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BIOLOGICAL PROPERTIES OF A RED PIGMENT PRODUCED BY THE NEMATOPHAGOUS FUNGUS *VERTICILLIUM SUCHLASPORIUM*

by

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Summary. *Verticillium suchlasporium*, a parasite of eggs of the cereal cyst nematode *Heterodera avenae*, produced diffusible pigments on the nutrient-rich media potato dextrose agar and malt extract agar but not on the nutrient poor medium corn meal agar. *V. suchlasporium* (isolate 10, CBS 464.88, ATCC no. 76547) produced a red pigment on potato dextrose agar when the fungus was plated together with *Arthrobotrys amerospora* and *Cylindrocarpon destructans*, the pigment being produced in the interaction zones between the colonies. The red pigment which was extracted from the agar using glacial acetic acid or chloroform, absorbed light at 510 nm, 355 nm and especially 200 nm. Four main spots with RFs 0.54, 0.31, 0.14 and 0.084 were resolved by thin layer chromatography using toluene/ethyl acetate/formic acid. The red pigment which inhibited the growth of *Cladosporium cucumerinum* spores in bioautographs, revealed the presence of mycotoxic compounds. Cysts of *Globodera rostochiensis* were placed on paper discs impregnated with potato root diffusate, sterile distilled water, acetic acid and an extract of the red pigment in acetic acid. After five days, significantly ($P<0.05$) fewer second-stage juveniles of *G. rostochiensis* were found hatched outside the discs treated with the pigment than in the rest of the treatments. The extract of the red pigment also significantly ($P<0.001$) reduced the mobility of *G. rostochiensis* juveniles compared with both untreated controls and acetic acid treated discs suggesting that the red pigment had a nematicidal role.

Dubos (1987) defined mycoparasitism as a broad term for all parasitic relationships exhibited by fungi on other fungi and reserved hyperparasitism '*sensu stricto*' for the antagonistic relationship (parasitic) exerted by fungi on other fungi which are themselves parasites of (on) higher plants. The phenomenon of antagonism is widespread amongst soil microorganisms including fungi. This is of great relevance when considering the effects of the soil microflora on biocontrol agents such as nematode fungal parasites. Tzean and Estey (1978) described several species of *Arthrobotrys* as mycoparasites or hyperparasites of plant pathogenic fungi e.g. *Rhizoctonia solani*. Sneh *et al.* (1977), in a survey for *Phytophthora* antagonists, found *V. chlamydosporium* parasitising oospores of several species of *Phytophthora* while Dayal and Barron (1970) found *V. psalliotae* parasitising the conidia of *Rhopalomyces elegans*, a parasite of nematode eggs. *V. chlamydosporium* has also shown antimycotical action against the rust fungi (Leinhos and Buchenauer, 1986). Several other species of the genus *Verticillium*, apart from *V. chlamydosporium* displayed mycoparasitic activities (Gams and Van Zaayen, 1982). Other fungi which have occasionally been isolated from *H. avenae* e.g. *Trichoderma viride* (Lopez-Llorca, 1988) are regarded as mycoparasites (Ferera-Cerrato, 1976; Elad *et al.*, 1983; Chet, 1987).

Papavizas (1987) stressed that before molecular biological approaches could be applied to improve biocontrol fungi, such as efficient systems for genetic manipulation (e.g. fungal plasmids), it is essential to understand their mechanisms of action, especially if these mechanisms are mediated by metabolites and also to determine the role of these substances in pathogenicity. In this paper, the antimicrobial characteristics and possible biological activities of a red pigment produced by the egg parasite *Verticillium suchlasporium* Gams and Dackman are studied. The possible pathogenic role of the pigment is considered.

Materials and methods

Verticillium suchlasporium isolate 10 (CBS 464.88, ATCC no. 76547) and isolate 19 were from infected eggs and *Cylindrocarpon destructans* (Zinssmeister) Scholten

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from cysts and eggs of the cereal cyst nematode *Heterodera avenae* Woll. isolated using a growth restricting medium (Lopez-Llorca and Duncan, 1986). These fungi were kept on corn meal agar (Oxoid) at 4 °C in the dark. *Cladosporium cucumerinum* Ell. and Arthur and *Arthrobotrys* sp. were donated by Dr. G.D. Lyon and Dr. E.P. Dashwood (Mycology and Bacteriology Department, SCRI) while *A. amerospora* Shenck, Kendrick *et* Pramer was from the Commonwealth Mycological Institute.

