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Nematocidal Activity and α-Terthiophene Content in Marigold Callus

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Tissue cultures of marigold (*Tagetes patula* L.) were established and a high nematocidal activity was detected in *n*-hexane extracts from the calli. The nematocidal activity varied with callus cell lines that were established on Murashige and Skoog (MS) basal medium supplmented with different concentrations of auxin and cytokinin. The highest nematocidal activity was found in the green solid callus on the medium with 0.1 ppm 1-naphthaleneacetic acid. The nematocidal activity and the level of α-terthiophene in the green calli were equivalent to those in the root of intact plants cultivated *in vitro*. HPLC analysis of *n*-hexane extracts from several callus cell lines indicated that the nematocidal activity was due predominantly to the level of α-terthiophene but other nematocidal components are also present.

It is well known that marigold plant decreases the population of nematodes in the soil.[1,2] Strong nematocides, α-terthiophene and its unstable derivatives, were found in marigold plants,[3,4] which have a common structure, 2, 2′-bithiophene. α-Terthiophene produces singlet oxygen under light illumination and the activated oxygen is toxic to organisms.[5] Recently, commercial utilization of phototoxins, such as α-terthiophene and other naturally occurring acetylenes, as pesticidal agents is suggested.[6] For screening novel and effective pesticidal components, we established callus lines of marigold as the materials. In this report, the production of nematocidal compounds in marigold calli was confirmed.

Materials and Methods

Plant materials. Seeds of marigold (*Tagetes patula* L.) were purchased from Sakata Seeds Co. Ltd. (Yokohama). Seeds were sterilized in a solution of NaClO, washed with sterilized water, and placed on MS medium[7] with 0.8% agar in glass bottles. Seedlings were cultivated aseptically.

Callus induction and culture. The cotyledons of the seedlings were excised and placed on the agarified MS medium with various concentrations of auxin [1-naphthaleneacetic acid (NAA) or 2, 4-dichlorophenoxyacetic acid (2, 4-D)] and benzyladenine (BA). They were kept at 25℃ under continuous light (*ca.* 3,000 lux: for 1 month. Calli that showed active growth were subcultured on the fresh medium with the same component at intervals of 1 month. The callus lines maintained for more than 3 months were used for the experimental materials.

Assay for nematocidal activity. *Caenorhabditis elegans* and *Pratylenchus penetrans* supplied by Shionogi & Co. Ltd. (Tokyo) were used for nematocidal activity. *C. elegans* and *P. penetrans* were maintained by the methods reported by Brenner[8] and Mitsui,[9] respectively. Calli grown up to 1 cm in diameter were collected, air-dried at room temperature in the dark, and the dried materials were put into and homogenized in a 50-fold excess by weight of *n*-hexane in a mortar. The homogenate was placed in a glass tube with snap cap and kept for 30 min at room temperature in the dark. It was then filtered and the filtrate was kept at 4℃ in the dark until use.

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The extract was serially subjected to twofold dilutions with *n*-hexane. Aliquots of 50 μl of extract and diluted samples were placed on slide glasses and dried up. The agar media, where nematodes had been cultured for 3 days, were cut into pieces about 1 cm square and placed with the upper surface in contact with the area where the samples had been applied. Then the agar piece was removed from the glass plate. In this way, 20 to 40 nematodes were placed on the area. The liquid medium (30 μl) for maintenance of nematode was placed on the treated area and the glass plates were kept in petri dishes with saturating humidity under white light (*ca.* 3,000 lux) for 8 h. The number of 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 was estimated by the dilution rate, at which 50% of nematodes were killed. The assay was conducted twice with each sample.

Determination of α-terthiophene. Hexane extracts of calli were analyzed by HPLC (Hitachi 3056, ODS column ; eluted with acetonitrile–water=4 : 1). The UV spectrum of the elute was monitored with a spectrophotometer (Hitachi Model L-4000) at 330 nm. The peak of α-terthiophene was identified by its retention time and coelution with a standard sample of α-terthiophene (Fluka). The amount of α-terthiophene was estimated in terms of the dry weight of calli.

Results and Discussion

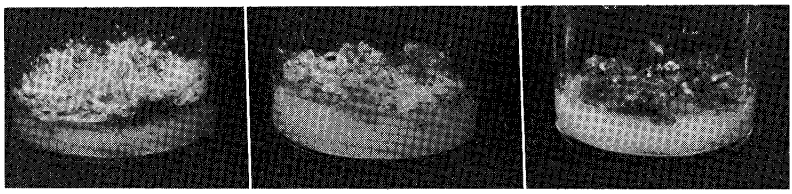
The calli which were derived from marigold cotyledons on the media with NAA and BA at different concentrations, showed various growth rate and morphological traits. We selected six callus cell lines that showed relatively active growth (**Table 1**). Three typical morphological traits of callus are shown in **Fig. 1**. Spontaneous formation of adventitious shoot on the green calli was observed during the subculture. Regenerants were obtained by replacing the shoots on MS medium without phytohormone (data not shown).

Previously, it has been reported that production of thiophene compounds was possible in cultured cells of *Tagetes*.[10-14] In these reports, the levels of total thiophene or some bithiophenes, bithienylbutinene (BBT), acetoxybutinylbithiophene (BBTOAc) and hydroxybutinylbithiophene (BBTOH), in the cells

Table 1. The level of α-terthiophene, the nematocidal activity, and the morphological traits of marigold callus lines.

