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Simple Detection of Potential Pollutant Effects on Fungal Growth, Sclerotia Formation and Germination

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The onset of unfavorable environmental conditions can trigger fungal differentiation processes that result in the formation of asexual or sexual reproductive structures. These various structures represent means for dispersal, propagation, and survival. Inhibition of their formation by pollutant chemicals can result in the organism's inability to adjust to natural environmental stresses and consequently reduce the possibility that the organism will reestablish itself following the development of favorable environmental conditions. Despite the importance of these factors in maintaining a viable fungal population, few reports have appeared in the literature describing the effects of toxic chemicals on these processes. Babich et al. (1982) have studied the toxicity of trivalent and hexavalent chromium to fungal sporulation and spore germination. Babich and Stotzky (1977) have investigated the sensitivity of fungal sporulation to cadmium and Schneider (1972) has examined cadmium inhibition of sporangium formation in a marine fungus. The effects of fungicides on spore germination has been examined by McCallan et al. (1954). Our studies have emphasized the effects of potential environmental toxicants on the formation and germination of fungal sclerotia.

Fungal sclerotia have been studied since the late 1800's (Brefeld 1877, De Bary 1887) and have been described as asexual, multicellular, firm resting structures (Chet and Henis 1975). Sclerotia of filamentous fungi are composed of condensed vegetative hyphae that have aggregated and become interwoven (Townsend and Willetts 1954). Sclerotia are different from fungal spores in that they are not considered reproductive structures (Burnett 1976). The extensive literature concerning the factors and processes involved in their formation has been compiled in several reviews (Chet and Henis 1975, Willetts 1971, Willetts 1972).

The effects of a number of environmental factors on the formation of sclerotia by six species of Aspergillus has been investigated by Rudolph (1962). In this work, mycelial growth and sclerotial

formation were quantified by rather simple methods of measuring the increase in the diameter of the mycelial mats grown on a solid medium and visually estimating the number of mature sclerotia. In this study we have refined these procedures to investigate the effect of potentially toxic elements (Cd, Cr, Cu, Ni, Zn, F, arsenate, borate, and ammonium ion). We have examined the effects of these compounds on sclerotia germination and formation, and mycelial growth.

MATERIALS AND METHODS

All chemicals are analytical reagent grade and were used without further purification. Ammonium sulfate, zinc sulfate ($7H_2O$), sodium sulfate, nickel (II) sulfate ($6H_2O$), cadmium (II) nitrate ($4H_2O$) and sodium borate were obtained from J. T. Baker Company. Chromium (III) sulfate (XH_2O) was obtained from Mallinkrodt. Sodium fluoride was obtained from Aldrich. Copper (II) sulfate ($5H_2O$) was obtained from Fisher Scientific. Potassium nitrate, sodium phosphate dibasic and potassium phosphate monobasic were obtained from Mallinckrodt. Magnesium sulfate was obtained from MCB Manufacturing Chemists. Sodium arsenate was purchased from Sigma. Glucose, yeast extract, casamino acids and agar were purchased from Difco Laboratories.

A solid agar medium was used to examine the effects of different test compounds on mycelial growth and sclerotia formation. The medium was prepared by combining aqueous solutions of test compounds, which were sterilized by membrane (0.22 μ) filtration, with autoclaved nutrient agar medium and dispensing the mixture to 150 x 15 mm petri dishes. To insure proper mixing, the sterile test solutions were warmed to 47°C before being added to the sterile agar medium, which was normally maintained at 47°C. The nutrient agar medium contained per liter; 1.0 g KNO_3, 0.5 g $MgSO_4$, 0.71 g Na_2HPO_4, 10.21 g KH_2PO_4 (or 80 mM phosphate buffer), 2 g glucose, 5 g casamino acids, 1 g yeast extract and 15 g agar. Before petri dishes were used in toxicity assays they were incubated overnight at 25°C to remove excess moisture from the agar surface.

