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Interaction of 2,4,5-trichlorophenylsulphonylmethyl thiocyanate with fungal spores

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Abstract

Interaction of a new fungicidal compound, viz. 2, 4, 5-trichlorophenylsulphonylmethyl thiocyanate with spores of Fusarium culmorum has been investigated.

The compound is readily taken up by spores and converted to the non-fungitoxic 2,4,5-trichlorophenylsuphinic acid, which is released into the ambient solution. Uptake of the thiocyanate can be markedly reduced by pretreatment of the spores with a thiol reagent like iodoacetic acid and slightly enhanced by pretreatment with a thiol, like dithiodiglycol. Moreover, the thiocyanate reacts with thiols in vitro by forming the same sulphinic acid. Hence, it is concluded that the title compound is able to react with fungal cell thiols.

However, addition of a thiol reagent does not affect the fungitoxicity of the thiocyanate. The absence of synergism suggests that the fungitoxicity of the thiocyanate is based at least partly on reaction with fungal cell thiols.

The thiocyanate investigated resembles the fungicide captan with respect to both uptake pattern and failure to give synergism with a thiol reagent. These observations suggest that the fungicidal compounds have a similar mode of action, as far as reaction with fungal cell thiols is concerned.

Introduction

Most present-day fungicides, acting as protectants, are able to inhibit germination of fungal spores. The selective toxicity of the fungicides, not harmful to the host, may be based on physical factors such as the permeability of the spore envelope. For this reason information about the interaction of fungicidal compounds with spores, as measured by several authors, may be fruitful in studies of fungicidal action.

Most fungicides, having a low toxicity to the spores, on a spore weight basis (Miller et al., 1953) act as non-specific agents on reactive groups like sulphydryl, amino or hydroxyl groups. Organic thiocyanates have been found to react with sulphydryl groups and the reaction was put forward as a possible mechanism of action (Zsolnai, 1962; Croshaw et al., 1966).

Recently a new group of fungicidal thiocyanates was synthesized by Dolman et al. (1969) in the laboratories of Philips-Duphar. The present study was undertaken to investigate the interaction of the title compound with fungal cells with particular reference to the interaction with cell thiols. Use is made of the procedure of Richmond and Somers (1966), who investigated the interaction of N-trichloromethylthio-4-cyclohexene-1,2-dicarboximide (captan) with thiols in conidia of *Neurospora crassa* by pretreatment of the spores with thiol reagents or thiols.

Materials and methods

Chemicals. 2,4,5-trichlorophenylsulphonylmethyl thiocyanate, 2,4,5-trichlorophenylsulphinic acid and N-trichloromethylthio-4-cyclohexene-1,2-dicarboximide (captan) were synthesized and purified in the laboratories of Philips-Duphar. Iodoacetic acid was obtained from Schuchardt (München, W. Germany), dithiodiglycol from Koch and Light (Colnbrook, England) and N-ethylmaleimide from Fluka (Switzerland).

Buffer solutions. Media were buffered with mixtures of citric acid and disodium hydrogen phosphate. Concentrations in reaction media were 0.020/0.010 (pH 3.0), 0.016/0.017 (pH 4.0) and 0.010/0.029 (pH 5.6) M citric acid/M phosphate.

Conidia. Conidia of *Fusarium culmorum* were washed from 10-day-old malt agar cultures, cultivated at 23 °C in the dark. Conidia of *Neurospora crassa* were harvested from cultures which were incubated for 5 days at 22 °C in the light. Spores were washed five times with distilled water by centrifugation.

Uptake experiments. Spores (3.4×10^6/ml) were gently shaken in a closed vessel at 20 °C with 25 ml of an aqueous solution containing buffer (pH 5.6), 1 % ethanol (v/v) and a non-toxic concentration of the fungicidal compound (0.013 mM). After appropriate time intervals the loss of compound from the supernatant was determined and the uptake is expressed as a percentage of the initial concentration removed from the external solution. After uptake experiments, spores were washed and tested on viability in a slide germination test.

Pretreatment of the spores. The same spore concentration and incubator shaking conditions were used for the reagent pretreatment of the spores. Spores were pretreated for 30 min, except with dithiodiglycol, which was incubated with spores for 3 h. Pretreatment with iodoacetic acid was performed at pH 3.0 to facilitate uptake of the acid, using a citrate phosphate buffer. Spores were washed twice with distilled water before uptake experiments.

