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

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In this paper the effects of indole-3-acetic acid (IAA) on growth of *Tagetes patula* hairy root cultures and secondary product formation are presented. The biosynthesis of thiophenes, sulfurous compounds with nematicidal activity, was inhibited by IAA application, as was evident from a decrease of [³⁵S]sulfur incorporation. The inhibition only occurred after the roots had developed numerous laterals as a result of auxin action. However, in roots cultured in the absence of IAA, there was no significant correlation between branching and thiophene accumulation. Therefore, development of lateral roots is not a sufficient condition for a low capacity to synthesize thiophenes. The highest rate of thiophene accumulation in the roots is found during the late exponential growth phase, when the weight increase of a root culture is at its maximum. Hence, growth and the production of thiophenes appear to be compatible in *T. patula* hairy roots.

Key words – Auxin, differentiation, French marigold, roots, secondary metabolism, *Tagetes patula*, thiophenes.

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Introduction

The production of secondary metabolites in plants is often restricted to specific tissues and organs during particular developmental stages. The morphological differentiation of plant cells into specialized cell types and tissues is accompanied by a chemical differentiation which establishes the metabolic pathway leading to secondary product formation. The relationship between morphological differentiation and secondary metabolism may be that formation of a specialized tissue is a prerequisite for secondary metabolism. This theory is substantiated by the observation that in callus cultures and in plant cell suspensions which do not differentiate into specialized structures, secondary metabolism is often low or even completely absent (Luckner 1980, Wiermann 1981, Rhodes et al. 1987, Yeoman 1987). Alternatively, in spite of the coincidence of the two processes, there

may be no causal relation between morphological differentiation and the establishment of a secondary metabolic pathway; common regulatory factors, e.g. phytohormones, may be involved.

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

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an agropine strain are characterized by both a hypersensitivity to auxin (Shen et al. 1988, Spanò et al. 1988) and an elevated IAA level (Deno et al. 1987). Auxin is considered as the main factor controlling the formation of hairy roots.

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

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

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

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

Materials and methods

Root cultures

Root line Tp9402 was obtained by transformation of *Tagetes patula* L. cv. Nana with *Agrobacterium rhizogenes* LBA 9402 (pRi 1855). Root line Tp9365 was obtained by transformation of the same *T. patula* cultivar

with *A. rhizogenes* LBA 9365 (pRi 8196). Transformation of the root lines was confirmed by their ability to grow on hormone-free medium, by the presence of agropine or mannopine in the tissue, and by Southern blotting of root DNA using the *EcoRI*-15 fragment of the Ri-plasmid pRi 1855 (Birot et al. 1987) as a probe.

Growth of isolated roots

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

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

Morphology of roots

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

Thiophene analysis

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

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

Identification of thiophenes

GC/MS: A capillary column (fused silica WCOT, coated with CP-Sil 5CB, 25 m \times 0.32 mm inner diameter; Chrompack, Middelburg, The Netherlands) was used to fractionate the samples. Carrier gas was He, and the flow rate 1.5 ml min^{-1} . Samples of 1 μl were injected with a splitting ratio of 1:10, at an injection port temperature of 250°C. The initial oven temperature was 100°C, the temperature was raised by 15°C min^{-1} to 280°C, and this temperature was maintained for 8 min. The electron impact method (EI) was used to ionize the fractions. Re-

corded spectra were compared with known spectra from the literature (Bohlmann et al. 1964, 1973, Groneman et al. 1984, Caniato et al. 1990, Bicchi et al. 1992). Fourier-transformed $^1\text{H-NMR}$ spectra were recorded on a spectrometer operating at 400 MHz. Samples were measured in CDCl_3 with tetramethylsilane as an internal standard. NMR spectra were compared with known spectra from the literature (Bohlmann and Kleine 1963, Atkinson et al. 1964, Bohlmann et al. 1964, 1965, Bohlmann and Berger 1965, Bohlmann and Zdero 1985).

