/ml}$, as determined from probit plot analysis of the data (Figure 15). The photoinduced growth inhibition observed in alpha-T treated samples was significant ($P = 0.05$) as compared to the controls, at concentrations of 0.1 $\mu\text{g/ml}$ or more (Appendix 6).

When either fungal culture which had been incubated in the presence of UV-A irradiation was examined by phase contrast microscopy 48 hrs after being placed in Fries medium containing levels of alpha-T between 1 and 100 µg/ml, cellular content of inocula was found to be granular and disrupted. Increasing the concentration of alpha-T led to more extensive granulation and cell lysis. Any concentration of alpha-T of 1 µg/ml or more completely prevented germination of conidia.

These data clearly demonstrate the phototoxic effects of alpha-T on fungal growth. The dose-response curves (+ UV-A) differed only slightly for the two fungi tested. This difference was not significant ($P = 0.05$) within the dose range tested (0.001 to 100 µg/ml) (Appendix 6).

Figure 14: Effect of alpha-T on mycelial growth of F. oxysporum var callistephi race 2 (FOVC race 2) and F. oxysporum f.sp. radicis lycoperisici (FORL) cultures incubated in the dark at 25°C. The data presented show the mycelial dry weights attained after exposure of the inocula to different concentrations of alpha-T for 72 hrs. Each point represents the mean of 5 samples. Bars represent standard deviations.

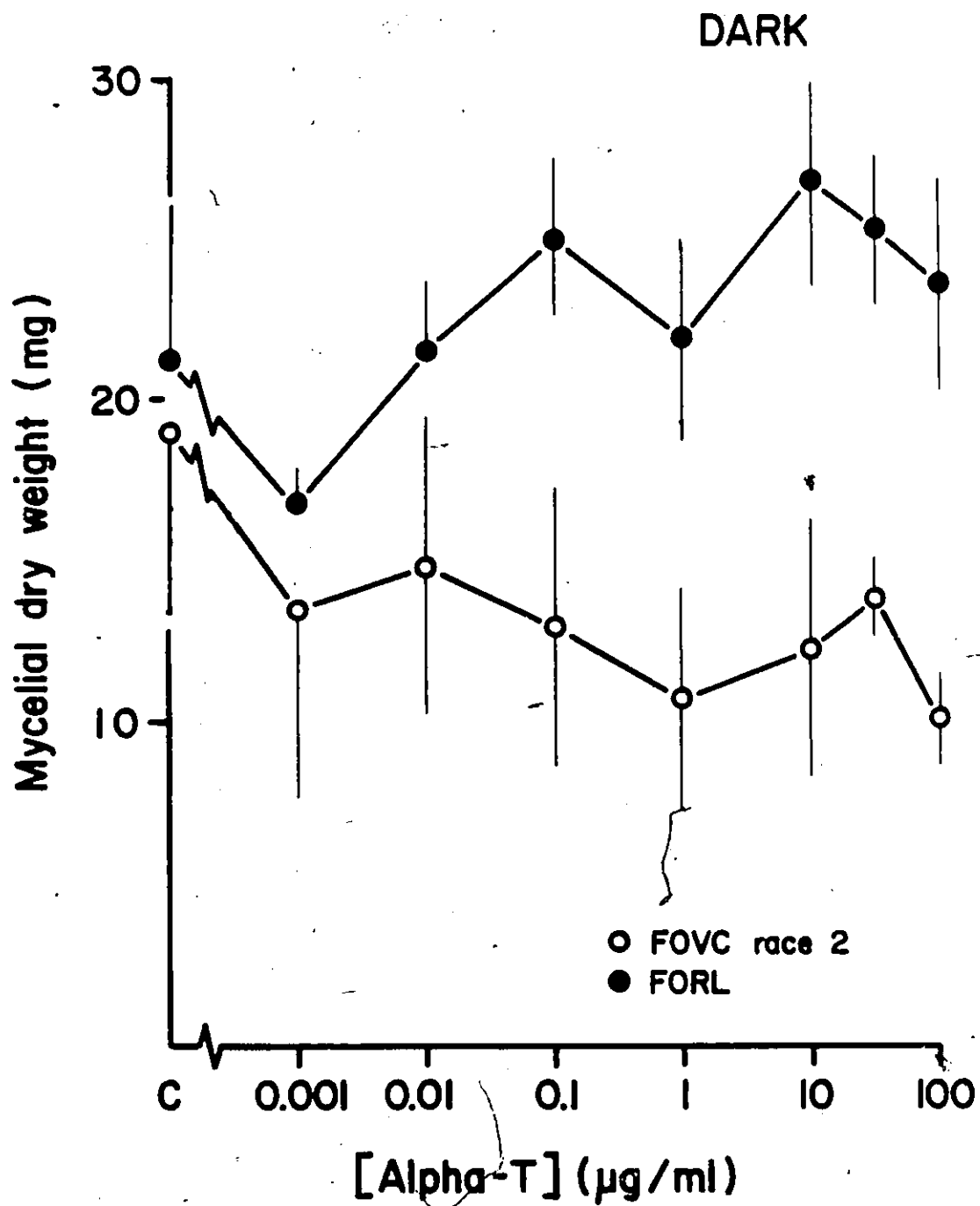
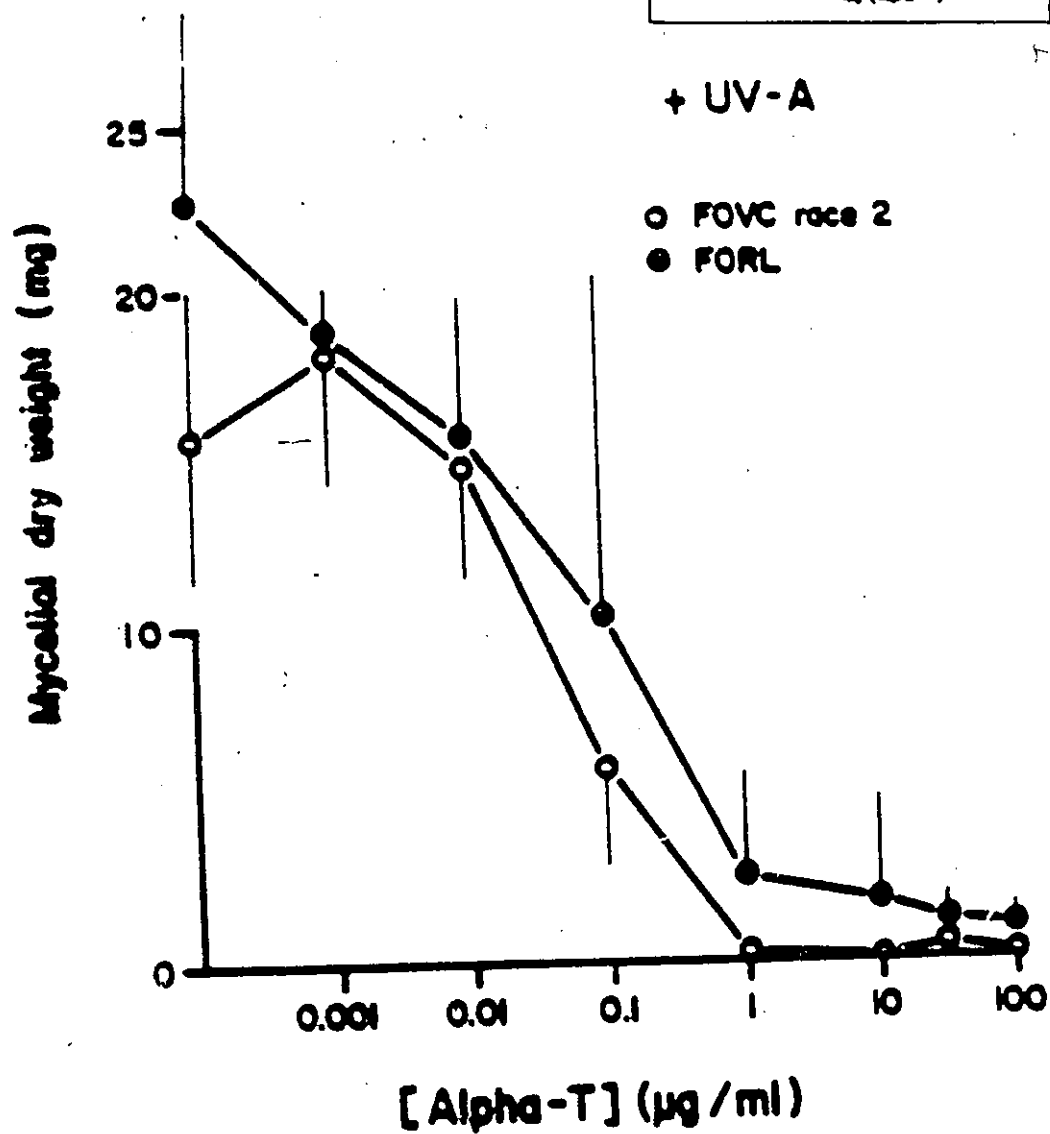
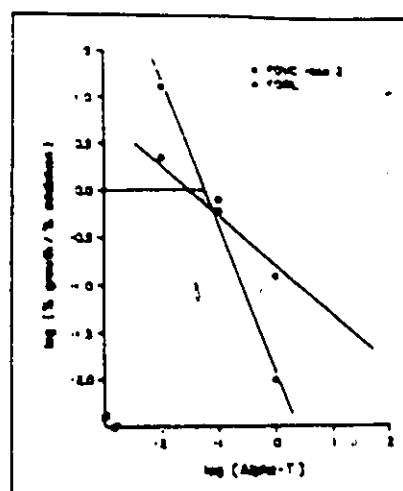