Analysis of compounds in the supernatant. After uptake by spores the thiocyanate was extracted from the supernatant with ether. Thiocyanate concentrations were determined in ether at 240 mμ ($\varepsilon = 11400$), using a recording UV spectrophotometer Optica Milano CF4R. The sulphinic acid formed was identified in the water layer. Captan was determined in the supernatant by the method of Burchfield and Schechtman (1958).

Slide germination test. Conidia were diluted with suspensions of the compound to be tested or with water in case of a viability test and a constant amount of cherry juice to a density of 10,000 spores/ml. Drops of the suspensions were put onto glass slides. Each slide was placed in a separate closed tube and incubated at 23 °C. After 24 h the minimum concentration at which germination was inhibited completely (MIC) was determined.

Assessment of mycelial growth. The compounds were finely divided and suspended in

0.5 ml of water and mixed with 4.5 ml of a molten agar medium containing 1 % glucose, 0.1 % $(NH_4)_2SO_4$, 0.05 % $MgSO_4$, 0.05 % NaCl and 2.5 % agar (w/v). The medium was buffered with a citrate phosphate buffer to maintain pH 4.0 and allowed to coagulate in horizontally placed tubes. The linear mycelial growth starting from punches (6 mm diameter) of a 3-day-old culture of *Fusarium culmorum* was assessed after one week's incubation at 23 °C.

Results and discussion

Uptake of the thiocyanate by fungal spores

2,4,5-Trichlorophenylsulphonylmethyl thiocyanate was taken up and subsequently converted by spores of *Fusarium culmorum* to the corresponding sulphinic acid (Fig. 1), which was identified by UV spectrum, IR spectrum and melting point of its S-benzylthiouronium salt being 180°. Other degradation products were not identified.

The acid formed was almost completely released into the ambient solution. Results are consistent with our finding that 2,4,5-trichlorophenylsulphinic acid was not taken up by spores. It was established by incubation of the thiocyanate with spore exudate that the reaction did not take place in the ambient solution. The sulphinic acid was found to be non-fungitoxic.

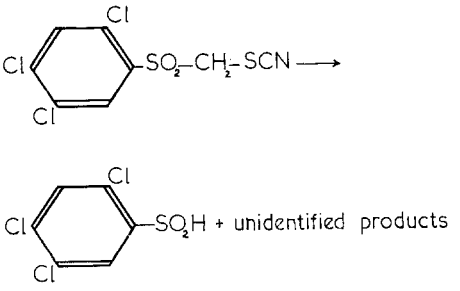

Fig. 1. Conversion of 2,4,5-trichlorophenylsulphonylmethyl thiocyanate to the corresponding sulphinic acid.

Fig. 1. Omzetting van 2,4,5-trichloorfenylsulfonylmethyl thiocyanaat in het overeenkomstige sulfinezuur.

The thiocyanate (0.01 mM), allowed to react with thiols like cysteine (0.1 mM) in neutral aqueous medium, was converted to 2,4,5-trichlorophenylsulphinic acid as well, suggesting the ability of the compound to react with fungal cell thiols. The thiocyanate did not react with other amino acids like tryptophan, serine, histidine, ornithine and tyrosine under the same conditions.

Evidence of reaction of the thiocyanate with cell thiols was obtained from experiments in which the uptake of the thiocyanate was influenced by pretreatment of the spores for 30 min with a thiol reagent. The uptake of the thiocyanate by spores of *Fusarium culmorum* is shown in Fig. 2. After 40 min about 80 % of the compound had been taken up and subsequently converted to the corresponding sulphinic acid. Conidia, pretreated with the familiar thiol reagent iodoacetic acid took up much smaller amounts of the thiocyanate. Fig. 2 shows that pretreatment with 3.4 mM iodoacetic acid reduced the uptake to a very large extent. Results present clear evidence of a relationship between thiocyanate uptake and amount of thiols available in the spores. The concentration of iodoacetic acid (0.86 mM) needed to reduce the uptake of the thiocyanate to at least 75 % appeared to be fungicidal, as was assessed by putting the spores after pretreatment to a slide germination test. This observation suggests that the fungi-

