Mycopathologia **125**: 157–162, 1994.
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Comparison of four media for the isolation of *Aspergillus flavus* group fungi

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Received 19 August 1993; accepted in revised form 20 October 1993

Abstract. Four agar media used to isolate aflatoxin producing fungi were compared for utility in isolating fungi in the *Aspergillus flavus* group from agricultural soils collected in 15 fields and four states in the southern United States. The four media were *Aspergillus flavus* and *parasiticus* Agar (AFPA, 14), the rose bengal agar described by Bell and Crawford (BCRB; 3), a modified rose bengal agar (M-RB), and Czapek's-Dox Agar supplemented with the antibiotics in BC-RB (CZ-RB). M-RB was the most useful for studying the population biology of this group because it permitted both identification of the greatest number of *A. flavus* group strains and growth of the fewest competing fungi. M-RB supported an average of 12% more *A. flavus* group colonies than the original rose bengal medium while reducing the number of mucorales colonies and the number of total fungi by 99% and 70%, respectively. M-RB was successfully employed to isolate all three aflatoxin producing species, *A. flavus*, *A. parasiticus* and *A. nomius*, and both the S and L strains of *A. flavus*. M-RB is a defined medium without complex nitrogen and carbon sources (e.g. peptone and yeast extract) present in BC-RB. M-RB should be useful for studies on the population biology of the *A. flavus* group.

Key words: Aflatoxins, *Aspergillus parasiticus*, *Aspergillus tamarii*, *Aspergillus nomius*, soil, fungal populations

Abbreviations: M-RB = Modified Rose Bengal Agar; CZ-RB = Czapeks Rose Bengal Agar; BC-RB = Bell and Crawford's Rose Bengal Agar; AFPA = *Aspergillus flavus* and *parasiticus* agar

Introduction

Fungi in the *Aspergillus flavus* group have long attracted interest due to their ubiquity and prevalence in many ecological niches, their use in industry and traditional food processing and capacity to produce an array of toxins [1]. The aflatoxins are the best known toxins produced by the *A. flavus* group [2]. These compounds are both highly toxic and carcinogenic and can have serious effects on human and domestic animal health when they contaminate foods and feeds [2, 3]. In many countries, these health concerns have fostered strict regulations on the aflatoxin content of agricultural commodities [4].

The economic and social importance of aflatoxins has led to increased interest in the study of populations of aflatoxin producing fungi [5]. Several different *A. flavus* strains have been identified and it has been suggested that strain incidence influences contamination severity [4, 6]. Other studies have suggested the use of atoxigenic *A. flavus* strains to competitively exclude toxigenic strains and thereby alter the toxigenicity of *A. flavus* populations and thus reduce the incidence of contamination [7–9]. These studies have

generated many questions on *A. flavus* group populations; to address these questions, the physiological and genetic characters of numerous *A. flavus* isolates must be examined, and the distribution of such characters determined.

There are several media useful for isolating *A. flavus* strains [5, 10, 11]. During the course of utilizing selected media to examine *A. flavus* populations, we have encountered occasional plant or soil samples for which the resident microbial population interfered with adequate enumeration and isolation of *A. flavus*. On these occasions, we empirically modified the rose bengal (BC-RB) medium of Bell & Crawford [10] to permit analysis of *A. flavus* populations in these difficult samples. This communication describes the relative effectiveness of the media (M-RB and CZ-RB) we and our cooperators use most often and compares these media to both the original medium (BC-RB) and to Pitt's AFPA [11].