To study the possible involvement of pigments in the antibiosis phenomena, *V. suchlasporium* and a trapping fungus were plated with several combinations on solid media of different nutrient content [potato dextrose agar (PDA), malt extract agar (MEA), Czapek Dox agar (CZ) and corn meal agar (CMA)].

A red pigment produced by *V. suchlasporium* (isolate 10) was extracted by cutting out the area of the agar containing the pigment and removing the mycelium. The agar plus the pigment it contained was cut into small fragments, placed in a filter paper cone (Whatman No. 1, 7 cm diameter) in a glass funnel (5 cm diameter), where several solvents of different polarity were tested as extractants for the pigment. Extracts of the pigment were collected and kept at 4 °C protected from the light. Pure oosporein, a red pigment from *Verticillium psalliotae* Treschow was a gift from Dr. M. Wainwright (Sheffield University, U.K.).

An acetic acid extract of the red pigment was chromatographed on 5x20 cm silica gel TLC plates (Merck) to separate possible different components in the following solvent mixtures: a) Toluene/ ethyl acetate / formic acid (5:4:1, v/v/v). b) n-Propanol/ 25% (aq.) ammonia (7:3, v/v). c) n-Butanol/ acetic acid/ water (60:25:15, v/v/v). d) n-Butanol/ ethanol/ 2N ammonia (60:20:20, v/v/v). e) Chloroform/ methanol (95:5, v/v). f) Ethyl acetate/ formic acid/ 2M HCl (85:6:9, v/v/v).

The (UV/VIS) spectrum of a crude chloroform extract of the red pigment was determined in a Pye Unicam spectrophotometer using chloroform as a blank.

TLC plates loaded with the red pigment, and developed in a particular solvent system, were left for at least 3 hours in a fume cupboard to evaporate all traces of remaining solvents that would interfere with the subsequent biological assay. *C. cucumerinum* was grown on PDA at 20 °C in the dark for 10-15 days. A spore suspension of this test organism was then prepared. Three plates were washed with 200 ml of Czapek-Dox liquid media. The spore suspension obtained was used to spray four (5x20 cm) TLC plates. The sprayed TLC plates were then incubated at 20 °C in the dark in a moist chamber (i.e. a plastic box with moist filter paper enclosed in a polythene bag). To prevent contamination, direct contact between the TLC plates and the moist paper was avoided. After 5-7 days the plates were checked for growth-inhibition areas.

The possible effects of the red pigment on nematode hatching were tested as follows: 23 mm diameter sterilised filter paper disks (Whatman No. 1) were soaked in 0.2 ml of the following solutions and liquids to test their effect on nematode hatching: *a)* sterile distilled water (SDW), *b)* potato root diffusate (PRD) prepared as in Forrest and Farrer (1983), and sterilised by filtration through a FlowPore D 0.2 µm (Flow Laboratories), *c)* glacial acetic acid, and *d)* an extract of the red pigment in acetic acid. The substances tested were applied on the discs in two loadings of 0.1 ml. When the paper discs were dried, they were placed in the centre of 6 cm plastic Petri dishes (Sterilin, Feltham, Middlessex, U.K.) containing 1% water agar freshly poured.

Globodera rostochiensis (Woll.) Behrens cysts (supplied by Dr. J.M.S. Forrest, Zoology Department, SCRI) were soaked in tap water for 15 days and rinsed for one hour in running tap water before use. Twenty-five cysts were placed on each paper disc. Five plates were prepared per treatment. Plates were then incubated at 20 °C in the dark and were checked daily for hatching of second-stage juveniles. Only juveniles outside the filter paper discs were counted.

A similar test to check the effect of the pigment on juvenile mobility/survival was conducted by placing 200 *G. rostochiensis* juveniles per disc instead of cysts. Filter paper discs were treated as for the hatching test but no PRD was used. Four plates were set up per treatment. Only juveniles outside discs were scored. Rigid-immobile nematodes were classified as dead, whereas motile and non-motile normal-shaped nematodes were classified as alive.

Results

The presence of diffusible pigments produced by *V. suchlasporium* and their colour varied with the growth medium used (Figs 1 and 2). Although the characteristics of the pigment varied for the same isolate in different media, pigments were usually produced on nutrient-rich media such as PDA or MEA and were not produced in nutrient-poor media (e.g. CMA) (Figs 2a, 2b and 2c).

