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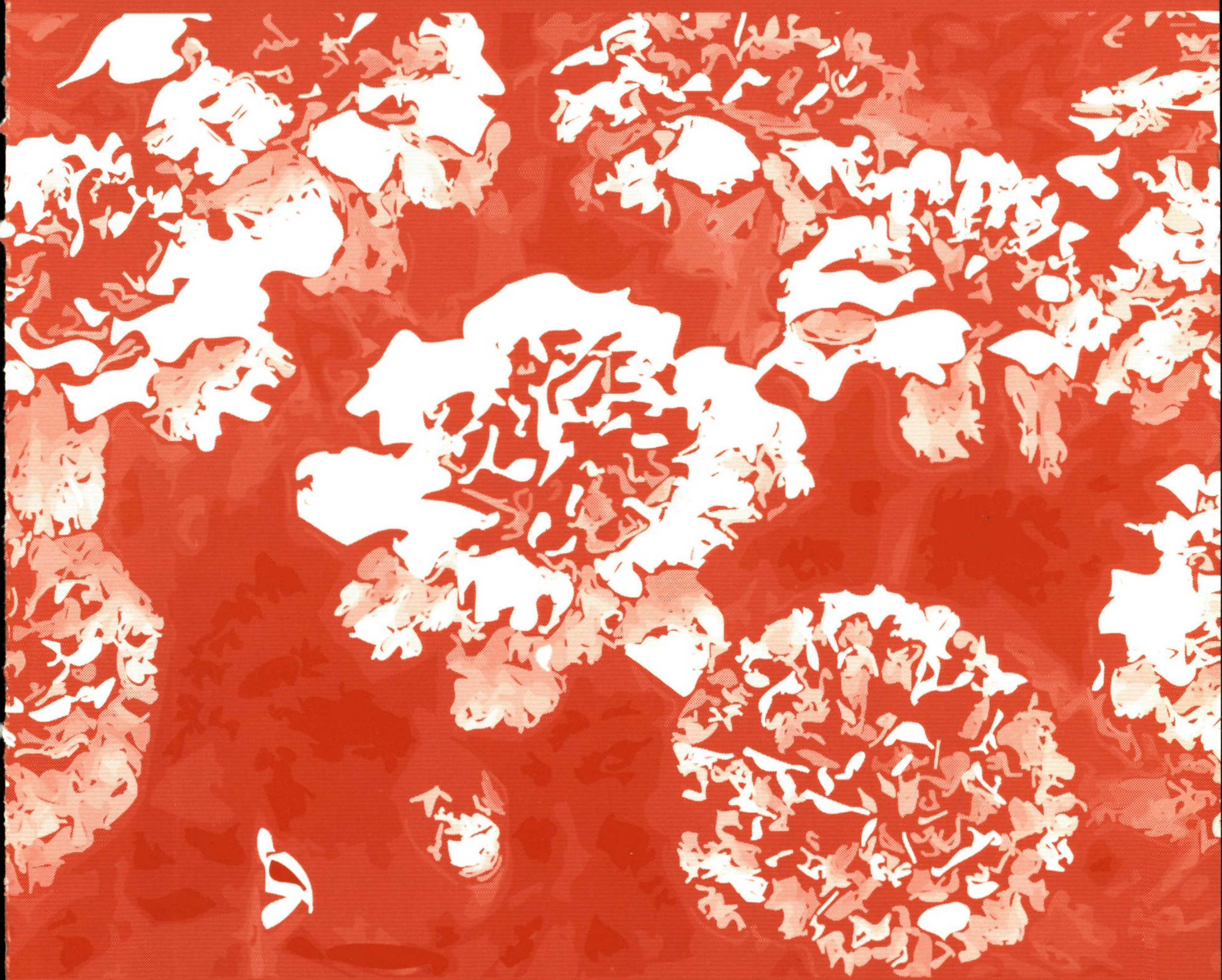

Randolph R.J. Arroo

Regulation of Thiophene Biosynthesis in *Tagetes patula* L.

Regulation of Thiophene Biosynthesis in *Tagetes patula* L.

Een wetenschappelijke proeve op het gebied van
de Natuurwetenschappen

PROEFSCHRIFT

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Preamble

Secondary metabolites, secondary metabolism

By the mid-nineteenth century it became apparent that living creatures are made of chemicals. The exact function of the different naturally occurring organic compounds in the chemistry of the living cell was unclear. However, it was assumed that not all these compounds were essential for the maintenance of life. In 1891 Kossel wrote ". chemistry must attempt to separate those compounds which are present, without exception, in a protoplasm capable of developing, and to recognise the substances which are either incidental or not absolutely necessary for life. Finding and describing those atom complexes to which life is bound comprises the most important basis for the investigation of life processes. I propose calling these essential components of the cell primary components, and those which are not found in every cell capable of developing, secondary. To decide whether a substance belongs to the primary or secondary components is extremely difficult in certain cases."

Originally, secondary compounds were not assumed to play a fundamental role in metabolism only because of their incidental occurrence. This statement, however, is of limited value because it may be the result of a lack of knowledge (Mothes, 1980). So-called secondary compounds may prove to be intermediates in primary metabolic pathways as had been the case with shikimic acid (Geissman & Crout, 1969) which is a key intermediate in the biosynthesis of aromatic compounds, or with squalene, a precursor of cholesterol biosynthesis. Hence, knowledge of the biosynthesis of a compound is needed to qualify it as a primary or secondary metabolite. Technical advances in chromatography and tracer techniques stimulated an increase in our knowledge of metabolic pathways and made it possible to incorporate the secondary metabolites in the whole of biosynthetic pathways.

A qualification of the products of secondary metabolic processes was avoided by Bonner and Galston (1952), when they distinguished between 'highways and byways

of metabolism' The highways included those metabolic processes needed for the formation of the plant body or the maintenance of its activities, *e g.* supply of energy, or catalysis of biochemical reactions. The elucidation of such fundamental processes in the plant itself was connected with appreciable difficulties, because in different species, or under different ecological conditions, these processes do not always proceed along the same routes (Doby, 1965). A further complication was that, in the sequence of reactions, side reactions frequently occur which lead to the byways of metabolism. These side branches of the primary metabolic pathways, the secondary metabolic pathways, lead to the formation of physiologically nonessential compounds generally known as secondary metabolites.

Whereas it has been difficult to assign a physiological role for the majority of plant secondary metabolites, there is increasing evidence that a considerable number of these substances play an ecological role in plants. For example, the colours or smell of flowers and fruits have been recognized to play a role in attracting animals to plants for pollination and seed dispersal. The red, pink, violet, magenta, and blue colours of flowers are caused by anthocyanins which accumulate extensively in the petals or sepals of flowers. Anthocyanins are also found in blueberries and other fruits. Flavonols are responsible for most of the clear or pale yellow flower colours, whereas the deeper yellows or oranges are usually due to carotenoids. The so-called essential oils of plants, including lemon, peppermint, rose, and lavender oils, are mostly terpenes.

Recent studies have indicated that plant phenolic compounds, excreted into the rhizosphere, play an important role in plant-microbe interactions (Peters & Verma, 1990). Flavonoids act as chemoattractants and induce *Rhizobium* nodulation genes that are required for symbiontic association with legumes (Maxwell *et al.*, 1989).

Another presumed ecological function of secondary metabolites is that of deterrent, *e.g.* the nettle poison of *Urtica dioica*. Flavonoids, and especially tannins, have a role as feeding deterrents, protecting plants from overgrazing by many animal species (Harborne, 1980). Ecdysteroids have been widely recognized as antifeeding agents against insects (Grunwald, 1980).

Many secondary metabolites have marked influences on animals and are thus thought to have a function as defence compounds. Because of their biological activity these compounds, or derivatives thereof, have often found an application as drugs. Podophyllotoxin, extracted from the rhizomes of *Podophyllum* species, is the natural source for the semi-synthesis of clinically applied cytostatics. Quinine, from the bark of *Cinchona* species, is used in treating malaria. Cocaine, from the leaves of *Erythroxylon coca*, and morphine, from the fruits of *Papaver somniferum*, are pain killers. Atropine, extracted from *Atropa bella-donna*, is a sedative and muscle relaxant. Strychnine, from the seeds of *Strychnos nux-vomica*, and coniine, from the juice of *Conium maculatum*, are violent poisons.

Scientific interest

As has been indicated above, the qualities that determine the ecological value of secondary metabolites are partly the same as those that determine their value for human application.

Many plant parts have long been appreciated as spices because of the flavours and fragrances they contain, *e.g.* cinnamon, cloves, nutmeg. However, some secondary metabolites may have adverse effects when they accumulate in high concentrations in foods, *e.g.* alkaloids in potato or cyanogenic glycosides in cassava. Toxic secondary compounds can also cause a problem in forage plants (Moyer *et al.* 1993; Tosi & Wittenberg, 1993). Manifestations of toxicity range from a marked reduction in animal performance and nutrient utilization to profound neurological effects and increased mortality (D'Mello, 1992). Therefore knowledge of the presence of secondary metabolites in food and forage plants is essential for agricultural research (Van Beek & Breteler, 1993).

Plants as a source for pharmaceutical products are the object of research in pharmacognosy. Phytochemical research has led to the isolation of numerous active compounds from medicinal herbs. Knowledge of the chemical structure of secondary plant products in many cases formed the basis for the chemical synthesis of

analogues. In this way secondary plant products still are the basis for development of many medicines.

Besides the interest in secondary plant products for human application, there is a growing interest in the function of these compounds in plants. The possible ecological role has already been mentioned. A group of compounds that are of particular interest is that of the phytoalexins. These compounds are defined as low molecular weight, antimicrobial compounds that are both synthesized by and accumulated in plants after exposure to micro-organisms (Müller & Börger, 1940; Paxton, 1981). Phytoalexins constitute a chemically heterogenous group of substances belonging to various classes of natural products (Ebel, 1986).

The enormous diversity in plant secondary products could only have arisen as the result of the fact that they are not essential for the maintenance of life in an individual plant. It seems likely that, in the evolution of families and species, certain chemical structures have descended in close association with the morphological characteristics on which the plant systematic is based (Bohlmann *et al.*, 1973). The distribution of secondary metabolites over plant species is of great importance for taxonomy. The argument for using secondary metabolites in an explanation of relationships between plants is that the distribution of these compounds is given by the enzymes within the plants, which again are given by the base sequences in the DNA (Christensen & Lam, 1990). If we want to use a secondary metabolite in a chemotaxonomic evaluation, it is necessary to know its biosynthesis, since the compound may easily have been biosynthesized by means of totally different enzyme systems.

The biosynthetic pathway leading to a secondary product can be manipulated without affecting the physiological processes needed for growth and development. Thus, secondary biosynthetic pathways provide a very suitable model system for the fundamental examination of general regulatory principles. For example, tissue specific gene expression can be studied since, in higher plants, formation of secondary products is expressed as a specific feature of certain organs or tissues, often only during restricted periods of their development (Luckner, 1980). In

specialized cells or tissues the facilities necessary for channelling of precursors and intermediates, *e.g.* special subcellular compartments (Cresswell *et al.*, 1989), co-occur with the synthesis of secondary metabolic enzymes. It is as yet unknown which internal signals trigger the programs of differentiation and development.

Biosynthesis of some secondary metabolites is induced by external signals, *e.g.* phytoalexins after exposure of plants to micro-organisms or flavonoids after UV-irradiation. The inducible biosynthetic pathways, as model system, have been of great value to enhance our knowledge of metabolic regulatory control and of signal transduction in plant cells (Hahlbrock & Scheel, 1989).

Plant cell and tissue culture

Somatic plant cells have shown to be totipotent (Muir, 1954) and are easy to propagate in simple, synthetic media. Plant cells, cultured *in vitro*, have been widely used to study secondary metabolism for about two decades because these suspension cultures offer several advantages over whole plants. They grow relatively fast and all cells are in close contact with the culture environment, which can be manipulated easily Furthermore, the culture system consists of relatively homogeneous cell types and isolation of metabolites from cell cultures has proven to be more convenient than with intact plants. However, cultured plant cells usually fail to produce, or yield low levels of secondary products characteristic of the intact plant (DiCosmo, 1990; Galneder & Zenk, 1990). The production of secondary metabolites often depends on the organization of plant cells into a morphologically differentiated state (Luckner, 1980; Wiermann, 1981; Berlin *et al.*, 1986; Rhodes *et al.* 1987; Yeoman, 1987).

Organized tissue cultures, especially root cultures, have been found to be useful in some cases when undifferentiated cell cultures fail to synthesize the compounds of interest (Yamada & Hashimoto, 1990). Although shoot and root cultures provide a vast repository of secondary plant products, often yielding amounts of these compounds greater than levels found in intact plants, they are usually slow growing

(DiCosmo, 1990). However, the availability of hairy root cultures obtained by genetic transformation with *Agrobacterium rhizogenes* (Tepfer, 1984), has put an end to this problem. Hairy root cultures can grow as fast as unorganized plant cell suspensions. Furthermore, hairy roots can express root-specific metabolic pathways as efficiently as normal root cultures or roots *in vivo* It has also been shown that hairy roots are responsive to stimuli such as elicitors and changes in the physical environment (Flores & Curtis, 1992).

Thiophenes

By the mid-twentieth century it was reported that the yellow petals of the 'Lemon' or 'African' variety of the common marigold (*Tagetes erecta* L.) contained a blue-fluorescing compound (Zechmeister & Sease, 1947) In a surprising reversal of the more common sequence of events, nature seemed to be imitating artifice The natural product was identical to a synthetic oligomer created just a few years earlier (Steinkopf *et al* , 1941) That compound was α-terthienyl. The physiological role of α-terthienyl in the plant remained problematical However, an ecological significance of α-terthienyl was proposed when it was discovered that the compound mainly accumulated in *Tagetes* roots and displayed a high nematicidal activity (Uhlenbroek & Bijloo, 1958). A second nematicidal compound from *Tagetes* was isolated shortly thereafter and identified as 5-(3-buten-1-ynyl)-2,2'-bithienyl (Uhlenbroek & Bijloo, 1959).

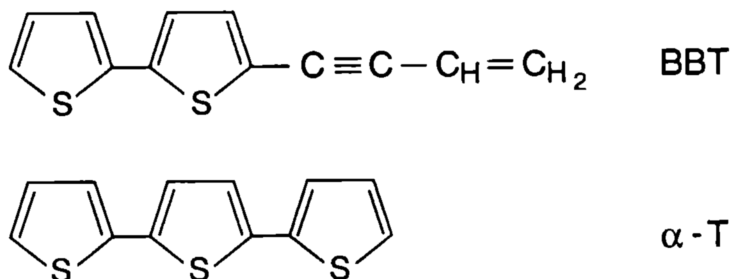

Fig. 1 Chemical structures of 5-(3-buten-1-ynyl)-2,2'-bithienyl (BBT) and α-terthienyl (α-T)

Chemotaxonomical interest then led to an extensive survey of the occurrence of thiophene derivatives in related plant species (comprehensive reviews *e.g.* Bohlmann *et al.*, 1973; Christensen & Lam, 1991; Christensen, 1992). Thiophenes appeared to be widely distributed in the Asteraceae (Compositae) family. However, the chemotaxonomical value of these compounds is limited since their occurrence is seemingly unrelated to morphological characters (Sørensen, 1977).

In addition to their well established nematicidal activity (Chitwood, 1993), thiophenes have been reported to be toxic at low dosages to fungi (DiCosmo *et al.* 1982; Kourany *et al.* 1988), insects (Nivsarkar *et al.*, 1992; Wells *et al.*, 1993), bacteria (Arnason *et al.*, 1981) and viruses (Marles *et al.*, 1992; Hudson *et al.* 1993). The proposed mode of action of thiophenes is based almost solely on studies with α-terthienyl, because this compound can be synthesized chemically and thus is available in large enough quantity to perform toxicological tests. The toxic effects of α-terthienyl are greatly enhanced by sunlight or artificial sources of near UV. The phototoxicity of this compound is due to the generation of singlet oxygen (Bakker *et al., 1979*) which causes the photodynamic disruption of membranes, which thus are the primary site of action in most organisms (Wat *et al.*, 1980). The mechanism of dark toxicity has not been elucidated, although models exist in which electronically excited species, like singlet oxygen, are generated by chemical or enzymatic processes (Cilento, 1980; Cilento & Adam, 1988).

In the toxicological structure-activity studies of thiophene derivatives much attention has been paid to the heterocyclic rings. However, most naturally occurring thiophenes possess acetylenic side chains that may also determine their biological activity. Non-phototoxic mechanisms have been described for polyacetylenes (Garrod *et al.* 1979; Arnason *et al.*, 1986), which are closely related to thiophenes. These compounds accumulate in membranes, and in the ground state may disrupt normal membrane fluidity. Whether this mode of action also applies for thiophenes is no known.

Thiophenes have been described as phytoalexins because of their biological activity, and low molecular weights (Kourany *et al.*, 1988). However, most

thiophenes are always present and not synthesized by and accumulated in plants only after exposure to micro-organisms. Hence not all thiophenes fulfil the criteria used to define phytoalexins.

The biosynthesis of thiophenes in plants is poorly understood. Labelling studies with several species of the Asteraceae have indicated that thiophenes are derivatives of polyacetylenes (Bohlmann & Hinz, 1965; Bohlmann *et al.* 1966), which in turn are oleic-acid-derived. Hence, the biosynthetic pathway leading to thiophenes can be considered as a side branch of fatty acid metabolism. The exact mechanism of sulphur incorporation into thiophenes has not yet been elucidated, but it is generally assumed that the thiophene biosynthetic route is also connected to the primary metabolism of sulphur. Thiophene interconversions have been the subject of several studies (Schulte & Foerster, 1966; Schulte *et al.*, 1968; Jente *et al.*, 1981; Jente *et al.*, 1988; Constabel & Towers, 1989). However, the sequence of metabolic steps in the turnover of thiophene derivatives is still unresolved. So, the biogenetic relations of these compounds remain a matter of discussion.

Enzymological studies of thiophene metabolism yielded the partial purification and biochemical characterization of three enzymes that are possibly concerned in bithienyl interconversions (Sutfeld & Towers, 1982; Pensl & Sutfeld, 1985; Metschulat & Sutfeld, 1987). At present it has not yet been established whether the enzymes are involved in a biosynthetic or in a degradative pathway.

Naturally occurring thiophenes in *Tagetes* mainly accumulate in the roots (Sutfeld, 1982; Downum & Towers, 1983; Tosi *et al.* 1988). The production of thiophenes in unorganized cell cultures is usually very low compared to that in roots (Norton *et al.*, 1985; Ketel, 1986; Croes *et al.*, 1989a). It was observed that a sharp rise in thiophene content coincided with the emergence of roots from calli. Root regeneration and the amount of thiophenes produced were found to be quantitatively related (Croes *et al.*, 1989a). Hairy roots, obtained after transformation with *Agrobacterium rhizogenes* expressed the thiophene metabolic pathways with an efficiency comparable to that of roots attached to the plant (Croes *et al.*, 1989b).

Scope of this thesis

The biosynthesis of secondary metabolites, here exemplified by thiophene biosynthesis, is a process that is under strict regulatory control. The regulatory principles that control thiophene biosynthesis are the subject of this thesis. Morphological differentiation in plants is often accompanied by chemical differentiation, *i.e.* the process of formation of specialized tissues is accompanied by the formation of specialized enzymes concerned in secondary metabolism. The processes co-occur, but are they causally related?

The first two chapters treat of the effects of some physiological factors, *viz.* hormones and nutrients, on secondary metabolism and root growth and development Chapter one deals with the question whether thiophene biosynthesis is directly affected by auxin or rather indirect as the result of an altered morphology of the producing tissue. In chapter two the mechanisms which regulate the flow of precursors through the pathways of primary and secondary metabolism are described.

In chapters three and four a part of the biosynthetic route leading to thiophene is established and a preliminary description of the possible cellular localization of thiophene metabolism is given. Detailed knowledge of the biosynthetic pathway of the compound under investigation - *i.e.* knowledge of the precursors, the intermediates, enzymes, possible location of biosynthetic steps in specialized cells or cellular compartments - is essential for a good understanding of the regulation of secondary metabolism.

In chapter five the role of thiophenes in the resistance of *T. patula* to soil-borne pathogens is discussed. The regulation of the secondary metabolic pathway appears to be inseparably linked with function of the metabolites produced.

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Chapter 1

Effect of exogenous auxin on root morphology and secondary metabolism in *Tagetes patula* hairy root cultures

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In this chapter the effects of indole-3-acetic acid (IAA) on growth of *Tagetes patula* hairy root cultures and secondary product formation are presented. The biosynthesis of thiophenes, sulphurous compounds with nematicidal activity, was inhibited by IAA application, as was evident from a decrease of [^{35}S]sulphur incorporation. The inhibition only occurred after the roots had developed numerous laterals as a result of auxin action. However, in roots cultured in the absence of IAA, there was no significant correlation between branching and thiophene accumulation. Therefore, development of lateral roots is not a sufficient condition for a low capacity to synthesize thiophenes.

The highest rate of thiophene accumulation in the roots is found during the late exponential growth phase, when the weight increase of a root culture is at its maximum. Hence, growth and the production of thiophenes appear to be compatible in *T. patula* hairy roots.

Introduction

The production of secondary metabolites in plants is often restricted to specific tissues and organs during particular developmental stages. The morphological differentiation of plant cells into specialized cell types and tissues is accompanied by a chemical differentiation which establishes the metabolic pathway leading to secondary product formation. The relationship between morphological differentiation and secondary metabolism may be that formation of a specialized tissue is a prerequisite for secondary metabolism. This theory is substantiated by the observation that in callus cultures and in plant cell suspensions which do not differentiate into specialized structures, secondary metabolism is often low or even completely absent (Luckner, 1980; Rhodes *et al.*, 1987; Wiermann, 1981; Yeoman, 1987). Alternatively, in spite of the coincidence of the two processes, there may be no causal relation between morphological differentiation and the establishment of a secondary metabolic pathway, common regulatory factors, *e.g.*

phytohormones, may be involved.