Materials and methods

Media preparation. Four media (BC-RB, AFPA, Rose Bengal Czapek's Medium and Modified Rose Bengal) were evaluated in the tests described here; the latter two were recently developed in our laboratory. Rose Bengal Czapek's Medium (CZ-RB) was described previously [5]; it is merely the antibiotics (dichloran, streptomycin and rose bengal) used in BC-RB added to Czapek's-Dox broth (Difco) with 2% Bacto agar (Difco). Modified Rose Bengal (MRB) is a defined medium containing the following per liter: 3.0 g sucrose, 3.0 g $NaNO_3$, 0.3 g KH_2PO_4, 0.7 g K_2HPO_4, 0.5 g $MgSO_4$ $7H_2O$, 0.5 g KCl, 10.0 g NaCl, 20 g Bacto agar, 50 mg Chloramphenicol, the micronutrients of Adye & Mateles [12], and the antibiotics contained in BC-RB [10]. The medium is made by combining the sucrose, inorganic salts, and micronutrients with 5 ml rose bengal stock in an appropriate volume of deionized water and adjusting the pH to 6.5. The mixture is then dispensed into media bottles, the agar is added and the mixture is brought to a boil, at this point the solution is stirred until the agar is melted and the Chloramphenicol is added. After autoclaving for 15 min at 120 °C, the medium is cooled on a stir plate to between 50 and 60 °C; at this point the dichloran (10 mg/L) and streptomycin (50 mg/L) are added and after stirring for 5 to 10 min the medium is poured (15–20 ml per 100 mm plate).

The micronutrients of Adye and Mateles consist of the following per liter: 0.7 mg $Na_2B_4O_7$ $10H_2O$ 0.5 mg $(NH_4)_6MO_7O_{24}$ $4H_2O$, 10 mg $Fe_2(SO_4)_3$ $6H_2O$, 0.3 mg $CuSO_4$ $5H_2O$. 0.11 mg $MnSO_4$ H_2O, 17.5 mg $ZnSO_4$ $7H_2O$. One ml of a stock solution (1,000×) was added to each liter of medium before autoclaving. Concentrated micronutrients were solubilized by acidifying the stock with HCl to about pH 2.0. Dichloran stock solution consisted of 250 mg technical dichloran (96.5%, NOR-AM Chemical Co., Wilmington, DE) dissolved in 20 ml acetone and brought to volume in a 250 ml volumetric flask with 95% acetone. Rose Bengal Stock consisted of 500 mg Rose Bengal moistened with 30 ml 95% ethanol and brought up to volume in a 100 ml volumetric flask with distilled water. Streptomycin stock consisted of 1.0 g streptomycin sulfate in 100 ml distilled water; this stock was filter sterilized before use. Chloramphenicol stock consisted of 2.5 g Chloramphenicol dissolved in 95% ethanol and brought up to volume with 95% ethanol in a 100 ml volumetric flask. The streptomycin and the micronutrient stocks were kept at −10 to −15 °C until use. The original RB and AFPA media were made as described previously [10, 11]. Both these media contain complex nitrogen and carbon sources (e.g. peptone and yeast extract).

Soils. Soils from agricultural fields were collected during the summers of 1989, 1990 and 1991. All fields were planted to either corn or cotton at the time of sampling. Soils were collected dry from the top 2 cm of the soil profile. Each sample was a composite of ten subsamples taken from locations at least 15 feet apart. Field locations are indicated in Table 1.

Table 1. Population levels of *Aspergillus flavus* group fungi detected in diverse soils with various isolation media and concentrations of Mucorales and total fungi detected by those media at soil dilutions optimal for isolation of *A. flavus*