On PDA, an orange pigment was produced when *Verticillium* was grown alone (Fig. 2d), but when the fungus was plated together with trapping fungi, a band of pigment (Fig. 2e, red pigment, and 2f, orange pigment) was produced in the zone of interaction between the colonies. This was also observed when CZ medium was used (Figs 2g and 2h). A red pigment was also produced when *V. suchlasporium* and *C. destructans* were grown together.

The red pigment (from *V. suchlasporium* isolate 10) was not extracted by water from the agar. It had only a low solubility in polar solvents such as ethanol and was only slightly soluble in butanol. The pigment was only extracted by glacial acetic acid or chloroform. The red pig-

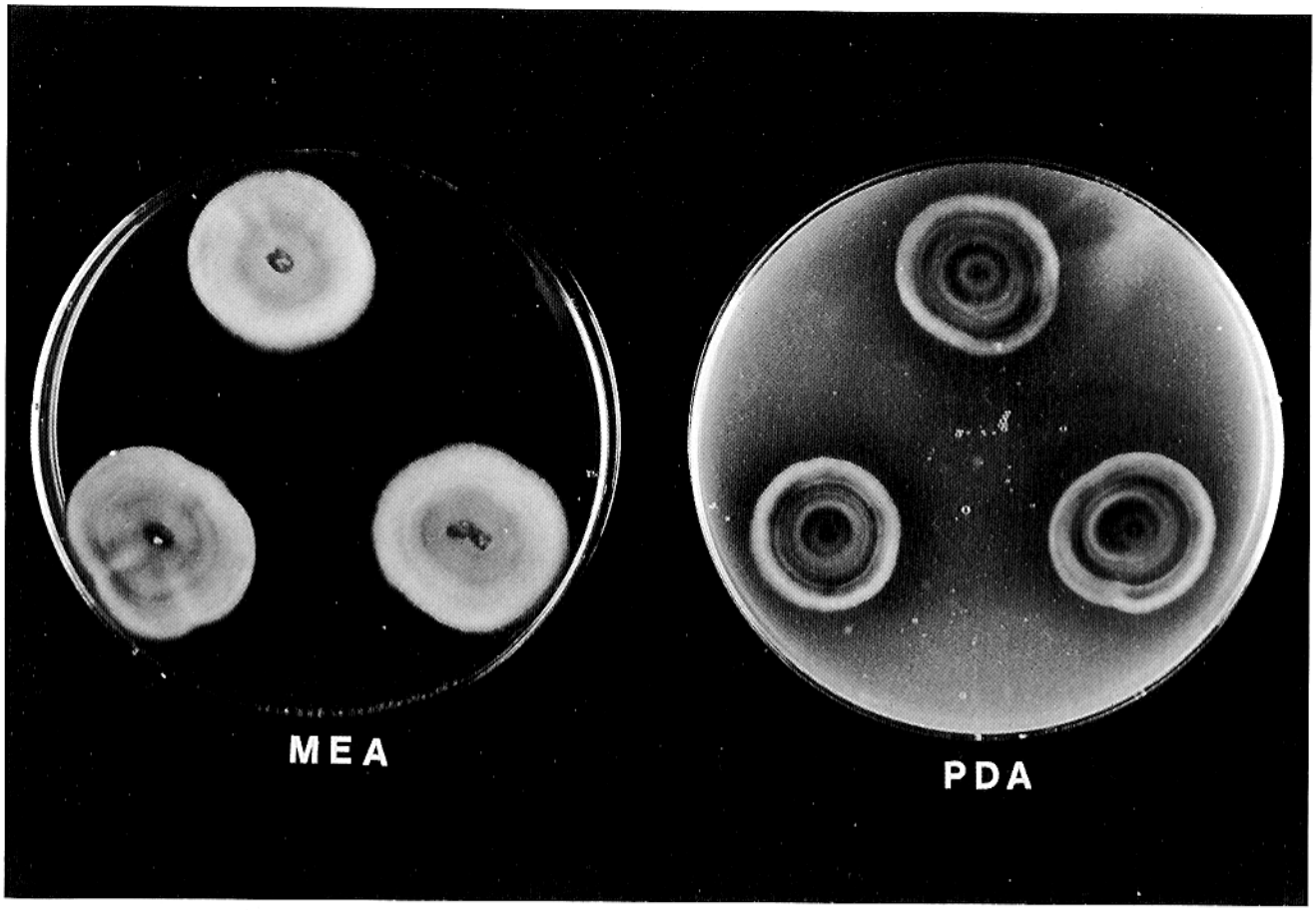

Fig. 1 - Effect of different growth media on pigment production by *Verticillium suchlasporium* (isolate 10) from *Heterodera avenae* infected eggs. The photograph shows the same isolate on two different media 10 days after inoculation. Abbreviations: malt extract agar (MEA) and potato dextrose agar (PDA).

