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Emerging antifungal azoles and effects on *Magnaporthe grisea*

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ABSTRACT

Derivatives of pyrazolo[1,5-*a*][1, 3, 5]triazine-2,4-dione,pyrazolo[1,5-c][1, 3, 5]thiadiazine-2-one, pyrazolo[3,4-*d*][1, 3]thiazine-4-one, and pyrazolo[3,4-*d*][1, 3]thiazine-4-thione were screened for antifungal activity against the causal agent of rice blast disease, *Magnaporthe grisea*. The compounds were tested at doses ranging from 10 to 200 $\mu g\,ml^{-1}$, using the commercial fungicide tricyclazole as reference compound. All triazine derivatives inhibited the growth and pigmentation of the mycelia less effectively than tricyclazole. The thiadiazine derivatives proved to be more effective than their triazine counterparts, but only 4-(butylimino)-7-methylpyrazolo[1,5-c][1,3,5]thiadiazine-2-one **(2h)** and 4-(cyclohexylimino)-7-methylpyrazolo[1,5-c][1,3,5]thiadiazine-2-one **(2j)** were more effective than tricyclazole. Pyrazolo[3,4-*d*][1,3]thiazine-4-one derivatives were active only at the highest doses, whereas members of the pyrazolo[3,4-*d*][1,3]thiazine-4-thione series inhibited fungal growth at the lowest concentrations used, at which tricyclazole had no effect. A dose-dependent mechanism might be responsible for this effect, with lipophilicity as the governing factor. Within a given set, the presence of a cyclohexyl or an *n*-butyl group generally increased antifungal activity, with respect to both growth inhibition and cell de-pigmentation of the mycelium, suggesting that a higher lipophilicity might improve transport inside the cells. SEM and TEM of *M. grisea* hyphae showed that treatment with the most active substance **(2h)** caused significant ultrastructural effects, particularly on the endomembrane system, suggesting a mechanism of action similar to that of most azole fungicides. Dissimilarities were also observed, with no alterations of the cell wall evident. In conclusion, several compounds showed greater inhibition than tricyclazole, and therefore provide useful new chemistry for control of *M. grisea* infections.

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Introduction

Within a programme to investigate the antifungal activity of nitrogen heterocycles, several compounds were synthesized and tested on phytopathogenic fungi of different taxa. Some of these compounds showed interesting properties, such as pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-thiones effective in controlling *Pythium ultimum* and *Rhizoctonia solani* (Giori *et al.* 1985), and derivatives bearing a trifluoromethyl group, whose activity was comparable to that of the reference commercial fungicides captafol and mancozeb (Vicentini *et al.* 1989; Mares *et al.* 2000; Vicentini *et al.* 2000).

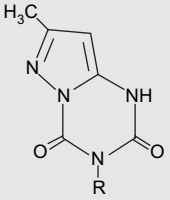
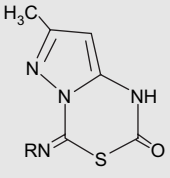
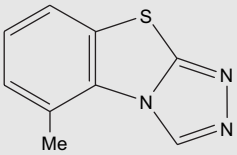
More recently, three sets of pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-thione, pyrazolo[3,4-*d*][1,3]thiazine-4-one/thione, and pyrazolo [1,5-*c*][1,3,5]thiadiazine-4-one/thione derivatives were screened for antifungal activity against the causal agent of rice blast disease, *Magnaporthe grisea,* some of which caused a remarkable inhibition of fungal growth (Vicentini *et al.* 2002). Among them, 7-methyl-2-phenylpyrazolo[1,5-*c*][1,3,5]thiadiazine-4-one inhibited the fungus even at the lowest dose tested (10 $\mu g\,ml^{-1}$), at which the reference commercial fungicide tricyclazole was completely ineffective. Moreover the same compound, when applied to rice plants at concentrations at which it was able to control fungal growth, did not exert any significant effects upon the plant itself.

Further analogues of tricyclazole were tested on the same fungal species and other fungi that are pathogenic for plants, animals and humans. However, these pyrazolo[3′,4′:4,5]thiazolo[2, 3-*c*]-1,2,4-triazole derivatives were less effective than tricyclazole, although, interestingly, they seem to possess a mechanism of action dissimilar to that of this fungicide (Mares *et al.* 2004).

Since the results obtained with tricyclazole analogues showed no improvement of the lead molecule, attention has been focused on four series of other new pyrazoles, in order to extend this screening programme for antifungal substances. These new compounds are derivatives of:

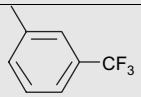
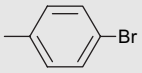
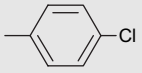
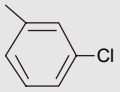
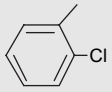
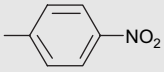
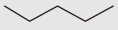
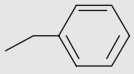
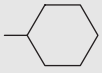
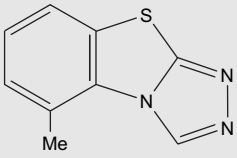
(1) pyrazolo[1,5-*a*][1, 3, 5]triazine-2,4-diones (**1g–l,** Table 1)
(2) pyrazolo[1,5-*c*][1, 3, 5]thiadiazine-2-ones (**2g–j,** Table 1)
(3) pyrazolo[3,4-*d*][1, 3]thiazine-4-ones (**3a–j,** Table 2)
(4) pyrazolo[3,4-*d*][1, 3] thiazine-4-thiones (**4a–j,** Table 3)

