CALLUS AND CELL CULTURE OF *TAGETES* SPECIES IN RELATION TO PRODUCTION OF THIOPHENES

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CALLUS AND CELL CULTURE OF *TAGETES* SPECIES IN RELATION TO PRODUCTION OF THIOPHENES

Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. C. C. Oosterlee,
in het openbaar te verdedigen
op woensdag 8 april 1987
des namiddags te vier uur in de aula
van de Landbouwuniversiteit te Wageningen

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STELLINGEN

I Totipotentie ondervangt niet de verschillende oorsprongs- en herinneringseffecten bij verschillende *Tagetes*-soorten.

Dit proefschrift (Hoofdstuk I).

II Voor de biosynthese van secundaire metabolieten is celdifferentiatie vereist.

Dit proefschrift (Hoofdstuk VII).

III Het gevaar dreigt, dat men de produktie van secundaire metabolieten in calli en celcultures gaat bestuderen zonder zich voldoende te realiseren wanneer en waartoe deze secundaire metabolieten in intacte planten worden gesynthetiseerd.

Dit proefschrift (Hoofdstuk IV).

IV Om de biosynthese van secundaire plantmetabolieten te kunnen reguleren is onderzoek naar de relatie tussen minerale huishouding en biosynthese van fytohormonen in planten en plantecelcultures onontbeerlijk.

Dit proefschrift (Hoofdstukken II en VI).

V De verklaring, die Wink geeft voor de afwezigheid van secundaire metabolieten in celcultures, n.l. de uitscheiding van (proteo-)lytische enzymen, is zeker niet zoals hij suggereert voor alle soorten cultures van toepassing. Bovendien is bedoelde verklaring in tegenspraak met recent door Banthorpe en medewerkers gepubliceerde resultaten betreffende de produktie en uitscheiding van enzymen door ongedifferentieerde calli en celcultures.

Wink, M. *Naturwissenschaften* 71: 635-636 (1984).
Banthorpe, D. V. et al. *Phytochemistry* 25: 629-636 (1986).

VI Een artikel, waarin men op grond van één enkele getalswaarde aangeeft wat de secundaire metabolietproduktie is in intacte planten om daarmee aan te geven dat celsuspensies meer of minder van dezelfde metabolieten produceren, zou geweigerd moeten worden.

Anderson, L. A. et al. *Planta Medica* 46: 25-27 (1982).
Scragg, A. H. and Fowler, M. W. In: *Cell Culture and Somatic Cell Genetics of Plants* (Ed. I. K. Vasil). Academic Press Inc. (London) Ltd. Vol. 2, p. 106 (1985). ISBN: 0-12-715002.

VII Men kan de produktiviteit van secundaire metabolieten van verschillende plantecelcultures op basis van droge-stofpercentages niet onderling vergelijken, zolang niet bekend is waarop deze percentages betrekking hebben.

Plantecelbiotechnologie in Nederland. Studierapport 14g. Nationale Raad voor Landbouwkundig Onderzoek. 's Gravenhage. Dec. 1985.

VIII Aangezien alleen al het weglaten van 2,4-D uit het groeimedium van vrije cellen van *Catharanthus roseus* een significante produktieverhoging van ajmalicine en serpentine veroorzaakte, kan aan de conclusie van Majerus en Pareilleux, dat immobilisatie van dergelijke cellen de produktie van ajmalicine verhoogt, geen betekenis worden toegekend.

Majerus, F. and Pareilleux, A. *Plant Cell Reports* 5: 302-305 (1986).

IX De door Yisraeli et al. opgestelde verklaring voor de reactivatie van de genexpressie bij gedemethyleerde genen in verschillende soorten spiercellen van Vertebraten draagt in belangrijke mate bij tot het antwoord op de vraag, hoe de verbinding 5-azacytidine als switch-punt functioneert bij de demethylering van genen in een crowngall bij planten, en draagt daarmee tevens in belangrijke mate bij tot een verklaring van de door Amasino et al. gevonden fenotypische variaties van crowngall regeneranten.

Yisraeli, J. et al. *Cell* 46: 409-416 (1986).
Amasino, R. M. et al. *Mol. Gen. Genet.* 197: 437-446 (1984).

X Milieugroepen, die terecht of onterecht voorzien zijn van een negatief imago, hebben nu nog een unieke kans dit imago om te buigen door constructief mee te werken aan de maatschappelijke voorbereiding op toekomstige, moderne biotechnologische toepassingen.

Samenvattend hoofdrapport: *Maatschappelijke aspecten van de biotechnologie* i.o.v. Hoofddirectie Wetenschapsbeleid, Ministerie van Onderwijs en Wetenschappen. Zoetermeer, 1985.

XI Bij de bepaling van de Km-waarde van de opname van ionen door intacte planten wordt de noodzaak om de activiteit en de concentratie van de ionen in het wortelmilieu gelijktijdig te bepalen vaak onderschat.

Ketel, D. H. and De Ruyter, A. W. *Proc. Ninth Intern. Plant Nutr. Colloq.* Vol. I, 288-293 (1982); CAB, UK (Ed. A. Scaife).

XII Het frequent doen verschijnen van slechts marginaal veranderde studieboeken bestemd voor LBO- en MBO-opleidingen en voor middelbare scholen heeft bij veel vakken niets te maken met nieuwe ontwikkelingen op kennisgebied, maar alles met het voor ouders en verzorgers onnodig duur maken van deze opleidingen.

XIII De manier waarop in het Noordwest-Overijsselse natuurgebied het toerisme wordt gestimuleerd staat haaks op de maatregelen, die men neemt om de flora en fauna aldaar te beschermen.

Stellingen, behorende bij het proefschrift: *"Callus and Cell Culture of* Tagetes *species in Relation to Production of Thiophenes"* door David Ketel.

Wageningen, 8 april 1987

Hoe lieflijk straalt Zijn schoonheid van omhoog.
Hier weidt mijn ziel met een verwonderd oog,
aanschouwende hoe schoon en zuiver is
Zijn licht, verlichtende de duisternis.

(psalm 27:2[b], berijmd)

VOORWOORD

Bij het verschijnen van dit proefschrift wil ik allereerst naar voren brengen dat de daarin vermelde, en door mij als onderzoeker te verdedigen resultaten boven tafel zijn gekomen door de inspanning van een team van mensen. Elk persoon van dit team heeft dus binnen de zijn of haar toegemeten taak aan dit proefschrift bijgedragen. Ik wil daarom in dit voorwoord beginnen met deze teamgenoten te noemen.

Omdat met name Marinus Jansen en Pieter Pikaar van meet af aan werkzaam zijn geweest in het project plantebiotechnologie, en juist zij in belangrijke mate hebben bijgedragen tot de ontwikkeling en uitvoering van een aantal methodieken en experimenten, bedank ik hen in de eerste plaats voor hun medewerking en collegialiteit. Hans Breteler, en daarna Dinie Lutke Willink zijn in een wat later stadium in de A_2-groep gekomen, maar hebben, respectievelijk, onmisbare leiding gegeven aan en assistentie verleend bij de uitvoering van het onderzoek. Michel van Duren was slechts een korte periode bij ons, maar heeft in die korte tijd veel voor mij gedaan. Dit proefschrift mag er dan ook, naar ik hoop, toe hebben bijgedragen, dat aangetoond is dat we er met elkaar de afgelopen jaren in zijn geslaagd een team te zijn, dat het hoofd kan bieden aan met name grote organisatorische problemen. Deze problemen hingen vaak samen met het levende materiaal waarmee we in ons onderzoek bezig zijn.

Mijn bijzondere waardering wil ik graag uitspreken voor mijn directe collega Dr. H. Breteler, die niet alleen in zijn functie als projectleider van ons team zich mijn persoonlijk lot en ook dat van mijn manuscripten heeft aangetrokken, maar daarnaast veel van mijn aanvankelijke beslommeringen uit handen heeft genomen. Daardoor kwam tijd vrij om me te kunnen concentreren op studie en schrijfwerk. Beste Hans, ik hoop dat we het onderzoek op het gebied van plantebiotechnologie binnen en buiten NOVAPLANT gezamenlijk kunnen blijven stimuleren. Het bedoelde vakgebied is een blijvend enthousiasme waard, omdat we weten welke perspectieven er in liggen opgesloten.

Aan mijn promotoren, Dr. J. Bruinsma en Dr. B. de Groot, ben ik veel dank verschuldigd. Uw beider betrokkenheid bij de voorbereiding van dit proefschrift, die op mij overigens zo verschillend overkwam (n.l. enerzijds de hoogleraar plantenfysiologie aan de Landbouwuniversiteit, en anderzijds de hoogleraar celgenetica en themaleider bij het ITAL), heeft er substantieel aan bijgedragen, dat het proefschrift zijn huidige inhoud heeft gekregen.

Dr. A. Ringoet, oud-directeur van het ITAL en mijn vroegere directe chef, heeft mij in het verleden sterk gestimuleerd en bovendien de gelegenheid geboden mij op het ITAL wetenschappelijk te ontplooien. Daarbij heeft hij niet nagelaten mij in staat te stellen de resultaten van het onderzoek te bewerken voor een proefschrift, tegelijkertijd echter de voorwaarde daaraan verbindend dat ik een aantal nevenactiviteiten diende af te stoten om mij te behoeden voor een te grote afmattingsslag. Waarde Ringoet, dank voor deze zorg, en die voor het welzijn van mijn gezin, dat, zo weet ik, vanwege bepaalde omstandigheden steeds veel aandacht bij U had.

Rie 't Hart-Versteeg heb ik met enige opzet nooit bij de afzonderlijke publikaties bedankt voor het typewerk. De totaliteit van het werk aan dit proefschrift geeft veel reden om dat nu hier te doen. Ook Wim van Lienden en Menno Drost wil ik graag bedanken voor de goede kwaliteit van het tekenwerk en het nemen van foto's.

Wanneer ik er andere proefschriften op nalees, blijk ik tot nog toe niet de enige gehuwde promovendus te zijn geweest, die de spanningen van het schrijven voor een proefschrift en alles wat daarbij komt, in mindere of meerdere mate wist af te wentelen op de hoofden en schouders van zijn of haar gezinsleden. Deze schrale troost heeft er echter allerminst toe geleid, dat de last van mijn soms verstrooide hersenen voor Ans in het bijzonder, maar ook in niet onbelangrijke mate voor onze kinderen, daar minder door is geworden. Dank zij jullie inventiviteit en begrip is het jullie goed gelukt om mij van tijd tot tijd ook weer tot de dagelijkse realiteit terug te voeren. Dat met name in het gezinsleven van alle dag plantebiotechnologie en afrikaantjes niet de belangrijkste rol spelen, is mij wel duidelijk geworden.

Acknowledgements
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CONTENTS

General Introduction

Plant cell biotechnology

Plant cell biotechnology developed since it was demonstrated that somatic plant cells are totipotent in vitro (Muir et al., 1954) and nutrient media were improved according to insights about the need of carbon, salts, vitamins, and growth regulators (Murashige and Skoog, 1962; Gamborg et al., 1968; Schenk and Hildebrandt, 1972; Nitsch, 1972). One of the outcomes was the successful production of secondary metabolites in callus cultures and cell suspensions reported by many investigators since about 1965 (e.g. Dalton et al., 1983; Scragg and Fowler, 1985). Others, however, mentioned disappointing results (e.g. Cashyap et al., 1978; Misawa and Suziki, 1982; Ellis, 1982; Tyler et al., 1986; Banthorpe et al., 1986).

In particular, when non- or low- producing cell cultures are considered, the failure of such production may be attributed to three different phenomena:

1) the positive correlation between differentiation and secondary-metabolite production (Yeoman et al., 1979; Wiermann, 1981; Lindsey and Yeoman, 1983; Banthorpe et al., 1986);
2) the negative correlation between the growth rate of cell cultures and the production of secondary metabolites (Brodelius and Mosbach, 1982; Lindsey and Yeoman, 1983 and 1985; Banthorpe et al., 1986);
3) problems involving genetic instability, called habituation (Meins et al., 1980; Fuller, 1984; Lindsey and Yeoman, 1985), selection (Singh et al., 1975; Vanzulli et al., 1980), and cell heterogeneity (Hall and Yeoman, 1986).

These problems indicate that, although insights into structure and function of DNA and genetic principles of plant cells increased rapidly, the poor progress in the adoption of plant cell biotechnology by the industry is mainly due to the deficiency of insights into the biochemistry and regulation of secondary metabolism.

A recent scope of plant cell biotechnology is the production of secondary metabolites with a high economic value on an industrial scale (Fowler, 1981), although most plant species apparently deliver non- or only low--producing cell cultures as yet (Ellis, 1982). This scope implicates aspects of molecular genetics, plant physiology, biochemistry, processtechnology, and down-stream processing. It is very difficult to study the problems met in each of these fields simultaneously with one type of cell culture. It requires the use of a diversified system of cell cultures of which the different parent plants contain the potency to produce similar secondary metabolites. Only such a system can adequately be used as an appropriate model-system.

Tagetes and a number of related species (Heleniae: Gommers, 1973) produce thiophene-biocides. Some reports dealing with callus cultures of T. erecta L. (Kothari and Chandra, 1986), T. patula L. (Norton et al., 1985), and T. minuta L. (Jain, 1977) indicated, that the plants of these species form a suitable diversified 'Tagetes-system' to study simultaneously the background of the failure or continuation of the production of secondary metabolites in calli and cell suspensions under mutually comparable conditions. The principles of the experimental methods for in vitro cultures are apparently simple and implicate the sterilization of suitable explants and the culture of calli and free cells and/or cell aggregates under axenic conditions (Street, 1977).

Thiophenes, and thiophene-related analytical aspects.

Probably because of the presence of thiophenes and thiophene derivatives (Atkinson et al., 1964), polyacetylenes (Sørensen, 1977), flavone glycosides (Ickes et al., 1973), terpenes (Bohrmann and Youngkan, 1968), and perhaps still other secondary metabolites, already the precolumbian Maya's in Southern America used Tagetes plants for religious and pharmaceutical purposes and to poisen animal (e.g. insects) and human ennemies (Neher, 1968).

Today, it is known that thiophenes are wide-spectrum biocides (Arnason et al., 1981; Champagne et al, 1984; Philogène et al., 1985) also tested as nematicides (Gommers, 1981). Under the influence of photodynamic processes, thiophenes produce singlet oxygen. This product causes the death for nematodes. There is good hope that thiophene-biocides eventually produced in liquid cultures can be applied in crops (Gommers et al., 1980; Gommers et al., 1982; Cooper and Nitsche, 1985). Because the reactions involved in the production of singlet oxygen in root cells in the darkness of soil is not

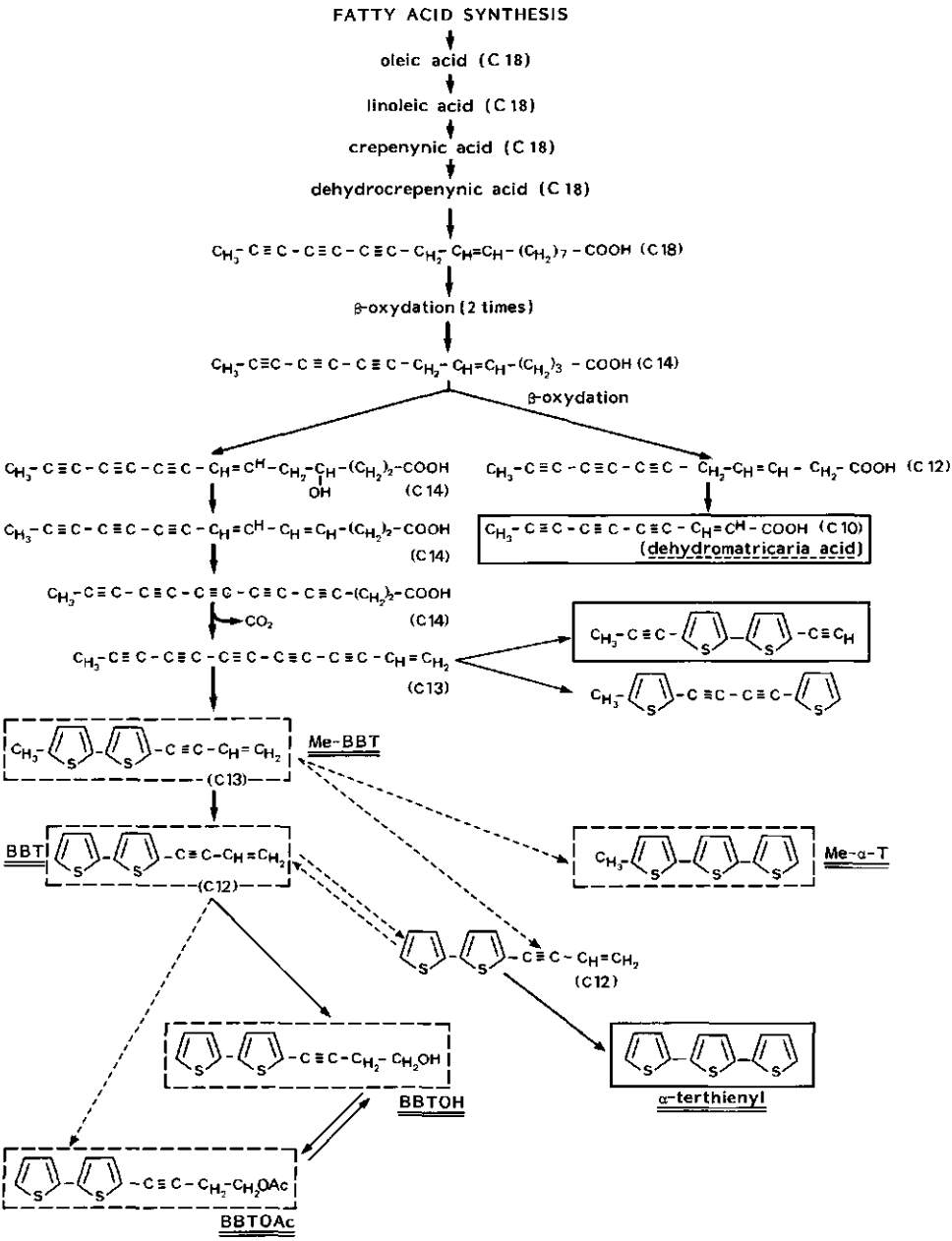

Fig. 1. Hypothesis for the biosynthesis of thiophenes, mainly based on the results from Bohlmann's group (e.g. Jente et al., 1981). See p. 14.

completely understood, it is not yet clear how to apply the thiophene-biocides in soils or crops. Therefore, thiophenes are not commercially produced until now.

Thiophene-biocides are secondary metabolites, which commonly occur in Tagetes species and other Compositae (Bohlmann and Zdero, 1973). However, the details of the way and the site of the biosynthesis of thiophenes in the plant systems are not understood. On the one hand, according to Jente et al. (1981), oleic acid and polyacetylenes are the precursors of thiophenes, such as α-T (2,2',5'2''-terthienyl) and BBTOAc [5-(4-acetoxy-1-butiny)-2,2'-bithiophene] (Fig. 1). On the other hand, according to Dr. R. Sütfeld (personal communication), it cannot be excluded that another way can probably be realized by the plant systems. For instance, the synthesis of the butinyl side-chain may be performed by condensation reactions of probably activated short-chain precursors. As yet unknown, sulphur-containing amino acids may then be involved in the synthesis of the thiophenic ring system. In this case the first products of the thiophene biosynthesis would rather be polar compounds such as BBTOH [5-(4-hydroxy-1-butinyl)-2,2'-bithiophene] than the non-polar compounds such as BBTOAc and α-T (Fig. 2., unpublished results provided by Dr. Sütfeld). Originally, Zechmeister and Sease (1947),

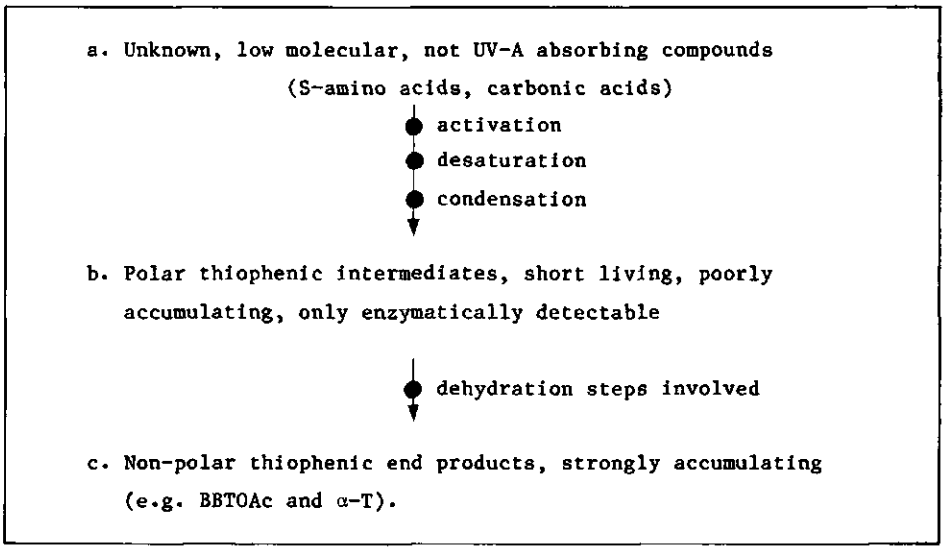

Fig. 2. Hypothetical scheme for the biosynthesis of thiophenic compounds based on physiological/biochemical investigations of Dr. R. Sütfeld (personal communication).

and Uhlenbroek and Bijloo (1958 and 1959) suggested that thiophenes mainly accumulate in the roots of intact plants. Later, Sütfeld (1982) reported that thiophenes are also distributed in stems and cotyledons of intact seedlings. These results indicate that thiophenes may occur in all organs of fully grown Tagetes plants, but the pattern of distribution may drastically differ between the several species. Although the concentration of secondary metabolites may vary impredictably with the age of the plants (Wiermann, 1981; Fuller, 1984), the non-polar characteristic of thiophenes gives rise to suppose that redistribution of these compounds in plants is of little importance. This aspect may be of particular importance for the selection of suitable explants.

Thiophene molecules differently absorb UV-light between 220 and 400 nm (Bohlmann and Zdero, 1985). Therefore, the presence of these compounds in solution can be readily determined spectrophotometrically. However, as long as purified thiophenes are not available the absolute amounts of accumulated thiophenes can not be determined. Under this condition the use of a high--speed spectrophotometer, which elucidates on-line the shapes of the different UV-spectra of thiophenes and also measures the amount of absorbed UV simultaneously is required. In particular, the combination with HPLC (Hostettman, 1984; Sütfeld, 1987) provides a sophisticated analytical method to analyse the complex picture of thiophene composition. It is supported by the analysis of a great number of other known as wel as unidentified naturally occurring thiophenes (Bohlmann and Zdero, 1985), not presented in Fig. 1. Standard samples and MS-spectra of thiophenes (e.g. α-T, BBT, and BBTOAc) became available only in the course of the present work.

The results presented in this thesis are related to the following topics:

a: the characterization of the growth and morphology of calli of different Tagetes species in the consecutive phases of their culture (chapter I);

b: the relationship between differentiation and thiophene production in calli of Tagetes species in combination with the nature of the medium (chapters II and III);

c: the selection of explants with respect to the age of the plants and the occurrence of thiophenes in intact plants and calli (chapter IV);

d: the influence of immobilization and environmental stress-conditions on the growth and secondary-metabolite production in cell suspensions of Tagetes species (chapter V);

e: the use of Agrobacteria to modify and control thiophene production in differentiated and undifferentiated neoplastic tissues of Tagetes species (chapter VI);

f: the production of thiophenes in liquid cultures with cell aggregates of T. patula (chapter VII).

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Chapter I

Effect of Explant Origin on Growth and Differentiation of Calli from *Tagetes* species

David H. Ketel, Hans Breteler and Bram de Groot
(J. Plant. Physiol. Vol. 118. pp 327 - 333, 1985)

SUMMARY

Tertiary leaf callus from 13-week old Tagetes minuta showed 5 to 6 fold higher fresh weight increment than callus from 3 to 7 week - old plants. No such differences were observed in the primary and secondary calli. Similarly, tertiary leaf callus of T. patula showed morphological differentiation that varied with the mineral nutrition to which intact plants had been exposed and that did not appear in the primary and secondary calli. No such effects were observed in T. minuta. These "origin" effects are discussed in relation to "memory" effects and to their application to plant biotechnical procedures.

