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Antifungal Activity of *Tagetes patula* Extracts

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Abstract

In the present paper the methanol extracts obtained from 10 cultivars of *Tagetes patula* were assayed on two phytopathogenic fungi: *Botrytis cinerea* and *Fusarium moniliforme. B. cinerea* showed a high dose-dependent inhibition, with a marked difference between light and dark treatment. *F. moniliforme* seems to be a more resistant test that does not appear to be affected by the different treatment conditions (light-dark) even at the highest dose. However, it can be asserted that *Tagetes patula* is a possible source of antifungal substances and that thiophene activity is, in general, strongly increased by UV-A irradiation. During the experiment the method of chromatographic plates was used to evaluate plant extracts bioactivity. The obtained data indicate that it is a rapid method than can be used as an alternative to Petri dish tests.

Keywords: Antifungal agents, *Tagetes patula*, thiophenes, TLC.

Introduction

Among the substances found in common plants, great attention has been focused on the constant presence of thiophenes in several species of Asteracee. These compounds are of interest as chemiotaxonomic markers (Bohlmann, 1988) and are also important as photosensitizers. In fact, several studies have shown that irradiating thiophenes at the appropriate wavelength (320–400 nm) imparts high photodynamic activity (Hudson & Towers, 1991 and references therein). Their light-dependent effects vs. viruses, bacteria, fungi, nematodes, insects and other organisms have been well documented (Gommers & Bakker, 1988; Hudson et al., 1986; Towers & Champagne, 1987; Zygadlo et al., 1994). The most highly studied of these compounds is α-terthienyl (α-T) that causes photodermatitis in humans (Rampone et al., 1986). On the contrary, due to its potent antifungal properties vs. dermatophytic fungi (Romagnoli et al., 1994), the same substance provides a topical, photochemotherapy approach for the treatment of dermatophytosis.

Tagetes patula L. is the thiophene-containing plant which has been studied the most (Towers, 1980). It contains four abundant biosynthetically-related thiophenes: 2,2′:5′.2″-terthienyl (α-T), 5-(3-buten-1-ynyl)-2,2′-bithienyl (BBT), 5-(4-acetoxy-1-butinyl)-2,2′-bithienyl (BBTOAc) and 5-(4-hydroxy-1-butinyl)-2,2′-bithienyl (BBTOH). Although these compounds are present in all plant tissues, the concentrations of the individual thiophenes may vary in relation to organ, plant differentiation (Tosi et al., 1988) and distribution of secretory cavities (Poli et al., 1995). A previous study determined the differences in the total and individual thiophene contents in 10 cultivars of one genus *Tagetes patula* (Mares et al., 2001). In this work, all 10 samples, composed of extracts drawn from a whole plant in full bloom, always presented a high percentage of α-T and BBTOH.

In the present paper, the methanol extracts obtained from the same 10 cultivars were assayed on two phytopathogenic fungi: *Botrytis cinerea* (Micheli) and *Fusarium moniliforme* (Sheld). These fungi were selected because they damage important crops. Besides assaying the biological activity of raw *Tagetes* extracts, this work was undertaken to determine whether the antifungal effectiveness was related to concentration in the extract of the individual thiophenes. During the experiment a rapid method was used to evaluate plant extracts bioactivity: chromatographic plates (Rahalison et al., 1991). This method was then compared to standard dish diameter inhibition techniques.

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Materials and methods

Chemicals

Plant material and extraction

Materials for the examination of thiophenes were obtained from 10 different cultivars of *Tagetes patula* L. growing in an experimental field owned by "Mazzoni vivai" in Tresigallo (Ferrara, Italy). The methanol extraction of thiophenes was performed as reported by Tosi et al. (1991). The doses used in the bioactivity tests were 5, 10 and 50 µg/ml, referred to the total thiophenes in each extract. The percentage of the individual thiophenes in these totals are reported in Table 1.

Thiophene standards

Reference compounds of α-T, BBT, BBTOH, and BBTOAc were kindly supplied by Prof. R. Rossi, University of Pisa, Italy. The standards were checked for purity by HPLC and GLC-MS and identified spectroscopically (IR, UV, NMR).

Microorganisms and growth media

The following fungi were tested: *Botrytis cinerea* (Micheli) (ATCC strain N° 48336) and *Fusarium moniliforme* (Sheld) (ATCC strain N° 36541) purchased from American Type Culture Collection (ATCC) (Rockville, Maryland, USA). The fungi were maintained at 4 °C as agar slants on potato-dextrose-agar (PDA, Difco).

