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Production of nematocidal compounds by hairy root cultures of *Tagetes patula* L.

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SUMMARY. Marigold (Tagetes patula L.) hairy roots induced by infection with Agrobacterium rhizogenes produced α-terthienyl when grown in darkness, and an n-hexane extract of the roots showed nematocidal activity. Depending on the hairy root line used, the level of α-terthienyl varied from 15 to 1268 μg per g dry weight, a level that corresponded to 0.15 to 12.7-fold that in intact roots. Analysis by HPLC indicated that the nematocidal activity was due predominantly to α-terthienyl. However, it is suggested that nematocidal compounds other than α-terthienyl are present in hairy roots cultured in the dark for long periods or in the light.

KEY WORDS: Hairy root-Nematocide-Tagetes patula L.- α-Terthienyl

INTRODUCTION

The marigold plant has a suppressive effect on the population of nematodes in the soil (Tyler 1938; Oostenbrink 1957; Oobayashi and Chikaoka 1973). Uhlenbroek and Bijloo (1958; 1959) identified several strong nematocides, α-terthienyl and some unstable derivatives, in marigold plants, and these nematocides seemed to be activated by illumination. The phototoxins, α-terthienyl and other naturally occurring acetylenes seem to have potential as pesticidal agents (Marchant 1987). The production of such compounds by cultured plant materials has been achieved but the productivity has not been high (Lam et al. 1989).

Recently, it has been noted that useful secondary compounds can be produced by hairy roots, generated by transformation with Agrobacterium rhizogenes. For example, the efficient production of atropine and scopolamine was demonstrated in hairy root cultures of Atropa belladonna (Kamada et al. 1986). In the present report we describe the production of nematocidal compounds, which include α-terthienyl, by marigold hairy root and suggest that nematocidal component(s) other than α-terthienyl are present in such roots.

MATERIALS AND METHODS

Plant materials. Seeds of marigold (Tagetes patula L.; Sakata Seed Co., Japan) were sterilized in a solution of NaOCl (1.3% of active chlorine), washed with sterilized water and placed on agar-solidified standard medium (Murashige and Skoog 1962) in glass bottles. Seedlings were cultivated aseptically under continuous light (3,000 lx.) at 25°C.

Induction and culture of hairy roots. Each seedling was decapitated and Agrobacterium rhizogenes 43057 (purchased from the American Type Culture Collect-

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ion, Rockville, Maryland) was applied to the cut end. After 3 weeks, the induced hairy root was cut off and placed on agar-solidified MS medium supplemented with 0.5 mg/ml Claforan (Hekist Japan Co., Tokyo) for suppression of the proliferation of Agrobacterium. After 2 weeks, tip portions 2 to 3 cm in length of newly formed roots on the medium were again cut off and placed on fresh layers of the same solidified medium. After 2 weeks the tip portions of growing roots were cut off once again and placed on solidified MS medium prepared without Claforan. When no growth of bacteria was observed for 2 weeks, we judged the roots to be axenic and cut off the 5- to 6-cm-long roots and put them each into 140 ml of liquid MS medium in 200-ml flasks. The roots were cultured at 25°C in the dark with rotary shaking at 100 rpm. Most lines of hairy roots were subcultured at two-week interval but a few lines that showed slow growth were subcultured at intervals of 4 weeks.

Assay of nematocidal activity. Caenorhabditis elegans and Pratylenchus penetrans, supplied by Shionogi & Co. Ltd. (Tokyo) were used for estimations of nematocidal activity. C. elegans and P. penetrans were maintained by the methods described by Brenner (1973) and Mitsui (1977), respectively. Hairy roots were harvested 2 weeks after inoculation, air-dried at room temperature in the dark, put into a 50-fold excess by weight of n-hexane and crushed with a pestle. After 30 min the extract was filtered and the filtered extract was put in a glass vial and kept at 4°C in the dark until use. The extract was serially subjected to two-fold dilutions with n-hexane. Aliquots of 50 ul of extract and diluted samples were placed on glass plates and evaporated to dryness in air. The agar medium on which nematodes had been cultured for 3 days was cut into pieces of about 1 cm^2 and placed with the upper surface of the agar in contact with the area where the sample had been dried. Then the piece of agar was removed from the glass plate. In this way, 20 to 40 nematodes were placed on the area. An aliquot of 30 ul of the medium used for maintenance of nematodes, without agar, was placed on the treated area on which nematodes had been placed and the glass plates were kept in petri dishes with saturating humidity under white light (ca. 3,000 lx.) for 8 h. The number of the wiggling nematodes and the total number were counted. The relationship between the dilution of the extract and the number of dead nematodes as a percentage of the total was graphed. The relative nematocidal activity of each hexane extract was estimated by reading from the graph the dilution, at which 50% of nematodes were killed. The assay was conducted twice with each sample.

