Effect of Agar on Inhibition of Spore Germination by Chemicals

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ABSTRACT

Both agar and nutrients decreased the inhibitory effect of cupric sulfate on spore germination. In general, chemicals were more inhibitory to spore germination in water than in water agar. The agar effect was more profound on cationic inhibition than on that due to anions or weakly dissociated compounds, although there were exceptions. The inhibitory effect of cupric sulfate on spore germination was inversely correlated with the amount of agar present. Agars from various sources and Bacto gelatin were all effective in reducing the fungistatic effect of cupric sulfate. Results suggested that binding of chemicals by agar may slow their rates of diffusion, and may account for the reduced activity of chemicals against spore germination in agar medium.

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A common method for determining fungitoxicity is a quantitative assay of spore germination on agar containing the test chemical (3, 4, 10, 14). In recent studies, we found that the mycelial growth could be increased by adding agar to liquid media (7). The purpose of this investigation was to evaluate the effect of agar on chemical activity against spore germination.

MATERIALS AND METHODS.—Conidia of *Calonectria crotalariae* (Loos) Bell & Sobers and *Helminthosporium maydis* Nisik. & Miyake were obtained by growing each fungus under continuous fluorescent light for 8 days at 24 C on V-8 juice agar (1). Sporulation of *Alternaria solani* (Ell. & G. Martin) Sor. was induced by growing the fungus on V-8 juice agar at 24 C for 4 days with light, followed by a 4-day incubation at 16 C (1). *Helminthosporium maydis* and *A. solani* were supplied by M. Aragaki. These fungi were used because their conidia germinate in water (5, 6).

Organic fungicides used were: methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate (Benlate); 2-(4-thiazolyl)-benzimidazole (Mertect); *N*-(trichloromethylthio)-4-cyclohexene-1, 2-dicarboximide (Captan); and a coordination product of zinc ion and manganous ethylenebis (dithiocarbamate) (Dithane M-45).

Chemicals tested were added singly to Bacto potato-dextrose agar (PDA), Bacto potato-dextrose broth (PDB), 1.5% Bacto water agar, or distilled water after autoclaving. Media with chemicals were adjusted to pH 5.5 with 1N HCl or 1N KOH and used immediately. Spore germination was tested in petri plates (90 × 15 mm) containing 20 ml of media and counted after incubation for 12 hours at 24 C. No growth of contaminants was observed on the media during counting. Three replicates were used, and the experiments were repeated at least once. Potency comparisons were made at ED_{50} which was the amount of a chemical capable of reducing spore germination to 50%, as determined from the dosage-response curve (10, 14). The ratio was calculated to the nearest whole number in terms of ED_{50} of a chemical in solid medium to that in liquid medium to facilitate comparison of activities.

RESULTS.—Both agar and nutrients decreased the inhibitory effect of cupric sulfate on spore germination of

TABLE 1. Effect of cupric sulfate in Bacto potato-dextrose agar (PDA), Bacto potato-dextrose broth (PDB), Bacto water agar (WA), and water on spore germination of three fungi

Fungus	ED_{50} (μg/ml)		ED_{50} ratio[a]	ED_{50} (μg/ml)		ED_{50} ratio[a]
	PDA	PDB	(PDA/PDB)	WA	Water	(WA/water)
Alternaria solani	380	130	3	72	0.36	200
Calonectria crotalariae	175	28	6	3.5	0.04	88
Helminthosporium maydis	90	82	1	0.58	0.04	15

[a]Ratio was calculated to the nearest whole number.