Hairy roots are an example of organized organ cultures, displaying a variety of specialized tissues. They usually accumulate the spectrum of secondary metabolites in levels as found in roots of the intact plant (Berlin *et al.*, 1990) and have been proposed as a system for the in vitro production of valuable plant metabolites (Flores, 1987). Hairy root cultures are obtained by transformation of dicotyledonous plants with either agropine- or mannopine-type strains of *Agrobacterium rhizogenes*. Mannopine strains confer an increased auxin sensitivity upon the plant tissue (Shen *et al.*, 1988; Spanò *et al.*, 1988). Since no auxin genes can be transferred by these strains, transformation does not lead to an elevated auxin level in the tissue (Cardarelli *et al.*, 1985). Tissues transformed with an agropine strain are characterized by both a hypersensitivity to auxin (Shen *et al.*, 1988; Spanò *et al.*, 1988) and an elevated IAA level (Deno *et al.*, 1987). Auxin is considered as the main factor controlling the formation of hairy roots.

The morphological differentiation processes caused by auxin in root cultures are typical long-term responses. Pericycle cells dedifferentiate (Karas & McCully, 1973) and lateral root primordia are initiated which increase in size through repeated cell divisions and subsequently, by cell elongation, emerge as lateral roots (MacIsaac *et al.*, 1989). At the same time meristematic activity in the root apex is inhibited, at least by exogenously applied auxin (Zeadan & MacLeod, 1984).

In addition to the effect of auxin on morphological differentiation, this hormone has been reported to reduce the concentration of secondary products in roots (Hashimoto *et al.*, 1986; Norton & Towers, 1986). This phenomenon may be used to study whether the suppression of product synthesis is causally related to changes in morphological differentiation. Secondary product accumulation also has frequently been shown to be negatively correlated with cell growth. The phytohormone auxin has been proposed to act as a regulator of the switch between cell growth and secondary product formation (Goddijn *et al.*, 1992;

Pasquali *et al.*, 1992).

Naturally occurring thiophenes, which are present in *Tagetes* species, are suiphur-containing heterocyclic compounds with biocidal activity. Transformed roots of *T. patula* accumulate the same thiophenes as roots attached to the plants. In roots, transformed with a mannopine strain of *A. rhizogenes*, extensive branching of the roots and a simultaneous decrease in thiophene accumulation, can be induced by exogenous auxin application. Rapidly growing and highly-branched roots, obtained after transformation of *T. patula* with an agropine strain of *A. rhizogenes* are low in thiophene content (Croes *et al.*, 1989).

Rapid growth and lateral root formation in *T. patula* hairy roots seem to be negatively correlated with thiophene production. Auxin appears to be the main factor controlling growth and morphology in hairy roots. This paper deals with the question whether the inhibition of thiophene biosynthesis is a direct effect of IAA or is the result of an altered root morphology or growth rate. To tackle the question, two types of hairy roots, either transformed by mannopine-type or agropine-type agrobacteria have been compared with respect to thiophene metabolism and growth in media with and without auxin.

Materials and methods

Root cultures.

Root line Tp9402 was obtained by transformation of *Tagetes patula* L. cv. 'Nana' with *Agrobacterium rhizogenes* LBA 9402 (pRi 1855) Root line Tp9365 was obtained by transformation of the same *T. patula* cultivar with *A rhizogenes* LBA 9365 (pRi 8196) Transformation of the root lines was confirmed by their ability to grow on hormone-free medium, by the presence of agropine or mannopine in the tissue, and by Southern blotting of root DNA using the EcoRI-15 fragment of the Ri-plasmid pRi 1855 (Birot *et al.*, 1987) as a probe.

Growth of isolated roots.

Roots were cultured in Gamborg's B5 medium (Gamborg, 1970) supplemented with 3% sucrose and 100 $\mu g\ l^{-1}$ biotin. The pH was adjusted to 5.75 before autoclaving. In all experiments where indole-3-acetic acid (IAA) was used, 10 mM 2-morpholinoethane sulphonic acid (MES) was added to the growth medium in order to stabilize the pH. The cultures were grown in 300-ml Erlenmeyer flasks with 100 ml medium on a rotary shaker (100 rpm) at 25°C in the dark and were subcultured every two weeks.

The growth of roots was followed in 50-ml Erlenmeyer flasks filled with 20 ml B5 medium inoculated with 10 fresh root tips, each approximately 1 cm in length. The cultures were grown on a rotary shaker (100 rpm) at 25°C in the dark. At intervals roots were taken out, blotted dry on tissue paper, and weighed.

Morphology of roots.

Petri dishes with an internal diameter of 9 cm with culture medium solidified with 0.5% Gellan gum, were inoculated with three fresh root tips with an approximate length of 1 cm. After 14 days the length of the root main axis was determined and the number of laterals counted.

Thiophene analysis

Root material was extracted as described earlier (Croes *et al.*, 1989). Thiophenes were recovered from the non polar fraction, and subsequently separated by high-performance liquid chromatography. Thiophene concentrations were calculated on the basis of molar absorption coefficients determined in this and other laboratories .

In some experiments, 1-cm-long tips were cut from the roots whereupon tips and mature parts were extracted separately.

Identification of thiophenes

GC/MS: A capillary column (fused silica WCOT, coated with CP-Sil 5CB, 25m x 0.32 mm ID) was used to fractionate the samples. Carrier gas was He, and the flow rate 1.5 ml min^{-1}. Samples of 1 μl were injected with a splitting ratio of 1:10, at an injection port temperature of 250°C. The initial oven

temperature was 100°C, the temperature was raised with 15°C min^{-1} to 280°C, this temperature was maintained for 8 min The electron impact method (EI) was used to ionize the fractions Recorded spectra were compared with spectra known from literature (Bohlmann *et al* , 1964, Bohlmann *et al* , 1973, Gronemann *et al* , 1984, Camato *et al* , 1990, Bicchi *et al* , 1992)

Fourier transformed ^{1}H-NMR spectra were recorded on a spectrometer operating at 400 MHz Samples were measured in $CDCl_3$ with tetramethylsilane as an internal standard NMR-spectra were compared with spectra known from literature (Bohlmann & Kleine, 1963, Atkinson *et al* , 1964, Bohlmann *et al* , 1964, Bohlmann *et al* , 1965, Bohlmann & Berger, 1965, Bohlmann & Zdero, 1985)

UV-absorption spectra were recorded in ethanol Absorption was measured between 210 and 600 nm Spectra were compared with known spectra from the literature (Uhlenbroek & Bijloo, 1959, Bohlmann & Herbst, 1962, Bohlmann & Kleine, 1963, Atkinson *et al* , 1964, Bohlmann & Berger, 1965, Bohlmann *et al* , 1965)

HPLC was performed on a Lichrosorb RP-18 column (particle size 7 μm) Column dimensions were 25 x 0 4 cm, the eluent was acetonitrile water (72 28, v/v) The flow rate was 1 5 ml min^{-1}, thiophenes were detected by their UV-absorption at 340 nm Every ten runs a standard mixture, consisting of four different thiophenes with known concentrations, was analyzed

Thiophene synthesizing capacity

Exponentially growing root cultures were labelled with [^{35}S]sodium sulphate (0 74 MBq ml^{-1}), and the sulphate concentration in the medium was raised to 30 mM to minimize label dilution by the internal sulphate pool After 4 h of incubation the roots were rinsed with ice-cold 100 mM Na_2SO_4 The fresh weight was determined and thiophenes were extracted Samples of the polar and the apolar fractions were mixed with scintillation fluid and counted The total radioactivity in the root was used as a measure for sulphate uptake The radioactivity in the organic phase of the extract was used to estimate thiophene synthesis Previous HPLC analysis had shown that over 95% of the ^{35}S counts in the organic phase are in thiophenes

Uptake of externally applied IAA

[5(n)^{3}H]IAA (940 TBq mol^{-1}) was purchased from Amersham, and purified shortly before use by two-dimensional chromatography on silica gel TLC plates

Roots were cultured in 50-ml Erlenmeyer flasks as described above, in medium supplemented with

130 Bq ml[1] [5(n)^{3}H]IAA at a chemical concentration of 10^{7} M At intervals of one or two days, the radioactivity of 50-μl medium samples was measured After 10 days, hormone uptake and conversion in the roots was determined (Peeters *et al* , 1991) Briefly, roots were rinsed with B5 medium supplemented with 10^{6} M unlabelled IAA, and then homogenized in MeOH The homogenate was centrifuged (5 min, 48 x *g*), the supernatant was collected and subsequently evaporated under N_2 at ambient temperature The residue was taken up in a small volume of MeOH and applied to a silica gel 60 TLC plate The plate was developed with $CHCl_3$ MeOH HOAc (75 20 5 v/v) as a solvent Finally the IAA spot and the rest of the lane were scraped off separately and the radio-activities were determined

Evaluation of data

All experiments were carried out at least twice Data points are means of 4 determinations ± the standard error of the mean (SEM)

Results

Growth and thiophene content of hairy roots.

In order to study the relation between culture growth and thiophene accumulation, both processes were monitored over a fifty-day-period. Hairy roots, grown as batch cultures, show a typical sigmoid growth curve (Fig. 1.1). It commences almost exponentially, then the net rate of increase declines, until finally there is no further change in culture size. During the exponential growth phase, the rate of accumulation of thiophene exceeded biomass production. As a consequence, thiophene concentration in the tissue increased during early culturing to reach its highest level at the end of the exponential phase. During transition to the stationary growth phase, thiophene level decreased in a characteristic two-step process observed in all accumulation experiments. The first step comprised a 40% decline followed by a plateau. Thiophene concentration resumed to drop when the culture became stationary. Meaningful comparisons between root lines

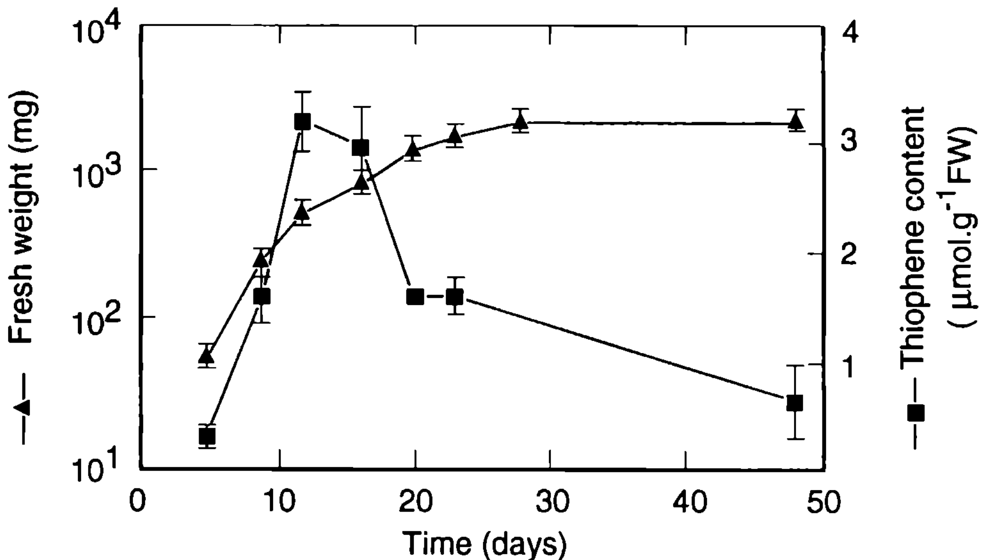

Fig. 1.1 Thiophene accumulation in the roots (squares) during growth of a hairy root culture (triangles).

can only be made when cultures are in the same growth phase. For this reason, only root cultures in the exponential phase of growth were compared in all further experiments.

The preceding results indicate a positive correlation between growth rate and accumulation of thiophenes. Thus it was expected that a rapidly growing root clone would accumulate higher amounts of thiophenes than a slow-growing clone. This expectation was tested by experiments in which a fast- and a slow- growing root culture were compared. Root clone Tp9402, which is transformed by an *aux*-genes containing agropine strain of *A. rhizogenes*, grows rapidly. Root culture Tp9365, transformed by a mannopine strain and lacking the *aux*-genes, is a slow-growing clone. In an attempt to complement the absence of the *aux*-genes in the mannopine-type transformant Tp9365, this root clone was also cultured in the presence of exogenously applied IAA.

The growth pattern of Tp9365 cultured in the presence of externally applied

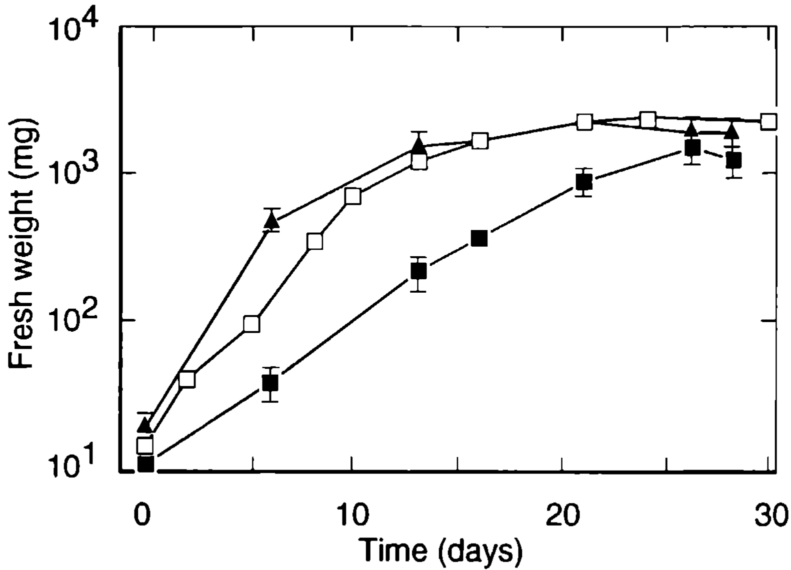

Fig. 1.2 Growth curves of *Tagetes patula* hairy root cultures Tp9402 in auxin-free growth medium (open squares), Tp9365 in auxin-free growth medium (solid squares), Tp9365 in growth medium with 10^{-7} M IAA (solid triangles)

IAA resembled that of the agropine-type root line Tp9402 grown in the absence of IAA (Fig. 1.2). In liquid medium the growth pattern of roots was exponential during the first 10 days. The biomass stabilized after approximately 20 days and did not appreciably decline upon prolonged incubation. All cultures, in spite of the differences in initial growth rate, ended up with the same biomass.

The maximum growth rate of the mannopine type root line Tp9365, cultured in the presence of auxin, did not differ significantly from that of the agropine type Tp9402, cultured in auxin-free medium. The time needed for these root cultures to double their fresh weight was approximately 37 h. Thiophene concentration in these relatively fast growing root cultures was significantly lower than that in Tp9365 cultured in auxin-free medium. The latter root culture needed twice as much time to double its weight (Tab 1.1).

Table 1.1 Growth rate (mean ± SE) and thiophene content (mean ± SE) of *Tagetes patula* hairy root cultures

Root culture	Doubling time during exponential growth phase (h)	Thiophene content (μmol g FW^{-1})
Tp9365 in B5	74 ± 23	2.7 ± 0.1
Tp9365 in B5 + 10^{-7} M IAA	38 ± 2	1.5 ± 0.4
Tp9402 in B5	37 ± 5	1.9 ± 0.5

Contrary to our expectations, the slow-growing clone Tp9365 without IAA treatment accumulated more thiophene per gram fresh weight than the rapidly growing clone Tp9402 or the IAA-treated Tp9365.

Effect of IAA application on growth and thiophene content.

It was observed that addition of IAA to liquid-grown Tp9365 cultures initiated the formation of high numbers of root primordia. Within two days, the primordia started to develop into lateral roots. Since the number of growing tips was high compared to non-auxin-treated roots, this process led to a significant increase in growth rate. The *aux*-genes containing root culture Tp9402 was also highly-branched, resulting in a high growth rate. Hence, it was supposed that thiophene content is inversely related to the number of lateral roots. The number of lateral roots formed, in turn, is dependent on auxin concentration. To substantiate these suppositions, hairy root cultures Tp9402 an Tp9365 were grown on agar plates at a range of IAA concentrations. After 14 days of growth, the number of lateral roots was counted and the length of the main axis was measured.

The effect of the auxin on elongation of the main root axis did not differ much for both root cultures. At concentrations above 10^{-8} M the root clones showed a

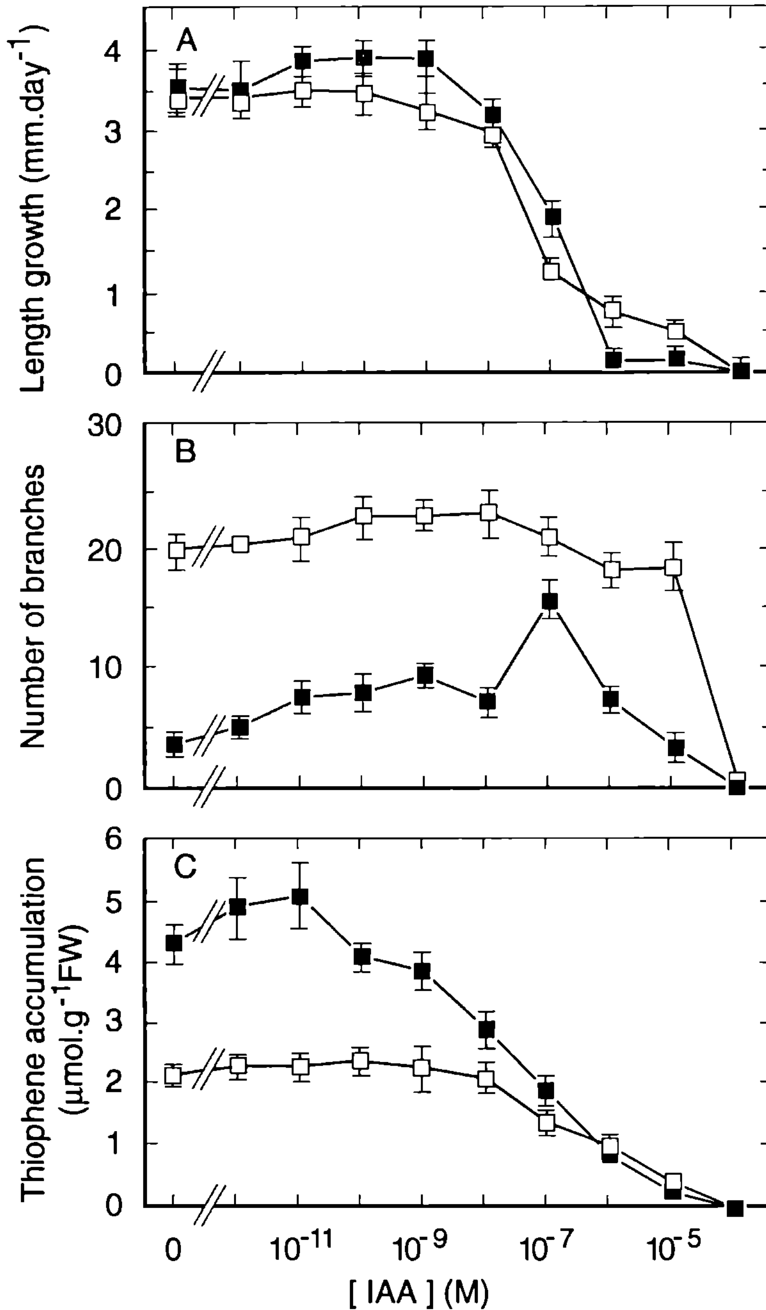

Fig. 1.3 Effect of exogenous IAA on elongation growth of the root main axis (A), formation of lateral roots (B), and thiophene accumulation (C) in the agropine-type root line Tp9402 (open squares) and the mannopine-type root line Tp9365 (solid squares). Roots were incubated on solidified medium and analyzed after 2 weeks

progressive decrease in elongation growth (Fig 1 3A) In contrast, the formation of laterals as a response to the external auxin concentration was different in the two types of roots Whereas this process in the agropine-type root was unaffected at all concentrations below 10^{5} M, the mannopine-type root clone Tp9365 showed an optimum in lateral root formation at 10^{7} M IAA (Fig 1 3B), at higher auxin concentrations the number of root primordia that were initiated still increased, but the primordia did not emerge as lateral roots The growth pattern of Tp9365 cultured at this IAA concentration again resembled that of the agropine-type root clone Tp9402 grown in hormone-free medium Along with the emergence of lateral roots, thiophene concentration in the tissue decreased The agropine-type roots in auxin-free medium as well as the mannopine-type roots cultured at 10^{7} M IAA, accumulated significantly less thiophenes than the hardly branched mannopine-type roots cultured in auxin-free medium This confirmed the observation that thiophene content is inversely related to branching

The preceding experiments support the hypothesis that the difference between mannopine type hairy roots and agropine type hairy roots lies basically in the response to the internal auxin level Exogenous application of IAA complemented the absence of T-DNA *aux*-genes and abolished the differences in growth and thiophene accumulation All further experiments are carried out only with the mannopine type root line Tp9365, because the effects of auxin application on root morphology and secondary metabolism are most explicit in this line

Although a correlation is found between lateral root formation and production of thiophenes, these processes need not be causally related Branching and secondary metabolism may be independently affected by auxin The dilemma was tackled by examining the relation between the number of lateral roots and thiophene concentration of Tp9365 cultured in a hormone-free medium Advantage was taken from the natural variation in the number of laterals per root system which ranged from zero to ten in the absence of auxin Individual roots were selected and assigned to four categories, according to the number of

Table 1.2 Relation between thiophene accumulation and number of laterals in *T patula* hairy root clone Tp9365 Roots were incubated solidified medium and analyzed after 2 weeks Thiophene accumulation (mean ± SE) of root tips, middle parts, and basal parts was measured separately

Number of	Thiophene accumulation (μmol g FW $^{-1}$)
0	2 3 ± 1 6
1 3	2 8 ± 2 0
4 7	1 7 ± 3 1
8 10	1 8 ± 0 2

laterals The roots were extracted, and the thiophene content was determined No correlation was found between the number of laterals and thiophene concentration in the roots (Tab 1 2) Thus, branching and thiophene metabolism must be independently affected by auxin For this reason it was necessary to study the effect of IAA on thiophene biosynthesis in more detail

Uptake and metabolism of externally applied IAA

The disadvantage of external application of auxin is that the internal hormone concentration may change with time due to exhaustion of the medium and inactivation inside the tissue To gain more insight in the presence of applied auxin in the tissue, *Tagetes* roots were cultured in the presence of [^{3}H]IAA and uptake of label during growth of the culture was monitored Initially, the labelled IAA was rapidly taken up by the roots, but the rate of uptake decreased when biomass increased (Fig 1 4) Analysis of the radiolabelled auxin in the roots showed that the amount of free IAA had decreased to less than 1% of the total radioactivity after 10 days Over 99% of the applied auxin was conjugated or otherwise metabolized

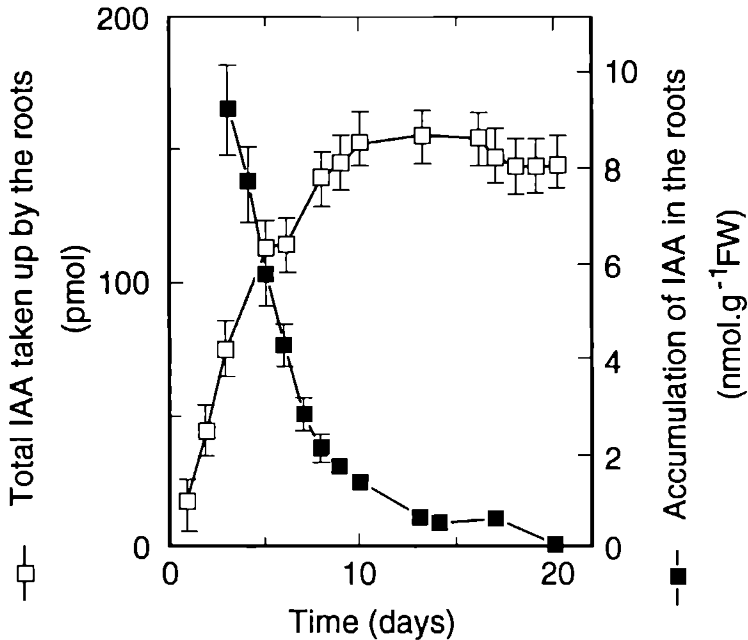

Fig. 1.4 Exogenously applied IAA taken up by a growing Tp9365 root culture (open squares) and the resulting internal levels of accumulated auxin (solid squares)

Effect of IAA on thiophene biosynthesis.