		A. flavus group (propogules/g)				Mucorales (propogules/g)				Total fungi (propogules/g)			
Field	State	M-RB	CZ-RB	BC-RB	AFPA	M-RB	CZ-RB	BC-RB	AFPA	M-RB	CZ-RB	BC-RB	AFPA
1	Alabama	332 a	204 a	409 a	307 a	9 b	51 b	255 a	0 b	3781 d	9946 b	12432 a	7119 c
2	Arizona	12 a	0 b	7 a	0 b	0 c	0 c	46 a	7 b	1124 b	1506 ab	1760 a	1936 a
3		5 a	0 a	5 a	0 a	0 a	5 a	55 a	0 a	1420 a	3560 a	3300 a	2730 a
4		318 a	413 a	461 a	318 a	0 a	0 a	0 a	0 a	588 c	3435 b	4771 b	7475 a
5		447 ab	563 a	447 ab	298 b	0 a	0 a	33 a	17 a	2217 b	6883 ab	7214 ab	10589 a
6		297 a	363 a	364 a	429 a	0 a	0 a	17 a	17 a	6011 b	8719 ab	10502 a	5746 b
7		338 a	411 a	484 a	338 a	0 a	0 a	73 a	97 a	1596 c	12379 b	23791 a	13023 b
8		107 a	47 a	107 a	142 a	0 b	95 a	214 a	71 a	2800 b	7546 a	5980 a	6692 a
9		499 a	998 a	458 a	874 a	0 b	499 a	707 a	41 b	2537 b	20300 a	18968 a	20466 a
10		2046 a	1675 ab	1369 b	1949 ab	0 c	419 ab	1160 a	16 bc	5348 b	10954 a	13402 a	17139 a
11		4934 a	2075 b	3669 b	2657 b	0 b	0 b	76 ab	228 a	8603 b	34615 a	34818 a	27733 a
12	Arkansas	49 a	28 a	33 a	0 b	0 a	0 a	0 a	0 a	1972 c	4931 a	3402 b	3172 bc
13	Mississippi	6 a	8 a	6 a	15 a	0 b	4 b	158 a	4 b	1117 c	3369 a	2643 ab	2160 b
14		41 a	21 a	36 a	0 a	0 b	10 b	128 a	0 b	1929 b	4844 a	3961 a	5973 a
15		3 a	3 a	5 a	3 a	3 b	5 b	71 a	34 a	862 b	1488 a	1222 a	1141 ab
Average		629	454	559	489	1	73	200	91	2794	8965	9878	8873

Media tested are described in detail in the text. M-RB - Modified Rose Bengal, CZ-RB = Czapek's Rose Bengal, BC-RB = Bell and Crawford's Rose Bengal, and AFPA = *Aspergillus flavus* and *parasiticus* agar. Optimal dilutions contained less than 250 propogules per milliliter.
Values are means of 4 replicates. Values for the same field and fungal group followed by the same letter do not differ significantly by the Man-Whitney U test.

Media comparisons. Soil was suspended in sterile water (50 g in 100 ml), stirred rapidly for 10 to 15 min, and the suspension was diluted in a ten-fold series (0 to 10^{-3}). Each dilution (0.1 ml) was spread on M-RB and the quantity of *A. flavus* group fungi in each soil was determined by colony counts after 3.5 days incubation at 30 to 31 °C. Subsequently, each soil was again assayed on each medium at a dilution considered optimal for isolation of *A. flavus*. These dilutions contained 10 to 250 propagules per ml. Media comparisons were replicated 4 to 5 times. On all rose bengal media, colonies were identified by characteristic growth pattern, retention of rose bengal in the mycelium and by characteristic production of green conidia after 3.5 days. On AFPA *A. flavus* and *A. parasiticus* isolates were identified by the pigmentation of colony undersides after 2 days, as previously described [11]. The number of fungal strains in the Mucorales growing on the various media and the total number of fungi were determined based on colony morphology.

To confirm that fungal strains isolated on M-RB were members of the *A. flavus* group, forty isolations were made from one soil from each state (4 fields, 160 isolates). Colony morphology, conidial characteristics, and toxin production were used to determine the *A. flavus* group species (*A. tamarii*, *A. flavus*, *A. nomius* or *A. parasiticus*) and if *A. flavus* the *A. flavus* strain (strain S or strain L) as previously described [12–15].

Statistical analysis. Statistical calculations were performed with ABSTAT, (Anderson-Bell, Parker, Colorado). Significant differences among media in detection of propagules of the various fungal groups were determined with the Mann-Whitney U test.