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

HPLC was performed on a Lichrosorb RP-18 column (particle size 7 μm). Column dimensions were 25 cm \times 0.4 cm, the eluent was acetonitrile:water (72:28, v/v). The flow rate was 1.5 ml min^{-1} , thiophenes were detected by their UV-absorption at 340 nm. Every 10 runs, a standard mixture, consisting of 4 different thiophenes with known concentrations, was analyzed.

Thiophene synthesizing capacity

Exponentially growing root cultures were labeled with [^{35}S]sodium sulfate (0.74 MBq ml^{-1}), and the sulfate concentration in the medium was raised to 30 mM to minimize label dilution by the internal sulfate pool. After 4 h of incubation the roots were rinsed with ice-cold 100 mM Na_2SO_4 . The fresh weight was determined and thiophenes were extracted. Samples of the polar and the apolar fractions were mixed with scintillation fluid and counted. The total radioactivity in the root was used as a measure for sulfate uptake. The radioactivity in the organic phase of the extract was used to estimate thiophene synthesis. Previous HPLC analysis had shown that over 95% of the ^{35}S counts in the organic phase are in thiophenes.

Uptake of externally applied IAA

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

Roots were cultured in 50-ml Erlenmeyer flasks as described above, in medium supplemented with 130 Bq ml^{-1} [5(n) ^3H]IAA at a chemical concentration of 0.1 μM . At intervals of one or two days, the radioactivity of 50- μl medium samples was measured. After 10 days, hormone uptake and conversion in the roots was determined (Peeters et al. 1991). Briefly, roots were rinsed with B5 medium supplemented with 1 μM unlabeled IAA, and then homogenized in MeOH. The homogenate was centrifuged (5 min, 48 g), the supernatant was collected and subsequently evaporated under N_2 at ambient temperature. The residue was taken up in a small volume of

MeOH and applied to a silica gel 60 TLC plate. The plate was developed with CHCl_3 :MeOH:HOAc (75:20:5, v/v/v) as a solvent. Finally the IAA spot and the rest of the lane were scraped off separately and the radioactivities were determined.

Evaluation of data

All experiments were carried out at least twice. Data points are means of 4 determinations \pm the standard error of the mean (SE).

Results

Growth and thiophene content of hairy roots

In order to study the relation between culture growth and thiophene accumulation, both processes were monitored over a 50-day-period. Hairy roots, grown as batch cultures, show a typical sigmoid growth curve (Fig. 1). It commences almost exponentially, then the net rate of increase declines, until finally there is no further change in culture size. During the exponential growth phase, the rate of accumulation of thiophene exceeded biomass production. As a consequence, thiophene concentration in the tissue increased during early culturing to reach its highest level at the end of the exponential phase. During transition to the stationary growth phase, thiophene level decreased in a characteristic two-step process observed in all accumulation experiments. The first step comprised a 40% decline followed by a plateau. Thiophene concentration resumed its decrease when the culture became stationary. Meaningful comparisons between root lines can only be made when cultures are in the same growth phase. For this reason, only root cultures in the exponential phase of growth were compared in all further experiments.

The preceding results indicate a positive correlation between growth rate and accumulation of thiophenes. Thus it was expected that a rapidly growing root clone would accumulate higher amounts of thiophenes than a slow-growing clone. This expectation was tested by experiments in which a fast- and a slow-growing root culture were compared. Root clone Tp9402, which is trans-