The internal levels of IAA are highest during the first days after exogenous application. Therefore, the effect of exogenously applied IAA on thiophene biosynthesis was expected to be strongest during this time. When the internal level of hormone would decrease due to dilution and inactivation, the effect on biosynthesis might wear off.

Exponentially growing roots cultured in auxin-free medium were transferred to either auxin-free medium or medium with 10^{-7} M IAA. One day after transfer, the rate of thiophene synthesis was determined by following the incorporation of radiolabelled sulphur. To localize the site of auxin action on thiophene biosynthesis, the synthesizing capacities of tips and proximal parts were measured

Table 1.3 Short-term effect of IAA on thiophene synthesis in root culture Tp9365 Roots that had grown for 24 h in medium supplemented with 10^{-7} M IAA were divided into 1-cm-long root tips, and other parts The synthesizing capacities (mean ± SE) of tips and proximal parts were measured separately

	Thiophene synthesis (nmol g $FW^{-1}\ h^{-1}$)
Tp9365 in B5	
Whole roots	7 ± 1
Root tips	10 ± 1
Other root parts	5 ± 2
Tp9365 in B5 + 10^{-7} M IAA	
Whole roots	7 ± 2
Root tips	12 ± 2
Other root parts	5 ± 1

Tab. 1.4. Long-term effect of IAA on thiophene synthesis in root culture Tp9365 Roots that had grown for 14 days in medium supplemented with 10^{-7} M IAA were divided into 1-cm-long root tips, and other parts The synthesizing capacities (mean ± SE) of tips and proximal parts were measured separately

	Thiophene synthesis (nmol g $FW^{-1}\ h^{-1}$)
Tp9365 in B5	
Root tips	9.0 ± 0.9
Other parts	2.8 ± 0.7
Tp9365 in B5 + 10^{-7} M IAA	
Root tips	3.1 ± 0.3
Other parts	2.2 ± 0.2

separately. No significant effect was detected within 24 h; neither the morphology nor thiophene synthesizing capacity had changed (Tab. 1.3).

In an other experiment, roots were cultured in auxin-free medium or in medium with 10^{-7} M IAA. After 14 days of growth, the rate of thiophene biosynthesis was determined. Auxin strongly reduced the thiophene synthetic capacity in the tips but barely affected the older parts. In this way, the hormone abolished the difference in synthesis rate between root tips and older parts (Tab. 1.4).

It was concluded that auxin had no short-term effect on thiophene synthesis. Only after prolonged growth in auxin-containing media, when the roots showed the heavily-branched phenotype, was thiophene synthesis affected.

Discussion

Growth and the expression of a secondary pathway appear compatible in *T. patula* hairy roots. Auxin induces the formation of excessive numbers of lateral roots, thereby causing an increase in the number of root tips which results in an increased growth rate. However, auxin also has a restraining influence on thiophene biosynthesis.

Hairy roots obtained after transformation of *T. patula* with an agropine-type strain of *A. rhizogenes* had a significantly lower thiophene content than roots obtained after transformation with a mannopine-type strain of the bacterium. The only functional difference between agropine- and mannopine-type transformants is the presence of bacterial genes encoding auxin synthesis. These bacterial *aux*-genes do not play a crucial role in the induction of hairy roots (Cardarelli *et al.*, 1987) but they clearly affect the morphology and the secondary product accumulation in the induced roots. The presence of the *aux*-genes in the agropine-type *T. patula* roots resulted in a highly-branched morphology and a decreased thiophene content, compared to the roots induced by a mannopine strain, which had no *aux*-genes on the T-DNA. The absence of the genes in the latter root

culture can be complemented by the exogenous application of IAA (Fig. 1.2). Hence, it can be concluded that auxin regulates both lateral root development and thiophene accumulation in *T. patula* roots.

The relatively high number of growing root tips in the highly-branched root cultures, which were low in thiophene content, resulted in a high growth rate of these cultures. This, however, does not automatically mean that a negative correlation exists between growth rate and rate of thiophene biosynthesis. The opposite is true: the highest rate of thiophene accumulation in the roots is found when the weight increase of the culture is at its maximum (Fig. 1.1).

A negative correlation was observed between the number of lateral roots formed after auxin application and the rate of thiophene biosynthesis per gram fresh weight. However, highly-branched roots that had not been cultured in the presence of exogenous IAA, did not show low thiophene accumulation (Tab. 1.2). Application of IAA did not lead to a significant repression of thiophene formation within 24 h. Only after a prolonged period of growth in the presence of IAA, when the root morphology had been altered, a decrease in thiophene biosynthesis was observed (Tab. 1.3, Tab. 1.4). Hence, a highly branched phenotype may be a necessary, but not a sufficient condition for a low capacity to synthesize thiophenes.

Exogenously applied IAA was rapidly taken up by the roots which resulted in high levels of accumulated auxin in the growing root culture during the first days after application. Within a day, a high number of root primordia was formed, resulting in roots with a hispid appearance. When the internal level of auxin decreased, due to a decreased auxin uptake and an increase in root biomass, the primordia differentiated into secondary roots. Conjugation leads to a decline in the level of the active hormone, because free IAA is the only biologically active form (Cohen & Bandurski, 1982). These observations indicate that exogenously applied IAA exerts its effect on root growth probably during the first days after application, because then the internal levels of the hormone are highest. That auxin application affects thiophene metabolism at a later stage is compatible with

a causal relation between morphological and chemical differentiation.

Our results support the hypothesis that the effect of auxin on growth is independent of its effect on thiophene biosynthesis and accumulation. Culture growth rate and secondary product formation are two amongst many developmental processes affected by auxin. The low secondary product accumulation in auxin-treated cell cultures probably is the result of an incorrect cell specialization, resulting in inefficient biosynthesis The elucidation of the relationship between morphology and metabolite concentration at the cellular level may enable us to define the exact role of plant growth substances in morphological and chemical differentiation.

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Chapter 2

Effect of sulphate limitation on thiophene biosynthesis in roots of *Tagetes patula*

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Species of the genus *Tagetes* accumulate thiophenes, sulphurous secondary metabolites. The accumulation of these compounds in *T. patula* was used as a model system to study the partitioning of sulphur between primary and secondary metabolism when sulphate supply was limited. Growth and thiophene accumulation were measured in root cultures incubated at various sulphate concentrations in the medium. A reduction in the concentration from 2 mM to 50 μM did not affect elongation growth, branching and biomass production within 8 d. In contrast, thiophene level had dropped to 45-65% of the control in the same period. The reduction in thiophene content was found to be due to a decline in biosynthetic capacity which amounted to 80-95% after 8 d. This capacity is restored when roots are transferred from the low-sulphate medium to the standard medium. The restoration takes more than 24 h and is suppressed by the transcription inhibitor cordycepin. It is concluded that the rate of thiophene synthesis is, at least for the major part, controlled at the level of transcription. Upon sulphate starvation, the pathway leading to incorporation of sulphur into thiophenes is down-regulated in favour of the primary metabolism supporting growth. In this way, *Tagetes* plants are able to tune secondary metabolism to the primary needs of the plant for growth and development.

Introduction

One of the fundamental areas of plant physiology and plant biochemistry is the relationship between the quantities of the essential nutrient elements and the metabolic changes in the plant. Most minerals are essential building materials, and some form part of indispensable catalysts. A deficiency in any element affects the metabolic system and thus disturbs the balance of chemical constituents that make up a plant. Some of these constituents do not play a basal

physiological role in growth and development. The supposed functions of these compounds, commonly named secondary metabolites, are as diverse as their structural variations. They may act as pollinator attractants or as herbivore repellents, as a food source for symbiotic bacteria or as biocides against pathogens. At present the biosynthesis of the secondary products and its *in vivo* regulation is poorly understood

All biosynthetic routes leading to secondary metabolites start from products of primary pathways At the points where the secondary routes branch off from primary metabolism, mechanisms must exist which regulate the amount of precursor drained away for the biosynthesis of secondary metabolites In the simplest model of precursor partitioning, there is no specific regulation of the flux of precursors into secondary metabolism a fixed proportion of the common precursor enters the secondary pathway in all cells where the metabolite is formed A second model involves an overflow mechanism The precursor is preferentially channelled into the primary pathway Secondary metabolism only proceeds at a substantial rate if there is an excess of precursor This mechanism will operate whenever two enzymes with different K_m values compete for the same substrate Enzymes in secondary pathways in vivo usually do not work under V_{max} conditions, due to limited substrate supply (Luckner, 1990) A third model includes an active mechanism functioning at the molecular level The precursor flow is diverted into the secondary route by induced enzyme synthesis or by activation of preexisting enzyme At least the former process requires specific gene expression. The regulated enzyme is usually, but not necessarily, the first enzyme of a biosynthetic side branch to secondary metabolism (Galneder & Zenk, 1990)

Immersed plant cell or tissue cultures are suitable to test the validity of the three models in a specific situation The surrounding nutrient solution provides a controlled environment to which the tissues are fully exposed. All precursors for primary and secondary metabolism may be supplied at defined concentrations. The metabolic flow can be monitored by using radiolabelled precursors

In the present study *Tagetes* hairy roots were used as a model system to study the regulation of a secondary pathway These roots accumulate the same secondary compounds as roots attached to the plants (Croes *et al* , 1989, Mukundan & Hjortso, 1990) These compounds, thiophenes (Zechmeister & Sease, 1947, Uhlenbroek & Bijloo, 1959, Bohlmann & Herbst, 1962, Atkinson, Curtis & Phillips, 1965), are characterized by the presence of one or more heterocyclic sulphur-containing rings The carbon skeleton is thought to be derived from polyacetylenes which in turn are synthesized from fatty acids (Bohlmann & Zdero, 1985, Christensen & Lam, 1990, Menelaou *et al* , 1991) The conversion of polyacetylenes to thiophenes probably occurs by addition of reduced sulphur from thiols to adjacent acetylenic groups (Bohlmann & Zdero, 1985) The sulphur atom ultimately comes from the sulphate provided in the medium Since sulphate is also an essential substrate for primary metabolism, limitation of this nutrient is expected to affect growth and development as well as thiophene synthesis Preliminary experiments had shown that in a completely sulphate-free nutrient solution all cell activity eventually stopped To extend these results and to establish the role of sulphate in the regulation of primary and secondary metabolism in *T patula*, we studied the effect of sulphate limitation on root cultures Root growth and morphology were used as parameters for primary metabolism, the accumulation and biosynthesis of thiophenes were taken as measure for secondary metabolism

Materials and methods

Root cultures

Most experiments were performed with root line Tp9402, which was obtained by transformation of seedlings of *Tagetes patula* L cv Nana with *Agrobacterium rhizogenes* LBA9402 (pRi1855) In some experiments a line of *T erecta* L (Te9402) transformed by the same bacterial strain was also

used Transformation of the roots was verified by Southern blotting using the EcoRI-15 fragment of the Ri-plasmid pRi 1855 as a probe (Birot *et al*, 1987)

Growth of isolated roots

Root cultures were maintained on hormone-free Gamborg's B5 medium (Gamborg, 1970) supplemented with 0 1 M sucrose and 100 μg l biotin The roots were grown in the dark at 25°C on a rotary shaker at 100 rpm in 50-ml Erlenmeyer flasks containing 20 ml medium The sulphate concentration in the medium was varied in some experiments by adding K_2SO_4, either to standard medium or to medium in which the sulphate had been substituted by chloride For the determination of the effect of sulphate concentration on tip elongation, branching, and thiophene accumulation, roots were grown on the same medium solidified with 0 2% gellan gum
In one experiment, nitrogen-free medium was prepared by omission of all nitrate and ammonium containing salts

Analysis of thiophenes and polyacetylenes

Thiophenes were analyzed as described previously (Croes *et al* 1989) Briefly, thiophenes were extracted from the roots in ethanol water (1 1, v/v) and purified by partitioning between this mixture and hexane *tert*-butylmethylether (1 1, v/v) Thiophenes were recovered from the hexane-ether fraction, subjected to HPLC on a reversed-phase C_{18} column (25 x 0 4 cm, particle size 7 μm), with acetonitrile water (72 28, v/v) at a flow rate of 1 5 ml min^{-1}, and quantified in the eluate on basis of UV absorption at 340 nm Polyacetylenes were extracted and analyzed as described for thiophenes, UV absorption was measured at 280 nm

Measurement of non-protein thiols

Thiol content was determined according to De Knecht *et al* (1992) Approximately 20 mg lyophilized roots were homogenized in 2 ml of a 0 2 M 5-sulphosalicylic acid solution with 6 3 mM diethylenetriaminepentaacetic acid at 0°C, using mortar and pestle and quartz sand The homogenate was centrifuged at 10000 ***g*** at 4°C for 10 min and the supernatants were immediately assayed for sulphhydryl groups For this purpose, 300 μl supernatant was mixed with 630 μl 0 5 M K_2HPO_4 and 25 ml 10 mM 5,5'-dithiobis[2-nitrobenzoic acid] (DTNB), at a final pH of 7 5 The absorbance was

measured at 412 nm. The values were corrected for the absorbance of the supernatants and of DTNB

Sulphate uptake and incorporation

$Na_2{}^{35}SO_4$ (37 TBq.μmol^{-1}) was obtained from Amersham To monitor the sulphate uptake during root growth, 720 Bq ml^{-1} of [^{35}S]sulphate was added to the standard medium. The capacity to synthesize thiophenes was determined by labelling nine-days-old root cultures in the exponential growth phase for two hours in growth medium containing 30 mM sodium sulphate and 370 kBq.cm^{-3} $Na_2{}^{35}SO_4$ The sulphate concentration in the medium was raised to this level to minimize label dilution by the internal sulphate pool in the roots After incubation roots were extracted as described above Over 95% of the label in the hexane-ether fraction was present in thiophenes Therefore, the radioactivity in this fraction was taken as measure of sulphur incorporation in thiophenes Unincorporated sulphur remained in the ethanol-water phase. Samples of both fractions were mixed with scintillation fluid and counted in a liquid scintillation counter

Statistical treatment

All data are the result of at least 4 independent determinations and are presented as means ± SE

Results

To determine the effects of sulphur limitation on growth and secondary product formation, we transferred a root culture of Tp9402 that had grown in standard liquid medium for two weeks to sulphate-free nutrient solution. The roots continued to grow at the same rate during the first 14 days and lagged behind the control afterwards (Fig. 2.1A). The amount of thiophenes per gram fresh weight steadily decreased during the whole culture period (Fig 2.1B). When, however, growing roots were transferred to nitrogen-free medium, growth immediately stopped (Fig 2.1A), but the amount of thiophenes per gram fresh weight remained stable (Fig 2.1B). Thus, a reduced growth rate does not automatically lead to a reduced amount of thiophene in the roots.

To further quantify the effects of sulphate, root tips were incubated on solid medium at a range of sulphate concentrations. After eight days of culture, the

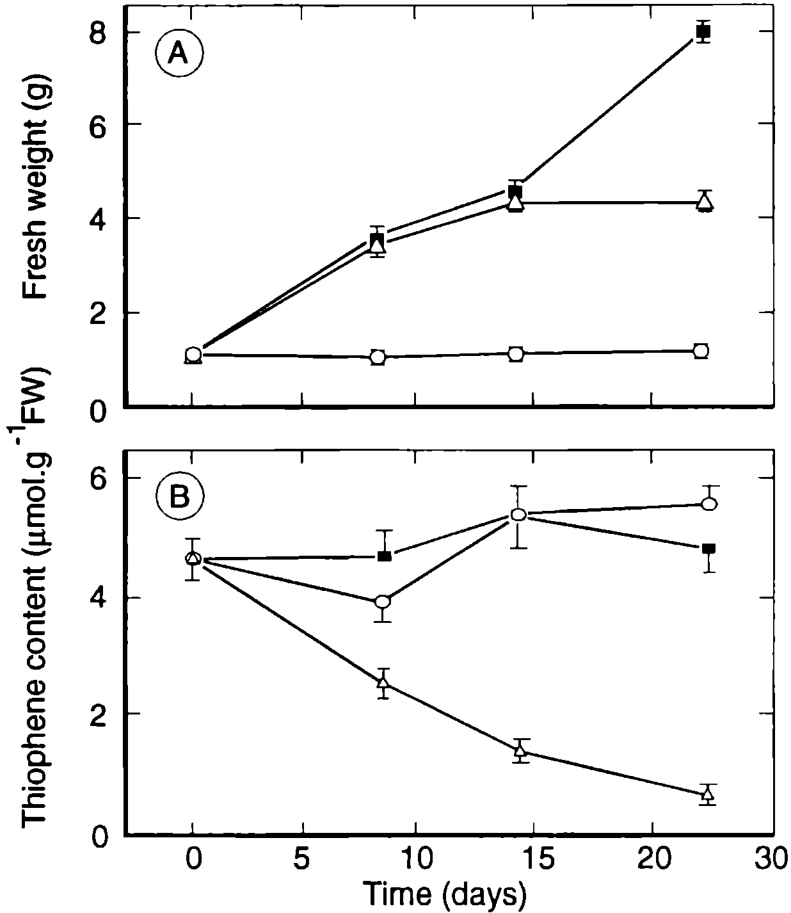

Fig. 2.1 Effect of sulphur and nitrogen starvation on root growth and thiophene accumulation in *Tagetes* root line Tp9402 Roots were first grown in liquid standard B5 medium for two weeks and then transferred to fresh medium (squares) or to deficient medium lacking nitrogen (circles) or sulphur (triangles) Biomass production and thiophene concentration were measured at weekly intervals Time zero is the moment of the medium shift Data are means of four determinations ± SE

elongation growth of the root and number of lateral roots did not differ significantly in the range of sulphate concentrations applied (Tab. 2.1). In contrast, the accumulation of thiophenes had dramatically decreased by lowering the sulphate concentration of the medium. Reduction of the sulphate content in

the growth medium from 2.0 mM to 0.1 mM thus seems to have no effect on primary metabolism, at least during the first eight days of culturing, whereas secondary metabolism is strongly reduced.

Roots in liquid nutrient solution showed a similar behaviour: A decrease of the sulphate concentration in the medium from 2.0 mM to 50 μM had no significant effect on root growth, nor on morphology whereas thiophene accumulation was reduced by 35% for *Tagetes patula* and 55% for *Tagetes erecta*. The effect of the low sulphate concentration on thiophene accumulation was even surpassed by its effect on biosynthesis. At the end of the incubation, the biosynthetic capacity of the roots in the low-sulphate medium had fallen to 7% (*T. patula*) and 3% (*T. erecta*) of the control values (Tab. 2.2).

Table 2.1 Effect of sulphate on root growth and thiophene accumulation on solid medium in *Tagetes* root line Tp9402. Root tips were incubated on solid B5 medium at various sulphate concentrations. Growth, branching and thiophene content of the roots were determined after 14 d. All data are means of six measurements ± SE.

[Sulphate] (mM)	Length of main root (cm)	Lateral roots formed	Thiophene accumulation (μmol.g FW^{-1})
0.1	6.5 ± 2	32 ± 11	0.6 ± 0.1
0.5	6.3 ± 1	34 ± 10	1.1 ± 0.2
2.0	6.7 ± 1	24 ± 8	2.3 ± 0.4

The discrepancy between the effects of sulphur starvation on accumulation and on synthesis suggests that shortage of sulphur strongly limits thiophene synthesis at the end of the culture period but much less at earlier stages. To test this sulphate uptake and biosynthetic capacity of roots incubated at 2.0 mM and at 50 μM sulphate were measured over a twelve-day-period. As in the previous experiment, sulphate limitation had no effect on biomass production (Fig. 2.2A-2.2B). The

sulphate concentration in the low-sulphate medium dropped to a final level of 2 mM within eight days; 96% of the sulphate had been taken up (Fig. 2.2B). In the high-sulphate medium only 25% of the total amount of sulphate had been taken up by the roots after twelve days (Fig. 2.2A). The capacity of the root to synthesize thiophenes at a low sulphate supply only started to decline after six days (Fig. 2.3) when the sulphate concentration in the medium was 30 $mmol.m^{-3}$ (Fig. 2.2B). The results show that the reduction in biosynthetic capacity in these roots gradually developed during culture. This explains why after eight days of sulphate limitation thiophene synthesis was more severely affected than thiophene accumulation.

