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Dichloran as an Inhibitor of Mold Spreading in Fungal Plating Media: Effects on Colony Diameter and Enumeration

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Problems associated with overgrowth by spreading molds are not addressed by currently recommended fungal enumeration media. Twenty-two fungi, including 12 mold and 3 yeast genera, were evaluated for the effects of dichloran (2,6-dichloro-4-nitroaniline), previously identified as a mold-spreading inhibitor, on colony diameter and enumeration. On malt agar or antibiotic-potato-dextrose agar (APDA), colony diameters were effectively reduced when dichloran was added. Colony diameters decreased as the dichloran concentration increased. Counts obtained with mixed mold spore suspensions were lower on APDA supplemented with 25 μg of dichloran per ml than on APDA and were higher than APDA with the addition of 5 μg of dichloran per ml (APDA-D-5). Overall counts of mixed and individual mold spore and yeast suspensions were higher in APDA-D-5 than in APDA. The additional advantages of APDA-D-5 may be useful in routine enumeration of fungi.

Selective agents, such as acidulants, dyes, or antibiotics, have been in use for many years in the isolation and enumeration of fungi to repress the bacterial growth normally associated with various samples, including foods (5, 13, 24). Acidulants and antibiotics are recommended additives to fungus enumeration media (1, 11), and a dye-antibiotic medium is recommended as an alternate medium in the isolation of fungi from foods (1).

Acidified fungus enumeration media have several inherent problems, such as spreading mold colonies (8), occasional bacterial growth (2), inhibition of sublethally injured fungi (9, 17), and precipitation of sample constituents (16). Several studies show that antibiotic-supplemented media are superior to acidified media in the enumeration of yeasts and molds from foods (3, 10). The increased pH of the antibiotic-supplemented media allows increased recovery of injured or stressed fungi (9, 12, 17, 20), which are of special concern in processed foods.

Various dyes, particularly rose bengal, control problems caused by spreading molds (7, 8, 18-21, 23), but may not inhibit bacteria sufficiently (15) or may inhibit colony formation by yeasts from food samples (15). In addition, rose bengal is incorporated into colonies of yeasts and molds (19) and, although assisting in the enumeration of small colonies, may require subculturing before identification can be made (15). Dichloran (2,6-dichloro-4-nitroaniline), used alone (6, 8) and in combination with rose bengal (2, 8), was found to effectively restrict mold colony diameters. King et al. (8) demonstrated that a medium containing 2 μg of dichloran per ml and 25 μg of rose bengal per ml is effective in reducing the colony diameters of various molds. Counts obtained with the medium used by King et al. for several food samples are generally greater than on acidified potato-dextrose agar. Dichloran alone (2 μg/ml) exhibits only limited control of mold spreading in their medium (8). In addition, dichloran aids in the recognition and identification of mycotoxic species of *Aspergillus* and *Penicillium* (14).

In this study, the effects of dichloran on colony diameter and enumeration of various fungi were investigated. The efficacy of dichloran was also evaluated in a standard fungus enumeration medium (antibiotic-potato-dextrose agar) for pure and mixed cultures of isolated fungi and with many food samples.

(A preliminary report of this study has been presented [O. E. Henson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, P9, p. 197].)

MATERIALS AND METHODS

Cultures. The fungi used in this study were: *Rhizopus oligosporus* NRRL 2710, *Kluyveromyces fragilis* NRRLY 1156, *Candida albicans* ATCC 753, *Saccharomyces cerevisiae* NRRLY 9763, *Fusarium graminearum* NRRL 5883, *Aspergillus oryzae* NRRL 2217, and *Trichoderma reesei* QM 9414. The following environmental isolates used in this study were: *Aspergillus niger*, *Aspergillus ochraceus*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Penicillium* sp., *Penicil-*

lium roqueforti, *Fusarium* sp., *Verticillium* sp., *Monascus* sp., *Neurospora* sp., *Mucor* sp., *Alternaria* sp., *Paecilomyces* sp., *Nigrospora* sp., and *Candida utilis*. Cultures were maintained on malt or Sabouraud-dextrose agar slants or plates at 0 to 4°C. Mold spore suspensions were prepared by lightly scraping spores off 4- to 5-day agar plate cultures into a 0.1% Tween 80 solution. Yeast suspensions were prepared from malt or Trypticase soy broth (BBL Microbiology Systems) cultures by suspending twice-washed cells in phosphate buffer (0.025 M).