Figure 15: Effect of alpha-T on mycelial growth of F. oxysporum var callistaphi race 2 (FOVC race 2) and F. oxysporum f.sp. radicis lycoperisici (FORL) cultures incubated at 25°C in the presence of UV-A irradiation (16:8 hr light:dark photoperiod, light intensity = 4 w/m²). The data presented show the mycelial dry weights attained after exposure of the inocula to different concentrations of alpha-T for 72 hrs. Each point represents the mean of 5 samples. Bars represent standard deviations. The insert represents the probit plot transformation of the data, with correlation values (r) of -0.99 and -0.98 for FOVC race 2 and FORL plots respectively. The EC₅₀ derived from these plots are 0.06 µg/ml and 0.03 µg/ml for FORL.



3.4.2 Metabolism of alpha-T in vitro

This experiment examined the ability of FORL to metabolize ^3H -labelled alpha-T (0.3 ug/ml) in shake culture in the dark.

Table 5 shows the distribution of radioactivity in the different fractions obtained from the ^3H -alpha-T treated FORL cultures or from the uninoculated controls containing ^3H -alpha-T only, expressed as activity of the labelled material recovered after 24 hrs. In the fungal cultures, the total recovery was only reduced to 86% since inevitable losses of radioactivity had occurred during extraction. Most of the label appeared in the combined hexane and ethyl acetate fractions (63.3%). A smaller proportion remained in the mycelial residue (32%) and some was present in the aqueous fraction (4.6%). The total recovery from uninoculated controls was 79% with most of the radioactivity found in the combined hexane and ethyl acetate fractions (92.2%).

Examination of TLC plates of extracts of the control, using hexane-ethyl acetate (1:1, v/v) as the solvent system (Figure 16) revealed that the location of radioactive material coincided with the location on the plates of the authentic alpha-T standard (band a, $R_f = 0.9$). The use of other solvent systems (hexane-ethyl acetate (17:3, v/v) or petroleum ether-acetone (19:1, v/v)) also gave corresponding R_f s. In cultures of FORL treated with ^3H -alpha-T, two bands (c and d, $R_f = 0.54$ and 0.19 respectively) were detected.

These bands contained only a very small percentage of the label (0.1-0.2%) and may represent either metabolites or chemical breakdown products caused by interaction with the fungus. Band b ($R_f = 0.61$) seen in both control and fungal extracts contained very low levels of radioactivity ($\leq 0.3\%$) and may represent some breakdown of alpha-T occurring during shaking in aqueous medium or during extraction of the medium.

These results indicate that under the experimental conditions employed, ^3H -alpha-T did not undergo significant levels of metabolism by FORL.

3.4.3 Fungitoxicity of thiophene standards

The 4 thiophenes tested: BBT-OH, BBT-OAc, BBT-2OAc and BBT caused complete inhibition of spore germination of FOVC race 2 and FORL at a concentration of 5 ppm in the presence of UV-A irradiation. When examined by phase contrast microscopy, most spores appeared dead, being lysed and with a granular content. The percentage germination of both control fungal treatments was greater than 70%.