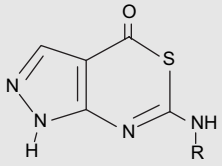
Table 1 – Antifungal activity of pyrazolo[1,5-*a*][1,3,5]triazine-2,4-dione and pyrazolo[1,5-*c*] [1,3,5]thiadiazine-2-one derivatives 1,2 and tricyclazole

Compound	R	Concentration ($\mu g\,ml^{-1}$)			
		20	50	100	200
1g		3.8 ± 0.6	7 ± 1.6	9 ± 1.9	10 ± 0.6
1h		8.7 ± 0.7	10 ± 0.9	25 ± 2.7	31 ± 0.7
1i		6.5 ± 1.4	12 ± 1.7	16 ± 0.4	17 ± 1.4
1j		11 ± 1.8	25 ± 0.8	42 ± 1.5	49 ± 1.2
1k		3.2 ± 0.8	4 ± 1.3	9 ± 1.1	15 ± 0.3
1l		0	0	2 ± 0.5	15 ± 1.3
2g		8.5 ± 1.6	14 ± 0.7	29 ± 1.7	46 ± 0.5
2h		46 ± 0.9	56 ± 0.6	80 ± 1.9	82 ± 1.4
2i		13.5 ± 0.8	15 ± 1.8	17 ± 0.3	18 ± 0.8
2j		19 ± 0.6	29 ± 1.8	73 ± 0.7	100 ± 0
Tricyclazole		12 ± 0.8	24 ± 1.4	62 ± 0.9	94 ± 1.4

Percentage inhibition of the growth of *Magnaporthe grisea* evaluated by measuring colony diameter 5 d after treatment; values are means of three trials made in triplicate ± SE.

Table 2 – Antifungal activity of pyrazolo[3,4-*d*][1,3]thiazine-4-one derivatives 3a–j and tricyclazole

Compound	R	Concentration (μg ml^{-1})			
		20	50	100	200
3a		2 ± 8.22	35 ± 6.4	48 ± 6.13	53 ± 0.96
3b		7 ± 3.4	25 ± 7.88	35 ± 5.12	36 ± 6.5
3c		3 ± 6.48	14 ± 1.5	37 ± 5.67	46 ± 4.34
3d		−10 ± 3.0	4 ± 2.5	9 ± 3.0	40 ± 7.04
3e		−43 ± 2.52	48 ± 15.06	60 ± 2.63	71 ± 1.5
3f		−10 ± 0.5	−7 ± 2.94	−5 ± 2.64	5 ± 3.32
3g		−9 ± 1.41	18 ± 3.77	36 ± 6.48	53 ± 7.59
3h		4 ± 2.38	22 ± 4.19	41 ± 2.98	67 ± 6.98
3i		40 ± 3.16	62 ± 1.5	75 ± 2.38	90 ± 1.7
3j		12 ± 2.75	41 ± 2.2	68 ± 4.42	81 ± 4.04
Tricyclazole		12 ± 0.8	24 ± 1.4	62 ± 0.9	94 ± 1.4



Data expressed as in Table 1.

The synthesis of compounds **1 g–l** (pyrazolo[1,5-*a*][1, 3, 5]triazine-2,4-dione derivatives) and **2 g–j** (pyrazolo[1,5-c][1, 3, 5]thiadiazine-2-one derivatives), and their activity as potential herbicides, were reported recently (Vicentini *et al.* 2004). Similar compounds like 1,3,4-oxadiazolo[3,2-*a*]-s-triazine-5,7-diones were found instead to possess biological activity against *Aspergillus niger* and *Fusarium oxysporum*, comparable with that of the commercial product Dithane M-45 (Global Headquarters, Dow Agro Sciences LLC9330 Zionsville Road, Indianapolis, IN 46268. Active ingredient: mancozeb; Mishra *et al.* 2000). The other two series **3a–j** (pyrazolo[3,4-*d*][1, 3]thiazine-4-ones) and **4a–j** (pyrazolo[3,4-*d*][1, 3] thiazine-4-thiones) showed some ability to act as photosynthetic electron transport inhibitors (Vicentini *et al.* 2005).

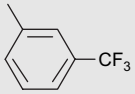
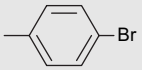
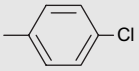
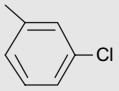
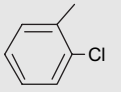
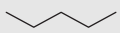
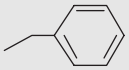
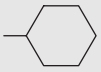
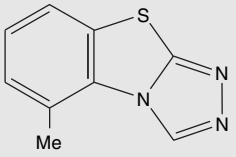
The present paper reports the evaluation of the four new series of compounds as possible antifungal substances. The purpose was to find new fungicides to inhibit the growth of *Magnaporthe grisea* (anamorph *Pyricularia oryzae (cooke) sacc.*) that causes disease in many species of the grass family, including rice (Zeigler *et al.* 1994). Information concerning their possible mechanisms of action was obtained by SEM and TEM of treated hyphae.