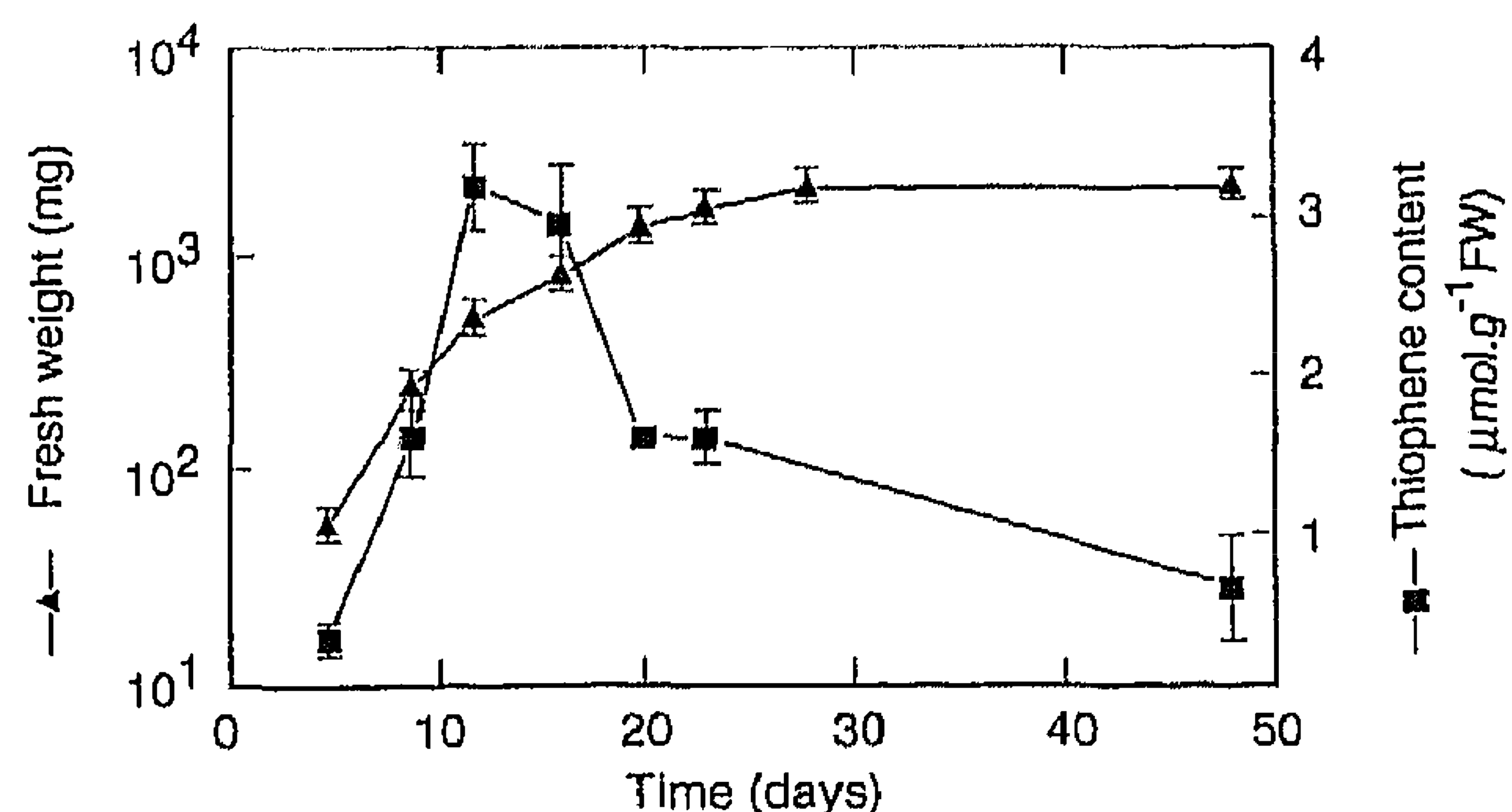


Fig. 1. Thiophene accumulation in the roots (■) during growth of a hairy root culture (▲). Means \pm SE, $n = 4$.

