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IN VITRO THIOPHENE PRODUCTION BY TRANSFORMED ROOT CULTURES OF *TAGETES LAXA (CABRERA).*

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ABSTRACT

The effect of different concentrations of carbohydrates, nitrogen, sulphate, plant growth regulators and elicitors on growth and thiophene accumulation by transformed root cultures of *Tagetes laxa (Cabrera)* was studied. The combinations of sucrose (30 g/l), nitrogen (60 mM), sulphate (150 mM) and the ratio N_{ox}:N_{red} 2:1 are the most appropriate combination to support growth and thiophene accumulation, which was increased by 90% when the cultures were elicited with homogenate of *Sclerotinia sclerottiorum* . The plant growth regulators used produced dedifferentiation with a decrease in thiophene biosynthesis.

INTRODUCTION

Thiophenes are heterocyclic sulfurous compounds which are vitally important since they have a variety of biocidal activity and are biodegradable (Chan et al, 1975; Hudson et al, 1986). *Tagetes laxa* (Cabrera), a South American native species which grows widely in the North-West of Argentina, is a source of these compounds. The contents and patterns of thiophenes have been studied in normal and transformed roots cultures. Both cultures have similar thiophene content but a different pattern. In fact, in hairy roots the maximum content corresponds to 5-(3-buten-1-inyl)-2, 2'-bithienyl (BBT) followed by 5-(4-acetoxy-1-butinyl) -2, 2'-bithienyl (BBTOAc), a-terthienyl (a-T) and 5-(4-hydroxy-1-butinyl)-2-2'-bithienyl (BBTOH). In seedling roots, however, the main thiophene accumulated was BBTOAc, followed by BBTOH, BBT and α-T (Rodriguez Talou et al, 1994). Since thiophenes are present in hairy roots, which are an appropriate in vitro system, we decided to use this system for our studies. The aim of this work is to study the effect of carbohydrates, nitrogen and sulphate concentrations, as well as plant growth regulators such as indole -3- acetic acid (IAA), 1- naphthylacetic acid (NAA), 6- benzyl amino purine (BAP) and gibberellins (GA_3, GA_7, GA_3), on growth and thiophene biosynthesis by hairy roots of *T. laxa*. In addition, results on elicitation are reported.

MATERIALS AND METHODS

Plant Material and Culture Maintenance

The hairy roots of *T. laxa* (Cabrera) clone 34 were used throughout this work. The origin of this line was described by Rodríguez Talou et al (1994). Cultures were maintained by subculturing at 14 day intervals in 50 ml of the MS mineral nutrient medium (Murashige and Skoog, 1962) with the addition of sucrose (30 g/l) and RT vitamin complex (Khanna and Staba, 1968). The pH was adjusted to 5.5. This medium will be referred to as MSRT medium. Growth was carried out at 24° ± 2° under darkness conditions.

Media Composition and Culture Conditions

In order to select the most appropriate carbohydrates for supporting *T. laxa* hairy root growth and thiophene production, the following carbohydrates were tested (at 30 g/l): glucose, sucrose, lactose, galactose, manitol and fructose . Sucrose at 5, 15, 60, 90, 120 and 150 g/l was also assayed. The carbohydrates were added to MSRT medium without sucrose.

To study the effect of nitrogen, MSRT medium with changes in nitrogen concentrations were used. Concentrations between 15 to 67.5 mM (Table 3) were tested. These concentrations were obtained by changing the KNO_3 concentration of MSRT medium which contains 60 mM of nitrogen supplied by KNO_3 and NH_4NO_3. In order to test the effect of oxidazed nitrogen to reduced nitrogen ratio (N_{ox}:N_{red}), several relationships were assayed. These ratios were obtained by modifications of KNO_3 and NH_4NO_3 concentrations in MSRT medium(Table 4).

Table 5 shows the concentrations of sulphate tested which were obtained by manipulation of $MgSO_4$ concentration in the MSRT medium. Mg^{++} concentration was kept constant by adding an appropriate amount of $MgCl_2$

To study the effect of plant growth regulators, auxins (IAA, NAA), cytokinins (BAP) and gibberellins (GA_3, GA_7,and GA_{13}) were added to MSRT medium at concentrations indicated in Table 6.

To study the effects of elicitors, homogenates of fungi which were prepared according to Quadri and Giulietti (1993), were assayed. The following strains belonging to our collection were used as elicitors: *Fusarium graminearum, Penicillium pinophillium, Curvalaria pallecens, Alternaria sp. and Sclerotinia sclerottiorum.* These were all maintained in Sabouraud dextrose (SD) (Oxoid CM41). The homogenate preparations were added to 14 day old transformed root cultures at a final concentration of 1% fresh weight in volume (FW/V). In the case of the homogenate of *S. sclerottiorum,* the following concentrations were used: 0.5, 1.0, 2.0, 4.0. The effect of elicitation was tested at 24, 48 and 72 hours after treatment.

