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Evaluation of a Broad Variety of Coumarins, Chromones, Their Furohomologues and Thione Analogues as Phototoxins Activated by UVA and Visible Light

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Abstract: The potential of a new kind of light-induced pesticide action was evaluated for a broad variety (32) of natural photosensitizers and their thione derivatives. The latter were synthesized to shift the absorption spectra towards the visible region and to increase the triplet and singlet oxygen quantum yields.

Qualitative and quantitative evaluation of growth inhibition of *Fusarium culmorum* (F. G. Smith) Sacc. produced by these photosensitizers under UVA and visible light was performed on silica gel plates and in liquid medium. The results show that the phototoxicity per excited molecule of the thione derivatives using UVA light was similar to that of their parent natural compounds. On the other hand, only the thione derivatives were photoactive under visible light irradiation.

These compounds show encouraging levels of phototoxicity against *F. culmorum*, both in liquid culture and on silica plates, and may have potential for use as photoactive pesticides.

Key words: photosensitizers, photoinhibition, photopesticides.

1 INTRODUCTION

Light-activated pesticides or photopesticides are compounds that, in the presence of UVA or visible light, cause damage to biological systems. These molecules belong to a more general group known as photosensitizers which absorb light and trigger the chemical modification of another molecule (the substrate or target) by various processes.

The first report of this kind of action is generally attributed to Raab at the end of the last century.¹ Raab found that some specific dyes, such as eosin, induced the killing of paramecia in the presence of light, which did not happen in the dark.² During this century dye-

sensitized photoreactions have been applied in many fields, of which by far the most important are the biomedical sciences, namely Photodynamic Therapy (PDT) and PUVA therapy.^{3,4} The agricultural importance of this type of compounds is also under study and several effects against pests, such as growth inhibition, have been observed.^{5,6}

Practical application of such compounds, as light-activated pesticides, should take into account that the sun emits largely visible radiation, so that shifting the absorption spectra of these compounds towards the visible region should greatly improve their efficacy.

Absorption of light by a sensitizer (Fig. 1; $^1\text{Sens}$) usually leads to the formation of a short-lived (ns) singlet excited state ($^1\text{Sens}^*$; opposite electron spins), which can either return directly to the ground state or

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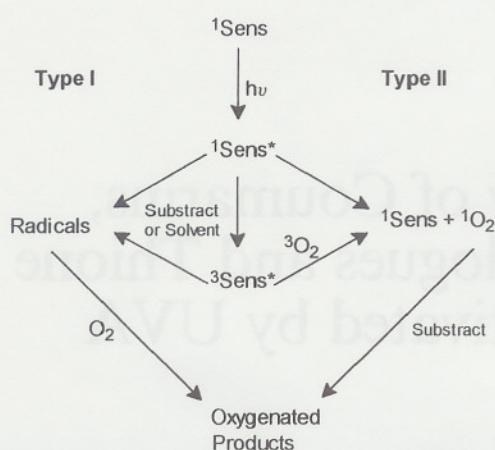


Fig. 1. Schematic photosensitization process.

form a much longer-lived (ms) triplet state (³Sens*; paired electron spins), which can have more chance to react. Consequently, in most natural and synthetic photosensitizers, the triplet state, directly, or the oxygen it sensitizes to the singlet chemically reactive state, are

the active phototoxic agents. Obviously the triplet formation quantum yield (number of triplet states/number of absorbed photons) is a determinant parameter of effectiveness.

Two photosensitization mechanisms, named Type I and Type II processes, are usually considered.⁷ In the Type I process, direct reaction between the excited sensitizer and substrate (amino acids, pyrimidine bases, unsaturated fatty acids, etc.) or solvent occurs, to give radicals or radical ions, which undergo further reactions with oxygen. The Type II process is characterized by energy transfer from the sensitizer to molecular oxygen (³O₂; triplet state) to form the highly reactive singlet molecular oxygen (¹O₂). These two processes are generally in competition as shown schematically in Fig. 1.