Culture media. Malt agar (MA) and antibiotic-potato-dextrose agar (APDA) were prepared according to manufacturer's instructions (Difco Laboratories). Dichloran (Aldrich Chemical Co.) was prepared as a stock solution dissolved in methanol and added at the appropriate concentration to MA or APDA before autoclaving.

Colony diameter determination. MA or APDA was spot inoculated with a spore or yeast suspension or from a 4- to 5-day MA colony. Plates were incubated at 25 to 30°C for 2 to 5 days, and colony diameters were measured with a caliper. All colony diameter measurements were made after growth for 5 days at 25 to 30°C, except for *Neurospora* sp. (2 days), *Mucor* sp. (3 days), *Nigrospora* sp. (4 days), *R. oligosporus* (3 days), and *T. reesei* (3 days). Measurements were made early with these molds to prevent complete overgrowth of the control plate. All colony diameter determinations were performed in duplicate.

Enumeration. Replicate mold spore and yeast suspensions were enumerated in duplicate by standard methods (1), using the control (APDA) or dichloran-supplemented medium. Data were statistically analyzed by Student's *t* test (4, 22).

Evaluation of food samples. Eighty-eight food samples, representing grains, grain products, dairy products, spices, condiments, fruits, vegetables, bread products, meats, and miscellaneous food ingredients were enumerated in duplicate by standard methods (1), using APDA and APDA containing 5 μg of dichloran per ml. Data were statistically analyzed by Student's *t* test (4, 22).

Experimental mold spore suspensions. The mixed-spore suspensions used for the comparative recovery from various dichloran-containing media had the following compositions: (A) *A. niger*, *A. oryzae*, *A. ochraceus*, *A. flavus*, and *A. fumigatus*; (B) *A. niger*, *A. fumigatus*, *Penicillium* sp., *T. reesei*, *Neurospora* sp., and *Mucor* sp.; (C) *Paecilomyces* sp., *Alternaria* sp., *A. fumigatus*, *Pencillium* sp., and *Fusarium* sp.; (D) *Nigrospora* sp., *F. graminearum*, *T. reesei*, and *Neurospora* sp.; and (E) *Neurospora* sp., *Mucor* sp., and *T. reesei*.

RESULTS

Effect of dichloran on colony diameter of various fungi. Colony diameter results of fungus spot-inoculated onto dichloran-containing media are shown in Table 1. Diameters are recorded as the percentage of the control, with

TABLE 1. *Mean colony diameter expressed as a percentage of the control of fungi grown on dichloran-supplemented media*

Culture	Colony diam (% control)							
	MA + dichloran						APDA + dichloran	
	1[a]	5	10	25	50	100	5	10
Aspergillus niger	81.1	44.6	14.6	13.6	11.9	9.3	34.7	10.0
A. ochraceus	90.9	88.1	64.9	51.7	48.7	48.3	84.3	55.9
A. fumigatus	94.1	42.4	26.3	10.9	10.2	11.5	30.1	13.0
A. flavus	81.9	58.4	49.4	41.1	40.5	40.8	64.8	47.2
A. oryzae	87.7	59.8	39.6	27.0	26.2	16.4	58.0	33.4
Penicillium sp.	84.7	34.3	21.5	10.2	8.9	9.1	31.0	33.2
P. roqueforti	86.9	83.9	15.4	7.4	7.9	6.5	58.2	19.2
Fusarium sp.	90.8	89.8	57.4	26.8	25.0	18.2	59.1	42.9
F. graminearum	NT[b]	78.8	NT	NT	NT	NT	85.6	32.2
Verticillium sp.	98.7	71.4	82.7	59.3	54.0	61.3	60.5	55.6
Monascus sp.	116.4	72.6	60.4	47.1	55.0	5.0	77.4	62.7
Neurospora sp.	68.9	46.7	8.6	3.4	3.6	3.8	43.9	3.6
Mucor sp.	99.2	40.6	68.1	38.6	28.0	25.3	22.9	20.3
Alternia sp.	86.2	41.7	14.8	9.0	6.9	8.5	55.4	7.2
Paecilomyces sp.	62.1	56.6	21.8	20.9	13.1	6.7	46.6	18.4
Rhizopus oligosporus	NT	21.0	17.5	NT	NT	NT	35.9	16.8
Trichoderma reesei	100.3	60.5	41.9	15.5	13.4	18.0	54.4	37.2
Nigrospora sp.	NT	100.1	NT	NT	NT	NT	114.6	NT
Kluyveromyces fragilis	103.2	90.2	90.3	85.5	75.8	74.2	100.0	NT
Saccharomyces cerevisiae	103.5	98.7	101.8	100.0	101.8	79.0	85.9	NT
Candida utilis	86.1	97.5	65.1	81.4	74.4	79.1	94.9	NT
C. albicans	98.2	98.6	91.1	96.4	91.1	78.6	108.7	NT

