Relation of Aspergillus flavus Colony Growth on Three Selective Media to Recovery from Naturally Infested Soil

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ABSTRACT

Three selective media, inhibiting the colony growth of *Aspergillus flavus* isolates either slightly, moderately, or severely in pure culture, were evaluated for isolating low populations of *A. flavus* from naturally infested soil. Recovery of *A. flavus* by the dilution-plate technique was approximately the same on slightly inhibitory 3% NaCl-Botran-amended medium, and on moderately inhibitory 3% NaCl-rose bengal-amended medium. *A. flavus* colonies on dilution plates were typically larger on 3% NaCl-Botran medium. Recovery of *A. flavus* from naturally infested soil on severely inhibitory 10% NaCl-rose bengal-amended medium was similar to that on 3% NaCl-rose bengal medium, but *A. flavus* colonies were larger on dilution plates of the former due to less crowding from undesired fungi. In contrast, recovery of *A. flavus* from nonsterile soil artificially infested with conidia was greater on media less inhibitory to *A. flavus* growth in pure culture. Conidia were one of the principal propagules of *A. flavus* in naturally infested soil. Conidial germination by *A. flavus* in pure culture was 88-100% within 24 hours on all three media. Severe growth inhibition of the desired fungus on a selective medium may not lead to reduced recovery, on a relative basis, from naturally infested soil.

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Several tests may be performed to determine the effectiveness of selective media for the isolation of fungi from soil. As indicated by Tsao (16), no selective medium is perfect, and performance of these tests is essential for determining the relative value of selective media. Some studies concerned with the evaluation of selective media incorporate tests on recovery from artificially or naturally infested soils, or tests on pure culture colony growth by the desired organism. Complete or partial inhibition of undesired organisms is an objective in developing a selective medium, but usually some inhibition of growth or propagule germination of the desired fungus by the antimicrobial chemical agents in a selective medium occurs as well. Many selective media presently in use are known to be quite inhibitory to some aspects of colony development in pure cultures of the desired organism (2, 7, 10, 13, 16), and in some cases concentrations of inhibitory agents have been reduced or the agents replaced (3, 13, 17, 18). Often this has been done arbitrarily and there has been little or no investigation of whether this inhibition in pure culture may result in poor recovery of the desired organism from naturally infested soils. This paper examines that problem and reports on the relation of *A. flavus* colony growth on three selective media to recovery of low populations of *A. flavus* from naturally infested soil.

MATERIALS AND METHODS.—Some of the principal components of the low water potential, malt-salt agar medium [page 45 of reference (19)], and the Bell and Crawford (1) medium containing Botran (2,6-dicloro-4-nitroaniline) were tested in this study for their effect on colony growth and conidial germination of *A. flavus*. Initially, various concentrations of NaCl were added to a basal medium (BM) of the following composition: 5 g peptone (Fisher Scientific Co., No. J-2007C), 10 g glucose, 1 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 20 g agar and 1 liter of distilled water (pH 5.2). When Botran was used, it was added in 3 ml of acetone. Rose bengal was added from a stock solution (2 g/600 ml distilled water). Streptomycin sulphate and chlortetracycline were added in aqueous solution to cooled (45 C) agar medium. For colony growth studies, the center of each plate was inoculated with a loop of dry conidia without scattering. After 4 days of incubation at 30 C, two colony diameter measurements were made in each of four plates. For conidial germination studies, 1 ml of a washed conidial suspension (1×10^4 conidia/ml) was pipetted onto and spread over each plate. Plates were incubated at 30 C, and percentage germination was based on two counts of 100 conidia per count in each of two plates. Washed conidia were prepared by harvesting conidia from 2-week-old potato-dextrose agar slant cultures with 45 ml of an inorganic salt solution (0.01 M sodium phosphate buffer, 0.05% KCl, 0.05% $MgSO_4 \cdot 7H_2O$, pH 5.7) plus 0.1 ml of 0.25% Tween 20 [polyoxyethylene (20) sorbitan monolaurate, Atlas Chemical Industries, Inc., Wilmington, Delaware]. The spore suspension (40-50 ml) was shaken in a 250-ml screw-cap Erlenmeyer flask on a wrist-action shaker (Burrell Corp., Pittsburgh, Pa.) operated at position 10 for 20 minutes. This was followed by washing the conidia three times (by centrifugation at 150 *g* for 20 minutes) with 45-ml portions of salt solution minus Tween 20.