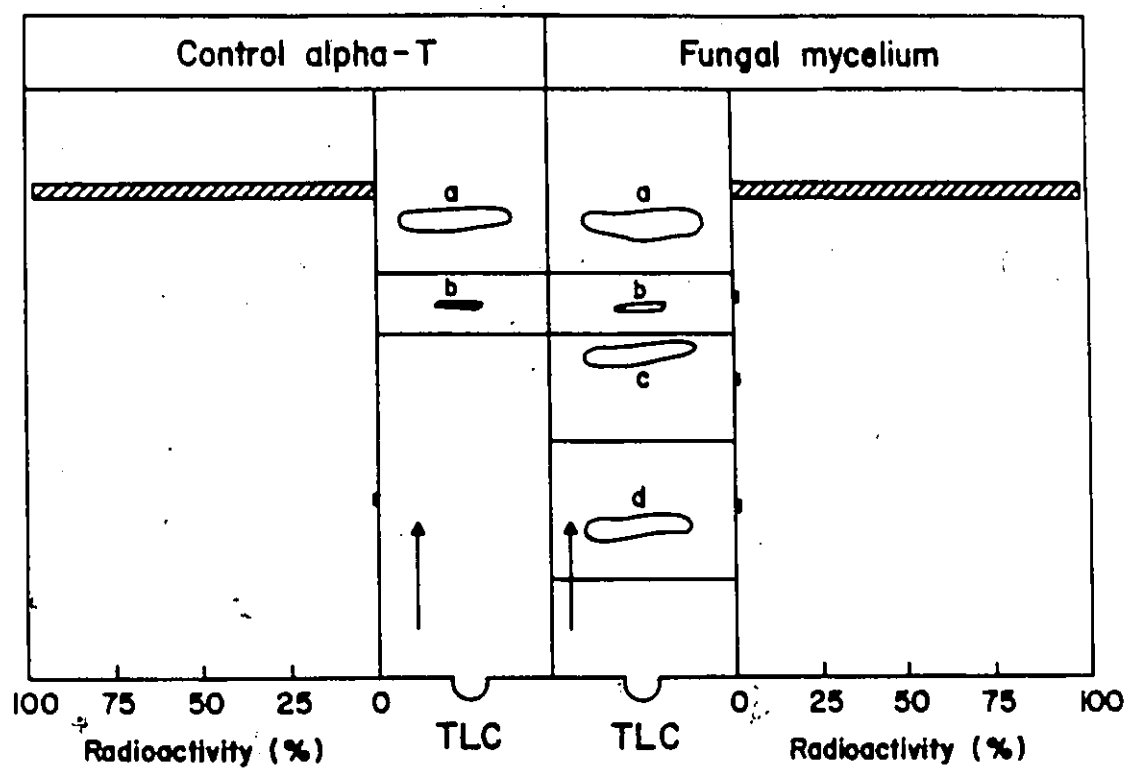
TABLE 5

DISTRIBUTION OF RADIOACTIVITY IN DIFFERENT FRACTIONS OBTAINED FROM CULTURES OF *F. oxysporum* f.sp. *radicis lycopersici* GROWN IN A LIQUID MEDIUM AT 25°C AND AFTER 48 HRS, SUPPLIED WITH ^3H -ALPHA-T, AND FROM UNINOCULATED CONTROLS CONTAINING ^3H -ALPHA-T ONLY. IN BOTH CASES, ^3H -ALPHA-T (10,000 DISINTEGRATIONS $\text{MIN}^{-1}/\text{UG}$) WAS ADDED TO GIVE A FINAL CONCENTRATION OF 0.3 UG/ML . RESULTS ARE EXPRESSED IN DISINTEGRATIONS $\text{MIN}^{-1}/\text{ML}$ OF ORIGINAL CULTURE RECOVERED AFTER 24 HRS INCUBATION WITH THE ^3H -LABELLED MATERIAL.

FRACTION	ACTIVITY ^a (disintegrations $\text{min}^{-1}/\text{ml}$ of original culture)	
	$\mu\text{FUNGAL MYCELIUM}$	UNINOCULATED CONTROL + dT
Mycelial residue	826 (32)	-
Aqueous fraction of filtrate	120 (4.6)	184 (7.7)
Combined ethyl acetate and hexane fractions of filtrate	1634 (63.3)	2186 (92.2)
Total	2580	2370
% Recovery	86%	79%

^a Values are means of duplicate samples. Values in brackets are the activities expressed as percentages of total recovered activity.

Figure 16: Schematic representation of thin layer chromatography of ethanolic extracts prepared from cultures of FORL mycelium incubated 24 hrs with ^3H -alpha-T (0.3 $\mu\text{g/ml}$) and from control uninoculated medium containing ^3H -alpha-T only. Horizontal bars represent ^3H activity of the various sections of the chromatogram. The activity of each section is expressed as a percentage of total activity recovered from the cultures. The values are means of duplicate samples. TLC solvent used was hexane-ethyl acetate (50:50, v/v).



Chapter IV

DISCUSSION

The present study provides evidence for the accumulation of thiophenes BBT-OH, BBT-OAc and Alpha-T in T. erecta seedlings as a response to applied stress under specific environmental conditions which seem to be related to plant age, growth conditions and the nature of the treatment agent. These thiophenes are highly phototoxic against the invading fungal pathogens F. oxysporum var callistephi race 2 (FOVC race 2) and F. oxysporum f.sp. radicis lycoperisici (FORL). These findings, in addition to evidence accumulated from previous studies on the toxicity of these compounds against bacteria, fungi and yeasts (sections 1.2.3.2 and 4.3), lead to the suggestion that this class of chemicals might be considered as a possible new class of phytoalexins.

4.1 Chemical identification of thiophenes from seedlings of Tagetes erecta

Several studies have examined the occurrence of thiophene derivatives in adult Tagetes species (Bohlmann et al., 1973 Bohlmann and Zdero, 1979; Groneman et al., 1984 Hogstad et al., 1984) or in calli and suspension-cultured cells of the marigold (Ketel et al., 1984 Norton et al., 1985), but

very few have dealt with young seedlings (Pensl and Sutfeld, 1985; Sutfeld, 1982; Downum and Towers, 1983). This is the first investigation, as far as is known, concerned with the analysis of thiophenes in seedlings of T. erecta. It has revealed the presence of 4 thiophenes: BBT-OAc, BBT, alpha-T (Figure 2) and compound 4 tentatively identified as being a polyine monothiophene (Figure 10) in extracts of whole young germinating seedling (4 to 8 days old). In roots and stems of older seedlings (14 to 26 days old) grown in aseptical medium, 3 thiophenes: BBT-OH, BBT-OAc and alpha-T were found (section 3.1).

The appearance of compound 4 only in the first days of growth and its subsequent disappearance from older plants lead to the suggestion that it could be a precursor of the other thiophene derivatives. In agreement with this suggestion is the fact that the tentatively identified monothiophenes (Figure 7) are believed to be important intermediates in the biosynthesis of naturally occurring thiophenes, being directly derived from the widespread tridecapentadyne by a reaction equivalent to the addition of H_2S (Figure 3) (Bohlmann and Zdero, 1985). These tentatively identified compounds were found in many species of the Asteraceae (Bohlmann and Zdero, 1985). One of them (Figure 7b) has been reported from one Tagetes species: T. paucilauba (Bohlmann and Hopf, 1973), but neither was ever isolated from T. erecta.

This investigation provides no evidence for the presence of BBT in lower stems and roots of T. erecta (14 to 26 days old) under the environmental conditions used. This does not correspond to previous profile analyses of thiophenes in T. erecta showing BBT to be an important constituent of the roots (Bohlmann et al., 1967 Bohlmann and Herbst, 1962; Hogstad et al., 1984). Differences in the growth stages of the plants, the environmental conditions employed and genetic differences in the material used might be the reason for the observed discrepancy.

Thiophene pattern in T. erecta seems therefore to depend upon the plant age, the environmental conditions used and the particular organ under study, in agreement with earlier observations made on Tagetes species (Sutfeld, 1982 Downum and Towers, 1983; Ketel et al., 1984 ,Norton et al., 1985).

4.2 Effects of various treatments on the fate of thiophenes in T. erecta

This study demonstrates that under very specific conditions, applied stress can cause the accumulation of thiophenes in T. erecta. This accumulation seems to be under the control of specific factors associated with the nature of the treatment agent, its ability to inflict damage on the plant tissue, the age and physiological state of the plant and the environmental conditions used.

In general, it was seen that severe treatment conditions causing rapid damage to the plant cells result in a reduced thiophene yield. Milder treatment, with the plant remaining in a relatively good physiological state, results in accumulation of thiophenes.

The failure of young seedlings of T. erecta (4 to 8 days old) to accumulate thiophenes after treatment with an abiotic agent (HgCl_2) (Figure 10) or with a biotic agent (A. taquetica) (Figure 11), could be due a) to the weak physiological state of the plant at the time of treatment, very young seedlings being more vulnerable and more easily penetrated and invaded by pathogens than older plants (Agris, 1977), and b) to unfavorable growth conditions, including external constraints such as shortage of mineral nutrients. These factors could have possibly limited the capacity of the plant for dynamic defense. The amount of stress imposed on the plant cells by biotic or abiotic treatment, added to the prevailing stress, have probably exceeded the elastic limits of the plant, causing rapid damage and death, thus preventing thiophene accumulation.