Key words: Callus culture, differentiation, memory effect, mineral nutrition, origin effect, plant age, Tagetes minuta, Tagetes patula.

ABBREVIATIONS

BM = basal medium; C_1, C_2, C_3 = primary, secondary, and tertiary callus, respectively; DM = dry matter; MS = according to Murashige and Skoog (1962).

INTRODUCTION

A predictable and reproducible relationship between biochemical traits of intact plants, calli, and suspended cells of the same species or cultivar is an obvious prerequisite for the practical use of plant biotechnology. The potential for this relationship is anchored in the totipotency of each cell, the genome of which is the genetic memory (cf. Thorpe, 1978) for all types of morphological and chemical differentiation that may occur during the development of the intact organism.

Sometimes impredictable traits become suddenly apparent in calli or suspended cells (Yeoman et al., 1980) and they might, on the one hand, be the consequence of genetic instability (Ikeda et al., 1981). On the other hand, it is possible that such traits are repressed in intact plants and expressed in only certain stages of their culture or under certain culture conditions (Aitchison et al., 1977).

Sometimes the term "memory process" (Thellier, et al., 1982) is used for this phenomena, because the underlying mechanism is not known. Presumably, some masked genetic regulation mechanism is responsible for the effects.

We herewith report two such effects, one of plant age and one of mineral nutrition, that were encountered during investigations into chemodifferentiation of cells and tissues of Tagetes species. They illustrate that "memory" phenomena may possess practical value in plant biotechnology and may act as new incentives.

MATERIALS AND METHODS

Plant cultivation. Seeds of Tagetes minuta L. (harvest 1982, kindly provided by the Botanic Garden of the University of Nijmegen) and of Tagetes patula L. cv. Nana furia (purchased from Tubergen Co.,

Lisse) were germinated in the dark at 22 °C on paper moistened with demineralized water. After one week the seedlings were transferred to well-aerated nutrient solutions, the composition of which is given in Table 1. Plants were grown in 16 h light (22 ± 1 °C, 30 W m^{-2}) / 8 h dark (19 ± 1 °C) cycles at 75 ± 5% RH.

Effect of age. T. minuta was grown in BM with $Ca(NO_3)_2$ (Table 1). This solution was circulated through a pH - stat, that kept the pH at 5.5 ± 0.2 with diluted KOH or H_2SO_4. Plants varying in age between 3 and 13 weeks were available simultaneously. Explants were prepared

	BM + NO_3^-	Hoagland & Snyder	Steiner
K^+	11	6	6.7
Ca^{2+}	10.5	10	10
Mg^{2+}	1	4	4
Cl^-	1.5	0	1
NO_3^-	10	15	11.9
$H_2PO_4^-$	1	1	1
SO_4^{2-}	10	4	6.9
total ions	45	40	41.4

Table 1. Composition of nutrient solutions (mmol of charge.dm^{-3}) used to grow Tagetes species. The media were slightly modified basal medium (Breteler et al., 1979) with $Ca(NO_3)_2$, Hoagland and Snyder (1933) solution, and Steiner solution (Steiner, 1968). Trace elements [(mol.dm^{-3}): 40 Fe^{3+}, 22 $B(OH)_3$, 6 Mn^{2+}, 1 Zn^{2+}, 0.16 Cu^{2+}, 0.04 $Mo_7O_{24}^{6-}$] did not differ between solutions.

from leaves sampled at random from each of at least 6 plants per age. No leaves were taken from the top and bottom segments of the main stem.

Effect of mineral nutrition. T. minuta and T. patula were grown for 7 weeks on various media (Table 1) prior to leaf sampling for explantation. Media were refreshed at 2-week intervals during which the pH rose from 5.0 ± 0.1 to 7.2 ± 0.2.

Callus culture. Excised leaves were submerged for 2 sec in 70% (v/v) ethanol and 13 min in 5% (w/v) $CaOCl_2$ and subsequently washed for 3, 10, and 30 min in fresh aliquots of sterile, demineralized water. Apical leaf sections of approximately similar size (1.5 cm long) were excised and placed upright in a tube (16 cm x Ø 2.5 cm) with 15 cm^3 MS medium, supplemented with 2% (w/v) sucrose, 0.5 $mg.dm^{-3}$ naphthylacetate, and 5 $mg.dm^{-3}$ benzyladenine, and solidified with 0.2% (w/v) Gelrite (Costar Ltd, Badhoevedorp, The Netherlands). The pH was adjusted to 5.9 with KOH prior to autoclaving (20 min, 120 °C). At least 40 leaf explants were used per treatment.

Axenic calli developed at 24 ± 1 °C and a 16 h photoperiod (5 Wm^{-2}). After 3 weeks the C_1 was cut into 3 pieces and partly subcultured. After 2 weeks the resulting C_2 was halved and subcultured to yield C_3, which was harvested after 2 weeks and partly suspended in liquid MS medium. After the various subcultures fresh calli were washed, weighed, lyophilized, and weighed again.

RESULTS

Effect of the age of the plant

Between days 5 and 7, callus growth on leaf explants of T. minuta became visible. After three weeks C_1 and C_2 calli were un-

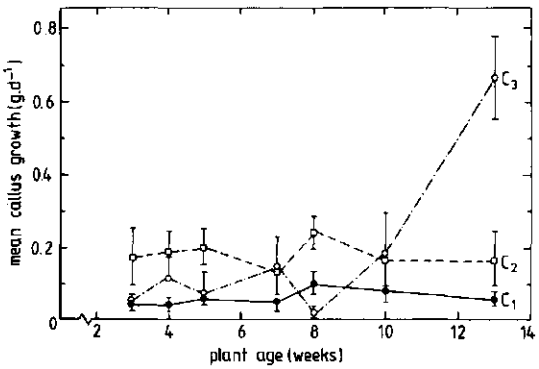

Figure 1. Growth rate of calli of Tagetes minuta from leaf explants of various ages (abscissa). C_1, C_2 and C_3 refer to the primary, secondary, and tertiary callus phases, respectively. Data are given as fresh weight $\pm$ SD (n = 15 for C_1, 5 for C_2 and 4 for C_3).

differentiated, firm, green tissues of 1 to 1.5 cm^3. At the end of the C_2 phase calli were green-yellow. Callus weight in C_1 and C_2 was not related to the age of the plant (Fig. 1). However, structure, volume, fresh and dry weight of C_3 calli depended on the age of the original plants (fresh weight data in Fig. 1). The tissue volume and fresh weight of C_3 calli from the younger plants increased slowly. These calli turned brown-green with firm apices resembling shoot initiation. Calli from the older plants were friable, pale green, and increased rapidly in weight and volume. On average, the fresh weight of C_3 calli from the oldest plants was 4 to 5 times that of the youngest plants.

The dry matter content of the calli increased with time in the callus of the younger plants, but decreased in the callus of the older plants (Fig. 2). The C_3 calli had a low dry matter content which was correlated with a good suspensibility of their cells.

Effect of mineral nutrition

Nutritional treatments of whole plants of T. patula (see Materials and methods) did not cause visual differences in C_1 and C_2 calli. Tertiary callus, however, showed various degrees of browning and growth retardation related to the nutrition of the original plant (Figure 3). Calli with a Hoagland and Snyder history turned dark brown to black. Those with a $(HM + NO_3^-)$ history became brown, and those with a Steiner history kept growing and were grey to green. The Steiner calli were friable and easily suspensible in liquid MS medium.

The appearance of callus (C_1, C_2 and C_3) of T. minuta did not respond differentially to the nutritional history of the original plants.

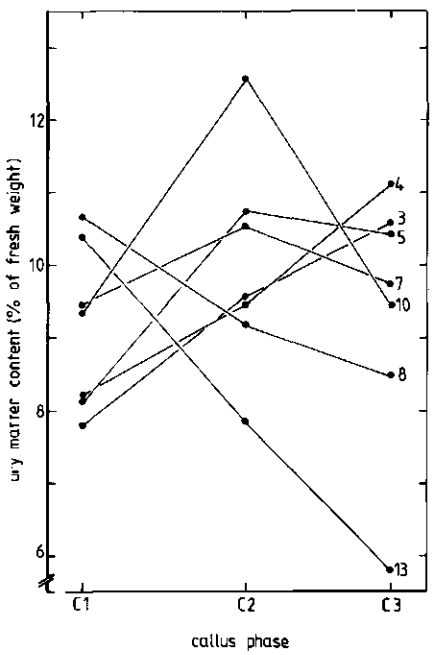

Figure 2. Dry matter content of calli of Tagetes minuta in various phases (C_1, C_2 and C_3). The calli were made of leaf explants of various ages, indicated (in weeks) in the figure.

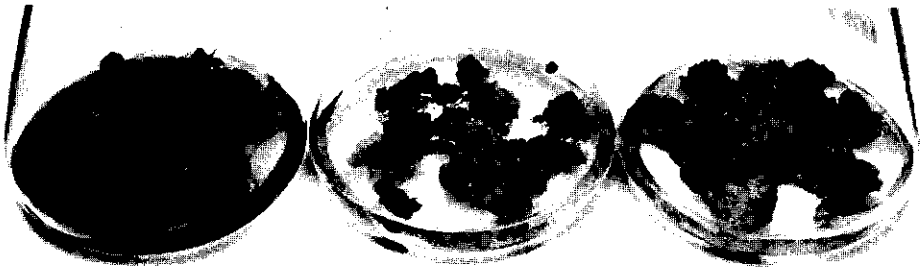

Figure 3. Tertiary callus of Tagetes patula originating from plants exposed to various nutrient solutions: left = Hoagland and Snyder solution, middle = basal medium + $Ca(NO_3)_2$, right = Steiner solution.

DISCUSSION

Callus weight of T. minuta varied considerably within treatments, presumably as a consequence of differences in initial weight, structure, and cross section of leaf explants (Yeoman and Mcleod, 1977). Variation in weight due to plant age, however, significantly exceeded the variation within treatments (Fig. 1). In C_1 and C_2 fresh weight increase was not systematically influenced by plant age, but significant differences in DM content were already noticeable at the first callus passage (Fig. 2). The DM content of the C_1 calli was positively correlated with the DM content of the original leaves, which was highest in the older plants and lowest in the younger plants (data not shown). The differential growth rate in C_3 is not a simple amplification of differences found in C_1, since the trend of higher DM production (data not shown) coincides with a decreasing DM content. The simultaneous increase of fresh weight and dry matter production in the C_3 calli from older plants might reflect a coordinated expression of plant age effects. In general, a low DM content is associated with a friable and readily suspensible callus (Aitchison et al., 1977).

Indeed, C_3 calli of the oldest plants could be excellently suspended, whereas calli of the youngest plants did not dissociate at all upon shaking in liquid medium. The effect of the nutrition of the intact plant in T. patula was also only conspicuous in C_3, not in C_1 and C_2, and this was highly reproducible. Main effects were on colour, texture (Fig. 3) and suspensibility.

For both phenomena reported here constituents originally present at different concentrations in the explants may have become diluted to critical levels that might have influenced growth rate and appearance of the calli. It should be noted, however, that the calli were kept on MS medium and grew well, implying that indispensable substances were readily available. The initial explant tissue had increased 100 to 400 fold in weight and volume by the end of the C_3 phase, indicating that the endogeneous supply of essential growth factors became progressively less important than the supply from the medium. Moreover, of the macronutrients present in the nutrient solutions, only the content of Ca^{2+} and Mg^{2+} differed (by about 20%) among the original leaves of plants from the different solutions, and this difference held for T. minuta as well (data not shown). Trace element supply was identical in all treatments (Table 1).

It is thus more likely that an age-related or nutrition-related property of the original plant, which was hidden for at least 5 weeks, became apparent (so-called memorized) after several callus passages. This aspect may affect the expression of parts of the genome of Tagetes species. However, this putative effect on genome expression seems to vary from one Tagetes species to another. A qualitative memory effect resembling the ones reported here was recently described by Margara and Piollat (1983) in begonia. They noted that explants of

callus, leaves, or roots were able to produce petal-like structures and colours, but only if the original explants had developed *in vitro* from petals. These authors suggest that vegetative tissues can preserve the memory of their floral origin. Other examples of the persistence of whole plant characteristics through cell and tissue culture have been provided by Sánchez de Jimenez and Fernández (1983), who found that calli from leaves and roots differed in glutamate synthase activity when exposed to glutamine as sole N source, but not in MS medium. However, Van Slogteren et al. (1983) found unstable expression of a T-DNA gene in a tobacco cell line transformed by *Agrobacterium tumefaciens*. It was shown that shoots from this line when transferred to media free of growth regulators and containing 5-azacytidine, showed constitutive expression of the unstable T-DNA gene and expression of two silent T-DNA genes as well. According to these authors hypomethylation of DNA may be the cause of switch-on of certain gene activities. It seems that the maintenance of a certain degree of methylation of the cytosine residues in the DNA of the original explant is a candidate for the exertion of memory effects under the altered conditions *in vitro*. It is difficult to trace the observed increase of moisture content and dry matter production in C_3 calli of T. *minuta* back to a particular property in the leaves of the older plants. The same holds for the observed effect of the mineral nutrition of the original *T. patula* plants. We suggest, therefore, that such reproducible effects be called "origin effects". The term "memory effect" might be reserved for the reappearance of particular differentiations present in the history of the callus or the original structure of the explant.

Apart from the significance of memory and origin effects in cultured plant cells and tissues for understanding genetic regulation, the phenomena may have an impact on large-scale production of certain plant cell compounds *in vitro* for commercial purposes, because they may interfere with stabile production. A better understanding of the phenomena, however, might lead to their directional application in practice.

Acknowledgements

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Chapter II

Morphological Differentiation and Occurrence of Thiophenes in Leaf Callus Cultures from *Tagetes* species: Relation to the Growth Medium of the Plants

David H. Ketel
(Physiol. Plant. Vol. 66. pp 392 - 396, 1986)

The morphology and the number and concentration of thiophene-like compounds were studied in leaves, roots and calli of Tagetes species grown with different nutrient solutions. The type of nutrient solution exerted no effect on the number of thiophene-like compounds, but altered the type of morphological differentiation and thiophene content of calli. Calli of T. minuta L. showed little differentiation and resulted in suspensible callus after two passages. Calli of T. erecta L. cv. Rose d'Inde differentiated rapidly and turned dark brown after one passage. The morphology of calli from T. patula L. cv. Nana furia was intermediate. Tertiary callus of T. patula contained more thiophene-like compounds and higher concentrations of them than did the corresponding calli of T. minuta. The content of thiophene-like compounds decreased after various callus passages, but the relative decrease varied between species.

Additional key words - Biocides, HPLC-UV detection, Tagetes erecta, Tagetes minuta, Tagetes patula, thiophenes.

INTRODUCTION

Tagetes species (marigolds) contain biocidal compounds of the thiophene group (Bohlmann et al. 1973, Gommers 1981) as non-polar products of secondary metabolism. Our group has been investigating the production of these biocides by Tagetes cell suspensions as a biotechnological model system. In general the use of plant cell cultures for the production of fine chemicals is hampered by the positive relationship between morphological differentiation and secondary metabolism (Wiermann 1981, Yeoman et al. 1980). At first sight the processes of gradual morphological dedifferentiation to obtain suspensible callus, on the one hand, and of secondary metabolite production, on the other hand, thus seem to be incompatible (Knoop and Beiderbeck 1983).

Preliminary observations (Ketel et al. 1985) showed that appearance and differentiation of Tagetes calli from leaf explants differ between species and are affected by the composition of the nutrient medium in which the original plants were grown.

The purpose of the present study was to examine the morphological differentiation and variation in the occurrence of thiophenes in callus from leaf explants from three Tagetes species grown in three nutrient solutions, in order to assess the validity of the correlation between morphological and chemical differentiation for our model system.

Abbreviations - BMN, basal medium supplemented with $Ca(NO_3)_2$; HPLC, high performance liquid chromatography; MS, Murashige and Skoog (1962) medium.

MATERIALS AND METHODS

Plant cultivation and callus culture

Seeds of Tagetes erecta L. cv. Rose d'Inde (purchased from Wouda Co., Steenwijk, The Netherlands), Tagetes patula L. cv. Nana furia (purchased from Tubergen Co., Lisse, The Netherlands) and Tagetes minuta L. (provided by the Botanic Garden of the University of Nijmegen) were germinated in demineralized water, and grown for 10 weeks in complete nutrient solutions. The solutions were Steiner (1968) solution, Hoagland and Snyder (1933) solution and BMN (Breteler et al. 1979). Plant cultivation is detailed in a previous paper (Ketel et al. 1985).

Each treatment comprised 20 tubes with one leaf explant each. Sterilization of the leaves and culture of primary, secondary and tertiary callus

(grown for 3, 2 and 2 weeks, respectively) proceeded under axenic conditions at 24 ± 1°C and a 16 h photoperiod (5 W m^{-2}) on MS medium as detailed by Ketel et al. (1985). Nine primary calli and halves of the remaining secondary calli were sectioned and subcultured per treatment. Organoid structures were removed before subculturing.

Sampling and chemical analysis

Regenerated roots and shoots were discarded and calli were washed with demineralized water. Roots, leaves and calli were frozen in liquid N_2 and freeze-dried (Durar Dry FTS, Stone Ridge, NY, USA).

One hundred mg of the dried and ground materials were extracted in the dark with cyclohexane for 12 h at 20 ± 3°C. The crude extracts were filtered through cyclohexane-washed quartz wool and a 0.22 µm filter. The filtrate was evaporated to dryness under a stream of N_2 and the residue dissolved in 2.5 ml cyclohexane. The purified extracts were stored at 4°C and analysed within 2 days.

HPLC was done by isocratic elution with hexane-dioxane (95:5 v/v) at a flow of 2.5 ml min^{-1} (Waters model 6000A pump, 10.3 Mpa, Waters µporasil-C18, PIN 27477 column, injection volume 100 µl). The eluate was scanned (190-400 nm) on-line with a Hewlett Packard 1040 high speed spectrophotometer and extinction values at the peak wavelength (330-380 nm) were used to quantify thiophene-like compounds (cf. Tab. 2). All organic solvents were of p.a. quality (Merck). Thiophenes were identified by retention time, UV spectrum (Bohlmann et al. 1973) and mass spectrum (M.A. Posthumus and D.H. Ketel, unpublished results). A standard curve for α-terthienyl (0.3 nM - 3 µM) prepared with our HPLC-UV equipment yielded good linearity at 351 nm. The molar extinction coefficients of natural thiophenes are comparable to those of α-terthienyl (Bohlmann et al. 1973). The HPLC-UV extinction values could thus be used to estimate the contents of thiophenes in the different samples. Nitrogen and inorganic compounds were analysed after digestion (N, K^+, Na^+, Ca^{2+}, Mg^{2+}, $H_2PO_4^-$) or extraction (NO_3^-, Cl^-, SO_4^{2-}) of dried leaf samples as described by Breteler and Hänisch ten Cate (1978).

RESULTS

Plants

Plants grew well on the three nutrient solutions. Only minor differences in yield were observed between nutrient treatments. T. erecta and T. patula grew best on the Hoagland and Snyder solution, whereas T. minuta grew best

on the BMN solution. Nitrogen and inorganic components of the leaves showed no differences between nutrient treatments of more than 10%, except for Ca^{2+} and Mg^{2+}, which differed by ca 20%. Magnesium was lowest in the leaves of BMN plants and highest in the leaves from Hoagland and Snyder plants. Calcium was lowest in the leaves of Steiner plants and highest in the BMN leaves (data not shown).

Callus

Callus induction was visible between 5 to 7 days after explantation in explants of all types. After three weeks the primary calli of T. erecta were highly differentiated, whereas those of T. minuta were not differentiated at all (Tab. 1). Differentiation in calli from T. erecta grown on BMN or

	Differentiated calli/Total calli					
	C_1		C_2		C_3	
	Root	Shoot	Root	Shoot	Root	Shoot
T. minuta						
BMN	0/20 (0)	0/20 (0)	0/9 (0)	0/9 (0)	0/6 (0)	0/6 (0)
Steiner	0/20 (0)	0/20 (0)	0/9 (0)	2/9 (22)	0/6 (0)	0/6 (0)
Hoagland	0/20 (0)	0/20 (0)	0/9 (0)	2/9 (22)	0/6 (0)	0/6 (0)
T. patula						
BMN	0/20 (0)	2/20 (10)	2/9 (22)	1/9 (11)	2/9 (22)	0/9 (0)
Steiner	0/18 (0)	0/18 (0)	2/9 (22)	1/9 (0)	6/9 (67)	0/9 (0)
Hoagland	1/19 (5)	0/19 (0)	1/9 (11)	1/9 (11)	5/9 (56)	1/9 (11)
T. erecta						
BMN	10/19 (53)	2/ 9 (11)	1/9 (11)	0/9 (0)		
Steiner	11/18 (61)	0/18 (0)	3/9 (33)	1/9 (11)		
Hoagland	4/19 (21)	12/19 (63)	2/9 (22)	1/9 (11)		

Tab. 1. Differentiation of roots or shoots from primary (C_1), secondary (C_2), and tertiary (C_3) calli from leaf explants of Tagetes spp. The plants were grown in different nutrient solutions. Differentiation as percentage of total calli is given in parenthesis. Some calli produced both roots and shoots. Evaluation after 21 (C_1), 35 (C_2), and 49 (C_3) days incubation at 24°C in the light.

Steiner solution was mainly root-like, whereas calli from plants grown with Hoagland and Snyder solution formed mainly shoots. Only a few primary calli of T. patula differentiated roots or shoots. Most calli of T. patula, however, had a cauliflower-like structure without clear differentiation. After three weeks, primary calli of T. erecta were brown, those of T. patula remained yellow, and calli of T. minuta were green.

During the second callus phase, the calli of T. erecta turned dark brown and stopped growing. Of 27 calli, the calli of T. erecta had formed 21 organoid structures, whereas these numbers were 9 and 4 in T. patula and T. minuta, respectively. At the end of the tertiary callus phase, nine differentiated organs (mainly roots) were observed in T. patula and no differentiation occurred in the calli of T. minuta.

Non-polar compounds

In the extracts of roots, leaves and calli, at least 16 different compounds were detected with HPLC retention times and UV-spectra similar to those of thiophenes (Fig. 1, Tab. 2). Roots of all Tagetes species contained 2 to 3 thiophenes, while leaves contained 3 to 4 other thiophenes in T. minuta, and only one other thiophene in T. erecta. Although no qualitative effects of the nutrient solutions could be detected the quantitative differences in the amounts of thiophenes can only be ascribed to nutritional differences.

In general, the number of thiophenes in primary calli of T. minuta decreased when compared with the leaves. In contrast, primary calli of T. patula and T. erecta contained a higher number of thiophene-like compounds than the corresponding leaves.

The number of thiophenes in primary and secondary calli from T. minuta plants grown on BMN or Steiner solution did not differ. However, in secondary calli from plants grown in Hoagland and Snyder solution no thiophenes could be detected. The number of thiophenes in secondary callus from BMN-grown T. patula was lower than in primary callus. Secondary callus of T. patula from plants grown on Steiner solution or Hoagland and Snyder solution, however, contained the same number of thiophenes as primary callus. Secondary calli of T. erecta were not extracted.

No thiophenes were detected in the tertiary callus of T. minuta and two thiophenes were detected in tertiary callus from Steiner-grown and BMN-grown T. patula.

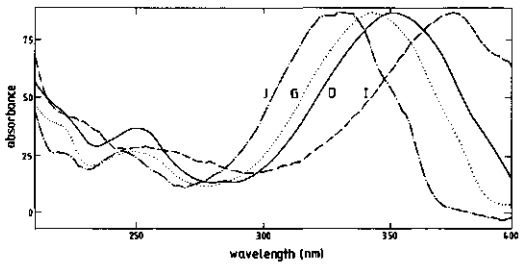

Fig. 1. Extinction spectra of four thiophene-like compounds detected in the cyclohexane extract of leaves of Tagetes minuta with a diode array high speed spectrophotometer. In this example the plant was grown in nutrient solution according to Steiner (1968). Mass spectral analysis of some extracts (M.A. Posthumus and D.H. Ketel, unpublished results) of leaves of Tagetes minuta indicated the apparent agreement of spectra D, G and I with compounds containing sulfur, and molecular masses of 262, 288 and 244, respectively. The molecular mass of the compound with spectrum J was unknown. Extinction values are represented in arbitrary units with the maximum extinctions for each compound equalized.