Five isolates of *A. flavus* were used to study conidial germination and colony growth response to chemical agents. Three were isolated from naturally infested Virginia peanut field soil, and two were isolated from peanut seed harvested in Virginia fields.

A naturally infested soil was obtained from each of two peanut fields in the vicinity of Holland, Virginia. Field soil I was a Woodstown loamy fine sand, had a pH of 5.7 (water-saturation percentage method), at 0.1 atmosphere contained 12% water, and contained 6.2 μg NH_4^--N plus 7.1 μg NO_3^--N/g soil. Field soil II was a Bertie fine sandy loam, had a pH of 5.7, at 0.1 atmosphere contained 21% water, and contained 5.4 μg NH_4^+-N plus 6.4 NO_3^--N/g soil. Soil samples were obtained from the top 10-cm layer of soil. Field soil I was used also to prepare artifically infested soil with washed conidia (prepared as above) at 10^3-10^4 conidia/g soil. This range is representative of the population levels of *A. flavus* in many naturally infested peanut field soils (1, 9, 12). Conidia were thoroughly hand-mixed into the soil with a spatula.

Artificially and naturally infested soils were assayed by the dilution plate technique. Samples (11-gram) were each placed in 250-ml screw-cap Erlenmeyer flasks containing 95 ml of sterile distilled water and 36 glass beads to obtain a 1-in-10 dilution. Flasks containing the soil suspension were shaken on a Burrell wrist action shaker at position 10 for 20 minutes. If needed, higher soil dilutions were prepared on a 10:90 basis. Soil dilutions to be assayed were placed in a sterile 250-ml beaker containing a magnetic stirring bar. One-milliliter samples were removed from agitating soil suspension and distributed over the surface of the test media. Separate soil samples were weighed out for dry-weight determinations. Dry weights were determined after 24 hours at 105 C. When soil suspensions were prepared by blending, rather than shaking, the same size samples were added to a sterile Waring Blendor containing 95 ml of distilled water. The soil suspension was blended at high speed for 2 minutes. Soil dilution plates were incubated at 35 C. Preliminary tests indicated colonies of *A. flavus* on dilution plates developed most rapidly at this temperature, and the optimum temperature for *A. flavus* conidial germination in glucose plus peptone is 30-35 C (14).

Estimates were made of the osmotic potential for the NaCl-amended media used based on the water activity found in the tables of Robinson and Stokes (15), and the formula for total water potential used by Manandhar and Bruehl (11). Solutions of 3% NaCl and 10% NaCl have water potentials at 30 C of −23.6 and −83.4 bars, respectively, and at 35 C water potentials of −24.1 and −84.8 bars, respectively. The basal constituents of the selective media would probably increase these values slightly, as found by Manandhar and Bruehl (11) for cornmeal-dextrose agar.

RESULTS.—*Influence of antimicrobial chemical agents on colony growth and conidial germination in pure culture.*—Tests indicated that additions of NaCl to the basal medium up to 3% did not inhibit, but sometimes stimulated colony growth by the five isolates of *A. flavus* (104 to 142% of colony diameter in BM). To this 3% NaCl-amended medium, chlortetracylcine (50 μg/ml) and streptomycin sulfate (50 μg/ml) were added to inhibit bacteria; these compounds had little or no effect on the colony growth of *A. flavus* isolates, either singly or in combinations with antifungal agents. Additions of compounds to inhibit undesired fungi resulted in various degrees of *A. flavus* inhibition. After testing several antifungal compounds and concentrations against *A. flavus* and undesired fungi, the following three combinations were selected for this study: (i) Botran, 1 μg/ml, plus 3% NaCl (inhibited *A. flavus* isolates slightly; mean = 81.8% and range = 76.6 to 88.2% of control); (ii) rose bengal, 33 μg/ml, plus 3% NaCl (inhibited three *A. flavus* isolates moderately and two severely; mean = 35.8% and range = 19.6 to 54.0% of control); (iii) 10% NaCl plus rose bengal, 33 μg/ml (inhibited all *A. flavus* isolates severely; mean = 18.8% and range = 12.6 to 23.1% of control). Except for the *A. niger* group of fungi, these three media (BM plus antibacterial antibiotics, NaCl and Botran, or rose bengal) either completely or severely inhibited the colony growth of undesired bacteria and fungi found in soils used in this study.

Conidial germination of all five isolates of *A. flavus* was complete (99-100%) on the 3% NaCl-Botran medium and on the 3% NaCl-rose bengal medium within 16 hours. On the 10% NaCl-rose bengal medium, conidial germination ranged from 84.5 to 95% at 16 hours, and from 87.5 to 97% at 24 hours. Thus, conidial propagules in soil may not be inhibited to any extent from germinating on these media.