Table 2.2 Effect of sulphate on root growth, thiophene biosynthesis and accumulation in liquid medium in two *Tagetes* root lines Root tips were incubated at two sulphate concentrations and analyzed after 8 d Thiophene synthesis was measured as the amount of [^{35}S]sulphur incorporated from sulphate in two hours All data are means of four determinations ± SE

[Sulphate] (mM)	Fresh weight (g)	Thiophene accumulation (μmol g FW^{-1})	Thiophene synthesis (nmol g FW^{-1} h^{-1})
T patula (Tp9402)			
0 05	0 25 ± 0 02	0 7 ± 0 2	1 1 ± 0 4
2 0	0 29 ± 0 05	1 1 ± 0 1	16 7 ± 0 9
T erecta (Te9402)			
0.05	0 30 ± 0.02	0 5 ± 0 2	0 5 ± 0 1
2 0	0 27 ± 0 03	1 1 ± 0 2	14 7 ± 2 7

The reduction in biosynthetic capacity at low sulphate concentration could mean that under these circumstances the secondary pathway leading to thiophenes is shut off by active regulation. However, biosynthesis could also be limited by a low availability of the donor compound from which the sulphur in the thiophene

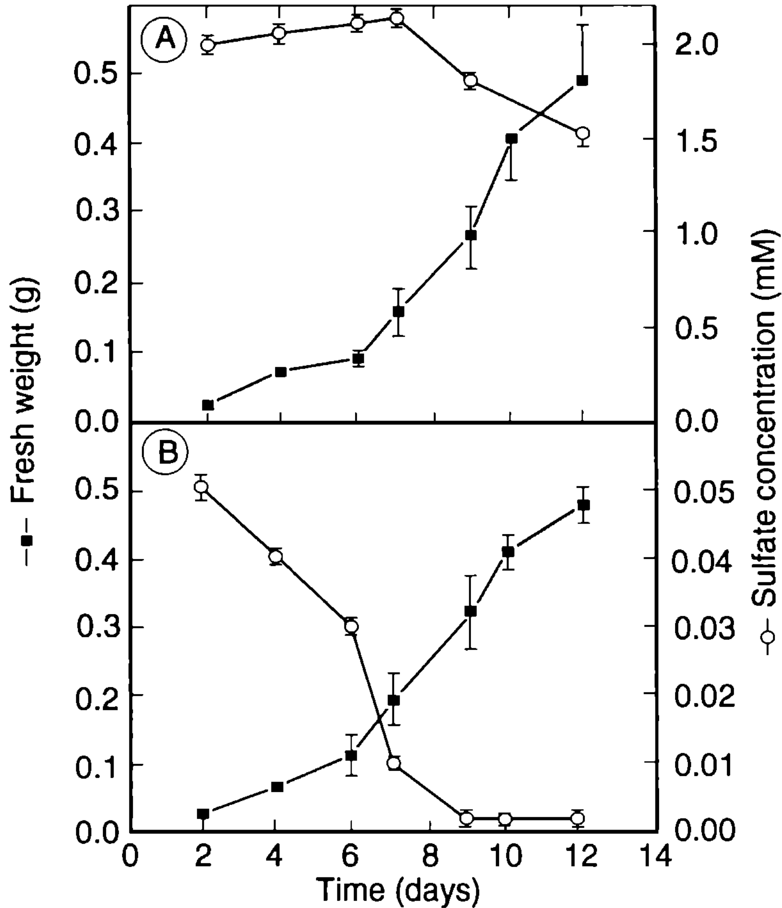

Fig. 2.2 Growth and sulphate uptake of *Tagetes patula* roots in liquid standard B5 medium (A) and in sulphate-deficient medium (B). Fifty-ml Erlenmeyer flasks, with 20 ml medium were inoculated with 10 root tips of approximately 1 cm length. The media contained 2 mM (A) and 50 μM (B) sulphate and 37 kBq.ml^{-1} $Na_2{}^{35}SO_4$. Biomass (solid squares) was determined at two-day intervals. Sulphate concentration in the medium (open circles) was followed by measuring the disappearance of radioactivity. Data are means of four measurements $\pm$ SE

ring is directly derived. In the latter case the concentration of thiol compounds would be expected to decrease at a restricted sulphate supply, and the non-sulphurous precursors in thiophene synthesis, the polyacetylenes, would be

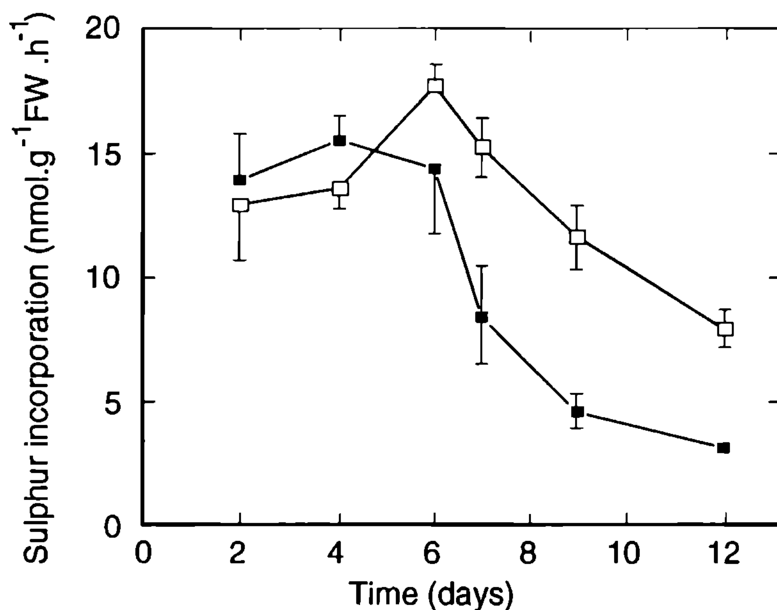

Fig. 2.3 Thiophene biosynthesis in *Tagetes patula* roots during culturing in liquid medium Roots were cultured for 12 d in medium containing either 2 mM sulphate (open squares) or 50 μM sulphate (solid squares) The capacity to synthesize thiophenes was determined at two day intervals Data are means of four measurements $\pm$ SE

Table 2.3 Effect of sulphate on thiol accumulation in *Tagetes* roots, cultured in liquid medium Root tips were incubated at two sulphate concentrations and analyzed after 8 d All data are means of four determinations $\pm$ SE

[Sulphate] (mM)	Thiol accumulation (μmol g FW^{-1})
0 05	0 20 $\pm$ 0 04
2 0	0 18 $\pm$ 0 03

expected to accumulate. To discriminate between these two possibilities, roots were cultured for 8 d at 50 μM sulphate and subsequently assayed for their thiol and polyacetylene content. The amount of non-protein thiols in the roots was not significantly lower than in the control (Tab. 2.3). Also, no increase in the number or amount of polyacetylenes was detected (data not shown). This shows that limitation of the sulphur donor was not likely to be the cause of the reduction in biosynthetic capacity. This corollary was substantiated by an experiment in which roots were cultured for 8 d at 50 μM sulphate and subsequently exposed to 2 mM. The capacity to synthesize thiophenes was measured at intervals during a 24-h period (Fig. 2.4).

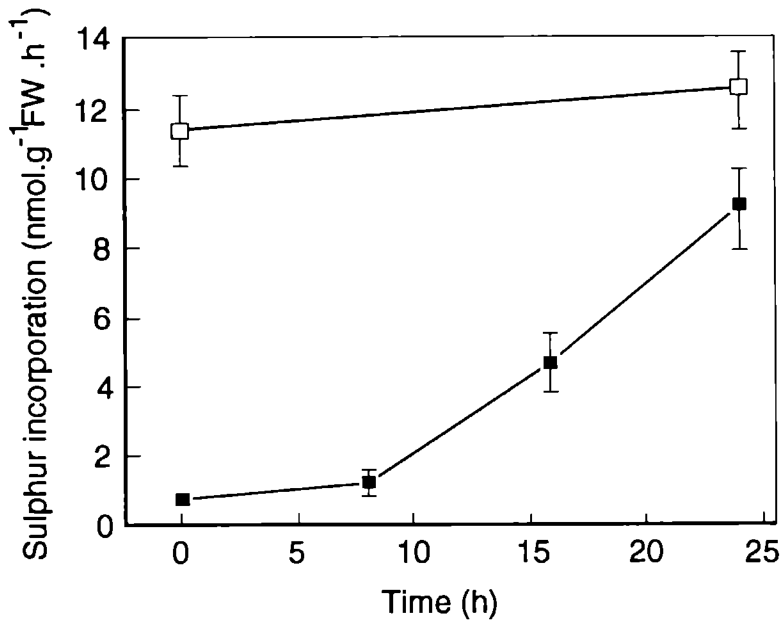

Fig. 2.4 Recovery of thiophene biosynthetic capacity of *Tagetes patula* roots starved for sulphate. Roots were precultured for nine days in medium containing either 2 mM sulphate (open squares) or 50 μM sulphate (solid squares) The tissue was then incubated in fresh medium with 2 mM sulphate. The capacity to synthesize thiophenes was determined at eight-hour intervals Data are means of four measurements $\pm$ SE.

The slow recovery found argued for a process of active regulation involving enzyme synthesis or modification. Additional evidence for such a mechanism was obtained when the experiment was repeated in the presence of cordycepin. A non-toxic concentration of 5 $\mu g.l^{1}$ of the transcription inhibitor completely prevented restoration of the thiophene biosynthetic capacity (Tab. 2.4). This indicates that for the restoration of thiophene biosynthesis transcription is a prerequisite. Cordycepin also reduced the rate of thiophene synthesis in control cultures that had been continuously cultured at 2 mM sulphate. This result suggests that in the absence of transcription the thiophene pathway is rapidly down-regulated.

Table 2.4 Effect of sulphate on the capacity of *Tagetes patula* roots to synthesize thiophenes Roots were precultured for 9 d in medium containing either 2 mM or 50 μM sulphate The tissues were then incubated for another 24 h in fresh medium with 2 mM sulphate Cordycepine was added to half of the cultures at 5 $\mu g\ l^{1}$ The incorporation of [^{35}S]sulphur in the thiophenes was measured at the time of the medium shift and at the end of the experiment. Data are means of four measurements ± SE

Time (h)	[Sulphate] during preculture (mM)	Cordycepine added	S incorporation (nmol g FW^{1} h^{1})
0	0 05	-	1 7 ± 0 4
	2 0	-	8 8 ± 0 9
24	0 05	no	5 3 ± 0 6
		yes	1 9 ± 0 3
24	2 0	no	10 1 ± 0 3
		yes	5 3 ± 0 6

Discussion

Sulphur partitioning between thiophene synthesis and primary requirements for growth and development in *Tagetes* roots is controlled by specific transcription. The roots use this mechanism to reduce the extent of their secondary metabolism and at the same time maintain the rate of primary metabolism when sulphate becomes limiting. The resulting decrease in thiophene biosynthesis is due to a breakdown of the thiophene synthesizing machinery.

Conditions of sulphate limitation did not instantly lead to starvation. The roots have an efficient mechanism to take up sulphate from the environment until the concentration has fallen to a very low level. This uptake assures the availability of a sulphate pool that acts as a reservoir (Clarkson *et al.*, 1983). Upon prolonged sulphate deficiency the roots still did not change their pattern of growth and development but thiophene synthesis was severely reduced. Hence, a mechanism must exist which preferentially down-regulates the flux of precursors into the thiophene biosynthetic pathway. The combination of continuous root growth with reduced thiophene synthesis indicates that sulphur is channelled away from the thiophene biosynthetic route into primary metabolism.

The 'overflow' model for precursor partitioning is not tenable in our model system because the pool of reduced sulphur compounds had not significantly decreased after 8 d of root growth in sulphate-deficient medium. No accumulation of polyacetylenic precursors was observed, which suggests that polyacetylene biosynthesis was also down-regulated as a direct or indirect consequence of sulphur deficiency.

Two lines of evidence indicate that the change in sulphur partitioning in sulphate-starved roots is an effect of a reduction in the activity of one or more enzymes involved in thiophene synthesis. Firstly, when roots grown at a low sulphate concentration are exposed to a non-limiting sulphate supply, the capacity for thiophene synthesis gradually rises. Yet, it takes more than 24 h for these tissues to acquire the rate of sulphur incorporation in thiophenes characteristic for

roots that had continuously grown without sulphate limitation. Such a long adaptation period would not be expected if only an overflow mechanism would regulate the rate of synthesis. Secondly, the gradual increase in thiophene synthesis is dependent on transcription as is concluded from the inhibiting effect of cordycepin.

A mechanism that regulates a biosynthetic pathway by modulation of transcriptional activity is only effective if the target enzyme is unstable. Evidence for this in the case of thiophene biosynthesis comes from the observation that addition of cordycepin to roots growing with an adequate sulphate supply, leads to a decline in thiophene synthesis within 24 h (Tab. 2.4). Thus continuous transcription is required for the thiophenes to be synthesized at a normal rate, which indicates that one or more enzymes involved are unstable. This implicates that, when sulphate becomes limiting, transcription of the genes coding for these enzymes is no longer sustained. When an adequate sulphate supply is restored, this transcription is resumed and the roots regain their thiophene synthetic capacity.

The nature of the signal-transduction mechanism which induces down-regulation of thiophene biosynthesis is still unclear. In pea cotyledons recovering from sulphur deficiency, renewed synthesis of specific mRNAs is induced by cysteine (Macnicol & Randall, 1987). This opens the possibility that the internal cysteine level regulates the formation of thiophenes at the transcriptional level thus affecting the balance between primary and secondary sulphur metabolism. Free cysteine normally is present in small quantities only (Buwalda *et al.*, 1988); glutathione is the predominant water-soluble, non-protein thiol compound (Rennenberg, 1982; Rennenberg & Lamoureux 1990). Whether the cysteine:glutathione ratio changed after sulphate depletion could not be determined with our thiol assay. Further elucidation of the regulation of enzymatic conversions in our model system has to await identification of the enzymes involved in the formation of the various thiophenes from their polyacetylenic precursors.

The diversity in the secondary biosynthetic routes is enormous and all are branches of primary pathways. In cases where a mineral is one of the substrates in the biosynthetic pathway, secondary product accumulation may pose a serious demand on the supply of a nutrient in the soil. Alkaloids, for instance, have been considered as storage compounds in which part of the nitrogen taken up by the roots is sequestered; a comparable role may be imagined for thiophenes with respect to sulphur This concept of the role of secondary metabolites almost necessarily requires that the needs of primary metabolism are satisfied first if the mineral supply in the soil is limiting. This, in turn, means that the partitioning of these elements in the plant is altered in favour of primary metabolism which leads to a reduction in the biosynthesis of the secondary metabolites.

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Chapter 3

Thiophene interconversions in *Tagetes patula* hairy root cultures

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On the basis of feeding experiments with ^{35}S-labelled intermediates, we present a modified biosynthetic pathway of the bithienyls in *Tagetes*. The monothiophene 2-(but-3-en-1-ynyl)-5-(penta-1,3-diynyl)-thiophene, which is present in small amounts in *Tagetes* hairy roots, is the presursor of all bithienyls that have been described for this species but not of α-terthienyl. The current hypothesis that 5-(3-buten-1-ynyl)-2,2'-bithienyl originates from 5'-methyl-5-(3-buten-1-ynyl)-2,2'-bithienyl after oxidative decarboxylation proved untrue. The latter compound is only converted into (5'-but-3-en-1-ynyl-[2,2']bithiophenyl-5-yl)-methyl acetate, probably via (5'-but-3-en-1-ynyl-[2,2']bithiophenyl-5-yl)-methanol. Substitution of the butenynyl side chain of 5-(3-buten-1-ynyl)-2,2'-bithienyl results in the formation of 5-(3,4-dihydroxy-1-butynyl)-2,2'-bithienyl and 5-(4-hydroxy-1-butynyl)-2,2'-bithienyl, which are subsequently converted into respectively 5-(3,4-diacetoxy-1-butynyl)-2,2'-bithienyl and 5-(4-acetoxy-1-butynyl)-2,2'-bithienyl.

The metabolically stable end-products of the bithienyl biosynthetic pathways are bithienyl-acetate esters. They are stored in a cell compartment where they are inaccessible to esterases, which are abundantly present in the *Tagetes* cells.

Introduction

Secondary products are not directly needed for the primary life functions of the plant and the biosynthetic pathway leading to a secondary product can be manipulated without affecting the physiological processes needed for growth and development. Therefore, secondary biosynthetic pathways provide excellent models to study regulation of plant metabolism at the molecular and physiological level. But, before the fundamental questions can be tackled, detailed knowledge of a secondary pathway is needed concerning bioconversions, enzymes and genes involved, and the way the products are transported or stored.

In the study of biosynthetic routes, plant cell cultures have been shown to be a

powerful tool (Hahlbrock & Scheel, 1989) Major advantages are the homogeneity of both the cell cultures and the environment in which they are suspended Radiolabelled precursors can be fed via the culture medium and their conversion by the submersed plant cells can be followed in time-course experiments A drawback is that cell suspension cultures often do not synthesize the same secondary products as intact plants Hairy-root cultures, however, normally produce the same secondary metabolites as roots on plants (Flores, 1987, Berlin, 1990, Mukundan & Hjortso, 1990) although the amounts are usually lower Thus hairy-root cultures are an excellent model system to study the synthesis of secondary products in roots

Most *Tagetes* species accumulate a range of thiophenes in their roots These compounds are characterized by one, two or three thiophene rings linked together by their α-carbons Formation of the thiophene rings is probably a two-step reaction (Bohlmann *et al* , 1973) The first step is the addition of a thiol to a diyne group, thus forming a thioether The second step would be cleavage of the thioether bond and concomitant ring closure (Fig 3 1) Although the thiophenes in *Tagetes* have been the subject of several studies, the biogenetic relations of these compounds are not completely known and their biosynthesis is poorly understood (Constabel & Towers, 1989)

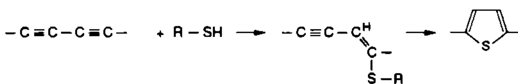

Fig 3.1 Formation of the thiophene ring in higher plants (after Bohlmann)

The close structural relationship of the naturally occurring thiophenes in *Tagetes patula* (Fig 3 2) led to the assumption that all these compounds are derivatives of the polyacetylene trideca-3,4,7,9,11-pentayn-1-ene (PYE) (Bohlmann & Hinz, 1965) Labelling studies with related plant species like *Echinops sphaerocephalus* L and *Buphthalmum salicifolium* L confirmed this assumption (Bohlmann & Hinz, 1965, Bohlmann *et al* , 1966) The biosynthetic pathway from PYE to the major thiophene derivatives comprises three types of

Me – C≡C – C≡C – C≡C – C≡C – C≡C – CH=CH₂ PYE

MeBBT

AcOCH₂BBT

BBT

BBT(OH)₂

BBTOH

BBT(OAc)₂

BBTOAc

α-T

Fig.3.2 Structures of the trideca pentaynene derived thiophenes accumulating in roots of *Tagetes patula*

biosynthetic steps thiophene-ring formation, demethylation, and substitution at the side chain

In the formation of bi- and terthienyls a monothiophene is expected as an intermediate Addition of sulphur to pentaynene can yield four possible monothiophenes, of which only the diynes 2-(but-3-en-1-ynyl)-5-(penta-1,3-diynyl)-thiophene (BPT) and 2-(hex-5-en-1,3 diynyl)-5(prop-1-ynyl)-thiophene are known to occur in plants (Bohlmann *et al* , 1973) On the basis of structural relationship only BPT can be the precursor of 5-(3-buten-1-ynyl)-2,2'-bithienyl (BBT) (Fig 3 3) Addition of sulphur to BPT would yield 5'-methyl-5-(3-buten-1-ynyl) 2,2'-bithienyl (MeBBT) which has been proposed to be converted to BBT after oxidative decarboxylation (Bohlmann & Kleine, 1963, Bohlmann *et al* , 1965, Bohlmann & Berger, 1965, Bohlmann *et al* , 1966, Bohlmann *et al* , 1973, Christensen & Lam, 1990) The presence of BPT has never been described for *Tagetes* However, mutagenesis of *Tagetes* seed yielded a mutant, deficient in BBT synthesis, which accumulates large amounts of BPT and MeBBT (Jacobs *et al* , in preparation) This pattern supports the hypothesis that BPT and MeBBT are intermediates in the synthesis of BBT

(Bohlmann & Hinz, 1965). Probably BPT also occurs in wild-type *Tagetes*, albeit in minute amounts.

Fig.3.3 Hypothetical biosynthetic pathway to the synthesis of BBT.

Feeding experiments with radiolabelled BBT indicated that this compound is readily converted into 5-(4-hydroxy-1-butynyl)-2,2'-bithienyl (BBTOH) (Jente *et al.*, 1988) and 5-(4-acetoxy-1-butynyl)-2,2'-bithienyl (BBTOAc) (Bohlmann & Hinz, 1965; Schulte & Foerster, 1966; Schulte *et al.*, 1968; Jente *et al.*, 1981). The diol 5-(3,4-dihydroxy-1-butynyl)-2,2'-bithienyl ($BBT(OH)_2$) may also be a BBT derivative (Bohlmann & Hinz, 1965). The mechanism of BBT-derivatization is not really established, but it seems probable that all derivatives are formed via an epoxidation of the vinyl group (Bohlmann *et al.*, 1973). Reduction of the epoxide moiety would yield the alcohol BBTOH, whereas hydrolysis would give $BBT(OH)_2$. The alcohols, in turn, may be converted into BBTOAc and 5-(3,4-diacetoxy-1-butynyl)-2,2'-bithienyl ($BBT(OAc)_2$) respectively. This concept is substantiated by the identification of acetyl transferases which catalyze the latter two reactions (Metschulat & Sütfeld, 1987; Sütfeld, 1988). However, esterases converting BBTOAc and $BBT(OAc)_2$ into the corresponding alcohols have also been identified (Sütfeld & Towers, 1982; Pensl & Sütfeld, 1985). It has not yet been established which enzymes are involved in biosynthetic or in degradative pathways, hence the sequence of metabolic steps in the turnover of thiophene derivatives is still unresolved.