Biological assays on Petri dishes and TLC

In a series of experiments the extract was added to the culture medium in the Petri dishes where the fungi were being grown. The diameter of the fungus growth was measured daily. Another series of experiments measured the growth inhibition ring of fungi grown on chromatography plates. The experiments on the Petri dishes were performed as described in Mares et al. (1990): in brief the cultures were grown at 26 ± 1 °C on PDA in the absence of light until they had reached the mid-log phase. Then the mycelia were transferred to dishes containing a medium with the extracts or the standards diluted to final concentrations of 5, 10 and 50 µg/ml. The fungi were kept in contact with the chemicals for 24 h in the dark; then half of the cultures were irradiated with UV-A, whereas the other half were kept in the dark. From this moment on, the growth rate was determined by measuring colony diameter (in millimeters) daily for 8 days, for *Botrytis*, and for 5 days for *Fusarium.* The controls, untreated fungi, contained 0.1% of the solvent (methanol). All determinations were performed in triplicate. The dishes were irradiated with UV-A light for 90 min using a black light blue fluorescent lamp (Sylvania, F 20, T 12-BLB) with a light intensity of 0.5 mW cm^{-2}.

Plant extracts were also assayed on *Fusarium moniliforme* using the thin-layer chromatography (TLC) bioassay method. In this case the extract was deposited on the chromatography plate support; the same doses were used as in the Petri dish experiments (5, 10 and 50 µg/ml). The method, introduced by Hanaus and Fuchs (1970), was similar to the one described by Rahalison et al. (1991). A µl dose of each extract, corresponding to the amounts added to the Petri dishes (5, 10 and 50 µg/ml), was spotted on silica gel 60 plates without fluorescence indicator (10 × 20 cm) (E. Merck, Darmstadt, Germany) and developed in 8 : 2 (v : v) hexane : ethyl acetate. After drying, the chromatograms were sprayed with a suspension of *Fusarium moniliforme*, obtained from a culture grown for 24 h in a PD broth and shaken at room temperature on a rotatory shaker at 200 rpm until the fungus had reached its exponential growth phase. The suspension, containing spores and small pieces of mycelium were sprayed using a FTC-313 B 1/8/ Piston-type oilless air compressor (Valex). Then, half of the thin-layer plates were irradiated with UV-A light, while the remainder were kept in the dark. All TLC plates were then incubated at 26 ± 1 °C for 24 h in a humid chamber.

Table 1. Total thiophene content and percentage of the individual thiophenes in 10 cultivars of *Tagetes patula* (mg/kg).

	Total thiophenes	α-T	BBTOH	BBT	BBTOAc
Cv n° 1	89.92	22.85 (25.42%)	37.05 (41.21%)	20.29 (22.57%)	8.89 (9.89%)
Cv n° 2	99	19.40 (19.59%)	58.40 (58.99%)	20.00 (20.20%)	1.32 (1.34%)
Cv n° 3	41.68	11.30 (27.11%)	15.79 (37.89%)	13.39 (32.14%)	1.18 (2.83%)
Cv n° 4	103.75	17.00 (17.34%)	46.36 (44.69%)	32.26 (31.10%)	7.05 (6.8%)
Cv n° 5	77.3	23.00 (29.75%)	24.20 (31.31%)	19.29 (24.96%)	10.8 (13.97%)
Cv n° 6	73.24	21.00 (28.67%)	30.00 (40.96%)	22.23 (30.36%)	0
Cv n° 7	50.00	11.04 (22.08%)	23.06 (46.13%)	16.05 (32.11%)	0
Cv n° 8	41.48	11.70 (28.20%)	14.57 (35.13%)	1.63 (25.65%)	4.56 (11.01%)
Cv n° 9	117.00	41.87 (35.79%)	34.20 (29.23%)	23.00 (19.65%)	18 (15.38%)
Cv n° 10	95.03	16.00 (16.84%)	51.00 (53.66%)	28.00 (29.47%)	0

Thiophene standards were also plotted at the same concentrations (5, 10 and 50 µg/ml). The controls had the solvent (methanol) deposited at the highest concentration used (30 µl).

