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Dichloran-Rose Bengal Medium for Enumeration and Isolation of Molds from Foods

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Overgrowth by spreading molds such as *Rhizopus* and *Mucor* species is a problem with fungal enumeration media used for foods. Thirty-one antifungal compounds were surveyed for their ability to selectively inhibit such fungi while allowing growth of mycotoxigenic molds and other species of significance in food spoilage. Dichloran (2,6 dichloro-4-nitroaniline) restricted growth of *Rhizopus stolonifer* while allowing satisfactory growth of the other test molds. Three *Rhizopus* and *Mucor* species were encountered that were not inhibited by dichloran; these were controlled by the addition of rose bengal. The optimal medium, designated DRBC, contained 2 μg of dichloran and 25 μg of rose bengal per ml. DRBC, in pure culture tests and with food samples, restricted the colony size of spreading molds and recovered a wider range of species in higher numbers than other enumeration media.

The development of media for isolation and enumeration of molds from foods has followed a pattern of refinement. Early selective media were acidified to suppress bacterial growth; acidified potato dextrose agar (APDA) is the most commonly used medium of this type (12). Later, the addition of antibiotics to media of higher pH permitted growth of a wider range of fungi and improved recovery of sublethally damaged fungal spores (2, 3, 5-7). A further refinement was the addition of rose bengal to inhibit rapidly spreading molds (1, 2, 4, 6, 8, 9). Rose bengal-chlortetracycline agar (RBC) (2) is the most effective fungal enumeration medium in current use.

In enumerating mold from foods, the problem of overgrowth of petri dish cultures by *Rhizopus* and *Mucor* spp. frequently occurs, preventing counting or isolation of other molds present. Various fungicidal compounds have been added to media to inhibit the growth of some fungi and to select for others (10, 14, 15, 16). The addition of dichloran (2,6-dichloro-4-nitroaniline) to rose bengal-streptomycin medium has allowed isolation of *Aspergillus flavus* from peanuts and soil by restricting other fungi, including *Rhizopus stolonifer* (1).

In this study, a number of fungicidal compounds were tested for selective activity against *Rhizopus*, *Mucor*, and other genera of rapidly growing fungi. A dichloran-amended modification of Jarvis RBC medium (2) has been developed suitable for enumeration and isolation of fungi from foods.

MATERIALS AND METHODS

Basal medium consisted of glucose, 10 g; peptone (Oxoid), 5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; K_2HPO_4 or KH_2PO_4, 1.0 g; agar, 15 g; distilled water, 1 liter; final pH was 7.2 with K_2HPO_4 and 5.6 with KH_2PO_4. Filter-sterilized chlortetracycline (Aureomycin, Lederle) was added to sterilized media just before pouring to give a final concentration of 10 μg/ml.

Rose bengal (5% [wt/vol] aqueous) was added in appropriate amounts to some media before autoclaving. Test antifungal compounds in either 95% ethanol or water were added to sterilized, tempered (50°C) media before pouring. Dichloran (2,6-dichloro-4-nitroaniline), thiabendazole (2-[4-thiazolyl]benzimidazole), pentachlorophenol, and pentachloronitrobenzene were obtained from Tokyo Kasei Co. Ltd., Tokyo, Japan; Mancozeb, Difolatan, Thiram 80, and Zineb 80 were from Lane Ltd., Sydney, Australia; and Benlate (50% [wt/wt] benomyl) was from Du Pont (Australia) Ltd., Sydney. Other compounds were laboratory reagent grade.

Experimental media were initially challenged with a mixed inoculum of *R. stolonifer* FRR 5 (500 conidia per ml), *A. flavus* FRR 3251, *Penicillium expansum* FRR 1718, and *Penicillium islandicum* FRR 1037 (200 conidia of each per ml) (FRR denotes the culture collection of the Commonwealth Scientific and Industrial Research Organisation Division of Food Research, North Ryde, New South Wales, Australia). The inoculum was surface plated, 0.1 ml/plate. Minimum inhibitory concentrations were determined by presence or absence of visible growth on plate surfaces

containing various concentrations of chemicals.