ment extracted in chloroform showed a peak of absorbance in the visible region at approx. 510 nm, another peak in the long UV region (approx. 355 nm) and the maximum of absorbance at short UV (near 200 nm).

An extract of the red pigment in glacial acetic acid did migrate on TLC in solvents (b) and (d) (see Materials and methods). In solvents (c), (e), (f) separation was observed although 'tailing' in the spots occurred. The best separation was found in non-polar solvent systems such as solvent (a). Fig. 3a shows a chromatogram developed for 45 min in solvent (a) of an acetic acid extract of the red pigment and oosporein. Oosporein did not run in this solvent mixture whereas the red pigment did. Four main spots were visible with RFs 0.54, 0.31, 0.14, 0.084 respectively. Some red compound remained in the origin. Fig. 3b shows the same TLC chromatogram as Fig. 3a but observed under UV light.

The bioautography of a TLC plate with two different loadings (5 and 10 µl) of an extract in acetic acid of the red pigment run in chloroform/methanol (95:5, v/v; solvent (f)) is shown in Fig. 4. The area near the solvent front showed a zone of complete growth inhibition of the test-organism used (*C. cucumerinum*) especially with 10 µl loading of the pigment. Near the origin of the chromatogram some inhibition was also apparent.

After five days, all treatments had significantly ($P<0.05$) different effects on the hatching of *G. rostochiensis* eggs (Table I) as measured by the numbers of second-stage juveniles found hatched outside the discs. Maximum values were found for potato root diffusate, then acetic acid, sterile distilled water and finally the extract of the red pigment. After 7 days the effects were similar, but the difference between potato root diffusate and acetic acid was not significant ($P<0.05$). Acetic acid and also the pigment, significantly reduced the mobility of juveniles of *G. rostochiensis* (Table II).