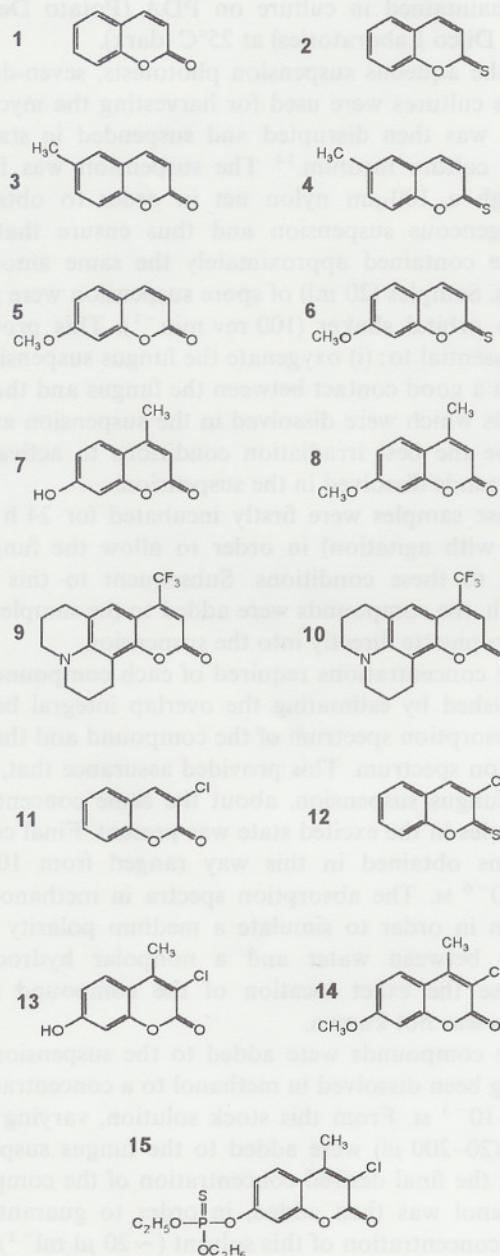
The photophysical properties of a large number of new thione analogues of coumarins, chromones and their furo derivatives (psoralens, khellin and visnagin) have recently been examined.⁸ The primary purpose of that work was to evaluate which of these compounds might have the photophysical characteristics to make