	Roots	Leaves	Primary callus	Secondary callus	Tertiary callus
T. minuta					
BMN	A153 B 51 C3	D160 G 72 I24 J 7	B 9 K 2	K4 L 3	–
Steiner	A199 B 96 C3	D110 G100 I16 J14	K 3 L 8	K2 L12	–
Hoagland	A106 B 96 C3	D114 G 75 I23	K 7 L13 N3	–	–
T. patula					
BMN	A 95 B178 C6	E 66 H 18	B103 C2 K19 M3 B37	K4	B14 C1
Steiner	A145 B248 C6	E 56 H 25	B 71 K 8 M2	K6 L46 O4	B53 C5
Hoagland	A159 B163 C6	E 62 H 12	B 10 M2	L 5 P39	–
T. erecta					
BMN	A 82 B 87 C2	F 10	B 6 K 3		
Steiner	A 22 B 72	F 21	B 16 C2 K 3		
Hoagland	A 91 B103 C3	F 31	K 2 L 4		

Tab. 2. Occurrence of thiophene-like compounds in roots, leaves and calli of *Tagetes* species, grown in different nutrient solutions. The 16 different compounds are indicated by letters with retention time (min) and peak wavelength (λ max in nm) in brackets: A (1.9, 343); B (3.6, 333); C (6.6, 331); D (1.8, 353); E (1.8, 349); F (1.8, 334); G (3.0, 345); H (3.3, 329); I (3.9, 377); J (14.0, 331); K (2.0, 340); L (3.8, 325); M (2.6, 325); N (4.5, 373); O (6.9, 323); P (4.4, 333). The level of the compounds is indicated by the number of milli-extinction units at λ max per 4 g of dry matter. A horizontal bar means that no thiophene-like compounds were detected. An example of 4 spectra is given in Fig. 1.

DISCUSSION

The morphogenic potency of the calli differed significantly among the three Tagetes species and so did the occurrence of thiophenes. The incidence of organoid structures (Tab. 1) indicates that the calli of T. erecta had the highest morphogenic potency, independent of the nutritional history of the plants. However, the type of morphological differentiation apparently depended to a large extent on the type of nutrition. The contents of Ca^{2+} and Mg^{2+} in

the explants differed by about 20% and this difference may have affected morphogenic expression in the calli. However, the original plant mass was 100 to 400 times diluted in the tertiary calli of T. patula (Ketel et al. 1985) and this renders any direct effect of macronutrients on morphogenesis unlikely. Calli of T. minuta had the lowest morphogenic potency and the friable structure of tertiary calli allowed a good suspensibility (Ketel et al. 1985). The morphogenic vigour of T. erecta has so far prevented the formation of stable cell suspensions of this species. The rapid differentiation of young primary calli of T. erecta could not be suppressed by varying the levels of naphthylacetate (1.3 - 21 μM) and indoleacetate (0.6 - 2.8 μM) in solid or liquid media (data not shown).

The morphogenic potency of calli of T. patula lies in between that of the other two species examined. However, a low frequency of organoid structures (Tab. 1) coincided with a cauliflower-like structure of the calli, indicating some degree of tissue organization. When shaken in liquid media calli of T. patula only dissociated into large aggregates.

Tertiary calli of T. minuta did not contain HPLC-UV-detectable levels of thiophenes. The decrease of the content of secondary metabolites (thiophenes) in calli and cell suspensions (Groneman et al. 1984) relative to intact plants is in agreement with a number of investigations in other plant species (Knoop and Beiderbeck 1983). This impoverishment of the secondary metabolite pattern may be due to the dedifferentiated state of the cells in calli and fine suspensions (Lindsey and Yeoman 1983, Misawa and Suzuki 1982). The behaviour of thiophenes (Tabs 1 and 2) in Tagetes agrees with this explanation.

Typical spectra of some of the 16 thiophene-like compounds that were found in the present experiments are given in Fig. 1. Mass spectrometry of some of the putative thiophenes indicated that their mass and number of S atoms per molecule corresponded with some known di- and trithiophenes (cf. Groneman et al. 1984).

Although the calli were obtained from leaf explants, the thiophene pattern of primary calli resembled that of roots rather than that of leaves (Tab. 2). The biochemical relationship between explants and their calli thus seems to be loosened by the dedifferentiation of the latter. This finding is in line with the observation that calli sometimes contain other secondary metabolites than do explant tissues (Wiermann 1981, Mulder-Krieger et al. 1982). The thiophene pattern in plants and calli, in contrast to morphological differentiation, was qualitatively unaffected by the nutritional history of the plants.

The occurrence of many different and in part yet unidentified thiophene--like compounds and the absence of authentic standards hindered a precise quantification of the thiophenes. However, based on the extinction coefficient of thiophenes (see Materials and Methods) we calculated the relative contents of thiophenes in the dry matter of the different samples (leaf value = 100%). Primary calli of T. minuta had only 4 to 11% of the thiophene content of leaves and this value was 0 to 3% in secondary callus and 0% in tertiary callus. The rapid decrease in thiophene content upon subculturing of callus explains the absence of thiophenes in cell suspensions (Groneman et al. 1984). A large variation occurred in the relative thiophene content of primary calli of T. patula (16 - 156%) and T. erecta (19 - 100%). This variation was related to the nutritional history of the plants, but even the lowest relative contents, found in calli from plants grown on Hoagland and Snyder solution, were significantly higher than in T. minuta. Relative thiophene contents of secondary and tertiary calli of T. patula were 13 to 69%, and 7 to 65%, respectively, and these figures were reproducible.

Alhough secondary calli of T. erecta were not analysed, it was noticed that they turned dark brown, due to the formation of phenols. The compounds were excreted into the solid medium. Phenols are also secondary metabolites and their presence seems to be related to a certain degree of morphological differentiation (Wiermann 1981). The high morphogenetic potency of T. erecta may thus cause toxic levels of phenols in secondary calli.

Notwithstanding variation in morphology and in thiophene patterns within the different combinations of species and nutritional history, the results show that a high degree of morphological dedifferentiation is apparently incompatible with the accumulation of thiophenes. However, in T. erecta and T. patula, morphological dedifferentiation only caused a quantitative decrease of the thiophene content and seemed not to suppress totally the presence of specific enzymes and substrates of the thiophene pathway. The induction of specific stress conditions, may thus intensify the synthesis and accumulation of thiophenes in suspended Tagetes cells. Moreover, new methods that yield stable suspensions of T. patula or T. erecta cells may also lead to thiophene production in suspension cultures. Both aspects are currently under investigation.

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Chapter III

Inorganic Nutrition of Callus Tissues of *Tagetes* species: The Effects on Morphogenesis and Accumulation of Thiophenes and other Non-polar Secondary Metabolites

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SUMMARY

The formation and morphology of callus were studied on leaf explants of Tagetes patula and Tagetes minuta, inserted into three different solid media with the inorganic composition of media used for soilless culture of intact plants, or into MS-medium. Callus formation responded differently to medium type and plant species. On all "plant media" callus formation occurred in only one of the two species. Which species died and which species proliferated depended on the type of plant medium. Growth of calli was curtailed on plant media as compared with MS-medium. However, transfer of secondary calli from plant media to fresh MS-medium restored a high growth rate.

Calli of T. patula had a higher level of non-polar metabolites and thiophenes (natural biocides from Tagetes plants) than calli of T. minuta. In T. patula calli the content of non-polar metabolites increased from primary to secondary callus and was highest when MS-medium was used. In contrast, T. minuta calli showed a decrease in non-polar metabolite content upon subculturing and this content was lowest on MS-medium. These trends of the contents of non-polar secondary metabolites parallelled the trends in morphological differentiation, that were observed when calli were cultured continuously on MS-medium (Ketel, Physiol. Plant. 66 (1986), 392) and confirm the positive correlation between morphological and biochemical differentiation.

It is concluded that the inorganic medium composition affected secondary metabolism only to a quantitative extent and that a proper inorganic composition of a medium together with the choice of the most suitable species are essential in the production of desired secondary metabolites in vitro.

Key words: Callus culture, growth, HPLC-UV detection, inorganic nutrition, morphogenesis, non-polar compounds, secondary metabolites, Tagetes minuta L., Tagetes patula L., thiophenes.

Abbreviations: BMN = basal medium with $Ca(NO_3)_2$; C_1, C_2, C_3 = primary, secondary, and tertiary callus, respectively; DM = dry matter; MS = according to Murashige and Skoog (1962).

INTRODUCTION

Nutrient media for plant growth differ in inorganic composition from those used in axenic cultures in vitro. In the latter cultures, the production of secondary metabolites, characteristic for the intact plant, is generally decreased or absent (Prenosil and Pedersen, 1983; Fuller, 1984). This phenomenon has been ascribed to the mutually excluding processes of growth and differentiation of the cells in vitro (Yeoman et al., 1980; Lindsey and Yeoman, 1983; Fuller, 1984). In Tagetes species, the accumulation of thiophenes (natural biocides) is very much reduced in vitro, particularly in T. minuta (Groneman et al., 1984; Ketel, 1986). The present research addresses the question whether the difference in inorganic nutrition between intact plants and callus tissues contributes to the difference in thiophene accumulation. The morphological differentiation and thiophene accumulation on three media designed for plant nutrition were compared with those on a medium with the inorganic composition as described by Murashige and Skoog (1962), the standard medium for many cultures in vitro.

MATERIALS AND METHODS

Cultivation of plants and callus.

Plants of Tagetes minuta L. (seeds provided by the Botanic Garden of the University of Nijmegen, The Netherlands) and Tagetes patula L. cv. Nana furia (seeds purchased from Tubergen Co., Lisse, The Netherlands) were grown during 9 weeks on BMN (Breteler et al., 1979). According to the inorganic composition of nutrient solutions used for the growth of intact plants (BMN, Hoagland & Snyder (1933) and Steiner (1968), Table 1), media were prepared for the growth of primary calli. A medium, containing macro elements similar to the MS-medium, was used as a control, and to restore the growth of C_2 calli (see Results). All media were supplemented with sucrose (2% w/v), naphthylacetate (0.5 $mg.dm^{-3}$), and benzyladenine (5 $mg.dm^{-3}$), solidified

	BMN	Hoagland & Snyder	Steiner	MS
K^+	11.0	6.0	6.7	20.0
NH_4^+	0	0	0	20.6
Ca^{2+}	10.5	10.0	10.0	6.0
Mg^{2+}	1.0	4.0	4.0	3.0
Cl^-	1.5	0	1.0	6.0
NO_3^-	10.0	15.0	11.9	39.4
$H_2PO_4^-$	1.0	1.0	1.0	1.2
SO_4^{2-}	10.0	4.0	6.9	3.0
total ions	45.0	40.0	41.4	99.2

Table 1: Macro-nutrients (mmol of charge. dm^{-3}) in media used to grow calli of Tagetes species. Trace elements ($mol.dm^{-3}$: 40 Fe^{3+}, 22 $B(OH)_3$, 6 Mn^{2+}, 1 Zn^{2+}, 0.16 Cu^{2+}, 0.04 $Mo_7O_{24}^{6-}$) did not differ between the media.

with Gelrite (0.2% w/v; Costar Ltd., Badhoevedorp, The Netherlands) autoclaved during 20 min at 120 C. The pH was adjusted to 5.9 with KOH prior to autoclaving.

Excised leaves were sterilized 2 sec in ethanol (70% v/v) and 13 min in $CaOCl_2$ (5% w/v), subsequently washed for 3, 10, and 30 min in fresh aliquots of sterile, demineralized water, and inserted into solidified media as described previously (Ketel et al., 1985). Of each species 30 C_1 calli per treatment were grown from leaf explants. After three weeks fifteen C_1 calli per treatment were cut into 3 pieces and subcultured on 40 cm of the different media in 250 cm erlenmeyer flasks. After 2 weeks 8 C_2 calli per treatment were halved and subcultered on MS-medium under similar conditions to obtain C_3 calli. Callus growth was done under axenic conditions at 24°C. Detailed growth conditions are given by Ketel et al. (1985).

Sampling and chemical analysis

Samples of callus were washed with demineralized water, pooled per treatment, frozen in liquid N_2, and freeze-dried (Durar Dry, FTS; Stone Ridge, New York, USA). One hundred mg of the dried and ground samples were

extracted in the dark with 2.5 cm^3 hexane during 12 h at room temperature. The crude extracts were filtered through hexane-washed quartz wool and a 0.22 μm filter. The filtrates were evaporated to dryness under a stream of gaseous N_2 and the residue dissolved in 2.5 cm^3 hexane. The purified extracts were stored at 4°C in the dark and analysed within 2 days.

HPLC of the extracts was done by isocratic elution with hexane-dioxane (95:5 v/v) at a flow of 1.5 cm^3 min^{-1} (Waters model 6000 A pump, 10.3 MPa; Serva 42360 column, Heidelberg, FRG; injection volume 0.1 cm^3). The eluate was scanned (230-400 nm) on-line with a Hewlett Packard 1040 A high speed spectrophotometer. Concentrations were estimated in terms of absorbance units at peak wavelengths (see Table 3). The hexane used was HPLC-grade (Fisons, Loughborough, UK). Sampling, extraction, and analysis yielded variation in the concentrations of analysed compounds between 2 and 6%.

RESULTS

The growth of callus

On BMN the explants of T. patula coloured brown and died, whereas the explants of T. minuta remained green and formed callus. On the Steiner and Hoagland & Snyder media, callus growth on leaf explants of T. patula became visible between 5 and 7 days. At T. minuta explants only roots without callus appeared when a Steiner or a Hoagland & Snyder medium was used. More and better developed roots were formed on Steiner medium than on a Hoagland & Snyder medium (Fig. 1).

Fig 1: Morphological development of 3-week-old leaf explants of Tagetes patula and T. minuta on BMN (I), Hoagland and Snyder medium (II) and Steiner medium (III).

The growth of C_1 and C_2 calli of T. patula on the Steiner and Hoagland & Snyder media, and of the C_2 calli of T. minuta on BMN, fell considerably behind that of the corresponding calli on MS-medium (Table 2). The texture of the C_1 calli that were grown on the "plant media" was less friable than of those grown on MS-medium. The dry matter content of the calli was highest on the plant media.

Time (weeks)	Species and callus phase		BMN	Hoagland & Snyder	Steiner	MS
	T. patula					
1-3	C_1(n=15)	FW:	-	0.45±0.32	0.40±0.23	1.63±0.90
		%DM:	-	13.4	14.8	6.0
4-5	C_2(n=7)	FW:	-	0.81±0.33	1.18±0.73	6.10±1.63
		%DM:	-	13.7	14.4	5.2
				MS	MS	MS
6-7	C_3(n=7)	FW:	-	3.54±2.76	2.82±2.60	11.8±5.24
		%DM:	-	9.8	10.7	4.7
	T. minuta					
1-3	C_1(n=15)	FW:	0.52±0.34	-	-	0.59±0.26
		%DM:	18.0	-	-	11.4
4-5	C_2(n=7)	FW:	0.81±0.30	-	-	3.80±1.74
		%DM:	14.2	-	-	10.4
			MS			MS
6-7	C_3(n=7)	FW:	1.54±0.52	-	-	6.33±1.60
		%DM:	13.6	-	-	9.4

Table 2: Fresh weight (FW, g per callus) and dry matter content (% DM) of primary (C_1), secondary (C_2) and tertiary (C_3) calli of Tagetes patula and T. minuta.
The C_1 and C_2 calli were grown on a BMN, Hoagland & Snyder, Steiner or MS-medium (Table 1). All C_3 calli were grown on MS-medium. No callus growth was obtained on leaf explants of T. patula on BMN and on leaf explants of T. minuta on Steiner and Hoagland & Snyder media. Total duration of the experiment was 7 weeks. FW data are given ±SD.

Upon subculturing, the fresh weight of C_2 calli on MS-medium increased 4 to 6-fold in two weeks. In contrast, the fresh weight of the C_2 calli grown on plant media was about doubled in this period. The latter calli turned brown, and green areas were only maintained in C_2 calli of T. minuta. However, when C_2 calli from plant media were transferred to MS-medium, the resulting C_3 calli grew prosperously again, although at very high variability (see SD in Table 2).

Non-polar compounds

C_1 calli of T.minuta grown on BMN contained one thiophene-like compound (K) and 5 other non-polar compounds (Fig. 2 and Table 3). The same thiophene-like compound also occurred in the C_2 and C_3 calli of T.minuta. The C_1 calli of this species grown on MS-medium, however, contained 2 thiophene-like compounds (E and M_1) that were different from the compound K. The mass-spectrum (M.A. Posthumus and D.H. Ketel, unpublished results) and UV-spectrum of the thiophene E corresponds with the methyl derivative of 5-(3-buten-1-enyl)-2,2'-bithienyl (cf. compound XVI in Bohlmann and Zdero, 1985). The thiophene K most likely corresponds with α-terthienyl, but its UV-spectrum was impure owing to the presence of compound 1 (Fig. 2). In addition, the different UV-spectra showed that different compounds sometimes had a similar HPLC retention time, e.g. K and 1 in Fig. 2. The transfer of C_2 calli from BMN to MS did not change the pattern of the non-polar metabolites (data not shown).

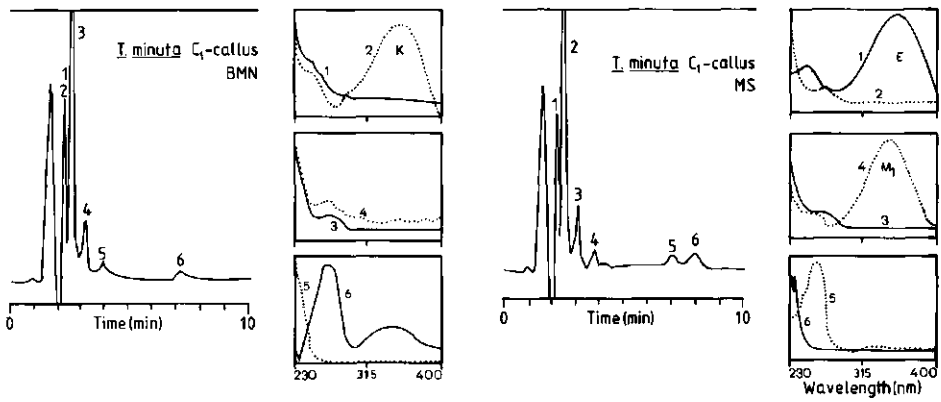

Fig. 2: HPLC chromatograms of hexane-extracts of primary (C_1) calli of *Tagetes minuta* grown on BMN (Table 1) or MS. In addition, the UV-spectra (230–400 nm) of the different compounds are presented to show the occurrence of different compounds in apparently similar chromatograms. Thiophene-like compounds are indicated by letters and indices according to the classification by Ketel (1986).

Independent of the type of mineral nutrition, the number of HPLC-UV detectable non-polar compounds occurring in the C_1 calli of T.patula amounted to 12 (Table 3). Whereas in the C_1 calli of T.patula grown on MS-medium 4 different thiophene-like compounds were found, the C_1 and C_2 calli of this species, when grown on Steiner or Hoagland & Snyder medium, contained only 2 or 3 of such compounds. Only the C_3 calli of T.patula with a previous Steiner nutrition contained 4 thiophene-like compounds, 3 of them having UV-spectra similar to those present in the C_1 calli grown on an MS-medium. The mass-spectrum (M.A. Posthumus and D.H. Ketel, unpublished results) of one of the thiophene-like compounds in the calli of T.patula corresponded with 5-(4-hydroxy-1-butenyl)-2,2'-bithienyl (data not shown; cf. compound XIV in Bohlmann and Zdero, 1985)

	Tagetes minuta				*Tagetes patula*						
	BMN and MS			MS	Steiner and MS			Hoagland and Snyder and MS			MS
	C_1	C_2	C_3	C_1	C_1	C_2	C_3	C_1	C_2	C_3	C_1
I	6	6	7	6	12	12	11	12	13	9	12
II	1	1	1	2	3	2	4	2	3	3	4
III	305	265	221	183	237	282	399	435	486	553	802
IV	19	24	31	49	60	17	191	27	34	197	512

Table 3: Total number of non-polar compounds (I), total number of thiophene-like compounds (II), sum of milli-absorbance units of the non-polar compounds (III), and sum of milli-absorbance-units of the thiophene-like compounds (IV) of primary (C_1), secondary (C_2), and tertiary (C_3) calli of *Tagetes minuta* and *T. patula*. C_1 and C_2 calli were grown on three different media with macro-element composition according to Steiner, Hoagland and Snyder and BMN (Table 1). No calli of *T. minuta* were obtained on Steiner and Hoagland and Snyder media and also no calli of *T. patula* when BMN was used. MS-medium was used as a control for C_1 calli, while all C_3 calli were grown on this medium.

Upon subculturing on the same medium, the concentration of some compounds decreased and sometimes fell below the limit of detection, while the concentration of others increased. Also new compounds appeared after subculturing. These trends differed between media and species. C_1 calli of T. patula grown on Steiner and Hoagland & Snyder media contained approximately a quarter and a half, respectively, of the concentration of non-polar compounds in similar callus grown on MS-medium(Table 3). In contrast, the concentration of these compounds in C_1 calli of T. minuta grown on BMN was about 70% higher than that in those grown on MS-medium. The total thiophene

content in the C_1 calli of T.patula was even more affected by growth on the plant media relative to MS, than the total content of non-polar metabolites. The thiophene content in T. minuta calli was very low and the data therefore do not permit any conclusion on the effect of medium composition. The trend in total non-polar and thiophene-like compounds, given for T. patula, was also manifest upon transfer of C_2 calli from plant media to MS-medium.

DISCUSSION

It is generally accepted that the poor production of secondary metabolites in cell cultures in vitro is connected with the non-differentiated state of dividing cells (Yeoman et al., 1980; Lindsey and Yeoman, 1983; Ketel, 1986). However, taking into account the differences in inorganic composition between media used for cultures in vitro (usually MS-medium) and for intact plants, the inorganic composition of the culture medium may also contribute to the decreased production of secondary metabolites, which occurs especially in T. minuta (Ketel, 1986).

The absence of callus and the formation of roots on leaf explants of T. minuta when grown on a Steiner or Hoagland & Snyder medium are the expression of a high potency to differentiate. The inorganic composition of BMN obviously suppressed root and allowed callus formation. In contrast, the leaf explants of T. minuta on BMN clearly formed calli without organoid structures, even after subculturing of the C_1 calli. Proliferation of cells was suppressed when the leaf explants of T. patula were grown on BMN.

Both the predomination of root production over shoot production in explants of T. minuta, and the very solid texture of the C_1 and C_2 calli (cf. high DM contents, Table 2) of both species may be the result of a so-called auxin effect that can also be the consequence of a relatively low cytokinin activity (Street, 1977; Ahuja et al., 1982). Although little is known about the direct relationship between the inorganic nutrition of callus and the regulation of the activity and the synthesis of auxins and cytokinins, it can be concluded that both the moderate differences between the plant media on the one hand, and the great differences between the plant media and MS-medium, on the other, considerably affected the regulatory mechanism of growth and morphogenesis of the different calli. The reported effects of different nitrogen sources on organ formation of calli (Sánchez de Jiménez and Fernandez, 1983), the requirement of relatively high nitrate concentrations to obtain well-growing and friable calli (Murashige and

Skoog, 1962; Knobloch and Berlin, 1983), and the influence of calcium transport on the activity of indoleacetate in embryogenesis (De Guzman and Dela Fuente, 1984), indicate that particularly the nitrogen source and the concentrations of Ca^{2+} and NO_3^- in the plant media (cf. Table 1) may have affected the morphogenesis and growth of the C_1 and C_2 calli. It is unknown whether differences in the concentrations of K^+ and Cl^- also affected the growth and development of the calli.

A higher number of non-polar compounds, in the calli of T. patula, than in the calli of T. minuta, including some thiophenes, has repeatedly been observed (Table 3; Ketel, 1986 and D.H. Ketel, unpublished results). This difference also held when plant media were used instead of MS-medium.

Upon subculturing, the total content of non-polar metabolites (estimated as sum of peak absorbances; Table 3) increased in T. patula, but decreased in T. minuta. This trend prevailed both with subculturing on plant media and when C_2 calli from plant media were transferred to MS-medium. It is unclear whether the relatively high and low contents of non-polar compounds in C_3 of T. patula and T. minuta, respectively, can be attributed to callus age or to the change in medium. The opposite trends in total non-polar metabolite content in the two species coincide with similar trends in morphological differentiation (see Results and Ketel, 1986). The present observations thus confirm the general positive relationship between biochemical and morphological differentiation. This relationship also holds for thiophenes in particular. The inorganic nutrition of the calli apparently is not a factor that switches thiophene accumulation on or off. The only effect of the difference in inorganic composition between the media tested here seems to be in the quantitative aspects.