[a] Dichloran concentration (micrograms per milliliter).
[b] NT, Not tested.

the control being either MA or APDA without dichloran. Methanol, the diluent for the dichloran, had no effect on fungal colony diameter (unpublished data). Dichloran had obvious effects on colony diameters of all molds tested, which increased as the dichloran concentration increased. Average mold colony diameter reductions on MA or APDA were 13, 41, 66, 74, 78, or 81% at 1, 5, 10, 25, 50, or 100 μg of dichloran per ml, respectively. Yeast colony diameter was much less affected, with reductions of 1.3 to 9.8% at 5 μg of dichloran per ml on MA (Table 1). At 5 μg of dichloran per ml, colony morphology and identification capability were unaffected. Higher dichloran concentrations made culture identification difficult without subculturing. Diameter reductions of mold grown on dichloran-supplemented MA were similar to those obtained on APDA. The common contaminating spreading molds, *Neurospora*, *Mucor*, *Paecilomyces*, *Rhizopus*, and *Trichoderma* spp., were reduced 56, 77, 53, 64, and 46%, respectively, on APDA containing 5 μg of dichloran per ml (APDA-D-5).

Recovery of individual spore suspensions on APDA and APDA-D-5. The recovery of individual yeast and spore suspensions on APDA and APDA-D-5 is shown in Table 2. Significantly higher counts ($P \leq 0.05$) were observed on the APDA-D-5 medium for cultures of *Aspergillus flavus*, *Fusarium* sp., *Penicillium roqueforti*, *A. ochraceus*, *Paecilomyces* sp., and *T. reesei*. A significant ($P \leq 0.05$) improvement was also observed in counts obtained on APDA-D-5 versus APDA when the combined results of all molds tested were compared.

TABLE 2. *Yeast or fungal spore recovery of suspensions plated on APDA and APDA-D-5*

Suspension	Mean $\log_{10}$[a] counts/ml	
	APDA	APDA-D-5[c]
Aspergillus niger	6.28	6.60
A. ochraceus[b]	5.89	6.13
A. fumigatus	6.50	6.86
A. flavus	5.52	6.52
A. oryzae	4.65	4.52
Penicillium sp.	5.64	5.83
P. roqueforti[b]	5.47	6.13
Fusarium sp.[b]	6.56	6.78
F. graminearum	3.40	3.39
Neurospora sp.	5.14	5.62
Mucor sp.	7.13	7.08
Alternaria sp.	2.96	3.03
Paecilomyces sp.[b]	6.51	6.92
Trichoderma reesei[b]	5.71	6.29
Nigrospora sp.	3.97	3.58
Kluyveromyces fragilis	6.70	6.59
Saccharomyces cerevisiae	4.95	5.24
Candida albicans	4.69	4.61
C. utilis	6.72	6.71

[a] Represents the mean of replicate counts performed in duplicate.

[b] Significant increase ($P \leq 0.05$) in recovery on APDA-D-5 versus unsupplemented APDA.

[c] Total recovery on APDA-D-5 was statistically greater ($P \leq 0.05$) than on APDA.

Recovery of mixed-spore suspensions on APDA and APDA plus various concentrations of dichloran. Table 3 shows the recovery of five separate mixed-spore suspensions on APDA supplemented with various concentrations of dichloran. Overall, a significant increase in counts ($P \leq 0.05$) was observed with APDA-D-5, whereas a significant decrease in counts ($P \leq 0.05$) was observed at a higher concentration of dichloran (25 μg/ml).

Recovery of yeasts and molds from various food samples plated on APDA and APDA-D-5. The results for the analysis of 88 food samples, using APDA and APDA-D-5, are shown in Table 4. No significant ($P \leq 0.05$) differences in counts between the two media for any food group or overall were observed. A general tendency was evident that counts at low contamination levels were slightly lower on APDA-D-5 than on APDA. This can be explained by the variability in counts of these samples in which a difference in one colony per plate can signify a large difference in count. Overall, both media allowed equivalent recovery of fungi from foods. In addition, colonies on APDA-D-5 were smaller, which aided in the isolation and identification of molds present in the foods.