TABLE 1
Trivial and Systematic Names of Compounds Studied

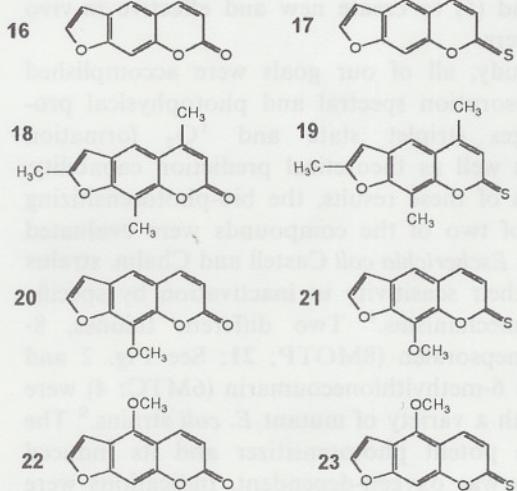
	Trivial or common name ^a	Systematic name
1	Coumarin	2H-chromen-2-one
2	Thionecoumarin	2H-chromene-2-thione
3	6-Methylcoumarin	6-Methyl-2H-chromen-2-one
4	6-Methylthionecoumarin	6-Methyl-2H-chromene-2-thione
5	7-Methoxycoumarin	7-Methoxy-2H-chromen-2-one
6	7-Methoxythionecoumarin	7-Methoxy-2H-chromene-2-thione
7	7-Hydroxy-4-methylcoumarin	7-Hydroxy-4-methyl-2H-chromen-2-one
8	7-Methoxy-4-methylcoumarin	7-Methoxy-4-methyl-2H-chromen-2-one
9	Coumarin 153	2,3,6,7-Tetrahydro-9-trifluoromethyl-1H,5H,11H-chromeno[6,7,8-i,j]quinolizin-11-one
10	Thionecoumarin 153	2,3,6,7-Tetrahydro-9-trifluoromethyl-1H,5H,11H-chromeno[6,7,8-i,j]quinolizine-11-thione
11	3-Chlorocoumarin	3-Chloro-2H-chromen-2-one
12	3-Chlorothionecoumarin	3-Chloro-2H-chromene-2-thione
13	3-Chloro-7-hydroxy-4-methylcoumarin	3-Chloro-7-hydroxy-4-methyl-2H-chromen-2-one
14	3-Chloro-7-methoxy-4-methylcoumarin	3-Chloro-7-methoxy-4-methyl-2H-chromen-2-one
15	Coumaphos	O-3-chloro-4-methyl-2-oxo-2H-chromen-2-yl O,O-diethyl phosphorothioate
16	Psoralen	Furo[3,2-g]chromen-7-one
17	Thioneopsoralen	Furo[3,2-g]chromene-7-thione
18	4,5',8-Trimethylpsoralen*	2,5,9-Trimethylfuro[3,2-g]chromen-7-one
19	4,5',8-Trimethylthioneopsoralen*	2,5,9-Trimethylfuro[3,2-g]chromene-7-thione
20	8-Methoxypsoralen* (8MOP)	9-Methoxyfuro[3,2-g]chromen-7-one
21	8-Methoxythioneopsoralen* (8MOTP)	9-Methoxyfuro[3,2-g]chromene-7-thione
22	5-Methoxypsoralen* (5MOP)	4-Methoxyfuro[3,2-g]chromen-7-one
23	5-Methoxythioneopsoralen* (5MOTP)	4-Methoxyfuro[3,2-g]chromene-7-thione
24	Thionechromone	4H-chromene-4-thione
25		2-Phenyl-4H-benzo[b]thiin-4-one
26		2-Phenyl-4H-benzo[b]thiine-4-thione
27	Flavone	2-Phenyl-4H-chromen-4-one
28	Thioneflavone	2-Phenyl-4H-chromene-4-thione
29	7,8-Dihydroxythioneflavone	7,8-Dihydroxy-2-phenyl-4H-chromene-4-thione
30	α -Naphthothioneflavone	2-Phenyl-4H-benz[h]chromene-4-thione
31	Khellin	4,9-Dimethoxy-7-methylfuro[3,2-g]chromen-5-one
32	Thionekhellin	4,9-Dimethoxy-7-methylfuro[3,2-g]chromene-5-thione

^a The numbering of substituents in the trivial names marked with an asterisk does not follow standard systematic usage. Since these names have been used elsewhere, they are retained here, but care must be taken in making comparisons with other compounds.