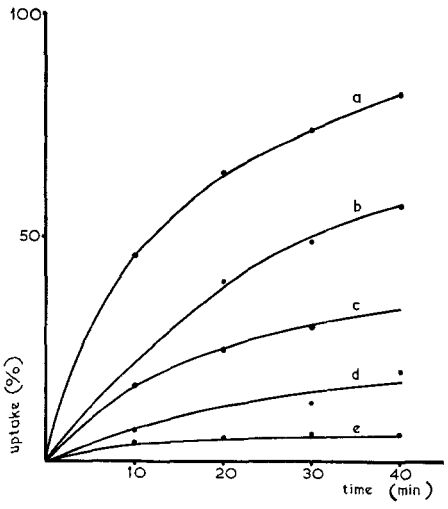

Fig. 2. Uptake of the thiocyanate by spores of *Fusarium culmorum*, pretreated with ioadacetic acid.
Uptake by unpretreated spores (a).
Uptake after pretreatment with 0.027 mM (b), 0.21 mM (c), 0.86 mM (d) and 3.4 mM (e) iodoacetic acid.

Fig. 2. Opname van het thiocyanaat door sporen van Fusarium culmorum, voorbehandeld met joodazijnzuur.

toxicity of iodoacetic acid is caused by reaction with those thiols, which are also blocked by the thiocyanate. It may be noted here that the fungicidal action of iodoacetic acid, determined directly in a slide germination test at the same pH (Table 1) was also found to be rather high. In Table 1 MIC's of some other compounds used in our experiments are mentioned.

The uptake of the thiocyanate could also be reduced by the fungicide captan, known for its ability to react with cell thiols (Lukens and Sisler, 1958). The uptake pattern of the thiocyanate after pretreatment with captan (Fig. 3) is similar to that obtained after pretreatment with iodoacetic acid. However, lower concentrations of captan were needed to give the same reduction of the uptake, this pointing to the better accessibility of the spores to captan in comparison with iodoacetic acid.

Similar results were obtained if spores were pretreated with the thiocyanate itself (Fig. 4.).

Pretreatment of spores with a thiol, viz. dithiodiglycol, chosen for its ability to penetrate the cells (Richmond and Somers, 1966) enhanced uptake of the thiocyanate

Table 1. Minimum inhibitory concentration (MIC) of some compounds used, as assessed in a slide germination test with conidia of *Fusarium culmorum*.

Compound	MIC	
	ppm	mM
The thiocyanate investigated	0.5	0.0015
Captan	1	0.003
Iodoacetic acid	500	2.7
Iodoacetic acid	15*	0.08*
N-ethylmaleimide	10	0.08
Dithiodiglycol	>1000	>7

* Assessed at pH 3.0. Other MIC's were determined at pH 5.6

Tabel 1. Minimum remmende concentratie van enkele gebruikte verbindingen, bepaald in een sporekiemingstest met sporen van Fusarium culmorum.

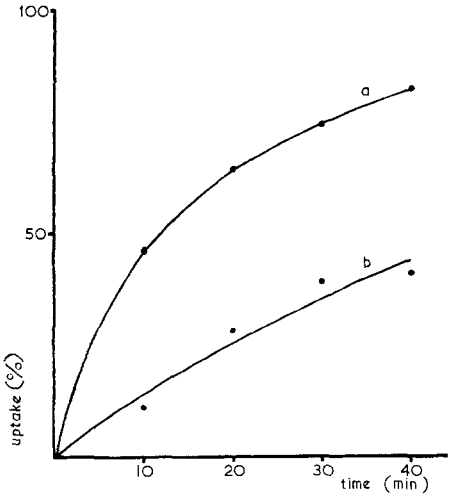

Fig. 3. Uptake of the thiocyanate by spores of *Fusarium culmorum*, pretreated with captan.
Uptake by unpretreated spores (a).
Uptake after pretreatment with 0.013 mM captan (b).

Fig. 3. Opname van het thiocyanaat door sporen van Fusarium culmorum, voorbehandeld met captan.

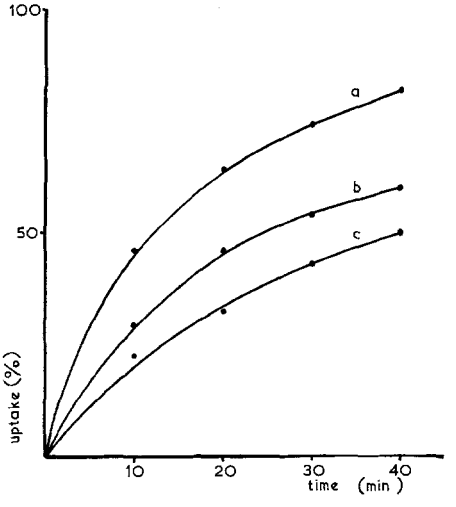

Fig. 4. Uptake of the thiocyanate by spores of *Fusarium culmorum*, pretreated with the thiocyanate itself.
Uptake bij unpretreated spores (a).
Uptake after pretreatment with 0.003 mM (b) and 0.013 mM (c) thiocyanate.