DISCUSSION

Because of the results of dichloran when used with rose bengal as a mold colony diameter

TABLE 3. *Recovery of mixed-spore suspensions on APDA and APDA containing various concentrations of dichloran*

Spore suspension[a]	Mean $\log^{b}_{10}$ counts/ml			
	0[c]	5[c,d]	10[c]	25[c,e]
A	5.72	5.82	5.58	5.64
B	6.18	6.43	6.00	4.78
C	5.96	6.51	5.97	6.04
D	6.30	6.48	5.65	5.72
E	5.53	5.93	5.63	4.92

[a] For spore suspension compositions, see the text.

[b] Represents the mean of replicate counts performed in duplicate.

[c] Dichloran concentration (micrograms per milliliter).

[d] Significant increase ($P \leq 0.05$) in recovery over unsupplemented APDA.

[e] Significant decrease ($P \leq 0.05$) in recovery over unsupplemented APDA.

TABLE 4. *Comparison of overall fungus recovery from food samples plated on APDA and APDA-D-5*

Sample	No. of samples	Mean log[a]10 counts/ml	
		APDA	APDA-D-5
Grain products	27	2.15	2.08
Dairy products	10	1.00	0.91
Spices and condiments	27	0.62	0.49
Vegetables and fruits	6	3.26	3.31
Bread products	7	0.48	0.33
Meats	6	4.56	4.54
Miscellaneous ingredients	5	2.91	2.90
Total	88	1.70	1.62[b]

[a] Represents the mean of duplicate counts performed on all products within the group.

[b] Not significantly different ($P \leq 0.05$) from the APDA counts.

inhibitor in previous studies (2, 8, 14), the effects of various concentrations of dichloran used alone on several fungi in selective and nonselective media were investigated. The greatest colony diameter inhibition occurred at high dichloran concentrations (Table 1), but a reduction in spore recovery at these levels was observed (Table 3). Dichloran was also an effective mold colony diameter inhibitor in MA, but the nonselectivity of MA excludes it from use in routine analysis of naturally contaminated samples. Because of the observed colony diameter reduction and increased spore recovery with dichloran at the 5-μg/ml level, this concentration was chosen for optimum use in enumeration media.

Dichloran-supplemented APDA has all of the previously established advantages of APDA, including selectivity (3), increased pH which allows recovery of stressed fungi (17), and the additional advantages of reduction of colony diameter, allowing greater ease and accuracy in enumeration and increased recovery of mold spores. Yeast colony diameters and enumeration were unaffected by dichloran.

The suitability of APDA-D-5 for use in fungus enumeration of naturally contaminated foods (Table 4) may reduce problems associated with the overgrowth of plates by spreading molds. APDA-D-5 may be especially suited to grain products because of the need to isolate and identify molds from grains. The reduced colony size on APDA-D-5 allows greater ease of mold isolation from foods. The use of dichloran in APDA is also important because of the use of APDA in standard fungus enumeration procedures (1, 11) and because of the wide acceptance of APDA for routine analysis of food products.

The significantly increased recovery of both mixed- and individual mold spore suspensions was somewhat surprising, although King et al. (8) reported slightly increased counts from foods with dichloran-containing media. A study in this laboratory with standardized low-spore inocula on APDA and APDA-D-5 indicated that increased counts were a result of improved counting efficiency owing to less interfering overgrowth by spreading molds (unpublished data). This improved ability to accurately count mold colonies on plates containing dichloran may be helpful in routine enumeration of molds from several sources.

A current standard method (11) recommends that counts for fungus enumeration plates be made after 3 and 5 days. This method also recognizes the common presence of spreading molds and suggests counting the reverse sides of overgrown plates. APDA-D-5 has been shown to effectively limit the colony diameter of several molds, including spreading molds, while allowing equivalent or overall increased recovery of mold spores in pure or mixed culture. This medium, besides having all of the advantages of APDA, would reduce or eliminate problems associated with spreaders. In addition, APDA-D-5 is similar to APDA in cost and could be readily utilized by any laboratory currently using APDA. A collaborative study evaluating and comparing the performance of APDA-D-5 with other fungus enumeration media will be reported elsewhere.

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