COUMARINS



PSORALENS



CHROMONES

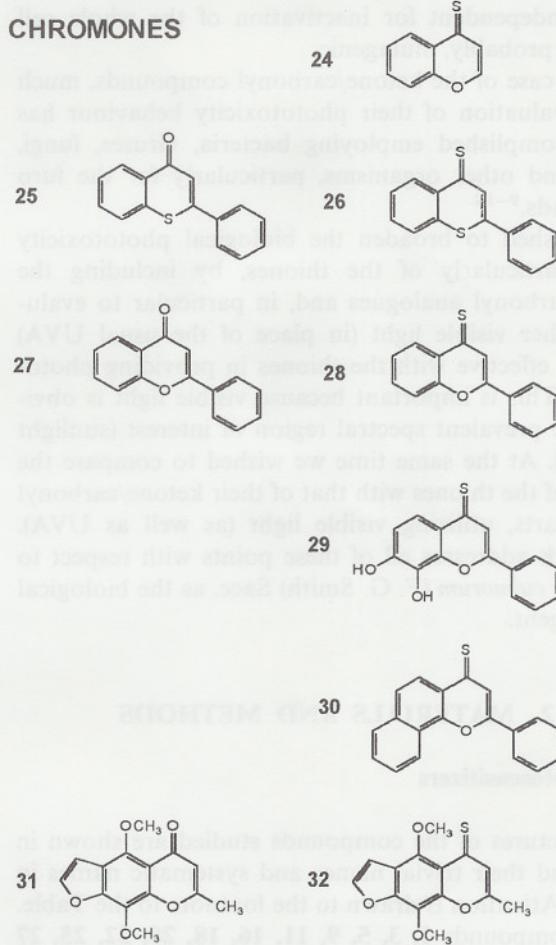


Fig. 2. Structures of compounds discussed. (See Table 1 for trivial and systematic names).

potentially good bio-photosensitizers. In addition we compared their photophysical properties with a significant number of parent ketone/carbonyl analogues, some of which are known to be bio-photosensitizers.

The specific goals were (1) to shift the absorption spectrum as far as possible into the visible region (or UVA), (2) to increase the quantum yield of triplet state formation, (3) to increase the quantum yield of singlet

oxygen (${}^1\text{O}_2$) formation, (4) to be able to predict via theoretical calculations the absorption spectral properties of the molecules of concern as well as charge densities, etc. and (5) to create new and effective in-vivo photosensitizers.

In that study, all of our goals were accomplished regarding absorption spectral and photophysical property changes (triplet state and ${}^1\text{O}_2$ formation increases), as well as theoretical prediction capability. On the basis of these results, the bio-photosensitizing capabilities of two of the compounds were evaluated using mutant *Escherichia coli* Castell and Chalm. strains differing in their sensitivity to inactivation by specific phototoxic mechanisms.⁹ Two different thiones, 8-methoxythioneopsonal (8MOTP; 21; See Fig. 2 and Table 1) and 6-methylthionecoumarin (6MTC; 4) were examined with a variety of mutant *E. coli* strains.⁹ The latter was a potent photosensitizer and its induced phototoxicity was oxygen-dependent. Indications were that the cell membrane was the target. On the other hand, 8MOTP, although an active phototoxin, was oxygen-independent for inactivation of the whole cell and was, probably, mutagenic.

In the case of the ketone/carbonyl compounds, much of the evaluation of their phototoxicity behaviour has been accomplished employing bacteria, viruses, fungi, insects and other organisms, particularly for the furo compounds.^{9–12}

We wished to broaden the biological phototoxicity study, particularly of the thiones, by including the ketone/carbonyl analogues and, in particular to evaluate whether visible light (in place of the usual UVA) could be effective with the thiones in providing phototoxicity. This is important because visible light is obviously the prevalent spectral region of interest (sunlight emission). At the same time we wished to compare the activity of the thiones with that of their ketone/carbonyl counterparts, utilizing visible light (as well as UVA). This work addresses all of these points with respect to *Fusarium culmorum* (F. G. Smith) Sacc. as the biological testing agent.

2 MATERIALS AND METHODS

2.1 Photosensitizers

The structures of the compounds studied are shown in Fig. 2 and their trivial names and systematic names in Table 1. Attention is drawn to the footnote to the Table.

The compounds 1, 3, 5, 9, 11, 16, 18, 20, 22, 25, 27 and 31 were purchased from Aldrich or Kodak and used without further purification. Coumaphos (15) was purchased from Riedel de Haen. The thione derivatives were synthesized as described elsewhere.⁸ The compounds 8, 7, 13 and 14 were synthesized as reported elsewhere.¹³

2.2 Biological tests

F. culmorum (laboratory standard strain) was grown and maintained in culture on PDA (Potato Dextrose Agar; Disco Laboratories) at 25°C (dark).