Older T. erecta seedlings (14 to 26 days old) grown under aseptic conditions reacted differently to infection by the two pathogenic fungi F. oxysporum f.sp. radicis lycopersici (FORL) and F. oxysporum var callistephi race 2 (FOVC race2). This was reflected in a) different amounts of damage caused to the host by the pathogen (Figure

12) and b) differences in the yields of the thiophenes BBT-OH, BBT-OAc and alpha-T in the infected tissues examined (Figure 13).

The more extensive and rapid colonization of T. erecta by FORL than by FOVC race 2, despite the fact that FORL is known as a tomato and not a marigold pathogen, may be explained by the high virulence of the isolate. It is not unusual for a virulent strain of wilt fungus to infect a wide variety of hosts (Bell and Mace, 1981). The environmental conditions employed, particularly the maintenance of the plants in aseptic medium, may have favored the development of the infection. Plants grown in sterilized medium often show decreased resistance to wilt diseases as compared to those grown in the field due to the lack of induced immunity caused by microorganisms or chemicals in the soil (Bell and Mace, 1981).

The major increases in the amounts of thiophenes occurring with time during the infection process in the roots and lower stems of moderately susceptible seedlings (FOVC race 2 infection) but not in highly susceptible seedlings (FORL infection) (Figure 13), lead to the suggestion that both types of host-fungal interactions affect the metabolic control system of thiophenes in different ways. Infection by FORL could prevent thiophene accumulation by a) suppressing synthesis, by the presence of a fungal suppressor preventing thiophene production or more probably by killing cells too

rapidly for synthesis to occur, b) enhancing the rate of degradation in the very damaged plant cells and c) metabolism by FORL.

Infection by FOVC race 2 could enhance thiophene accumulation as a result of a) the presence of a fungal or endogenous elicitor for thiophene production, b) a disorganized pattern of growth, c) the absence of a fungal suppressor of thiophene production and d) other interactions that preceded and led to thiophene accumulation.

Changes of thiophene content of plant cells may depend upon alterations in the activity of the enzymes responsible for their synthesis. The only enzyme concerned with thiophene metabolism that has been characterized is 5-(4-acetoxy-1-butyryl)-2,2'-bithiophene : acetate esterase isolated from T. patula and responsible for the conversion of BBT-OAc to BBT-OH (Sutfeld and Towers, 1982).

It is suggested that the observed accumulation of BBT-OH with time in FOVC race 2 infected tissues might be a direct consequence of an increase in the activity of this enzyme. This is sustained by the fact that the increase in the amounts of BBT-OH occurring in infected tissues 4 days after treatment with FOVC race 2 is accompanied by a simultaneous decrease of the BBT-OAc content of the infected plant, relative to day 0 levels (Figure 13a,b). This time-course change in the thiophene levels is not observed in healthy control plants.

Several workers have reported increases in the amounts of certain enzymes occurring during fungal infection. Accumulation of furanoterpenes (Uritani et al., 1976), sesquiterpenes (Corsini and Pavék, 1980), stilbenes (Fritzemeier and Kindl, 1981), isoflavonoids (Rathmell and Bendall, 1971; Dixon and Bendall, 1978; Dixon and Lamb, 1979) and furanocoumarins (Hauffe et al., 1986) have been associated with increased enzyme activity.

The results obtained in this investigation are substantiated by the results of a recent study examining thiophene production in crown galls of T. patula produced by Agrobacterium tumefaciens (strains A208, A277) infection or by transformed callus tissues derived from them (Norton et al., 1985). BBT-OH and BBT-OAc were recovered in higher yields from galls and callus tissues than from normal uninfected plant parts. The high standard deviations obtained in their data led the authors to suggest that the irregularities of thiophene production were possibly due to disorganized patterns of growth, and that it was impossible to predict the amount of thiophenes in these transformed tissues.

It is possible that the high standard deviations observed by these authors may have been caused by the environmental growth conditions (plants were grown in greenhouses), rather than by an unpredictable thiophene profile. In addition, little information was obtained on the time-course

changes of thiophene levels in the transformed tissues, since thiophene contents were measured only at two separate times (August and November harvest). The authors did not relate the morphological appearance of the different infected tissues to their thiophene content and did not comment on the possible implication of the accumulated thiophenes in defense against microbial invasion.

4.3 Fungitoxicity and metabolism of thiophenes

4.3.1 Effect of Alpha-terthienyl on fungal growth

The biological activity of alpha-T against fungi has already been studied quantitatively (Di Cosmo et al., 1982). In the presence of near UV light, photoinduced toxicity of this compound was observed toward a variety of ascomycetous and oomycetous fungi, with EC_{50} values ranging from 0.07 to 750 $\mu\text{g/ml}$, as measured using inhibition of radial mycelial growth across an agar surface. In the dark, alpha-T was not significantly toxic against several fungal species tested but some, as for example Colletotrichum sp., were highly inhibited by the thiophene ($EC_{50} = 6 \text{ ug/ml}$).

The present study indicates that both spores and mycelium of FOVC race 2 and FORL are highly sensitive to alpha-T in the presence of near UV irradiation, in agreement with Di Cosmo et al. (1982), and both fungi seem to be equally affected by the toxic thiophene. In the dark, only the growth of FOVC race 2 is inhibited by the presence of this

compound (section 3.4.1). Possible mechanistic explanations accounting for the differential sensitivity of the two fungi to alpha-T in the dark include : a) differential cell wall permeability to the thiophene, b) differential sensitivity of the site of action, c) existence of a metabolic bypass for the process inhibited by alpha-T in FORL but not in FOVC race 2 and d) differential detoxification of alpha-T before or after its entry into the fungal cells. These processes might not operate in the presence of near UV light when the strong photosensitizing action of alpha-T might mask the more subtle biological effects seen in the dark.

This is the first report on alpha-T causing protoplasmic granulation and lysis of fungal cells (FORL and FOVC race 2). No previous microscopical studies have been made on the fungitoxic effects of this compound, despite the evidence for its antifungal activity. Various other naturally occurring antifungal substances have been shown to cause a similar type of activity in other organisms. They include kievitone, orchinol, phaseollin (Smith, 1982) and phenylheptatriyne (Bourque et al., 1985), which are all membrane-damaging compounds. Similarly, the extensive research done on the mechanism of action of alpha-T supports the fact that cellular membrane damage is the primary target of alpha-T photosensitization (see section 1.2.3.2).

As an antifungal compound, alpha-T appears to be particularly toxic in the presence of near UV light. It should be

noted that the near UV radiation levels from the lamps used are not as high as those from the sun, incident at the surface of the earth. Until now, only a few naturally occurring compounds have been attributed an EC_{50} value of less than 1 $\mu\text{g/ml}$. Even some of the most active fungitoxic secondary plant substances such as phaseollin (Skipp and Bailey, 1976), kievitone (Smith, 1976), capsidiol (Bailey et al., 1976) and wyerone epoxide (Hargreaves et al., 1976) have been reported to have antifungal activities at around 10 $\mu\text{g/ml}$.