Fig. 4. Opname van het thiocyanaat door sporen van Fusarium culmorum, voorbehandeld met het thiocyanaat zelf.

to a certain extent (Fig. 5). This again indicates a reaction of the fungicidal compound with thiols in fungal cells.

Experiments concerning uptake of the thiocyanate as influenced by pretreatment with iodoacetic acid and dithiodiglycol were also carried out with conidia of *Neurospora crassa*. Results proved to be similar to those obtained with conidia of *Fusarium culmorum*.

In a similar way interaction of captan with spores of *Fusarium culmorum* pretreated with iodoacetic acid (Fig. 6) or with the thiocyanate (Fig. 7) was investigated. Apart from some quantitative differences, the uptake pattern of captan (Fig. 6 and 7) is similar to that of the thiocyanate (Fig. 2 and 4), strongly suggesting that the same kinds of interaction are involved. It has to be noted that results with uptake of captan after pretreatment with iodoacetic acid are similar to those obtained by Richmond and Somers (1966), who investigated interaction of captan with cell thiols of *Neurospora crassa*.

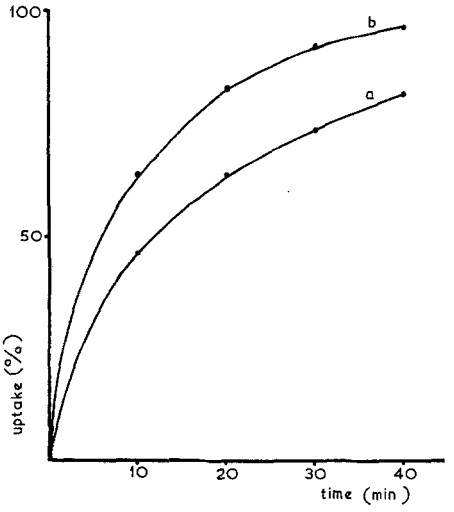

Fig. 5. Uptake of the thiocyanate by spores of *Fusarium culmorum*, pretreated with dithiodiglycol.
Uptake by unpretreated spores (a).
Uptake after pretreatment with 4.0 mM dithiodiglycol (b).

Fig. 5. Opname van het thiocyanaat door sporen van Fusarium culmorum, voorbehandeld met dithiodiglycol.

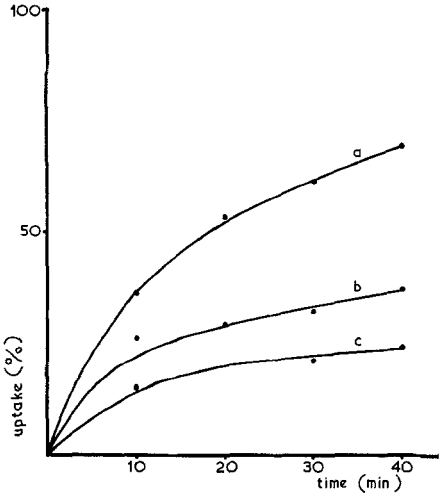

Fig. 6. Uptake of captan by spores of *Fusarium culmorum*, pretreated with iodoacetic acid.
Uptake by unpretreated spores (a).
Uptake after pretreatment with 0.027 mM (b) and 0.21 mM (c) iodoacetic acid.

Fig. 6. Opname van captan door sporen van Fusarium culmorum, voorbehandeld met joodazijnzuur.

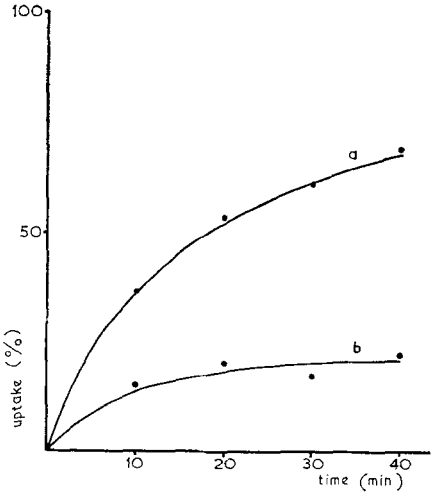

Fig. 7. Uptake of captan by spores of *Fusarium culmorum*, pretreated with the thiocyanate.
Uptake by unpretreated spores (a).
Uptake after pretreatment with 0.013 mM thiocyanate (b).