In the aqueous suspension phototests, seven-day-old fungus cultures were used for harvesting the mycelium, which was then disrupted and suspended in standard liquid culture medium.¹⁴ The suspension was filtered through a 100-μm nylon net in order to obtain an homogeneous suspension and thus ensure that each sample contained approximately the same amount of spores. Samples (20 ml) of spore suspension were placed on an orbital shaker (100 rev min⁻¹). This procedure was essential to: (i) oxygenate the fungus suspension, (ii) obtain a good contact between the fungus and the compounds which were dissolved in the suspension and (iii) achieve the best irradiation conditions to activate the compounds dissolved in the suspension.

These samples were firstly incubated for 24 h (25°C dark, with agitation) in order to allow the fungus to adapt to these conditions. Subsequent to this initial growth, the compounds were added to the samples, with a micropipette directly into the suspension.

The concentrations required of each compound were established by estimating the overlap integral between the absorption spectrum of the compound and the lamp emission spectrum. This provided assurance that, in the final fungus suspension, about the same concentration of species in the excited state was present. Final concentrations obtained in this way ranged from 10^{-4} to 2×10^{-6} M. The absorption spectra in methanol were chosen in order to simulate a medium polarity somewhere between water and a nonpolar hydrocarbon because the exact location of the compound in the fungus was not known.

The compounds were added to the suspension after having been dissolved in methanol to a concentration of 10^{-2} – 10^{-3} M. From this stock solution, varying quantities (20–200 μl) were added to the fungus suspension to get the final desired concentration of the compound. Methanol was then added, in order to guarantee the same concentration of this solvent (~ 20 μl ml⁻¹), in all samples.

There were twelve 20-ml samples for each compound tested. Half of them were kept in the dark and the remaining ones were irradiated. Blank samples, either without any compound or with only methanol, were also tested both in the dark, and under irradiation. These consisted of eight samples without compound (four in the light and four in the dark) and eight samples with methanol (of the quantity needed to make up the test solutions) which consisted of four in the light and four in the dark. The samples without compound were used to evaluate whether the fungus was growing properly. The samples with methanol were used to evaluate the effect of methanol on the growth. 8-MOP (20) was

used as control due to its well known phototoxic behaviour.

After 72 h continuous irradiation, the results were obtained as a difference between dry weights of irradiated and non-irradiated samples.

For the silica gel phototests, all the compounds were dissolved in methanol in equal concentration (10^{-3} M); again 8-MOP was used as control. Aliquots of these solutions were applied in triplicate on TLC plates with a micropipette. Spots of ~ 1 cm in diameter were obtained. This was easy to achieve because the R_F -values (methanol) of the tested compounds are all within the range 0.6–0.7. The plates were sprayed with the fungus suspension (prepared as described above) and subsequently placed in humid chambers, one of the chambers being irradiated and the other kept in the dark, at 25°C for 72 h. Control plates which had not been irradiated permitted us to distinguish between dark toxicity and phototoxicity. Inhibition areas were revealed as white spots, and the relative diameters were considered as measures of the relative phototoxicities. In order to be sure that the results did not reflect different abilities of the compounds to diffuse on the silica plates in contact with the moisture of the humid chamber, the following test was performed. Plates with ~ 1 cm spots of the compounds were eluted with water and, after being dried, the spots were revealed by UV light. Irrespective of the compound tested, the spots were only slightly dis-

placed (in accordance with the small R -values in water) and their sizes were not significantly changed.

2.3 Instrumentation

The light sources were five fluorescent lamps of UVA light (Philips TLK 40W/09N; emission range ~ 310 –440 nm; $\lambda_{\text{max}} = 354$ nm; $73 \mu\text{mol m}^{-2} \text{s}^{-1}$) or visible light (Osram L18w/10 Daylight; emission range ~ 350 –730 nm; $\lambda_{\text{max}} = 485$ nm; $48 \mu\text{mol m}^{-2} \text{s}^{-1}$). In some cases visible (VIS) or UVA irradiation was selected using a Xenon arc lamp (150 W) with a set of appropriate filters (cut-off at 400 nm and 320 nm, respectively).