In conclusion, the present results show that both the selection of a suitable plant species and an adequate callus medium contribute to the production of desired secondary metabolites in vitro.

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Chapter IV

Distribution and Accumulation of Thiophenes in Plants and Calli of Different *Tagetes* species

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ABSTRACT

The diversity of thiophenes (natural biocides) and the differences between the concentrations of these compounds in the leaves and roots of Tagetes erecta L., T. patula L. cv. Nana furia, and T. minuta L. (marigolds) indicated at least the presence of two different sites of accumulation: leaves and roots. Leaf explants of Tagetes, however, are used by preference to obtain callus cultures. Once subcultured, secondary (C_2) calli of T. patula obtained from leaves of 4 to 7 weeks old plants, contained higher amounts of accumulated thiophenes (up to 80% of the amounts in the leaves) than original (C_1) or twice subcultured calli (C_3). The concentrations of thiophenes in C_2 calli of T. minuta were about half those of C_1 calli, while the concentrations of thiophenes of C_1 calli amounted to 1 to 2% of the leaf values. Most of the C_3 calli of T. minuta did not contain thiophenes at all. Although C_1 calli of T. erecta also contained considerable amounts of thiophenes, the C_2 calli died, most likely owing to high levels of accumulated polyphenolic compounds. The combination of species effects and the physiological state of plants and calli provides adequate information to decide whether Tagetes calli are able to produce thiophenes or not. It is concluded that the ability to produce thiophenes does not depend on the organ used, but on the genetic information present in the species, and on the physiological state of plants and calli, particularly their age.

Keywords: callus, explant selection, Tagetes erecta, Tagetes minuta, Tagetes patula, thiophenes.

Abbreviations: C_1, C_2, C_3 = primary, secondary, and tertiary callus, respectively; DM = dry matter; HPLC = high performance liquid chromatography; MS = according to Murashige and Skoog (1962).

INTRODUCTION

The capability of the original explant to accumulate secondary metabolites may not provide clear indications as to the production of the same metabolites by in vitro cultures (Speake et al., 1964; Zenk et al., 1977; Zieg et al., 1983). This problem was considered with respect to the potential production of thiophenes in calli and cell cultures. Thiophenes had been characterized as natural nematicides (Gommers, 1981), or as wide-spectrum biocides (Arnason et al., 1981; Champagne et al., 1984; Philogène et al., 1985), and frequently occur in the leaves and the roots of different Tagetes (marigolds) species (Bohlmann, et al. 1973).

With respect to the selection of explants to induce the formation of callus, roots are less suitable than leaves; in addition, there is evidence that the stage of development and the physiological state of intact plants also may determine the yield of thiophenes in the different organs and calli (Sütfeld, 1982; Norton et al., 1985). These aspects, and the observation that the selection of the appropriate explants is limited by their ability to survive rigid sterilization procedures and to form friable and dedifferentiated callus (Yeoman and Macleod, 1977; Ketel et al., 1985), prompted research into the relation between the accumulation of thiophenes in roots and leaves of different Tagetes species, and thiophene accumulation in callus from these organs. The ultimate aim of the study was to select criteria for the choice of explants for thiophene-producing calli.

MATERIALS AND METHODS

Plant cultivation and callus culture. Seeds of Tagetes erecta L. and T. minuta L. (both wild types) and of T. patula L. cv. Nana furia were germinated in the dark and seedlings grown (24 $\pm$ 1 ^{o}C, RH 75 $\pm$ 5%, 16 h photoperiod at 30 Wm^{-2}) in a liquid basal medium (Breteler et al. 1979), supplemented with 5 mM $Ca(NO_3)_2$. The species were cultivated in succession. Thirty leaves from at least 6 plants at a range of times from 2 to 14 weeks, were used to perform explants. The excised leaves were surface-sterilized in ethanol (70% v/v) and $CaOCl_2$ (5% w/v) and the upper third part of each leaf section was individually inserted with the cut base into 15 cm^3 MS-medium (Murashige and Skoog, 1962) in glass tubes. The MS-medium was supplemented with sucrose (2% w/v), naphthylacetate (0.5 $mg.dm^{-3}$), and benzyladenine (5 $mg.dm^{-3}$), and solidified with Gelrite (0.2% w/v; Costar Ltd., Badhoevedorp, The Netherlands). Incubation conditions were 24 $\pm$ 1 ^{o}C,

RH 75 ± 5%, and continuous light (5 Wm^{-2}). From 30 primary calli (C_1), obtained 3 weeks after explantation, 15 calli were harvested and 15 calli subcultured on fresh MS-medium during 2 weeks to yield secondary calli (C_2). Five C_2 calli were also harvested and also the remainings subcultured on fresh MS-medium during 2 weeks to yield 10 tertiary calli (C_3). At each subculturing the organoid structures were removed from the calli.

Sampling and chemical analysis. Simultaneously with sampling for explants, washed and blotted roots, and leaves of intact plants were weighed, pooled and cut coarsely. Accurately weighed samples of up to 20 g, varying from ca. 10 to 100% of the total weight of the different organs of the various groups of plants, were frozen in liquid nitrogen and freeze-dried (Durar Dry FTS, Stone Ridge, NY, USA) during 48 h. In addition, harvested calli stripped of organoid structures, were washed with demineralized water, and treated as described above.

Accurately weighed samples (ca 0.1 g) of dried material were dispersed into 5 cm^3 hexane (Fisons, Loughborough, UK) and extracted during 12 h at 20 ± 3 °C in the dark. The crude extracts were quantitatively filtered through hexane-washed quartz wool and a 0.22 µm filter, successively. The filtrates were evaporated to dryness under a current of N_2 and the residues dissolved in 2.5 cm hexane. The purified extracts were stored at 4 °C in the dark and analysed within 2 weeks. All organic solvents used were of HPLC-grade.

HPLC was done by isocratic elution with hexane-dioxane (95 : 5 v/v) at a flow of 2.5 cm^3 min^{-1} (Waters model 6000A pump, 10.3 MPa; Waters µporasil-C_{18} PIN 27477 column; injection volume 100 µl). The eluate was scanned (190 to 400 nm) on-line with a Hewlett Packard 1040 high speed spectrophotometer. The UV-spectra of the extracted compounds were compared with those of previously (GC/MS-) identified thiophenes (M.A. Posthumus and D.H. Ketel, unpublished results). Spectra of putative thiophenes were compared with spectra of other thiophenes and polyacetylenes (Bohlmann and Zdero, 1985). Extinction values were actively recorded in the milliabsorbance range, and assumed to be similar for different natural thiophenes (Norton et al., 1985). The peak wavelengths of the different spectra (ca 320-380 nm) were used to quantify the different thiophenes. A standard of α-terthienyl was used as a reference.

Sampling, extraction and analysis as described here yielded a variation in the concentration of individual thiophenes of 2 to 6%.

RESULTS

Plants. Fresh and dry weight increments of the different Tagetes plants indicated an exponential phase between 4 and 7 weeks followed by linear growth for several weeks (data not shown). The oldest plants of T. erecta (10 weeks) and T. patula (14 weeks) were just flowering, whereas the oldest plants of T. minuta (13 weeks) were still in the vegetative phase at the end of the experiment. The first samples were taken at a seedling stage of ca 0.5 g. per plant (week 0), the final ones at a plant fresh weight of 50 to 70 g. During this period the dry matter content of the leaves increased from 10 to 15%, that of the roots from 5 to 7%.

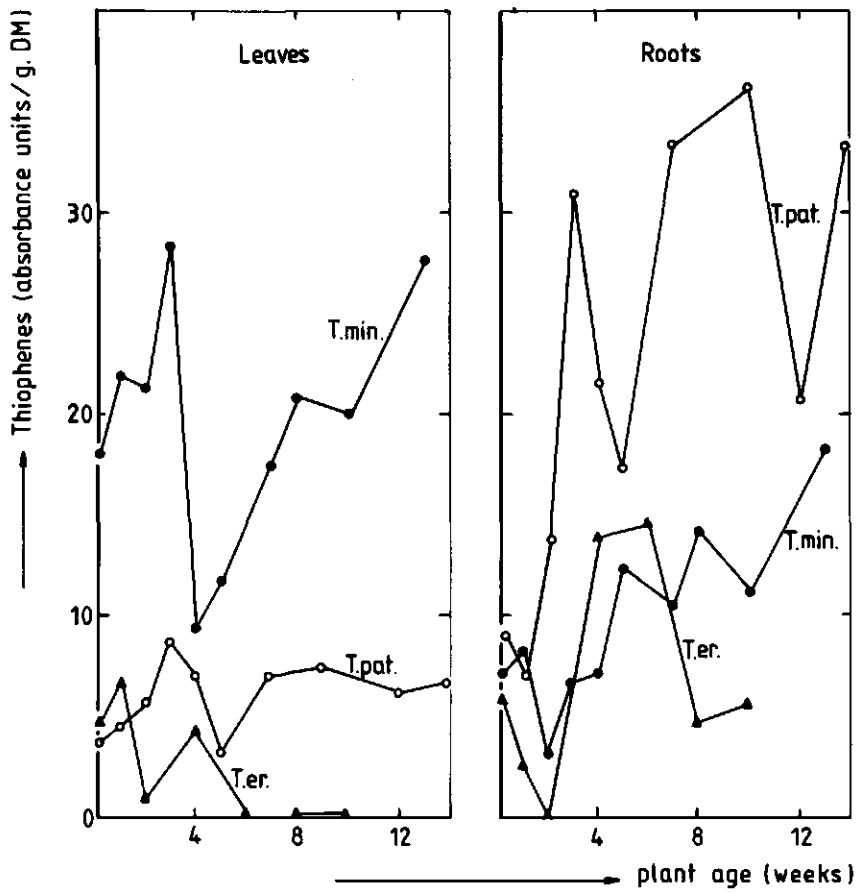

Fig. 1. Total amounts of thiophenes extracted from leaves and roots of Tagetes erecta ( ), T. patula (o), and T. minuta ( ) plants. The absorbance values of the individual thiophenes are given in Table 1. See p. 61.

The occurrence of thiophenes in the leaves and roots was found to vary drastically between the different Tagetes species. Also some thiophenes appeared only incidentally (Table 1). As presented in Figure 1, the total amount of thiophenes in the leaves and in the roots varied considerably and inconsistently. Table 1 further shows that a number of thiophenes only occurs in the leaves or in the roots. In general, the diversity and the content of thiophenes were highest in the leaves of T. minuta and lowest in the leaves of T. erecta. Young plants of T. erecta mainly contained thiophenes in the leaves, whereas in older plants the thiophenes mainly accumulated in the roots. In the roots the diversity and the content of thiophenes were highest in T. patula, whereas for the leaves this was the case with T. minuta.

Callus. Because roots turned out to be unsuitable for callus growth, their analysis had to be abandoned and in the further study only leaf explants were used. Primary leaves of 1 and 1 and 2 weeks old seedlings of T. patula and T. minuta, respectively, did not survive the sterilization procedure. Most of the explants of 4 weeks old plants of T. patula were lost owing to microbial contamination. The remaining C_1 calli were only used to grown C_2 and C_3 calli; therefore, C_1 and C_2 calli of T. patula were not available to perform analysis of thiophenes. After three weeks incubation of the explants, most of the C_1 calli of T. erecta showed organoid structures, mainly roots. The C_1 calli of T. patula were cauliflower-like, green-yellowish and friable, whereas the dark-green C_1 calli of T. minuta were firm and only a few of them showed some small, shoot-like organs. A few days after the C_1 calli of T. erecta were subcultured, the C_2 calli turned dark-brown and stopped growth. C_2 calli of T. patula and T. minuta maintained their typical C_1 structure.

The results of the analyses of thiophenes in the different calli, presented in Table 2, show that only two different thiophenes occurred in the C_1 calli of T. erecta. However, their thiophene content in general surpassed that of the C_1 calli of the other Tagetes species examined.

C_2 and C_3 calli of T. patula maintained relatively high concentrations of thiophenes if compared with C_2 and C_3 calli of T. minuta. Moreover, in general, the concentrations of the thiophenes in C_2 and C_3 calli of T. patula were higher than those in the C_1 calli.

TABLE 1. *Milli-absorbance values of thiophenes in the hexane-extracts of leaves and roots of* Tagetes erecta, T. patula, *and* T. minuta *with respect to the age of the plants*

The absorbance values per 100 mg DM were computed at U.V.-peak-wavelength (c. 320–380 mm) of the extinction spectra. Thiophenes (A–P) are listed according to a system used by Ketel (1986). At week 0 the seedlings were transferred to the nutrient solution.

	Plant age (weeks)	Leaf thiophenes								Root thiophenes							
T. erecta		A_1	B	B_1	F					A_1	B	C_1	F	K			
	0	137	—	2	336					193	—	—	398	—			
	1	128	—	360	189					30	—	58	173	—			
	2	—	—	10	95					—	—	—	—	—			
	4	—	340	—	95					90	590	123	—	600			
	6	—	—	—	—					—	33	463	—	968			
	8	—	—	—	—					—	255	78	—	153			
	10	—	15	—	—					30	103	305	83	55			
T. patula		E	H_1	K	K_1	L	N			G	H_1	I_1	J_1	L_1	M_1	N	P
	0	121	—	226	—	21	4			—	85	—	44	—	—	—	777
	1	163	—	242	18	19	3			—	—	—	142	—	30	—	531
	2	292	—	250	—	26	2			—	18	—	1128	—	—	—	237
	3	95	—	626	92	49	—			—	18	546	2417	—	18	—	93
	4	173	—	496	—	32	7			—	23	104	1921	—	45	—	63
	5	182	—	117	—	40	—			—	29	335	1295	—	—	6	74
	7	250	9	333	—	116	—			4	21	296	2919	12	65	—	42
	9	664	—	—	—	80	2			16	23	745	2636	—	79	2	147
	12	519	—	—	—	98	—			14	23	450	1237	16	47	—	284
	14	494	13	63	—	100	—			10	63	800	2139	25	41	13	226
T. minuta		D	D_1	E_1	F_1	G	G_1	I	K	A	A_1	C	G	I	M		
	0	555	157	—	—	460	—	432	200	700	—	10	—	—	10		
	1	1088	105	—	—	488	—	339	161	540	240	—	25	—	25		
	2	1521	—	31	—	363	—	222	—	125	75	—	125	—	—		
	3	2350	—	60	47	160	—	215	—	435	115	35	65	25	—		
	4	535	—	35	—	190	10	170	—	460	165	20	65	10	—		
	5	795	—	20	—	140	—	220	—	920	85	—	215	10	—		
	7	1325	—	50	—	220	—	150	—	950	—	—	80	10	—		
	8	1590	—	45	25	265	15	130	—	1375	—	—	40	10	—		
	10	1050	—	140	35	520	20	235	—	970	—	—	130	—	—		
	13	1505	—	135	55	1000	—	65	—	1710	—	10	85	—	10		

TABLE 2. *Milli-absorbance values of thiophenes in the hexane-extract of primary (C_1), secondary (C_2), and tertiary (C_3) calli of* Tagetes patula *and* T. **minuta** *and of primary calli of* T. erecta *with respect to the age of the plants*

C_1, C_2 and C_3 calli were grown on MS-medium, supplemented with growth regulators, during 3, 2 and 2 weeks, respectively, and sampled at the time of transfer. The absorbance values per 100 mg were computed at U.V.-peak wavelength (*c.* 320–380 nm) of the extinction spectra. Thiophenes (A–L) are listed according to a system used by Ketel (1986). ? = unknown thiophenes.

	Plant age (weeks)	Callus thiophenes																		
		C_1						C_2						C_3						
T. erecta		C_1	?				Σ													
	1	28	35				63													
	2	—	18				18													
	4	—	13				13													
	6	56	18				74													
	8	28	5				33													
	10	—	8				8													
T. patula		I_1	?	?	H_1	L	Σ	?	?	H_1	L	L_1	Σ	?	?	H_1	I_1	?	L	L_1	Σ
	2	—	—	—	7	87	94	—	—	—	—	—	0	—	—	—	—	3	49	—	52
	3	—	15	—	17	25	57			42	29		71	11	16	28	—	6	86	—	147
	4													—	—	34	173	—	51	3	261
	5	—	7	—	11	17	35	92	8	50	93	13	256	—	5	9	29	—	28	3	74
	7	11	—	—	—	2	13	11	—	41	12	1	65								
	9	—	—	3	2	—	5	—	—	6	—	—	6	2	5	6	—	—	54	4	71
	12	—	5	—	—	—	5	14	—	5	—	—	19	—	—	9	—	—	—	—	9
	14	—	—	—	—	14	14	—	—	—	—	—	0	—	—	—	—	—	—	—	0
T. minuta		A_1	?	B	B_1	D	Σ	B_1	D	E_1	?	G	Σ	B_1	G						Σ
	3	—	25	—	—	15	40	—	5	—	—	5	10	—	—						0
	4	10	—	—	—	—	10	—	5	5	—	—	10	—	—						0
	5	—	—	—	—	10	10	5	5	—	—	—	10	—	—						0
	7	—	—	15	—	15	30	35	10	—	—	—	45	—	—						0
	8	—	—	—	35	10	45	20	5	—	—	—	25	—	—						0
	10	—	—	—	15	30	45	—	5	—	5	—	10	—	—						0
	13	10	—	—	55	—	65	30	5	—	—	—	35	5	15						20

Only one or two different thiophenes occurred frequently in the C_1 and C_2 calli of T. minuta. Conspicuously, compounds B and B_1 in T. minuta calli did not occur in leaves and roots of the original plants. The HPLC retention time and UV-spectra of both substances clearly matched with those of leaf and root thiophenes of T. erecta.

Table 3 shows that the concentration of the thiophenes in the C_2 and C_3 calli of T. patula was high with respect to the concentration of these compounds in the leaves, especially when the age of the original plants amounted to 3 to 7 weeks, when the plants were in their phase of exponential growth, and the thiophene production in the plants was low (Figure 1). In contrast, the concentration of the thiophenes in the C_2 calli of T. minuta was generally lower than that in the C_1 calli, while only two different thiophenes with low absorbance values were detected in the C_3 calli, which were obtained from the oldest plants.

DISCUSSION

The results, presented in Table 1, indicate the presence of at least two different sites of thiophene accumulation in plants of Tagetes species with a normal pattern of development. The differences of the thiophene pattern between leaves and roots of young and older plants, and the variation of the thiophene concentrations suggest that the accumulation of individual and total thiophenes are not related in the two organs. This may be due to a restriction of the transport of the non-polar thiophenes via the aqueous phloem and xylem flows. Apparently, roots and leaves have their own thiophene biosynthesis. The roots turned out not to be able to produce suitable calli in our medium. They might be useful sources for callus growth in other media, but this was not tested by us.

Moreover, comparison of the tables 1 and 2 shows that the thiophene pattern in callus from leaves is not an exact copy of the thiophene pattern in these leaves, but expresses the potency of the whole thiophene-related part of the genome of the entire plant (see also Ketel, 1986). This expression coincides with a lower state of functional and anatomical differentiation of callus (Aitchison et al. 1977) and explains the occurrence of compounds which are not typically accumulated in the original organs (Mulder-Krieger et al. 1982). Therefore, in Tagetes the nature of the origin of the organ from which the explants are derived is not a criterion for the production of the thiophenes in the callus.

	Plant age (weeks)	thiophene concentration in callus / thiophene concentration in leaves (%)		
		C_1	C_2	C_3
T. erecta	1	9	-	-
	2	17	-	-
	4	3	-	-
	6		-	-
	8		-	-
	10	53	-	-
T. patula	2	16	0	9
	3	7	8	17
	4	-	-	32
	5	10	76	22
	7	2	9	8
	9	1	1	10
	12	1	3	2
	14	2	0	0
T. minuta	3	1	0	0
	4	1	1	0
	5	1	1	0
	7	2	3	0
	8	2	1	0
	10	2	1	0
	13	3	1	0

Table 3. Thiophene concentrations in primary (C_1, secondary (C_2), and tertiary (C_3) calli of Tagetes erecta L., Tagetes patula L. and Tagetes minuta L. as percentages of the total thiophene content in the original leaves. Leaf explants were taken from different Tagetes plants which were grown 14 (T. patula and T. minuta) and 10 weeks (T. erecta) on basal medium (Breteler et al., 1979) with 5 mM $Ca(NO_3)_2$). See p. 66.

The pattern of levels of secondary metabolites, in particular thiophenes, in plants (Figure 1) most likely reflects unpredictable periods of variable activity in the synthesis and breakdown of these substances (Schulte et al. 1968, Wiermann 1981, Sütfeld 1982). However, no reflection of the resulting fluctuations of thiophene contents were recovered in the C_1 calli. The sum of the concentrations of the thiophenes in the C_1 calli of T. minuta amounted to 1 to 3% of the sum of the concentrations of the thiophenes in the leaves (Table 3). In addition, the gradual decrease of thiophenes in the C_2 and C_3 calli of T. minuta agrees with the general pattern of the occurrence of secondary metabolites in calli and cell suspensions of dicotyledonous plants (Lindsey and Yeoman, 1985). In the C_1 calli of T. patula the sum of the concentrations of the thiophenes decreased from 16 to 1% of the leaf values with respect to the age of the plants. Whereas leaves of T. erecta did not contain thiophenes after four weeks of culture, the calli from the older leaves continued to produce thiophenes. In conclusion, there is no relationship between the thiophene production of calli and that of the original organ of which explants were obtained.

Nevertheless, the leaf, as the most suitable organ for callus formation, offers good prospects for metabolite production.

The second conclusion, drawn form Table 3, is that the potency of callus to synthesize thiophenes depends on the species (see also Ketel et al., 1985), as well as on the physiological state of the original plants, particularly their age. This includes that, in general, an accurate analysis of secondary metabolites in different callus generations will be necessary to select plant species and explants as suitable candidates for the production of desired compounds with a cell culture system. On the basis of the present results, a cell culture from ca 5-weeks old T. patula plants appears to be a good material for thiophene production.

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Chapter V

Effects of Immobilization and Environmental Stress on Growth and Production of Non-polar Metabolites of *Tagetes minuta* Cells

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ABSTRACT

Tagetes minuta (marigold) cells were entrapped under sterile conditions in agarose, κ-carrageenan, agar and alginate. The effects of different supports on the growth rate of the entrapped cells during incubation for one week under standard conditions [I] were studied. In the second part (week 2 and 3) of the experiment the effects of low temperature (10°C) [II], intermittent N_2 gassing [III], and omission of carbohydrates from the medium [IV] - superimposed on that of entrapment - on growth rate and the production of non-polar secondary metabolites were investigated. Compared to free cells, the impact of agarose on growth during the first week was nil, while the inhibition of growth increased in the order κ-carrageenan, agar, alginate, probably as a result of increasing rigidity of the support.

In the second period the plant cells clearly had reached the stationary phase of the growth cycle in all cases. Again the pattern of growth on agarose closely followed that of free cells, i.e. small increase in case I and III, and a small decrease under the other two conditions. Low temperature [II] had the greatest effect on

cell growth and cell release, probably as a result on gel structure at this temperature. Similar to the effects on growth, the impact on secondary metabolite production was most pronounced in the case of alginate combined with low temperature. Both the omission of carbohydrates, and N_2 gassing resulted in low concentrations of non-polar compounds in the media. The major trend observed was shift away from mainly intracellular compounds in the case of free cells to mainly extracellular compounds in the case of entrapped cells at 10°C.

Keywords: agar, agarose, alginate, κ-carrageenan, entrapment, environmental conditions, immobilization, plant cell suspension, secondary metabolites, Tagetes minuta.

INTRODUCTION

Tagetes species (marigolds) contain a wide variety of organic compounds, including aromatics, for which the plants were already used as a source in the 16th century by the Mayas[1]. Other secondary metabolites, e.g. natural biocides, such as thiophenes[2], also render cell suspensions of this genus potentially attractive for biotechnological production purposes. However, cell suspensions of Tagetes minuta grown on MS-medium[3], do not produce detectable concentrations of non-polar thiophenes.[4] This negative result may be due to the low extent of cytological differentiation[5] and/or the high growth rate of suspended T.minuta cells.[6] Stress conditions, that affect the growth rate of the cells, have been reported to stimulate the synthesis of secondary metabolites.[7]
Therefore we examined the influence of stress conditions, in particular immobilization, on growth and synthesis of non-polar secondary metabolites in cells of T.minuta. Systematic studies with plant cells immobilized in solid supports are scarce[7,8] and no data on the immobilization

of cells from Tagetes or other biocide-containing species are available. In this report the results of the effects of stress, induced by entrapment in different supports (alginate, κ-carrageenan, agarose and agar) and environmental conditions (10°C, periodical N_2 gassing and the omission of carbohydrates from the medium), and interactions thereof on T.minuta cells are presented.