Materials and methods

Chemicals

All the compounds (series **1–4**) were synthesized in the laboratories of the Department of Pharmaceutical Science,

Table 3 – Antifungal activity of pyrazolo[3,4-*d*][1,3]thiazine-4-thione derivatives 4a–j and tricyclazole

Compound	R	Concentration (μg ml^{-1})			
		20	50	100	200
4a		21 ± 8.22	24 ± 2.5	52 ± 4.54	53 ± 2.75
4b		−17 ± 5.0	5 ± 10.07	27 ± 5.67	40 ± 6.65
4c		−3 ± 1.7	12 ± 1.91	17 ± 3.77	18 ± 2.54
4d		8 ± 2.08	24 ± 2.0	29 ± 4.19	34 ± 1.0
4e		3 ± 2.21	14 ± 6.32	26 ± 4.7	37 ± 7.5
4g		0 ± 8.22	11 ± 2.5	54 ± 4.54	70 ± 1.63
4h		14 ± 1.89	18 ± 2.94	35 ± 4.78	36 ± 1.71
4i		46 ± 2.44	46 ± 2.21	49 ± 3.31	50 ± 2.45
4j		43 ± 4.08	47 ± 4.54	59 ± 2.06	68 ± 3.09
Tricyclazole		12 ± 0.8	24 ± 1.4	62 ± 0.9	94 ± 1.4

Data expressed as in Table 1.

University of Ferrara, as described previously (Vicentini *et al.* 2004; Vicentini *et al.* 2005)

Fungal isolate

Magnaporthe grisea (T.T. Herbert) Yaegashi & udagawa (ATCC 64413) was purchased from the American Type Culture Collection (Manassas, VA) and maintained at 4 °C as agar slants on potato dextrose agar (PDA; Oxoid, UK).

Evaluation of antifungal activity

To evaluate the ability of the compounds to inhibit fungal growth, cultures of *Magnaporthe grisea* were obtained by transplanting 10 mm diam, mycelium discs, from a single culture in stationary phase. These were incubated at 26 ± 1 °C on PDA (pH 5.6 ± 0.2), on thin sterile sheets of cellophane (BeP Italia, Gorizia, Italy), until the logarithmic phase of growth was reached, and then transferred to Petri dishes containing the medium supplemented with the compound to be tested. Each compound was dissolved in dimethyl sulphoxide [DMSO 99.5 % (v/v)], and a dilution was added aseptically to the medium at 45 °C to obtain a final concentration of 10, 20, 50, 100 and 200 μg ml^{-1}. The DMSO concentration in the final medium was adjusted to 0.1 % (v/v). Control media contained equivalent quantities (0.1 %) of DMSO. The growth rate was determined by measuring colony diameter daily for 5 d after transfer of the fungus onto dishes containing the test compound. Three replicates were used for each concentration. Growth inhibition was expressed as the mean of values obtained in three independent

experiments. For comparison, the same concentrations (10–200 μg ml^{-1}) of the commercial fungicide tricyclazole (Beam, Dow AgroSciences, Italy) were also tested.

Antifungal activity of each compound was also evaluated by determining the capacity to inhibit mycelial pigmentation, by means of visual and photographic evaluation of treated cultures, in comparison with untreated controls of the same age. All observations were made on the tenth day after the transfer of *M. grisea* onto the treated media, because only after this time period was a regular pigmentation of the mycelium seen in the untreated control cultures. Tricyclazole was used as a reference compound, as the mechanism of action of this fungicide is related to a de-pigmentation of the fungal cell (Woloshuk *et al.* 1983).

Electron microscopy

The morphological changes induced by treatment with the compound inhibiting cell growth most effectively, **2h,** were studied by TEM and SEM. The youngest mycelial cells from untreated colonies and from colonies treated for 24 h with 10 and 100 μg m1^{-1}were harvested and routinely processed as described in Mares *et al.* (2002). The hyphae were fixed with 6 % (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7, for 3 h at 4 °C, washed with buffer, and postfixed overnight with 1 % (w/v) osmium tetroxide (OsO_4) in the same buffer. The samples were then dehydrated in a graded series of ethanol concentrations and embedded in Epon–Araldite resin. Sections were cut with an LKB Ultratome III ultramicrotome, stained with uranyl acetate and lead citrate, and observed with a Hitachi H 800 electron microscope (Hitachi, Tokyo) at an accelerating voltage of 100 kV.

For SEM analysis, hyphae were fixed in glutaraldehyde as for TEM, then briefly postfixed in 1 % OsO_4, rapidly dehydrated in a graded series of acetone concentrations, critical point-dried and gold-coated in a Sputter-coater S 150 (Edwards, Crawley, West Sussex, UK). Observations were made with a Siemens Autoscan scanning electron microscope at an accelerating voltage of 20 kV.

Results

The ability of the new pyrazoles to inhibit the growth of *M. grisea* was evaluated at concentrations of 20–200 μg ml^{-1}, a range in which the reference compound tricyclazole was found to exert an increasing effect. Results are summarized in Tables 1–4.

Pyrazolo[1,5-a][1, 3, 5]triazine-2,4-dione series 1g–l

These derivatives were marginally effective at all doses tested, being less effective than that of the reference fungicide tricyclazole (Table 1). Interestingly, the presence of either a cyclohexyl or a *n*-butyl group (compounds **1j** and **1h**) improved the antifungal activity, but which never reached that of tricyclazole.

