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Dyes As Fungal Inhibitors: Effect on Colony Diameter

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The effects of a wide range of concentrations of 13 dyes on the colony diameters of nine fungal strains (including members of the Deuteromycetes and Zygomycetes) were evaluated. Auramine at a concentration of 50 ppm (50 μg/ml), methylene blue at a concentration of 500 ppm, gentian violet at a concentration of 5 ppm, and phenol red at a concentration of 50 ppm performed as well as the commonly used dyes dichloran at a concentration of 2 ppm and rose bengal at a concentration of 50 ppm in that they allowed adequate colony development of the Deuteromycetes strains tested and controlled rapidly spreading fungi.

The effects of acidulants, antibiotics, fungicides, dyes, and essences on growth and enumeration of fungi have been studied by many authors. The incorporation of these compounds into culture media has been recommended to inhibit bacterial growth (3, 7, 14, 21, 22), to reduce the colony diameters of spreading fungi (5, 11, 12, 14–16, 21), and, in some cases, to develop a selective and/or differential medium (2–5, 8, 9, 13, 20, 23–26). Overgrowth of petri dish cultures by *Rhizopus* and *Mucor* spp. is a problem with fungal enumeration media because it prevents counting or isolation of the other molds present. Various attempts have been made to improve fungal enumeration media. Rose bengal may be added to some general culture media to avoid formation of excess aerial mycelium (14, 17, 21), but some yeast and mold strains may be inhibited completely by this dye (14). Dichloran, alone and in combination with rose bengal, has been shown to inhibit spreading fungi and to limit colony diameters of other genera in fungal enumeration media (11, 15). After consideration of all of the media developed for counting colony-forming units of fungi in foods, we concluded that one ideal medium does not exist. In daily routine examinations the use of a standardized medium is recommended for comparisons of data and reproducibility (10). This fact prompted us to investigate the effects of some dyes currently used in microbiology in staining procedures, in selective media, or as pH indicators on the colony diameters of various fungi in order to find a range of dye concentrations that restrict growth of rapidly spreading fungi while allowing satisfactory growth of other test fungi or in order to select growth of a given fungal species.

Cultures. The fungi used in this study were *Aspergillus parasiticus* NRRL 2999, *Alternaria alternata* CCFVB 252 (CCFVB is the Culture Collection of the Veterinary Faculty of Barcelona), *Aspergillus flavus* CCFVB 255, *Cladosporium herbarum* CCFVB 438, *Fusarium oxysporum* CCFVB 300, *Penicillium verrucosum* var. *cyclopium* CCFVB 417, *Absidia corymbifera* CCFVB 332, *Mucor racemosus* CCFVB 334, and *Rhizopus stolonifer* CCFVB 333. The last three organisms are Zygomycetes, while the rest are Deuteromycetes. Cultures were maintained on 2% malt extract agar (MEA) slants at 4°C.

Dyes. The following 13 dyes were studied: auramine (TCI, Tokyo, Japan), bromothymol blue (Panreac, Barcelona, Spain), basic fuchsin (Panreac), dichloran (TCI), gentian violet (Panreac), malachite green (Panreac), methylene blue (Panreac), methyl red (Panreac), phenol red (Panreac), rose bengal (TCI), safranin (Panreac), sudan black (Panreac), and water blue (Panreac). Stock solutions of the dyes were made with distilled water. Because of the relatively low solubility of some dyes in water, they were first dissolved in a minimal amount of 95% ethanol and then made to volume with distilled water.

Culture media. We used MEA (20 g of malt extract [Difco], 1.0 g of peptone [Difco], 20 g of dextrose [Merck], and 20 g of agar [Difco] in 1 liter of distilled water) as the basal and control medium. An appropriate amount of each dye solution was added to MEA before autoclaving. Disposable 9.0-cm petri dishes containing 20 ml of medium were used. The final dye concentrations in culture media ranged from 0.25 to 5,000 ppm (0.25 to 5,000 μg/ml) depending on the dye assayed. Sudan black, phenol red, methyl red, and safranin were tested only at concentrations up to 50 ppm because it was difficult to obtain good solubility at higher concentrations.