Further tests of the media were made with foods from local stores and food processing plants. Foods were sampled by stomaching (13) 40 g in 200 ml of 0.1% peptone-water for 30 s or by weighing into peptone-water and shaking periodically for 0.5 h (12) and then surface plating, 0.1 ml/plate.

Media effective against most *Mucorales* were further tested to determine their action on a wide range of genera. Plates were spot inoculated by the methods of Pitt (11) with one, four, or nine cultures per 90-mm plate, depending on the expected colony diameters. Genera studied are listed in Table 6; the species of *Aspergillus* and *Penicillium* included but not listed on Table 6 were: *A. candidus* FRR 93, *A. chevalieri* FRR 547, *A. clavatus* FRR 1314, *A. flavus* FRR 3251, *A. fumigatus* FRR 163, *A. ochraceus* FRR 1558, *A. parasiticus* FRR 2999, *A. sydowi* FRR 1997, *A. terreus* FRR 1910, *A. versicolor* FRR 499, *A. wentii* FRR 1589, *P. brevicompactum* FRR 1100, *P. chrysogenum* FRR 1951, *P. citrinum* FRR 805, *P. crustosum* FRR 3468, *P. cyclopium* FRR 1465, *P. digitatum* FRR 1313, *P. expansum* FRR 1718, *P. glabrum* FRR 1496, *P. griseofulvum* FRR 989, *P. islandicum* FRR 1037, *P. janczewskii* FRR 1985, *P. oxalicum* FRR 1058, *P. phoeniceum* FRR 1233, *P. purpurogenum* FRR 75, *P. roqueforti* FRR 1525, *P. rubrum* FRR 1625, *P. sclerotiorum* FRR 1202, and *P. simplicissimum* FRR 1182.

All experiments both with pure culture inocula and with foods were carried out in duplicate. All plates were incubated at 25°C and examined after 3, 4, and 5 days, but counted or measured at 4 days, except as noted.

RESULTS

Survey of antifungal compounds for selective inhibition of *Rhizopus*. Antifungal compounds were tested in the basal medium without rose bengal. Table 1 lists the 31 compounds tested, showing the approximate minimal inhibitory concentrations against *R. stolonifer*, *A. flavus*, *P. expansum*, and *P. islandicum*. Eleven had a lower minimal inhibitory concentration for *R. stolonifer* than for the other three fungi. Only two of these compounds, dichloran and pentachloronitrobenzene, showed sufficient specificity to warrant further testing. Because pentachloronitrobenzene is considered a cancer-suspected agent, it was not given further consideration. *R. stolonifer* grew better than the other fungi on media containing Benlate, Difolatan, thiabendazole, and Zineb. These compounds may be useful for selective isolation of *Rhizopus* species.

Further testing of dichloran. Table 2 shows the effect of various concentrations of dichloran added to basal medium on the growth of *R. stolonifer*. RBC medium (2) (pH 5.6) was included for comparison. After 2 days, colonies on the medium containing 1.25 μg of dichloran per ml were 1 to 4 mm in diameter and readily countable. After 4 days, colonies were 4 to 18 mm in diameter, and plates with inocula greater than 50 sporangiospores per plate could no longer be counted.

Dichloran restricted colony diameters of all the test fungi, and also markedly affected conidiogenesis in some cases. At a concentration of 5 μg/ml, conidial development in *A. niger* was inhibited almost completely, whereas a concentration of 2.5 μg/ml allowed almost normal conidiogenesis.

On the basis of the above observations and further experiments with 1, 2, and 3 μg of dichloran per ml (data not shown), 2 μg of dichloran per ml was chosen as giving good balance between growth and inhibition of the fungal species studied. A series of experiments showed that dichloran could be added to the basal medium before sterilization without affecting fungal counts.