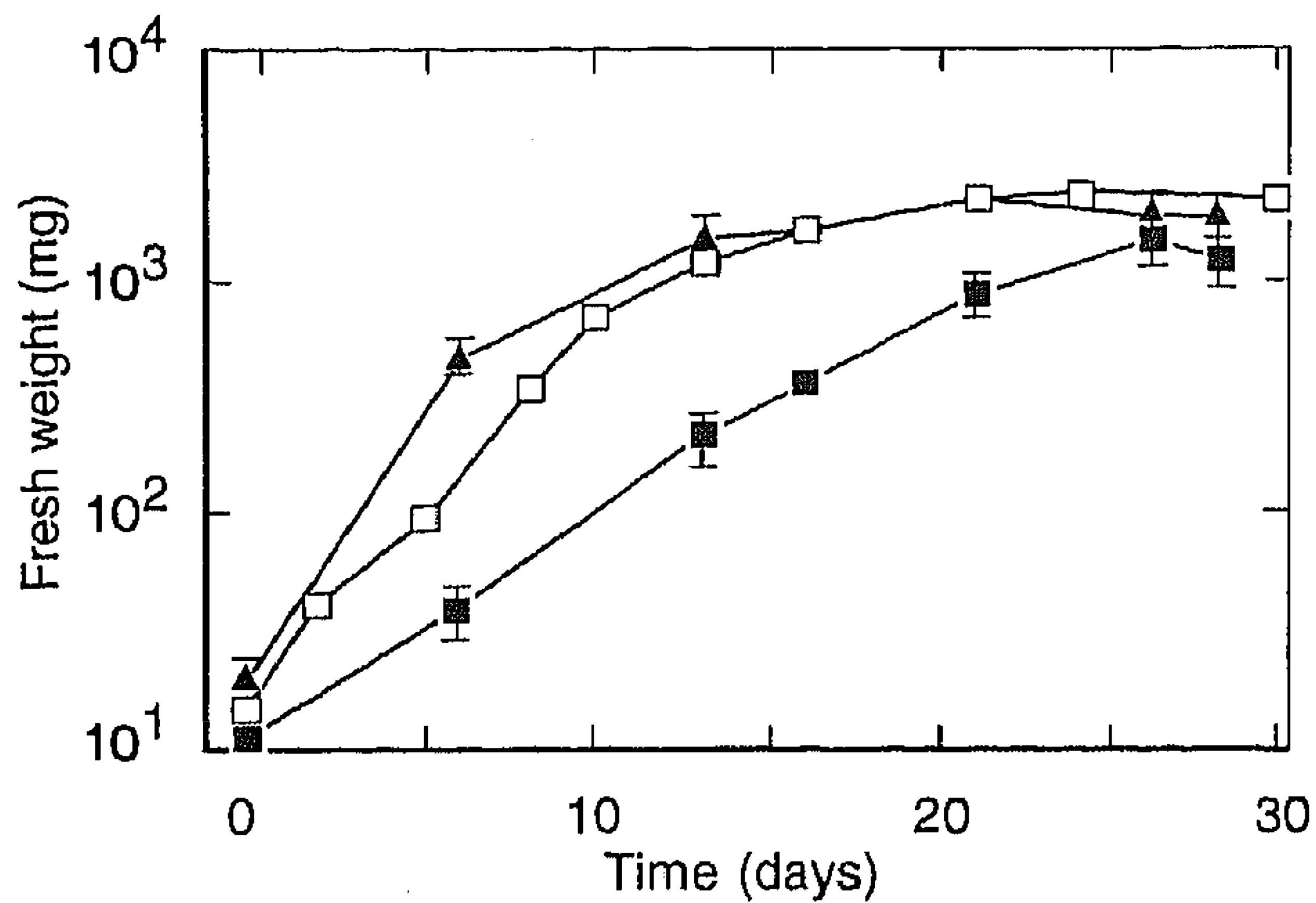


Fig. 2. Growth curves of *Tagetes patula* hairy root cultures. Tp9402 in auxin-free growth medium (□), Tp9365 in auxin-free growth medium (■), and Tp9365 in growth medium with 0.1 μM IAA (▲). Means \pm SE, $n = 4$.

formed by an *aux* genes-containing agropine strain of *A. rhizogenes*, grows rapidly. Root culture Tp9365, transformed by a mannopine strain and lacking the *aux* genes, is a slow-growing clone. In an attempt to complement the absence of the *aux* genes in the mannopine-type transformant Tp9365, this root clone was also cultured in the presence of exogenously applied IAA.

The growth pattern of Tp9365 cultured in the presence of externally applied IAA resembled that of the agropine-type root line Tp9402 grown in the absence of IAA (Fig. 2). In liquid medium the growth pattern of roots was exponential during the first 10 days. The biomass stabilized after approximately 20 days and did not decline appreciably upon prolonged incubation. All cultures, in spite of the differences in initial growth rate, ended up with the same biomass.