In this paper the presence of BPT, the 'missing link' in the biogenesis of bithienyls, will be described in isolated *Tagetes* roots. Furthermore the hypothetical,

but still unresolved, steps in the biosynthetic pathway, *i.e.* oxidative decarboxylation of MeBBT to BBT and the conversion of BBT into BBTOAc and BBT(OAc)$_2$ via BBTOH and BBT(OH)$_2$, will be investigated in experiments in which ^{35}S-labelled intermediates were fed to *Tagetes* hairy roots.

Materials and methods

Root cultures

Hairy root line Tp9402 was obtained by transformation of *Tagetes patula* L. cv. 'Nana' with *Agrobacterium rhizogenes* LBA 9402 (pRi1855). Root line A-II-5 was obtained by transformation of a *Tagetes erecta* mutant, which accumulated BPT and MeBBT, with the same *Agrobacterium* culture (Jacobs *et al.* in preparation).

Growth of isolated roots

Roots were maintained in liquid Gamborg's B5 medium (Gamborg, 1970), supplemented with 3% (w/v) sucrose and 100 μg l^{-1} biotin. The cultures grew in 50-ml Erlenmeyer flasks filled with 20 ml medium, on a rotary shaker (100 rpm) at 25°C in the dark and were subcultured every two weeks.

Kinetics of thiophene biosynthesis

Tips of 1 cm were cut from roots precultured for 8 d and incubated in sulphate-free medium for 2 h. Carrier-free [^{35}S]sulphate (0.8 MBq.ml^{-1}) was then added to the culture. The thiophenes were extracted at intervals and separated by HPLC. The specific activities of each of the compounds were calculated from the radioactivity and the chemical amount present in the peaks.

Preparation of radiolabelled precursors

Roots that had been grown in B5 medium with $Na^{35}SO_4$ (specific activity 40 GBq mol^{-1}) synthesized radiolabelled thiophenes. Root line Tp9402 was used for the production of ^{35}S-labelled BBT, BBTOAc, and $AcOCH_2BBT$. The amount of $BBT(OAc)_2$ in these roots was too small to allow isolation of enough material for precursor-feeding experiments. The mutant root line A-II-5 was used for the production of labelled BPT and MeBBT After fractionation of the root extracts (Norton *et al.* 1985), thiophenes were recovered from the petrol phase This phase was fractionated on a column of 4 g silica gel G-60 (Merck) by stepwise elution with 30-ml fractions of 0, 10 and 40% diethyl ether in petrol. The fractions were evaporated to dryness, dissolved in ethanol and purified by reverse-phase HPLC Purity of the isolated compounds was confirmed by analytical HPLC and TLC The alcohols BBTOH and $HOCH_2BBT$, which are minor metabolites in the root, were made by saponification of the corresponding acetate esters (Jente *et al* , 1981).

Formylation of BBT via the Vilsmeier-Haack reaction gave formyl-BBT In this reaction 5 mg BBT was mixed with 5 mg $POCl_3$ and 5 mg N-methyl-formanilide for 20 min at 50°C. After cooling, 1 ml of a 1 M sodium acetate solution and 2 ml ethyl acetate were added. After mixing for 30 min the mixture was shaken with 6 ml hexane:*t*-butylmethyl ether (1:1, v/v) The hexane layer was evaporated under nitrogen at ambient temperature, the residue was taken up in 200 μl ethyleneglycolmonomethyl ether. The structure of the chemically synthesized compound was confirmed by GC/MS and ^1H-NMR The procedure was repeated using [^{35}S]BBT as starting material The identity of [^{35}S]formyl-BBT was confirmed by HPLC analysis, using the non-labelled compound as reference

Precursor feeding

In the feeding experiments the labelled precursors, dissolved in 100 μl ethanol, were added to 10 ml 0.1% Tween-20 in Gamborg's B5 medium supplemented with 3% (w/v) sucrose and 100 μg/l biotin, the radioactivity of the incubation medium was 2 kBq ml^{-1} Eight-days-old root cultures were placed in these emulsions and incubated for 40 hours Then roots were rinsed with unlabelled B5 medium and thiophenes extracted as described above

The organic phase was fractionated with reverse-phase HPLC, thiophenes were monitored by UV absorption at 340 nm. Fractions of 0 5 ml were collected, mixed with scintillation fluid and radioactivity was measured

Identification of thiophenes

GC/MS. A capillary column (fused silica WCOT, coated with CP-Sil 5CB, 25m x 0.32 mm ID) was used to fractionate the samples Carrier gas was He, and the flow rate 1 5 $ml.min^{-1}$. Samples of 1 μl were injected with a splitting ratio of 1:10, at an injection port temperature of 250°C The initial oven temperature was 100°C, the temperature was raised with 15°$C.min^{-1}$ to 280°C, this temperature was maintained for 8 min The electron impact method (EI) was used to ionize the fractions Recorded spectra were compared with spectra known from literature (Bohlmann *et al* , 1964; Bohlmann *et al* , 1973; Groneman *et al.*, 1984; Caniato *et al.*, 1990; Bicchi *et al* , 1992). Since BBTOH and $HOCH_2BBT$ are not stable at the oven temperatures used, these compounds were acetylated prior to GC/MS identification This was done by dissolving the alcohols in acetic acid anhydride and subsequent heating at 70°C for 1 h. The anhydride was removed by evaporation.

Fourier-transformed ^1H-NMR spectra were recorded on a spectrometer operating at 400 MHz Samples were measured in $CDCl_3$ with tetramethylsilane (TMS) as an internal standard NMR-spectra were compared with spectra known from literature (Bohlmann & Kleine, 1963; Atkinson *et al.*, 1964; Bohlmann *et al.*, 1964; Bohlmann *et al.*, 1965, Bohlmann & Berger, 1965; Bohlmann & Zdero, 1985).

UV-absorption spectra were recorded in ethanol. Absorbtion was measured between 210 and 600 nm. Spectra were compared with known spectra from the literature (Uhlenbroek & Bijloo, 1959; Bohlmann & Herbst, 1962; Bohlmann & Kleine, 1963; Atkinson *et al* , 1964; Bohlmann & Berger, 1965; Bohlmann *et al.*, 1965).

HPLC was performed on a Lichrosorb RP-18 column (particle size 7 μm). Column dimensions were 25 x 0.4 cm, the eluent was acetonitrile water (72:28, v/v). The flow rate was 1.5 $ml.min^{-1}$, thiophenes were detected by their UV-absorption at 340 nm.

TLC. strongly lipophilic thiophenes were separated on Al_2O_3 by elution with petrol, thiophenes of moderate polarity on silica gel 60 F_{254} by elution with benzene:chloroform (9:1, v/v) (Curtis & Phillips, 1962). Alternatively thiophenes of moderate polarity were separated on silica gel 60 F_{254} by elution with hexane·ethyl acetate (9:1, v/v), which resulted in similar retardation factors.

Protein extraction

All steps were carried out at 0-4°C under exclusion of UV- or daylight. Root material was homogenized in liquid nitrogen using a mortar and pestle. The powder was suspended in 0 1 M potassium phosphate buffer pH 6 5, 50 mM potassium ascorbate, 0.25 M sucrose, 1 mM dithiothreitol (DTT) and 0.6% (w/v) polyvinylpolypyrrolidone (PVPP) After continuous stirring for 15 min, PVPP and insoluble residues were removed by filtration on Whatman GF/C glass fibre filters The filtrate was centrifuged (15 min,

28,000 x *g*) The supernatant was fractionated by addition of solid $(NH_4)_2SO_4$ Proteins precipitating in a range from 40% to 80% $(NH_4)_2SO_4$ saturation were centrifuged (30 min, 28,000 x *g*), and the pellet was dissolved in 0 1 M potassium phosphate buffer, pH 8 0

Esterase assay

The BBTOAc acetate esterase assay was carried out according to Sutfeld & Towers (1982) The mixture (300 μl) contained 9 nmol substrate and 0 3 mg bovine serum albumin (BSA) in 0 1 M potassium phosphate buffer, pH 8 0 After incubation at 30°C for 2 h, the reaction was stopped by addition of 600 μl hexane *t*-butylmethyl ether (1 1, v/v), followed by thorough mixing After centrifugation (5 min, 10,000 x *g*) the organic layer was collected, hexane *t*-butylmethyl ether was evaporated under nitrogen at ambient temperature, and the residue was taken up in ethanol for HPLC analysis

RESULTS

Formation of the thiophene rings

All thiophenes that have been described in *T. patula* roots could be identified in the HPLC chromatogram of an extract from the *T patula* hairy root line Tp9402 (Fig 3.4) Moreover, after culturing the hairy roots in the presence of ^{35}S-labelled sulphate or cysteine, radioactive label could be traced in two minor peaks that had not yet been identified as thiophenes. One peak was identified as BPT on the basis of HPLC retention time and GC/MS pattern that were compared with those of authentic samples The other compound could not yet be identified

The presence of BPT in the root extract makes it plausible that this compound is a precursor in polythienyl biosynthesis. To prove this idea we fed [^{35}S]BPT to Tp9402 hairy roots. After 48 h the roots were extracted and the extracts fractionated with HPLC. Radioactive label was found in all bithienyl-containing fractions, but not in 2,2'·5',2"-terthienyl (α-T). This indicates that the bithienyls, but not terthienyl, are derived from BPT.

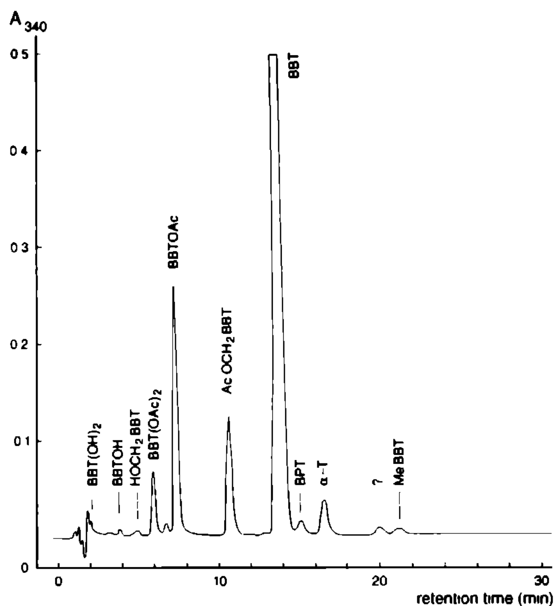

Fig. 3.4 The HPLC elution profile of an extract of two-week-old *Tagetes patula* hairy roots grown in Gamborg's B5 liquid culture medium. The absorption was read at 340 nm.

Feeding [^{35}S]MeBBT to the roots led to the formation of labelled (5'-but-3-en-1-ynyl-[2,2']bithiophenyl-5-yl)-methyl acetate (AcOCH$_2$BBT), whereas no radioactive BBT or other bithienyls were detected (Tab. 3.1). This result sheds doubt on the hypothesis of BBT formation by oxidative decarboxylation of MeBBT. A considerable amount of radioactivity (20%) was present in a fraction that did not contain any of the known thiophenes.

To clarify the point whether MeBBT is converted into BBT via oxidative decarboxylation, we synthesized the presumed intermediates in this decarboxylation reaction, (5'-but-3-en-1-ynyl-[2,2']bithiophenyl-5-yl)-methanol (HOCH$_2$BBT) and 5'-formyl-5-(3-buten-1-ynyl)-2,2'-bithienyl (CHOBBT). Feeding the radiollabeled aldehyde or the alcohol both lead to the formation of [^{35}S]AcOCH$_2$BBT, while no [^{35}S]BBT could be detected. When [^{35}S]AcOCH$_2$BBT was fed, it was taken up by the roots but not converted.

Table 3.1 Conversion of thiophenes in transformed roots of *Tagetes patula* Eight-day-old root cultures were placed in an emulsion containing ^{35}S-labeled thiophenes, 2 kBq ml^{-1} After 40 h of incubation the roots were rinsed and thiophenes were extracted The radioactivities of the individual thiophenes were measured after separation by HPLC and expressed as percentage of the total activity in the extract The values are the mean of at least three determinations, the standard error was 5%

Precursor	Isolated compound									
	Distribution of activity in the root extract (%)									
	BBT-$(OH)_2$	BBT OH	$HOCH_2$ BBT	BBT $(OAc)_2$	BBT OAC	$AcOCH_2$ BBT	BBT	BPT	α-T	Me BBT
BBTOH	--	2	--	3	87	--	1	--	--	--
$HOCH_2BBT$	--	--	3	--	--	81	--	--	--	--
BBTOAc	--	5	1	4	85	--	--	--	--	--
$AcOCH_2BBT$	--	--	5	3	1	70	--	--	--	--
BBT	--	1	--	16	20	--	61	--	--	--
BPT	3	21	6	2	10	18	8	4	--	22
MeBBT	1	1	1	1	--	31	--	--	--	46

The preceding experiments indicated that the thiophene biosynthetic pathway branches at BPT. The end product of one branch is $AcOCH_2BBT$, which is derived from MeBBT. The other branch leads to the five demethylated bithienyls.

Side chain substitutions

[^{35}S]BBT is readily converted into [^{35}S]$BBT(OH)_2$, [^{35}S]BBTOH, [^{35}S]$BBT(OAc)_2$, and [^{35}S]BBTOAc. After feeding of [^{35}S]BBTOH, incorporation of label into BBTOAc was high. When [^{35}S]BBTOAc was fed to the roots, it was taken up but not converted. The results indicate that the acetate ester is produced from BBT via the alcohol. During root growth, BBTOAc hardly underwent any turnover and thus

can be considered as a metabolically inactive end product.

To obtain additional information on the order in which the individual bithienyls are synthesized *in vivo*, carrier-free [^{35}S]sulphate was fed to a growing root culture and the specific activity of the thiophenes that were formed, was determined at intervals. The bithienyls appeared in the order BBT, BBTOH/BBT$(OAc)_2$, and BBTOAc/$AcOCH_2$BBT (Tab. 3.2). The specific activities at 15 to 60 min. decreased in the same order. The amounts of BPT, MeBBT, $HOCH_2$BBT, and BBT$(OH)_2$ were too small to accurately determine their specific activities.

Table 3.2 Synthesis of individual thiophenes in transformed roots of *Tagetes patula* Tips were cut from exponentiallly (8 days) growing roots and preincubated in sulfate-free medium for 2 h Carrier-free [^{35}S]sulfate, 0 75 MBq ml^{-1}, was then added and the roots were extracted after various periods The radioactivities and the chemical concentrations of individual thiophenes were measured after separation by HPLC

Time after $Na_2{}^{35}SO_4$ addition (min)	Specific activity in thiophenes (GBq mol^{-1})				
	BBTOH	BBT$(OAc)_2$	BBTOAc	$AcOCH_2$BBT	BBT
15	0 1	0 3	0	0	0 7
30	0 8	1 2	0	0	2 4
60	16 2	16 2	8 9	10 1	35 4

These results show that the sequence of thiophene formation is BBT, BBTOH/BBT$(OAc)_2$, BBTOAc/$AcOCH_2$BBT. Three of these thiophenes, *i.e.* BBT, BBTOH, and BBTOAc, are part of the same branch in the biosynthetic pathway. The sequence of formation and the differences in specific activities of these three compounds indicated that BBT is converted into BBTOH, which is subsequently converted into BBTOAc

The preceding results indicate that a BBTOAc:acetate esterase, as found by Sütfeld and Towers (1982), is not active in *Tagetes* hairy roots. However, a protein extract of the growing roots converted BBTOAc into BBTOH. In addition, a protein extract from *Rubia tinctorum* hairy roots (Rubiaceae) showed the same esterase activity whereas commercially available porcine liver esterase too was able to catalyze this reaction. These experiments showed that BBTOAc can be a substrate for unspecific esterases. In spite of the presence of esterases in the root cultures, of which high activity could be shown *in vitro*, the rate of conversion of BBTOAc into BBTOH was very low *in vivo*. Similar results were obtained with the other acetyl ester, $AcOCH_2BBT$.

Discussion

The only monothiophene in *Tagetes*, BPT, is the precursor of all bithienyls in this species. This implies that the formation of the first thiophene ring in bithienyl biosynthesis is strictly regulated with respect to its position. The formation of the second ring occurs in two ways. First, addition of a thiol compound to the diyne moiety of BPT results in the formation of MeBBT. This reaction scheme is the same as for the formation of the first ring. Therefore both processes may be catalyzed by the same enzyme. In the alternative pathway, that leads to BBT, the methyl group of the monothiophene is removed prior to the closure of the second thiophene ring. The oxidative removal of a methyl group directly linked to a triple bond is a reaction described for only a few species of the subtribe Coreopsidinae (Heliantheae) (Christensen & Lam, 1991). Demethylation of BPT would lead to the C_{12} compound 2-(but-en-1-ynyl)-5-(but-1,3-diynyl)-thiophene, which has been found to accumulate in *Eclipta* species (Heliantheae) (Christensen & Lam, 1990). If the enzyme that catalyses the formation of the thiophene ring in *Eclipta* is able to form a second ring

after demethylation, then BBT would be expected as the main accumulation product in this species However, the occurrence of BBT in *Eclipta* has not been described Therefore, it is unlikely that the enzyme involved in the formation of the second ring of BBT is the same as in the other thiophene ring formations

The hypothesis that BBT originates from MeBBT after oxidative decarboxylation (Bohlmann & Kleine, 1963, Bohlmann *et al* , 1965, Bohlmann & Berger, 1965, Bohlmann *et al* , 1966, Bohlmann *et al* , 1973, Christensen & Lam, 1990) proved untrue MeBBT is an intermediate in the formation of $AcOCH_2BBT$ in *Tagetes* We propose a reaction scheme involving first oxidation of the methyl group to form $HOCH_2BBT$, then acetylation of the alcohol MeBBT is detected in wild-type *Tagetes* only in small amounts This indicates that the oxidation step occurs fast $HOCH_2BBT$ is present in wild-type roots, again in very small amounts compared to its derivative $AcOCH_2BBT$

Fig. 3.5 The biogenetic relations of the thiophenes in Tagetes

The precursor for BBTOH and BBTOAc is BBT We found no evidence for the conversion of MeBBT into BBTOH and BBTOAc as proposed by Jente *et al* (1981) This incongruity can be explained by a reinterpretation of their original results In the column and TLC steps used (Jente *et al* , 1981) the alcohols BBTOH and $HOCH_2BBT$ are not separated since their retardation factors differ too little in these separation systems The same is true for the corresponding acetate esters However, with reversed-phase HPLC the four compounds are easily separated Since the introduction of this technique, several authors have described a major absorption peak attributable to $AcOCH_2BBT$ (Downum & Towers, 1983, Pensl & Sutfeld, 1985, Sutfeld, 1987, Ketel, 1986) Though this compound was already known from *Flaveria* (Bohlmann & Kleine, 1963) and *Buphthalmum* (Bohlmann *et al* , 1966), its presence has only recently been reported for *Tagetes* (Caniato *et al* , 1990)

It is unlikely that BBTOH and BBTOAc are biosynthesized separately as was suggested by Jente *et al* (1988) Two lines of evidence support this conclusion After feeding radiolabelled sulphate, the label is incorporated into BBTOH first and then into BBTOAc, which results in a higher specific activity of BBTOH Moreover, when ^{35}S-labelled BBTOH is fed to roots, it is almost completely converted into [^{35}S]BBTOAc within 40 h The conclusion that BBTOH is the direct precursor of BBTOAc is in agreement with results of Metschulat & Sutfeld (1987) who showed the activity of a specific acetyl transferase catalyzing this conversion

The bithienyl acetate esters BBTOAc, $BBT(OAc)_2$ and $AcOCH_2BBT$ are not converted *in vivo* (Tab 3 1) and thus are metabolically inactive end products of the bithienyl biosynthetic pathway It has been stated that radio-tracer experiments cannot give insight to the actual reaction mechanisms and that the elucidation of biosynthetic pathways is only possible by structure determination of the enzymatically formed intermediates and by characterization of the single enzymes or enzyme systems involved in these reactions (Zenk, 1980) This is only partly true, since by studying bioconversions only *in vitro*, it can not be discerned whether a particular enzyme is involved in a biosynthetic or in a degradative pathway

Although protein-extracts of *Tagetes* roots contain esterases that degrade bithienyl acetate esters (Sütfeld & Towers, 1982; Pensl & Sütfeld, 1985), this reaction does not take place *in vivo*. Therefore we conclude that these esterases are not involved in bithienyl biosynthesis.

The bithienyl acetate esters are probably stored in a certain cell compartment (Sütfeld, 1988). Disruption of the compartmentalization may lead to degradation by esterases, thus forming the corresponding alcohols. Compartmentalization of thiophenes may also explain why BBT, which is only an intermediate in a biosynthetic pathway, is the main accumulation product in *Tagetes* roots. After [^{35}S]BBT feeding, the distribution pattern of radiolabelled thiophenes differed from the distribution pattern of accumulated thiophenes. Relatively more BBT was converted into BBTOAc than would be expected on the basis the ratio of the BBT over the BBTOAc content. This indicates that two BBT-pools exist which are located in different cell compartments. One is metabolically active and rapidly converted, the other metabolically stable and remaining unaffected.

The elucidation of bithienyl biosynthesis is a prerequisite to study its regulation. The biosynthetic pathway leading to the bithienyls and to α-terthienyl is highly branched. At every branching point enzymes may be active which regulate the flow of precursors, which will directly affect amounts of end products formed. Thus, different thiophene spectra in closely related species may be attributed to changes in enzyme activities. In order to identify enzymes that catalyze individual steps in bithienyl biosynthesis, bioassays should be developed which carry out these conversions in an *in vitro* cell-free system. Identification of the enzymes and isolation of the corresponding genes will render powerful tools to study the fundamental principles underlying regulation of chemical differentiation in higher plants.

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Chapter 4

Cellular distribution of thiophenes in *Tagetes patula* L.