MATERIALS AND METHODS

Cell suspensions

A stock cell suspension of Tagetes minuta L. was grown for almost two years in our laboratory through regular subculturing on MS-medium[3] (4.72 g dm^{-3}), supplemented with myo-inositol (0.1 g dm^{-3}), sucrose (20 g dm^{-3}), N-benzyladenine (5 mg dm^{-3}), naphthylacetate (0.5 mg dm^{-3}) and polyvinylpyrrolidone (MW 40,000; 1 g dm^{-3}). The pH of the medium was brought to 5.8 with KOH solution before autoclaving (20 min, 120°C). The stock cell suspension was cultivated in a 1 dm^3 Erlenmeyer flask, containing 500 cm^3 medium, and subcultured at 2-weekly intervals. The flask was shaken continuously (Gallenkamp Orbital Shaker, 120 rpm, amplitude 2.5 cm) at 24±1°C, in continuous light (5 W m^{-2}), at a relative humidity of 75±5 %. "Standard conditions" in the text refer to these conditions. Preparation of suspensible callus from leaves has been described in an earlier paper.[9]

Immobilization procedures

All manipulations with plant cells were performed under axenic conditions. Prior to entrapment, the cell suspension was sieved (grid diameters 5.0, 2.8 and 1.4 mm) using an NaCl solution (9 g dm^{-3}). Only the fraction smaller than 1.4 mm was used.

A sodium alginate solution (type Kelmar, Kelco Division of Merck, USA; 36 g dm^{-3}), autoclaved at 115°C for 20 minutes, was mixed with the cell suspension to a final alginate concentration of 24 g dm^{-3}. The mixture was extruded dropwise into a stirred 0.2 M $CaCl_2$ solution. The beads were left for 2 h in the aerated $CaCl_2$ solution before transfer to fresh MS-medium. The same procedure was used with a solution of sodium-κ-carrageenan (Genugel X-0828, Copenhagen Pectin Factory, Denmark; 30 g dm^{-3}), autoclaved at 121°C for 20 minutes, whereby the final κ-carrageenan concentration, after mixing with the cells, was 20 g dm^{-3}. These beads were hardened in a 0.2 M KCl solution. For entrapment in agar (Difco Laboratories, Detroit, USA) and in agarose (type VII, Sigma, St. Louis, USA), solutions containing 75 g dm^{-3} of each of these supports were autoclaved at 121°C for 20 minutes. Solutions were mixed with the cells at 44°C and 33°C, respectively, until final support concentrations of 50 g dm^{-3} were obtained. Agar and agarose, were extruded dropwise into a continuously shaken flask which contained refined and cooled (5°C) soy oil. The beads were separated from the oil and washed with an NaCl solution (9 g dm^{-3}) before transfer to MS-medium. The fresh weight of the cells with respect to fresh weight of the beads of the different supports varied between 14 an 27%.

Other experimental procedures

After one week of storage in a 1 dm^3 flask (containing 500 cm^3 medium) under standard conditions, entrapped and free cells, and cell-free beads were divided over eighty 250 cm^3 flasks, each containing 45 cm^3 of fresh MS-medium and an accurately weighed amount of beads (10-15 g) or free cells (4-5 g fresh weight). From the remaining cultures samples were taken to determine fresh and dry weight. The flasks were divided over twenty treatments of 4 flasks each, and kept for 2 weeks under standard con-

ditions, 10°C, 6 h of N_2 gassing per day, and no carbohydrates. One flask of each treatment was retained as a spare in case of microbial contamination. The experiment was finished 2 weeks after the cells and the beads were exposed to the different environmental conditions. At that moment the beads, the free cells or, in the case of cell release, a mixture of beads and released cells were separated from the media by filtration, and washed with demineralized water. Beads were separated from released cells by sieving. Fresh and dry weight (freeze-drying for 48 h) were determined so that the dry weight increment of the cells in the beads could be calculated.

The loading factor (volume of cells/total bead volume) was determined after 0, 1, and 3 weeks. The difference in weight between loaded and non-loaded beads yielded the cell fresh weight, and on the assumption that the specific density of fresh cells is 1 g cm^{-3}, the cell volume was calculated. In addition, the mean diameters of the beads were measured and used to calculate the total bead volume.

Analytical procedures

Filtered media were shaken for 12 h at 20±3°C in the dark with double the volume of hexane (HPLC-grade, Fisons, Loughborough, UK). Samples (100 mg) from the various dried and ground cells and beads were extracted with aliquots of 2.5 cm^3 hexane, and the crude extracts were filtered through hexane-washed quartz wool. The filtered hexane extracts of cells, beads, and media were evaporated to dryness under a flow of N_2 gas and the residues were dissolved in 1 cm^3 of hexane.

HPLC was performed by isocratic elution with hexane:dioxane (95:5, v/v) at a flow of 1.5 cm^3 min^{-1} (Waters Model 6000A pump, 10.3 MPa, Serva Silica column

42360, Heidelberg, FRG). The injection volume was 0.1 cm^3. The eluate was scanned (215-400 nm) on-line with a Hewlett Packard 1040A high-speed spectrophotometer. The signal wavelengths of the detector were routinely set at 225, 270 and 350 nm, so that essentially all non-polar compounds that were present in the extracts could be detected simultaneously. Eluted peaks were classified on the basis of their UV-spectra and retention times. If a peak with a characteristic spectrum occurred at least twice in the three replicates of similarly treated material, and the light absorbance was ten times higher than that of the background (cell-free control beads), the signal in question was listed as a compound.

RESULTS AND DISCUSSION

The process of entrapment

Entrapment of the cells took about 3 h for each support to yield 350 g of homogeneous beads. The beads had an initial diameter of 2.8 mm and a fresh weight of about 11 mg. The dry weight of cells in the loaded beads was in the range 0.1 mg (in agar) to 0.2 mg (in κ-carrageenan) per bead at the start of the experiment. Under the axenic conditions employed for the preparation of beads no infection occurred during the first week of the experiment and from the 80 subcultures used in the second and third week, only 5 had to be discarded because of microbial infection.

Growth

During the first week of incubation dry weight of the free cells increased 15-fold (Table 1), corresponding with an average doubling time of 1.8 days. Entrapment in agarose did not affect the dry weight increment of the cells. Entrapment in the other supports inhibited cell growth and the dry weight increment decreased in the

Table 1 Dry weight increment (DW) of cells of *Tagetes minuta* and loading factor (×100, LF = volume of cells/total bead volume) of the cells and beads of different supports at the start, after 1 week and after 3 weeks under different conditions as given in the Table. DW at week 1 is given relative to that at $t = 0$, and DW ± standard deviation ($n = 3$) at week 3 is given relative to that at week 1. LF is given in absolute values. In treatments with entrapped cells DW data refer to entrapped cells only. Released cells were discarded

		Incubation time (weeks)					
		0	1	3			
			Environmental conditions				
			Standard	Standard	10°C	N_2 gas	No carbohydrates
Free cells	DW	1.0	15.0 ± 2.0	2.0 ± 0.3	0.7 ± 0.3	2.7 ± 0.3	0.8 ± 0.2
Agarose	DW	1.0	15.0	1.7 ± 0.4	0.8 ± 0.1*	1.3 ± 0.1	0.5 ± 0.1
	LF	16	74	79	56	79	68
κ-Carrageenan	DW	1.0	9.2	0.6 ± 0.0*	1.5 ± 0.1	0.8 ± 0.1*	0.6 ± 0.0*
	LF	20	68	57	70	67	58
Agar	DW	1.0	5.4	0.7 ± 0.0	0.8 ± 0.1	0.3 ± 0.1	0.4 ± 0.0
	LF	14	48	41	32	20	37
Alginate	DW	1.0	2.3	0.9 ± 0.1	1.5 ± 0.1	0.8 ± 0.1	0.4 ± 0.0
	LF	27	46	43	46	41	35

*Considerable amounts of cells were released from the beads. The weight of released cells was not taken into account for the calculation presented here (see Results and discussion)

order κ-carrageenan, agar, alginate. The increment of the loading factor of the beads decreased in the same order, but not proportionally as result of increasing bead diameters. The ratio between the increase of the loading factor and the increase of the dry weight was highest in alginate and lowest in agarose (Figure 1), indicating that the rigidity of the alginate[10] is highest. This rigidity of alginate may very well result in a certain strain on the plant cells leading to the stimulation and excretion of secondary metabolites, a phenomenon especially observed with alginate-entrapped plant cells, e.g. L-DOPA by cells of Mucuna pruriens.[11] It seems unlikely that the diffusion limitation of oxygen is a factor that caused the difference in increment of the dry weight of cells in the different supports, as it has been reported[12]. It is likely that transport of oxygen in all the supports that we examined is of comparable magnitude. Recent experiments with T.patula cell aggregates of various sizes also point to another diffusional limitation than that of oxygen, in the production of thiophenes.

In the second experimental phase (weeks 2 and 3) the cell growth rate was much lower than in the first phase. In free cells the dry matter increment during weeks 2 and 3 varied between 0.7 and 2.7 (Table 1), on the average corresponding with a doubling time of 18 days. As

expected, these cells had reached their stationary growth phase. This was also the case for the entrapped plant cells which mostly showed a decreased rate of increase in dry cell weight. Accordingly, in most treatments the

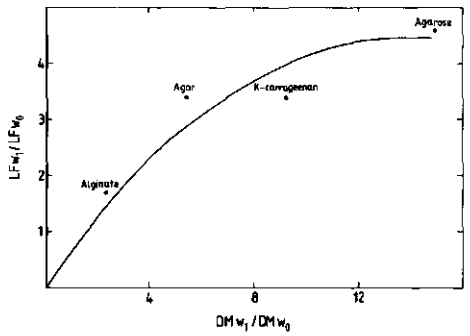

Figure 1. Relationship between the relative increase of dry matter (DM_{w1}/DM_{w0}) of cells of Tagetes minuta and of the loading factor (LF_{w1}/LF_{w0}) of beads with these cells during the first week of the experiment. The cells were entrapped in different supports as indicated in the figure. Suffixes w1 and w0 refer to the values measured after 1 week, and at the beginning of the experiment, respectively. The line of best fit was calculated with a second degree polynome.

cells turned yellowish at the end of the experiment, indicating chlorophyll breakdown and cell aging, the initial cell suspension being dark green.

During weeks 2 and 3, "growth" in agarose most closely followed that of the free cells for all 4 conditions. Cell release from this support was observed at 10°C. For κ-carrageenan, on the other hand, only at 10°C was no cell release observed. Also with respect to growth the treatment at 10°C had the greatest impact. At 10°C growth was only observed in cells entrapped in alginate and κ-

carrageenan. This all indicates a different gel structure at 10°C, which can be expected with these types of gels.

In the treatments indicated with an asterisk in Table 1, cells were released from the beads, and survived as single cells and small cell aggregates. This has been used by Morris and Fowler[13] to produce fine cell suspensions, and invalidates an absolute comparison of cell growth rates between all treatments. The fresh weight of cells released from agarose beads at 10°C was 2.1 times that of the entrapped cells after 3 weeks. This ratio was 3.8, 2.0, and 1.5 in κ-carrageenan under standard conditions, N_2 gassing and omission of carbohydates, respectively (data not shown).

Secondary metabolites

At the end of the experiment cells grew slowly, if at all. Accumulation of secondary metabolites, which has been postulated to occur especially during the last part of the growth cylcle,[14] thus could be studied with little interference of differences in cell growth. At the end of the experiment, cells and media were analyzed for non-polar compounds. Twenty-two presumptive compounds with a retention time varying between 1.6 and 13.8 minutes, and absorption peaks between 215 and 400 nm were consistently found in cells and media (data not shown). Compounds released into the medium were more polar (high retention time) than intracellular compounds (low retention time). Cells entrapped in agarose, agar and alginate contained no compounds with a high polarity (retention time exceeding 5 min). None of the compounds showed a UV-spectrum corresponding with that of thiophenes.[6,15]

When the number of non-polar compounds in entrapped cells is compared with that in free cells, it is clear that a shift occurs from intracellular compounds in case of free cells, to extracellular compounds for the

entrapped cells (Table 2). In agreement with the findings for growth, entrapment in agarose has the least impact on the number of compounds released into the medium. On the average, the concentration of non-polar compounds in the medium increases in the order of κ-carrageenan, agar, alginate. The stimulation of the number and the concentration of non-polar compounds in the medium by alginate entrapment may be related to the rigidity of this support, which has been suggested to stimulate the accumulation of other secondary metabolites as well[11]. When the effect of different conditions is considered, the 10°C treatment caused the most pronounced increase of the number and concentration of the non-polar compounds in the medium. Not mentioned in Table 2 are the following more specific details. Entrapment in κ-carrageenan and alginate led to the accumulation of a cellular compound that was not detected in free cells. In addition, entrapment led to at least 3 (in agarose) and at most 7 (in alginate) compounds in the medium that did not occur in the medium of free cells. Free cells produced a lower total (cells + medium) concentration of non-polar compounds than entrapped cells, when both were grown at 10°C. In a medium without carbohydrates the total content of non-polar compounds was low to zero, except for entrapment in alginate. The concentration of non-polar compounds was also low in the N_2-gassed media. The similarity in the effect of N_2 gassing and omission of carbohydrates on the concentration of non-polar compounds in the medium suggests a relation between cell respiration and the release of non-polar metabolites to the medium. The content of non-polar compounds in the medium was drastically increased by entrapment. The total content of non-polar compounds in the medium was highest in alginate entrapment at 10°C. In this treatment and in κ-carrageenan at 10°C the highest number of non-polar compounds was found in the medium.

Table 2 Number and concentration of non-polar compounds in cells and media of free and entrapped cells of *Tagetes minuta* under conditions indicated in the table at the end of the experiment (3 weeks after entrapment). The total concentration of the compounds is tentatively given in absorbance units (at peak wave lengths) per g dry cell mass or per dm^3 medium

		Number of non-polar compounds		Concentration of non-polar compounds	
		Cells	Medium	Cells	Medium
Free cells	A*	7	1	8	2
	B	6	2	177	3
	C	10	0	9	0
	D	8	0	95	0
Agarose	A	3	2	513	10
	B	6	3	62	396
	C	5	0	17	0
	D	5	0	93	0
κ-Carrageenan	A	7	1	2562	3
	B	6	6	23	356
	C	5	1	68	47
	D	7	0	76	0
Agar	A	4	1	373	2
	B	5	3	42	643
	C	4	1	388	7
	D	5	1	1220	1
Alginate	A	4	4	167	22
	B	4	6	16	713
	C	3	4	15	23
	D	4	2	24	29

* A, B, C and D refer to the standard conditions, low temperature (10°C), N_2 gassing and the omission of carbohydrates in the medium, respectively

CONCLUSION

Evidence from the literature supports the hypothesis that a negative relationship exists between the growth rate of cell suspensions and the production of secondary metabolites.[6,16,17] Combining the data on growth and on secondary metabolites presented here, it is clear that in T.minuta cells not all compounds are affected to the same degree or in the same direction by the retardation of growth. Obviously none of the conditions used in the described experiments could replace the natural stimuli for cytological and biochemical differentiation of T.minuta cells, which result in the synthesis of thiophene-biocides. Further systematic investigations on the effect of entrapment and environmental conditions are needed for a specific stimulation of synthesis and/or release of these compounds.

Recent experiments with another Tagetes species (T.patula[18]) showed that natural cell immobilization due to aggregation[19] may be an alternative for artificial entrapments.

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Chapter VI

Accumulation of Thiophenes in Tissues of *Tagetes* species Genetically Transformed with *Agrobacterium* species

David H. Ketel and Mark Gibbels

SUMMARY

Agrobacterium tumefaciens en A. rhizogenes strains (wild-type and mutant strains with respect to the biosynthesis of auxin and cytokinin) were used to inoculate the stem of in vitro grown axenic plants of Tagetes erecta, T. patula and T. minuta to study the accumulation of thiophenes in neoplastic tissues. Untransformed cells of primary and secondary explants of axenic stems, grown on MS-medium with and without appropriate amounts of exogenously supplied growth regulators were used as controls. In addition, a plasmidless strain of A. tumefaciens was used as a control.

The formation of crown galls and tumours with or without rhizoid differentiations ('roots') was in agreement with general differentiation patterns of calli of these plants, but also implied contrasts with respect to the growth of neoplastic tissues on the stem of other dicotyledonous plants. Although oncogenes clearly stimulated the growth of tumours and roots, no nopaline or octopine dehydrogenase activity could be detected in such tissues.

Thiophene-biocides accumulated mainly in 'roots', indicating that the coherence between morphogenesis and secondary metabolism also occurred in transformed tissues. After one passage the thiophene content in 'roots' showed a greater decrease than that in tumours or calli. This trend was pronounced significantly in tissues infected with wild-type A. tumefaciens. In this material the thiophene concentration was higher in unorganized tissues than in organized tissues. Our results show that genetic transformation opens perspectives to study growth and morphogenesis of unorganized tissues with an intact mechanism to synthesize thiophenes, and may be used to obtain 'root' cultures in vitro with a high degree of stability.

INTRODUCTION

The occurrence of thiophene-biocides in different calli of Tagetes erecta, T. patula, and T. minuta (Marigolds) makes the use of phylogenetically related tissues attractive to study the production of secondary metabolites in solid and liquid culture systems. Under standard conditions (MS-medium supplemented with appropriate amounts of growth regulators; Ketel et al., 1985) calli of T. erecta show a strong potency to differentiate, in contrast to calli of T. patula and T. minuta. On the one hand, the observed difference can be used to study the relation between tissue morphogenesis and the biosynthesis of thiophenes in calli, but, on the other hand, from the calli of T. erecta it is difficult to initiate a liquid culture system.

Friable and undifferentiated tissues of T. erecta can be obtained from crown galls appearing after infection of intact plants with Agrobacteria (Riker and Hildebrandt, 1951). Therefore, genetic transformation with this type of bacteria may yield tissues of T. erecta suitable for liquid cultures. Although unorganized tissues often do not accumulate secondary metabolites (Yeoman et al., 1979; Ketel, 1986[b]), the capacity for secondary metabolism, particularly the biosynthesis of thiophenes, is still present in tumours of T. patula (Norton et al., 1985). This contrast prompted us to investigate: (1) the interaction of Agrobacteria with Tagetes species; (2) the effect of genetic transformation on the occurrence and accumulation of different thiophenes in neoplastic tissues.

Key words: Agrobacterium rhizogenes, Agrobacterium tumefaciens, calli, crown galls, differentiation, DNA-transformation, growth regulators, stem explants, Tagetes erecta, Tagetes minuta, Tagetes patula, thiophenes, tumours.

Abbreviations: BA: benzyladenine; DM: dry matter; MS-medium: according to Murashige and Skoog (1962); NAA: naphthylacetic acid.

MATERIALS AND METHODS

Culture of axenic plants.

Axenic plants were cultured from sterilized seeds of Tagetes erecta L. cv Rose d'Inde (purchased from Wouda Co., Steenwijk, The Netherlands) and Tagetes patula L. cv nana furia (purchased from Tubergen Co., Lisse, The Netherlands) of which the corolla was removed, and from sterilized seeds of Tagetes minuta L. (wild type, and originally provided by the botanic garden of the University of Nijmegen, The Netherlands). The seeds were sterilized with ethanol (70% v/v; ca 30 sec) and $CaOCl_2$ (1% w/v; ca 30 min) and subsequently washed with sterile demineralized water for 10, 20 and 30 min. Only sunken seeds were used, and four plants per tube (16 x Ø 2.5 cm, 15 cm^3 MS-medium) were grown. The MS-medium was supplemented with sucrose (2% w/v), and Gelrite (0.2% w/v; Costar Ltd., Badhoevedorp, The Netherlands), and autoclaved for 20 min at 120 °C (pH adjusted to 5.9 with KOH prior to autoclaving). During germination the seeds were kept in the dark for 5 days at 24 ± 1 °C. The seedlings were grown at 24 ± 1 °C, 80 ± 5% RH, and a 16 h photoperiod (10 Wm^{-2}) for 4 weeks. At that time the thickness of the stem of the plants allowed pricking with a needle without severe damage. In order to avoid root damage, pricking with needles was performed while the plants remained in situ in the tubes. Sixteen plants of each Tagetes species were used per treatment. The treated plants were kept for 8 days under the same conditions as before.

In the text the terms 'root', crown gall, tumour and callus are used to indicate a transformed tissue with a rhizoid structure, a transformed tissue with an undifferentiated neoplastic structure on the stem of an intact plant, a transformed tissue with an undifferentiated neoplastic structure grown on MS-medium, and an untransformed undifferentiated tissue also grown on MS-medium, respectively.

Bacterial strains.

Plants were infected with wild-type and mutant strains of Agrobacterium species listed in Table 1. Plants treated with the avirulent strain of A. tumefaciens LBA 288, and axenic plants were used to control bacterial virulence and genetic transformation, respectively.

Strain	Plasmid	Opine	Neoplastic tissue
A. tumefaciens:			
LBA 288	plasmidless	-	-
LBA 4001	pTi Ach5	octopine	crown gall, rough
LBA 4060	pTi AL108	octopine	crown gall + shoots
LBA 8370	pTi T37	nopaline	crown gall + teratomata
A. rhizogenes:			
LBA 9402	?	mannopine	hairy roots

Table 1. Characteristics of wild-type and mutated Agrobacterium strains (Hooykaas, 1979) and morphology of tumours on Kalanchoë daigremontiana. The selected strains were used to examine the production of thiophenes in genetically transformed tissues obtained from infected plants of different Tagetes species.

The culture of explants.

Smooth stems and stems with calli and neoplastic structures as 'roots' or crown galls at the infection site were cut in 3 to 4 cm segments, containing the infection sites. The explants of each tube were transferred to one petri-dish (Ø 9.4 x 1.6 cm), which contained 25 cm^3 solid MS-medium. All explants with bacterial infection were transferred to MS-medium, supplemented with sucrose, Gelrite, and carbenicillin (0.1 mg cm^{-3}: Sigma, St. Louis, U.S.A.). Axenic explants were divided into 2 similar groups and were transferred to MS-medium supplemented with 0.5 $mg.dm^{-3}$ NAA and 5 $mg.dm^{-3}$ BA, or to MS-medium without exogenous growth regulators. Before transfer the control explants were incised over their total length to improve the condition for a wound reaction.

The explants were cultivated during 3 weeks under the same condition as before (phase I). Before transfer to MS-medium all the neoplastic structures, and half of each tumour were removed from the plants. Where only clusters of roots had appeared at the infection site, the remainders of the stems were removed and the root masses were cut into two approximately equal parts. To obtain two approximately equal parts of the tumours and 'roots' the attached parts of the stem were removed and a transversal cut was made through the gall tissue. One part of the tumours and 'root' masses was used for analysis and the other part was subcultured during 2 weeks (phase II) under similar conditions as in phase I. To compare the amounts

of neoplastic tissues and calli in phase I and II (Table 3) the fresh weights of the different tissues obtained in phase II were multiplied by 2.

All treatments with plants and explants were executed with sterilized tools and under sterile conditions.

Sampling and analysis.

At the end of phase I and phase II the harvested 'roots' and tumours were washed with demineralized water, and blotted. After these treatments the fresh weight of the different tissues per explant was determined. Per treatment small samples of the different tissues from each explant were pooled, weighed and dried (80 ^{o}C, 48 h) to determine the DM content. The remaining parts of the tissues were also weighed, frozen with liquid nitrogen and minced. Thiophenes were extracted with acetone (ca 5 cm^3 per g fresh weight) in the dark during 24 h at 20 $\pm$ 3 ^{o}C in continuously shaken vials. After extraction, the acetone phase was quantitatively decanted, filtered through hexane-washed quartz wool, and evaporated with a current of N_2 up to a final volume of approximately 0.5 cm^3. Hexane (5 cm^3, HPLC grade) was added to the residues and the biphasic solutions were shaken again during 1 h. Subsequently, the non-polar (hexane) and polar (aqueous acetone) phases of the solutions were separated with a pipette and the hexane fractions (which contained the thiophenes) were filtered quantitatively through a 0.22 m filter. The filtrates were evaporated to dryness under N_2 gas, and the residues were dissolved in 0.25 cm^3 hexane.