TABLE 2. Effect of different chemicals in water and 1.5% water agar on spore germination of *Alternaria solani*

Chemical	ED_{50} (μg/ml)		ED_{50} ratio[a] (water agar/water)
	Water agar	Water	
Cations:			
Cupric sulfate	72	0.36	200
Zinc chloride	500	6.0	83
Zinc sulfate	980	21	47
Aluminum chloride	860	20	43
Ferrous sulfate	24	3.0	8
Mercuric chloride	0.58	0.42	1
Anions:			
Sodium arsenate	2,200	115	19
Sodium chloride	1,000	92	11
Potassium chloride	1,000	270	4
Sodium nitrite	1,350	1,200	1
Boric acid	2,100	2,200	1
Weakly dissociated compounds:			
Dithane M-45	2.2	0.70	3
Mertect	1,000	440	2
Benlate	1,750	1,900	1
Captan	4.1	4.4	1
Actidione	0.50	0.36	1
Pimaricin	0.50	0.56	1

[a]Ratio was calculated to the nearest whole number.

TABLE 3. Effect of the mineral content in Czapek's agar and broth on spore germination of three fungi

Fungus	Spore germination (%)				
	Czapek's agar	Czapek's agar minus sucrose	Czapek's broth	Czapek's broth minus sucrose	Distilled water
Alternaria solani	100	26	98	7	98
Calonectria crotalariae	89	33	93	11	93
Helminthosporium maydis	100	30	81	11	70

H. maydis, A. solani, and *C. crotalariae* (Table 1). In the presence of nutrients, the agar effect on chemical activity was also greatly reduced. For example, when *A. solani* was used, the ED_{50} ratio of cupric sulfate in water agar and in water was 200, while that in PDA and PDB was only 3. Since the effect of nutrients on fungicide activity is well documented (2), only the agar effect was further investigated using *A. solani* as the test fungus. In general, chemicals were more effective in water than in water agar, and the agar effect was more profound on cations than on anions or weakly dissociated compounds, although there were exceptions (Table 2).

Since the ED_{50} values for $FeSO_4$ and KCl in water are below the amounts called for in Czapek's media (15), we also studied the effect of mineral content in Czapek's agar and broth on spore germination. Conidia of *A. solani, C. crotalariae,* and *H. maydis* were not inhibited either on Czapek's agar or in Czapek's broth. However, when sucrose was omitted, the mineral content in both media was inhibitory (Table 3). Agar also decreased the inhibitory effect of minerals in Czapek's solution on spore germination.

To study the effect of agar concentration on chemical inhibition, spore germination of *A. solani* was tested on various concentrations of Bacto water agar containing 10 μg/ml of cupric sulfate. The inhibitory effect of cupric sulfate on spore germination was inversely correlated with the amount of agar present (Fig. 1). Agars from various sources at 1.5% and Bacto gelatin at 12%, a commonly-used concentration, were tested to determine their effects on activity of cupric sulfate against *A. solani.* All the solidifying agents tested reduced the fungistatic effect of cupric sulfate, compared to that in water. The ED_{50} of cupric sulfate in Bacto agar, Bacto purified agar, Bacto Noble agar, Calbiochem agar, Bacto gelatin, and water was 53, 12, 14, 215, 1,250, and 0.28 μg/ml, respectively.

On agar, only the contact surface of spores was exposed

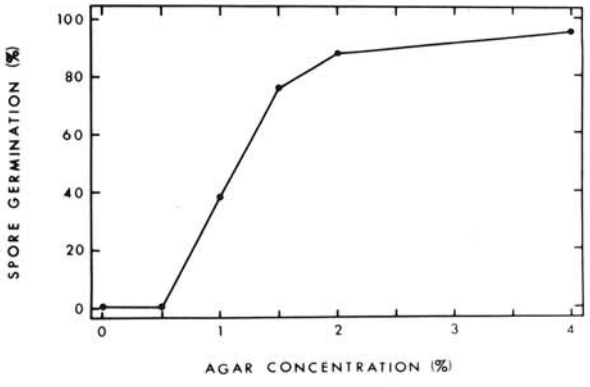

Fig. 1. Germination of *Alternaria solani* conidia on various concentrations of Bacto water agar containing 10 μg/ml cupric sulfate.