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Thiophenes are sulphur-containing, fatty acid-derived secondary metabolites that accumulate in the roots of *Tagetes patula*. Since plastids play a major role in fatty acid synthesis and are the location of assimilatory sulphate reduction in roots, it was assumed that these organelles are also the site of thiophene biosynthesis. Cell fractionation experiments were performed to substantiate this assumption. Our results indicate that thiophenes accumulate in the plastid membranes.

Introduction

To fully understand how a plant metabolic pathway is regulated, knowledge of the various bioconversions and the enzymes catalyzing these conversions is not sufficient. This information needs to be accompanied by information how metabolites are shuttled from one cellular compartment to another during the operation of the pathway. Spatial organization is one of the most important regulatory features in secondary metabolism (Luckner, 1980).

The principle site of biosynthetic activity in plants is the plastid. Plastids are found in all plant cells, although their mature morphology is very variable and they may serve a variety of functions. It is clear that plastids represent the major, perhaps exclusive, site of *de novo* fatty acid synthesis (Harwood, 1988; Kader & Laurent, 1990). Chloroplasts isolated from *Chrysanthemum flosculosum* L. (Bohlmann & Schultz, 1968) and from *Carthamus tinctorius* L. (Ichihara & Noda, 1977), have been shown to convert tritium-labeled oleic acid into a polyacetylene. Hence, dehydrating enzymes, necessary for the triple bond formation, are chloroplast-bound.

The formation of completely reduced sulphur compounds from inorganic sulphate, a characteristic that is unique for green plants and many microorganisms, is also localized in the plastids (Brunold & Suter, 1989). However, plant mitochondria should be investigated as well (Schmidt & Jäger,

1992) since the enzymes of the sulphate activation sequence have been detected in algal mitochondria (Brunold & Schiff, 1976; Saidha *et al.*, 1985).

Thiophenes are heterocyclic sulphurous secondary compounds which have attracted considerable attention due to their broad spectrum of biological activities (Chitwood, 1993; Hudson *et al.*, 1993; Wells *et al.*, 1993). These compounds mainly accumulate in the roots of several species of the Asteraceae. The biosynthesis of these compounds is not known in detail, but it is generally assumed that the thiophene ring is formed by addition of a reduced sulphur compound to the triple bonds of a polyacetylene (Bohlmann & Zdero, 1985). The plastids are the site where thiophene precursors are formed. Therefore, plastids may be the subcellular location of thiophene biosynthesis.

The major thiophene that accumulates in roots of *Tagetes patula* is BBT. This compound is probably stored in a subcellular compartment. Upon elicitation BBT is rapidly converted into a polar derivative, $BBT(OH)_2$, and secreted (Chapter 5). The enzymes that catalyze the conversion of BBT, as well as BBT itself, may be localized in the plasmamembrane, since this membrane is the receptor for environmental signals and may be involved in certain host-pathogen interactions.

The considerations about thiophene localization given above are highly speculative. In a first attempt determine the compartmentalization involved in thiophene metabolism, cellular distribution of thiophenes in roots of *Tagetes patula* was examined.

Materials and methods

Root cultures

Experiments were performed with root line Tp9402, which was obtained by transformation of seedlings of *Tagetes patula* L. cv. "Nana" with *Agrobacterium rhizogenes* LBA9402 (pRi1855) Transformation of the roots was verified by Southern blotting using the EcoRI-15 fragment of the Ri-plasmid pRi 1855 as a probe (Birot *et al.*, 1987).

Growth of isolated roots

Root cultures were maintained on hormone-free Gamborg's B5 medium (Gamborg, 1970) supplemented with 3% (w/v) sucrose and 100 $\mu g\ l^{-1}$ biotin The roots were grown in the dark at 25°C on a rotary shaker at 100 rpm in 50-ml Erlenmeyer flasks containing 20 ml medium

Analysis of thiophenes

Thiophenes were extracted and analyzed as described previously (Croes *et al* , 1989) Briefly, thiophe nes were extracted from the roots in ethanol water (1 1, v/v) and purified by partitioning between this mixture and hexane *tert*-butylmethylether (1 1, v/v) Thiophenes were recovered from the hexane-ether fraction, subjected to HPLC on a reversed phase C_{18} column (25 x 0 4 cm, particle size 7 μm), with acetonitrile water (72 28, v/v) at a flow rate of 1 5 ml min^{-1}, and quantified in the eluate on basis of UV absorption at 340 nm

Isolation of plasmamembranes

Plasmamembranes were isolated according to Larsson (1985) Hairy roots (150 g) were frozen with liquid nitrogen, mixed with a spoonfull of quartzsand, and subsequently homogenized with a mortar and pestle The slurry was mixed with 275 ml of 0 5 M sucrose, 50 mM HEPES/KOH, pH 7 5, 5 mM ascorbic acid, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulphonyl fluoride (PMSF), and 0 6% (w/v) polyvinylpolypyrrolidone The homogenate was filtered through four layers of 240 μm nylon cloth Centrifugation of the homogenate, at 10,000 *g* for 15 min, yielded a 10,000 *g*-pellet and a supernatant A microsomal pellet was obtained from the supernatant by centrifugation at 45,000 *g* for 45 min This 45,000 *g* pellet was suspended in a total volume of 10 ml in 0 33 M sucrose, 3 mM KCl, 5 mM potassium phosphate, pH 7 8, and the suspension was collected in the tube of a Potter-Elvehjem homogenizer and homogenized by gently stirring the pestle Then 9 0 g of the homogenate was added to 27 0 g of a previously prepared phase mixture to give a 36 0 g phase system with a final composition of 6 2% (w/w) Dextran T500, 6 2% polyethylene glycol 3350, 0 33 M sucrose, 3 mM KCl, 5 mM potassium phosphate, pH 7 8 The phase system is mixed by 20-30 gentle inversions of the tube and phase settling accelerated by centrifugation in a swinging bucket centrifuge at 1500 *g* for 3 min The upper phase was collected a repartitioned twice with fresh lower phase The final upper phase was diluted to a total volume of 210 ml with 0 33 M sucrose, 5 mM MES/Tris, pH 6 5 The plasmamembranes were collected by centrifugation at 100,000 *g* for 90 min

Isolation of organelles

All procedures were carried out at 4°C. Roots were homogenized in one volume of 50 mM tricine/KOH pH 7.9, 0.33 M sorbitol, 1 mM EDTA, 1 mM $MgCl_2$, 0.1% (w/v) BSA, 0.5% (w/v) PVPP, (buffer I) by rapid slicing with a razor blade followed by gentle maceration in a mortar and pestle. The slurry was filtered through four layers of nylon tissue (pore size, 240 μm) and centrifuged at 200 *g* for 3 min. to remove intact cells, nuclei and cell debris. The resulting supernatant was underlaid with 0 5 ml buffer I and 50% Percoll. Centrifugation was carried out in a swing-out rotor at 4000 *g* for 20 min. The resulting pellet was gently resuspended in 5 ml 50 mM tricine/KOH pH 7.9, 0.33 M sorbitol, 0 1% (w/v) BSA, (buffer II). The suspension was underlaid with a discontinuous Percoll gradient consisting of 5 ml 5%, 5 ml 10%, and 5 ml 25% Percoll in buffer II (v/v). After centrifugation in a swing-out rotor at 4000 *g* for 30 min. The Percoll gradient was divided into 1-ml aliquots which were analyzed separately

Protein determination

Protein content in the homogenates and extracts was determined with the method of Bradford (1976) Commercially available dye (Biorad) was used and the test was performed according to the manufacturer's instructions.

Marker enzyme assays

K^+-stimulated, Mg^{2+}-dependent, vanadate sensitive ATPase The K^+-stimulated increment in ATPase activity in combination with vanadate sensitivity were used as markers for plasma membranes (Hodges & Leonard, 1974; Gallagher & Leonard, 1982) The assay was carried out in a 50 μl reaction volume containing 1 mM Tris-ATP, 1 mM $MgSO_4$, 40 mM MES/Tris buffer pH 5.9, 250 mM sucrose, 0.01% (w/v) Triton X-100, 50 mM KCl (when present), 75 mM Na_3VO_4 (when present), and 1 μg of membrane protein After incubation for 20 min. at 30°C, the reaction was stopped by addition of 50 μl icecold 10% (w/v) TCA. The proteins were allowed to precipitate for 10 min. at 0°C After centrifugation at 10,000 *g* for 10 min., the inorganic phosphate content of the supernatant was determined according to Fiske and Subbarow (1925).

Glutamate synthase (EC 2.6 1.53) This marker was assayed according to Emes and Fowler (1979). Cuvettes contained 100 μmol tricine buffer pH 7 5, 5 μmol α-ketoglutarate, 2 μmol L-glutamine, 83 nmol NADH and extract in a total volume of 1 ml. The decrease in OD_{340} resulting from oxidation of

NADH was taken as a measure for enzyme activity

Nitrite reductase (EC 1 7 7 1) The reaction was carried out aerobically in a 10-ml test tube, according to Losada and Paneque (1971) In this tube 1 ml test sample was mixed with ml 300 μl 0 5 M Tris HCl pH 8 0, 200 μl 20 mM sodium nitrite, 200 μl methyl viologen, and 300 μl of a freshly made solution of 2 5% (w/v) sodium dithionite in 0 29 M $NaHCO_3$ After incubation for 10 min at 30°C, the reaction is stopped by vigorous shaking on a vortex mixer untill the dithionite is completely oxydized and the dye becomes colorless Then the nitrite content is determined a 1 ml of 1% (w/v) sulphanilamide in 3 M HCl and 1 ml *N*-(1 naphtyl)ethylenediamine is added to a 2-ml aliquot of a 100-fold dilution of the reaction mixture, and the volume is made up to 5 ml with water After 10 min the OD_{540} is determined, and the nitrite content is calculated from a standard curve

Shikimate oxidoreductase (EC 1 1 1 25) The increase in OD_{340}, as a result of NADPH formation during the oxidation of shikimate to 3-dehydroxyshikimate, was determined according to Fiedler and Schultz (1985) In a cuvet were mixed 500 μl 200 mM glycine KOH pH 9 5, 50 μl 8 mM NADP, and 400 μl test sample After preincubation for 7 min at ambient temperature, the reaction was started by addition of 50 μl 24 mM shikimate The increase on OD_{340} was followed for 6 min

Cytochrome c oxidase (EC 1 9 3 1) The oxidation of reduced cytochrome c was determined by following the decrease in OD_{550} Cytochrome c was reduced by addition of a small quantity sodium dithionite The solution was desalted over a Dowex column The reaction was started by addition 50 μl organelle fraction to 2 95 ml 0 1 M potassium phosphate buffer of pH 7 2, 0 05 mM reduced cytochrome c (initial OD_{550} = 0 8)

Cytochrome c reductase A mitochondrial fraction was split in two Reductase activity of one fraction was determined in an isotonic solution, 10 mM potassium phospate buffer pH 6 8, 0 7 M mannitol, 0 1% (w/v) BSA, 1 mM KCN, 5 mM NADH, 0 05 mM oxidized cytochrome c (initial OD_{550} = 0 1) The activity of the other fraction was determined after osmolysis of the mitochondria in a hypotonic solution, 5 mM potassium phosphate buffer pH 7 2, 1 mM KCN, 5 mM NADH, 0 05 mM oxydized cytochrome c (initial OD_{550} = 0 1) The ratio in reductase activities of the two fractions was taken as a measure of mitochondrium intactness

Results

In a first attempt to localize thiophene accumulation in *Tagetes* cells, a root homogenate was fractionated by differential centrifugation The thiophene content of the different fractions was determined The protein content of the fractions was taken as a measure of the amount of plant cell material. Thiophene content of the

Table 4.1 Difference in thiophene distribution between subcellular fractions of *Tagetes patula* root tissue

Fraction	Organelle	Thiophene content (mmol g protein^{1})
10 000 *g* pellet	plastids/mitochondria	8 0
50,000 *g* supernatant	cytoplasm	0 01
50,000 *g* pellet		
PEG fraction	plasmamembrane	0 02
Dextran fraction	intracellular membranes	1 8

fractions is expressed as nmol thiophene μg protein^{1}

The 10,000 x *g* pellet, which contains the bulk of plastids and mitochondria (Larsson 1985), was the main site of thiophene accumulation (Tab 4 1) The 50,000 x *g* supernatant, the cytoplasmatic fraction, was almost devoid of thiophenes The 50,000 x *g* pellet was partitioned over a two-phase system consisting of polyethylene glycol 3350 and dextran T500 (Larsson, 1985) Plasmamembranes accumulate in the polyethelene glycol phase The presence of plasmamembranes in this fraction was confirmed by the activity of K^{+}-stimulated, Mg^{2+}-dependent, vanadate sensitive ATPase (results not shown) The fraction contained very little thiophene In contrast, the thiophene content in the dextran fraction, which mainly contains intracellular membranes, was a hundred times higher This was taken as evidence that thiophenes accumulate in the intracelluar membranes

Subcellular organelles of root tissue homogenate were separated on a discontinuous percoll gradient After centrifugation, the gradient was divided into 1-ml fractions The thiophene content and marker enzyme activity of the different fractions was determined Since BSA was used in the homogenation buffer, a routine determination of protein was impossible and thiophene content in each fraction was expressed as nmol ml^{1}

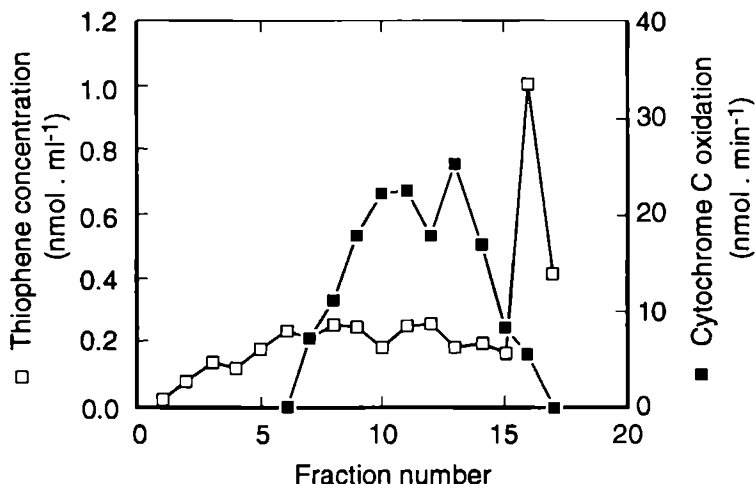

Fig. 4.1 Distribution of thiophenes and cytochrome c oxidase activity afer cell fractionation on a discontiuous Percoll gradient

Maximal activity of the mitochondria marker enzyme cytochrome C oxidase was found at the interphase of 10% and 25% percoll (Fig. 4.1, fraction 13), whereas the highest thiophene content was found in the pellet (Fig. 4.1, fraction 17). Hence, the mitochondrial fraction did not coincide with the presence of thiophenes. The intactness of the mitochondria preparation was determined by latency of cytochrome C reductase activity. Activities in isotonic buffer and in hypotonic buffer were equally high, therefore it was concluded that the isolated mitochondria were broken.

The plastids now remained as the most likely subcellular organelle in which thiophenes accumulate. To finally prove this assumption, three different marker enzymes were used to identify the plastid-containing fractions, *viz.* glutamate synthase, nitrite reductase, and shikimate oxidoreductase. However, no clear enzyme activity could be detected in any of the fractions.

Discussion

Our results lead to the preliminary conclusion that thiophenes in *T. patula* hairy roots accumulate in the plastids, adsorbed at the plastid membrane. The plasmamembrane and the mitochondrial membranes are ruled out as possible sites for thiophene accumulation.

After differential centrifugation, the particulate fraction was greatly enriched in thiophenes. This indicated that thiophenes are not located in the cytosol. Partitioning of the microsomal fraction in an aqueous dextran-polyethylene glycol two-phase system, yielded a plasmamembrane fraction with a purity higher than 90% (Larsson, 1985). This fraction hardly contained any thiophene. So, it is concluded that thiophenes do not accumulate in the plasmalemma.

The fraction containing the mitochondrial marker enzyme cytochrome c oxidase did not coincide with the fraction containing the highest amount of thiophenes after density gradient centrifugation. The mitochondrial fraction was able to reduce cytochrome c in an isotonic buffer solution. When mitochondria are intact, the cytochrome is unable to reach the reducing enzymes which are situated on the inner mitochondrial membrane. Therefore, it can be concluded that the mitochondria were ruptured during isolation.

Whereas the mitochondrial marker enzymes used are membrane bound, the plastid markers nitrite reductase, glutamate synthase, and shikimate oxidoreductase are all localized in plastids and not adsorbed at the plastid membrane (Losada & Paneque, 1971; Emes & Fowler, 1979; Leuschner & Schultz, 1991). When the plastids are ruptured during isolation, like the mitochondria were, marker enzyme activity would be found in the non-particulate, soluble fraction. This explains why no plastid markers are found in the percoll gradient. The plastids were broken during extraction and only the plastid membranes, containing the accumulated thiophenes, were recovered after density gradient centrifugation.

Whether root plastids are capable of synthesizing thiophenes cannot yet be

decided This has to await the isolation of intact plastids in which thiophene biosynthesis can be monitored by following the conversion of radio-labelled precursors

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Chapter 5

Thiophene interconversions in elicitor-treated roots of *Tagetes patula* L.

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Thiophenes are well known for their phototoxicity. The compounds, however, mainly accumulate in the root where photo-activation is not likely to occur. Therefore, the question was addressed whether thiophenes could play a role in defence against micro-organisms in the dark.

A heat-stable, cell-free extract from the vascular wilt fungus *Fusarium oxysporum* induced biosynthesis of relatively hydrophilic thiophenes in root cultures of *Tagetes patula*. The thiophenes formed were partially excreted into the culture medium. Similar results were obtained with *T. patula* seedlings. The excreted thiophenes proved to inhibit fungal growth in the absence of light and thus may play a role in the biochemical defence against soil-borne pathogens.

Introduction

Secondary plant products are by definition 'substances which are incidental or not absolutely necessary for life' (Kossel, 1891). Although secondary products are often physiologically nonessential compounds, they may play a significant ecological role (Harborne, 1977). This is particularly true for the phytoalexins, low molecular weight, antimicrobial compounds which are synthesized by and accumulated in plants after exposure to micro-organisms (Müller & Börger, 1940; Paxton, 1981). Phytoalexin synthesis is induced by elicitors which can be complex carbohydrates from fungal or plant cell walls, lipids, microbial enzymes, or polypeptides (Ebel, 1986). The accumulation of a specific phytoalexin is an example of a metabolic shift, resulting from a change in the activities of the enzymes controlling the biosynthesis of secondary products (Luckner & Diettrich, 1990).

Investigations on elicitor-induced secondary metabolite formation in plants have been performed for the main part with callus or suspension cultures derived from phytoalexin-forming plant species. The concept of phytoalexin induction,

however, is a phytopathological one and thus inseparably linked to the differentiated plant (Eilert, 1989). The response to elicitation is often tissue-specific, as is exemplified by potato cultivars which, upon infection, accumulate sesquiterpenoid phytoalexins in tubers but not in leaves (Rohwer *et al.*, 1987).

A particularly well-suited system to study the role of phytoalexins in plant-pathogen interactions are hairy root cultures, obtained after infection of dicotyledonous plants with *Agrobacterium rhizogenes*. These root cultures provide all the conveniences of *in vitro* cell cultures, but at the same time show all the characteristics of roots attached to plants.

Members of the genus *Tagetes* accumulate a relatively broad spectrum of biochemically related thiophenes. These are heterocyclic sulphur-containing secondary metabolites which are characteristic for members of the Asteraceae (Bohlmann *et al.*, 1985). The main site of thiophene accumulation in *Tagetes* is the root. In *T. patula* roots the most abundant thiophene is 5-(3-buten-1-ynyl)-2,2'-bithienyl (BBT) (Uhlenbroek & Bijloo, 1959). Part of the BBT is converted (Jente *et al.*, 1981; Jente *et al.*, 1988) into 5-(3,4-dihydroxy-1-butynyl)-2,2'-bithienyl ($BBT(OH)_2$) (Pensl & Sütfeld, 1984) or 5-(4-hydroxy-1-butynyl)-2,2'-bithienyl (BBTOH) (Atkinson *et al.*, 1965). These two derivatives do not appear in large amounts and are thought to represent short-lived intermediates in bithienyl metabolism (Pensl & Sütfeld, 1984). This supposition is substantiated by the rapid conversion (Chapter 3 of this thesis) of $BBT(OH)_2$ and BBTOH into their corresponding acetyl-esters 5-(3,4-diacetoxy-1-butynyl)-2,2'-bithienyl ($BBT(OAc)_2$) (Pensl & Sütfeld, 1984) and 5-(4-acetoxy-1-butynyl)-2,2'-bithienyl (BBTOAc) (Bohlmann & Herbst, 1962; Bohlmann *et al.*, 1964). Other bithienyls that occur in *Tagetes* roots are 2-(but-3-en-1-ynyl)-5-(penta-1,3-diynyl)-thiophene (BPT), 2,2':5',2"-terthienyl (α-T) (Uhlenbroek & Bijloo, 1958), 5'-methyl-5-(3-buten-1-ynyl)-2,2'-bithienyl (MeBBT) (Groneman *et al.*, 1984) and its derivatives (5'-but-3-en-1-ynyl-[2,2']bithiophenyl-5-yl)-methanol ($HOCH_2BBT$) and (5'-but-3-en-1-ynyl-[2,2']bithiophenyl-5-yl)-methyl acetate ($AcOCH_2BBT$) (Chapter 3 of this thesis).