Absorption spectra were determined in a Beckman DU-70 Spectrophotometer. Emission spectra of the light sources and solar emission were recorded using the emission monochromator and photomultiplier of a SPEX Fluorolog fluorimeter and corrected for the wavelength response of the apparatus.

3 RESULTS AND DISCUSSION

In Fig. 3, the absorption spectra of some representative natural photosensitizers (coumarins, chromones, psoralens and flavones) and their synthetic thione analogues are shown. Note the dramatic effect of the insertion of sulfur in place of oxygen in the ketone/carbonyl group on the spectral absorption range. Clearly, the thione

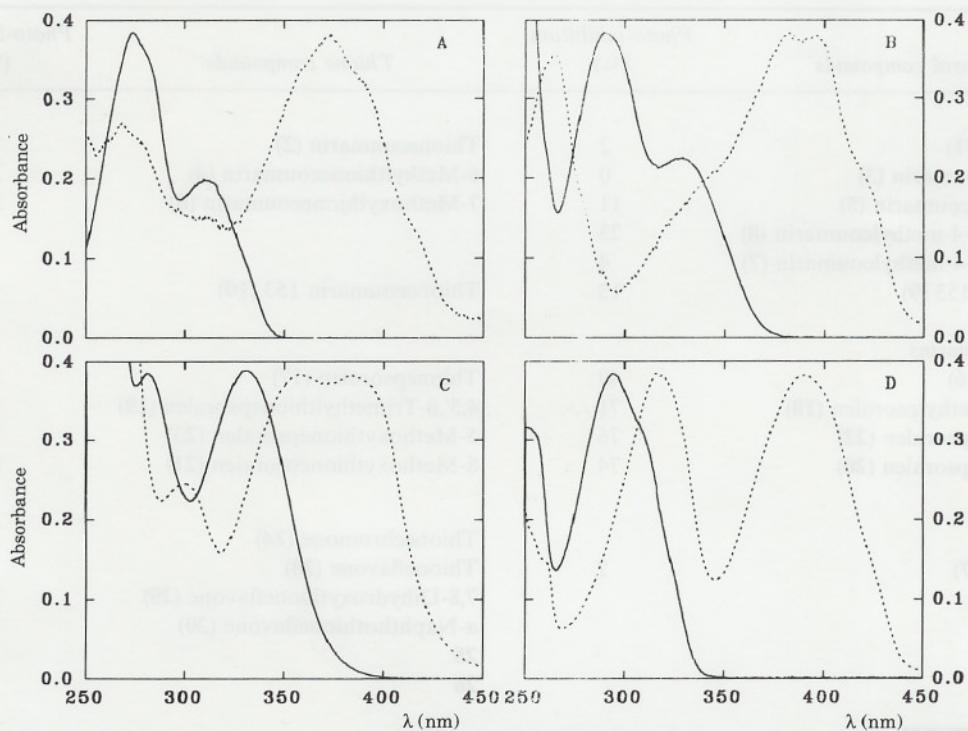


Fig. 3. Absorption spectra of (—) the natural compounds, and (---) their thione analogues in methanol: A, coumarin (3.6×10^{-5} M) and thione coumarin (3.27×10^{-5} M); B, psoralen (3.2×10^{-5} M) and thione psoralen (2.1×10^{-5} M); C, khellin (7.0×10^{-5} M) and thione khellin (4×10^{-5} M); D, flavone (1.5×10^{-5} M) and thione flavone (1.8×10^{-5} M). The concentrations were chosen in order to equalize the absorbance maxima to 0.4.

absorption strongly overlaps with natural (sun) light emission, in contrast with that of their natural counterparts. Moreover, the fluorescence quantum yields of the thione derivatives fall to negligible values, due to a large increase of their triplet formation quantum yields.⁸ Consequently, these compounds will be potentially much more photoactive because of (1) their large sunlight absorption and (2) their larger triplet and singlet oxygen quantum yields as well as their longer excited state lifetimes (triplet lifetimes are normally more than three orders of magnitude larger than singlet lifetimes).