Thiophenes were separated and characterized with HPLC (injection volume of the samples 0.1 cm^3) in association with a diode array detector (200 - 400 nm) as described by Ketel (1986[a]). The concentrations of the different thiophenes were calculated in terms of absorbance-units per g DM at the peak-wavelengths between 320 - 380 nm.

Sampling, extraction, and analysis of thiophenes yielded a variation between 2 and 6%.

Octopine and nopaline in roots and calli were determined according to the method described by Otten and Schilperoort (1978).

RESULTS

Growth of plants and explants.

Responses to infection on the intact plants were different for the three Tagetes species. Plants of T. minuta and T. patula showed a higher frequency of response at the site of infection than plants of T. erecta (Table 2). Apparently, similar effects occurred when the different Tagetes

	Plant species:								
Strains of	T. erecta			T. minuta			T. patula		
Agrobacterium	a	b	c	a	b	c	a	b	c
Control	12	0	-	16	0	-	12	0	-
LBA 288	12	3	r*	16	0	-	9	0	-
LBA 4001	12	7	cg+r	15	12	cg	12	10	cg
LBA 4060	12	6	r	14	11	cg	.	.	.
LBA 8370	12	0	-	13	9	cg	.	.	.
LBA 9402	12	7	r	14	11	r	.	.	.

Table 2. Number (b), and type (c) of neoplastic tissues formed on the site of infection of a number of plants (a) of different Tagetes species and caused by wild type and mutant strains of Agrobacterium tumefaciens and A. rhizogenes. Parent plants were cultured from sterilized seeds and kept under axenic conditions for 4 weeks. The neoplastic tissues formed were crown galls (cg), roots (r), or roots with a terminal swelling (r*). The points (.) in the table refer to not executed treatments.

species were treated with A. tumefaciens LBA 4001 and A. rhizogenes LBA 9402. However, fewer crown galls and more 'roots' were formed on T. erecta plants. In contrast to the results with other species (Table 1), only roots were formed when T. erecta plants were treated with LBA 4060 (Fig. 1) and LBA 288 (control), and no neoplastic outgrowth appeared after treatment with LBA 8370. In agreement with expectation crown galls appeared on T. minuta plants in the cases of LBA 4060 (Fig. 1) and LBA 8370 infection, and, apparently, no response was observed after treatment with LBA 288. Pricking with sterile needles did not result in newly formed tissues in each of the Tagetes species.

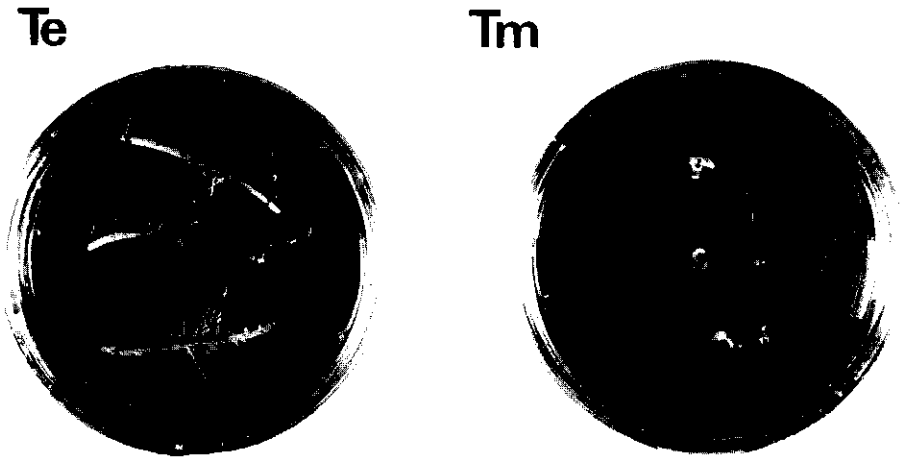

Fig. 1. Neoplastic outgrowth of cells on stem explants of Tagetes erecta (Te) and T. minuta (Tm) due to infection with Agrobacterium tumefaciens LBA 4060.

At the end of phase I the formation of 'roots' on the explants of T. erecta exceeded the formation of 'roots' on the explants of T. minuta (Table 3). The magnitude of this difference varied between the bacterial treatments. A. rhizogenes LBA 9402 induced only 'roots', and continuous growth of 'roots' was observed in the successive phases of the experiment as was expected. The fresh weight of 'roots' per explant of T. erecta and T. patula infected with LBA 4001 surpassed the fresh weight of the 'roots' of similarly treated T. minuta explants approximately 10 times. The predominant crown gall formation upon LBA 4001 treatment, observed on intact plants of T. patula and T. minuta, continued during phase I and phase II. After treatment with LBA 4060 and LBA 8370 the trend of tumour formation of the explants of T. erecta and T. minuta in phase I was the same as on the intact plants. The amounts of calli formed during phase I and II on the control explants which were provided with exogenously supplied growth regulators, was in agreement with the growth of Tagetes calli under standard conditions.

Owing to an abundant growth of initially released bacteria of the strain LBA 9402, all explants infected with this strain had to be discarded.

Treatment:	Tagetes erecta						Tagetes minuta						Tagetes patula					
	phase: I			II			I			II			I			II		
	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c
Control + horm.	6	0.52	0	6	1.23	0.12	6	0.28	0	6	0.74	0	6	0.94	0	4	3.49	0.02
Control - horm.	6	0	0.24	6	0	0.37	6	0	0.01	6	0	0	6	0	0.02	5	0.64	0.54
Control LBA 288	9	0	0.11	6	0	0.79	16	0	0.04	11	0	0.02	9	0	0.05	8	1.65	0
LBA4001	7	0.16	0.11	6	0.84	0.03	12	0.22	0.01	12	1.07	0	10	0.48	0.09	7	4.59	0.23
LBA4060	6	0	0.29	6	0	2.50	11	0.12	0.04	9	1.81	0.02	.	.	.	.	.	.
LBA8370	12	0	0.03	9	0	0.03	9	0.16	0.03	9	1.58	0	.	.	.	.	.	.
LBA9402	7	0	0.45	.	.	.	11	0	0.26	.	.	.	.	.	.	.	.	.

Table 3. Fresh weight of tumours and 'roots' obtained from stem explants of Tagetes erecta, T. minuta and T. patula at the end of two passages (phase I and phase II). The duration of phase I and phase II was 2 and 3 weeks, respectively. Before explantation the stems of the different plants were inoculated with different wild-type and mutant strains of Agrobacterium tumefaciens and A. rhizogenes (see Table 1). Axenic explants and explants treated with plasmidless cells (LBA 288) were used as controls. The letters a, b, and c represent: number of explants (a), the fresh weight (in g) per tumour, (b); the fresh weight (in g) of roots per explant, (c).

Neoplastic tissues in phase I and II showed particular differentiations; conspicuous 'root' formation occurred in all treatments of T. erecta with the exception of LBA 4001 and LBA 8370 (Table 3). After treatment with LBA 4001, LBA 4060, and LBA 8370 and with hormones, the formation of calli and tumours on explants of T. minuta increased during phase II, while root formation decreased like in the control with LBA 288. The development of calli, tumours and 'roots' in T. minuta from phase I to phase II deviated from the two other species for as far as the incomplete comparison with T. patula allows the conclusion. In general, callus and tumour development was stronger after both of the treatments as well as in the two controls of T. patula during the whole experiment. In addition, 'root' formation at T.

patula did not follow the pattern of 'root' formation at T. erecta and T. minuta; in the control without hormones there is similarity between T. patula and T. erecta, whereas in the treatment LBA 288 there is similarity between T. patula and T. minuta. Finally, 'root' formation in T. patula treated with LBA 4001 was stronger than in the same treatment in T. erecta and T. minuta.

Nopaline, octopine, and thiophene analyses.

No evidence was found for the presence of nopaline or lysopine dehydrogenase activity in extracts of calli, tumours, and roots at the end of phase II.

The number of presumptive thiophenes found in calli, tumours, and 'roots' of the different explants at the end of phase I varied from 4 to 15 (Table 4). The on-line spectroscopy with the diode array detector showed reproducible shapes of the UV-spectrum of the thiophenes in calli within the tretaments (Fig. $2^{A,B}$). A low reproducibility of the shape of the UV-spec-

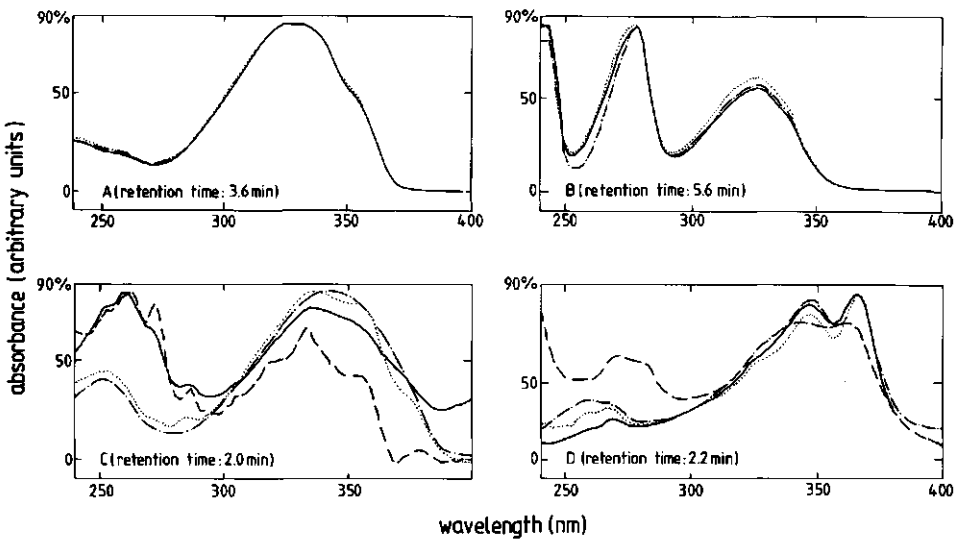

Fig. 2. UV-spectra of four thiophenes which occurred in calli (compounds A and B) and tumours (compounds C and D) of Tagetes erecta. The figures give an example of the difference between the reproducibility (n=4) of the UV-spectra of compounds which occurred in both genetically untransformed (A and B) and genetically transformed (C and D) tissues.

Treatment:	Tagetes species	Number of putative thiophenes				Sum of absorbance units per g DM				R/T	
		Phase: I		II		I		II		I	II
		Tumour	Roots	Tumour	Roots	Tumour	Roots	Tumour	Roots		
Control											
+ horm.	T.e.	5	-	7	8	13	-	21	67	-	3.2
	T.m.	4	-	1	-	12	-	2	-	-	-
	T.p.	11	-	8	3	37	-	15	37	-	2.5
Control											
- horm.	T.e.	-	9	-	6	-	41	-	26	-	-
	T.m.	-	5	-	-	-	149	-	-	-	-
	T.p.	-	9	14	11	-	112	94	121	-	1.3
Control											
+ LBA 288	T.e.	-	9	-	9	-	53	-	48	-	-
	T.m.	-	8	-	1	-	61	-	2	-	-
	T.p.	-	10	14	-	-	102	51	-	-	-
LBA 4001	T.e.	4	8	3	1	3	77	16	6	25.8	0.4
	T.m.	7	5	2	-	8	87	13	-	10.9	-
	T.p.	9	10	7	6	11	56	13	7	5.1	0.5
LBA 4060	T.e.	-	12	-	5	-	45	-	16	-	-
	T.m.	9	10	4	1	6	118	3	7	19.6	2.3
	T.p.	.	.	.	.	.	.	.	.	.	.
LBA 8370	T.e.	-	8	-	1	-	82	-	1	-	-
	T.m.	6	8	5	-	9	116	1	-	12.9	-
	T.p.	.	.	.	.	.	.	.	.	.	.
LBA 9402	T.e.	-	15	.	.	-	44	.	.	.	.
	T.m.	-	10	.	.	-	17	.	.	.	.
	T.p.	.	.	.	.	.	.	.	.	.	-

Table 4. Number and concentration of thiophenes in absorbance units at peak-wavelength of the different compounds in 'roots' (R), and tumours and calli (T) obtained from stem explants of Tagetes erecta (Te), T. minuta (Tm), and T. patula (Tp). Stems of axenic plants were inoculated with wild-type and mutants of Agrobacteria (see Materials and methods). Axenic explants and explants treated with plasmidless bacteria (LBA 288) were used as controls. Phase I and phase II refer to two consecutive periods of growth (2 and 3 weeks, respectively) of the explants after subculturing.

tra of compounds with identical retention time, was observed in tumours within the treatments (Fig. $2^{C,D}$). Thiophenes accumulated in the tissues of the different controls were not consistently found in the tissues from the explants of the transformation treatments. 'Roots' derived from LBA 9402 and LBA 4060 infected plants contained a higher number of thiophenes than axenic tissues (Table 4). In phase I, both the number, and the total concentration of thiophenes in 'roots' exceeded these in the corresponding tumours and calli. At the end of phase II the tissues contained thiophenes which generally showed the same retention-time and UV-spectrum as those in the corresponding tissues harvested at the end of phase I. However, especially in 'roots' both the number and the total concentration of thiophenes were drastically lower at the end of phase II, and thiophene concentrations in calli and tumours at the end of phase II tended to be higher than in the corresponding 'root' tissues, in particular in tumours that resulted for LBA 4001 transformation. However, for a part the results with T. minuta show an opposite tendency.

DISCUSSION

Axenic plants of Tagetes erecta, T. minuta, and T. patula infected with wild-type and mutant strains of Agrobacterium tumefaciens and A. rhizogenes responded differently at the site of infection (Table 2). In particular, on T. minuta the induction of crown galls and 'roots' was in agreement with infection response reported for many dicotyledonous species, e.g. Kalanchoë daigremontiana (Hooykaas, 1979) and Nicotiana tabacum (Van Slogteren, 1983). It is well known that LBA 4060 and LBA 8370 bacteria induce tumours on which shoots and teratomata appear, respectively. These differentiations depend on species and culture conditions (Joos et al., 1983). Therefore, the absence of shoots and teratomata on Tagetes plants infected with the strains used is no indication of a deviating response. However, the formation of 'roots' upon treatment with LBA 4060, observed on T. erecta explants (Fig. 1), has not been reported previously, to our knowledge. The potency of T. erecta to form 'roots' upon wounding is shown in the control treatments. This potency makes particularly conceivable why the negative response to the LBA 8370 treatment in our material is due to suppressed 'root' formation. Obviously, 'root' formation is the only developmental pathway as a response to hormonal stimulation. A similar effect of LBA 8370 has been observed on potato tuber discs treated with NAA; it was the only A. tumefaciens strain that suppressed 'root' induction upon infection of the discs (Ch.H. Hänisch ten Cate, unpublished results).

Besides the typical effects of the bacterial strains on Tagetes plants, spontaneous root formation of T. erecta plants and, in contrast, the formation of unfriable crown galls on T. minuta plants, reflect variation of differentiation tendencies between species. Also tissue cultures on various media show strongly different reactions of T. erecta as compared to T. minuta, and T. patula (Ketel, 1986[a]). In genetically-transformed tissues the acitivity of oncogenes as well as that of residing genes may contribute simultaneously to growth and morphogenesis of neoplastic tissues. In addition, because nopaline and lysopine dehydrogenase activity was not detected in the neoplastic tissues, it seems that the capability of such tissues to grow in the absence of added growth regulators in the medium is the only evidence of transformation. However, even this property may be exhibited by untransformed tissues, a phenomenon known as habituation. It is most unlikely, however, that no T-DNA is present anymore in the final cultures in our experiments. The use of DNA-probes as a conclusive method to establish the integration of T-DNA in the eukaryotic genome should provide final evidence of genetic transformation.

The consistency of the response to infection in the consecutive phases of the experiment (Table 3) indicates that the ratio cytokinin/auxin was not drastically changed during the phases I and II. This phenomenon is of biotechnological relevance with respect to the improvement of the stability of plant cell cultures, in general. In addition, in treatments where 'roots' were formed rather than shoots - particularly in the controls LBA 288, MS-medium without growth regulators, and LBA 4060 treatments - the synthesis of cytokinin would be low with respect to the synthesis of auxin (Reinert et al., 1977; Weiler and Spanier, 1981). The relationship, however, between the amount of synthesized phytohormones in cells and the activity of genes coding the synthesis of these substances, is not clear. Moreover, the formation of untransformed roots on leaf explants of T. minuta inserted on media with relatively low nitrate and high calcium concentrations (Ketel, 1986[a]) apparently caused a similar balance between cytokinin and auxin as transformation did, e.g. with LBA 4060 bacteria. The conclusion is drawn that, in contrast to regulation under environmental conditions which mostly coincides with unsuitable growth conditions, genetic transformation may give similar effects under suitable growth conditions.

Table 4 shows that in phase I the 'roots', i.e. the differentiated tissues, contained the highest amounts of thiophenes if compared with tumours, i.e. the undifferentiated tissues. When 'roots' and tumours were present simultaneously, it was calculated that the concentration of accumulated thiophenes in differentiated tissues surpassed the concentration of accumulated thiophenes in undifferentiated tissues 5 to 25 times. This pattern of thiophene accumulation occurred before and after transfer of the explants. This phenomenon indicates that the coherence between morphogenesis and secondary metabolism, recently emphasized in particular for T. erecta by A.F. Croes (unpublished results), apparently occurs also in transformed tissues. Phytohormones are then suitable candidates for a regulatory role in differentiation patterns.

After one passage of the various explants the difference between thiophene accumulation in differentiated and undifferentiated tissues became approximately 5 to 8 times smaller (Table 4). However, after infection with LBA 4001 thiophene accumulation mainly occurred in undifferentiated tissues. This trend was never observed in calli of Tagetes before and is also in disagreement with numerous observations in many other species (Yeoman et al., 1979; Kothari and Chandra, 1986). Our finding opens the possibility that genetic transformation of DNA may also be used as a simple method to change radically the original relationship between morphogenesis and secondary metabolism.

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Chapter VII

Accumulation of Thiophenes by Cell Cultures of *Tagetes patula* and the Release of 5-(4-Hydroxy-1-butinyl)-2,2′-bithiophene into the Medium

David H. Ketel

ABSTRACT

Secondary and tertiary calli of Tagetes patula were used to start liquid cultures. Both types of calli contained thiophene-biocides, mainly BBT and BBTOAc, but secondary calli contained about 3 times higher concentrations of thiophenes than tertiary calli.

Batch-wise cultured cells formed coarse cell aggregates (3-8 mm), the volume of which was doubled each 15 days (μ ca 5% d^{-1}).

In contrast to callus cultures grown on solid medium, cell aggregates grown in liquid medium released thiophenes. The release into the medium was at least 2.3 $mg.dm^{-3}$ in 90 days by an average amount of 100 g of fresh cell aggregates. Release of thiophenes was mainly as BBTOH. In the liquid medium the ratio between the amount of water-soluble BBTOH and the sum of the amounts of non-polar thiophenes (BBT and BBTOAc) varied between 2 and 4, whereas this ratio was less than unity in the cell aggregates. The amount of thiophenes recovered in the medium corresponded to 0.02% of the cell dry weight.

The productivity of the liquid cultures was not related to the productivity of the original calli; neither did the thiophene production correspond with the growth rate of the biomass of the liquid cultures. The results indicate that not the selection of calli for thiophene content, but rather the selection of calli with the ability to form cellaggregates is of relevance for the production of thiophenes in liquid cultures.

INTRODUCTION

Thiophenes, secondary metabolites occurring in composites like Tagetes (marigolds) species (1), are wide-spectrum biocides (2). Their identification as the nematicidal principle in a number of species (3,4,5) opened perspectives, on the one hand, to employ thiophenes to suppress nematode populations in soils and crops and, on the other hand, to use callus and cell cultures for the production of thiophenes. Analysis of calli of three Tagetes species (T. erecta, T. minuta and T. patula) indicated that only secondary and tertiary calli of T. patula yield suitable material for thiophene-producing cell cultures (6). I here report on the accumulation of thiophenes in the calli of T. patula when grown on solid medium, and present the first results of a liquid-culture system that releases relatively high amounts of thiophenes into the medium.

Abbreviations: BBT: 5-(but-3-en-1-inyl)-2,2'-bithiophene; BBTOAc: 5-(4-ace--toxy-1-butinyl)-2,2'-bithiophene; BBTOH: 5-(4-hydroxy-1-butinyl)-2,2'-bi--thiophene; C_1, C_2, C_3: primary, secondary and tertiary calli, respectively; DM: dry matter; MS-medium: according to Murashige and Skoog, (8); FW: fresh weight; PCV: packed cell volume; PCV%: PCV as % of total volume; μ: relative growth rate.

MATERIALS AND METHODS

Explants.

Plants of Tagetes patula L. cv Nana furia (seeds from Tubergen Co., Lisse, The Netherlands) were grown during 5 weeks in basal medium (7) supplemented with 5 mM $Ca(NO_3)_2$, in 16 h light (30 Wm^{-2}) at 24 $\pm$ 1 °C and 75 $\pm$ 5% RH. Forty fully-expanded leaves were randomly collected from ten plants, sterilized for 2 sec in ethanol (70% v/v) and 5 min in $CaOCl_2$ (5% w/v), and subsequently washed for 3, 10, and 30 min in fresh aliquots of sterile, demineralized water. The top third parts of the leaves were inserted upright into autoclaved (20 min, 120 °C) MS-medium (8), containing sucrose (2 % w/v), naphthylacetate (0.5 $mg.dm^{-3}$), benzyladenine (5 $mg.dm^{-3}$) and myo--inositol (100 $mg.dm^{-3}$), and solidified with Gelrite (0.2 % w/v; Costar Ltd., Badhoevedorp, The Netherlands), and adjusted to pH 5.9 with KOH prior to autoclaving, as previously described (9).

Callus culture and experimental design.

Callus culture proceeded under axenic conditions at 24 ± 1 °C and a 16 h photoperiod (5 Wm^{-2}) in Erlenmeyer flasks (250 cm^3), each containing 30 cm^3 solidified medium. Three successive series were grown for 3, 2, and 2 weeks, hereafter designated as C_1, C_2, and C_3 calli, respectively.

Out of 36 C_1 calli that developed axenically on the 40 explants, 5 were taken at random to determine fresh and dry weights. The remaining calli were cut into three equal parts; two parts were transferred to fresh solid medium to produce C_2 calli, while the third part (approximately 30 mg DM) was used for the assay of thiophenes (see Table 1; n=31). The 2 pieces of callus were put in one Erlenmeyer flask. One of the 2 pieces of the 16 C_2 callus lines was used for thiophene assay (see Table 1; n=16). Both pieces of callus of 5 and 10 lines were used to determine fresh and dry weights, and for starting two liquid C_2 cultures, respectively. With respect to both liquid cultures the C_2 calli of five Erlenmeyer flasks with the lowest thiophene content found in the parental C_1 callus were combined to start one culture (C_2-L). Similarly, the C_2 calli of five Erlenmeyer flasks with the highest content found in the parental C_1 callus were combined to start the other culture (C_2-H).

The remaining parts of the 16 C_2 callus lines were cut into three equal parts to produce C_3 calli and also transferred (3 per Erlenmeyer flask) to fresh solid medium. Pools of C_3 callus lines were made from five callus lines with the lowest thiophene contents in the C_1 and C_2 calli and from five callus lines with the highest contents to start two new liquid cultures: C_3-L and C_3-H, respectively. Of each callus line 2 pieces of callus were selected as starting material for these two cultures, while the remaining parts were used for thiophene determination (see Table 1; n=11). Four C_3 callus lines containing 3 pieces of callus, and one C_3 callus line containing 2 pieces of callus were used to determine fresh and dry weights.

Liquid cultures.

Pools of 'low' and 'high'-producing C_2 (ca. 15 g FW) and C_3 (ca. 20 g FW) callus lines as described above were minced with a scalpel. The minced calli were suspended in about two volumes of liquid medium, with the composition given under 'Explants', but without Gelrite. The 4 cultures were continuously agitated (Gallenkamp Orbital Shaker, 120 rpm, 2.5 cm amplitude) and kept at 24 ± 1 °C under continuous light (5 Wm^{-2}). During the first 9 days, the medium was refreshed daily to cope with an intense browning. From day 9 on,

the refreshment was every two days and the packed cell volume, PCV (10), was determined simultaneously.