The compounds are known as powerful photosensitizers (Bakker *et al* , 1979) When the role of thiophenes in plant defense is discussed, the phototoxic activity after exposure to sunlight or artificial sources of UV-light is emphasized However, the role of light in a host-pathogen interaction that occurs in the rhizosphere must be negligible Because of their toxicity at low dosages to plant pests such as fungi, insects (Wells *et al* , 1993) and nematodes (Chitwood, 1993), thiophenes have been described as phytoalexins (Kourany *et al* , 1988) However, most thiophenes are always present and not synthesized by and accumulated in plants only after exposure to micro-organisms Hence, not all thiophenes fulfil the criteria used to define phytoalexins

Root cultures of *Tagetes* accumulate the same thiophenes as roots attached to plants (Croes *et al* , 1989, Mukundan & Hjortso, 1990a, Menelaou *et al* , 1991, Sáenz-Carbonell *et al* , 1993) Root cultures are amenable to elicitation upon treatment with fungal elicitors, thiophene production increased (Mukundan & Hjortso, 1990b, Buitelaar *et al* 1992)

The present investigation is intended to study the elicitation of thiophenes from a phytopathological point of view and thus to evaluate the role of thiophenes in the resistance of *T patula* to soil-borne pathogens As a model system, hairy root cultures were elicited with an extract of the vascular wilt fungus *Fusarium oxysporum* The excretion of water-soluble thiophenes is reported and the antifungal effect of thiophenes in the absence of light is established

Materials and methods

Plant material

Achenes of *Tagetes patula* L cv 'Nana' were planted in moist vermiculite and germinated in total darkness at 25°C for 2 d The seedlings were then transferred to white light (photoperiod 16 h day^{-1} 12 W m^{-2}) and cultivated further at 25°C on vermiculite in half strength Hoagland's solution

(Hoagland & Snyder, 1933)

The hairy root culture Tp9402 was obtained after transformation of seedlings of *T. patula* L. cv 'Nana' with *Agrobacterium rhizogenes* LBA9402 (pRi1855). Transformation of the roots was verified by Southern blotting using the EcoRI-15 fragment of the Ri-plasmid (Birot *et al.*, 1987) as a probe.

Root cultures were maintained on hormone-free medium consisting of Gamborg's B5 salts (Gamborg, 1970) supplemented with 3% (w/v) sucrose and 100 $\mu g\ l^{-1}$ biotin. The roots were grown in the dark at 25°C on a rotary shaker at 100 rpm in 50-ml Erlenmeyer flasks containing 20 ml medium.

Fungal culture and elicitor preparation

The fungal strain *Fusarium oxysporum* F14 was kindly supplied by the Department of Microbiology of the Agricultural University of Wageningen. The fungus was routinely maintained on 1% (w/v) soytone (Difco) solidified with 0.2% (w/v) gellan gum (Gelrite) in a 9-cm Petri dish at 25°C in the dark.

For the preparation of elicitor, a 5-mm agar-disc from a full-grown culture was used to inoculate 50 ml of a 1% (w/v) soytone solution in a 300-ml Erlenmeyer flask. After 14 days of growth in the dark at 25°C without shaking, the mycelium was separated from the medium with a sieve and washed several times with demineralized water. The mycelium residue was suspended in demineralized water and homogenized. The homogenate was filtered over a Whatman GF/C filter, and the carbohydrate content of the filtrate was determined. The filtrate was lyophilized and stored at -80°C. Before use it was dissolved in water and autoclaved (120°C, 20 min).

Elicitor treatment of root cultures and plants

In the elicitation experiments, 8-day-old root cultures were transferred to 50-ml Erlenmeyer flasks containing 20 ml B5 medium, supplemented with sucrose and biotin as described above, with an elicitor concentration of 0.1 mg glucose equivalents ml^{-1}. After 2 days incubation, in the dark, at 25°C, on a rotary shaker at 100 rpm, the roots were extracted for thiophene determination.

In some experiments 22-day-old plants were placed in Erlenmeyer flasks filled with half-strength Hoagland's solution supplemented with 0.1 mg glucose equivalents ml^{-1} of elicitor. The bottoms of the flasks were covered with aluminum foil to reduce entry of light into the root zone. After 2 days incubation in white light (photoperiod 16 h day^{-1}, 12 W m^{-2}) at 25°C, plants were extracted for thiophene determination.

Effect of thiophenes on mycelial growth

Paper discs (diameter, 5 mm) were impregnated with ethanolic solutions containing 200 nmol of either BBT, BBTOH, or BBTOAc As a control a paper disc was impregnated with pure ethanol The ethanol was allowed to evaporate and the discs were placed on top of soytone agar in a 9-cm Petri dish Then the agar was inoculated with hyphae of *F oxysporum* and placed in the dark at 25°C After an incubation period of 14 days the agar plates were visually examined

Preparation of radiolabelled thiophenes

Roots that had been grown in B5 medium with tracer amounts of $Na^{35}SO_4$ (final specific activity 0 4 TBq mol^{-1}) synthesized radiolabelled thiophenes Root line Tp9402 was used for the production of ^{35}S-labelled BBT and BBTOAc The alcohol BBTOH, which is a minor metabolite in the root, was made by saponification of BBTOAc (Bohlmann & Herbst, 1962) The amount of $BBT(OAc)_2$ in the roots was too small to allow for isolation of enough material for precursor feeding

After fractionation of the root extracts, thiophenes were recovered from the organic phase and further purified by reverse-phase HPLC Purity of the isolated compounds was confirmed by analytical HPLC

Precursor feeding

In the feeding experiments, the labelled precursors, dissolved in 100 μl ethanol, were added to 10 ml 0 1% Tween-20 in Gamborg's B5 medium supplemented with 3% (w/v) sucrose and 100 μg l^{-1} biotin The radioactive concentration of the incubation medium was 2 kBq ml^{-1} Eight-days-old root cultures were placed in these emulsions and incubated for 2 days Then roots were rinsed with unlabelled B5 medium and thiophenes were extracted as described below

The organic extract was fractionated with reverse-phase HPLC, thiophenes were monitored by UV absorption at 340 nm Fractions of 0 5 ml were collected, mixed with scintillation fluid and radioactivity was measured

Thiophene analysis

Thiophenes were analyzed as described previously (Croes *et al* , 1989) Briefly, thiophenes were extracted from the roots in ethanol water (1 1, v/v) and purified by partitioning between this mixture and hexane *t*-butylmethylether (1 1, v/v) Thiophenes were recovered from the hexane-ether fraction, and finally dissolved in ethanol

HPLC analysis was performed on a Lichrosorb RP-18 column (particle size 7 μm) Column dimensions were 25 x 0 4 cm, the eluent was acetonitrile water (72 28, v/v) The flow rate was 1 5 ml min^{-1}, thiophenes were detected by their UV-absorption at 340 nm

Absorption spectra from 210 nm to 600 nm were recorded in ethanol

Determination of the carbohydrate content

The carbohydrates were quantified by the dinitrosalicylic acid reagent method, modified by Miller (Miller, 1959) The reagent consisted of 1% (w/v) 3,5-dinitrosalicylic acid, 0 2% (w/v) phenol and 0 05% (w/v) Na_2SO_3 in 1% (w/v) NaOH The colour tests were made with 400 μl aliquots of reagent added to 400-μl aliquots of test samples The mixtures were heated for 15 min in a boiling water bath Immediately thereafter, 130 μl 40% (w/v) Rochelle salt ($KNaC_4H_4O_6$ $2H_2O$) was added, to stabilize the colour The mixture was cooled and the absorption was read at 575 nm A calibration curve made with glucose standards was used as a reference

Statistical treatment

All data are the result of at least three independent determinations and are presented as means ± SE

Results

Effect of thiophenes on mycelial growth

The first experiment was designed to test the antifungal activity of thiophenes present in *Tagetes* roots Paper discs, impregnated with either BBT, BBTOH or

BBTOAc, were placed on agar medium in a Petri dish. Then the medium was inoculated with hyphae of *F. oxysporum* and placed in the dark. After one week, mycelium had formed covering the agar (Fig. 5.1). Around the BBTOH-impregnated discs, a mycelium-free zone was observed. The BBTOAc-impregnated paper discs were also free of mycelium, indicating a growth-inhibiting effect. The BBT-discs did not inhibit fungal growth and were fully covered with mycelium.

Other thiophenes that naturally occur in *Tagetes* were not tested, because of the limited availability of purified samples of these compounds.

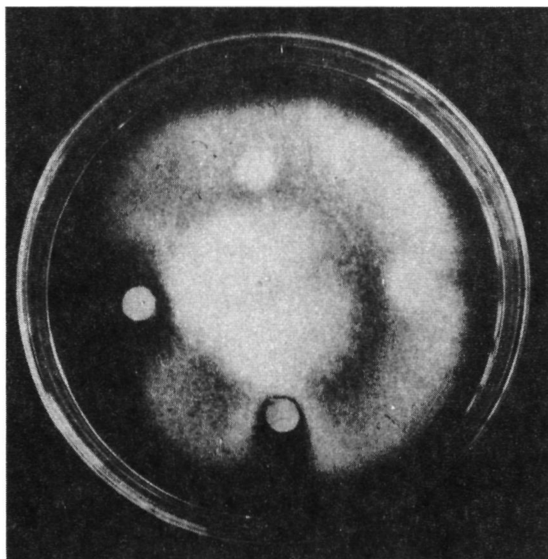

Fig. 5.1 Effect of different thiophenes on mycelium growth of *F. oxysporum*. Paper discs were impregnated with ethanolic solutions of BBTOH (left), BBT (right), BBTOAc (bottom), or ethanol only (top). Discs were placed on agar and, after evaporation of the ethanol, the agar plate was inoculated with fungal hyphae. After incubation for 14 d at 25°C in the dark, growth of the fungus was evaluated.

Effect of a fungal elicitor on thiophene accumulation.

If the accumulation of thiophenes is an active defense mechanism, then exposure of roots to a fungal elicitor is expected to trigger the synthesis of the biologically most active thiophenes. Treatment of *T. patula* roots with the elicitor did not lead to a significant increase in the total amount of thiophenes (Tab. 5.1). However, the concentration of some thiophenes, *viz.* BBTOH, $BBT(OAc)_2$ and BBTOAc,

increased after elicitor treatment Furthermore, extracts of elicitor-treated roots contained a compound, which had an HPLC-retention time shorter than that of BBTOH This compound was identified as $BBT(OH)_2$ by comparison with a genuine sample of this diol, obtained by hydrolysis of $BBT(OAc)_2$ The HPLC retention times of the two compounds and their UV-spectra were identical

The optimal elicitor concentration was 0 1 mg carbohydrate ml^{-1} medium and the optimal incubation time of the elicitor was 2 days

Table 5.1 Thiophene contents of hairy roots of *T patula* before and after elicitation with a cell wall extract of *F oxysporum*

Compound	Concentration in control root (nmol g FW^{-1})	Concentration in elicitor treated root (nmol g FW^{-1})
BBTOH	3 ± 1	15 ± 3
$HOCH_2BBT$	5 ± 2	7 ± 1
$BBT(OAc)_2$	37 ± 10	74 ± 2
BBTOAc	77 ± 12	136 ± 8
$AcOCH_2BBT$	102 ± 28	103 ± 8
BBT	919 ± 242	779 ± 140
α T	72 ± 22	75 ± 5
Total thiophene	1215 ± 314	1188 ± 167

Effect of elicitor treatment on thiophene biosynthesis

All thiophenes whose levels increased upon elicitation, are derivatives of BBT, as had previously been shown by radiotracer studies (Jente *et al* 1981, 1988, Chapter 3 of this thesis) Thus, the accumulation of these compounds may result from an increased conversion rate of their common precursor

Trace amounts of [^{35}S]BBT were fed to roots and the rate of conversion was

determined In the control roots, 20% of the radiolabelled tracer was converted into BBTOH and BBTOAc, an equal amount was converted into $BBT(OH)_2$ and $BBT(OAc)_2$. A small portion (0.4%) of the activity was present in extracellular $BBT(OH)_2$ (Tab. 5.2). In the elicitor-treated root, 73% of the labelled BBT was converted into $BBT(OH)_2$ and $BBT(OAc)_2$ whereas 8% was converted into BBTOH and BBTOAc. Analysis of the culture medium revealed that, after elicitation, 26% of the radioactivity was present in extracellular $BBT(OH)_2$ (Tab. 5 2).

Table 5.2 Distribution of radioactivity over thiophenes after $[^{35}S]$BBT feeding of root cultures

Compound	Radioactivity present in roots (%)	Radioactivity present in culture medium (%)
Control roots		
BBT	61 2 ± 3 0	0 0 ± 0 0
$BBT(OH)_2$	0 2 ± 0 1	0 4 ± 0 1
BBTOH	0 4 ± 0 0	0 1 ± 0 0
$BBT(OAc)_2$	16 4 ± 0 1	0 0 ± 0 0
BBTOAc	19.7 ± 0 1	0 0 ± 0 0
Unidentified	1 6 ± 0 3	0 0 ± 0 0
Elicitor-treated roots		
BBT	5 8 ± 1 9	3 7 ± 1 5
$BBT(OH)_2$	3 6 ± 0 7	25 7 ± 6 4
BBT(OH)	2 5 ± 0 3	1 7 ± 0 9
$BBT(OAc)_2$	41 6 ± 8 3	0 0 ± 0 0
BBT(OAc)	3 5 ± 0 7	0 0 ± 0 0
Unidentified	11 9 ± 2 7	0 0 ± 0 0

The increase in the amount of hydroxy bithienyls is relatively higher than that of the acetate esters. Since BBTOH and $BBT(OH)_2$ are the direct precursors of the acetate esters BBTOAc and $BBT(OAc)_2$, the extent of the conversion of the alcohols can be determined from the ratios BBTOH:BBTOAc and $BBT(OH)_2$:$BBT(OAc)_2$. Thus it can be deduced that, in the control roots, over 95% of the alcohols are converted into the corresponding acetoxy bithienyls. After elicitation, the conversion of the alcohols had decreased to 45% for BBTOH and 58% for $BBT(OH)_2$.

The relatively high increase of the hydroxy bithienyls after elicitation could be the result of either a lower rate of esterification or a higher rate of the reverse reaction, the hydrolysis of acetoxy bithienyls. Feeding experiments in which trace amounts of [^{35}S]BBTOH were fed to root cultures confirmed that, in the absence of elicitor, over 95% of this bithienyl is converted into BBTOAc. In the presence of elicitor the conversion decreased to 77% (Tab. 5.3). When [^{35}S]BBTOAc was fed, in the control culture 2% was converted into BBTOH. After elicitation a small but significant increase in the rate of this conversion was measured (Tab. 5.4).

Table 5.3 Distribution of radioactivity over thiophenes after [^{35}S]BBTOH feeding of root cultures of *T. patula*

Compound	Radioactivity present in control roots (%)	Radioactivity present in elicitor treated roots (%)
BBTOH	3.9 ± 0.4	22.8 ± 10.1
BBTOAc	96.1 ± 0.4	77.2 ± 10.1
Unidentified	0.0 ± 0.0	0.0 ± 0.0

Table 5.4 Distribution of radioactivity over thiophenes after [^{35}S]BBTOAc feeding of root cultures of *T patula*

Compound	Radioactivity present in control roots (%)	Radioactivity present in elicitor-treated roots (%)
BBTOH	1 9 ± 1 2	8 2 ± 4 5
BBTOAc	91 3 ± 3 2	81 0 ± 10 4
Unidentified	6.8 ± 3 8	10 8 ± 9 3

The preceding experiments showed that, in root cultures, elicitation led to an accumulation of the hydroxy bithienyls. These compounds were partly excreted into the growth medium (Tab 5.2). In plants, the thiophenes that are excreted from the cells upon elicitation could have a twofold function They could either be transported through the vascular system, and thus provide systemic resistance, or be secreted into the rhizosphere, and there create an environment unfavourable for pathogens.

To find out whether the results obtained after elicitation of root cultures also apply to intact plants, radiolabelled BBT was fed to *T. patula* plants, hydroponically cultured either in the presence or absence of elicitor. The conversions of the labelled compound and the distribution of label in the plants were determined. Again, elicitation induced an increase in the formation of BBTOH and $BBT(OH)_2$ in the roots (Tab. 5.5). No significant changes were detected in other plant parts after elicitation. Excretion of $BBT(OH)_2$ from the roots led to a threefold increase of this thiophene in the culture medium.

Table 5.5 Distribution of radioactivity over thiophenes after $[^{35}S]$BBT feeding, and localization of the labelled thiophenes over 3-week-old seedlings of *T patula*

Control plants

		Localization		
Compound	Medium	Roots	Hypocotyl	Leaves
BBT	0 0 ± 0 0	59 6 ± 4 0	4 3 ± 1 9	0 1 ± 0 0
$BBT(OH)_2$	1 0 ± 0 0	0 5 ± 0 0	0 1 ± 0 1	0 0 ± 0 0
BBTOH	0 5 ± 0 1	3 4 ± 0 5	0 5 ± 0 4	0 0 ± 0 0
$BBT(OAc)_2$	0 0 ± 0 0	1 1 ± 0 1	0 1 ± 0 1	0 0 ± 0 0
BBTOAc	0 0 ± 0 0	23 8 ± 1 9	5 0 ± 2 0	0 1 ± 0 1

Elicitor-treated plants

Compound	Medium	Roots	Hypocotyl	Leaves
BBT	0 0 ± 0 0	65 8 ± 6 3	4 5 ± 2 0	0 2 ± 0 1
$BBT(OH)_2$	3 3 ± 0 9	4 2 ± 1 0	0 1 ± 0 1	0 0 ± 0 0
BBTOH	0 3 ± 0 0	4 3 ± 1 4	0 5 ± 0 1	0 0 ± 0 0
$BBT(OAc)_2$	0 0 ± 0 0	1 5 ± 0 2	0 1 ± 0 1	0 0 ± 0 0
BBTOAc	0 0 ± 0 0	10 0 ± 1 6	5 2 ± 2 4	0 1 ± 0 1

Discussion

The biochemical basis of the defence mechanism conferring disease resistance on *T. patula* was investigated. Treatment of *T. patula* hairy roots with an elicitor from the vascular wilt fungus *F. oxysporum* resulted in a metabolic shift, leading to an elevated synthesis of relatively hydrophilic hydroxy bithienyls. Part of the bithienyls formed was excreted into the culture medium. Hydroxy bithienyl proved to be an effective inhibitor of fungal growth in the absence of light.

In elicitor-treated roots the amounts of $BBT(OH)_2$, BBTOH, $BBT(OAc)_2$, and BBTOAc were significantly higher than those in the control roots. These results agree with those of Kourany *et al.* (1988). We did not detect a significant change in the levels of the other thiophenes. Root cultures of *T. patula* have been found to increase their amount of BBT upon elicitation, provided that the right conditions are chosen (Mukundan & Hjortso, 1990b; Buitelaar *et al.*, 1992). Such an arduously inducible mechanism is not likely to play a role in active defense against micro-organisms, especially since BBT is already abundantly present in non-elicited roots. Moreover, compared to BBTOH and BBTOAc, the fungicidal activity of BBT was relatively low. Therefore BBT, the main bithienyl in the roots of *T. patula*, probably does not function as a phytoalexin. More likely, the role of BBT in the roots is that of common precursor for the antimicrobial compounds that are synthesized, since the biosynthesis of the BBT-derivatives $BBT(OH)_2$, BBTOH, $BBT(OAc)_2$, and BBTOAc increases after exposure of the roots to the fungal extract.

BBT → epoxide → $BBT(OH)_2$ → $BBT(OAc)_2$; epoxide → BBTOH → BBTOAc

Fig. 5.2 Bioconversion of BBT, after Bohlmann *et al* (1973)

The derivatives of BBT are thought to be formed via an epoxidation of the vinyl group (Fig. 5.2) (Bohlmann *et al.*, 1973). Reduction of the epoxide moiety yields BBTOH whereas hydrolysis gives $BBT(OH)_2$. The latter reaction is particularly increased after elicitation. Normally, the concentration of hydroxy bithienyls in the roots is very low because they are readily acetylated. The acetoxy bithienyls are metabolically inactive, *i.e.* they are not degraded in living cells (Chapter 3 of this thesis). After elicitation the rate of acetylation apparently did not increase to the same extent as the hydroxylation of BBT (Tab. 5.2, Tab. 5.4). Elicitation led to a slight increase in degradation of the acetoxy compounds (Tab. 5.4), but this degradation alone could not explain the strong accumulation of alcohols (Tab. 5.2). Thus, the amount of hydroxy bithienyls increased relatively faster than that of the acetoxy bithienyls (Tab. 5.1) mainly because of the enormous increase in the hydroxylation of BBT and the absence of a proportional rise in acetylation. The esterases catalyzing the conversion of BBTOAc to BBTOH (Sütfeld & Towers, 1982), and of $BBT(OAc)_2$ to $BBT(OH)_2$ (Pensl & Sütfeld, 1984), and the acetyltransferases catalyzing the inverse conversions (Metschulat & Sütfeld, 1986) probably only play a minor role in the accumulation of hydroxy bithienyls.

That BBTOH and $BBT(OH)_2$ are more toxic than BBT is understandable because these more polar compounds diffuse faster through an aqueous environment than BBT. This argument is substantiated by the observation that BBTOH is biocidal to *Fusarium* at a visible distance from the place of application whereas BBT had no impact on fungal growth. Hence, it would be favourable to produce hydroxy bithienyls in response to fungal attack. After elicitor treatment of *T. patula* roots the hydroxy bithienyl, particularly $BBT(OH)_2$, were excreted into the culture medium. In plants these compounds probably exert their fungicidal activity in the rhizosphere, in the absence of light. Hence, we must conclude a mechanism of dark toxicity. Such a mechanism has been described for several polyacetylenes, compounds that are structurally closely related to thiophenes. Tests in which antifungal properties of polyacetylenes were assessed, using agar medium, showed that the activity of a polyacetylenic diol, falcarindiol,

was especially high (Kemp, 1978). This suggests that activation of a precursor molecule by hydroxylation is a more general mechanism to give rise to a potent phytoalexin. The destructive ability of falcarindiol is probably due to its hydrophobic nature. The aliphatic terminal moiety of this compound is adsorbed on the hydrophobic groups of the phospholipids in the biological membranes and at some critical concentration, the lipid bilayer is destroyed, due to the formation of mixed falcarindiol/phospholipid micelles (Garrod *et al.*, 1979). Other polyacetylenes that accumulate in membranes and disrupt membrane fluidity have been described (Arnason *et al.*, 1986).

Transformed root cultures provide an excellent model system for the investigation of plant-pathogen interactions with soil-borne pathogens. The results obtained with our root cultures were shown to be representative for the actual situation in intact plants. Moreover, cultured roots are easy to propagate in simple, synthetic media and provide a suitable system for elucidating the enzymology of root-specific biosynthetic pathways. Further elucidation of the mechanisms regulating the metabolic shifts after elicitation have to await the isolation and identification of the enzymes or genes involved in secondary metabolism.