Pyrazolo[1,5-c][1, 3, 5]thiadiazine-2-one series 2 g–j

These derivatives have the same substituents as four of the compounds in the previous group (Table 1). With the exception of **2i**, they proved on the whole to be more effective than their triazine counterparts, and always exerted a dose-dependent inhibition of fungal growth. As with series **1g–l,** the presence of a cyclohexyl **(2j)** or a *n*-butyl **(2h)** group resulted in an increased effectiveness, that was generally greater than that of tricyclazole, and evident even at the lower doses. In particular, **2h** caused about 46 % growth inhibition compared with 12 % for tricyclazole at 20 μg ml^{-1}, whereas at the highest concentration of 200 μg ml^{-1} the inhibition was slightly lower (82 %) than that of the reference compound, (94 %). In contrast, fungal growth was inhibited completely by the highest concentration of **2j**.

Pyrazolo[3,4-d][1, 3]thiazine-4-one series 3a–j

The compound bearing a cyclohexyl group **(3j)** also showed good inhibitory activity within this group of derivatives (Table 2). However, in this case the most effective compound **(3i)** contained a benzyl group, and at 200 μg ml^{-1} caused an inhibition of growth similar to that of tricyclazole. The derivatives with butyl **(3h)** and ethyl **(3g)** moieties were less effective, as were those moleculessubstituted with a halogen in various positions, with the exception of **3e,** that has Cl in position 2. At low concentrations, compounds **3d–g** stimulated growth. This was most pronounced with **3e,** whose growth was increased by 43 % at a concentration of 20 μg ml^{-1}.

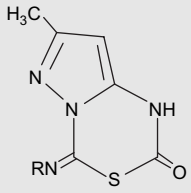
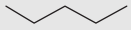
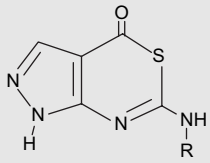
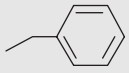
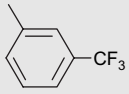
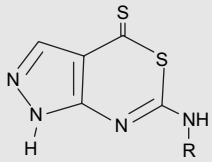
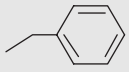
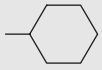
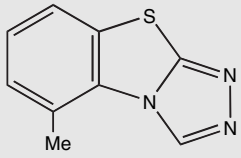
Pyrazolo[3,4-d][1, 3]thiazine-4-thione series 4a–j

The derivatives of this series have exactly the same substituents as their isosteres **3a–j** (Table 3). The compound **4f,** with R = nitrophenyl, was produced with a low chemical yield that was insufficient to perform the *in vitro* experiments. As a whole, they were not as effective as the corresponding substances of the previous series, with the exception of **4g,** that produced a 70 % inhibition at 200 μg ml^{-1}. The substitution in R = cyclohexyl **(4j)** once again showed a positive effect that, nevertheless, was lower than the corresponding **3j**. It is noteworthy that **4i** and **4j** at low doses showed high inhibition values, whereas the effect did not increase with concentration. Compounds **4b** and **4c** showed a slight growth stimulation at the lowest concentration.

For the compounds showing the highest activity at the lowest dose of 20 μg ml^{-1}, further experiments were performed by treating *M. grisea* with a lower dose of 10 μg ml^{-1} (Table 4). At this concentration, **2h** was the most effective substance on all fungi showing a greater inhibition than tricyclazole, which had no effect.

Many of these new compounds, belonging to all four groups of molecules, were able to induce macroscopic modifications of the pigmentation of *M. grisea* (Table 5). Using this parameter as an index of antifungal activity, compounds **2h** (R = *n*-butyl) and **3i** (R = benzyl) were the most active, causing substantial de-pigmentation of the hyphae at the lowest dose of 10 μg ml^{-1}, similar to tricyclazole (Fig 1 A–C for **2h, 3i,** and tricyclazole, respectively). Significant effects were also caused, in decreasing order, by compounds **2j** and **3j** (R = cyclohexyl), **3f** (R = 4-nitrophenyl) and **4i** (R = benzyl). Interestingly, some other substances that had little effect on growth, such as **1k** (R = phenyl), **1j** (R = cyclohexyl), **2i**

Table 4 – Antifungal activity of selected pyrazolo[1,5-*c*][1,3,5] thiadiazine-2-one and pyrazolo[3,4-*d*][1,3]thiazine-4-one/thione derivatives 2–4 and tricyclazole

Compound		R	Concentration ($\mu g\ ml^{-1}$) 10
2h			42 ± 0.8
3i			23 ± 3.59
4a			12 ± 2.5
4i			39 ± 1.0
4j			30 ± 2.5
Tricyclazole			0

Data expressed as in Table 1.

(R = benzyl), **3e** (R = 2-chlorophenyl) and **4h** (R = *n*-butyl), were able to induce a de-pigmentation of the mycelium, in particular at the highest doses of 100 and 200 $\mu g\ ml^{-1}$. For comparison, the decrease of colour intensity in *M. grisea* mycelia induced by the reference compound tricyclazole at 10 $\mu g\ ml^{-1}$ is also shown (Fig 1C).