The high phototoxicity of alpha-T indicates that it has a potential as a protectant fungicide. Its lipophilic nature combined with the fact that it is much less effective inside the plant (in the dark) make it an inefficient systemic fungicide. But further investigations are required, especially to assess the selective toxicity of this compound to the fungus as compared to the host plant.

4.3.2 Metabolism of Alpha-T in vitro

This study does not provide any substantial evidence for the in vitro metabolism of alpha-T by FORL (Figure 16).

Studying the metabolic conversion of a natural substance by a microbial agent requires that the appropriate environmental conditions be chosen. It is possible that the nutrient status of the medium employed and the period of incubation of the fungal mycelia in the presence of ^3H -alpha-T, might have influenced the regulation of the alpha-T metabolism.

Some cases exist where environmental factors have been shown to influence the degradation of a secondary plant metabolite by a fungus. For example, in vitro degradation studies of pisatin have indicated that the nature and the concentration of the carbohydrate source in the medium affected the rate of metabolism of this substance by F. solani f.sp. pisi, suggesting that synthesis of pisatin degrading enzymes were subjected to catabolite repression (De-Wit Elshove and Fuchs, 1971; Van Etten and Barz, 1981). Most degradation studies have been done on short term experiments (<24 hrs) but longer incubation times were needed in order to observe breakdown of pisatin by F. solani f.sp. pisi (De Wit-Elshove and Fuchs, 1971). Further studies on the metabolism of alpha-T by fungi should take into account these considerations.

4.3.3 Fungitoxicity of thiophene standards

The spore germination bioassay used in this study to test the fungitoxicity of thiophene standards is one of the most extensively and widely used of the fungicide test methods (Pelletier, 1977). The results of this test (section 3.4.3) demonstrate the antifungal activity of the thiophenes BBT-OH, BBT-OAc and BBT occurring in T. erecta and of the thiophene reference compound BBT-2OAc not detected in this plant but found in T. patula (Pensl and Sutfield, 1985). Five µg/ml of each of these standards completely prevented germination of FORL and FOVC race 2 conidia in the presence

of near UV light. The antifungal spectra of these thio-phenes, as well as their fungitoxic activity in the dark were not investigated owing to the very limited amounts of each compound which were available.

The antifungal action of BBT has been previously demonstrated (Di Cosmo et al., 1982). This compound was toxic in the dark against a variety of fungal species including Aspergillus niger ($EC_{50} = 200 \mu\text{g/ml}$), Pythium aphanidermatum ($EC_{50} = 20 \mu\text{g/ml}$) and Saprolegnia sp. ($EC_{50} = 20 \mu\text{g/ml}$), but its activity was enhanced in the presence of UV-A irradiation. The phototoxic action of BBT-OH, BBT-OAc and BBT has been reported for Escherichia coli, Pseudomonas aeruginosa and Saccharomyces cerevisiae (Downum et al., 1983), but under the conditions of the experiment, these compounds were not effective in the dark. The phototoxic activity of BBT-2OAc had never been examined. This is the first report on the photosensitization effect of BBT-2OAc, as well as the antifungal activity of the two bithiophene derivatives BBT-OH and BBT-OAc.

The mode of action of these compounds has not been extensively studied. Only BBT has received some attention. BBT seems to require oxygen for expression of its toxicity (Mc Lachlan et al., 1984). In contrast to alpha-T, this compound was more toxic toward the repair-deficient E. coli strain than toward the repair competent E. coli strain, suggesting that cellular DNA may be a molecular target for the

photoactivated process (Downum et al., 1983). It would be surprising however, if alpha-T and BBT which are structurally very closely related, have very different modes of action. Further structure-activity relationships are needed in order to obtain more detailed information upon the exact mechanism of action of these thiophene derivatives.

4.4 Thiophenes: Role in disease resistance in T. erecta.

From the present study, it is clear that treatment of T. erecta seedlings with a mild pathogen (FOVC race 2) causes a marked accumulation of thiophenes in the infected plant cells (Figure 13). This finding, in combination with the antimicrobial properties of these compounds (section 3.4) lead us to suggest that thiophenes might be considered as a new class of phytoalexins since the two criteria: antimicrobial activity and accumulation following microbial infection, define a compound as phytoalexins (Paxton, 1981). Clearly, more evidence on the non-photosensitizing effects of BBT-OH and BBT-OAc is required for a definitive assessment of their role as phytoalexins.

The other biotic and abiotic treatments utilized do not result in this accumulation. Thus, there seems to be some degree of specificity associated with the accumulation of thiophenes. This specificity has been observed for other types of secondary plant substances. For example, in potato tubers, only Phytophthora infestans compatible and incompati-

ble races, as well as arachidonic and eicosapentanoic acids isolated from this fungus, were able to cause accumulation of sesquiterpenes phytoalexins (Bloch et al., 1984). Other fatty acids (Bostock et al., 1981), biotic (Lisker and Kuc, 1977) and abiotic agents (Currier, 1975; Varns, 1970; Varns et al., 1971a) did not cause phytoalexin accumulation in potato.

Fungal-induced host metabolites with antimicrobial properties are believed to play a role in the resistance of plants to many phytopathogens (Cruickschank, 1963). This study indicates that more than one post-infectionally accumulated compound may be involved since BBT-OH, BBT-OAc and Alpha-T were all seen to ultimately accumulate in FOVC race 2 infected tissues (Figure 13). This is not rare in the case of phytoalexins (Thomas and Allen, 1970; Allen and Thomas, 1971b; Varns et al., 1971b; Higgins and Smith, 1972). For example, phaseollin, phaseollidin, phaseollinisoflavan and kievitone phytoalexins were found to accumulate in bean tissues in response to infection by some fungal and bacterial pathogens as well as some viruses (Bailey, 1973; Smith et al., 1976).

It seems a logical consideration that the adverse chemical environment created by the high levels of these thiophene derivatives present in and around the area of fungal attack, would play a role in the inhibition of the attacking pathogen. In assessing this possible role, it is necessary

to relate the toxicity of thiophenes in vitro to the levels at which they accumulate in the infected tissues. On the basis of these considerations, a possible role of BBT-OH, BBT-OAc and alpha-T on the limitation of FOVC race 2 development in treated marigolds can be visualized.

If any involvement of thiophenes occurred in the inhibition of the FOVC race 2 infection process, BBT-OH would be primarily responsible since this compound attained relatively high levels in the early stages of infection (216 ng/seedling in infected seedlings as compared to 31 ng/seedling in controls, at day 8 post-treatment).

A close examination of the thiophene kinetics in the Tagetes-FOVC race 2 interaction as compared to the Tagetes-FORL interaction (Figure 13) reveals the coincidental and comparable thiophene pattern 4 days after inoculation, different from that of the control plants. This is significant in that it apparently demonstrates the similar initial reaction of the infected plants to cope with the fungal invasion in terms of thiophene metabolism. But the subsequent fate in the thiophene content of infected cells markedly differed for both interactions, decreasing with time in the rapidly spreading and damaging FORL infection and drastically increasing with time in the milder infection. The possible reasons for this differential fate of thiophenes have been discussed (section 4.2).