The relative UVA-phototoxic activities of the compounds against *F. culmorum* are given in Table 2 (aqueous suspension) and Table 3 (silica plates).

In Table 2, where the results refer to equal concentrations of excited state species, the following observations can be made:

(i) In general, the thione compounds display photoactivity levels comparable to those of their parent natural compounds. Exceptions to this trend are thionecoumarin 153 (10), thioneepsoralen (17) and thionekhellin (32).

(ii) Three thionecoumarins are phototoxic, in contrast to their parent compounds. This behaviour parallels that found with bacteria, where 6-methylthionecoumarin (4) was active and 6-methylcoumarin (3) was not.⁹

(iii) 7-Methoxy-4-methylcoumarin (8) is active while 7-hydroxy-4-methylcoumarin (7) is inactive. Similar results were obtained by others with hydroxy-*v* methoxy-substituted psoralens, namely 5-hydroxypsoralen (inactive) *v* 5-methoxypsoralen (22) (active)¹⁵ and also for 8-hydroxypsoralen (hydroxy analogue of 20) (inactive) *v* 8-methoxypsoralen (20) (active).¹⁶ This contrasting behaviour is most probably due to differences in partition of the compounds between water and target. The hydroxycoumarins exist in the anionic form at neutral pH, and will be much more water-soluble.¹⁷

(iv) The psoralens show more phototoxicity than any other family of compounds. This is also true for the thioneepsoralens.

(v) Among the chromones tested, flavone (27) was inactive, as were its thione analogue (28) and the corresponding compounds based on the benzothiin nucleus, 25 and 26. However, three other thionechromones, 24, 29 and 30 showed some activity. The furanochromone, khellin (31) was slightly active, but its thione analogue, 32, was inactive.

The degrees of photoinhibition produced by natural compounds and their thione derivatives when irradiated with UVA or VIS light on silica gel plates are compared in Table 3. The following observations can be made:

TABLE 2

Photo-inhibition of *Fusarium culmorum* after Three Days Growth in Suspension as Evaluated from Dry Weight on Mycelium under UVA light

<i>Natural compounds</i>	<i>Photo-inhibition (%)</i>	<i>Thione compounds</i>	<i>Photo-inhibition (%)</i>
<i>Coumarins</i>			
Coumarin (1)	2	Thionecoumarin (2)	11
6-Methylcoumarin (3)	0	6-Methylthionecoumarin (4)	24
7-Methoxycoumarin (5)	11	7-Methoxythionecoumarin (6)	23
7-Methoxy-4-methylcoumarin (8)	23		
7-Hydroxy-4-methylcoumarin (7)	4	Thionecoumarin 153 (10)	3
Coumarin 153 (9)	13		
<i>Furanocoumarins</i>			
Psoralen (16)	49	Thioneepsoralen (17)	20
4,5'-8-Trimethylpsoralen (18)	71	4,5',8-Trimethylthioneepsoralen (19)	58
5-Methoxypsoralen (22)	76	5-Methoxythioneepsoralen (23)	73
8-Methoxypsoralen (20)	74	8-Methoxythioneepsoralen (21)	65
<i>Chromones</i>			
Flavone (27)	2	Thionechromone (24)	32
		Thioneflavone (28)	2
		7,8-Dihydroxythioneflavone (29)	23
		α -Naphthothioneflavone (30)	11
		25	0
		26	0
<i>Furanochromones</i>			
Khellin (31)	17	Thionekhellin (32)	1

The value presented for each compound is the average of at least four different experiments. The error of the method using this particular biological target is 11% (in absolute value).