Determination of α-terthienyl. Hexane extracts of the hairy roots were analyzed by HPLC (ODS column, eluted with a mixture of acetonitrile and water at a ratio of 4:1; Hitachi Model 3056). The UV spectrum of the elute from the column was monitored with a spectrophotometer (Hitachi Model L-400) at 330 nm. The peak of α-terthienyl was identified by its retention time and coelution with a standard sample of α-terthienyl (Fluka). The amount of α-terthienyl was estimated in terms of the dry weight of hairy root. The hexane extract was fractionated according to the peaks in the profile of absorbance at 330 nm and the fractions were assayed for nematocidal activity.

Detection of opines. Fresh hairy root (ca. 0.5 g fresh weight) was ground in a small tube with distilled water. After centrifugation (10,000 x g, 5 min) the supernatant was spotted on a piece of Whatman 3MM paper (Whatman K.K., Tokyo). Electrophoresis and detection of opines was conducted as previously reported (Tepfer and Tempe 1981).

RESULTS AND DISCUSSION

As a result of infection with Agrobacterium rhizogenes, many hairy roots were formed on the decapitated seedlings. Each root was separately placed on solidified medium with Claforan.

After subculture and elimination of bacteria, 34 lines of hairy root cells were established in MS liquid medium without phytohormones. The markers of transformation, agropine and mannopine, were detected clearly in ten of the lines examined and in the rest of lines either agropine or mannopine were detected at low levels (data not shown). All of the 34 lines are considered to be trans from this result and from the fact that each grew continuously in the medium without phytohormones.

The intact root of a marigold seedling, cultivated aseptically on MS medium in a bottle, contains 16.4 ug of α-terthienyl per g dry weight. The intact root of an adult plant grown in the field, contains approximately 100 ug α-terthienyl per g dry weight (Uhlenbroek and Bijloo 1958). One line of hairy root cells (No. 1 in Table 1) showed 12- and 80-fold higher level of α-terthienyl than those in intact roots cultivated in the field and in vitro, respectively.

Table 1. The levels of α-terthienyl, growth rates and productivity of the various lines of marigold hairy root cultured in the dark.

Line No.	α-Terthienyl (μg/g d.w.)	Growth[a)] (mg)	Productivity[b)] (μg)
1	1267.9	9.6	12.2
2	553.2	16.0	8.8
3	489.9	29.7	14.6
4	392.8	24.4	9.6
5	378.8	29.1	11.0
6	338.0	24.7	8.3
7	298.3	27.2	8.1
8	220.7	13.6	3.0
9	217.8	17.2	3.7
10	201.2	24.5	4.9
11	178.2	20.5	3.7
12	151.8	34.9	5.3
13	149.4	144.3	21.6
14	144.6	87.0	12.6
15	131.2	23.3	3.1
16	116.0	26.0	3.0
17	106.4	33.6	3.5
18	99.6	79.0	7.9
19	92.6	48.6	4.5
20	88.4	25.8	2.3
21	76.3	32.9	2.5
22	67.4	70.5	4.8
23	65.0	58.3	3.8
24	64.0	36.7	2.3
25	59.4	89.5	5.3
26	56.8	67.2	3.8
27	48.2	119.0	5.7
28	45.4	75.0	3.4
29	42.1	69.7	2.9
30	41.8	121.6	5.1
31	33.9	162.1	5.5
32	27.9	127.7	3.7
33	20.4	133.4	2.7
34	15.1	108.3	1.6

a)represents the final dry weight. Initial dry weight was approximately 2 mg.
b) (α-terthienyl content) x (growth) x 0.001

The final dry weight of hairy roots from the different lines after 14 days in culture varied from 9.6 to 162.1 mg (Table 1). The lines containing α-terthienyl at higher concentrations seemed to have lower growth rates. Therefore, the extent of variation in the productivity of α-terthienyl among the lines was not as great as that in the levels of α-terthienyl in the hairy roots. The accumulation of a large amount of α-terthienyl may suppress the growth of hairy roots.