The limitation of the FOVC race 2 infection could be associated with the accumulation of BBT-OH, BBT-OAc and alpha-T to amounts which might be expected to inhibit mycelial growth based on the bioassay data. The concentration of BBT-OH in the tissues of moderately susceptible T. erecta 12 days after inoculation with FOVC race 2 is 464 ng/seedling equivalent to 6.96 $\mu\text{g/g}$ fresh weight (since 15 seedlings = 1 g fresh weight). This would, according to in vitro studies that 5 $\mu\text{g/ml}$ of BBT-OH completely prevented germination of FOVC race 2 conidia in the presence of UV-A irradiation, and equating $\mu\text{g/g}$ fresh weight of tissues with $\mu\text{g/ml}$, result in a total inhibition of FOVC race 2 conidial germination in the infected tissues and probably also inhibition of mycelial growth. It is unlikely that the levels of BBT-OH present in controls at day 12 (37ng/seedling or 0.55 $\mu\text{g/g}$ fresh weight) be as efficient in inhibiting fungal growth. It should be pointed out that the fungitoxicity of BB-OH in the dark was not investigated due to the limited amount of material available.

Similarly, alpha-T had antifungal activities in vitro toward FOVC race 2 causing 50% growth inhibition at 0.06 $\mu\text{g/ml}$ in the presence of near UV light and 43% growth inhibition at 1 $\mu\text{g/ml}$ in the dark. The amount of alpha-T isolated from FOVC race 2 infected tissues 12 days after inoculation (267 ng/seedling equivalent to 4 $\mu\text{g/g}$ fresh weight) corresponds to 67 times the EC_{50} value in the presence of light and 4 times the approximate EC_{50} value in the dark.

But such estimations, despite their popularity in the area of phytoalexin research, may mean very little. It is risky to draw conclusions pertaining to the biological effectiveness of in situ concentrations from in vitro assays since the environments in which the compounds exist in the two situations are quite different. The present results are based on experiments done in an artificial system and there are undoubtedly problems in trying to extend them to naturally infected whole plants in which the spatial and temporal distribution of the infecting fungus, the interactions with local microflora and the components of the host's response must be much more complex. It should also not be forgotten that assays to test antifungal activity were made separately for each thiophene, whereas they occur together in T. erecta tissues. It is not known whether additive or synergistic effects on fungal growth would result from this interaction. Furthermore, this analysis does not take into account unequal distribution of the thiophenes within the plant tissues. Phytoalexins in various wilt infected plants are apparently exuded into gums, gels or tylose walls of infected xylem vessels which may block phytoalexin movement in the xylem vessel, thus limiting its diffusion and permitting its accumulation in regions of infection (Bell and Mace, 1981). If this is the case for thiophenes, they would accumulate in these structures at many fold higher concentration than those in extracts of entire tissues.

4.5 Future research

It is the concentration of thiophenes in the cells in contact with the advancing hyphae that is biologically important. Such estimations are technically difficult. Detailed histochemical studies coupled with biochemical studies are needed in order to clarify the role of thiophenes in the time-space relationships of the dynamic process that contain wilt fungi in T. erecta.

Nothing is known on the biochemical basis of the modification in the metabolism of thiophenes occurring in T. erecta plant tissues following infection by pathogens or exposure to other kinds of stress. Information is especially needed concerning the basis of differentiation in thiophene metabolism between highly susceptible and moderately susceptible host-pathogen interaction. The question of the involvement of specific elicitors or suppressors in this metabolic regulation is an important theme for future research.

✓ The two T. erecta - F. oxysporum host-pathogen systems described in this study might serve as a valuable model for the characterization of enzymes involved in thiophene metabolism, as well as for the evaluation of the role of thiophenes in the coordinated process of resistance in T. erecta.

Finally, it is believed that the synthesis of some thiophenes might be light induced (Sutfeld, 1982). Since thio-

phenes are powerful photosensitizers, the examination of the relationships between light duration, thiophene yields and disease resistance of T. erecta to fungal pathogens would be an interesting area of research. One study has recently demonstrated that the susceptibility of T. erecta to A. tagetica was decreased by increasing exposure to light in the humidity chambers (Cotty and Misaghi, 1985). It remains to be determined whether thiophenes play a role in this increase in plant resistance.

Chapter V

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APPENDIX 1

Composition of the Media Used in this Studya) Hoagland's nutrient solution (from Hoagland and Arnon, 1938)

Ingredient	Weight (g)	Distilled H ₂ O (ml)	Use in preparation of nutrient solution (ml)
<u>Salt</u>			
KH ₂ PO ₄	136.09	1000	1
KNO ₃	101.10	1000	5
Ca (NO ₃) ₂ 4H ₂ O	236.16	1000	5
MgSO ₄	120.39	1000	2
<u>Micronutrients</u>			
H ₃ SO ₄	2.86		
MnCl ₂ 4H ₂ O	1.86	Distilled H ₂ O to 1000 ml.	
ZnSO ₄ 7H ₂ O	0.22	1 ml is used in preparation	
CuSO ₄ 5H ₂ O	0.88	of nutrient solution.	
H ₂ MoO ₄ H ₂ O	0.02		
<u>Iron solution</u>			
Ferric tartrate	5.0	1000	1

To make 1 l. of full strength Hoagland's nutrient solution, use the required amount of salt, add the required amount of micronutrients solution and iron solution and make up to 1000 ml with distilled H₂O.

b) Potato Sucrose Agar (PSA) (from Booth, 1977)

- * 500 ml potato extract
- 20 g sucrose
- 20 g agar
- 500 ml distilled water

The water and potato extract are mixed together and the sucrose and agar added. The mixture is heated slowly until the agar is dissolved and the pH adjusted if necessary to 6.5 with calcium carbonate.

- * Potato extract is prepared from 1800 g of mature main crop potatoes peeled and diced and suspended in muslin in 4500 ml of water and boiled for 10 min. The potatoes are then discarded and the liquor placed in large glass containers and autoclaved at 15 psi for 20 min. It can be stored in a refrigerator for use as required.

c) FRIES MEDIUM (modified from Miller and Adams, 1971)

1g KH_2PO_4
 5g $(\text{NH}_4)_2\text{C}_4\text{H}_4\text{O}_6$
 0.2g MgSO_4
 0.1g NaCl
 20g Sucrose

Make up to 1000 ml with distilled water and then add 5 ug biotin, 1 ml of solution A and 1 ml of solution B.

PREPARATION OF SOLUTIONS A AND B:

Ingredients	Weight (mg)	Distilled H ₂ O (ml)
<u>Solution A</u>		
(Na ₂ B ₄ O ₇ · 10H ₂ O)Na	8.8	100
(NH ₄) ₆ MoO ₂₄ · 4H ₂ O	6.4	
Zn SO ₄ · 7H ₂ O	8.8	
CuCl ₂	27	
MnCl ₂ · 4H ₂ O	72	
<u>Solution B</u>		
FeCl ₃ · 6H ₂ O	96	100

APPENDIX 2

NAMES, MOLAR AND SPECTRAL CHARACTERISTICS OF THE THIOPHENE REFERENCE COMPOUNDS. THE UV ABSORPTION MAXIMA ARE FOLLOWED BY PUBLISHED EXTINCTION COEFFICIENT IN PARENTHESES. THE MASS SPECTRAL DATA SHOW THE RELATIVE INTENSITIES OF m/e PEAKS.