The effects of individual compounds on mycelial growth and sclerotia formation were assayed in the following manner. Assays were initiated by placing a single mature sclerotium in the center of each of three replicate agar plates amended with individual test compounds of various concentrations, which ranged from 0 (control) to 500 ppm. Inoculated plates were subsequently incubated (10 to 14 d) at 25°C until mycelial growth had reached the edge of control plates. At this time the diameter of the mycelial mat was measured and the sclerotia were counted. The level of inhibition was expressed as the percentage of control values.

Mature seed sclerotia were isolated from 2 week old agar dish cultures of an Aspergillus species isolated from soil. Seed sclerotia were harvested by irrigating plates with sterile distilled water and dislodging the sclerotia with a rubber spatula. After harvesting the sclerotia were washed with sterile distilled water to remove mycelia attached to the sclerotia surface. When no attached mycelia could be visibly detected, the sclerotia were air dried. Sclerotia prepared in this manner can be stored at room temperature up to at least one year without loss of viability.

RESULTS AND DISCUSSION

In some fungi, hyphae become interwoven to form sclerotia, small hyphal aggregates, which have the ability to resist adverse conditions for longer periods than the ordinary hyphae of the mycelium. When sclerotia forming fungi are maintained on solid medium, sclerotia are formed in concentric circles as the fungal mycelia grow radially from the plate center. Sclerotia formation is initiated following a reduction in available nutrients (Wang and Tourneau 1971), the formation of staling products (Bedi 1958), and/or initiation of other possible biochemical controls (Hadar et al. 1983). We have used this property of a sclerotia forming species of Aspergillus to develop a bioassay to measure the effects of the potentially toxic elements (Cd, Cr, Cu, Ni, Zn, F, arsenate, borate, and ammonium ion) on mycelial growth and sclerotia formation and germination.

Inorganic cations generally inhibited sclerotia formation better than the anions examined (Tables 1 and 2). Five of the six inorganic cations tested inhibited the formation of sclerotia (Table 1); only ammonium ion was not inhibitory. Cadmium was the most effective inhibitor followed in order by Zn > Cu > Cr > Ni. Nickel the least inhibitory cation completely prevented sclerotia formation at 200 ppm. Anions showing the greatest inhibition (Table 2) were: BO_3^{3-}, which completely eliminated sclerotia formation at concentrations of 300 ppm or greater, and F^-, which was 50% inhibitory at 500 ppm. Surprisingly, arsenate was not inhibitory.

Only cations, at the concentrations examined, inhibited mycelial growth. Nickel, the least effective inhibitor of sclerotia formation, was the strongest inhibitor of radial mycelial growth and sclerotia germination (Table 3). Nickel inhibited fungal growth 90% at 200 ppm and prevented germination at concentrations of 300 ppm or greater. Copper inhibited fungal growth 90% at 300 ppm and was the only other inorganic ion to inhibit germination albeit at 500 ppm, the highest concentration tested. Zinc inhibition steadily increased over the entire concentration range reaching 60% inhibition at 500 ppm. Cadmium inhibition was

Table 1. Effect of Cations on Fungal Sclerotial Formation.

Cation Concentrations	Sclerotia Formation (% of Control)[a]					
ppm	Cd^{2+}	Zn^{2+}	Cu^{2+}	Cr^{3+}	Ni^{2+}	NH_4^+
0	100 ± 0	100 ± 2	100 ± 4	100 ± 20	100 ± 31	100 ± 17
15	<1	14 ± 3	11 ± 3	72 ± 11	83 ± 19	97 ± 17
30	<1	14 ± 1	32 ± 9	32 ± 7	53 ± 19	92 ± 13
100	<1	9 ± 1	20 ± 7	<1	23 ± 12	95 ± 13
150	<1	<1	6 ± 7	<1	11 ± 4	94 ± 12
200	<1	<1	3 ± 4	<1	<1	114 ± 14
300	<1	<1	<1	<1	<1	109 ± 14
500	<1	<1	<1	<1	<1	121 ± 25

[a] mean ± standard deviation, n=3

Table 2. Effects of Anions on Fungal Sclerotial Formation.