C_2-cultures (ca 50 cm^3 initial total volume) were started in 250 cm^3 Erlenmeyer flasks (a), and subsequently grown in 0.5 (b) and 1 dm^3 (c) Erlenmeyer flasks. Under the conditions a, b, and c, the final total volume of the cultures was ca 125, 250 and 500 cm^3, respectively, and the cell volume was kept at 30, 50 and 50% (v/v), respectively. The C_3-cultures were initiated in 0.5 dm^3 flasks and next transferred to 1 dm^3 flasks in which they were treated as the C_2-cultures. In each size of Erlenmeyer flask the cultures were grown for 15 days.

When the total volume of the cell cultures in 1 dm^3 flasks had attained 0.5 dm^3, 60 cm^3 of the cells were transferred to a new flask and replenished with fresh liquid medium, up to a total volume of 0.5 dm^3 (ca 14 PCV%). Twice a week the agitation of the cultures was stopped to determine the PCV of the cell mass, and to refresh 250 cm^3 liquid medium. When the PCV in such a flask was 250 cm^3, about 60 cm^3 of cells were again transferred to a new flask, thus maintaining growth cycles between 14 and 50 PCV%. Parts of decanted liquid medium and cells were used for thiophene analysis.

Extraction of thiophenes.

All extraction procedures were performed at room temperature, and dim light was used only during sample manipulation. When the up-scaling procedures were finished, and growth cycles from 14 to 50 PCV% in 1 dm^3 flasks were started, samples of 100 cm^3 of decanted and paper-filtered liquid medium were taken upon refreshment of that medium (usually twice a week). These samples were extracted overnight at 22±2 °C by shaking with 30 cm^3 hexane. The hexane fractions, containing the released thiophenes, were evaporated to dryness under a current of N_2 and the residues dissolved in 0.25 cm^3 hexane.

Washed, blotted, and weighed samples of harvested calli and cell aggregates were used to determine the DM content after freeze drying. Simultaneously, other samples (1-2 g FW) of the calli and cell aggregates, treated similarly, were frozen with liquid nitrogen and dispersed in acetone (ca 5 cm^3 per g fresh weight). The frozen tissues were crushed with a pestle and shaken overnight. The extracts were quantitatively filtered through hexane-washed quartz wool and the water-acetone mixtures evaporated under a current of N_2 to a final volume of ca 0.5 cm^3. Five cm^3 hexane were added to the residues and the vials were shaken again for 1 h. The water-acetone phase in the vials was removed with a pipette and the hexane fraction con-

taining 50 ± 5% of the total amount of thiophenes, was filtered (0.22 μm) and evaporated under a current N_2 to dryness. Each residue was dissolved in 0.25 cm^3 hexane. The various compounds showed small differences of recovery which did not affect the final results.

Analysis of thiophenes.

HPLC was done by isocratic elution with hexane:dioxane (95:5 v/v) as previously described (11). The eluate was scanned (220-400 nm) on-line with a high-speed spectrophotometer (Hewlett Packard 1040 A detection system).

The computed UV-spectra and HPLC retention times of the different substances as well as the GC/MS analysis of a number of naturally occurring and purified thiophenes (M.A. Posthumus and D.H. Ketel, unpublished results) were used to characterize thiophenes in calli, cell aggregates and liquid media.

In addition, based on the only small differences between the molar extinction coefficients of the prepondering thiophenes (12; R. Jente, personal communication), a standard curve of α-terthienyl was used to calculate the concentrations of thiophenes, both in calli and cell aggregates and released into the liquid medium. Sampling, extraction and analysis as described here yielded a variation in the concentration of individual thiophenes of 2-6%.

RESULTS

Callus cultures.

Leaf explants formed cauliflower-like calli, with low DM content (on the average 7.2, 6.0, and 5.8% in C_1, C_2 and C_3 calli, respectively). In particular, the friability of the C_2 and C_3 calli allowed easy mincing of these tissues to initiate liquid cultures.

Mainly 4 different thiophenes (Fig.1) occurred in the various callus phases, BBT and BBTOAc being the prepondering compounds (Table 1). The high content of BBT in C_2 calli caused a higher total thiophene content in these calli than in C_1 and C_3 calli. The increase was ca 2 and 3 times, respectively. Table 1 further shows the great variation of the contents of the thiophenes. This variation in the C_1 and C_2 calli (Table 2) was used to select 'high' (H) and 'low' (L) callus-lines as described under 'Materials and Methods", and eventually to obtain 4 different liquid cultures, indicated as C_2-H, C_2-L, C_3-H, and C_3-L. Variation in the total content of thiophenes between the individual calli within each group was less than 10 percent.

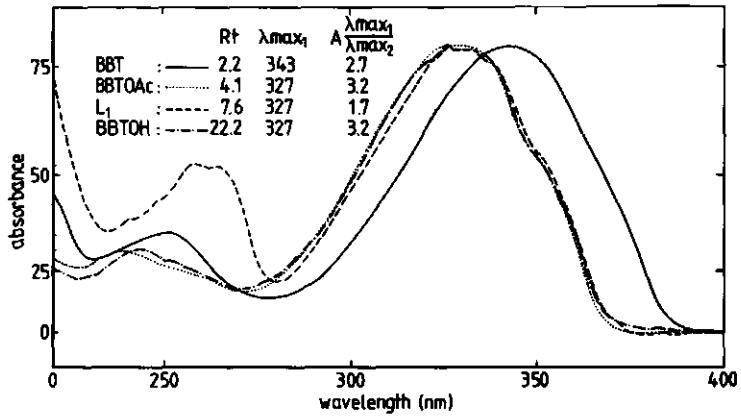

Fig. 1. Normalized absorption spectra of BBT, BBTOAc, BBTOH and L_1 (an unidentified thiophene, provisionally listed according to a system used by Ketel (11) to compare putative thiophenes) extracted from calli and liquid cultures of Tagetes patula (see also Tables 1 and 2). Rt and λ max, in the figure indicate the HPLC retention times (min) and peak wavelengths (nm), respectively. The absorbance is given in arbitrary units.

Callus phase	Thiophenes (µg per gDM ± SD): BBT	BBTOAc	BBTOH	L_1	Total
C_1 (n = 31)	4.5 ± 4.5	29.5 ± 18.5	3 ± 1.5	1 ± 0.5	38
C_2 (n = 16)	44 ± 24.6	27.5 ± 14.5	1.2 ± 0.5	1.5 ± 1	74
C_3 (n = 11)	6.5 ± 2	19 ± 8.5	1.5 ± 0.5	1 ± 0.5	28

Table 1. Thiophene accumulation in primary (C_1), secondary (C_2), and tertiary (C_3) calli of Tagetes patula grown on solid MS-medium under standard conditions (see Materials and methods) for 3, 2, and 2 weeks, respectively.

Ultimate liquid culture	Preceding callus	Thiophenes (µg per g DM): BBT	BBTOAc	BBTOH	L_1	Total
C2-H	C_1	3	46	4.5	1	54.5
	C_2	66	40	1.5	2	109.5
C2-L	C_1	1.5	12	3	0.5	17
	C_2	20	17	1.5	1.5	40
C3-H	C_1	7.5	47	1.5	1	57
	C_2	51	45	1.5	2.5	100
	C_3	5	38	2	3	48
C3-L	C_1	1.5	6.5	3	0.5	11.5
	C_2	31.5	8	1	0.5	41
	C_3	5.5	11.5	1	1	19

Table 2. Thiophene contents in secondary (C_2) and tertiary (C_3) calli of Tagetes patula used to obtain two 'high' (H) and two 'low' (L) producing liquid cultures. C_2 and C_3 liquid cultures were initiated from 5 minced calli each. The calli used were selected according to the results presented in Table 1.

Liquid cultures.

Initially, rapid browning of the growth medium urged daily washing of the submerged callus fragments and refreshment of the medium during the first weeks of liquid culture. After 3 weeks the frequency of medium refreshment could be gradually decreased to 3 times a week. The removal of dark-brown

cell aggregates and the loss of small cell aggregates during these refreshments impeded reliable measurements of the real volume increment of the biomass during the preliminary phases of the cell cultures in 0,25 and 0,50 dm^3 Erlenmeyer flasks.

Although the loss of cell aggregates during medium refreshment was considerably reduced when the cultures were grown in 1 dm^3 Erlenmeyer flasks, the volume of the sedimented cell aggregates (Fig. 2) could not be assessed with high accuracy. The calculated doubling time of the biomass was ca 15 days (μ: 5%d^{-1}) during at least 3 consecutive growth phases (Fig. 3). The DM content of the cell aggregates gradually increased by 40 to 45% and the growth of cell aggregates of the C_2 and C_3 liquid cultures declined suddenly after 4 and 3 cycles (ca 100 and 70 d), respectively. No significant differences in structure, appearance and growth rate were observed between cultures derived from C_2 and C_3 calli.

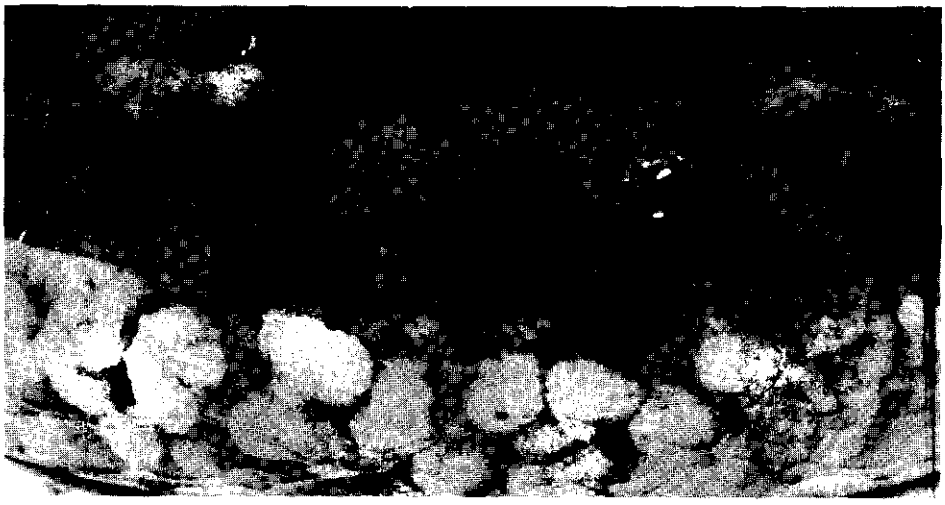

Fig. 2. Cell aggregates (3-8 mm) of Tagetes patula formed in batch cultures with liquid MS-medium (see Materials and methods). The aggregates accumulated and released thiophenes during different growth cycles according to the data presented in Tables 3 and 4.

The concentrations of glucose and nitrate, the osmolarity and the pH of the media did not change drastically in the 3.5 days intervals of medium refreshment (data not shown).

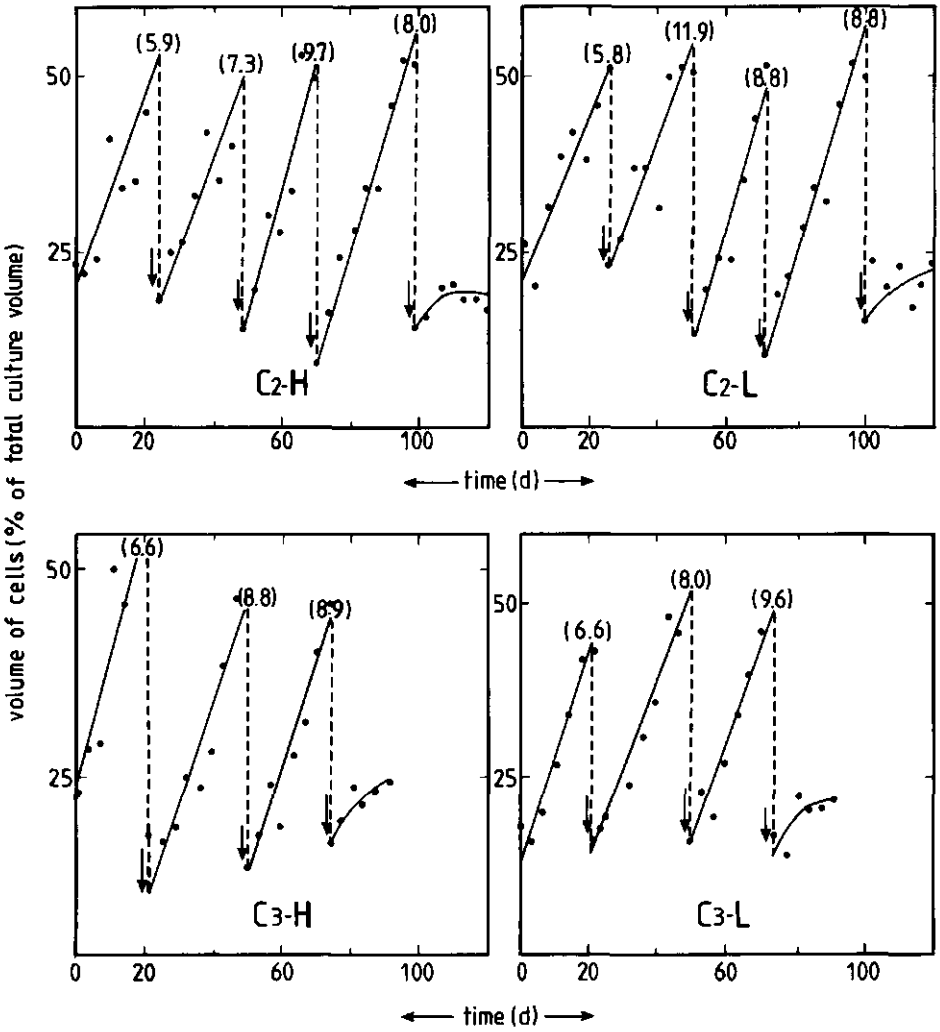

Fig. 3. Growth of the four liquid cultures of Tagetes patula in 1 dm^3 Erlenmeyers. Two liquid cultures (C_2-H and C_2-L) were obtained from secondary callus and two liquid cultures (C_3-H and C_3-L) from tertiary calli. The calli originally differed in thiophene content (H = 'high'; L = 'low'). Dilution of cell aggregates in the Erlenmeyer flasks to ca 14 PCV% was done when the volume of the cell aggregates was ca 0.25 dm^3 (= 50% of the total volume of the liquid culture). Measurements of the PCV were done twice a week. DM content of the cell aggregates, in % of fresh weight (between brackets) was determined at the end of each growth cycle.

Thiophenes.

During consecutive growth cycles in 1 dm^3 flasks cell aggregates from liquid cultures and the remaining cellfree liquid media contained thiophenes within the same spectrum and HPLC retention time. The amount of non-polar thiophenes (BBT, BBTOAc, and the presumptive thiophene L_1) and the amount of the more water-soluble BBTOH gradually decreased throughout the experiment (Table 3). The thiophene production of the L-cultures was not always lower than that of the H-cultures and generally, the productivities did not differ to a large extent. The ratio (BBT + BBTOAc)/ BBTOH in the cell aggregates increased with culture age from ca 3 to more than 10 because the content of BBTOH decreased more rapidly than the content of non-polar thiophenes.

In the cell-free media from all types of liquid cultures BBTOH was the preponderant thiophene during all growth cycles in 1 dm^3 flasks (Table 4). Calculated per culture, and averaged over all growth cycles, the total amount of BBTOH excreted into the liquid medium surpassed the average amount of BBTOH accumulated in the cell aggregates 125 to 2000-fold.

Over various growth cycles with an average cell density of 90 g FW dm^{-3} (5-8 g DM dm^{-3}) at least 2.2 (C_2) and 2.4 mg (C_3) thiophenes, corresponding with 10 to 15 µg dm^{-3} per day, were recovered from the growth medium. In addition, the quantity of BBTOH in the media of both types of liquid cultures amounted to about 75% of the total amount of thiophenes in the media.

Growth cycle	Thiophenes in cell aggregates (µg per g DM)			
	C_2-H	C_2-L	C_3-H	C_3-L
1	220 (157)	176 (88)	58 (15)	49 (12)
2	122 (19)	31 (3)	11 (10)	31 (13)
3	61 (3)	44 (5)	18 (1)	4 (3)
4	30 (0.1)	1 (0.2)		

Table 3. The sum of three non-polar thiophenes (BBT, BBTOAc and L_1) and the content of BBTOH (between brackets) in cultured cell aggregates at the end of three or four growth cycles in liquid medium. The duration of each growth cycle varied between 18 and 24 days. Cell aggregates were obtained from 'high' (H) and 'low' (L) thiophene producing secondary (C_2) and tertiary (C_3) calli (see Table 2).

Growth cycle	Culture age (d)	Thiophenes (µg dm^{-3})							
		C_2-H		C_2-L		C_3-H		C_3-L	
1	2	0	(49)	0	(31)	3	(55)	2	(63)
	4	0	(32)	0	(30)	4	(20)	2	(76)
	7	1	(32)	3	(53)	14	(51)	37	(71)
	11	15	(102)	95	(186)	21	(82)	39	(79)
	14	67	(178)	54	(161)	27	(90)	45	(120)
	18	133	(283)	131	(303)	39	(151)	48	(172)
2	21	78	(466)	94	(471)	130	(510)	100	(405)
	25	86	(258)	126	(323)	110	(441)	146	(401)
	28	102	(161)	57	(192)	53	(277)	61	(337)
	32	32	(17)	61	(71)	20	(157)	44	(183)
	35	7	(6)	14	(5)	13	(63)	33	(116)
	39	1	(5)	6	(3)	1	(14)	8	(35)
	42	1	(5)	4	(1)	0	(1)	14	(37)
3	46	4	(43)	9	(26)	1	(29)	21	(207)
	49	1	(12)	1	(12)	0	(16)	23	(166)
	53	1	(10)	1	(10)	0	(6)	8	(57)
	56	0	(6)	0	(3)	0	(2)	3	(32)
	60	0	(3)	1	(3)	0	(5)	0	(8)
	63	0	(3)	0	(0)	0	(2)	0	(11)
4	67	2	(12)	0	(3)	-	-	-	-
	70	1	(5)	0	(2)	-	-	-	-
	73	1	(6)	1	(2)	-	-	-	-
	77	0	(11)	0	(4)	-	-	-	-
	80	1	(1)	0	(4	-	-	-	-
	84	0	(1)	1	(1)	-	-	-	-
	87	0	(0)	0	(0)	-	-	-	-

Table 4. Non-polar thiophenes (BBT, BBTOAc) and BBTOH (between brackets) in the growth medium of different liquid cultures of Tagetes patula. The liquid cultures were obtained from selected secondary (C_2) and tertiary (C_3) calli that originally contained 'high' (H) or 'low' (L) concentrations of thiophenes. Samples were taken twice a week at the refreshments of part of the total volume (0.5 dm^3) of the cultures for three or four growth cycles. Each growth cycle started with fresh medium. - = not determined.

DISCUSSION

Callus cultures.

The growth of C_1, C_2 and C_3 calli of Tagetes patula, their structure, DM content, and thiophene concentration were in agreement with earlier observations (6,9,13). In contrast to other Tagetes species examined (11,13), the callus culture of T. patula resulted in suspendable callus with a significant thiophene content. The concentrations of the preponderant thiophenes, BBT and BBTOAc, in C_2 calli (Table 1) was not much different from the concentrations in intact leaves (data not shown). However, release of thiophenes by the calli into the solid medium was never observed. Jente et al. (14) suggested that BBTOAc and BBT in intact plants of T. patula are end-products of different routes of thiophene synthesis, whereas BBTOH might be a precursor of BBTOAc. The high concentration of BBT and BBTOAc in all calli, on the one hand, and the low concentration of BBTOH and the presumptive thiophenic compound L_1, on the other, do not conflict with this hypothesis. In addition, the concentration of BBTOH in intact leaves was frequently below the level of detection, while the concentration of BBTOH amounted to only 0.005% of the DM of calli.

Liquid cultures.

Pale-yellowish coloured fragments of calli of T. patula gradually formed coarse cell aggregates (3-8 mm) when shaken in liquid medium (Fig. 2). Cell aggregation occurred throughout the experimental period (ca 6 months). By comparison, minced calli of T. minuta consistently formed liquid cultures with free cells and small aggregates (ca 5-20 cells, data not shown). The start of liquid cultures with minced calli was accompanied by rapid and intense browning of the medium, probably owing to the release of phenolic compounds by wounded cells (15,16). Preliminary experiments proved that frequent removal of brown medium and washing of the cell aggregates was urgent to prevent death of these aggregates during the initial phase of liquid culture. The new cell aggregates, which developed in the course of the culture, did not release brownish substances, and showed increasing viability. Therefore, the frequency of washing was also gradually decreased until a rate required for refreshment of the medium and replenishment of nutrients.

When half of the total volume of the liquid cultures was replaced by fresh medium twice a week in the later phase of the experiment, the culture

conditions enabled a continuous growth of the biomass. Longer intervals between refreshments were not examined. Because the density of the cultures was reduced before the stationary phase was reached (Fig. 3), and apparently before the liquid medium changed to inviable conditions, the successive batch cultures provided a system for continuous growth. Probably due to the assay of the cell mass by volume the typical phases of a normal growth cycle of cells could not be discerned. The mean relative growth rate of the cultures was constant for ca 20 days (Fig. 3) and did neither differ between cultures from C_2 or C_3 calli, nor between cultures derived from calli with low or high thiophene contents. Nevertheless, the DM content of the cell aggregates increased gradually upon ageing. This phenomenon was probably related to the secretion of mucilaginous substances around the cells as could be observed under the microscope, and to an increase of the cell density (17). Therefore, the formation of cell aggregates was similar to a natural method of entrapment (18). However, after 3 or 4 cycles the cultures suddenly stopped growth, which may be caused by hardening of the secreted substances.

Production of thiophenes in liquid cultures.

The production of alkaloids in fine cell suspensions of Catharanthus roseus (19) illustrates that the selection of calli with high contents of secondary metabolites can be an appropriate method to obtain cell cultures in liquid medium, which continue the production of the compounds in question. In cultures of T. patula, however, both type and content of thiophenes in calli and the corresponding liquid cultures were poorly related (Tables 2, 3, and 4). This poor relationship may be explained by the heterogeneity of the calli (20), which can be observed clearly with the microscope. The results, presented in this paper, clearly indicate that in such a case the selection of calli does not result in high or low producing cell cultures. Experiments with fine cell suspensions of T. minuta (21), with calli of T. erecta (6), and with selected sizes of cell aggregates of T. patula (22) clearly illustrate that for Tagetes systems only aggregation may be a key to a certain degree of cellular differentiation as a prerequisite for the synthesis of secondary metabolites (23,24). For callus cultures like T. patula it may be important that the capacity to form cell aggregates is the determinant for high levels of thiophenes in liquid cultures.

Cell aggregates of T. patula were able to accumulate and release thiophenes during at least 150 days. However, the kinetics of release during

consecutive growth was not related to the increase of the biomass in the different liquid cultures. It rather seems, that the release of thiophenes, particularly that of the BBTOH, was stopped immediately after a short period of stimulation (Table 4). The stimulation of the production and release of thiophenes, mainly BBTOH, was apparently due to complete refreshment of the liquid medium. During subsequent partial medium refreshments the thiophene level in the media reflected dilution only. So it may be, that the release of thiophenes is sensitive to inhibition by external BBTOH. The same phenomenon probably explains the pattern of the release of conyferyl aldehyde into the liquid medium as studied with liquid cultures of Matricaria chamomilla (25). In conclusion frequent washing of cell aggregates may be a prerequisite to reinduce the release of BBTOH and other secondary metabolites into the liquid medium. Especially the stimulation of the release of secondary metabolites is attractive from a biotechnological point of view (18,26). Therefore the study of the kinetics of the release of BBTOH and other thiophenes opens perspectives to develop adequate production systems.

Although the amount of released BBTOH corresponded to at least 0.02% of the DM of the cell aggregates and thus was low with respect to the formation of other secondary metabolites in cell culture systems of other species (27) this amount surpassed considerably the amount of this compound in intact leaves and calli. In addition, owing to the presence of light and oxygen, essential factors for the growth of many liquid cultures, considerable amounts of thiophenes might have been broken down. Therefore, separate production conditions and other measures to protect thiophenes from breakdown may enhance the yield of released thiophenes.

It is remarkable that cell aggregates in liquid cultures as well as roots from T. patula seedlings have high levels of BBTOH (28), whereas callus cultures and upper parts of plants do hardly contain this compound. The presence of an aqueous environment may stimulate the accumulation and possibly the release of this compound, which may act as a defensive chemical of the plant under natural conditions (2). F.H. Gommers (personal communication) found that the nematicidal activity of BBTOH is comparable with that of other natural nematicides, such as α-terthienyl.