Recoveries of A. flavus from naturally and artificially infested soils.—Preliminary tests with naturally infested Virginia peanut field soils indicated that low soil dilutions (10^{-1}) were required to isolate *A. flavus* (5). In three experiments, 3% NaCl-rose bengal medium was compared to 3% NaCl-Botran medium. Recovery of *A. flavus* was similar on both media. Populations of 3.6 (soil I), 13.8 and 10.0 (both in soil II) propagules/g soil were obtained in the three experiments for 3% NaCl-rose bengal medium, and populations of 2.2 (soil I), and 12.0 and 12.4 (both in soil II) were obtained for 3% NaCl-Botran medium. Populations in each experiment were based on *A. flavus* colony counts on 50 plates after three days of incubation at 35 C. Total *A. flavus* colony count on the two media for all experiments was 135 and 133, respectively. *Aspergillus flavus* colony size on 3% NaCl-Botran medium was typically larger (range, 2-11 mm) than on 3% NaCl-rose bengal medium (range, 2-9 mm). Colony-size of undesired fungi was about the same on both media. When 10% NaCl-rose bengal was compared to 3% NaCl-rose bengal for naturally infested soil, recovery of *A. flavus* on the two media was also similar. In two experiments, populations of 10.8 and 15.6 (both in soil II) propagules/g soil were obtained for 3% NaCl-rose bengal medium, and populations of 12.4 and 14.8 propagules/g soil were obtained for 10% NaCl-rose bengal medium. Populations in each experiment were based on *A. flavus* colony counts on 25 plates after six days incubation at 35 C. However, *A. flavus* colony diameter on soil dilution plates was much greater on 10% NaCl-rose bengal medium (range, 7-17 mm) than on 3% NaCl-rose bengal medium (range, 3-7 mm). Much greater inhibition of other fungi was observed on 10% NaCl-rose bengal, than on 3% NaCl-rose bengal. Less interference

from undesired fungus colonies, together with the long incubation period of six days, resulted in the larger *A. flavus* colony size on 10% NaCl-rose bengal medium. Three days of incubation was inadequate for large *A. flavus* colony development of 10% NaCl-rose bengal medium due to its slow growth rate on this medium. *A. flavus* colonies were typically the largest colonies produced by all fungi on all three of the media used to assay naturally infested soil. Most fungi had colony diameters one-half or less of that of *A. flavus* with the exception of *A. niger* which produced colonies about the same size and frequency as *A. flavus* on dilution plates.

The origin of colonies on soil dilution plates of naturally infested soil (soil II) was determined by microscopic observation of plates early in colony development (within 24 hours) following gentle washing of excess soil off plates with distilled water to improve microscopic observations. By this time, most germinating propagules of *A. flavus* should have penetrated the agar medium to avoid the accidental removal of propagules from washing. Knowledge of the nature of propagules in soil is important to assessments of selective media performance because all propagule types of a given fungus may not germinate equally well on the same selective medium. These observations indicated that many of the colonies were derived from single conidia, but that the origin of other *A. flavus* colonies was not clear, being obscured by small soil particles or small pieces of organic matter. In no case were colonies derived from sclerotia.

Nonsterile soil artifically infested with *A. flavus* was used to examine the effectiveness of the selective media in recovering the fungus from conidial propagules in soil. In a comparison of 3% NaCl-rose bengal medium and 3% NaCl-Botran medium, the latter medium yielded a higher recovery of *A. flavus* (6,800 and 8,100 propagules per gram of soil, respectively). Populations are based on *A. flavus* colony counts on 20 plates after 3 days incubation at 35 C. Total *A. flavus* colonies counted were 135 and 161, respectively. Colony-size of *A. flavus* on 3% NaCl-Botran was double that on 3% NaCl-rose bengal (10-28 mm and 5-13 mm, respectively). In a separate study, the recovery on 10% NaCl-rose bengal medium and the same medium containing rose bengal but no NaCl (a medium moderately inhibitory to *A. flavus*) was determined for two artificially infested soils. Due to little interference from undesired fungi, media not containing NaCl could be used for plating soil at high dilution (10^{-3}). For two artificially infested soils, both prepared from field I, recovery of *A. flavus* on rose bengal medium was 3, 300 and 8,800 propagules/g soil, and on 10% NaCl-rose bengal medium was 1,800 and 4,200 propagules/g soil, respectively. *A. flavus* colony-size was much greater on the former medium after three days. For all selective media examined, *A. flavus* typically had by far the largest colony-size of all fungi growing on soil dilution plates.