One of the most effective compounds, 4-butyl-7-methyl-pyrazolo[1,5-*c*][1,3,5]thiadiazine-2-one **(2h),** was chosen for further study by electron microscopy. This choice was made because it was one of the four substances **(2j, 2h, 3i, 3j)** that induced an inhibition of growth higher than 80 % at 200 $\mu g\ ml^{-1}$, and produced the greatest inhibition at the lowest dose, 10 $\mu g\ ml^{-1}$. Moreover, **2h** was one of the substances that caused a clear de-pigmentation of the mycelium. SEM observation of the external, and thus youngest, portions of mycelia from colonies in untreated controls (Fig 2A) showed hyphae with typical tapered apices and a smooth surface (Fig 2B). Portions of the mycelium from the inner, older regions of colonies showed hyphae often aggregated into cords (Fig 2C), with a surface covered by globular structures of various size (Fig 2D). After treatment with the lowest dose of **2h** (10 $\mu g\ ml^{-1}$), the hyphae lost their linearity, often forming unusual ring-like structures (Fig 2E). In these samples, approximately 40 % of the apices appeared ruptured (Fig 2F). When *M. grisea* was treated with the highest dose (100 $\mu g\ ml^{-1}$), hyphal apices appeared swollen, with a globular shape, in addition to the changes mentioned above (Fig 2G).

TEM showed untreated controls with a normal cytoplasm where ribosomes, endoplasmic reticulum, lipid droplets and nuclei were visible (Fig 3A). A thick wall surrounded the hyphae and grew centripetally to form septa with associated Woronin bodies (Fig 3B). The most significant alteration in all the treated samples was the presence of many membranous structures arranged around the periphery of the cytoplasm (Fig 3C). Similar structures, likely to be portions of rough endoplasmic reticulum, were also visible near nuclei, which had an altered ultrastructure (Fig 3D). Frequently the karyoplasm was separated from the nuclear envelope so that a space was visible (asterisk in Fig 3D). This nuclear anomaly was noted in fungi treated with both 10 (20 % of the observed hyphae) and 100 $\mu g\ ml^{-1}$ (about 50 % of the observed hyphae). The nuclei also often showed wide breaks in their envelope, so that the nuclear and the cytoplasmic contents were in contact. Other anomalies were an increased aggregation of glycogen granules and the presence of polymembranous bodies, that were absent in the controls (Fig 3E). The latter were of various sizes and shapes, located in the cytoplasm (Fig 3F) often near developing septa (Fig 3E),

Table 5 – De-pigmentation of *Magnaporthe grisea* colonies after treatment with the series of pyrazolo derivatives (see Tables 1–4)

Compound	Concentration (μg ml^{-1})			
	20	50	100	200
1g	–	–	–	–
1h	–	–	+	+
1i	–	–	–	–
1j	–	+	+	++
1k	–	–	+	+++
1l	–	–	–	–
2g	–	–	–	+
2h	++	++	+++	+++
2i	–	–	+	++
2j	+	++	+++	+++
3a	–	–	–	–
3b	–	–	–	–
3c	–	–	–	–
3d	–	–	–	–
3e	–	+	++	+++
3f	+	++	++	+++
3g	–	–	–	–
3h	–	–	+	+
3i	++	++	+++	+++
3j	+	+	++	++
4a	–	–	–	+
4b	–	–	–	–
4c	–	–	–	–
4d	–	–	–	–
4e	–	–	–	–
4g	–	–	–	–
4h	–/+	+	+	++
4i	+	++	+++	+++
4j	–	–	+	+

–, No de-pigmentation; +, ++, +++ indicate increasing degress of de-pigmentation; –/+ indicates that de-pigmentation is not present in all replicates.

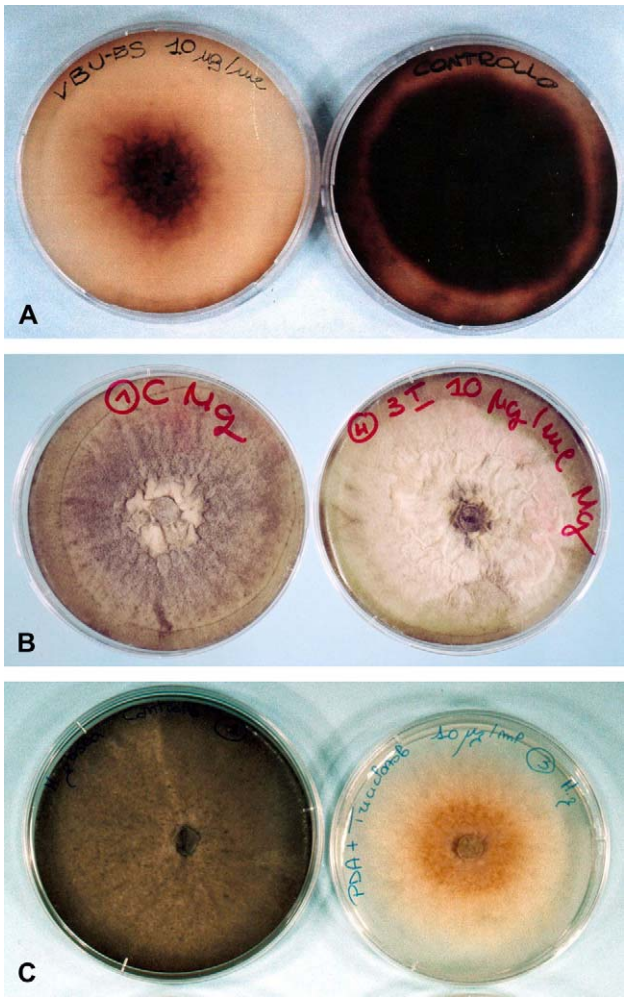


Fig 1 – De-pigmentation of *Magnaporthe grisea* mycelium after treatment with compounds 2h (A), 3i (B) and tricyclazole (C) at 10 μg ml^{-1}. Treated plate on left in A and on right in B and C.

and seemed to consist of many convoluted membrane cisternae. No alteration in the shape or texture of cell walls was apparent in the treated hyphae.