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Summary and Conclusions

Besides their well-known function as border plants, french marigolds (*Tagetes patula* L.) have found application as a natural defence system against plant-pathogenic nematodes. This application has made *Tagetes* an object of study in agricultural research. In 1958 it was discovered that these plants form thiophenes, which are toxic at low doses to nematodes and other plant pathogens.

Thiophenes are sulphur-containing secondary metabolites that mainly accumulate in the roots of *Tagetes* species. Although the thiophenes in *Tagetes* have been the subject of several studies, the biogenetic relations of these compounds are not completely known and their biosynthesis is poorly understood. The factors that regulate the formation of thiophenes are also largely unknown. The principles that regulate the formation of thiophenes in *Tagetes* roots are the subject of this thesis.

The biosynthesis of thiophenes in unorganized suspension cultures is very low compared to plant roots. Hairy root cultures, obtained after transformation of *Tagetes patula* with *Agrobacterium rhizogenes*, form thiophenes in amounts comparable to those in roots attached to plants. Since the effects of transformation with *A. rhizogenes* are mainly the result of altered auxin-sensitivity of the plant tissue, the effects of this phytohormone on root growth and secondary product formation were studied (Chapter 1).

The biosynthesis of thiophenes was inhibited by IAA application, as was evident from a decrease of [^{35}S]sulphur incorporation. The inhibition only occurred after the roots had developed numerous laterals as a result of auxin action. However, in roots cultured in the absence of IAA, there was no significant correlation between branching and thiophene accumulation. Therefore, development of lateral roots is not a sufficient condition for a low capacity to synthesize thiophenes. This observation suggests a direct involvement of auxin with thiophene biosynthesis. However, it remains very difficult to make causal connections between auxin application and secondary metabolism. Hormones in general affect a range of developmental processes. Therefore, a clear discrimination between direct and

indirect effects of hormone application will always be problematical.

The biosynthetic routes leading to secondary metabolites start from products of primary pathways. At the points where the secondary routes branch off from primary metabolism, mechanisms must exist which regulate the amount of precursor drained away for the biosynthesis of secondary metabolites. The accumulation of thiophenes in *T. patula* was used as a model system to study the partitioning of sulphur between primary and secondary metabolism when sulphate supply was limited (Chapter 2). A reduction in the concentration from 2 mM to 50 μM did not affect elongation growth, branching and biomass production within 8 days. In contrast, a decline in biosynthetic capacity was found which amounted to 80-95%. This capacity is restored when roots are transferred from the low-sulphate medium to the standard medium. The restoration takes more than 24 h and is suppressed by the transcription inhibitor cordycepin. It is concluded that sulphur distribution is, at least for the major part, controlled at the level of transcription. Upon sulphate starvation, the pathway leading to incorporation of sulphur into thiophenes is down-regulated in favour of the primary metabolism supporting growth.

A further elucidation of the regulation of enzymatic conversions in the model system has to await identification of the enzymes involved in the formation of thiophenes from their polyacetylenic precursors. Of special interest in this respect are the enzymes involved in the formation of the thiophene ring. This formation is probably a two-step reaction (Bohlmann *et al.*, 1973), first addition of a thiol to a diyne group, then cleavage of the thus formed thioether bond and concomitant ring closure. The thioether bond may be cleaved by the action of a specific C-S lyase. A number of these enzymes have been described by the group of Mendel Mazelis in California (*e.g.* Hamamoto & Mazelis, 1986; Staton & Mazelis, 1991).

At present ten different thiophenes have been described in *Tagetes patula*. The exact biosynthetic pathway of these different components is not known yet in detail. For a good understanding of the regulation of secondary metabolism in *Tagetes*, this

knowledge is indispensable. On the basis of feeding experiments with ^{35}S-labelled intermediates, we present a modified biosynthetic pathway of the bithienyls in *Tagetes* (Chapter 3).

To fully understand how a plant metabolic pathway is regulated, knowledge of the various bioconversions and the enzymes catalyzing these conversions is not sufficient. This information needs to be accompanied by information how metabolites are shuttled from one cellular compartment to another during the operation of the pathway.

Thiophenes are sulphur-containing, fatty acid-derived secondary metabolites that accumulate in the roots of *Tagetes patula*. Since plastids play a major role in fatty acid synthesis and are the location of assimilatory sulphate reduction in roots, it was assumed that these organelles are also the site of thiophene biosynthesis. Cell fractionation experiments were performed to substantiate this assumption (Chapter 4). Our results indicate that thiophenes accumulate in the plastid membranes.

The regulation of secondary product formation is inseparably linked to the function of these compounds. Thiophenes are well known for their phototoxicity. The compounds, however, mainly accumulate in the root where photo-activation is not likely to occur. Therefore, the question was addressed whether thiophenes could play a role in defence against micro-organisms in the dark (Chapter 5).

A heat-stable, cell-free extract from the vascular wilt fungus *Fusarium oxysporum* induced in root cultures of *Tagetes patula* the biosynthesis of relatively hydrophillic thiophenes which proved to inhibit fungal growth in the absence of light. The thiophenes formed were partially excreted into the culture medium. Similar results were obtained with *T. patula* seedlings. The excretion of hydrophillic thiophenes may play an important role in the biochemical defence against soil-borne pathogens.

The tools of molecular biology may provide further insight into the principles

regulating secondary metabolism. However, these techniques should not be used without a thorough knowledge of the physiology of the model system used. Only the combination can lead to a full understanding of the regulation of secondary metabolic pathways in plants.

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Samenvatting

Secundaire plantestoffen

Rond het midden van de negentiende eeuw werd duidelijk dat levende wezens zijn opgebouwd uit chemische bouwstenen De precieze functie van de verschillende organische verbindingen die samen de levende cel vormen was nog onduidelijk Er werd echter wel al van uitgegaan dat niet alle stoffen essentieel waren voor de instandhouding van het leven In 1891 maakte Kossel, in een voordracht voor het Berlijns physiologisch genootschap, voor het eerst onderscheid tussen primaire en secundaire verbindingen Primaire verbindingen werden omschreven als 'die stoffen waarvan het leven afhankelijk is en welke zonder uitzondering voorkomen in iedere cel die in staat is tot groei en ontwikkeling' Daarnaast bestonden secundaire verbindingen welke werden gedefinieerd als 'die verbindingen welke slechts incidenteel voorkomen en niet noodzakelijk zijn voor de instandhouding van het leven'

Bij primaire verbindingen moet gedacht worden aan stoffen die noodzakelijk zijn voor de normale levensverrichtingen, ofwel de fysiologie, van planten Voorbeelden zijn koolhydraten, vetten en eiwitten Secundaire verbindingen spelen geen rol van betekenis in de fysiologie van de plant Chemisch gezien kunnen deze stoffen van zeer uiteenlopende aard zijn alkaloiden, flavonoiden, anthrachinonen, terpenen, etc

Hoewel het moeilijk is een fysiologische rol toe te schrijven aan de meeste secundaire verbindingen in planten, wordt steeds meer aangenomen dat deze verbindingen een rol spelen in de oecologie van de plant Bijvoorbeeld de kleur en geur van bloemen en fruit spelen een belangrijke rol bij het aantrekken van dieren wat noodzakelijk is voor de verspreiding van stuifmeel of zaad Bloemkleuren worden veroorzaakt door secundaire verbindingen die ophopen in kroon- of kelkbladeren, voor de rode, roze, violette en blauwe tinten zijn anthocyanen verantwoordelijk, de gele en oranje kleuren worden veroorzaakt door flavonolen of carotenoiden Geuren als die van sinaasappel, pepermunt, roos of lavendel, worden

veroorzaakt door terpenen.

Recent onderzoek heeft aangetoond dat secundaire verbindingen, die door de wortels worden uitgescheiden, een belangrijke rol spelen in de communicatie tussen planten en micro-organismen. Zo spelen flavonoïden een rol bij het aantrekken van de knolletjesbacteriën *Rhizobium*, welke van essentieel belang zijn voor de stikstofbinding in de bodem.

Een andere veronderstelde oecologische functie van secundaire verbindingen is die van afweermiddel, bijvoorbeeld het gif uit de brandnetel. Flavonoiden, en vooral tanninen, maken planten onaantrekkelijk als voedsel en bieden zo bescherming tegen overbegrazing. Ecdosteroïden worden algemeen beschouwd als stoffen met een insectenwerende werking. Strychnine, uit de braaknoot (*Strychnos nux-vomica*), en coniine, uit het sap van de gevlekte scheerling (*Conium maculatum*), zijn zeer sterke vergiften. Afweerstoffen die voorkomen in voedselgewassen kunnen een schadelijk effect hebben als ze in te hoge concentraties ophopen, bijvoorbeeld alkaloïden in aardappel of blauwzuurvormende verbindingen in cassave.

Wetenschappelijk belang

De belangstelling voor secundaire plantaardige verbindingen is in eerste instantie een gevolg van het feit dat voor de toepassing van planten deze stoffen uiterst belangrijk zijn. Van oudsher worden plantedelen als specerijen gebruikt vanwege de erin aanwezige geur- en smaakstoffen (kruidnagels, kaneel), of er worden reuk- en kleurstoffen (lavendelolie, anthrachinonen) uit gewonnen. Daarnaast vormen planten een belangrijke bron voor geneesmiddelen. Podophyllotoxine, gewonnen uit de wortelstokken van *Podophyllum* soorten, is de natuurlijke grondstof voor de bereiding van klinisch toegepaste cytostatica. Kinine, uit de schors van de kinaboom (*Cinchona succirubra*), wordt gebruikt in de behandeling van malaria. Cocaïne, uit de bladeren van *Erythroxylon coca*, en morfine, uit de zaaddozen van *Papaver somniferum*, zijn pijnbestrijders.

Het streven naar de toepassing van natuurlijke bestrijdingsmiddelen heeft de belangstelling voor fundamenteel onderzoek naar de oecologische rol van secundaire verbindingen sterk doen toenemen.

Verder maakt het feit dat vele secundaire stoffen voor de primaire levensfucties van de plant niet direct noodzakelijk zijn, secundaire stofwisselingsroutes zeer geschikt om de fundamentele principes van stofwisselingsregulatie op genetisch en fysiologisch niveau te onderzoeken.

De vorming van sommige secundaire producten treedt alleen op na toediening van een bepaalde prikkel (bijvoorbeeld infectie of UV-straling). Dit maakt de biosyntheseroutes van deze stoffen tot ideale modelsystemen voor fundamenteel onderzoek naar signaalontvangst en -overdracht in planten.

Plantencel en -weefselculturen

Bij de bestudering van de vorming van secundaire plantestoffen wordt meestal gebruik gemaakt van sterk vereenvoudigde modelsystemen.

Eén van de meest gebruikte systemen is de zogenaamde callus- of suspensiecultuur, hierbij worden planten als losse cellen gekweekt in een voedingsoplossing. De gekweekte plantencellen groeien relatief snel en iedere cel staat in nauw contact met de omringende vloeistof, waarvan de samenstelling op eenvoudige wijze veranderd kan worden. Verder is een plantecelcultuur redelijk homogeen van samenstelling en is gebleken dat stoffen makkelijker uit celculturen zijn te isoleren dan uit hele planten. Een nadeel is dat de secundaire verbindingen die zo karakteristiek zijn voor de hele plant, in suspensieculturen niet, of slechts in zeer geringe mate, gevormd worden. De vorming van secundaire verbindingen is vaak afhankelijk van een bepaalde graad van organisatie van cellen, van de vorming van weefsels of organen.

Georganiseerde weefselculturen, zoals wortel- en scheutculturen, vormen wel de secundaire verbindingen die in ongeorganiseerde culturen afweezig blijven, maar ze

groeien normaliter erg langzaam. Dit laatste probleem kan overkomen worden door genetische modificatie van planteweefsel. Bacteriën van het geslacht *Agrobacterium* kunnen genen overbrengen naar plantencellen. Door *Agrobacterium rhizogenes* worden genen overgebracht die plantencellen aanzetten tot de vorming van zogenaamde adventiefwortels. Deze genetisch gemodificeerde wortels kunnen in voedingsoplossing net zo snel groeien als de ongeorganiseerde suspensieculturen en vormen dezelfde stoffen als wortels aan de plant.

Afrikaantjes als modelsysteem

Afrikaantjes (*Tagetes*), zijn niet alleen bekend als perkplanten maar kennen ook toepassing in de natuurlijke bestrijding van plantenaaltjes. Deze toepassing heeft de belangstelling gewekt van landbouwkundige onderzoekers. Eind jaren vijftig heeft men vastgesteld dat Afrikaantjes thiofenen vormen, stoffen die al in kleine hoeveelheden giftig zijn voor aaltjes en andere plantenbelagers.

Thiofenen zijn zwavelbevattende secundaire verbindingen die vooral in de wortels van Afrikaantjes worden gevormd. Hoe deze verbindingen in de plant precies worden gevormd is nog grotendeels onbekend. Ook is nog onbekend welke factoren een rol spelen in de regulatie van thiofeenvorming. De principes die de vorming van thiofenen in *Tagetes* wortels reguleren vormen het onderwerp van dit proefschrift.

Ongeorganiseerde celculturen vormen slechts zeer geringe hoeveelheden thiofeen vergeleken met wortels. Wortelculturen, verkregen na genetische modificatie van afrikaantjes met behulp van *Agrobacterium rhizogenes*, vormen thiofenen in dezelfde mate als wortels die nog aan de plant zitten. De genen die door de bacterie op de plant worden overgebracht veranderen de gevoeligheid van de plant voor het hormoon auxine. In hoofdstuk 1 wordt het effect beschreven van auxine op de groei van *Tagetes patula* wortelculturen en op de vorming van secundaire verbindingen. Toevoeging van het hormoon leidt tot de vorming van grote hoeveelheden zijwortels

en tot remming van de thiofeenvorming (Fig. 1.3).
In wortels die gekweekt waren in voedingsoplossing zonder auxine, kon geen verband gevonden worden tussen wortelvertakking en thiofeenvorming (Tab. 1.2). Dus, alleen de vorming van zijwortels is geen voldoende voorwaarde voor de verminderde thiofeenvorming.

Voor de vorming van secundaire plantenstoffen worden chemische bouwstenen gebruikt die ook nodig zijn voor de vorming van primaire verbindingen. Er moet in de cel een mechanisme bestaan dat regelt welke hoeveelheid van de bouwstenen gebruikt wordt voor de vorming van primaire en welke voor de vorming van secundaire verbindingen. In hoofdstuk twee wordt beschreven wat het effect is van zwavelgebrek in de voedingsoplossing op de groei van wortelculturen en op de vorming van de zwavelhoudende secundaire verbindingen in *Tagetes patula*. Uiterlijk was geen verschil zichtbaar tussen wortelculturen die acht dagen in een normale voedingsoplossing waren gekweekt en wortelculturen die acht dagen in zwavelarme oplossing waren gekweekt. De vorming van thiofenen, echter, was na acht dagen groei in een zwavelarme omgeving met 80-95% afgenomen (Tab. 2.2). De thiofeenvorming kwam weer terug op het oude niveau als wortels van een zwavelarme naar een normale voedingsoplossing werden overgezet (Fig. 2.4). Na toevoeging van 5 $\mu g.l^{-1}$ van de transcriptieremmer cordycepine, trad geen herstel van de thiofeenvorming op (Tab. 2.4), terwijl de wortels wel normaal doorgroeiden. Hieruit werd geconcludeerd dat de zwavelverdeling in de cel op het niveau van DNA-transcriptie gereguleerd wordt.

Er zijn op het moment tien verschillende thiofenen beschreven in *Tagetes patula*. Het was nog niet in detail bekend hoe al deze verschillende verbindingen gevormd worden. Gedetailleerde kennis is echter onmisbaar in verder onderzoek naar de regulatie van thiofeenvorming. In hoofdstuk drie wordt beschreven hoe een deel van de biosyntheseroute is ontrafeld. Er werden via de voedingsoplossing radioactief gelabelde bouwstenen toegediend. Na verloop van tijd werd bepaald welke door de

wortelculturen gevormde verbindingen het radioactieve label opgenomen hadden (Tab. 3.1). Een overzicht van de resultaten wordt gegeven in Fig. 3.5.

Om volledig te kunnen begrijpen hoe de vorming van een secundaire verbinding in een plant verloopt, is het niet voldoende om alleen te weten hoe de ene stof in de andere wordt omgezet of welke enzymen bij de omzetting een rol spelen. Ook de precieze plaats van de vorming van de verschillende verbindingen is belangrijk. Een plantecel is namelijk verdeeld in verschillende compartimenten en tijdens de vorming van secundaire verbindingen worden tussenproducten regelmatig van het ene naar het andere celcompartiment gesluisd.

Thiofenen zijn zwavelbevattende, van vetzuur afgeleide secundaire verbindingen. Er werd verondersteld dat de plastiden het compartiment vormden waar de thiofeenvorming plaats zou vinden omdat plastiden een belangrijke rol spelen in de vetzuursynthese en deze organellen ook het compartiment vormen waar zwavelreductie plaatsvindt in wortels. Om de veronderstelling hard te maken werden celfractioneringsexperimenten uitgevoerd, beschreven in hoofdstuk vier. Onze resultaten wijzen er op dat thiofenen ophopen in de membranen van de plastiden.

De regulatie van de vorming van secundaire verbindingen is onlosmakelijk verbonden met de functie van deze verbindingen. In hoofdstuk vijf wordt beschreven hoe de vorming van thiofenen beïnvloed wordt door blootstelling van de wortels aan plantenziekteverwekkende micro-organismen. Een extract van de schimmel *Fusarium oxysporum*, de veroorzaker van wortel- en stengelrot, werd toegevoegd aan de voedingsoplossing waarin *Tagetes*-wortels gekweekt werden. De toevoeging leidde tot de vorming door de wortels van wateroplosbare thiofenen (Tab. 5.2), welke een sterk remmende werking hadden op schimmelgroei (Fig. 5.1). De thiofenen werden uitgescheiden in de voedingsoplossing. Soortgelijke resultaten werden behaald met *T. patula* kiemplanten. De uitscheiding van wateroplosbare thiofenen speelt zeer waarschijnlijk een belangrijke rol in de afweer van de plant tegen ziekteverwekkende bodemorganismen.

Bedankt !

Schijnbaar onbeduidende voorvallen kunnen onvoorspelbaar grote gevolgen hebben; een vleugelslag van een vlinder in Brazilië kan een storm veroorzaken in Texas (Lorenz, 1979).

Welke voorvallen leidden uiteindelijk tot de resultaten die in dit boekje staan beschreven? Was het de deskundige en persoonlijke begeleiding? Waren het de stimulerende discussies, de technische ondersteuning en de goede sfeer op het lab? Misschien de kritische houding en de niet te stoppen stroom van ideeën en spitsvondigheden van de studenten die een nimmer opdrogende bron van inspiratie vormden? De loftuitingen van omstanders? Of waren het de dichterlijke woorden 'Boldly go where no-one has gone before' (Roddenberry, 1964) die telkens weer moed gaven?

Het is allemaal heel goed mogelijk, maar wie verzekert mij dat de sleutel tot het beschrevene niet zit in al de andere dingen die ik hoorde, zag, beproefde en mat, in de vergissingen of meest idiote opinies, in de onafgemaakte zinnen? In de afgekeurde manuscripten? In de adviezen die niet gevolgd werden? In het schamperen van omstanders? Of in het besef dat iedere gedachte die ik niet beschrijf verloren gaat?

Wie aan een promotie-onderzoek begint, kan slechts gokken wat er vier jaar later op papier zal komen te staan. Om de onzekerheid te bezweren heeft de promovendus, net als iedere doorgewinterde gokker, een systeem waaraan hij vasthoudt tijdens het spel. Als, na veel onbegrijpelijke rituelen, de inspanning leidt tot de uitreiking van een prijs, zal een buitenstaander ten onrechte denken dat het bedachte systeem het juiste was.

Het onderzoek is een verzameling, een enorme opeenhoping bewegingen en denkbeelden. Het meeste gaat voor altijd verloren en het kan niet anders of de keuze van wat wordt vastgelegd of verloren gaat is het gevolg van wanhopige willekeur.

Door het feit dat het een blijft en het ander verdwijnt, kan niemand ontkennen dat er een verschil is tussen die twee. Toch wordt daardoor het altijd weer opnieuw gepleegde bedrog niet gerechtvaardigd, dat juist datgene wat blijft bij elkaar zou horen, dat het een *systeem* zou vormen (Hermans, 1953).

Een toevallige samenloop van omstandigheden heeft bepaald welke denkbeelden uiteindelijk in dit proefschrift terecht zijn gekomen. Ik vlij me met de gedachte dat ik hiermee in een lange onderzoekstraditie sta. Zonder de uitvinding van de badkuip had Archimedes zijn boek over drijvende lichamen wellicht niet geschreven. Als Isaac Newton indertijd in één van de Britse kolonies onder een kokospalm had geslapen, had hij zijn 'Philosophiae naturalis principia mathematica' zeker niet geschreven. Had Alexander Fleming netter gewerkt, dan had hij de penicilline niet ontdekt.

De oude Grieken vermoedden het al en tegenwoordig wordt het door natuurwetenschappelijke experimenten ondersteund: alles komt voort uit chaos. Ik bedank iedereen die de afgelopen jaren heeft bijgedragen aan de chaos, met name diegenen die dit in slechts zeer geringe mate deden.

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Curriculum vitae

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Practical experience:	1989-1994 PhD research at the Catholic University of Nijmegen, Department of Experimental Botany Regulation of thiophene synthesis in *Tagetes patula* L.
	1988-1989 Research technician at the Utrecht University Hospital (AZU), Department of Clinical Immunology Screening of human serum samples for aberrant expression of immunoglobulins
	Research technician at the Institute for Drinking-Water Inspection (KIWA), Department of Microbiology Analysis of factors that stimulate growth of *Aeromonas* spec. in the Dutch waterworks
	1986-1988 The Netherlands Organization for Applied Research (TNO), Department of Biotechnology Electrofusion of fungal protoplasts. Production of lignans by *Linum flavum* cell and tissue cultures

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