Results and discussion

The inhibition of *Botrytis* growth (see Table 2) was always directly proportional to the dose, both in the experiments performed in the light and in the dark. All extracts taken from the 10 cultivars showed photoinduced activity. In fact, the fungi treated with extracts containing 5 and 10 µg/ml of thiophene and subsequently irradiated showed less growth than did the corresponding sample treated with the same concentration and kept in the dark. Only the extract obtained from cultivar no. 10 gave an unusual effect at these lower doses: growth increased. On the other hand, such behavior was observed in a previous work where some dermatophytes were treated with BBTOH (Romagnoli et al., 1998). On the contrary, the fungi treated with a dose of 50 µg/ml and kept in the dark had a greater overall growth inhibition than did those which were irradiated. Most likely, this effect is due to the greater percentage of BBTOH in the total thiophenes, as indicated in Table 1. In fact, treatment in the dark using extract no. 9, containing a lower BBTOH content than the other extracts, showed greater growth than did the corresponding sample treated in the light. As previously reported by Romagnoli et al. (1998), it may be that, at the highest concentration, BBTOH is toxic, even without light stimulus. Treatment in the dark with extracts no. 3 and 7 at 50 µg/ml actually blocked *Botrytis* growth altogether: this may be related to the synergism between the presence of α-T, BBTOH and BBT; in particular, a higher concentration of the latter is present in these extracts (Table 1).

Fusarium moniliforme appears to be a more resistant test. In fact, although the same doses were used, growth was higher and total inhibition was never achieved. On the other hand, the higher resistance of this phytopathogen to fungicides was previously observed upon treatment with synthetic fungicides, such as new pyrazole-Pyrimidines (Mares et al., unpubl.). On the whole, the data in Table 3 indicate a dose-dependent action and the thiophene photoactivation is generally less marked than on *Botrytis*. Moreover, this fungus does not appear to be at all affected by the different treatment conditions (light-dark) even at the highest dose. Indeed, at the highest dose, only extracts 2, 4 and 6 showed an evident difference in inhibition between the samples exposed to the light and those kept in the dark. Nevertheless, the results were not uniform for all the different cultivars: in fact, the treatment with some extracts at a dose of 50 µg/ml inhibited *Fusarium* more in the dark that they did in the light (1, 3 and 7), partially replicating the effect observed with *Botrytis*, although less pronounced.

Tests on chromatography plates were also performed on *Fusarium*. The data for these tests indicate that it is a rapid method that can be used as an alternative to Petri dish tests. Even after just 24 h from inoculation the plate already showed inhibition rings visible as clear zones where the fungus did not grow. These zones corresponded to bands related to two distinct groups of thiophenes: BBTOH + BBTOAc and BBT + α-T (Table 4).

Nevertheless, the inhibition rings differed depending on which cultivar was used. On the whole, a marked difference could be seen in the behavior of the two blocks, corresponding to the points in the run for BBTOH + BBTOAc and BBT + α-T. For both the blocks, the inhibition rings were greater on the irradiated plates than on those kept in the dark and were in line with the doses used.

Differences were seen depending on the dose: at 5 µg/ml inhibition rings could be seen around the BBTOH + BBTOAc spot (even in the plates kept in the dark) although they were always smaller than those found on the irradiated plates. At the same concentration, around the BBT + α-T spot, *Fusarium* growth was inhibited only in the light; the sole exception being for extract no. 8 where a clear zone

Table 2. Percentage inhibition rate of *Botrytis cinerea* on the 8th day of treatment in Petri dishes.

	Controls		5 µg/ml		10 µg/ml		50 µg/ml	
	Light	Dark	Light	Dark	Light	Dark	Light	Dark
Cv n° 1	–	–	64.3	38.0	76.1	40.5	84.4	83.5
Cv n° 2	–	–	36.0	7.8	66.4	21.9	70.4	75.0
Cv n° 3	–	–	42.3	33.0	52.3	43.0	80.8	100.0
Cv n° 4	–	–	54.6	12.7	64.0	31.8	77.4	79.4
Cv n° 5	–	–	50.5	29.1	51.4	34.2	68.0	76.0
Cv n° 6	–	–	45.4	0	60.8	20.7	73.2	82.5
Cv n° 7	–	–	75.0	24.5	80.0	35.9	94.0	100.0
Cv n° 8	–	–	64.5	11.5	74.1	36.5	82.7	77.0
Cv n° 9	–	–	55.8	13.2	75.0	21.0	83.7	77.4
Cv n° 10	–	–	16.7	+14	23.0	+7.9	39.6	41.3

Table 3. Percentage inhibition rate of *Fusarium moniliforme* on the 5th day of treatment in Petri dishes.