Combinations of dichloran and rose bengal. When the basal medium plus 2 μg of dichloran per ml (DC2) was challenged with a variety of molds from foodstuff, three species, *R. arrhizus*, *Mucor racemosus*, and *M. circinelloides*, were encountered which spread even in the presence of 40 μg of dichloran per ml. To overcome this problem, media containing various concentrations of dichloran and rose bengal were tested. The interactions of these two compounds on mold growth are shown in Tables 3 and 4. Table 3 lists colony counts obtained, and they are not statistically different. Lower counts generally reflected partial overgrowth of plates. The two highest levels of rose bengal in combination with dichloran effectively controlled the diameter of the *Rhizopus* and *Mucor* colonies (Table 4). On the basis of both colony counts and control of *Rhizopus* and *Mucor* colony diameters (Tables 3 and 4 and similar data), a combination of 2 μg of dichloran and 25 μg of rose bengal per ml in basal medium, pH 5.6, was chosen for further study and designated DRBC medium. RBC contains 50 μg of rose bengal per ml in basal medium.

Enumeration of yeasts and molds from foods. Table 5 shows counts of yeasts and molds obtained when several food samples were plated on DRBC and RBC. DC2 and APDA were included for comparison. Colony development of various molds was partially inhibited by 50 μg of rose bengal per ml, especially at pH 5.6. Average mold counts on RBC were lower than on DRBC at pH 5.6, but similar at pH 7.2. In the presence of 25 μg of rose bengal per ml (DRBC), this inhibitory effect was less marked. Counts of yeasts were lower at pH 5.6, but mold counts were unaffected by pH in DRBC.

Figure 1 shows the effectiveness of DRBC as

TABLE 1. *Screening of antifungal compounds for selective inhibition of R. stolonifer*

Compound	Concn tested (μg/ml)		Minimum inhibitory concn (μg/ml) for:	
	Highest	Lowest	*R. stolonifer*	*Asperigillus* and *Penicillium* spp.[a]
Benlate	100	2.5	50	<2.5
Dichloran	500	1.25	2.5	12.5
Difolatan	500	0.1	2	<.1
Diphenyl	5,000	125	1,250	1,250
Mancozeb	500	12.5	12.5	12.5
Thiabendazole	100	2.5	50	<2.5
Thiram 80	500	1.25	1.25	5
Zineb	500	12.5	250	125
Phenol	10,000	250	500	500
Pentachlorophenol	25[b]	1	2	5
4-Cinnamylphenol	100[b]	5	10	25
Cinnamic acid	1,000	25	250	500
Sodium pentachlorophenate	500	1.25	6	12.5
Pentachloronitrobenzene	100[b]	5	<5	50
Sodium 4-hydroxybenzoate	1,000	25	>1,000	>1,000
N-propyl-4-hydroxybenzoate	1,000	25	100	100
Salicilic acid	1,000	25	500	500
Benzene sulfonic acid	1,000	25	>1,000	>1,000
Gentian violet	100	2.5	5	10
Malachite green	1,000	0.63	12.5	25
Mercurochrome	100	2.5	>100	>100
Rose bengal	500	12.5	250	500
Cadmium chloride	100	25	100	25
Cobalt chloride	100	25	100	<50
Cupric sulfate	1,000	25	100	500
Dipotassium chromate	1,000	25	100	100
Sodium acetate[c]	5,000	125	1,250	1,250
Sodium benzoate[c]	1,000	25	1,000	>1,000
Sodium lactate[c]	5,000	125	>5,000	>5,000
Sodium propionate[c]	1,000	25	>1,000	>1,000
Potassium sorbate[c]	1,000	25	>1,000	>1,000

[a] *A. flavus, P. expansum,* and *P. islandicum.*

[b] Five concentrations of compound were tested in the range indicated; otherwise, six concentrations or more were tested.

[c] Acidified medium (pH 3.5).