Colony diameter determinations. Media were spot inoculated with spore suspensions of each fungus studied. Mold spore suspensions were prepared by lightly scraping spores off 7-day-old MEA plate cultures into a 0.0001% Tween 80 solution. For each colony two diameters, measured at right angles to one another, were averaged to give the mean diameter for that colony. All measurements except those for Zygomycetes strains were made after 7 days at 28°C. For the Zygomycetes strains, measurements were made after 2 days to prevent complete overgrowth of the control plate. All colony diameters were determined by using at least four replicates for each strain. Inhibition was measured as the percent decrease in colony diameters compared with the colony diameters in the control medium (MEA) in the absence of dye. The percentage of reduction was calculated as follows: % reduction = 100 − (diameter on medium with dye/diameter on MEA). In general, all of the dyes had obvious effects on the colony diameters of the fungal strains tested; these effects increased as the dye concentration increased, especially with dichloran, rose bengal, auramine, bromothymol blue, basic fuchsin, gentian violet, malachite green, methylene blue, and water blue (Fig. 1). Nevertheless, in some cases (bromothymol blue, basic fuchsin, methylene blue, and water blue) it was necessary to test high concentrations of dyes. Dyes such methyl red, safranin, sudan black, and phenol red were tested only at concentra-

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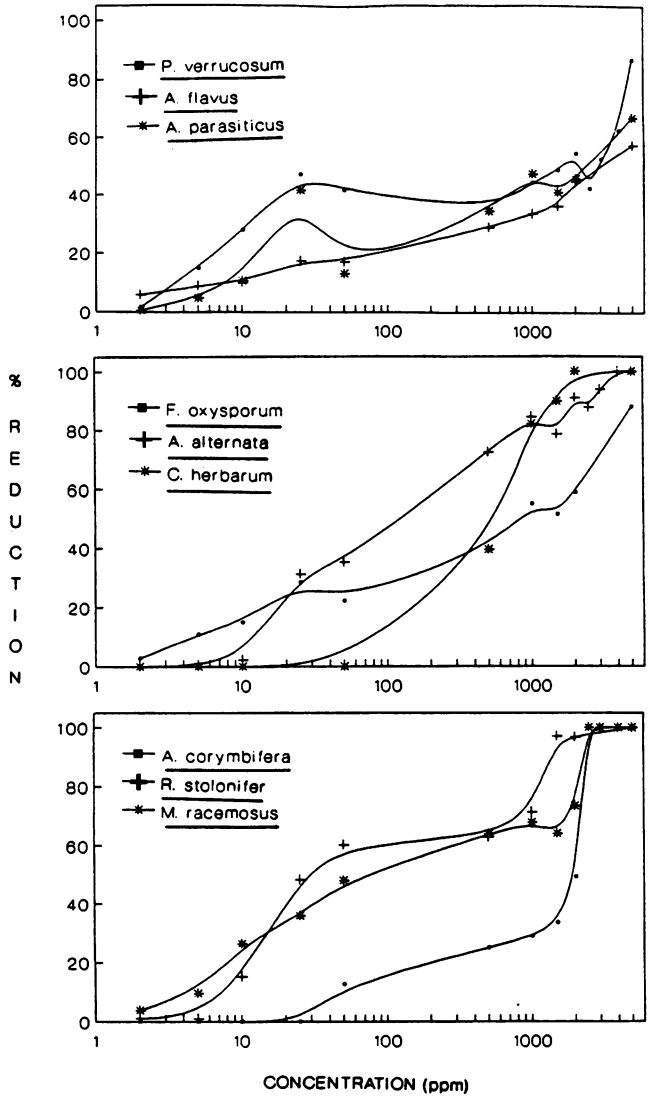

FIG. 1. Percentages of reduction of colony diameters of fungal strains caused by various concentrations of auramine.

tions up to 50 ppm because of their low solubility at higher concentrations. With the exception of phenol red, these dyes produced small reductions in colony diameters in the strains tested.

The discussion of the results obtained below is based mainly on the effects of dyes on Zygomycetes strains; we looked for dye concentrations that allowed adequate colony development of the Deuteromycetes strains tested while controlling rapidly spreading fungi. Dichloran and rose bengal have been reported as mold-spreading inhibitors at different concentrations (usually 2 and 50 ppm, respectively) (1, 14). The most effective concentrations of the 13 dyes which we studied are summarized in Table 1. The mean percentages of reduction for Zygomycetes and Deuteromycetes strains are also included in Table 1. The mean percentage of reduction of colony diameters of Zygomycetes strains obtained with dichloran at a concentration of 2 ppm and rose bengal at a concentration of 50 ppm were 40.2 and 45.6%, respectively. Similar results were obtained with

TABLE 1. Percentages of reduction of colony diameters of fungal strains determined at the most effective concentrations of dyes[a]