The maximum growth rate of the mannopine-type root line Tp9365, cultured in the presence of auxin, did not differ significantly from that of the agropine type Tp9402, cultured in auxin-free medium. The time needed for these root cultures to double their fresh weight was approximately 37 h. Thiophene concentration in these relatively fast-growing root cultures was significantly lower than that in Tp9365 cultured in auxin-free medium. The latter root culture needed twice as much time to double its weight (Tab. 1).

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

Effect of IAA application on growth and thiophene content

It was observed that addition of IAA to liquid-grown Tp9365 cultures initiated the formation of high numbers of root primordia. Within two days, the primordia started

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

The effect of the auxin on elongation of the main root axis did not differ much for both root cultures. At concentrations above 10^{-8} M the root clones showed a progressive decrease in elongation growth (Fig. 3A). In contrast, the formation of laterals as a response to the external auxin concentration was different in the two types of roots. Whereas this process in the agropine-type root was unaffected at all concentrations below 10^{-5} M, the mannopine-type root clone Tp9365 showed an optimum in lateral root formation at 10^{-7} M IAA (Fig. 3B); at higher auxin concentrations the number of root primordia that were initiated still increased, but the primordia did not emerge as lateral roots. The growth pattern of Tp9365 cultured at this IAA concentration again resembled that of the agropine-type root clone Tp9402 grown in hormone-free medium. Along with the emergence of lateral roots, thiophene concentration in the tissue decreased. The agropine-type roots in auxin-free medium as well as the mannopine-type roots cultured at 10^{-7} M IAA, accumulated significantly less thiophenes than the hardly branched mannopine-type roots cultured in auxin-free medium. This confirmed the observation that thiophene content is inversely related to branching.

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

Although a correlation is found between lateral root

Tab. 1. Growth rate and thiophene content of *Tagetes patula* hairy root cultures. Means \pm SE, $n = 4$.

Root culture	Doubling time during exponential growth phase (h)	Thiophene content ($\mu\text{mol g}^{-1}$ FW)
Tp9365 in B5	74 \pm 23	2.7 \pm 0.1
Tp9365 in B5 + 0.1 μM IAA	38 \pm 2	1.5 \pm 0.4
Tp9402 in B5	37 \pm 5	1.9 \pm 0.5