TABLE 2. *Effect of dichloran concentration on R. stolonifer colony numbers after 2 days*

Estimated *Rhizopus* spores per plate	Counts in presence of dichloran (μg/ml):				Counts on RBC
	5	2.5	1.25	0	
1,000	0	0	384	NC[a]	NC
100	0	0	55	NC	40
50	0	1	21	NC	12
10	0	0	6	2	4

[a] NC, Plate completely overgrown and not countable.

TABLE 3. *Interaction of dichloran and rose bengal on mold growth: average mold count*[a]

Dichloran concn (μg/ml)	Avg mold count with rose bengal (μg/ml):			
	50	25	10	0
3	25	29	32	28
2	22	36	28	
1	18	27	27	
0	24[b]			

[a] Two samples of peanuts and one sample of sultanas.

[b] RBC medium.

an inhibitor of *Rhizopus* and *Mucor* species in comparison with other media. The effect of pH is also demonstrated.

Spot inoculation of test media. To determine whether the test media would allow adequate colony development of food spoilage molds while controlling rapidly spreading fungi, plates were spot inoculated with fungi repre-

senting 14 genera (Table 6). Little variation was observed in colony diameters of *Aspergillus* and *Penicillium* spp. on the seven media tested; RBC at pH 5.6 was the most inhibitory. Most other genera studied showed a wider range of growth patterns. The *Botrytis* sp. did not grow in RBC at pH 5.6, and *Wallemia sebi* did not grow either on RBC at pH 5.6 or on APDA. Growth was generally better at pH 7.2 than at pH 5.6, but the higher-pH medium did not adequately control the spreading fungi. DRBC at pH 5.6, the medium of choice, provided satisfactory selective inhibition of all the spreading fungi tested, except *Trichoderma viride*, while permitting growth of all other genera and species examined.

DISCUSSION

A survey of antifungal compounds for selective inhibition of *Mucorales* showed dichloran to be the best choice. Dichloran alone failed to inhibit spreading of some fungi, but reduced aerial growth. The combination of dichloran and rose bengal in a fungal enumeration medium gave the most satisfactory recovery of molds from foods, both in number and in variety of species isolated.

The influence of pH in enumeration media was investigated when a discrepancy between the observed pH and expected pH of Jarvis RBC medium (2) was discovered. RBC as originally formulated had a final pH of 5.6 rather than 7.2 as quoted by Jarvis. Jarvis (personal communication) has confirmed that the original formulation should have indicated the use of K_2HPO_4,

TABLE 4. *Average Rhizopus and Mucor colony diameter with dichloran and rose bengal*[a]

Dichloran concn (μg/ml)	Avg colony diam (mm) with rose bengal (μg/ml):			
	50	25	10	0
3	5	14	45	90
2	20	11	24	
1	12	23	30	
0	63[b]			

[a] One sample of peanuts and one sample of sultanas. BC medium.

TABLE 5. *Comparison of media for counts of molds or yeasts per gram from foods*

Medium	pH	Mold count[a]	Yeast count[b]
RBC	5.6	70	1
	7.2	94	50
DRBC	5.6	102	45
	7.2	95	76
DC2	5.6	114	83
APDA	3.5	59	68

[a] Average of six foods: black pepper, white pepper, sage, spice powder, wheat, and minced meat.

[b] Average of four foods: raisins, dried currants, grapes (two samples).