To examine the recovery efficiency of *A. flavus* from artificially infested soil, the population in soil based on computations from hemacytometer counts of the washed spore suspension was compared to data based on colony counts on dilution plates using 3% NaCl-Botran medium. As *A. flavus* conidia frequently clump together, even after shaking in Tween 20, both total conidia and total units (clumps of conidia and single conidia) were counted on the hemacytometer. The results indicated that recovery efficiency is very high. When nonsterile soil was infested with 1,790 *A. flavus* conidia (A) or 900 *A. flavus* units (B) per gram soil, 1,500 colonies of *A. flavus*/g soil were recovered on diluted plates. The former was based on 12 hemacytometer counts, and the latter was based on colony counts of 20 dilution plates for each of six soil samples after 3 days of incubation at 35 C. Percentage recovery based on A was 85.5% whereas recovery based on B was 170%. It appears that many of the clumps of conidia break up during incorporation of conidia into soil and during the blending used for soil dilution preparation. This makes a more precise determination of absolute recovery difficult.

DISCUSSION.—A critical test for the performance of selective media is the relative recovery of suspected low populations (less than 10 propagules/g soil) of the desired fungus from naturally infested soil, when more than 99.9% of the population of total fungi in the soil may consist of undesired fungi. When naturally infested soils with very low *A. flavus* populations were examined, differences in relative recovery for media varying in inhibition to *A. flavus* growth were not apparent in our experiments. Thus, severe inhibition of colony growth of the desired fungus on selective media (as determined in pure-culture tests) may not result in reduced recovery, on a relative basis, from naturally infested soil.

A key factor in these results would appear to be that all three media tested allowed high or complete conidial germination by *A. flavus* in pure-culture tests. However, for nonsterile soil artifically infested with conidia, the selective media less inhibitory to *A. flavus* colony growth yielded the higher relative recovery. Soil fungistatic factors, although important to *A. flavus* conidial germination (4, 8), should not restrict conidial germination on dilution plates in the presence of glucose and peptone in selective media. Differences in propagule type, fungus clones, and degree of competition from undesired fungi may account, in part, for the different results found for artificially and naturally infested soil. Different fungus isolates (13), as observed here for *A. flavus,* or different propagules of the same fungus (18) have different reactions to the same selective medium. Conidia appeared to be not the only *A. flavus* propagule type in the naturally infested soils studied. Interference from undesired fungi on dilution plates was much greater for naturally infested soil than for artificially infested soil.

Conceivably, some slow-germinating, aged propagules of *A. flavus* in naturally infested soil may have been overgrown by other fungi on the less inhibitory media. In contrast, it is possible that not all natural *A. flavus* propagules (conidia and hyphal fragments, possibly) did not germinate on the highly inhibitory medium, 10% NaCl-rose bengal. The effect of these factors may not be large, however, since the recoveries from naturally infested soil on the test media were, as indicated, remarkably similar. To our knowledge, this is the first study to compare pure culture colony growth by isolates of the desired fungus on several selective media to recovery from naturally infested soil.

Colony size of the desired fungus versus colony size of undesired fungi on dilution plates prepared from naturally infested soil may be one indicator of the effectiveness of selective media, also. Small pinpoint

colonies of the desired fungus are not only difficult to detect, but suggest that the fungus may be overgrown in other cases if colonies of undesired fungi are large and numerous. *A. flavus* colonies, along with *A. niger*, were typically the largest colonies on dilution plates of the three media tested here. Because of reduced competition from undesired fungi and the longer incubation time used for 10% NaCl-rose bengal medium, *A. flavus* colonies were larger than expected (from pure-culture data) on dilution plates of naturally infested soil.

All three media can be recommended for isolating *A. flavus* from naturally infested soil, but, in extensive testing, we have used the 3% NaCl-Botran medium most often because rose bengal is not as stable in storage (6, and G. J. Griffin, *unpublished*). Only in one instance when a large number of colonies of *Rhizopus* spp. were present on the 3% NaCl-Botran medium plates (from soil adjacent to decomposing rye) was this medium unsatisfactory. Increasing the Botran concentration to 2 μg/ml solved this problem and had a small effect on *A. flavus* colony size on dilution plates. Use of NaCl in selective media may be helpful in isolating from soil other fungi tolerant of low water potential.

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