Taxon	% Reduction in colony diameter caused by:												
	Dichloran (2 ppm)	Rose bengal (50 ppm)	Auramine (50 ppm)	Bromothymol blue (50 ppm)	Basic fuchsin (500 ppm)	Gentian violet (5 ppm)	Methylene blue (500 ppm)	Malachite green (1 ppm)	Methyl red (50 ppm)	Phenol red (50 ppm)	Safranin (50 ppm)	Sudan black (50 ppm)	Water blue (5,000 ppm)
P. verrucosum	4.3	0	41.9	2.9	8.9	6.9	25.7	13.9	11.0	13.5	13.3	20.8	6.1
Aspergillus flavus	7.2	24.9	17.2	8.0	16.6	17.2	18.6	4.5	7.3	9.6	14.2	10.8	29.2
Aspergillus parasiticus	6.5	29.5	13.1	3.8	13.0	31.7	15.4	17.8	2.5	0.9	3.3	3.3	11.4
F. oxysporum	0	20.8	22.1	0	5.3	37.4	16.7	7.9	9.5	18.2	14.4	14.0	0
Alternaria alternata	8.9	42.3	35.2	10.1	60.5	43.0	46.4	45.3	14.2	15.8	20.4	23.6	31.7
C. herbarum	0	0	0	18.3	90.7	0	15.0	96.4	16.6	14.6	0	20.1	0
Absidia corymbifera	25.0	15.1	12.5	12.0	68.8	13.9	54.2	42.8	7.8	26.2	33.0	17.1	60.6
R. stolonifer	90.0	65.2	60.2	40.4	33.8	49.0	18.8	58.4	16.1	48.0	6.6	50.5	98.6
M. racemosus	5.7	56.4	48.0	23.9	69.6	74.6	40.4	70.7	28.9	38.7	7.3	32.1	67.5
Deuteromycetes[b]	4.5	19.6	21.6	7.2	32.5	22.7	23.0	31.0	10.2	12.1	10.9	15.4	13.1
Zygomycetes[b]	40.2	45.6	40.2	25.4	57.4	45.8	37.8	57.3	17.6	37.6	15.6	33.2	75.6

[a] Complete fungal inhibition data for all 13 dyes can be obtained from M.R.B.
[b] Mean percentages of reduction for strains belonging to this group.

auramine at a concentration of 50 ppm, gentian violet at a concentration of 5 ppm, methylene blue at a concentration of 500 ppm, and phenol red at a concentration of 50 ppm. Dichloran at a concentration of 2 ppm produced only a slight inhibition of the Deuteromycetes strains tested (less than 5%), and rose bengal at a concentration of 50 ppm produced a mean percentage of reduction of 19.6%. In both cases, *Alternaria alternata* was the most inhibited strain, especially with rose bengal (42.3%). The dyes which produced greater colony diameter reductions of the Zygomycetes strains tested than of the Deuteromycetes strains tested (similar to the reductions produced by dichloran at a concentration of 2 ppm and rose bengal at a concentration of 50 ppm) were phenol red at a concentration of 50 ppm and auramine at a concentration of 50 ppm (although the value for *P. verrucosum* var. *cyclopium* was 41.9%), as well as methylene blue at a concentration of 500 ppm and gentian violet at a concentration of 5 ppm (although at these levels, reductions of *Alternaria alternata* colony diameters of 46.4 and 43%, respectively, were observed, which were similar to the reductions in colony diameters produced by rose bengal at a concentration of 50 ppm). Basic fuchsin at a concentration of 500 ppm was also effective as a Zygomycete colony diameter inhibitor, but greater inhibition of Deuteromycetes strains, especially *Alternaria alternata* (60.5%) and *C. herbarum* (90.7%), was obtained. Water blue at a concentration of 5,000 ppm produced mean percentages of reduction for the Zygomycetes and Deuteromycetes strains tested of 75.6 and 13.1%, respectively. However, the medium was too opaque to be of practical value. Dyes such a sudan black, methyl red, and safranin at a concentration of 50 ppm (maximum concentration tested), as well as bromothymol blue at a concentration of 50 ppm, produced lower mean percentages of reduction of Zygomycetes colony diameters than dichloran at a concentration of 2 ppm and rose bengal at a concentration of 50 ppm. Malachite green at a concentration of 1 ppm produced large reductions in the colony diameters of Zygomycetes strains (57.3%), but *C. herbarum* was highly inhibited (96.4%), so the mean percentage of reduction for Deuteromycetes strains was much too high (31%).