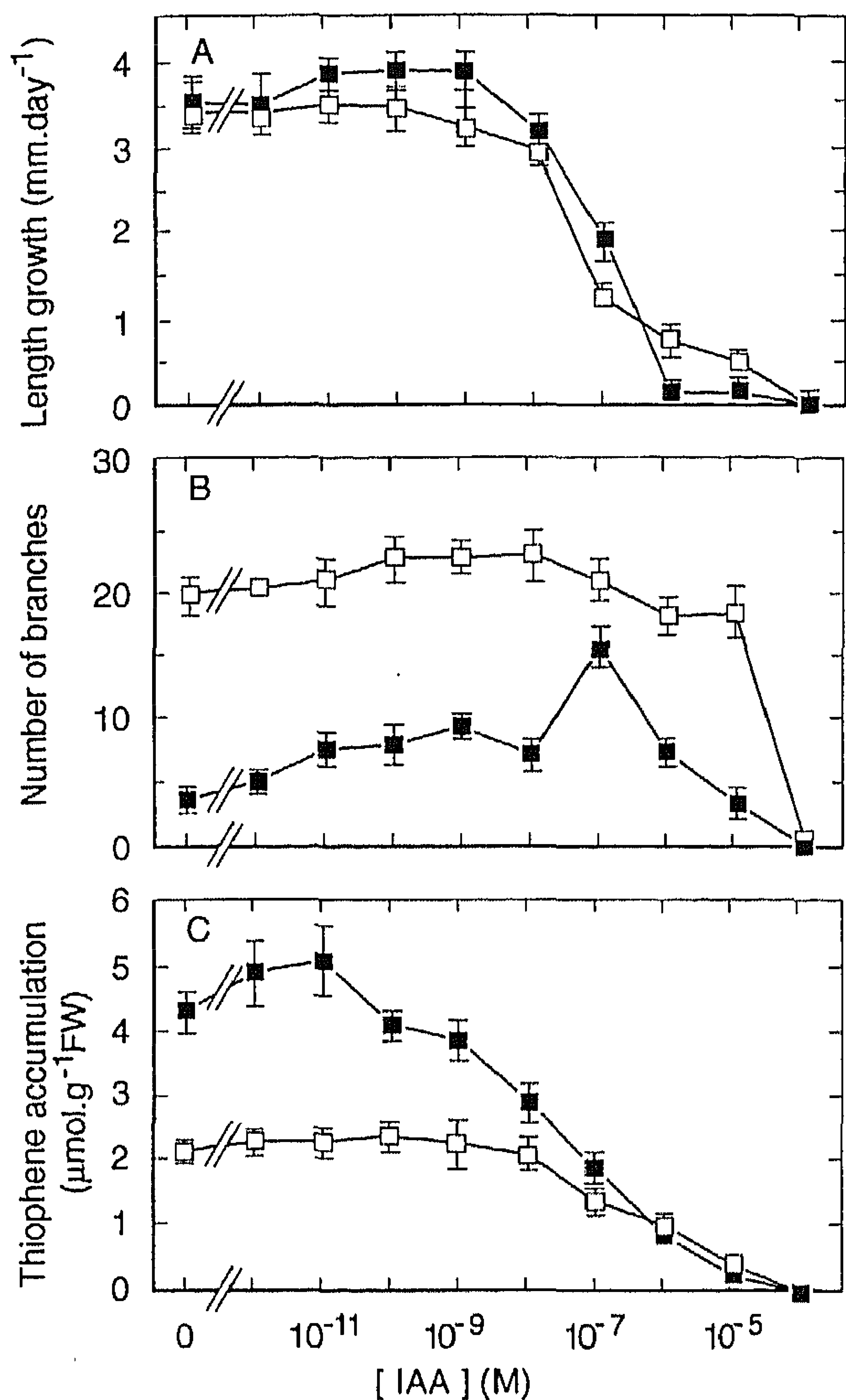


Fig. 3. Effect of exogenous IAA on elongation growth of the root main axis (A), formation of lateral roots (B), and thiophene accumulation (C) in the agropine-type root line Tp9402 (□) and the mannopine-type root line Tp9365 (■). Roots were incubated on solidified medium and analyzed after 2 weeks. Means \pm SE, $n = 10$.

formation and production of thiophenes, these processes need not be causally related. Branching and secondary metabolism may be independently affected by auxin. The dilemma was tackled by examining the relation between the number of lateral roots and thiophene concentration of Tp9365 cultured in a hormone-free medium. Advantage was taken of the natural variation in the number of laterals per root system which ranged from 0 to 10 in the absence of auxin. Individual roots were selected and assigned to four categories, according to the number of laterals. The roots were extracted, and the thiophene content was determined. No correlation was found between the number of laterals and thiophene concentration in the roots (Tab. 2). Thus, branching and thiophene metabolism must be independently affected by auxin. For this reason it was necessary to study the effect of IAA on thiophene biosynthesis in more detail.

Tab. 2. Relation between thiophene accumulation (mean \pm SE, $n = 10$) and number of laterals in *T. patula* hairy root clone Tp9365. Roots were incubated in solidified medium and analyzed after 2 weeks.

Number of laterals	Thiophene accumulation ($\mu\text{mol g}^{-1}$ FW)
0	2.3 ± 1.6
1-3	2.8 ± 2.0
4-7	1.7 ± 3.1
8-10	1.8 ± 0.2