All experiments were carried out in 250 Erlenmeyer flasks containing 40 ml of the corresponding medium and inoculated with 0.10-0.15g fresh weight of the leading 2-3 cm of root tips of 19 day old hairy roots. The Erlenmeyer flasks were incubated on a rotary shaker at 100 rpm at 24° ± 2° under darkness conditions. After 14 days of culture, growth index (GI) and thiophene concentrations were determined.

Analytical methods

Thiophene extraction and HPLC analysis were performed as described by Norton et al (1985). For FW determinations, root tissues were separated from the medium by filtration through glass fibre paper (Whatman GF/A) under vacuum and weighed. The GI is defined as the harvested FW at 14 days of culture divided by the inoculum FW. Four replicates were used in all determinations and statistical Scheffe analysis was conducted in each test.

RESULTS AND DISCUSSION

Effect of sucrose, sulphate and nitrate

Sucrose is the carbohydrate which best supported growth of *T. laxa* hairy roots (Table I), whereas glucose and fructose produced the highest values of thiophene specific productivity. These results suggest that the following two step process could be carried out: in the first step sucrose could be used as carbon source to obtain a high value of biomass. And, afterwards, a second step using glucose or fructose could be employed for thiophene production. This observation was also reported by Jung et al (1992) for catharantine biosynthesis by *Catharanthus roseus* hairy roots. Lactose, galactose and mannitol did not support growth of *T. laxa* hairy roots.

Table 1: Effect of carbohydrates on growth and thiophene biosynthesis by transformed root cultures of *T. laxa* (clone 34) at 14 days of culture.

Carbohydrate (30 g/l)	Growth Index (GI)	Specific productivity mg/g FW
Sucrose	10.9±1.20	1090±150
Glucose	1.92±2.00	2540±384
Fructose	2.3±0.30	2730±410

All the sucrose concentrations tested were able to support growth (Table 2) but concentrations higher than 60 g/l produced a decrease in growth and thiophene production. No changes in thiophene pattern were observed In contrast with our results Norton et al (1986) reported increases in acetylene production when C:N ratio is increased. They suggest that this increment is due to stimulation of fatty acid synthesis such as oleic acid, a precursor of polyacetylenes and thiophenes.

Table 2: Effect of sucrose concentration on growth and thiophene biosynthesis by transformed root cultures of T. laxa (clone 34) at 14 days of culture.

Concentration g/l	Growth Index (GI)	Specific Productivity mg/g FW
30	11.3±1.3	1120±156.8
60	15.5±1.8	930±111.6
90	10.5±1.3	370±38.1
120	5.7±6.2	330±39.6
150	1.9±2.4	340±44.2

Table 3 shows the effect of nitrogen concentration on growth and thiophene accumulation by transformed roots of *T. laxa* (clone 34). Nitrogen concentrations up to 60 mM (which is the nitrogen content present in MSRT medium), reduced GI and thiophene accumulation. No changes in thiophene pattern were observed. Payne et al

Table 3. Effect of nitrogen content on growth and thiophene accumulation in transformed root cultures of *T. laxa* (clone 34) at 14 days of culture.

Media	Nitrogen content (mM)	Growth index (GI)	Thiophene content (ug/g fr. wt.)
MSRT	60.00	10.95±1.28	1205±160
	67.50	5.23±0.44	406± 59
	63.70	7.47±1.55	376± 58
	61.50	8.65±1.82	716±109
	60.70	8.90±1.63	945±137
	50.40	14.80±2.27	930±112
	45.90	16.63±1.24	708±101
	30.00	17.35±2.57	626± 81
	15.00	18.68±2.77	517± 75

(1987), who studied growth and hyoscyamine production in hairy root cultures by *D. stramonium* reported the same effect.

Concerning the N_{ox}:N_{red} ratio (Table 4), a relationship different to 2:1 (MSRT medium) affected growth and thiophene production negatively. When there is preeminence of N_{red} (medium 5) the effect is more dramatic. This can be attributed to the fact that ammonium uptake in plant cells is carried out by an H^+ antiport mechanism which produces stress by lowering pH (2.0-3.0).

Concerning sulphate concentrations, two clear effects were observed. Concentrations above those of MSRT did not affect either growth or thiophene pattern but concentrations below those present in the MSRT medium produced a significative decrease in the thiophene content, with no change in thiophene pattern (Table 5).

Table 4: Influence of Nox:Nred on growth and thiophene specific yield in transformed root culture of T. laxa (clone 34) at 14 days of culture.

Medium	Nox:Nred ratio		Growth Index (GI)	Thiophenes content Mg/gFW
MS	2	1	11.08±1.10	1209±182
1	1	0	13.31±1.57	817±92
2	0	1	2.20±0.31	817±89
3	1	2	9.05±1.03	930±88
4	3	1	15.30±1.75	913±91
5	1	3	6.30±075	127±14

In all media the total nitrogen content is 60 mM (the same of MSRT medium). N_{ox} corresponding to nitrate. N_{red} corresponding to ammonia.