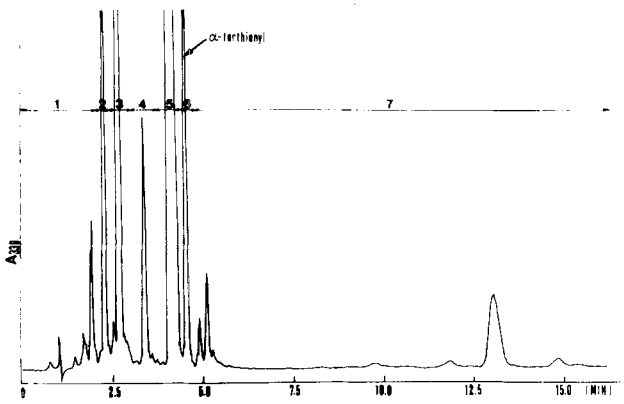

Fig. 1. HPLC pattern of n-hexane extract of marigold hairy root cultured in the dark. The horizontal arrows indicate the extents of each fractionated elute.

Figure 1 shows the profile of elution after HPLC of the n-hexane extract of one line of hairy root cells (No. 28 in Table 1) which had been cultured in the dark for 14 days. The elute was separated into 7 fractions (Nos. 1 to 7 in Fig. 1 and Table 2). Each fraction was evaporated to dryness and solubilized in the original volume of n-hexane. The relative nematocidal activity of each fraction and of fractions diluted into n-hexane was assayed as described in Materials and Methods (Table 2). Fraction 6, which contained the highest amount of α-terthienyl, had the highest nematocidal activity. However, fractions 3 and 4 also showed considerable activity. This result indicates that while the nematocidal activity was mainly dependent on α-terthienyl, there were nematocidal compounds other than α-terthienyl in the extract of the hairy root.

Table 2. The nematocidal activity of various fractions after HPLC of an n-hexane extract of marigold hairy root cultured in the dark.

Fraction No.[a)]	Nematocidal activity[b)]
1	5
2	7
3	35
4	62
5	4
6	370
7	3

a)same as that indicated in Fig. 1.
b)estimated as described in Materials and Methods.

We examined the growth rate and the level of α-terthienyl in one line of hairy root cells (No. 28 in Table 1). Growth continued until the 28th day but the level of α-terthienyl was highest on the 14th day and decreased thereafter (Table 3). The level of α-terthienyl in hairy root cells cultured for 7 days in the light was very low, suggesting that light inhibited the accumulation of α-terthienyl in the hairy root cells. The levels of α-terthienyl in the hairy root cultured for 28 days in the dark and in hairy root cultured for 7 days in the light were 8.7 and 1.1 μg per g dry weight, respectively, values which are very low when compared to the highest level recorded, namely, 45.4 μg per g dry weight (Table 3). However, the nematocidal activities (in the units described in the Methods) of the extracts from the two cultures of hairy root were estimated at 360 and 350, respectively, which are about 60% of nematocidal activity of the hairy root cells that contained α-terthienyl at the highest concentration measured. These results suggest the presence of nematocidal compounds other than α-terthienyl in the hairy root cells. Studies of the nematocidal compounds in hairy roots cultured in the light are in progress.

Table 3. The levels of α-terthienyl and nematocidal activity of extracts of hairy root under various conditions.

Days in culture	Light conditions	α-Terthienyl (μg/g d.w.)	Nematocidal activity[a)]
7	darkness	31.4	520
14	darkness	45.4	560
21	darkness	23.5	420
28	darkness	8.7	360
7	light	1.1	350

a)estimated as described in Materials and Methods.

P. penetrans, a species of nematode responsible for frequent root lesions, was also used to assay for the nematocidal activity of the hexane extracts from hairy roots. Ten-fold higher concentrations of nematocides were required to obtain 50% mortality with this species as were required with C.elegans (data not shown).

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