to chemicals in the medium, whereas in water the whole spore surface was exposed. To determine if this was the basis for spores to be less sensitive to chemicals on agar, disks of cellophane containing *A. solani* conidia were placed on agar or water amended with various concentrations of cupric sulfate. Cupric sulfate was still more effective in water than in agar. The ED_{50} of cupric sulfate in water and agar was 5.6 and 59 μg/ml, respectively. Germination of *A. solani* conidia both upon and submerged in agar containing various concentrations of cupric sulfate was also compared. Spores were embedded by placing them on solidified agar in a petri plate and then covering them with a thin layer of molten agar at 45 C. The ED_{50} of cupric sulfate was 78 μg/ml with spores on the surface and 73 μg/ml with spores embedded. Apparently, the reduced sensitivity of spores to chemicals in agar relative to that in water was not due to differences in spore surface area exposed to the media.

Saturated solutions of safranin and fast green dyes were prepared to compare the rate of diffusion of compounds in agar and water. Agar disks (1.5% Bacto water agar, 14 mm in diameter) were immersed in the solutions for 2 hours, removed and blotted to eliminate excess dyes, and then placed on cellophane on agar or water in petri dishes. Both dyes diffused throughout the entire plates of water within 4 hours. On agar, the dyes had diffused only approximately 5 cm from the disks after 48 hours.

Effect of agar powders and solidified agar pieces on spore germination of *A. solani* in chemical solutions was also studied. Powders of Bacto agar and pieces (approximately 5 × 5 × 5 mm) of solidified 3% Bacto agar were added to cupric sulfate or mercuric chloride solutions at a rate equivalent to 1.5% agar. Mercuric chloride was used because its inhibition to spore germination was not affected by agar. Like chemicals in solidified agar, the inhibitory effect of cupric sulfate was reduced by agar powders and solidified agar pieces (Table 4). Mercuric chloride toxicity was not changed. Cupric sulfate and mercuric chloride solutions containing 1.5% agar powders also were shaken gently for 1 hour and centrifuged at 1,800 rpm for 20 minutes to remove agar particles, before spore germination of *A. solani* was tested. Cupric ions, but not mercuric ions, apparently were bound by agar powders. After treatment of solution with agar powders, the ED_{50} of cupric sulfate increased from 0.34 to 59 μg/ml, whereas that of mercuric chloride remained unchanged (approximately 0.52 μg/ml).

DISCUSSION.—Agar decreased the inhibitory effect on spore germination of 10 out of 17 chemicals tested. These results were in accord with those of Ram et al. (13) who also showed that fungicides generally were more effective against fungal growth in liquid than in agar medium. Since all the solidifying agents tested were effective in reducing the inhibitory effect of cupric sulfate, it is suggested that whenever the activity of a chemical is tested in a solid medium, the possible interference by the solidifying agent should be taken into consideration. Effect of nutrients in agar media on toxicity of metals (8) and interference by impurities in agar in physiological studies of fungi (9, 12) also had been reported.

Both safranin and fast green dyes diffused faster in water than in agar. Moreover, shaking of cupric sulfate solution for 1 hour with agar powders also reduced its activity. These results suggest that binding of chemicals by agar may result in slow diffusion of these substances in agar which in turn may account for the reduced activity of chemicals against fungal spore germination in agar medium. Not all chemicals tested were affected by agar, probably because agar did not adsorb all of these chemicals. Mercuric chloride was one of these. The inhibitory effect of this chemical remained unchanged after shaking of the solutions with agar powders. Agar exists principally as a calcium salt of the sulfuric acid ester of a galactan (11). Therefore, binding of a chemical by agar probably could result from replacement of the calcium portion of the agar by this chemical.

TABLE 4. Effect of agar powders and solidified agar pieces on inhibition of spore germination of *Alternaria solani* by cupric sulfate and mercuric chloride

Treatment	ED_{50} (μg/ml)	
	Cupric sulfate	Mercuric chloride
Chemical solution + agar powders	55	0.53
Chemical solution + solidified agar pieces	59	0.51
Chemical solution	0.34	0.44
Chemical in solidified agar	66	0.56

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