Fig. 7. Opname van captan door sporen van Fusarium culmorum, voorbehandeld met het thiocyanaat.

Table 2. Fungitoxicity of the thiocyanate, mixed with iodoacetic acid, assessed by measurement of linear mycelial growth of *Fusarium culmorum*.

Conc. thiocyanate (ppm)	Linear mycelial growth (mm)			
conc. iodoacetic acid (ppm):	0	1	3	10
0	48	47	46	47
0.3	24	25	34	28
1	20	19	18	14
3	8	9	7	7

Tabel 2. Fungitoxiciteit van het thiocyanaat, gemengd met joodazijnzuur, bepaald door meting van de lineaire myceliumgroei van Fusarium culmorum.

The fungitoxicity of the thiocyanate, as influenced by addition of thiol reagents

The fungitoxicity of the thiocyanate was investigated in relation to its reaction with fungal cell thiols, the fungicidal action being assessed in a mycelial growth test using *Fusarium culmorum*. It appeared that iodoacetic acid added to the thiocyanate could not influence its fungitoxicity (Table 2). Nor could addition of another thiol reagent viz. N-ethylmaleimide influence the fungitoxicity of the thiocyanate. If the reaction of the thiocyanate with cell thiols were a detoxication, synergism could arise due to the sites of loss being blocked, the thiocyanate being largely available to the sites of action. However, failure of synergistic action with a thiol reagent indicates that the reaction of the thiocyanate with cell thiols cannot merely be considered as a detoxication. It is suggested that fungicidal action should be based at least partly on reaction with sulphydryl groups.

Similar experiments were carried out with captan. Likewise, iodoacetic acid did not influence the fungicidal action of captan (Table 3).

Consequently the thiocyanate investigated resembles captan with respect to both uptake pattern and failure to give synergism with a thiol reagent. It is suggested that the thiocyanate and captan have a similar mode of action, as far as reaction with fungal cell thiols is concerned.

Table 3. Fungitoxicity of captan, mixed with iodoacetic acid, assessed by measurement of linear mycelial growth of *Fusarium culmorum*.

Conc. captan (ppm)	Linear mycelial growth (mm)			
conc. iodoacetic acid (ppm):	0	1	3	10
0	48	47	46	47
3	33	34	37	37
10	18	23	22	20
30	2	2	1	1

Tabel 3. Fungitoxiciteit van captan, gemengd met joodazijnzuur, bepaald door meting van de lineaire myceliumgroei van Fusarium culmorum.

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Samenvatting

De interactie van 2,4,5-trichloorfenylsulfonylmethyl thiocyanaat met schimmelsporen

De interactie van een nieuwe fungicide verbinding nl. 2,4,5-trichloorfenylsulfonylmethyl thiocyanaat met sporen van *Fusarium culmorum* is onderzocht.

De verbinding wordt goed door sporen opgenomen en omgezet in het niet fungitoxische 2,4,5-trichloorfenylsulfinezuur, dat aan de buitenoplossing wordt afgestaan. Opname van het thiocyanaat kan in aanzienlijke mate worden verlaagd door voorbehandeling van de sporen met een thiolreagens zoals joodazijnzuur, en enigszins worden verhoogd door voorbehandeling met een thiol zoals dithiodiglycol. Voorts reageert het thiocyanaat met thiolen in vitro onder vorming van hetzelfde sulfinezuur. De conclusie wordt getrokken, dat het thiocyanaat in staat is te reageren met thiolen van de schimmelcel.

Toevoeging van een thiolreagens heeft echter geen invloed op de fungicide werking van het thiocyanaat. Het uitblijven van synergisme suggereert, dat de fungitoxiciteit van het thiocyanaat tenminste gedeeltelijk is gebaseerd op reacties met thiolen in de schimmelcel.

Het onderzochte thiocyanaat gelijkt op het fungicide captan niet alleen in opnamepatroon maar ook wat betreft het uitblijven van synergisme met een thiolreagens. Deze waarnemingen suggereren dat de fungicide verbindingen hetzelfde werkingsmechanisme hebben, althans voorzover het reacties met thiolen van de schimmel betreft.

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