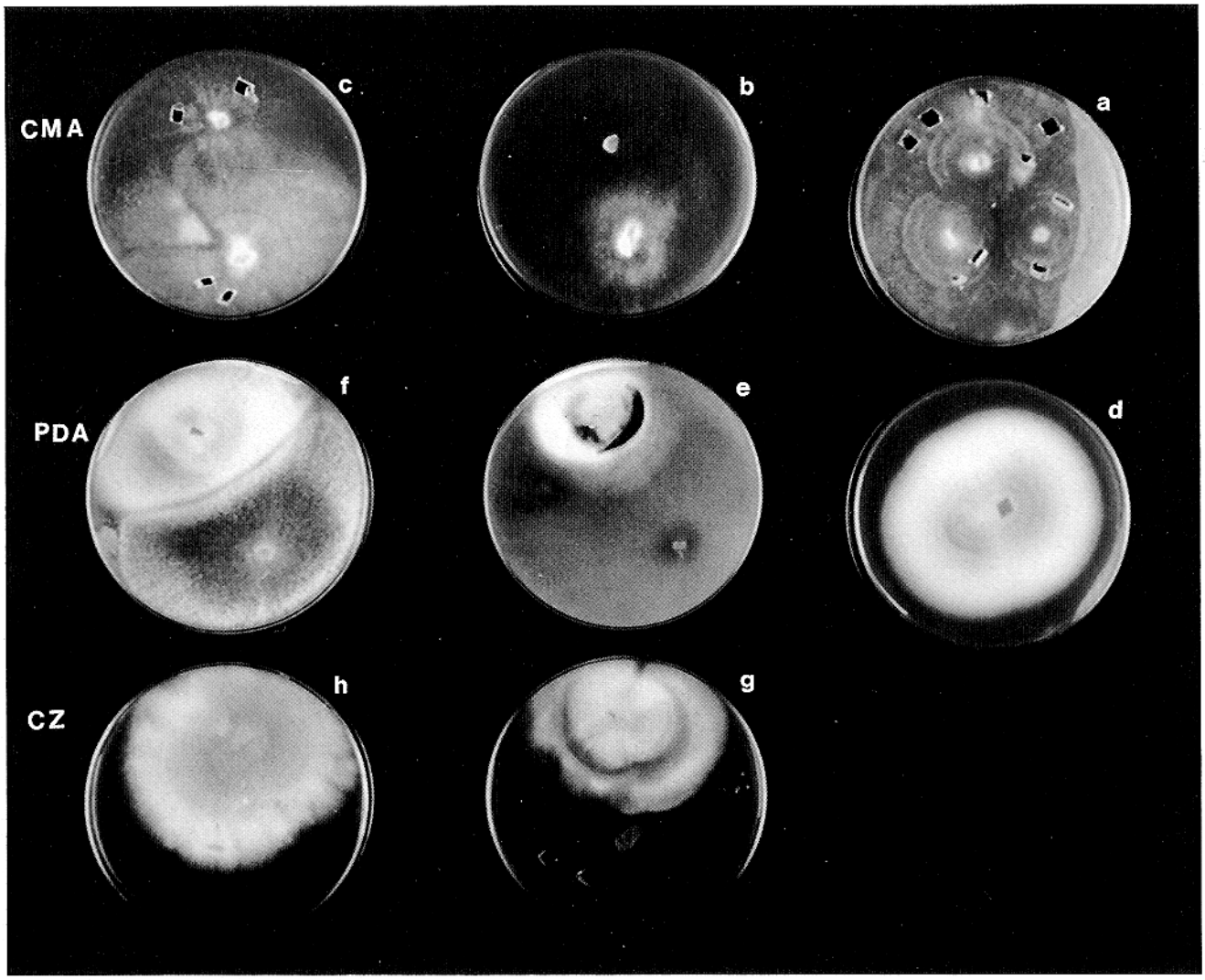

Fig. 2 - Effect on pigment production by *V. suchlasporium* (isolate 10) (V) of two trapping fungi: *Arthrobotrys sp.* (A) and *A. amerospora* (Aa). a) V alone on corn meal agar (CMA). b) V and A (CMA). c) V and Aa (CMA). d) V alone on potato dextrose agar (PDA). e) V and A (PDA). f) V and Aa (PDA). g) V and A on Czapek-Dox agar (CZ). h) V and Aa (CZ).

Discussion

The production of pigments by *V. suchlasporium* was affected by the nutrient content of the medium used. Pigments were produced when the fungus grew alone, but when plated together with other fungi they were produced in the interaction zones between the colonies, suggesting that the pigments are sometimes a response to the presence of these other fungi. When the red pigment was studied, its extraction and behaviour in TLC solvent mixtures suggest that the pigment was non-polar. The high absorbance in the UV region suggests the presence of benzene rings. The presence of several bands by TLC would suggest more than one substance present in the pigment. The red pigment was not oosporein, a pigment produced by *V. psalliotae* and other fungi with similar chemical characteristics and with antibiotic properties (Lloyd *et al.*, 1955; Vining *et al.*, 1962; Takeshita and Anchel, 1965; Wainwright *et al.*, 1986), because the red pigment and oosporein behaved differently in TLC. Fungistatic naphthoquinones were also found in *V. agaricinum* (Link) Corda (Osman and Valadon, 1984). Minato *et*