Anion Concentrations	Sclerotia Formation (% of Control)[a]		
ppm	F^-	AsO_4^{3-}	BO_3^{3-}
0	100 ± 11	100 ± 3	100 ± 17
15	82 ± 10	87 ± 9	100 ± 14
30	85 ± 8	104 ± 4	103 ± 13
100	97 ± 14	93 ± 3	97 ± 17
150	95 ± 15	94 ± 9	91 ± 12
200	104 ± 13	103 ± 4	82 ± 12
300	82 ± 15	113 ± 4	<1
500	56 ± 8	112 ± 9	<1

[a] mean ± standard deviation, n=3

variable. Inhibition was highest at 15 to 30 ppm, tended to decrease to 200 ppm, and increased up to the final concentration of 500 ppm. All other inorganic ions did not inhibit radial mycelial growth over the entire concentration range of 0 to 500 ppm.

Table 3. Effect of Cations on Radial Mycelial Growth.

Cation Concentrations	Radial Mycelial Growth (% of Control)[a]					
ppm	Cd^{2+}	Zn^{2+}	Cu^{2+}	Cr^{3+}	Ni^{2+}	NH_4^+
0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
15	32 ± 1	90 ± 2	100 ± 0	100 ± 0	96 ± 2	100 ± 0
30	26 ± 2	89 ± 0	100 ± 0	100 ± 0	92 ± 4	100 ± 0
100	64 ± 0	80 ± 6	100 ± 0	100 ± 0	79 ± 7	100 ± 0
150	64 ± 2	73 ± 6	100 ± 0	100 ± 0	57 ± 3	100 ± 0
200	66 ± 2	63 ± 4	81 ± 4	100 ± 0	11 ± 0	100 ± 0
300	54 ± 0	56 ± 4	11 ± 2	100 ± 0	ng	100 ± 0
500	46 ± 0	43 ± 2	ng[b]	100 ± 0	ng	100 ± 0

[a] mean ± standard deviation, n=3

[b] ng = no germination

In all cases, sclerotial formation was more sensitive to inhibition than was radial growth, i.e., sclerotia formation was inhibited at inorganic ion concentrations that were not inhibitory to mycelial growth. Similar results were found by Babich and Stotzky (1977) for Cd inhibition of fungal sporulation. Sporulation was inhibited at Cd concentrations that did not effect the growth of Aspergillus niger, Tricoderma viride, and Rhyzopus stolonifer. However, a similar correlation was not found with chromium (III). Babich et al. (1982) observed that Cr at 200 ppm did not inhibit sporulation by Aspergillus giganteus and Penicillium vermiculatim was only inhibited 52%, whereas we observed complete inhibition of sclerotia formation at this concentration. The apparent differences in Cr inhibition may be the result of differences in the growth medium used in the two laboratories. Babich and Stotzky (1980) showed that the use of different media markedly affected the growth response of Botrytis cinerea challenged with 1000 ppm Pb.

Fungal toxicity assays previously used (Starkey and Waksman 1943, Drucker et al. 1979, Rabie et al. 1981, Babich et al. 1977, Babich et al. 1982) have generally required that test cultures be inoculated from freshly grown precultures; that the fungal mycelia be harvested, dried and weighed to determine dry weights of liquid cultures; or that spores be counted using a Petroff-Housser bacterial counter. The fungal toxicity assay described herein is simple and requires a minimum of advance preparation. The assay does not require the maintenance of a viable fungal culture because sufficient sclerotia need only be harvested on a yearly

basis. Stored in the dry state, sclerotia will remain viable for one to two years. After initiating the assay by simply placing a mature sclerotia in the center of an agar plate, germinating sclerotia are identified, fungal growth is determined by measuring the diameter of the fungal colony and developing sclerotia are easily counted by visual observation. Thus the three fungal parameters - germination, growth and sclerotia formation - can be assessed in a single assay.

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