NAME	MOLECULAR WEIGHT	FORMULA	CHEMICAL STRUCTURE	UV-MAXIMA (m)	IR (1/cm)	MS (m/e 10%)
5-(4-hydroxy-1-butenyl)-2,2'-bithienyl (RRT-OH)	234	$C_{12}H_{10}S_2$		333 (22700) 327 (22500) 240 (6600)	3650, 3600, 3060, 1050, 845	-
5-(4-acetoxy-1-butenyl)-2,2'-bithienyl (RRT-OAc)	279	$C_{14}H_{12}O_4S_2$		333 (21800) 325 (21500) 246 (5540)	-	43(47), 95(116), 108(114), 149(111), 171(114), 203(18), 216(100), 217(18), 218(22), 279(19)
5-(1,4-diacetoxy-1-butenyl)-2,2'-bithienyl (RRT-2OAc)	334	$C_{16}H_{14}O_6S_2$		332 (22800) 325 (22700) 239 (6500)	2240, 1760, 1240	43(100), 219(30), 232(74), 274(42), 334(18)
5-(4-buten-1-ynyl)-2,2'-bithienyl (RRT)	216	$C_{12}H_8S_2$		346 (27800) 254 (9750)	3070, 2200, 1600, 975, 920, 850	95(13), 171(118), 215(11), 216(100), 217(18)
2,2':5',2''-terthienyl (M-T)	248	$C_{12}H_8S_3$		350 (22400) 252 (9100)	830, 800, 690	69(18), 124(110), 127(19), 171(114), 203(18), 248(100), 249(18), 250(15)

APPENDIX 3Statistical analysis of Figure 10 (a to d)

Note: Means followed by the same letter in a row or by the same number in a column are not significantly different ($p = 0.05$) in Duncan's multiple range test.

Analysis of Fig. 10(a)

Days post-treatment	Mean concentration of BBT-OAc ($\mu\text{g}/\text{seedling}$) for HgCl_2 exposure times of:				
	0 min	10 min	30 min	60 min	120 min
1	0.35 ₁ ^a	0.31 ₁ ^a	0.21 ₁ ^b	0.15 ₁ ^b	0.16 ₁ ^b
2	0.60 ₂ ^a	0.33 ₁ ^b	0.31 ₂ ^b	0.20 ₁ ^c	0.14 ₁ ^d
3	0.71 ₃ ^a	0.41 ₁ ^b	0.27 ₂ ^c	0.17 ₁ ^c	0.18 ₁ ^c

Analysis of Fig. 10(b)

Days post-treatment	Mean concentration of BBT ($\mu\text{g}/\text{seedling}$) for HgCl_2 exposure times of:				
	0 min	10 min	30 min	60 min	120 min
1	0.23 ₁ ^a	0.21 ₁ ^a	0.22 ₁ ^a	0.18 ₁ ^a	0.22 ₁ ^a
2	0.18 ₁ ^a	0.14 ₁ ^{ab}	0.13 ₁₂ ^{ab}	0.14 ₂ ^{ab}	0.11 ₂ ^b
3	0.19 ₁ ^a	0.14 ₁ ^{ab}	0.11 ₂ ^{ab}	0.11 ₂ ^{ab}	0.09 ₂ ^b

Analysis of Fig. 10(c)

Days post-treatment	Mean concentration of compound 4 (ug/seedling) for HgCl ₂ exposure times of:				
	0 min	10 min	30 min	60 min	120 min
1	0.31 ₁ ^a	0.31 ₁ ^a	0.28 ₁ ^a	0.27 ₁ ^a	0.31 ₁ ^a
2	0.28 ₁ ^a	0.24 ₂ ^{ab}	0.22 ₂ ^{bc}	0.20 ₂ ^{bc}	0.17 ₂ ^c
3	0.21 ₁ ^a	0.15 ₃ ^{ab}	0.12 ₃ ^b	0.14 ₃ ^{ab}	0.16 ₂ ^{ab}

Analysis of Fig. 10(d)

Days post-treatment	Mean concentration of Alpha-T (ug/seedling) for HgCl ₂ exposure times of:				
	0 min	10 min	30 min	60 min	120 min
1	0.10 ₁ ^a	0.10 ₁ ^a	0.12 ₁ ^a	0.10 ₁ ^a	0.09 ₁ ^a
2	0.14 ₁ ^a	0.13 ₁₂ ^a	0.12 ₁ ^a	0.08 ₁ ^b	0.09 ₁ ^b
3	0.11 ₁ ^{ab}	0.17 ₂ ^a	0.10 ₁ ^{ab}	0.05 ₂ ^b	0.10 ₁ ^b

APPENDIX 4

Statistical Analysis of Figure 11 (a to d)

Note: Means followed by the same letter in a row not significantly different ($p = 0.05$) in one way analysis of variance.

Means followed by the same number in a column are not significantly different ($p = 0.05$) in Duncan's multiple range test.

Analysis of Fig. 11(a)

Days post-inoculation	Mean concentration of BBT-OAc ($\mu\text{g}/\text{seedling}$) in <u>I. erecta</u> :	
	Controls	Infected with <u>A. tagetica</u>
0	0.35 ₁ ^a	0.35 ₁ ^a
1	0.49 ₂ ^a	0.38 ₁ ^a
2	0.47 ₂ ^a	0.23 ₂ ^b
3	0.54 ₂ ^a	0.21 ₂ ^b
4	0.54 ₂ ^a	0.12 ₃ ^b

Analysis of Fig. 11(b)

Days post-inoculation	Mean concentration of BBT ($\mu\text{g}/\text{seedling}$) in <u>I. erecta</u> :	
	Controls	Infected with <u>A. tagetica</u>
0	0.16 ₁₂ ^a	0.16 ₁ ^a
1	0.17 ₂ ^a	0.16 ₁ ^a
2	0.12 ₁ ^a	0.11 ₂ ^a
3	0.13 ₁₂ ^a	0.08 ₃ ^b
4	0.13 ₁₂ ^a	0.05 ₄ ^b

Analysis of Fig. 11 (c)

Days post-inoculation Mean concentration of compound 4 (ug/seedling) in T. tagetica:

	Controls	Infected with <u>A. tagetica</u>
0	0.13 ₁ ^a	0.13 ₁₂ ^a
1	0.16 ₁ ^a	0.15 ₁ ^a
2	0.12 ₁ ^a	0.11 ₂₃ ^a
3	0.15 ₁ ^a	0.09 ₃ ^b
4	0.13 ₁ ^a	0.05 ₄ ^b

Analysis of Fig. 11 (d)

Days post-inoculation Mean concentration Alpha-T (ug/seedling) in T. erecta:

	Controls	Infected <u>A. tagetica</u>
0	0.06 ₁ ^a	0.06 ₁ ^a
1	0.06 ₁ ^a	0.07 ₁ ^a
2	0.09 ₁ ^a	0.09 ₁ ^a
3	0.07 ₁ ^a	0.07 ₁ ^a
4	0.07 ₁ ^a	0.05 ₁ ^a

APPENDIX 5

Statistical Analysis of Fig. 13 (a to c)

Note: Means followed by the same letter in a row or by the same number in a column are not significantly different ($p = 0.05$) in Kruskal-Wallis multiple range non parametric test.