Discussion

Among the four series of compounds tested, the pyrazolo-triazine derivatives **(1g–l)** were least effective in reducing the growth of *Magnaporthe grisea*, always giving less inhibition than the reference fungicide tricyclazole. The pyrazolo[1,5-*c*][1, 3, 5]thiadiazine-2-one derivatives **(2g–j)** showed higher efficacy than the triazine counterparts, indicating that the presence of a thiadiazine ring may act as amplifier for fungistatic activity.

Comparison of pyrazolo[3,4-*d*][1,3]thiazine-4-one **(3a–j)** and the pyrazolo[3,4-*d*][1,3]thiazine-4-thione **(4a–j)** derivatives, showed that the former were more effective at the highest doses, whereas the latter showed excellent activity at concentrations as low as 10 μg ml^{-1}. Assuming these compounds traverse the fungal plasma membrane by a concentration-dependent passive diffusion process, the lipophilic thione derivatives might more readily reach the intracellular concentration required to cause inhibition, hence their activity does not increase further at higher doses. In contrast, the more hydrophilic, less favourably transported, -one derivatives required a greater concentration in the external medium to give the same effect.

Within a given set of compounds, the presence of either a cyclohexyl (**1j, 2j, 3j** and **4j**) or a *n*-butyl (**1h, 2h** and **3h**) group generally results in an increased effectiveness of the compounds, with respect to both growth inhibition and de-pigmentation of the mycelium. Interestingly, similar results were obtained in a previous study on some of the same triazines and thiadiazines, that showed the compounds **1j, 1h, 2j** and **2h** are also the most active in inhibiting photosynthetic electron transport in blue-green and eukaryotic algae, and isolated spinach chloroplasts (Vicentini *et al.* 2004). The new data reported here on antifungal activity also show the higher effectiveness of the thiadiazine on the triazine ring, and an increase in biological activity with a cyclohexyl or a *n*-butyl group in position 3. Taken together, these data suggest lipophilicity is an important factor, in that it may facilitate trans-membrane transport and attainment of a critical