	Controls		5 µg/ml		10 µg/ml		50 µg/ml	
	Light	Dark	Light	Dark	Light	Dark	Light	Dark
Cv n° 1	–	–	34.8	23.8	40.6	26.4	53.6	58.3
Cv n° 2	–	–	31.1	18.0	36.2	21.4	54.5	40.0
Cv n° 3	–	–	37.7	29.8	43.5	40.5	65.9	75.0
Cv n° 4	–	–	50.7	35.7	56.7	34.3	71.6	57.2
Cv n° 5	–	–	31.9	27.4	36.3	31.0	58.0	58.4
Cv n° 6	–	–	38.8	35.7	53.7	35.7	77.6	55.7
Cv n° 7	–	–	34.5	20.0	43.1	22.7	65.5	66.7
Cv n° 8	–	–	29.3	18.7	39.7	24.0	56.9	53.4
Cv n° 9	–	–	26.1	21.4	33.4	25.0	50.8	46.0
Cv n° 10	–	–	44.8	29.6	47.8	40.0	65.7	57.2

Table 4. Inhibition rings (mm) of *Fusarium moniliforme* on silica gel plates after 24 h of treatment with thiophene extracts from the 10 cultivars.

			TREATED					
			5 µg/ml		10 µg/ml		50 µg/ml	
		CONTROLS	Light	Dark	Light	Dark	Light	Dark
Extract 1	BBTOH + BBTOAc	0	17	5	20	7	25	8
	BBT + α-T	0	36	–	36.5	3	55	8
Extract 2	BBTOH + BBTOAc	0	17	8	20	12.5	25	20
	BBT + α-T	0	36	–	36.5	–	55	–
Extract 3	BBTOH + BBTOAc	0	29	4	39	6	45	7
	BBT + α-T	0	30	–	33	–	36	–
Extract 4	BBTOH + BBTOAc	0	32	15	41	20	50	25
	BBT + α-T	0	20	–	30	–	40	–
Extract 5	BBTOH + BBTOAc	0	29	3	39	4	45	20
	BBT + α-T	0	30	–	33	4	36	10
Extract 6	BBTOH + BBTOAc	0	20	5	22	8	25	12
	BBT + α-T	0	30	–	40	6	50	13
Extract 7	BBTOH + BBTOAc	0	14	5	16	7	30	9
	BBT + α-T	0	33	–	35	10	40	20
Extract 8	BBTOH + BBTOAc	0	30	13	40	17	45	20
	BBT + α-T	0	30	11	35	24	40	26
Extract 9	BBTOH + BBTOAc	0	15	3	17	3	31	4
	BBT + α-T	0	28	–	30	–	38	–
Extract 10	BBTOH + BBTOAc	0	24	2	26	–	33	–
	BBT + α-T	0	51	–	56	–	40	–

was also seen in the plate kept in the dark. This is in agreement with the presence of BBTOH in the first band, thiophene that has proved biologically active even in the dark (Romagnoli et al., 1998) as opposed to α-T whose biological activity is strictly related to irradiation (Mares et al., 1990; Hudson & Towers, 1991). Comparison to pure standards further confirms this (Table 5). In fact, of the 4 standard thiophenes, only BBTOH yielded an inhibition ring even in the dark and at all doses tested; the other three only were active upon irradiation.

Increasing the extract concentration to 10 and 50 µg/ml, in 5 of the 10 samples, yielded clear zones indicating a lack of fungus growth. These corresponded to the BBT + α-T band even in the plates kept in the dark. However, except for cultivar no. 8, they were always much smaller than those in the samples exposed to UV-A light (Table 4). The inhibition

Table 5. Inhibition rings (mm) by pure standards of *Fusarium moniliforme* on silica gel plates after 24 h.

	5 µg/ml		10 µg/ml		50 µg/ml	
STANDARDS	Light	Dark	Light	Dark	Light	Dark
BBTOH	15	8	32	11	35	13
BBTOAc	10	–	23	–	33	–
BBT	10	–	23	–	33	–
α-T	15	–	30	–	35	–

zones were bigger as the concentration increased. It may be that this is not due so much to the presence of the two light-activated thiophenes in the band, α-T and BBT, but rather to the presence of other, as yet unidentified, antifungal substances in the extract whose activity is not related to light. The treatment at the lowest concentration (5 µg/ml) shows no sign of such activity because the concentration is too low; however, such activity does become evident as the concentration increases. The fact that not all the cultivars behave in the same manner could indicate that, while still members of the same species, there may be differences in the chemical components, perhaps derived from environmental changes or pathologies.

In conclusion, it can be asserted that 1) *Tagetes patula* is a possible source of antifungal substances, and 2) thiophene activity is, in general, strongly increased by UV-A irradiation. The observation that the methanol extract is active on the two fungi tested "*in vitro*" even at relatively low concentrations makes it a likely alternative to the synthetic chemical compounds used to treat or prevent phytopathogens. The chromatography plate method can be usefully employed in screening phytoconstituents as antimycotics since it is faster than the classic Petri dish method and is also more selective as the various constituents can be chromatographically separated.

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