Results and discussion

Of the four media compared, on average M-RB detected the most *A. flavus* group propagules and permitted growth of the fewest competing fungi (Table 1). By supporting fewer competitive fungi, M-RB reduced the incidence of potential contaminants of isolations and thus facilitated our population studies. All fungal strains (160 isolates from soil collected in four fields) isolated on M-RB were members of an *A. flavus* group species. In both M-RB and CZ-RB, complex nitrogen sources were replaced with nitrate as the sole nitrogen source. This was directed at members of the Mucorales which complicated isolations from certain soils. Counts of Mucorales averaged 99% lower on M-RB than on the original BC-RB (Table 1). Relatively low Mucorales counts also occurred on AFPA, this partly may be attributed to rapid growth of competing fungi on AFPA; rapid growth of competing fungi also made isolations from AFPA more difficult. AFPA was developed from Aspergillus Differentiation Medium [16] primarily for rapid detection and enumeration of *A. flavus* and *A. parasiticus* [11, 16]. However, this medium has been used to isolate *A. flavus* group fungi from soils and agricultural commodities [16].

Both Chloramphenicol and streptomycin were included in M-RB because alone, neither adequately restricted bacterial occupants of certain soils. Bacterial growth occasionally interfered with isolation of fungi from soil on BC-RB and CZ-RB (which contain only Streptomycin), in the current study. Bacterial growth in soil dilutions plated on AFPA, which contains only chloramphenicol, has previously been described [16].

Sodium chloride concentrations were not increased above 1% in M-RB because preliminary results agreed with previous researchers [10,17] that increased NaCl resulted in fewer and slower developing colonies of *A. flavus* on dilution plates. Increased NaCl concentrations did not interfere with isolations from infected seed, concentrations of 7.5% NaCl are routinely employed for isolation of *A. flavus* from infected corn kernels [18].

Fungal strains had to be transferred from all the rose-bengal media to permit reliable identification of both *A. flavus* group species and the S strain of *A. flavus*.

Several thousand isolates of *A. flavus* group fungi have been isolated in the author's laboratory from soils and agricultural commodities with

the M-RB medium [6]. These isolates include *A. tamarii*, *A. parasiticus*, *A. nomius* and both the S and L strains of *A. flavus*. About four hundred of these isolates were seeded onto AFPA to test the reliability of the AFPA color reaction for identifying *A. flavus* and *A. parasiticus*. All isolates of *A. flavus* and *A. parasiticus* tested produce the color reaction described by Pitt et al. [11] and the medium was very useful for positive confirmation of *A. tamarii* which produced numerous brown conidia and a brown reverse on this medium within 10 days. However, 5 of 6 aflatoxin producing isolates of *A. nomius* that were isolated on M-RB failed to produce the color reaction produced by *A. flavus* and *A. parasiticus* on AFPA. Similarly, the type strain of *A. nomius* (NRRL 13137) and three other isolates from the National Center for Agricultural Utilization Research in Peoria (NRRL 6552, NRRL 6107, NRRL 5919) failed to produce the characteristic color reaction. Thus AFPA was successful at detecting its targeted species but, it failed to detect all aflatoxin producing fungi. This may not be a problem for many users because, in our experience, *A. nomius* occurs far less frequently than either *A. flavus* or *A. parasiticus* in most locations. On AFPA most *A. flavus* group isolates did not sporulate at the time of plate reading. This was inconvenient for transferring the isolates and those working in our laboratory preferred to see conidial heads when evaluating colonies.

In this laboratory, the M-RB medium has been used to successfully isolate *A. flavus* group fungi from diverse soils ranging from those listed in Table 1 to soils collected in Asia. M-RB has also been useful in isolating diverse *A. flavus* group fungi from numerous cottonseed samples originating from ten cotton producing US states, as well as from brazil nuts, peanuts, pistachios, and corn. Reduced selection of strains within the *A. flavus* group by M-RB as compared to other isolation media and increased inhibition of fungi outside the *A. flavus* group make M-RB a useful tool for studies on the population biology of the *A. flavus* group.

Acknowledgments

The author thanks Dr SW Peterson for providing cultures from the Agricultural Research Service Culture Collection and Mrs DL Downey and Miss LA Fortune for technical assistance.

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