Analysis of Fig. 13(a)

Days post-inoculation	Mean concentration of BBT-OH (ng/seedling) in <u>T. erecta</u> :		
	Controls	Infected with FOVC race 2	Infected with FORL
0	<17 ^a ₁	<17 ^a ₁	<17 ^a ₁
4	<17 ^a ₁	63 ^b ₂	70 ^b ₂
8	31 ^a ₁₂	216 ^b ₃	21 ^a ₁
12	37 ^a ₂	464 ^b ₄	<17 ^c ₁

N.B.: Values <17 are below the detection limit of the assay method.

Analysis of Fig. 13(b)

Days post-inoculation	Mean concentration of BBT-OAc (ug/seedling) in <u>T. erecta</u> :		
	Controls	Infected with FOVC race 2	Infected with FORL
0	0.97 ^a ₁	0.97 ^a ₁	0.97 ^a ₁
4	0.76 ^a ₂	0.30 ^b ₂	0.24 ^b ₂
8	0.81 ^a ₂	0.84 ^a ₁	0.06 ^b ₃
12	0.84 ^a ₁₂	2.64 ^b ₃	0.08 ^c ₃

N.B.: Values <31 are below the detection limit of the assay method.

Analysis of Fig. 13(c):

Days post-inoculation	Mean concentration of Alpha-T (ng/seedling) in <u>T. erecta</u> :		
	Controls	Infected with FOVC race 2	Infected with FORL
0	<31 ^a ₁	<31 ^a ₁	<31 ^a ₁
4	37 ^a ₁	32 ^a ₁₂	38 ^a ₂
8	<31 ^a ₁	47 ^b ₂	<31 ^a ₁
12	100 ^a ₂	267 ^b ₃	<31 ^c ₁

N.B.: Values <31 are below the detection limit of the assay method.

APPENDIX 6

STATISTICAL ANALYSIS OF FIGS. 14, 15

NOTE: Means followed by the same letter in a row or by the same number in a column are not significantly different ($P = 0.05$) in Duncan's multiple range test and in T-tst respectively.

Statistical Analysis of Fig. 14

Treatment group	Mycelial dry weights (mg) for α -T concentrations (ug/ml):							
	0	0.001	0.01	0.1	1	10	50	100
FOVC race 2 - UV	19.0 ^a 1	13.5 ^{ab} 1	14.9 ^{ab} 1	13.0 ^b 1	10.8 ^{bc} 1	12.4 ^b 1	14.0 ^{ab} 1	10.2 ^{bc} 1
FORL - UV	21.1 ^a 1	16.8 ^b 1	21.6 ^a 1	25.2 ^{ab} 2	22.0 ^a 2	27.1 ^b 2	25.5 ^{ab} 2	28.8 ^{ab} 2

Statistical Analysis of Fig. 15

Treatment group	Mycelial dry weights (mg) for α -T concentrations (ug/ml):							
	0	0.001	0.01	0.1	1	10	50	100
FOVC race 2 + UV	15.7 ^a 1	18.0 ^a 1	14.6 ^a 1	5.8 ^b 1	0.2 ^c 1	0 ^c 1	0.3 ^c 1	0 ^c 1
FORL + UV	22.7 ^a 1	18.9 ^{ab} 1	15.7 ^b 1	10.3 ^c 1	2.5 ^d 1	1.9 ^d 1	1.1 ^d 1	1.0 ^d 1

APPENDIX 7

2

VALUES OF F (F), DEGREES OF FREEDOM (DF), AND PROBABILITIES (P) OBTAINED FROM ANALYSIS OF VARIANCE (ANOVA) OF FIGURE 10

LENGTH OF TREATMENT WITH $HgCl_2$

COMPOUND	0 min			10 min			30 min			60 min			120 min		
	DF	F	P	DF	F	P	DF	F	P	DF	F	P	DF	F	P
BET-OAC	8	40.8	.000	8	2.9	.133	8	11.3	.009	8	1.6	.286	8	2.6	.153
BET	8	1.0	.436	8	2.2	.194	8	3.8	.084	8	23.3	.001	8	10.7	.011
Compound 4	8	2.7	.145	8	30.9	.001	8	34.6	.001 ²	8	13.6	.006	8	15.3	.004
alpha-T	8	0.8	.487	8	5.6	.043	8	0.6	.569	8	0.1	.888	8	10.9	.010

DATE POST-TREATMENT

COMPOUND	1			2			3		
	DF	F	P	DF	F	P	DF	F	P
BET-OAC	14	10.2	.001	14	171.5	.000	14	91.9	.000
BET	14	0.9	.476	14	1.6	.254	14	2.4	.122
Compound 4	14	1.0	.455	14	7.1	.006	14	2.1	.154
alpha-T	14	0.6	.668	14	6.8	.006	14	4.4	.026

TOTAL INTRINSECS OF FREEDOM (DF), F RATIOS (F) AND SIGNIFICANCE OF F (P)
OBTAINED IN ONE-WAY ANALYSIS OF VARIANCE OF FIGURE 11

DAYS AFTER INOCULATION WITH A. alternata

COMPOUND	0				1				2				3				4			
	DF	F	P		DF	F	P		DF	F	P		DF	F	P		DF	F	P	
NET-DMC	5	0	1	.5	16.0	.016	.5	32.1	.005	5	454.6	.000	5	152.2	.001					
NET	5	0	1	.5	0.1	.879	.5	0.0	.435	5	8.9	.041	5	24.0	.008					
Compound 4	5	0	1	.5	0.1	.910	.5	0.1	.778	5	15.1	.018	5	40.7	.003					
alpha-T	5	0	1	.5	0.4	.561	.5	0.0	1.00	5	0.0	1.00	5	4.5	.101					

VALUES OF F (F), DEGREES OF FREEDOM (DF) AND PROBABILITIES (P) OBTAINED
FROM ANALYSIS OF VARIANCE (ANOVA) OF FIGURE 11

TREATMENT

COMPOUND	CONTROLS				INFECTED WITH <u>A. alternata</u>			
	DF	F	P		DF	F	P	
NET-DMC	14	0.4	.003		14	30.4	.000	
NET	14	2.4	.117		14	49.2	.000	
Compound 4	14	1.1	.413		14	14.1	.000	
alpha-T	14	1.5	.274		14	1.2	.366	

STATISTICAL ANALYSIS OF FIGURE 13. VALUES OF CHI-SQUARE AND SIGNIFICANCE (P)

TREATMENT

COMPOUND	CONTROL		POVC race 2		FORL	
	Chi-square	P	Chi-square	P	Chi-square	P
BET-OH	8.1	.004	9.7	.022	9.1	.028
BET-OAC	6.7	.081	8.2	.042	9.4	.024
alpha-T	8.2	.042	8.0	.046	8.8	.032

DAY

COMPOUND	0		4		8		12	
	Chi-square	P	Chi-square	P	Chi-square	P	Chi-square	P
BET-OH	0	1	5.8	.034	6.1	.050	6.6	.038
BET-OAC	0	1	5.8	.055	5.6	.061	6.3	.044
alpha-T	0	1	1.5	.464	6.8	.034	6.6	.038

TOTAL DEGREES OF FREEDOM (DF), F RATIOS (F) AND SIGNIFICANCE OF F (P)
OBTAINED IN ONE-WAY ANALYSIS OF VARIANCE OF FIGURES 14 AND 15.

TREATMENT GROUP	DF	F	P
FORL + UV	39	25.7	.000
FOVC race 2 + UV	39	38.4	.000
FORL - UV	39	5.8	.000
FOVC race 2 - UV	39	2.1	.080