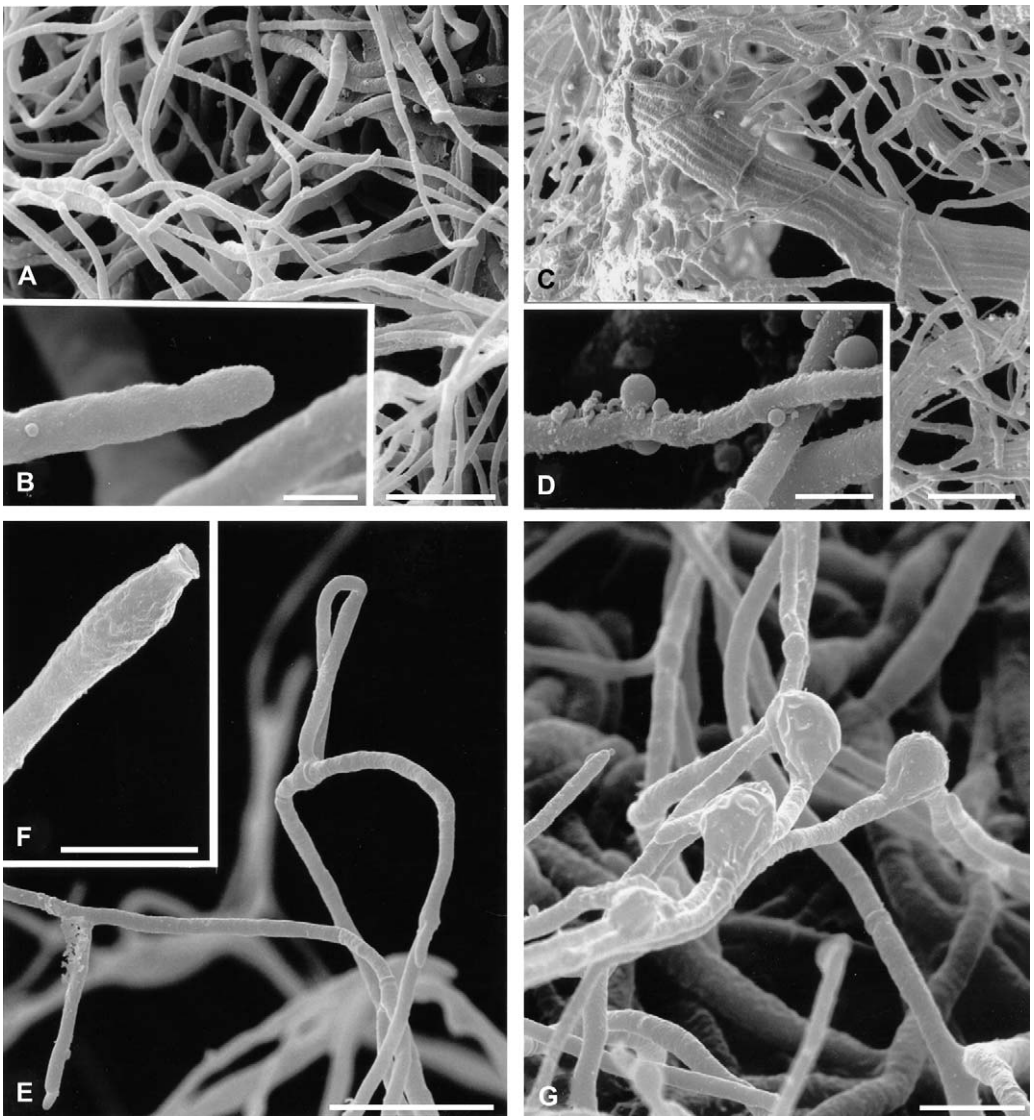


Fig 2 – SEM of *Magnaporthe grisea* hyphae. (A) Hyphae from youngest portions of untreated control mycelium. Bar = 10 μm. (B) Apical hypha with smooth surface from control mycelium. Bar = 1 μm. (C) Older central zone of control mycelium with aggregated hyphae. Bar = 20 μm. (D) Hypha from central zone showing surface structures. Bar = 2 μm. (E) Unusual ring-like hypha in youngest portion of mycelium after treatment with 10 μg ml^{-1} of 2h. Bar = 10 μm. (F) Hypha with ruptured apex from mycelium treated as in E. Bar = 4 μm. (G) Hyphae treated with 100 μg ml^{-1}of 2h, with swollen hyphal apices. Bar = 5 μm.

concentration inside the cell. Similar factors might contribute to the increased biological activity observed for the benzyl substituent.

It is also noteworthy that some of these substances inhibit the growth of *M. grisea* at 10 μg ml^{-1}, a concentration at which the reference compound is completely ineffective, and at 50 μg ml^{-1} six of them (**3j** and **4j**, **3e**, **4i**, **2h** and **3i**) are two- to threefold more effective than tricyclazole. The latter is normally applied on rice plants at a concentration of 200 μg ml^{-1}, and the possibility of using lower rates of fungicides may represent a significant reduction of environmental impact.

The mechanism of action of the most active compound, **2h,** is indicated by the data from electron microscopy. Many studies have described the morphology of *M.grisea*, in relation to the important diseases that this fungus causes in many species of grasses, and in rice in particular (Howard & Valent 1996; Bourett & Howard 1990 and references therein). The pathogen forms specialized cells, called appressoria, to directly penetrate the epidermis of the host leaf. A strong adhesion capacity and a pentaketide-derived melanin pigment are essential for appressorium functionality (Howard & Ferrari 1989). The role of melanin is well known as an important determinant of pathogenicity and virulence in *M. grisea* and other phytopathogenic fungi (Butler *et al.* 2001). Thus melanin synthesis represents a suitable target to control the potential pathogenic capacity of many fungi. The fungicide tricyclazole (5-methyl-1,2,4-triazolo[3,4-*b*] benzothiazole), patented by Eli