After an increase of the level of released thiophenes during the initial phase of the liquid cultures (2-20 days), both accumulation and discontinuous release of BBTOH decreased drastically. These decreases are generally

observed in producing cell suspensions (18,29). In our experiments the increase of the DM content of the cell aggregates (Fig. 3) parallelled the decreased production of thiophenes. Both phenomena indicate the gradual approach of the final stage. Selection of friable cell aggregates during the liquid culture as recently performed in fresh cultures of T. patula yielded a continuation of the accumulation and release of thiophenes for at least 9 months.

In conclusion, the liquid culture of naturally-formed cell aggregates of T. patula presents an attractive system for biotechnological studies because the requirements for product synthesis, product accumulation, and product release are met under standard growth conditions.

Experiments under controlled conditions in different types of fermentors and with different aggregate sizes are now in progress.

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Conclusions and Prospects

The research in this thesis demonstrated large variations in secondary-metabolite production in the plants as sources of explantation as well as in tissues *in vitro*. The factors underlying such variations are poorly understood, at present. Therefore, to achieve at reproducible biotechnological production systems of valuable secondary metabolites, empirical approaches with some taxanomically related plant species are recommendable.

This approach may be elaborated along the following lines:

a. the analysis of the different organs of taxanomically related plant species in relation to the age of the plants grown under standard conditions;

b. the selection of suitable explants (roots, stems, leaves) to obtain callus and cell cultures under conditions commonly used for methods *in vitro*;

c. emphasis should be laid on a low potential of differentiation and a high friability of the callus to obtain granular liquid cultures. These characteristics of calli have to be examined in various passages of callus cultures to observe systematically origin, memory and species effects;

d. the effect of plant age and callus phase on the content of desired secondary metabolites should be analysed in order to select high-yielding explant tissues and suspendable calli with a high metabolite content, respectively.

The absence or presence of secondary metabolites in a plant organ does not always predict the absence or presence of similar compounds in calli. In general, when smooth calli are obtained and desired compounds remain absent after some passages of callus culture, there is little perspective that cell suspensions reinitiate the synthesis of desired compounds in growth

media. As long as reliable methods are not available to improve the structure and the potential of such calli to produce desired metabolites, only trial and error may open some perspectives, until the underlying mechanisms (morphological and chemo-differentiation, growth, etc.) are fully understood.

The results obtained provide the possibilities to start the following projects:

a) The development of continuous cultures with genetically transformed roots to obtain rapidly liquid cultures which produce stable amounts of thiophenes. Emphasis has to be laid on the selection of roots which differ in habitus to investigate the relationship between the morphology of the roots and the release of thiophenes into the medium.

b) The use of thiophene-producing liquid cultures of T. patula and non-thiophene-producing cell suspensions of T. minuta to investigate the stimuli which influence the production of thiophenes in plants, calli, and cell suspensions and the release of these compounds into the medium. In particular, the nature of cell aggregates has to be studied accurately with respect to the effect of the entrapment of the cells and to the production of thiophenes. In addition, embedding of cells has to be studied in comparison with other conditions which elicitate thiophene biosynthesis. The results from these studies may be used to know the appropriate conditions for thiophene production in fermentors.

c) Treatment of nematodes which occur in the infected vegetative organs of plants (tubers, bulbs, etc.) with water-soluble thiophenes (a.o. BBTOH) released into the medium of liquid cultures to examine the nematicidal activity and the molecular stability of these compounds.

d) The types of research mentioned above may stimulate further investigations into appropriate conditions for down-stream processes.

Abstract

The production of thiophene-biocides by cell cultures in vitro was simultaneously investigated with Tagetes erecta, T. patula and T. minuta. The calli from which the liquid cultures had to be derived differed between species in the appearance of organoid structure, texture, and colour, independently of the nutrition of the plants and explants. In particular, the difference between the friability of calli of different species is obviously related to the expression of the activity of silent genes in only a late phase of the callus and/or the cell suspension culture. Therefore 'origin effects' may eventually determine the suitability of calli to initiate liquid cultures (Chapter I). The differences between calli, however, showed that the production of thiophenes in the calli was positively related with the measure of differentiation. Rapidly growing and fine granular cell suspensions, for instance obtained from smooth calli of T. minuta, did not produce thiophenes (Chapters II and III).

Differentiated calli of T. erecta did not provide suitable material to initiate cell cultures in liquid medium. However, minced cauliflower-like calli of T. patula with irregularly occurring small root- or shoot-like differentiations, formed large cell aggregates (3-8 mm) in liquid media. These cell aggregates accumulated non-polar thiophenes and released spontaneously relatively high amounts of a water-soluble thiophene (BBTOH) into the medium (Chapter VII). Apparently, the increased morphological de-differentation of calli runs parallel with a decreased production of thiophenes in the cell suspensions derived from them. The long-term accumulation of thiophenes in cell aggregates and the release of such compounds into the mediom open perspectives for the commercial production of such compounds under fermentor conditions.

Embedding of the fine granular suspension cells of T. minuta in alginate resulted in the release of secondary metabolites into the liquid medium, but did not provide adequate conditions to reinitiate the production of thiophenes (Chapter V). In contrast, naturally formed cell aggregates which can be considered as a natural system of entrapment, as formed by T. patula cells, obviously provide suitable conditions for the production of thiophenes.

Genetic transformation of intact Tagetes by means of infection with wild-type and mutant strains of Agrobacterium tumefaciens and A. rhizogenes, induced neoplastic outgrowth of various organized and unorganized tissues without added growth regulators(Chapter VI). The change in this potential may be related to an altered synthesis of endogenously formed phytohormones. The species-dependent relationship between morphological differentiation and thiophene production persisted in all transformed tissues examined.

In conclusion, the results of the present experiments on thiophene production in cell cultures of Tagetes species support the view that, despite the totipotency of plant cells (Chapter IV), major differences exist between closely related species in the ability to serve as a biotechnological unit in vitro. Consequently, extensive research to adapt a certain recalcitrant plant species for plant cell biotechnology should be avoided by looking for a better producing species.

Samenvatting en plaats binnen het onderzoek

Tagetes planten (afrikaantjes) zijn algemeen bekende tuinplanten, behorend tot de familie der Composieten, en komen oorspronkelijk uit Zuid- en Midden--Amerika. Bovendien is bekend, dat al vroeg in de geschiedenis bepaalde Tagetes-soorten met name door de Maya's voor allerlei rituele en praktische doeleinden werden gebruikt. Kennelijk bezaten deze planten dus stoffen op grond waarvan ze voor vele doeleinden, zoals genezing, vergiftiging en insektenbestrijding konden worden gebruikt. In West-Europa worden al sinds lang bepaalde soorten, zoals Tagetes erecta en Tagetes patula, veredeld en gekweekt als sierplant.

Alhoewel er enkele voor-oorlogse publikaties zijn, die al suggereerden dat afrikaantjes een dodelijke werking hadden op nematoden (aaltjes: dierlijke parasieten, die o.a. verantwoordelijk zijn voor een aantal ziekten bij planten, b.v. de aardappelmoeheid), werd pas na de Tweede Wereldoorlog op grond van praktijkvoorbeelden duidelijk, dat er inderdaad in afrikaantjes stoffen voorkomen, die deze bijzondere aaltjesdodende eigenschap hebben. In het begin van de jaren 60 werden deze stoffen door Nederlandse onderzoekers uit de wortels van afrikaantjes geïsoleerd en als thiofeenverbindingen geïdentificeerd. Het afrikaantje als zodanig kreeg echter nooit dié bekendheid als ziekte-onderdrukker welke op grond van dié eigenschappen zou mogen worden verwacht, en bleef niet meer dan een gewaardeerde tuinplant. Daarnaast is ook de gebruikswaarde van de groep van thiofeenverbindingen tegengevallen. Pogingen om de stoffen synthetisch te maken en als nematicide door de grond te mengen bleken niet te slagen.

Slechts aan een gering aantal onderzoekers, zowel in Nederland als daarbuiten, is de werking van thiofenen niet ontgaan. Met name aan de Technische Hogeschool in West-Berlijn is in de jaren 1970-1980 veel onderzoek gedaan omtrent de vraag hoe de plant deze secundaire metabolieten maakt. Aanzienlijke vooruitgang werd geboekt toen enkele jaren geleden aan de Universiteit

te Münster de enzymen werden gevonden die van invloed zijn op de eindfase van de syntheseroutes. Slechts gedeeltelijk is de biochemische syntheseketen nu bekend.

De synthese van thiofenen start waarschijnlijk vanuit de vetzuuropbouw (zie Fig. 1, pag. 13), waarna een aantal polyacetylenen (koolstofketens met 3-voudige bindingen) worden gevormd. Ten slotte ontstaan er verbindingen met één of meer moleculaire ringstructuren waarin het zwavelatoom.

Omtrent de vraag waarom thiofenen, wanneer ze zonder meer door de grond worden gemengd, geen nematoden doden, is onderzoek gedaan in Nederland. Daaruit bleek dat het licht op een bepaald moment in een keten van enkele chemische reacties een belangrijke rol speelt. De ingewikkeldheid van dit proces maakt het tot dusver moeilijk om natuurlijke en synthetische thiofenen als bestrijdingsmiddel toe te passen als biocide. Een groep onderzoekers in Canada toonde inmiddels aan, dat thiofenen niet alleen aaltjes maar ook bepaalde soorten bacteriën en schimmels kunnen doden, m.a.w. thiofenen kunnen worden opgevat als potentiële biociden met een vrij breed werkings-spectrum.

Behalve problemen, die nog vastzitten aan de toepassing van thiofenen in de land- en tuinbouw, zijn er een aantal wetenschappelijke aspecten, die het gewenst maken om juist de produktie van deze stoffen met behulp van biotechnologische methoden te onderzoeken. Deze aspecten zijn in te delen in een drietal biotechnologische aandachtsgebieden waarbinnen nog veel onderzoek gedaan zal moeten worden. De bedoelde aandachtsgebieden zijn:

- de problematiek, die bestaat rondom het in stand houden en vermeerderen van het levende materiaal dat de gewenste secundaire metabolieten moet produceren onder biotechnologische omstandigheden;
- het uitzoeken van de omstandigheden waaronder en de manier waarop dat levende materiaal de bedoelde secundaire metabolieten optimaal produceert;
- de manier waarop de secundaire metabolieten kunnen worden gewonnen, opgewerkt en gezuiverd.

Gaandeweg is duidelijk geworden, dat de Tagetes-soorten met hun thiofenen een goed onderzoeksysteem vormen dat als een model kan worden ingezet voor het oplossen van een aantal fundamentele problemen, die de plantecelbiotechnologie in het algemeen thans nog kent.

Plantecelbiotechnologische produktie vereist, dat er onder steriele omstandigheden over een systeem van delende cellen wordt beschikt. Dit systeem moet in staat zijn om beter gereguleerd, en meer van bepaalde

stoffen te produceren dan de intakte plant. De cellen moeten daarbij continu in beweging gehouden worden in een vloeibaar voedingsmedium. Door een aantal omstandigheden te optimaliseren kan de produktie van de gewenste stof(fen) door de cellen worden verhoogd en uiteindelijk stijgen boven die van de cellen in een intakte plant. Het systeem toegepast op commerciële schaal, zou dus de mogelijkheid bieden om beter in te spelen op o.a. marktontwikkelingen en veranderende economische en politieke situaties. In commerciële opstellingen van het systeem spreekt men over fermentoren of bioreactoren met een inhoud van 50 liter of meer; op laboratoriumschaal werkt men met kleine bioreactoren (2-5 liter inhoud) of met erlenmeyers (0.1-3 liter inhoud).

Voordat echter over losse cellen van een plant kan worden beschikt, is er een tussenstap nodig, de zgn. callusfase. Callus is te omschrijven als een ongeorganiseerd delingsweefsel. Callus wordt verkregen door stukjes van een bepaald orgaan van de plant af te snijden, te steriliseren en dan over te brengen naar een vast voedingsmedium. Door de aktiviteit van bepaalde groeiregulatoren (auxine en cytokinine) wordt de deling van de cellen aan het wondvlak gestimuleerd en ontstaat er binnen enige tijd (meestal 1 tot 2 weken) een uitgroeisel dat enkele cm^3 groot kan worden. Vaak is het nodig het callus te vermeerderen, indien er over voldoende cellen voor een celsuspensie moet worden beschikt. Dit kan door het enkele weken oude callus in stukjes te snijden en deze stukjes over te zetten op vers groeimedium. Een aantal produktieproblemen, die optreden in de celsuspensiecultures hangen samen met een aantal fundamentele problemen, die voortkomen uit eigenschappen van de plant en het callus.

In Hoofdstuk I worden resultaten gepresenteerd, die aangeven dat calluscellen duidelijk andere eigenschappen vertonen dan de plant. Dit betekent, dat deze calluseigenschappen niet van eigenschappen van de plant zijn af te leiden, maar wel erfelijk aanwezig zijn. Dergelijke eigenschappen kan men aanduiden met sluimerende eigenschappen. In Hoofdstuk II wordt bediscussieerd, dat het tot expressie komen van deze eigenschappen in calli biotechnologisch gezien gunstige en ongunstige kanten kan hebben. Zo is voor Tagetes minuta het tot expressie komen van de zachtheid van het weefsel gunstig te noemen, terwijl voor Tagetes patula bleek, dat onder overeenkomstige omstandigheden de calli donkerbruin worden en afsterven als gevolg van een plotselinge stijging van het polyfenolgehalte. Deze eigenschappen van het callus werden niet direct na de overenting van het plantmateriaal waargenomen maar

na 2x overenten en werden "oorsprongeffecten" genoemd. Ze zijn niet te vereenzelvigen met "soortspecifieke effecten" en "herinneringseffecten". Van een oorsprongeffect moet gebruik gemaakt worden wanneer van Tagetes minuta celsuspensies moeten worden verkregen.

In Hoofdstuk II is mede nagegaan of bepaalde verschillen tussen verschillende media voor de plantekweek nog van invloed zijn op de groei en de ontwikkeling van calli en de produktie van thiofenen. Inderdaad bleek dat voedingsverschillen van de intakte planten opvallende morfologische effecten bij de calli kunnen veroorzaken. Ook deze effecten waren afhankelijk van de hiervoor beschreven soorteigenschappen. De gevonden verschillen konden in verband worden gebracht met veranderingen in de interne hormoonconcentraties in de verschillende explantaten. Omdat ook de thiofeenproduktie zich bleek te hebben aangepast aan de veranderingen in differentiatie van de calli, kon het verschil tussen plante- en callusvoeding niet verantwoordelijk worden gesteld voor het lage thiofeengehalte in de calli van T. minuta.

Op de ontwikkeling en groei van callus en de produktie van thiofenen wordt in het bijzonder ingegaan in Hoofdstuk III. Op een veel gebruikt groeimedium (MS-medium), waaraan een voedingssuiker, vitaminen, auxine en cytokinine zijn toegevoegd, blijkt dat calli van T. erecta na enige tijd spontaan en vrij massaal gaan differentiëren, terwijl calli van T. patula dat veel minder doen en die van T. minuta deze neiging nauwelijks of niet vertonen. Mede in overeenstemming met gegevens uit recente literatuur over andere systemen kon worden vastgesteld, dat, kennelijk in samenhang met genoemde verschillen in neiging tot differentiatie, het thiofeengehalte in calli van T. erecta en T. patula in de regel vele malen hoger was dan in calli van T. minuta.

Hierdoor kon tevens worden verklaard waarom snel groeiende en zeer fijne celsuspensies van T. minuta geen thiofenen produceren. Wij stelden daarbij tevens vast, dat deze eigenschap zeer stabiel is. Voor de hypothese, dat de afwezigheid van thiofenen rechtstreeks en alleen maar een gevolg is van de ongedifferentieerde toestand in de celsuspensiecultures, is evenwel nog geen bewijs geleverd. Immers ook andere factoren dan morfogenetische processen kunnen een rol spelen bij de tijdelijke uitschakeling van metabolische processen in de plant.

Wanneer de voeding, die normaliter gebruikt wordt voor planten, ook gebruikt wordt voor de voeding van calli bleek, met behoud van de typische soort-eigenschappen, deze 'vreemde' callusvoeding de groei en morfologie van

de calli zeer sterk te bepalen. In samenhang met morfogenetische effecten werden eveneens veranderingen van de thiofeengehalten aangetroffen. Bovendien kon worden vastgesteld, dat naast deze verschuivingen van de thiofeengehalten een aantal andere secundaire metabolieten zich dienovereenkomstig gedroegen. Eén van de conclusies was, dat met name het gebruik van MS-medium ook dit maal niet verantwoordelijk is voor het lage thiofeengehalte in de calli van T. minuta.

In Hoofdstuk IV zijn resultaten gegeven van experimenten, die te maken hebben met de vraag naar het meest geschikte uitgangsstadium van de intakte plant om bepaalde organen te gebruiken als explantaat. Zowel groeistadium van de plant als type orgaan staan in verband met de vraag waar en het tijdstip waarop de gewenste secundaire metabolieten worden gevormd. Ogenschijnlijk gaat daarbij de voorkeur uit naar die organen waar de desbetreffende metabolieten in de plant worden gesynthetiseerd, terwijl de tijd van explanteren ook ogenschijnlijk het gunstigst is op het moment dat deze verbindingen in hoge mate worden aangemaakt. De meest gunstige situatie zou daarom zijn een hoge syntheseaktiviteit in die jonge organen, die als explantaat de beste eigenschappen hebben.

Thiofenen blijken voor te kunnen komen in bladeren en in wortels van de drie onderzochte soorten. Omdat het niet waarschijnlijk is dat deze relatief weinig in water oplosbare verbindingen via het waterige sap in het floeem (zeefvaten) en xyleem (houtvaten) in de stengels kunnen worden getransporteerd, lijkt het aannemelijk dat er sprake is van op z'n minst twee afzonderlijke syntheseplaatsen in de plant. Deze gedachte werd onderkend door het feit, dat de thiofeensamenstellingen in het blad en in de wortel niet gelijk zijn. Bovendien was er geen verband tussen het stijgen en dalen van het thiofeengehalte in beide organen wanneer dit gedurende 10 tot 15 weken wordt gevolgd. Daarom kan er in dit opzicht geen echte voorkeur worden uitgesproken voor de keus van één van beide organen, zij het, dat er over het algemeen van bladexplantaten gemakkelijker callus valt te verkrijgen dan van wortelexplantaten. Bovendien blijken er in callus zowel typische blad- als wortelthiofenen voor te komen. Een verklaring daarvoor kan zijn een meer algeheel tot expressie komen van de erfelijke eigenschappen (het "geheugen" van de plant) in weinig gespecialiseerde weefsels. Dit verschijnsel illustreert een duidelijk herinneringseffect, dat zich op grond van herkenbare continuering van genexpressie onderscheidt van de hiervoor genoemde oorsprongeffecten.

De resultaten kunnen als volgt worden samengevat: de hoogste thiofeengehalten komen voor in secundaire calli van T. patula, die aanvankelijk afkomstig zijn van bladexplantaten van 4 tot 7 weken oude planten. Dergelijke twee weken oude secundaire calli zijn over het algemeen zacht en het best te omschrijven als kleine, meestal geelgroen tot lichtbruin gekleurde, bloemkoolachtige weefsels, die weinig wortel- en spruitdifferentiaties vertonen.

Gelet op de kwaliteit van deze secundaire calli en hun synthesecapaciteit van thiofenen lijkt vooral een dergelijk type callus een goede kandidaat te zijn om producerende suspensiesystemen te ontwikkelen.

In Hoofdstuk V is nagegaan of niet-thiofeen producerende fijne celsuspensies wederom tot thiofeenproduktie zijn aan te zetten door het aanbrengen van zgn. stress-condities. Over het algemeen zijn deze condities er op gericht de snelheid van aanwas van nieuwe cellen te beperken. In dit experiment werd gewerkt met enkele fysische en chemische factoren die deze stress kunnen induceren. In de meeste behandelingen kon wel een duidelijke groeiremming worden vastgesteld, maar dit had geen nieuwe start van de thiofeensynthese tot gevolg. Ofschoon met name immobilisatie van losse cellen van andere plantesoorten soms wel tot positieve resultaten aanleiding heeft gegeven, moet de conclusie zijn dat de toepassing van stress niet altijd het gewenste resultaat oplevert.

Gelet op de resultaten die met name uit de Hoofdstukken IV en V naar voren zijn gekomen moet de vraag onder ogen gezien worden of er andere methoden te vinden zijn om de positieve correlatie tussen differentiatie en secundair metabolisme te ontkoppelen. Deze vraag wordt in Hoofdstuk VI aan de orde gesteld. Verandering van de erfelijke eigenschappen van plantecellen door bacteriën (Agrobacterium tumefaciens) blijkt misschien zelfs op eenvoudige wijze een dergelijk gewenst effect te kunnen veroorzaken. De infectie met een wild-type en met mutant-stammen van Agrobacteriën bij Tagetes species blijkt daarnaast in sommige gevallen getransformeerde uitgroeisels te veroorzaken, die opmerkelijk zijn gezien het feit, dat dezelfde bacteriestamnmen bij andere dicotyle planten (o.a. Kalanchoë en tabak) andere typen uitgroeisels opleveren. Wanneer echter de uitgroeisels bij alle onderzochte stammen onderling worden vergeleken blijken de opmerkelijke uitgroeisels bij Tagetes in morfologische zin sterk te worden bepaald door de verschillende eigenschapen van de gebruikte Tagetes species. Op deze wijze kan worden aangetoond dat met name T. erecta een goede kandidaat is voor de produktie van opmerkelijke, genetisch getransformeerde wortelweefsels. Dergelijke wortelweefsels kunnen eventueel worden gebruikt voor de produktie van stabiele wortelcultures in vloeibaar medium.

In tegenstelling tot celsuspensies van T. minuta bleken vloeistofculturen van T. patula, die niet-getransformeerd materiaal bevatten, spontaan thiofeen te produceren (Hoofdstuk VII). Tegelijk met de produktie van thiofenen viel in de bedoelde cultures de vorming van grote celaggregaten op. Dergelijke aggregaten komen niet voor in de fijne celsuspensies van T. minuta. Bovendien bleek een goed in water oplosbaar thiofeen (BBTOH), dat in planten en callus niet of slechts in geringe mate voorkomt, in veel grotere mate in het vloeibare medium te worden uitgescheiden. Dit thiofeen kan worden beschouwd als een voorlaatste stap in de synthese van het slecht in water oplosbare BBTOAc, dat één van de eindprodukten is van de thiofeensynthese. Van het goed in water oplosbare BBTOH-molekuul werd de aaltjesdodende werking in vitro vastgesteld. Juist deze verbinding blijkt dus unieke eigenschappen te hebben en aan alle in het begin genoemde voorwaarden te voldoen. Inmiddels kon worden vastgesteld, dat voor de spontane produktie van thiofenen de aanwezigheid van vrij grote celaggregaten een vereiste is.

Dank zij de in dit proefschrift beschreven resultaten zal gericht onderzoek verder mogelijk zijn om de regulatie van de produktie van secundaire metabolieten en in het bijzonder van thiofenen te beheersen en om nieuwe openingen te vinden voor de verdere ontwikkeling van de plantebiotechnologie in het algemeen.

Curriculum Vitae

De auteur van dit proefschrift werd geboren op 6 maart 1941 te Amsterdam. In 1962 behaalde hij het Ing. diploma van de Chr. Hogere Landbouwschool te Ede. Na het behalen van de aan deze opleiding verbonden onderwijsbevoegdheid in 1963, vervulde hij zijn militaire dienstplicht. In 1965 werd hij aangesteld als assistent bij de Landbouwhogeschool en trad in 1968 in dezelfde funktie in dienst van het ITAL te Wageningen. Inmiddels specialiseerde hij zich als botanisch assistent (STOVA-diploma, 1968) en als biochemisch laboratorium medewerker (STOVA-diploma, 1972). In 1973 liet hij zich als student van de bijzondere MO-studie biologie inschrijven aan de Rijks Universiteit van Utrecht en voldeed het doctoraalexamen (cum laude) in 1978 (hoofdvakken plantenfysiologie en genetica, bijvak pedagogiek). Zijn dienstverband op het ITAL bleef in die tijd gehandhaafd en werd na 1980 formeel omgezet in de rang van wetenschappelijk medewerker binnen het project plantebiotechnologie. In 1981 werd daadwerkelijk begonnen met het in dit proefschrift beschreven onderzoek.