Uptake and metabolism of externally applied IAA

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

Effect of IAA on thiophene biosynthesis

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

Exponentially growing roots cultured in auxin-free

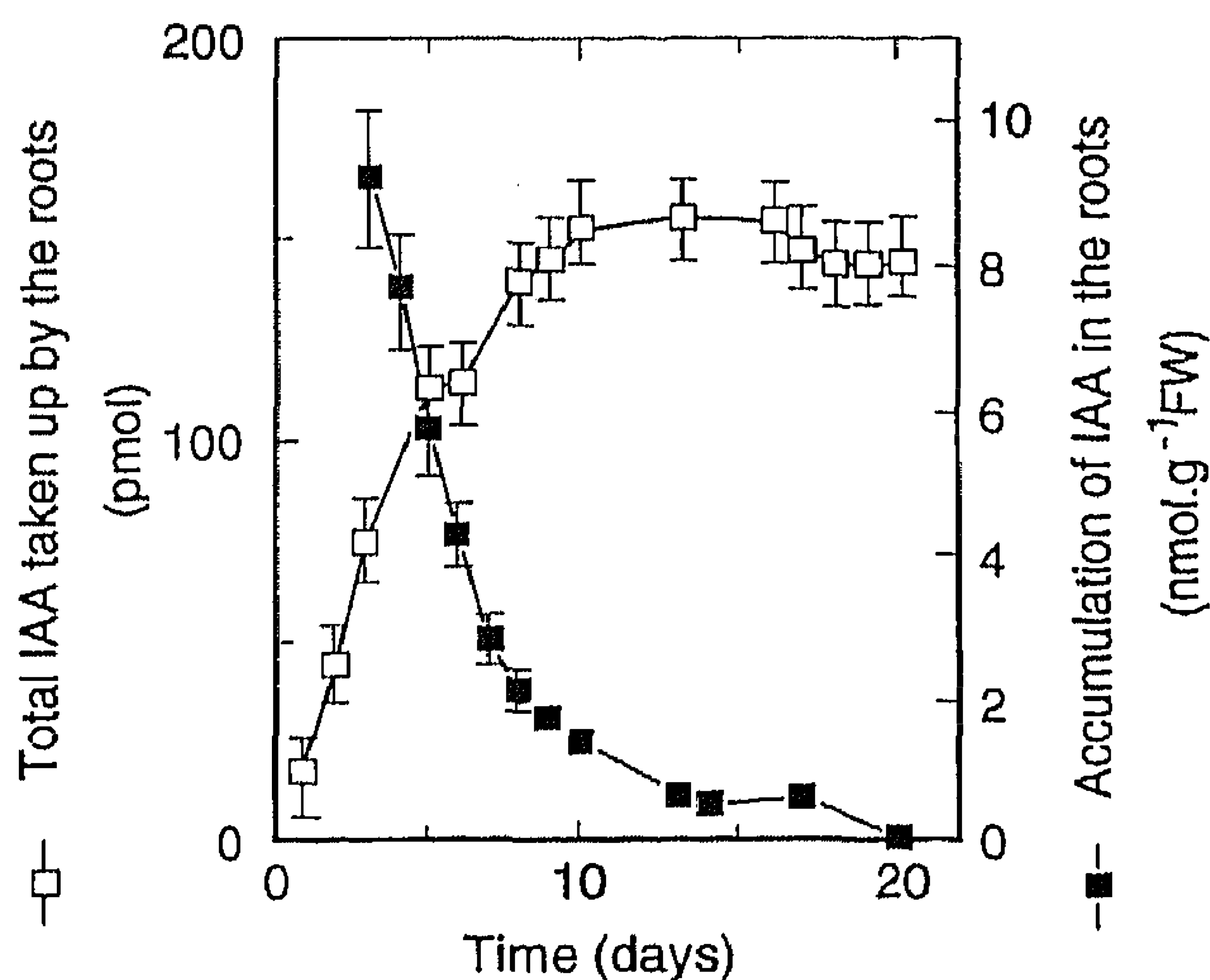


Fig. 4. Exogenously applied IAA taken up by a growing Tp9365 root culture (□) and the resulting internal levels of accumulated auxin (■). Means \pm SE, $n = 10$.

Tab. 3. Short-term effect of IAA on thiophene synthesis in root culture Tp9365. Roots that had grown for 24 h in medium supplemented with 0.1 μM IAA were divided into 1-cm-long root tips, and other parts. The synthesizing capacities (mean \pm SE, $n=4$) of tips and proximal parts were measured separately.