Effect of plant growth regulators

Table 6 shows the effect of exogenous plant regulators on growth and thiophene production by *T. laxa* hairy roots. In the case of IAA, none of the concentrations tested affected growth. This fact could be explained taking into account the results reported by Croes et al (1989), who found a compensatory effect at different concentrations of IAA while working with transformed root cultures of *T. patula*. At low concentrations, IAA had only a weak effect on lateral roots, whereas at high concentrations, IAA was strongly inhibitory of root-tip elongation with a clear stimulatory effect on lateral root formation. Concerning the thiophene content, high concentrations of IAA (10 mM) produced a significative decrease due to the dedifferentiation observed.

Table 5. Effect of sulphate concentration on growth and thiophene accumulation in transformed roots cultures of *T. laxa* (clone 34) at 14 days of culture.

Media	Sulphate concentration (mM)	Growth index (GI)	Thiophene content (ug/g fr.wt.)
MSRT	1.50	10.68±1.70	1180±190
	7.50	11.95±2.01	826±160
	6.00	11.28±1.90	992±149
	4.50	11.60±2.12	1125±171
	3.00	10.42±2.05	1106±159
	1.00	11.21±1.52	920± 70
	0.38	11.75±2.03	891± 74
	0.19	10.57±1.89	587± 38

* Concentration of sulphate in MSRT medium.

NAA affected both growth and thiophene accumulation. At high concentrations (1.0-10.0 mM) the effect was more dramatic with changes in root morphology, dedifferentiation and callus formation.

Regarding cytokinins, low concentrations of BAP (0.01 to 0.1 mM) did not affect either growth or thiophene production, but higher concentrations (0.5 and 1.0 mM) produced callus formation and a decrease in thiophene accumulation. The different responses to GA_3, GA_7, GA_{13}, could be attributed to different endogenous gibberellin activities in hairy roots. GA_7 presented the most pronounced effect on growth and thiophene production; GI was doubled and the total amount of thiophene content decreased to 1/3 of the value reached in MSRT control medium. The increase in growth is the result of an increase in the amount of branching and root elongation rate (data no shown). The decrease in thiophene accumulation could be attributed to the fact that the carbon flux is oriented to primary metabolism instead of secondary metabolism. The influences of GA_7 seen on growth of transformed root cultures of *T. laxa* extend the observations of Ohkawa et al (1989) and Pitta-Alvarez et al (1995) who worked with transformed root cultures of *D. innoxia and Brugmansia candida* respectively.

Table 6. Effect of auxins, cytokinins and gibberellins on growth index and thiophene accumulation in transformed root cultures of *T. laxa* at 14 days of culture.

Hormones	Concentration (mM)	Growth index (GI)	Thiophene content (mg/g fr. wt.)
IAA	0.00	10.28±1.31	1081±149
	0.01	10.87±2.25	1039±157
	0.10	10.03±2.31	952±185
	1.00	10.88±2.17	404± 65
	10.00	11.60±2.39	206± 35
NAA	0.10	12.82±1.60	319± 40
	1.00	22.20±2.85	69± 10
	10.00	19.57±2.70	99± 14
BAP	0.01	11.00±1.68	1230±165
	0.05	10.83±1.75	1190±170
	0.10	11.90±2.05	1050±162
	0.50	7.20±1.01	405± 57
	1.00	3.90±0.41	150± 22
GA3	0.10	9.05±1.40	1070±152
	1.00	12.00±1.58	1050±160
	10.00	9.80±1.00	1090±154
GA7	0.10	25.00±3.50	288± 40
	1.00	23.07±3.00	300± 43
	10.00	20.80±3.40	252± 41
GA13	0.10	12.11±1.90	802±119
	1.00	13.44±1.89	712±115
	10.00	15.12±2.21	721±118

The use of elicitors, such as fungi homogenates of *F. graminearum, P. pinophillium, C. pallecens, Alternaria sp* did not affect significantly thiophene accumulation and its excretion to the medium after 24, 48 and 72 hours of treatment (data not shown), but the roots darkened. This change is due presumably to the oxidation and polymerization of phenolic compounds which are also produced in response to elicitation (Quadri and Giulietti, 1993). Only the homogenate of *S. sclerotiorum* affected thiophene accumulation depending on the concentration used

Table 7: Effect of elicitation with homogenate *S. sclerottiarum* on transformed root culture of *T. laxa* (clone 34) after 48 hours

Elicitor concentration 9% FW/V	GI	Thiophene content mg/gFW
00	11.02±1.30	1154±153
0.5	11.90±1 60	1900±204
1.0	12.30±1.80	2135±228
2.0	11.02±0.95	1020±170
4.0	9.30±1.03	1030±168

(Table 7). The maximum thiophene accumulation was obtained at 48 hours of elicitation. Little excretion (0.5% of the total thiophene content) was detected in the culture medium.

We can conclude that the combination of sucrose, nitrogen and sulphate contents present in MSRT medium is the most appropriate for thiophene production by hairy root of *T. laxa* (clone 34). Plant growth regulators used produced dedifferentiation with a decrease in thiophene biosynthesis. With respect to elicitation, the use of homogenate of *S. sclerottiorum* may be an interesting strategy for increasing the thiophene biosynthesis by hairy roots of *T. laxa* (clone 34).

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