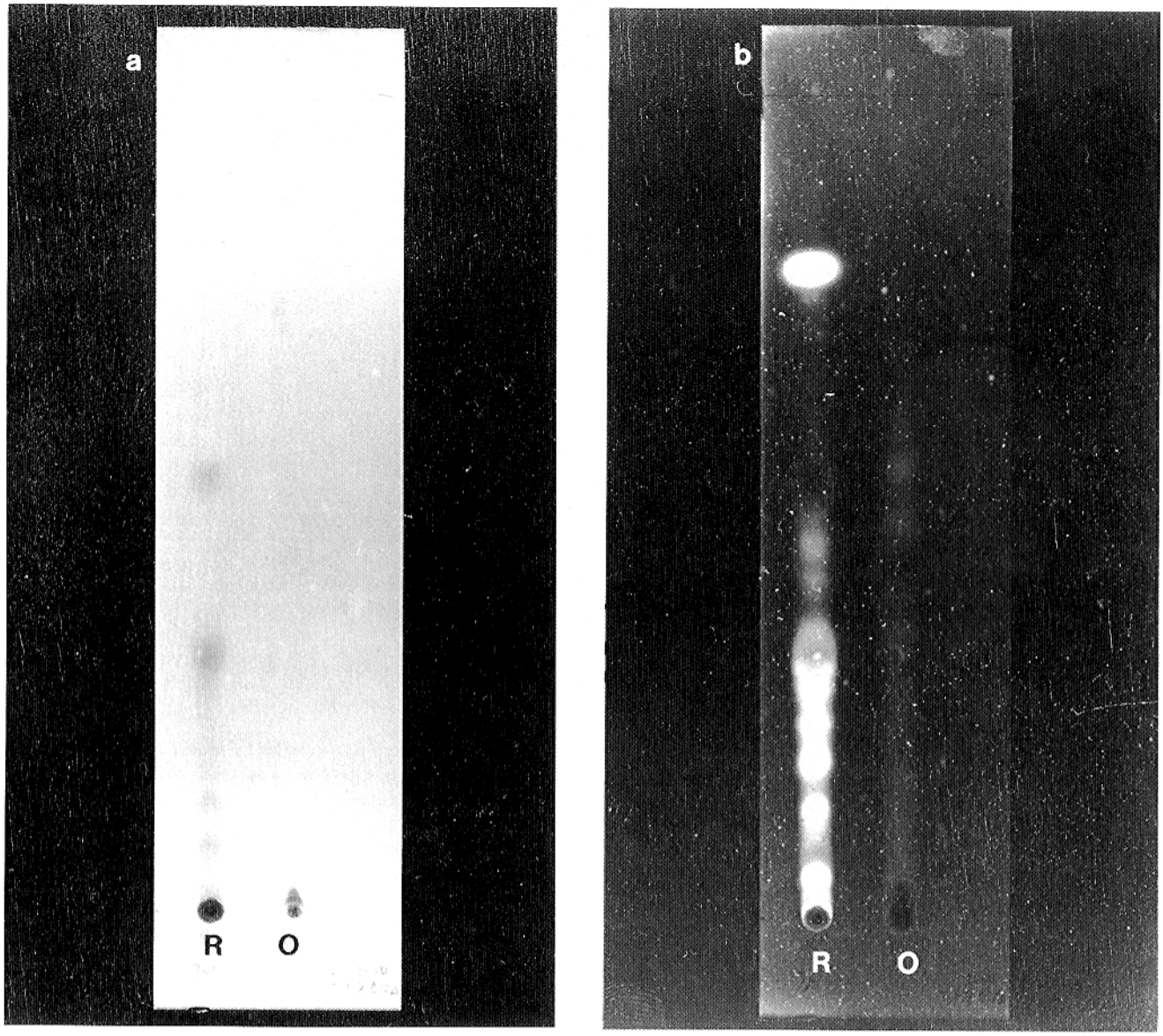

Fig. 3 - Thin layer chromatography (TLC) of a red pigment (R) produced by *V. suchlasporium* (isolate 10) and oosporein (O), a red pigment produced by *V. psalliotae*, in toluene/ethyl acetate/formic acid (5:4:1, v/v/v) developed for 45 min. (a); same TLC plate under UV light (b).

al. (1971, 1973), found a new family of antibiotic compounds in a species of *Verticillium* which they named 'Verticillins' after the genus. Possible relationships between these antagonistic substances and the pigments studied here, still remain to be investigated.

The pigment, after being extracted in acetic acid was processed by gas chromatography/mass spectrometry (GC/MS) and was identified as di-n-octyl-phthalate (Dr. van Eijk, C.B.S., The Netherlands, pers. comm.). However, phthalates are ubiquitous impurities found in MS, since they are common components of plasticizers (tubing, cap liners, gaskets) and chromatography column packings (McLafferty, 1980). Therefore, a more detailed investigation is required, in order to ascertain the structure of the substance (or substances) of which the pigment consists.

The red pigment, when bioautographed using *C. cucumerinum* as test organism, revealed the presence of mycotoxic compounds. *V. chlamydosporium* also showed antimycotical action against rust fungi (Leinhos and Buchenauer, 1986).