Henson (11) performed an exhaustive study on the effects of dichloran on the colony diameters of various fungi and reported similar results for *R. stolonifer* and a *Mucor* sp. but greater reductions in colony diameters for *Aspergillus flavus*, a *Penicillium* sp., a *Fusarium* sp. and an *Alternaria* sp. than the values obtained in our study. King et al. (15) demonstrated that a medium containing dichloran at a concentration of 2 ppm and rose bengal at a concentration of 25 ppm was more effective in reducing the colony diameters of various fungi than rose bengal (50 ppm)–chlortetracycline agar and media containing dichloran at a concentration of 2 ppm. Andrews and Pitt (1) formulated dichloran (2 ppm)–chloramphenicol–peptone agar and, in agreement with our results, obtained an 85% reduction in *R. stolonifer* colony diameters and less than 20% reductions in colony diameters for *Fusarium* sp., *Alternaria* sp., *Cladosporium* sp., *Aspergillus flavus*, and *Penicillium viridicatum*. We found no references to the antifungal activities of the remaining dyes tested, with the exception of gentian violet. Various authors (18, 19, 26) have tested gentian violet for its inhibitory effect on the growth of aflatoxigenic strains. The use of this dye as a possible fungal inhibitor in poultry feed has also been reported (6, 27). Chen and Day (6) studied the antifungal activity of this substance at various concentrations (5, 50, 500, and 5,000 ppm). These authors reported that gentian violet at a concentration of 500 ppm in potato dextrose agar inhibited the growth of the fungus species tested (*Aspergillus flavus*, *Aspergillus fumigatus*, *Candida albicans*, *Fusarium moniliformis*, and *Penicillium camemberti*). In our case, a final concentration of 75 ppm was enough to inhibit all of the fungal strains tested except *Aspergillus flavus*, which required 1,000 ppm.

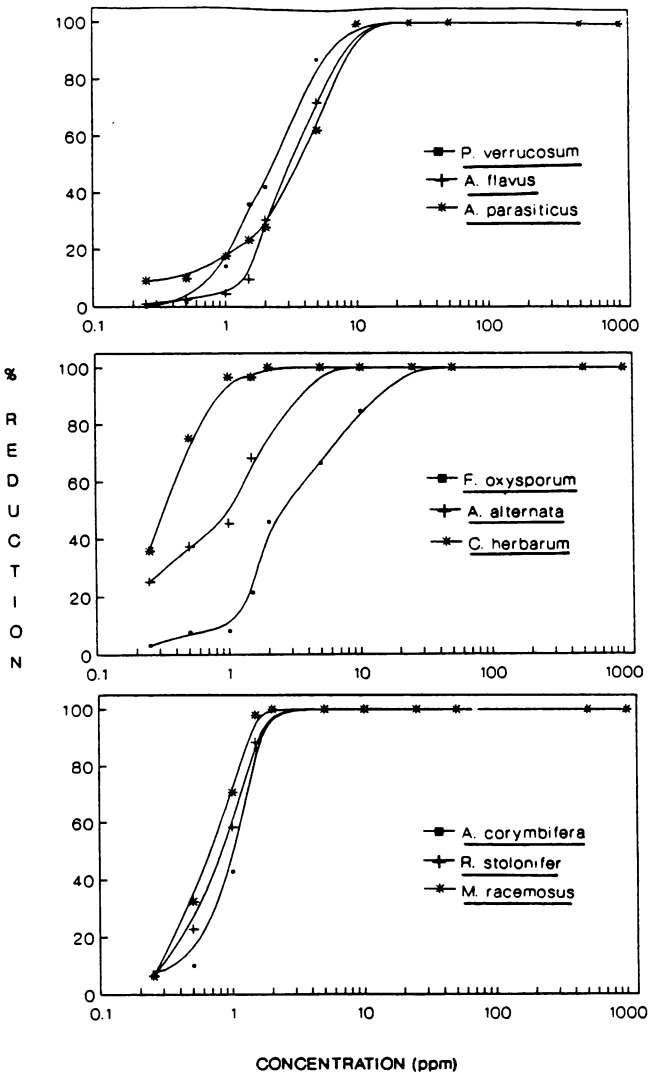

FIG. 2. Percentages of reduction of colony diameters of fungal strains caused by various concentrations of malachite green.

From the results obtained in this study, it is clear that auramine at a concentration of 50 ppm, gentian violet at a concentration of 5 ppm, phenol red at a concentration of 50 ppm, and methylene blue at a concentration of 500 ppm performed as well as dichloran at a concentration of 2 ppm and rose bengal at a concentration of 50 ppm. Otherwise, the data obtained with malachite green (Fig. 2) show that the Zygomycetes strains were inhibited at a concentration of 2 ppm and that at a concentration of 10 ppm all of the remaining organisms except *F. oxysporum* were completely inhibited. Further studies will be made on the suitability of this dye in selective media for *F. oxysporum*. Results obtained in this study could make possible the development of new fungal enumeration media or selective media.

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