Line No.	Culture condition	Form	α-terthiophene (μg/g, d. w.[b])	Nematocidal activity[a]	
				C. elegans	*P. penetrans*
1	NAA 0.1	green solid	20.3	410	30
2	NAA 1	white with root	3.7	120	5
2	NAA 1 (Dark[d])	white with root	23.0	290	—[c]
3	NAA 1 BA 3	yellow-green soft	ND[e]	3	ND
4	NAA 0.1 BA 3	yellow-green soft	1.0	90	—
5	2,4-D 0.1 BA 1	yellow-green soft	ND	3	—
Root[f]	—	—	16.4	420	—

[a] Estimated as described in MATERIALS AND METHODS.
[b] d. w.: dry weight.
[c] —: not examined.
[d] cultured in the dark for 2 months.
[e] ND: not detected.
[f] obtained from seedlings cultivated aseptically in a bottle.



a b c

Fig. 1. Three marigold callus cell lines showing the typical morphological traits. White and rooty (a), yellow-green and soft (b), green and solid (c) calli maintained on MS media supplemented with NAA 1 ppm, NAA 1 ppm and BA 3 ppm, and NAA 0.1 ppm, respectively.

were found to be considerable; however, that of α-terthiophene was very low or not examined. We examined the level of α-terthiophene in the established callus cell lines (**Table 1**). In the light culture condition, the highest level of α-terthiophene was shown in the green solid callus (No. 1) on MS medium supplemented with 0.1 ppm of NAA. The lower level was shown in the white callus (No. 2) on the medium with 1 ppm of NAA. When the No. 2 cell line was cultured in the dark, the level of α-terthiophene was much higher than when cultured in the light. The level of α-terthiophene of No. 1 calli cultured in the light or that of No. 2 calli cultured in the dark exceeded that of intact roots of the seedlings aseptically cultured on solidified MS medium. In the calli (Nos. 3 to 5) cultured in the presence of BA or 2, 4-D instead of NAA, the level of α-terthiophene was very low or not detectable. These results suggest that both BA and illumination have inhibitory effects on the accumulation and/or the production of α-terthiophene.

The level of α-terthiophene in the calli of *Tagetes* was previously shown in only one report,[10] in which α-terthiophene content in *T. erecta* callus on MS medium with 10 μM BA and 1 μM NAA in the light was 1.1 nmol per gram fresh weight. In our case (**Table 1**), Nos. 1 and 2 (dark) cell line contained 20.3 and 23.0 μg α-terthiophene per gram dry weight, which could be converted into 5.7 and 4.3 nmol per gram fresh weight, respectively. The lower level in the previous report[10] would be due to the inhibitory effect of BA or light as described above. The inhibitory effect of light or cytokinin on total thiophene accumulation in the calli of *T. minuta* and *T. erecta*[10] has been demonstrated.

Ketel and Breteler[14] suggested that cell specialization and close cell-to-cell contact were prerequisite for the production of thiophene compounds in cultured cells of *Tagetes*. In the present study, too, the level of α-terthiophene in the light culture condition seemed to be correlated with the morphological traits of calli (**Table 1**). The green solid callus, in which cell-to-cell contact seemed to be close, showed the higher level of α-terthiophene than yellow-green soft callus or the callus with adventitious root.

In the previous reports,[10-14] the biocidal activity in the products of the cultured *Tagetes* cells had not been examined. We designed an assay method for estimating the nematocidal activity in the calli. As shown in **Table 1**, the nematocidal activity in the calli was roughly proportional to their level of α-terthiophene. However, the proportionality was not applicable to some results. For example, the extract from No. 2 calli (light) contained α-terthiophene at a concentration of 0.038 μg/ml, and the nematocidal activity of the extract was estimated at 120 in the unit described in Materials and Methods. The nematocidal activity of the *n*-hexane containing the same concentration (0.038 μg/ml) of authentic α-terthiophene was 46 in the unit. Also in a preliminary result, the nematocidal activity of *n*-hexane extract from leaf was equal to that of root but α-terthiophene was not detectable in the extract. These results suggest the presence of some nematocidal compounds besides α-terthiophene.

As shown in **Table 1**, *C. elegans* was more sensitive to the nematocide in the samples than *P. penetrans*. However, it is suggested that the nematocidal compounds effective on *C. elegans* were also effective on *P. penetrans*. This suggests that a nematocidal assay using *C. elegans* is applicable for screening effective nematocides to *P. penetrans*, one of the most important root lesion nematode species.

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マリーゴールドカルス中の殺線虫活性と α-ターチオフェン含量

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マリーゴールド（*Tagetes patula* L.）で確立された組織培養系カルスのヘキサン抽出物中に高い殺線虫活性が検出された．異なるホルモン条件下で誘導，継代されたカルス系統間で，その殺線虫活性は大きく異なった．最も高い活性は 0.1 ppm の NAA を含む MS 寒天培地上で誘導，継代された緑色カルスにおいて見られた．その殺線虫活性および α-ターチオフェン含量は試験管内栽培された植物体の根においてみられるものに匹敵した．いくつかの異なる系統のカルスのヘキサン抽出物を，HPLC 分析した結果，殺線虫活性はおもに α-ターチオフェン含量に相関するものの，α-ターチオフェン以外の殺線虫物質の存在をも示唆した．