Root parts	Thiophene synthesis (nmol g ⁻¹ FW h ⁻¹)
Tp9365 in B5	
Whole roots	7 \pm 1
Root tips	10 \pm 1
Other root parts	5 \pm 2
Tp9365 in B5 + 0.1 μM IAA	
Whole roots	7 \pm 2
Root tips	12 \pm 2
Other root parts	5 \pm 1

medium were transferred to either auxin-free medium or medium with 0.1 μM IAA. One day after transfer, the rate of thiophene synthesis was determined by following the incorporation of radiolabeled sulfur. To localize the site of auxin action on thiophene biosynthesis, the synthesizing capacities of tips and proximal parts were measured separately. No significant effect was detected within 24 h; neither the morphology nor thiophene synthesizing capacity had changed (Tab. 3).

In another experiment, roots were cultured in auxin-free medium or in medium with 0.1 μM IAA. After 14 days of growth, the rate of thiophene biosynthesis was determined. Auxin strongly reduced the thiophene synthetic capacity in the tips but barely affected the older parts. In this way, the hormone abolished the difference in synthesis rate between root tips and older parts (Tab. 4).

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

Discussion

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

Hairy roots obtained after transformation of *T. patula* with an agropine-type strain of *A. rhizogenes* had a significantly lower thiophene content than roots obtained after transformation with a mannopine-type strain of the bacterium. The only functional difference between agropine- and mannopine-type transformants is the presence of bacterial genes encoding auxin synthesis. These bacterial *aux* genes do not play a crucial role in the induction of hairy roots (Cardarelli et al. 1987) but they clearly affect the morphology and the secondary product accu-

mulation in the induced roots. The presence of the *aux* genes in the agropine-type *T. patula* roots resulted in a highly-branched morphology and a decreased thiophene content, compared to the roots induced by a mannopine strain, which had no *aux* genes on the T-DNA. The absence of the genes in the latter root culture can be compensated for by the exogenous application of IAA (Fig. 2). Hence, it can be concluded that auxin regulates both lateral root development and thiophene accumulation in *T. patula* roots.

The relatively high number of growing root tips in the highly-branched root cultures, which were low in thiophene content, resulted in a high growth rate of these cultures. This, however, does not automatically mean that a negative correlation exists between growth rate and rate of thiophene biosynthesis. The opposite is true: the highest rate of thiophene accumulation in the roots is found when the weight increase of the culture is at its maximum (Fig. 1).

A negative correlation was observed between the number of lateral roots formed after auxin application and the rate of thiophene biosynthesis per gram fresh weight. However, highly-branched roots that had not been cultured in the presence of exogenous IAA, did not show low thiophene accumulation (Tab. 2). Application of IAA did not lead to a significant repression of thiophene formation within 24 h. Only after a prolonged period of growth in the presence of IAA, when the root morphology had been altered, was a decrease in thiophene biosynthesis observed (Tabs 3 and 4). Hence, a highly branched phenotype may be a necessary, but not a sufficient condition for a low capacity to synthesize thiophenes.

Exogenously applied IAA was rapidly taken up by the roots which resulted in high levels of accumulated auxin in the growing root culture during the first days after application. Within a day, a high number of root primordia was formed, resulting in roots with a hispid appearance. When the internal level of auxin decreased, due to a decreased auxin uptake and an increase in root biomass, the primordia differentiated into secondary roots. Conjugation leads to a decline in the level of the active hormone, because free IAA is the only biologically active form (Cohen and Bandurski 1982). These observations

Tab. 4. Long-term effect of IAA on thiophene synthesis in root culture Tp9365. Roots that had grown for 14 days in medium supplemented with 0.1 μM IAA were divided into 1-cm-long root tips, and other parts. The synthesizing capacities (mean \pm SE, $n=4$) of tips and proximal parts were measured separately.

Root culture	Thiophene synthesis (nmol g ⁻¹ FW h ⁻¹)
Tp9365 in B5	
Root tips	9.0 \pm 0.9
Other parts	2.8 \pm 0.7
Tp9365 in B5 + 0.1 μM IAA	
Root tips	3.1 \pm 0.3
Other parts	2.2 \pm 0.2

indicate that exogenously applied IAA probably exerts its effect on root growth during the first days after application, because by then the internal levels of the hormone are highest. That auxin application affects thiophene metabolism at a later stage is compatible with a causal relation between morphological and chemical differentiation.

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

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