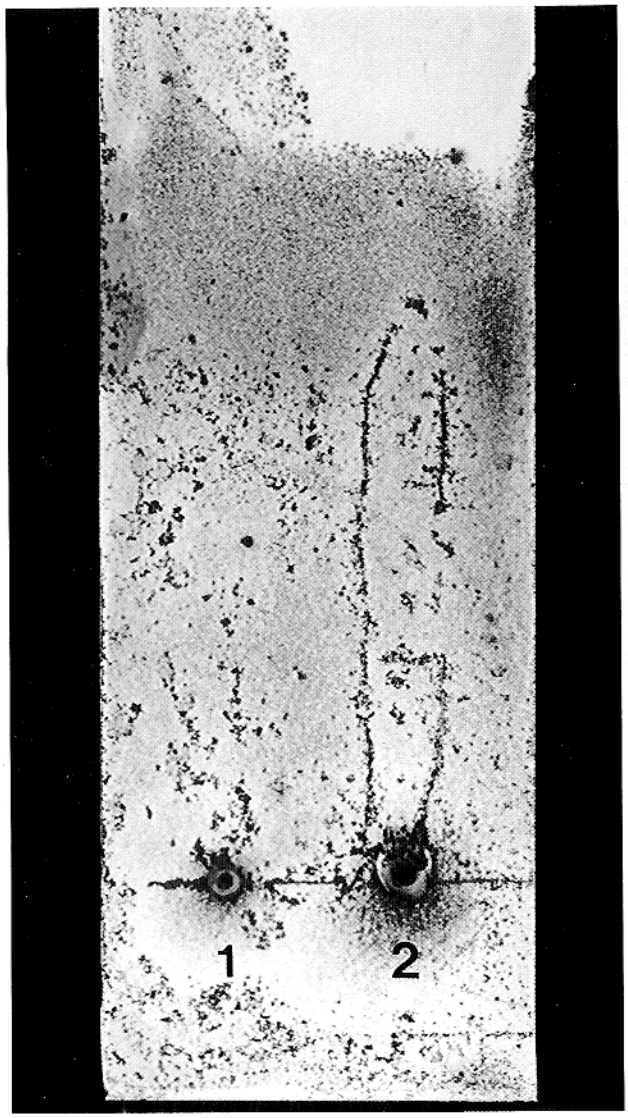

Fig. 4 - Bioautography of a TLC plate with 5 µl (1) and 10 µl (2) of an extract in acetic acid of a red pigment produced by *V. suchlasporium* (isolate 10), showing antimycotical activity against *Cladosporium cucumerinum*.

When paper discs with nematode cysts were impregnated with acetic acid alone, hatching was promoted (as measured by the numbers of juveniles per plate outside treated discs) to a similar degree as did PRD (see Table I). When discs were impregnated with an extract in acetic acid of the red pigment, the numbers of juveniles found hatched outside the discs were considerably reduced (similar to those of SDW discs). There are two possible reasons for this: either the red pigment reduces hatching, neutralising the positive action of acetic acid, or it promotes hatching and the nematodes hatched are killed (nematicidal action), so that fewer juveniles are found outside treated discs. The same extract affected nematode mobility (perhaps even survival), providing evidence for a nematicidal role for the red pigment. *V. lecanii*, a similar entomopathogenic species, is also reported to produce secondary metabolites with insecticidal activities (Claydon and Grove, 1982). More research, however, is necessary to assess the biological activity of the pigment against nematodes.

TABLE I - *Effect of an extract of a red pigment of Verticillium suchlasporium (isolate 10, CBS 464.88, ATCC no. 76547) on the hatching of* Globodera rostochiensis *juveniles from cysts.*

Treatment	Days after starting the test	Juveniles hatched/plate (average values for 5 plates/treatment)
SDW	5	1
	7	2
PRD	5	26
	7	17 ns
A	5	9
	7	25ns
A+P	5	0
	7	0

LSR (5 days) = 2.0387 / LSR (7 days) = 2.91

SDW = sterile distilled water, PRD = sterile potato root diffusate, A = glacial acetic acid, A+P = acetic acid extract of the pigment; LSR = least significant ratio; ns = not significantly different values ($P<0.05$).

TABLE II - *Effect of an extract of a red pigment from* V. suchlasporium *(isolate 10) on juvenile survival and mobility for* G. rostochiensis.

Treatment	Total Juveniles (alive + dead)/plate outside treated discs (Average of 4 plates/treatment)
Control (untreated)	31
Acetic acid	7
Acetic acid + red pigment	1

LSR = 1.684. All values significantly different ($P<0.01$)

LSR = least significant ratio.

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