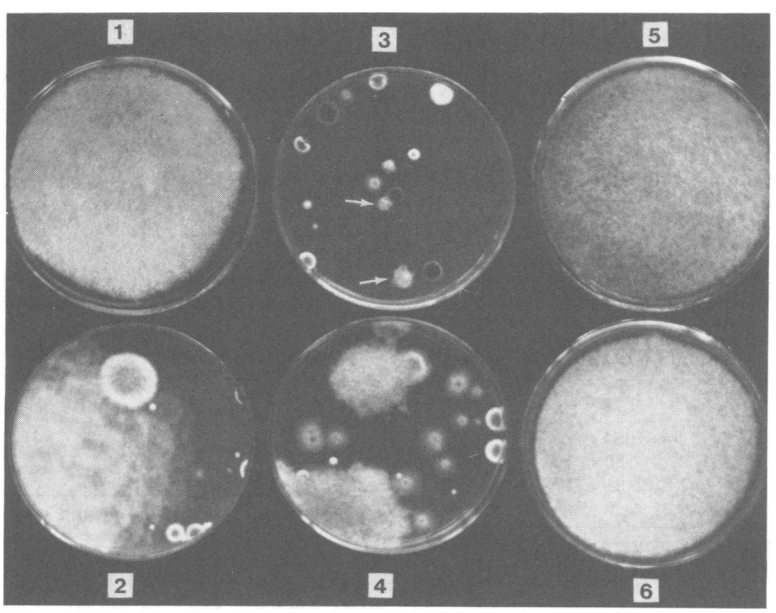

FIG. 1. *Comparison of mold growth on plates inoculated with raw peanut washings: 1, APDA; 2, DC2 (pH 5.6); 3, DRBC (pH 5.6); 4, DRBC (pH 7.2); 5, RBC (pH 5.6); 6, RBC (pH 7.2); Arrows on plate 3 indicate colonies of R. stolonifer.*

TABLE 6. *Average colony diameters of cultures on test media*[a]

Organism	Avg colony diam (mm) on:						
	RBC		DRBC		DC2 (pH 5.6)	Basal (pH 5.6)	APDA (pH 3.5)
	pH 5.6	pH 7.2	pH 5.6	pH 7.2			
Aspergillus (11 species)	12 (4–23)[b]	18 (10–27)	13 (6–22)	15 (6–24)	16 (6–26)	18 (5–28)	17 (4–33)
Penicillium (18 species)	11 (3–17)	16 (10–27)	12 (8–19)	13 (8–16)	16 (8–22)	20 (10–26)	19 (8–30)
Other genera							
Wallemia sebi FRR 1471	0	5	4	ND[c]	4	4	0
Cladosporium sp. 56-1	11	21	12	ND	13	19	16
Epicoccum nigrum FRR 1986	11	26	14	ND	24	25	22
Helminthosporium sp. 26-10	3	15	6	ND	15	17	>12
Botrytis sp. 26-8	0	38	5	14	20	46	48
Fusarium sp. 71-4	16	53	18	ND	42	49	35
Byssochlamys fulva FRR 4	17	27	19	ND	40	50	42
Paecilomyces varioti FRR 555	18	30	16	26	36	ND	36
Mucor circinelloides FRR 2109	>70	>70	19	40	>70	>70	>70
Mucor racemosus FRR 1988	15	42	16	ND	>70	>70	>70
Rhizopus arrhizus FRR 2062	40	>70	16	32	>70	>70	>70
Rhizopus arrhizus FRR 2099	>70	95	6	8	>70	>70	>70
Syncephalastrum racemosum FRR 1155	>70	>70	10	24	>70	>70	>70
Trichoderma viride FRR 57	52	>90	64	>90	ND	ND	ND

[a] Average of duplicate colonies.
[b] Data in parentheses are ranges.
[c] ND, Not determined.

not KH_2PO_4. The higher-pH media allowed faster growth of food spoilage molds but did not inhibit spreading molds as effectively as did those at pH 5.6. Rose bengal was found to be more inhibitory at the lower pH, and this had a marked effect on yeast counts when the rose bengal concentration was 50 μg/ml.

Bell and Crawford (1) proposed a medium with 25 μg of rose bengal per ml and 5 or 10 μg of dichloran per ml for the isolation of *A. flavus*. Their medium did not inhibit *A. flavus* but restricted growth of other fungi (1). RBC medium (2) is somewhat inhibitory to molds because of the higher concentration of rose bengal (50 μg/ml) and suffers from occasional overgrowth of spreading fungi. For the dual purpose of enumeration and identification of molds in foods, colony diameters around 10 mm are desirable. This rate of growth was most effectively achieved with DRBC medium, pH 5.6 (Table 6). DRBC gave an acceptable balance between growth and inhibition of the molds commonly present in a wide range of foods.

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