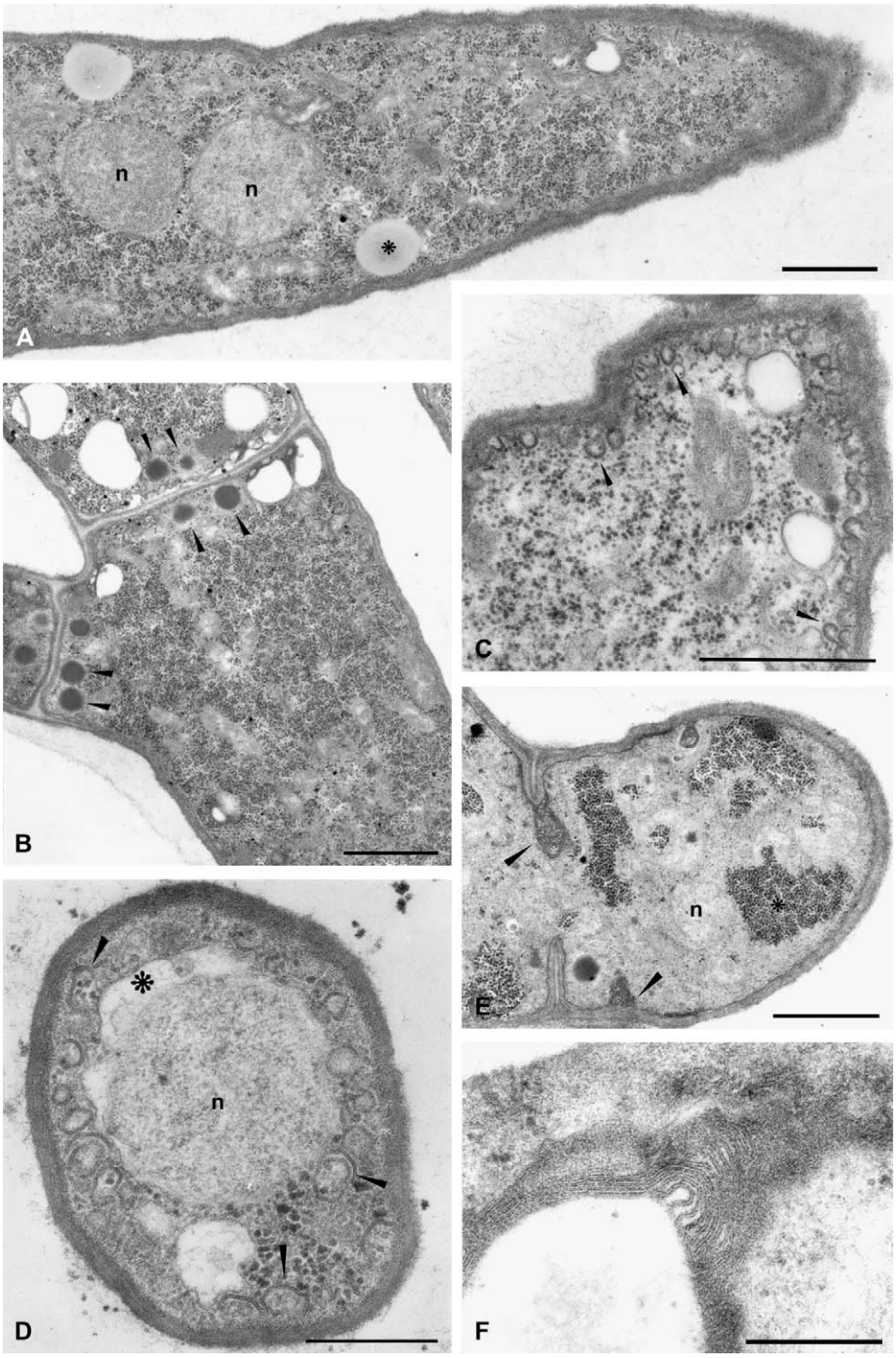


Fig 3 – TEM of *Magnaporthe grisea* hyphae. (A) Median section of an untreated control hypha with nuclei (n) and lipid droplets (*). Bar = 1 μm. (B) Untreated hypha, with Woronin bodies (arrowheads) adjacent to the septa. Bar = 1 μm. (C) Portion of hypha treated for 24 h with 10 μg ml^{-1} of 2h, with many membranous structures (arrowheads) adjacent to the plasma membrane. Bar = 1 μm. (D) Hypha treated for 24 h with 100 μg ml^{-1} of 2h with a space (*) between the karyoplasm (n) and nuclear envelope, which was also associated with membranous structures (arrowheads), as in Fig C. Bar = 2 μm. (E) Hypha treated for 24 h with 100 μg ml^{-1} of 2h, showing indistinct nuclei (n), aggregates of glycogen granules (*) and poly-membranous bodies (arrowheads) adjacent to an incomplete septum. Bar = 1 μm. (F) Detail of poly-membranous body of convoluted membrane cisternae in hypha treated with 100 μg ml^{-1} of 2h. Bar = 2 μm.

Lilly, protects the rice epidermis from penetration by fungal hyphae by inhibiting melanin synthesis in appressorial cells (Froyd *et al*. 1976; Woloshuk *et al*. 1983). The de-pigmentation that takes place as a consequence has been studied in detail for *M. grisea* (Bailey 1986; Bell & Wheeler 1986; Howard & Valent 1996). De-pigmentation was evident after treatment with several of the compounds tested here, including **2h**. Surprisingly, no consequent alterations in the cell wall of *M. grisea* were observed with SEM and TEM, except at hyphal tips. However, this could simply reflect the short duration (24 h) of contact between the mycelium and the compound before the samples were harvested for TEM observations, whereas visual observations for pigmentation of the mycelia were made ten days after treatment.

In contrast, several sub-cellular alterations were observed at the cytoplasmic level. Some indicated only a variation in metabolism, such as the clustering of glycogen granules seen after treatment, but the most significant alterations concerned the endomembrane system. Treatment with **2h** induced a re-arrangement of membranes surrounding the nucleus, causing the breaking of the nuclear envelope and intermingling of the cytoplasmic and nuclear contents. Moreover, in treated cells membrane vesicles (secretory?) and polymembranous bodies appear. These effects suggest that compound **2h** acts at the membrane level, with a mechanism similar to that of most azolic fungicides. Their mode of action generally relates to interference in the synthesis of ergosterol for incorporation in the fungal membrane, subsequent accumulation of 14 α-methylsterols (Vanden Bossche 1990), and a decrease in the ratio of saturated:unsaturated fatty acids (Vanden Bossche & Marichal 1992), which in turn leads to a disorganization of cellular membranes.

Alterations to the cell wall are usually found after treatment of fungi with azole derivatives (Borgers 1988; Mares *et al*. 2002). Only the high percentage of lysed tips of *M. gipsea* hyphae (40 %) seem to suggest wall alterations after treatment with **2h,** which is likely to be the primary cause of growth inhibition. The presence of hyphae with swollen tips may reflect a weakening of the cell wall. Alternatively, these structures resemble chlamydospores, whose formation may allow survival through the development of resistance forms. The structure and function of these apical swellings merits further investigation.

In summary, it may be concluded that the four series of azole derivatives tested possess a remarkable inhibitory ability, in several cases comparable to or even higher than the reference fungicide. It has also been demonstrated that the presence of a cyclohexyl or a *n*-butyl group increases the biological activity of these molecules, and that of the 29 substances tested the most effective is 4-butyl-7-methylpyrazolo[1,5-*c*][1,3,5]thiadiazine-2-one **(2h).** This compound appears to be an interesting new chemical for control of the rice blast agent at doses lower than those of tricyclazole, through a different mode of action that will be explored further in future research.

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