TABLE 3
Photo-inhibition of *Fusarium culmorum* after Three Days Growth on Silica Gel Plates as Evaluated from the Diameter of the Inhibition Zones, under UVA and VIS light

Natural compounds	Photo-inhibition (cm)		Thione compounds	Photo-inhibition (cm)	
	UVA ^a	VIS ^b		UVA ^a	VIS ^b
<i>Coumarins</i>					
Coumarin (1)	0	0	Thionecoumarin (2)	1.3	0.5
6-Methylcoumarin (3)	0	0	6-Methylthionecoumarin (4)	1.4	1.0
7-Methoxycoumarin (5)	1.3	0	7-Methoxythionecoumarin (6)	1.0	1.4
7-Methoxy-4-methylcoumarin (8)	1.5	0			
7-Hydroxy-4-methylcoumarin (7)	0	0			
3-Chlorocoumarin (11)	0.7	0	3-Chlorothionecoumarin (12)	1.3	1.3
3-Chloro-7-hydroxy-4-methylcoumarin (13)	0	0			
3-Chloro-7-methoxy-4-methylcoumarin (14)	1.1	1.1			
Coumaphos (15)	0	0			
<i>Furanocoumarins</i>					
Psoralen (16)	1.9	0 ^c (2.0)	Thionepsoralen (17)	1.9	1.4
4,5',8-Trimethylpsoralen (18)	1.9	0 ^c (1.8)	4,5',8-Trimethylthionepsoralen (19)	2.1	1.6
5-Methoxypsoralen (22)	3.5	0 ^c (2.0)	5-Methoxythionepsoralen (23)	3.4	1.7
8-Methoxypsoralen (20)	3.6	0 ^c (2.2)	8-Methoxythionepsoralen (21)	3.0	1.5
<i>Furanochromones</i>					
Khellin (31)	1.4	0	Thionekhellin (32)	0	0

^a Philips lamps.

^b Osram lamps.

^c Xenon lamp with cut-off filter.

(i) Thionecoumarins show phototoxicity either under UVA or VIS light irradiation, while the corresponding natural coumarins are active only under UVA irradiation. The thione compounds are either more active with VIS than with UVA light or equally active.

(ii) Thionekhellin (32), is UVA and VIS inactive, and khellin (31) is only UVA active, as expected.

(iii) Thionepsoralens are the most phototoxic thiones, both with UVA and VIS excitation. With psoralens, some photoactivity was observed when using the Osram lamps. However, careful filtering of a Xenon lamp output in order to avoid the ultraviolet region showed that VIS light activity occurs exclusively in the cases of the thiones. Therefore, it is clear that the Osram lamps in fact emit a small amount of UVA light.

Negative results were obtained in dark with all the tested compounds, except for the psoralens and thionepsoralens, which showed some residual activity.

4 CONCLUSIONS

From these results it may be concluded that the thione compounds represent an effective improvement, relative to their natural counterparts, with respect to potential application as photopesticides. The improvements

include (1) significant absorption of sunlight without loss of photoactivity per excited molecule, (2) increase in the triplet and singlet oxygen quantum yields⁸ (most phototoxic mechanisms involve the triplet state directly or indirectly),¹⁵ (3) good efficiency (they are photoactive against *F. culmorum*, which has been referred to as difficult to control)¹⁸ and (4) probable bio- or photodegradability (thiones are efficiently hydrolysed back to their natural parent compounds under varying environmental conditions).¹⁹ Therefore, although there are still several open questions regarding the photoactivity mechanisms, such as the location of the photosensitizer in the fungus, and the kinetics of photo/thermal degradation of these compounds, we think that these results are encouraging for the possible utilization of thione analogues of natural compounds in the control of phytopathogenic fungi.

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